Analysis of N-Glycans Released from Proteins of Therapeutic and Clinical Significance Using Capillary Electrophoresis and Liquid Chromatography Coupled to Mass Spectrometry

A Thesis Presented By
Victoria Berger

to
The Department of Chemistry and Chemical Biology

In Partial Fulfillment of the Requirements for the Degree of
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ABSTRACT OF THESIS

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ABSTRACT

N-linked glycosylation is a prevalent post-translational modification which modulates the physical, chemical and biological properties of proteins. Glycosylation is important to monitor in both disease and biotherapeutic products.

A growing part of the biopharmaceutical sector are monoclonal antibody (mAb) based therapeutics, typically of the immunoglobulin G (IgG) class which contain a conserved glycosylation site at asparagine-297 in the crystallizable fragment (Fc) region. This site can be occupied by 32 possible glycan structures (in human serum IgG) that affect the function of the molecule. Therefore due to the vast microheterogeneity that can arise, biopharmaceutical products must be analyzed during and after manufacturing to verify the glycan chains or monosaccharides present. One approach to this analysis is by using capillary zone electrophoresis (CZE) with laser induced fluorescence (LIF) to monitor the abundance of individual monosaccharides from released and hydrolyzed N-glycan chains on the mAb biotherapeutic. This quantitative approach was applied to nine biotherapeutic monoclonal antibodies. The original procedure, as obtained from the literature, was further optimized, using sample clean-up protocols and dilution, to reduce noise present due to the sensitivity of the fluorescence detector and ultimately to improve the separation of the monosaccharide peaks. The resulting data showed that the therapeutics contained high levels of fucosylation with variable levels of galactosylation consistent with human glycosylation.

To investigate alterations in sialylated glycans that are associated with disease, a previously developed fluoride-mediated negative ion microfluidic chip LC-MS method can be applied; however, the glycosidic bond between the sialic acid and underlying glycan is labile and easily lost during mass spectrometric ionization. To minimize the charge on the sialic acids and therefore, to enable the application of our fluoride-mediated method, a charge neutralization reaction such as methylation or methyl amidation of the carboxylic acid can be used. A variety of reagents were tested to methylate the carboxylic acid on the sialic acid; however, none of the
methylation procedures showed completed conversion of the carboxylic acids to methyl esters. A methyl amidation approach was also undertaken with (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyAOP). This approach proved to be promising as it showed complete conversion for both linkages (α(2,3) and α(2,6)) on disialyllacto-\(N\)-tetraose, a disialylated milk sugar. When the procedure was further applied to a disialylated, complex \(N\)-glycan, an incomplete reaction was seen indicating the further need for optimization of the reaction conditions. After further optimization, PyAOP will be applied to investigate glycosylation on alpha-1 acid glycoprotein, a heavily sialylated glycoprotein involved in immune response and implicated in disease progression.
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I would also like to thank the other members of the Karger Lab: Dr. Shujia Daniel Dai, Zhenke Jack Liu, Siyang Peter Li, Siyuan Serah Liu, Chen Li, Simion Kreimer, Dr. Sandor Spisak, Dr. Alexander Ivanov, Somak Ray and Yuanwei Abby Gao. Everyone was available for any questions that I had and were more than willing to help me. In addition, I would like to thank members of the Hancock research group who have always been available to answer questions about instrumentation: Fateme Tousi, Francisca Gbormittah and KyOnese Taylor.

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reminded me of what I could accomplish if I only focused. Without their love and support, I
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LIST OF ABBREVIATIONS

2-AA 2-Aminobenzoic Acid
2-AB 2-Aminobenzamide
2-AP 2-Aminopyridine
A2 Disialylated, Galactosylated, Biantennary glycan
A3 Trisialylated, Galactosylated, Triantennary glycan
ACN Acetonitrile
ADCC Antibody-Dependent Cellular Cytotoxicity
ANTS 2-Aminonaphthalene Trisulfonic Acid
APTS 8-aminopyrene-1,3,6-trisulfonic Acid
Asn Asparagine
C Constant
CDC Complement-Dependent Cytotoxicity
CDG Congenital Disorders of Glycosylation
CE Capillary Electrophoresis
CFG Consortium for Functional Glycomics
CGE Capillary Gel Electrophoresis
CHO Chinese Hamster Ovary
CID Collision Induced Dissociation
CZE Capillary Zone Electrophoresis
DMSO Dimethyl Sulfoxide
DSLNT Disialyllacto-N-tetraose
Endo Endoglycosidase
ECD Electron Capture Dissociation
EIC Extracted Ion Chromatogram
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron Transfer Dissociation</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic Flow</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-Binding Fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment Crystallizable Region</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier Transform-Ion Cyclotron Resonance</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanidine Diphosphate</td>
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<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic Acid</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylgalcosamine</td>
</tr>
<tr>
<td>Gln</td>
<td>Glucosamine</td>
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<tr>
<td>HILIC</td>
<td>Hydrophilic Interaction Liquid Chromatography</td>
</tr>
<tr>
<td>HPAEC-PAD</td>
<td>High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>i.d.</td>
<td>Internal Diameter</td>
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<tr>
<td>IdoA</td>
<td>Iduronic Acid</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>IS</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>KDN</td>
<td>Deaminated Neuraminic Acid</td>
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<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar Electrokinetic Chromatography</td>
</tr>
<tr>
<td>MeI</td>
<td>Methyl Iodide</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry, Mass Spectrometric, Mass Spectrometer</td>
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<tr>
<td>MTT</td>
<td>3-Methyl-1-(p-tolyl)triazene</td>
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<tr>
<td>NaF</td>
<td>Sodium Fluoride</td>
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<td>NaOH</td>
<td>Sodium Hydroxide</td>
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<td>NeuAc</td>
<td>N-acetylneuraminic acid</td>
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<tr>
<td>NeuGc</td>
<td>N-glycolylneuraminic acid</td>
</tr>
<tr>
<td>NH₄F</td>
<td>Ammonium Fluoride</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer Diameter</td>
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<tr>
<td>PGC</td>
<td>Porous Graphitized Carbon</td>
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<tr>
<td>PNGase F</td>
<td>Peptide-N-Glycosidase F</td>
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<tr>
<td>PyAOP</td>
<td>(7-azabenzotriazol-yloxy)trispyrriolidinophosphonium hexafluorophosphate</td>
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<tr>
<td>Q-TOF</td>
<td>Quadrupole Time-of-Flight</td>
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<tr>
<td>RF</td>
<td>Radio Frequency</td>
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<tr>
<td>RP</td>
<td>Reversed Phase</td>
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<td>Tetrahydrofuran</td>
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<tr>
<td>TOF</td>
<td>Time of Flight</td>
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<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>UPLC</td>
<td>Ultra-Pressure Liquid Chromatography</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Variable</td>
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CHAPTER ONE
INTRODUCTION TO GLYCANS AND GLYCAN ANALYSIS

Glycosylation is an extremely important post-translational modification that can be found on a wide variety of biological molecules including proteins and lipids. Its importance arises from its involvement in a wide variety of biological processes including host-pathogen interactions, immune response and cell-cell interactions.¹ Many protein binding interactions are also mediated by glycan epitopes which are composed of 2-10 monosaccharides such as the blood group antigens.² Glycosylation is a term that refers to the addition of glycan or oligosaccharide chains that contain a diverse group of monosaccharide building blocks.

1.1 Building Blocks of Glycosylation

All glycan chains are composed of a variety of monosaccharides that are covalently linked in many different ways. Monosaccharides are the smallest units that build up oligosaccharides or glycan chains. The terms glycans and oligosaccharides can be used interchangeably in the field of glycobiology. The structure of a monosaccharide is of a polyhydroxy aldehyde or polyhydroxy ketone containing either five or six carbons.¹ An exception to this are the sialic acids which are a family that are composed of nine carbon sugars.³ The monosaccharide can exist in either an open or a cyclic hemiacetal form.¹ Monosaccharides are usually depicted in the cyclic form in a chair conformation. Typically the rings are shown as pyranosic (six membered); however, the structure can also be shown in the furanosic form (five membered).¹ In addition, the monosaccharide structures can exist in two enantiomeric forms: D and L.³ Vertebrate organisms generally have D-monosaccharides except for L-fucose and L-iduronic acid.³

Monosaccharides also contain another chiral carbon center after they cyclize to form hemiacetals. This location, typically carbon-1 (C-1), can contain one of two anomers: alpha (α)
or beta (β). Anomericity is defined in relation to the highest numbered chiral carbon center. If both C-1 and the highest numbered chiral carbon are fixed in opposite directions (i.e. axial up and axial down), then the anomericity is alpha. On the other hand, if both positions are fixed in the same direction (i.e. axial up at highest chiral carbon and equatorial at C-1) then the anomericity is beta (Figure 1.1).

![Image of glucose anomericities](image)

**Alpha (α)**

**Beta (β)**

*Figure 1.1: A comparison of both anomericities of glucose. The image on the left shows the α anomer and the image on the right depicts the β anomer.*

The anomeric carbon of the monosaccharide can combine with any hydroxyl on a neighboring monosaccharide forming a glycosidic bond. Since monosaccharides contain many hydroxyl groups, there are many possible linkages that can be formed which is further complicated by the fact that the linkages are also determined by the anomericity (α or β) of the non-reducing end monosaccharide. These factors give a large diversity of possible glycan structures.

Despite the vast diversity of available monosaccharides, only ten are commonly seen in mammals. These monosaccharides are xylose (Xyl), glucose (Glc), galactose (Gal), mannose (Man), N-acetylglicosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), glucuronic acid (GlcA), iduronic acid (IdoA) and sialic acids, with the most common sialic acid in humans being N-acetyleneuraminic acid (NeuAc or Neu5Ac) (Figure 1.2).
Figure 1.2: Nine of the most common monosaccharides in mammals. All of the monosaccharides depicted are in the D-enantiomer except for fucose which is the L-enantiomer. The wavy line indicates that the monosaccharide can be in either anomeric form. Adapted from Reference Two.²

All of these sugars are composed of six carbons with the exception of xylose (a five carbon sugar) and sialic acid (nine carbon sugars). Many of the monosaccharides are epimers of each other meaning that they differ in configuration around a single chiral carbon.³ Differences between the monosaccharides can be seen in the positioning (axial or equatorial) of the hydroxyl groups. The monosaccharides in the glycan chain can also be further modified by other modifications such as sulfation, acetylation and phosphorylation.¹
1.2 Glycosylation Types and Biosynthesis

Glycans can be added to proteins through two mechanisms: interaction with the hydroxyl group of a serine or threonine residue or the amide group on an asparagine residue. These two mechanisms give rise to O- and N-linked glycans, as described by their linkage to the protein backbone.

O-glycan chains or individual monosaccharides such as GalNAc, GlcNAc, Xyl, Man and Fuc can be transferred to serine or threonine residues.\textsuperscript{1} O-linked glycans are added to proteins one monosaccharide at a time through glycotransferase proteins specific to various monosaccharides in the Golgi apparatus.\textsuperscript{1, 3} O-glycosylation can also occur in the cytosol and nuclear space and is important in regulating transcription.\textsuperscript{4} The first monosaccharide that is linked to the amino acid is a GalNAc by one of more than 24 GalNAc transferases.\textsuperscript{1} These GalNAc transferases have differences in their specificity which leads to a fine-tuned control of O-glycosylation.\textsuperscript{1} This monosaccharide is further elongated by other transferases to generate eight possible O-glycan cores (Figure 1.3). The main focus of this thesis is on the analysis of N-glycans; therefore, O-glycans will not be further discussed in terms of analytical approaches for their analysis. It should be noted, however, that O-glycan analysis is still very challenging since there is no release method comparable to the ease in which N-glycans can be released from proteins, due to the lack of a general and efficient O-glycanase.
N-linked glycans are added to asparagine residues that are within the sequon Asn-Xaa-Ser/Thr (Xaa denotes any amino acid except for proline). Despite the appearance of an N-linked glycosylation site in a protein, not all sequon sites are occupied, leading to a degree of macroheterogeneity. This heterogeneity arises from enzyme competition during the biosynthesis of N-glycan chains.

N-glycan biosynthesis first begins on the cytosolic side of the membrane of the endoplasmic reticulum (ER). A GlcNAc residue and phosphate molecule are transferred from uridine diphosphate to membrane bound dolichol-P. Dolichol is a lipid that is composed, in a linear fashion, of five carbon isoprene units. The number of isoprene units varies among various organisms. After the transfer of GlcNAc and phosphate, the chain is further elongated by the action of other uridine diphosphate (UDP) sugar complexes and guanosine diphosphate (GDP) sugar complexes. One more GlcNAc is added to the chain by UDP-GlcNAc and five mannose residues are added by GDP-Man. The chain is then translocated into the lumen of the
ER where it is further elongated with four mannoses and three glucose residues. The precursor to mature N-glycan chains is then bound to unfolded proteins at the sequon using an oligosaccharyltransferase (Figure 1.4).³

Figure 1.4: The synthesis of the dolichol-P-P-GlcNAc₂Man₉Glc₃ glycan precursor. Reprint from open access Essentials of Glycobiology (2nd edition) on the NCBI bookshelf, Chapter 8, 2009.³

After the glycan has been added to the protein, trimming of glucose and mannose residues begins with glucosidases and mannosidases in the ER.⁵ During this trimming, glucose residues can be added and removed as part of a quality control mechanism in protein folding, the calnexin/calreticulin pathway.⁵ Further trimming and addition of monosaccharides is accomplished in the Golgi (Figure 1.5).³ Some of the modifications that are possible include antenna elongation with galactose and GlcNAc residues, bisecting GlcNAc residues, capping of antenna with sialic acids and fucosylation.
The final result of the biosynthetic pathway is a complex glycan. This is not the only major type of N-glycans; there are two other major types, hybrid and oligomannose (Figure 1.6).
Each glycan type has the same chitobiose core but different variations in the rest of the antenna structure. The core is composed of two GlcNAc residues attached to three mannose residues. Oligomannose glycans contain only mannose residues attached to the core and indicate that the glycan did not go through the complete biosynthetic pathway. Hybrid N-glycans have one antenna that is complex and the other that only contains mannose residues. This also indicates incomplete biosynthesis. The complex glycan is the structure found at the end of the biosynthetic pathway. It is composed of antennae that begin with GlcNAc residues and are further modified by the addition of other monosaccharides.

There is vast microheterogeneity seen among glycans on proteins because of the various types of glycans that are possible and the addition of modifications. Microheterogeneity arises from tissue specific regulation of the genes that encode the proteins and enzymes involved in the biosynthetic pathway.⁶

Many different glycan isomers are possible. Glycans can have the same overall structure but contain different linkages. A variety of linkages are possible due to the abundance of
hydroxyl groups that can undergo linkage formation. In addition, glycans can be positional isomers. This is commonly seen with a monosaccharide that is present on different arms of the glycan (Figure 1.7). All aspects of linkage and positional isomers can further complicate the structural interpretation of glycans.

Figure 1.7: An example of two positional isomers in which the galactose residue is on the α-3 mannose arm or on the α-6 mannose arm.

1.3 Monosaccharide Symbol Systems

There are two main systems used to depict glycans and their underlying monosaccharide linkages and composition. One system that is used is the Oxford symbol notation. This system gives underlying information on both the linkage positions and types. In addition, it can be used in a black and white format since all of the symbols for the various monosaccharides are different and do not require color (Figure 1.8).
Figure 1.8: A depiction of the Oxford symbol notation for glycans including both monosaccharides and linkage position.\textsuperscript{7}

The second system is the Consortium for Functional Glycomics (CFG) system. This system is typically represented by colors and various shapes (Figure 1.9).

Figure 1.9: The ten most common monosaccharides found in humans using the Consortium for Functional Glycomics symbols.
The common shapes indicate whether the monosaccharides are hexoses or \( N \)-acetylhexosamines and the colors indicate their true identity. This system can also show linkages if they are written in but the connections between monosaccharides do not inherently show the linkages as the Oxford system does. In addition, since the symbols are in color, care must be taken when using black and white reproductions so that the differences can be seen. This thesis will use the CFG system nomenclature when showing glycan structures for ease of interpretation.

1.4 Glycans in Disease

Glycan deficiencies and changes are implicated in a large variety of diseases. One major group of disorders, congenital disorders of glycosylation (CDG), are a result of mutations in the genes that encode the proteins used in the biosynthetic pathway of glycosylation.\(^6\) The resulting glycosylation is defective and has severe clinical implications.\(^6\) There are currently 45 diseases in the CDG relating to both \( N \)- and \( O \)-linked glycans.\(^8\) The disorders involve a vast multitude of organ systems; however, a large proportion also contain a neurological component.\(^8\) Due the complexity and widespread effects of these disorders, they are still poorly treatable and require further research efforts.\(^8\)

Changes in glycosylation are also implicated in a large variety of disorders and diseases. For example, in rheumatoid arthritis there is decreased galactosylation on immunoglobulin G whereas in cystic fibrosis there is undersialylation and overfucosylation of plasma membrane glycoconjugates.\(^6\)

Altered glycosylation has been widely documented in various cancers.\(^9\) It is still unknown whether these changes seen in glycosylation are a result of the cancer or a cause.\(^{10}\) Results have indicated that some expression of certain glycosyl epitopes promotes cancer progression whereas expression of other glycosyl epitopes actually suppresses cancer progression.\(^{10}\) Analysis revealed that in breast cancer samples, an increase in sialylation and
fucosylation on glycans was seen.\textsuperscript{9} However, in gastric cancer it was shown that levels of fucosylated non-and mono-sialylated glycans were decreased. Research is continually being done on other cancers to assess the implications of glycosylation changes.

1.5 Glycan Analysis

Glycans can be analyzed either after release from proteins or attached to proteins (glycopeptides). The latter analysis is more challenging due to limitations in instrumentation and methods; however, improvements are constantly being made so that this approach becomes more feasible.\textsuperscript{1} Ideally, analyzing the glycopeptide would give both glycan and site specific information on the protein which is beneficial to understand changes in function and the implications in disease. This thesis will focus on techniques that are used for released glycan analysis, which are more commonplace for glycan characterization. The three main techniques that are used for analysis of released glycans are chromatographic, mass spectrometric and electrophoretic methods. Many of these techniques have also become coupled such as liquid chromatography-mass spectrometry (LC-MS). These couplings provide even more information than one dimension alone would.

1.5.1 Glycan Release

\textit{N}-linked glycans are typically released enzymatically using either peptide-N-glycosidase F (PNGase F) or peptide-N-glycosidase A (PNGase A).\textsuperscript{1} PNGase F cleaves the bond between the GlcNAc and the asparagine residue except in cases of a fucose attached to the core GlcNAc via an α(1,3) linkage.\textsuperscript{1} The addition of fucose in this manner is normally found in plant and insect glycoproteins.\textsuperscript{1} PNGase A can be used for this modification because it will cleave with this fucose modification present.\textsuperscript{1}

Three other enzymes that will cleave glycans but leave one GlcNAc residue attached to the protein are endoglycosidase (endo) D, H and F. Endo D will cleave all classes of \textit{N}-glycans
whereas endo H will only cleave oligomannose and hybrid N-glycans.\textsuperscript{1,11} There are three Endo F enzymes: F1, F2 and F3.\textsuperscript{11} F1 will cleave oligomannose or hybrid glycans while F2 will cleave oligomannose and biantennary complex glycans.\textsuperscript{11} F3 will only cleave biantennary and triantennary complex glycans in addition to the presence of only the chitobiose core.\textsuperscript{11}

\textit{N}-glycans can also be released chemically using hydrazinolysis.\textsuperscript{12} This method releases the glycan from the protein through first a $\beta$-elimination mechanism and then a further reaction with the hydrazine.\textsuperscript{12} Although this method can be used, an enzymatic method is better due to the high risk involved with hydrazinolysis.\textsuperscript{12} One of the drawbacks of the chemical method is the use of anhydrous hydrazine.\textsuperscript{12} This compound is extremely toxic and also very explosive therefore requiring great care for the reaction, whereas the enzymatic approach only requires standard safety precautions.\textsuperscript{12} \textit{N}-glycans can also be released chemically using reductive $\beta$-elimination with sodium hydroxide and sodium borohydride.\textsuperscript{1} This approach results in the conversion of the reducing end of the glycan to an alditol which prevents further derivatization with fluorescent labels limiting the sensitivity of detection.\textsuperscript{1}

1.5.2 Chromatographic Analysis

\textit{N}-glycans are commonly analyzed using high performance liquid chromatography (HPLC) or even now ultra-pressure liquid chromatography (UPLC). A UPLC system offers increased separation efficiency over classic HPLC due to the smaller particles which allow for a higher peak capacity.\textsuperscript{13} In addition, the UPLC system is designed to minimize dead volumes which decreases band broadening and yields sharper peaks.

Since most glycans are not UV absorbing or contain fluorophores, they must first be derivatized using various labels. The most common labels are 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (2-AP), 2-aminonaphthalene trisulfonic acid (ANTS) and 8-aminopyrene-1,3,6-trisulfonic acid (APTS) (Figure 1.10).\textsuperscript{12}
Each of these labels can be used in various situations. 2-AB is widely used because it lacks a negative charge and allows for a broad range of chromatographic techniques. 2-AA does contain a charge which means that the charge can be utilized in various methods, including both ion polarities in mass spectrometric analysis. The trisulfonic acid derivatives are mainly used in capillary electrophoresis techniques. The labels are attached to the glycans through reductive amination. In reductive amination, the primary amine on the label reacts with the aldehyde on the reducing end of the glycan (the end that is normally attached to a protein) to form an imine or Schiff base. This imine further undergoes reduction to a secondary amine that links the glycan and the label (Figure 1.11).
Figure 1.11: A general reaction scheme for reductive amination using the reducing end GlcNAc and 2-aminobenzoic acid (2-AA).

There are two main stationary phases or chromatographic mechanisms that are used for glycan analysis: hydrophilic interaction liquid chromatography (HILIC) and porous graphitized carbon (PGC).

HILIC columns contain highly polar stationary phases such as bare silica particles, amine-, hydroxyl-, amide-bonded or zwitterionic particles or polymer monoliths. The polar glycans interact with the hydrophilic stationary phase through a partitioning mechanism into a hydration layer, which is tightly bound to the stationary phase when present in a high solvent environment. It is also thought that ion exchange and dipole-dipole interactions can play a role in retention. The eluting solvent in these separations is water; glycans are loaded into the column with a high organic mobile phase composition and eluted with increasing percentages of aqueous solvent. The separation is based on the number of polar groups in a glycan, and therefore, the elution can be predicted based on the glycan composition and associated size.
Derivatized glycans might be less retained on HILIC columns if the label is hydrophobic; however, this interaction, of the label with the stationary phase, has been shown to be minimal.\textsuperscript{13} Knežević et. al. found that retention on HILIC stationary phases is through the interaction of the glycan with the amide phase and not from contributions of the attached labels through experiments using three fluorescent labels concurrently.\textsuperscript{14} HILIC also has the ability to separate isomer peaks which can be beneficial in analysis.

HILIC can be run either coupled with a mass spectrometer or on its own. When it is run by itself, the glycans must be derivatized in order to be seen with either a UV detector or with a fluorescence detector.

When HILIC is run as an analysis method by itself, a labeled ladder of glucose oligomers such as dextran is also run (Figure 1.12).\textsuperscript{13}

![Figure 1.12: An example of a 2-AB labeled dextran ladder at various flow rates run using a Waters UPLC system with 1.7 μm HILIC resin. Reprinted from Journal of Chromatography B, 878, Joomi Ahn, Jonathan Bones, Ying Qing Yu, Pauline M. Rudd, Martin Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction](image-url)
The dextran ladder allows for retention time based normalization of the analytical data by conversion to glucose units, helping to minimize technical and experimental variation. The sample in question can then be compared to the glucose units for glycan identification since a library of standard glycans can be created that shows typical elution in terms of glucose units. The glucose units also allow for reproducible and normalized elution since all samples are run against the standard. This procedure can eliminate day to day variation when running samples.

Porous graphitized carbon is also growing in popularity as a stationary phase used in the analysis of glycans. PGC is a highly hydrophobic stationary phase that retains glycans through both hydrophobic and polar interactions. Planarity, inducible polarity and charge also contribute to retention on PGC. The eluting solvent in PGC chromatography is organic; therefore, samples are loaded in a high concentration of aqueous solvent and then eluted using increasing organic percentages. Ionic strength in the mobile phase is necessary for the complete elution of charged glycans such as sialylated glycans. PGC is also very sensitive to pH and temperature which can change its retention characteristics. PGC is capable of separating anomers which might or might not be ideal for analysis. Anomer separation can increase the complexity of the resulting chromatogram making analysis more difficult; however, anomer separation also gives more information on the glycan structure. Reducing the glycans to alditols, to eliminate the anomeric separation, decreases the sensitivity of further mass spectrometric analysis in addition to limiting the ability of further derivatization. PGC columns are commonly used in coupled methods with mass spectrometry.

Both HILIC and PGC can also be used in solid phase extraction experiments for clean-up of glycans before analysis. HILIC is especially useful in this regard since its elution is pure water.
without the need for salts or acids. This is amenable for subsequent mass spectrometric detection without any further steps.

Other chromatographic techniques include reversed phase liquid chromatography (RPLC), anion exchange chromatography and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Reversed phase columns can only be used if the glycans are first derivatized with a hydrophobic agent, such as 2-aminoacridone. The most common RP stationary phase is octadecyl silica in which an 18 carbon chain is attached to the particle. Some common labels that are used are the neutral tags; 2-AB, 2-aminoacridone and 2-AP. Anion exchange chromatography can be used as a fractionation technique prior to subsequent analysis. At basic pH, glycans become deprotonated allowing retention on the anion exchange column and subsequent separation. Glycans must be labeled with a UV or fluorescent tag to be detected on an anion exchange column. HPAEC-PAD is a separation in which the pH is extremely basic causing the hydroxyls on the glycans to become deprotonated. The charged glycans are then separated and subsequently detected using amperometry. This technique is normally not used in conjunction with mass spectrometers because a high level of salt is needed for elution. The advantage to this technique is that the glycans do not need to first be derivatized but the disadvantages are that the baselines are extremely unstable and can vary from day to day and between buffer preparations. The technique is highly sensitive to contaminants which decreases the sensitivity of the analysis.

1.5.3 Mass Spectrometric Analysis

Mass spectrometric (MS) methods of analysis are popular for glycan analysis due to their low sample consumption and high sensitivity. There are two principle ionization methods that are used in mass spectrometric (MS) analysis: electrospray ionization (ESI) and matrix assisted laser desorption (MALDI). Although these are both considered soft ionization techniques,
MALDI is harsher than ESI and results in fragmentation of labile glycan linkages, such as the linkage to sialic acids, resulting in loss of structural information. Glycans can be analyzed as native glycans or as derivatized glycans depending on the aim of the experiment. Some of the derivatization tags can increase the ionization efficiency of the glycan because of the added hydrophobic moiety which in turn increases the sensitivity of the analysis.

Another commonly employed technique for increasing ionization efficiency is the use of permethylation where the hydrogens on the hydroxyl, amine and carboxyl groups are replaced by methyls resulting in methyl ethers and esters, which decreases the intermolecular hydrogen bonding. Permethylation further stabilizes the labile bond between sialic acids and the underlying glycan in addition to improving glycan retention on reversed phase columns. In addition, when permethylated glycans undergo fragmentation, the resulting ions are termed “scars” and give unique masses based on the lack of a methyl or methoxy group where the monosaccharide was attached to the glycan chain. Resulting interpretation of the fragment ions will give information on the linkage of the monosaccharides in the glycan chain.

Glycan analysis can be performed in both the positive and negative polarity. Often times, neutral glycans are run in the positive mode and show improved sensitivity especially with sodium adduction. Charged glycans, on the other hand, are analyzed using negative polarity because of the improved sensitivity with this technique and negatively charged groups. Glycans can also be deprotonated through anion adduction and analyzed in negative mode. Monosaccharide rearrangements have been reported in the positive ion mode whereas no reports have yet been made on rearrangements in the negative ion mode.

A variety of mass analyzers can also be used for the analysis of glycans. One type of mass analyzer that is commonly used is time of flight (TOF) due to its resolution, sensitivity, and good signal to noise ratios. Ion trap instruments can also be utilized for multiple fragmentation experiments. Ion traps offer high sensitivity in addition to the ability to repeat the
isolation/fragmentation process multiple times resulting in a great deal of structural information.\textsuperscript{21}

Often times, fragmentation of glycan structures by various strategies is used during MS experiments to gain more information about the underlying glycan structure. One type of fragmentation is collision induced dissociation which breaks the glycosidic bonds.\textsuperscript{1} The use of CID with positive ion polarity mainly results in the cleavage of the glycosidic bond rather than cross ring cleavages.\textsuperscript{2} However, in negative ion, CID results in both glycosidic cleavage and cross ring fragmentation which give more detailed information on the glycan composition.\textsuperscript{2} Other fragmentation mechanisms such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) are also being applied to glycan analysis because of the difference in mechanism of fragmentation.\textsuperscript{1} The basis for the mechanism of activated electron dissociation (ECD and ETD) is the transfer of an electron to the precursor ion resulting in the formation of a radical.\textsuperscript{2} The radical can then undergo further rearrangement causing cleavage of the glycosidic bond or sugar ring.\textsuperscript{2} ECD is limited in its application because it requires a Fourier-transform ion cyclotron resonance (FT-ICR) MS system which is both complex and expensive.\textsuperscript{21} ETD, on the other hand, is more widely applied since it can be performed on quadrupole-ion trap instruments.\textsuperscript{21} ETD also does not target labile bonds, allowing sialic acids to stay bound to the underlying glycan chain.\textsuperscript{21} One disadvantage of ETD is that it requires higher charge states (greater than 2) for generating cross ring cleavages.\textsuperscript{21}

\textbf{1.5.4 Coupled Techniques}

Liquid chromatography (LC) is often coupled to mass spectrometry to add another dimension of separation which will increase the amount of information obtained.\textsuperscript{24} LC can be used to separate the various glycan structure isomers allowing for increased sensitivity in the mass spectrometer because of improved ionization efficiency. Increased sensitivity is the result of fewer glycan ions entering the mass spectrometer at the same time due to their prior
separation compared to directly injecting glycans into the mass spectrometer. Using HILIC or PGC as the LC stationary phase, can allow for isomer differentiation which cannot be determined by mass alone in the mass spectrometer.

A nanoLC-MS based analysis with a porous graphitized carbon stationary phase was performed on N-glycans from epithelial ovarian cancer, resulting in the detection of over 100 distinct N-linked glycan compositions using the coupled methodology. The resulting analysis of the epithelial ovarian cancer patients compared to normal patients showed altered glycosylation trends that yielded seven potential biomarkers. ESI-MS with a zwitterionic HILIC phase was also applied to derivatized glycans released from human serum IgG. The methodology allowed for increased separation between isomeric glycans. LC-MS analysis using a microfluidic chip packed with graphitized carbon was also utilized to analyze N-linked glycans from human serum. The analysis yielded the annotation of nearly 200 glycans in human serum based upon a computational assignment of all possible structures to a given m/z value within the allowed mass accuracy.

1.5.5 Electrophoretic Techniques

Capillary electrophoresis (CE) is a common technique that can be used to separate glycans based on charge with low sample consumption (nanoliter injection volumes). Like HPLC, glycans must first be derivatized in order to be detected and also most commonly to impart a charge. There are a variety of modes to capillary electrophoresis that arise from varying separation conditions including capillary zone electrophoresis, micellar electrokinetic chromatography and capillary gel electrophoresis.

Separation in capillary zone electrophoresis (CZE) can be controlled by changing the electroosmotic flow, the directional bulk flow generated from the electric field when ions are attracted to the charged capillary surface. The separation conditions can be effectively modified by changing the background electrolyte used and the direction of the EOF. When the
system is run without flow (arheic conditions), the separation of analytes is controlled by their charge to hydrodynamic radius allowing for separation of isomers.\textsuperscript{27}

Other electrophoretic techniques that have been applied for glycan analysis include micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE).\textsuperscript{12} With CGE, the capillary is often filled with a viscous solution or gel and the EOF is minimized.\textsuperscript{28} Under these conditions, the glycans are separated based on their charge but also on their size as larger molecules are retarded more than smaller molecules.\textsuperscript{28} In MEKC, detergents are used in the buffer that will form micelles with other detergent molecules.\textsuperscript{28} Uncharged glycans will be able to interact with the detergents and will migrate at the same rate as the micelle when interacting with the micelle.\textsuperscript{28} The effective migration of the glycan will be determined by the residence in the micelle, based on hydrophobic interactions.\textsuperscript{28}

These techniques can give overall glycan composition information but not structural information unless exoglycosidases are applied. Exoglycosidases can be employed to systematically and specifically remove monosaccharides and record the change in migration. There are a multitude of exoglycosidases that are commonly employed (Figure 1.13).\textsuperscript{1} Exoglycosidase digestions can also be applied with liquid chromatography techniques.

\textbf{Figure 1.13: A depiction of the structural analysis of human serum IgG. A) A depiction of commonly used exoglycosidases for structural characterization: ABS (Arthrobacter ureafaciens sialidase) releases α(2-3/6/8)-linked terminal NeuAc and NeuGc; NAN1 (Streptococcus

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pneumoniae sialidase) releases α(2,3)-linked terminal NeuAc and NeuGc; BTG (bovine testes β-galactosidase) releases terminal galactose with β(1-3/4) linkages; SPG (S. pneumoniae β-galactosidase) releases terminal galactose in β(1,4) linkages; AMF (almond meal α-fucosidase) releases α(1-3/4) terminal fucose residues (non-reducing end) except α(1,6) core fucose; BKF (bovine kidney α-fucosidase) releases α(1-2/6) terminal fucose more efficiently than α(1-3/4) linked fucose; GUH (β-N-acetylglucosaminidase from S. pneumoniae) releases β(1,4)-linked GlcNAc to mannose but not a bisecting β(1,4)-linked GlcNAc to mannose. B) HILIC profiles of 2-AB labeled glycans released from human serum IgG showing subsequent peak shifts from exoglycosidase digestions. Reprinted by Permission from Macmillan Publishers Ltd: [Nature Chemical Biology] (Marino K; Bones J; Kattla JJ; Rudd PM, A systematic approach to protein glycosylation analysis: a path through the maze. Nat. Chem. Bio. 2010, 6, 713-723.), 2010.¹

1.5.6 Microfluidic Devices

Microfluidic systems manipulate very small amounts of fluids (nanoliter range) using channels that are only hundreds of micrometers at most.²⁹ These small devices offer a wide range of advantages including the use of only small quantities of samples and reagents, high resolution and sensitive separations and detections, low cost and short analysis time.²⁹ The devices are commonly made using photolithography with polymeric compounds to create the channels necessary for separation.²⁹ The use of polymers allows for easier fabrication of the pumps and valves within the devices.²⁹ These devices have been applied to both the field of capillary electrophoresis and HPLC.²⁹

Agilent Technologies offers a commercially available microfluidic device that contains two integrated columns: enrichment column and analytical column.³⁰ The device also contains an integrated ESI emitter tip allowing for direct coupling to a mass spectrometer.³⁰ The commercial chip offers high reproducibility and facile operation allowing many research groups to easily implement the nanoscale technology.³⁰
The field of capillary electrophoresis has also benefited from the use of microfluidic devices. The channels in these devices can be fabricated into various designs, such as spirals, to increase the length of the capillary without greatly increasing the size of the chip. When performing injections on these devices, various voltages are applied across a “T” structure of channels allowing a small amount of sample to be redirected down the capillary for detection and the rest of the sample sent to waste. This type of sample injection is termed pinched injection. The time of voltage difference on the “T” can be manipulated to inject more or less sample for subsequent analysis. Another advantage of CE microfluidic devices is that a greater electric field can be generated in the small separation column allowing for increased resolution and analysis time. Application of these principles allowed for separation of structural isomers of glycans in less than 1.5 minutes with a peak capacity of ~200.

1.6 Conclusions

The field of glycomics is rapidly growing as new instrumentation and methods emerge and evolve. Mass spectrometry has played an important role in allowing characterization of glycans in a rapid manner. As the role of glycosylation in disease is further investigated, analytical methods will be increasingly important in diagnosing diseases and monitoring them. The future of glycan analysis is in glycopeptide or glycoprotein analysis, which will allow both characterization information and site specific information. Both pieces of information are needed to fully understand the impact of glycosylation changes in proteins and disease.
1.7 References

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CHAPTER TWO

MONOSACCHARIDE ANALYSIS OF BIOThERAPEUTIC PRODUCTS

2.1 Introduction

Biopharmaceutical products are a fast growing sector of the pharmaceutical market with sales reported at $99 billion globally in 2009. More than one third of these biological therapeutics, that are either approved or in clinical trials, are glycoproteins. Glycosylation occurs on over 50% of proteins in the human body indicating its importance in stability of proteins, aiding in protein targeting and ligand recognition, regulating the half-life in serum of a protein and helping proteins fold.

One class of glycoproteins that are being utilized as therapeutics is monoclonal antibodies (mAb). Monoclonal antibodies comprise four of the top five selling biotherapeutics with sales around $38 billion in 2009. These molecules are extremely specific for their target antigen which is why they are so prominent in the biopharmaceutical sector especially in the field of treating cancer.

There are five main antibody classes in humans: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD) and immunoglobulin E (IgE) that share similar structures with immunoglobulin (Ig) domains. Differences between the classes arise in the location and abundance of N-linked glycosylation sites and the characteristics of the linkers that connect the domains. In the human body, IgG is predominant in human serum, circulating at 10-15 mg/mL, with a catabolic half-life of approximately 21 days. The majority of licensed intact mAbs being used as therapeutics are based on IgG with a large proportion based on specifically IgG1, a subclass of IgG. The differences between the four IgG subclasses arise from differing γ-chain sequences and disulfide bridging patterns which result in varying effector functions and concentrations in serum. IgG1 accounts for sixty percent of IgG present in normal human serum.
Each IgG molecule is composed of two heavy and two light polypeptide chains that come together through both covalent (disulfide bonds) and non-covalent interactions to form a “Y” shaped structure (Figure 2.1).³

*Figure 2.1: A) The α-carbon backbone of the IgG molecule with the light chains designated in blue and the heavy chains designated in red. B) A pictorial of the structure of IgG with light chains in blue and heavy chains in red. In addition, the disulfide bridges are shown that attach the chains together. V refers to the variable region and C refers to the constant region for each chain, heavy (H) and light (L). Figure A is reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery]⁷, 2009. Figure B is adapted from Lehninger Principles of Biochemistry.⁸*

The domains of the IgG chains form through covalent and non-covalent interactions to form three independent protein sites that are connected through a flexible hinge region.⁷ Two of the protein sites are called antigen-binding fragment (Fab) regions and form the top part of the IgG molecule.⁷ The last protein site is called the fragment crystallizable (Fc) region which forms
the stalk of the “Y”. The Fc region is very important in binding to ligands present on cells of the innate immune system that subsequently activate clearance and transport.7

Each Fc and Fab region contain both constant (C) and variable (V) regions.9 Constant regions allow immunoglobulins to be categorized into various groups since all members of the group will express the same constant regions.9 Variable regions are specific to individual antibodies and dictate which antigen or antigens the antibody may bind.9 Within the IgG class there are a multitude of variable region sequences determining antigen specificity but only one constant region sequence.

On the Fc region in IgG, there is a highly conserved N-glycosylation site at asparagine (Asn) 297 on each of the C_{H2} domains.9 The glycan chains present at Asn 297 on both C_{H2} domains can either be the same (symmetric) or different (asymmetric).5 The glycan chain at this site is a complex biantennary glycan that has a heptasaccharide core termed G0 that can be further modified by the addition of various monosaccharides (Table 2.1).3

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<th>Glycan Name</th>
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<td>Go</td>
<td>![Go Glycan]</td>
</tr>
<tr>
<td>G1</td>
<td>![G1 Glycan]</td>
</tr>
<tr>
<td>G2</td>
<td>![G2 Glycan]</td>
</tr>
</tbody>
</table>

Table 2.1: The three most common glycan core structures found on IgG. These structures can be further modified by the addition of fucose, sialic acid and bisecting GlcNAc monosaccharides. The symbols that are used are as follows: blue square (GlcNAc), green circle (mannose) and yellow circle (galactose).
There are a total of 32 possible glycoforms, in human serum IgG, that are based on either the structures of G0, G1 or G2 with further addition of either fucose, sialic acids or bisecting GlcNAc residues. In humans, most of the glycan chains on IgG’s are fucosylated at the core with equal distributions of either zero, one or two galactose monosaccharides. There is a low abundance of bisecting GlcNAc monosaccharides seen in addition to a low occurrence of sialylation.

The N-glycan chains are extremely important for stability of the IgG molecule because of many hydrophobic and polar non-covalent interactions with the C\textsubscript{H}2 domain. In addition, N-glycans are also important for the effector functions of the IgG’s. When truncation experiments were performed on the glycan chain, it was shown that structural integrity and functionality were greatly compromised and even lost in some cases. There is also glycosylation present in the Fab domain of some IgG molecules. Approximately 30% of polyclonal IgG in humans contain N-linked glycans on either the variable light or heavy regions. This glycosylation has various influences on antigen binding including positive, negative or neutral effects.

IgG molecules bind to membrane bound Fc\gamma receptors and the C\textsubscript{1q} component of complement. Two of the main effector functions for IgG molecules that bind to Fc\gamma receptors are antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the Fc portion of the IgG binds to a Fc\gamma receptor on a monocyte, macrophage or natural killer cell while the Fab region is bound to a target cell. The binding of the Fc region causes the activation of the innate immune system cells to kill the target cell. In the CDC pathway, the IgG is bound to a target cell through the Fab region, and the Fc region activates the C1 component of complement. This activation further causes a cascade of reactions which result in disruption of the cell membrane of the target cell. Both the ADCC and CDC pathway are dependent on the glycosylation of the IgG. The ADCC pathway is utilized in antibody therapeutics to target and kill cancer cells.
When IgG molecules contain only G0 glycan chains, the GlcNAc residue is exposed which can be bound by the mannose receptor targeting the antibody to be removed from circulation. If the glycan chains are sialylated, this can inhibit binding to mannose receptors and increase the half-life of the antibody in circulation allowing for longer action. However, this modification can also result in lower ADCC activity due to reduced antibody receptor interaction but improved CDC binding. It was also shown that removal of core fucose on glycans attached to the IgG molecule, increases the ADCC activity by up to 100 fold. This increase arises because when fucose is present on the core of the glycan chain, it hinders binding to FcγRIIIA which elicits the ADCC pathway. In a contrary report, non-fucosylated rituximab showed a stronger ADCC response. These contradictory findings could indicate a balance between binding various Fcγ receptors and the involvement of fucose residues in binding to elicit the ADCC response. Increased ADCC was also seen for glycans containing bisecting GlcNAc. This observation can be explained by the ability of a bisecting GlcNAc to inhibit the fucosyltransferase that adds α(1,6)-fucose to the core. It has also been shown that agalactosylated IgG glycan chains can trigger the CDC pathway but in some cases can also reduce the activity. These contradictory reports further indicate that there is most likely a balance between various receptors and their requirements for binding.

The various modifications to glycan chains are important to consider when producing biotherapeutic products. Most biotherapeutic products are produced using mammalian cell lines such as Chinese Hamster Ovary (CHO) and mouse myeloma cells (NSO, SP2/0). Escherichia coli systems cannot be used to produced glycoprotein products because they will not add any glycan moieties. The mammalian cell lines produce, in general, glycan chains that are highly fucosylated (>90%) and hypogalactosylated. In addition, both CHO and murine cell lines can potentially add sugars that are immunogenic in humans such as N-glycolylneuraminic acid (NeuGc) and galactose-α(1,3)-galactose. These variations in biotherapeutic products will produce some IgG’s that will have an improved response in the patient due to the ADCC and
CDC pathways; however, the IgG’s could also produce an immune response that could further harm an already immunocompromised patient. It has also been theorized that an increased efficacy could ultimately result in unwanted or increased side effects.7

When using mammalian cell lines, the processing must be tightly regulated. The glycan produced can be influenced by the conditions used during growth such as the culture media composition, culture format and dissolved oxygen content.4 In addition, the glycan can also be influenced by downstream processing.4 All of these factors must be considered when producing IgG biotherapeutics.

The future of biotherapeutic production might include post-translational engineering such as glycan engineering.1 Glycan engineering can be used to optimize the ADCC and CDC responses from a monoclonal antibody.1 It has already been reported that engineered antibodies contain a glycocomponent that is more homogeneous than what is found on antibodies from CHO cells.4 This homogeneity was shown to have a higher ADCC capacity.4 Glycoengineering will also eliminate the addition of immunogenic sugars added to the monoclonal antibody therapeutics.

Since there are a variety of glycan modifications that can alter the function or activity of the biotherapeutic, it is extremely important to monitor the glycosylation throughout manufacturing. One method that can be easily applied to the analysis of glycans on biotherapeutics is monosaccharide analysis using capillary zone electrophoresis (CZE) with laser induced fluorescence (LIF).

Capillary zone electrophoresis employs the use of a bare fused silica capillary coated with polyimide.11 Since polyimide does not transmit UV light or lasers, the coating must be removed in the area that detection will occur in order to be able to detect separated compounds.11 This removal produces a very fragile area in the capillary.

The interior of the capillary is composed of fused silica groups (siloxanes) that are further washed with sodium hydroxide (NaOH). NaOH hydrolyzes the fused silica to Si-O-
groups (deprotonated silanols) which are utilized in the method. During analysis, water in the buffer will electrostatically pair with the negative silanol groups causing a layer of immobile positive charge. The positive charges are then further hydrated by the water present in the buffer.\textsuperscript{11} When an electric field is applied, with the cathode (negatively charged electrode) at the outlet and the anode (positively charged electrode) at the inlet, the positive charges will move toward to the cathode. This causes a bulk flow of the buffer towards the electrode, termed electroosmotic flow (EOF) (Figure 2.2).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{capillary_electrophoresis.png}
\caption{The surface of the bare fused silica capillary becomes negatively charged after treatment with NaOH resulting in a layer of negative charges that are neutralized by the addition of a layer of positive charges from the buffer. When an electric field is introduced, the EOF moves the bulk liquid to the cathode.}
\end{figure}

EOF is a significant factor in capillary electrophoresis because of the high surface to volume ratio.\textsuperscript{11} In monosaccharide analysis, the EOF is maximized to aid in separation of the monosaccharides.

In capillary electrophoresis, plug flow is seen which is a result of the decrease in lateral diffusion caused by the narrow capillary.\textsuperscript{11} Plug flow further increases the resolution of separated compounds. In addition, the narrow capillary reduces temperature differences that are seen between the wall and center allowing for better separations.\textsuperscript{11} During analysis, the
addition of electric current causes an increase in temperature in the capillary which will further cause the current to rise.\textsuperscript{11} This heating process is known as Joule heating and is minimized by flowing a liquid around the capillary that can remove the added heat and maintain a steady current.\textsuperscript{11} It is important to stabilize the capillary temperature to achieve reproducible separations between runs.\textsuperscript{11}

The buffer used in capillary electrophoresis analysis can greatly affect the separation. A variety of buffers can be utilized such as borate based buffers or highly alkaline buffers in the case of glycan analysis.\textsuperscript{12} At a high pH, borate molecules undergo interaction with free hydroxyl groups to form tetrahydroxyborate ions (B(OH)\textsubscript{4}^-).\textsuperscript{12} The tetrahydroxyborate ions can then further reversibly complex with the hydroxyl groups on the monosaccharides.\textsuperscript{12} The degree of complexation is greatly affected by the structure of the sugar including any present charged groups, the location of the hydroxyls in relation to each other (cis versus trans), open versus closed ring and anomericity.\textsuperscript{12} These varying levels of complexation allow for different electrophoretic mobilities on varying monosaccharides, which can increase resolution.\textsuperscript{12} Complex formation generally increases as the concentration of the borate is increased and the pH is also increased.\textsuperscript{12} An optimal pH for analysis of monosaccharides is between 10 and 11.\textsuperscript{12}

Since monosaccharides are not innately UV or fluorescence sensitive, they must first be derivatized.\textsuperscript{13} Derivatization is performed through reductive amination with a fluorophore through first the production of a Schiff base and then further reduction with sodium cyanoborohydride.\textsuperscript{14}
Figure 2.3: A reaction scheme depicting derivatization with primary amine dyes. GlcNAc is derivatized using 8-aminopyrene-1,3,6-trisulfonic acid (APTS) through the formation of a Schiff base which is further reduced.

There are a large variety of moieties that can be used for derivatization; however, sulfonated aromatic amines will introduce permanent anionic charges which can increase the migration speed of the derivatized glycans.\textsuperscript{14} 8-aminopyrene-1,3,6-trisulfonic acid (APTS) is an example of a sulfonated aromatic amine that will introduce three negative charges onto the monosaccharide at a basic pH as seen in Figure 2.3.\textsuperscript{14}

Since derivatized monosaccharides now have negative charges from the derivatization and complexation with borate, they will not be attracted to the cathode. Instead, the monosaccharides will migrate against the established EOF towards the positively charged anode. Since the EOF is maximized, the bulk flow will be strong enough to move the monosaccharides toward the cathode despite their repulsive charges. This process results in monosaccharide separation and enhances the resolution seen in the electropherogram (effectively similar to a longer column). Such high resolution is required to separate the
monosaccharides, which have the same basic structure but vary in the location of the hydroxyl groups.

Capillary electrophoresis is a technique that can be easily applied to the analysis of monosaccharides released from biotherapeutic products. Although monosaccharide analysis will not provide direct evidence of the structure of the glycan chain, inferences can be made based on the percentages of monosaccharides present. Of great interest as well is the comparison of fucose to galactose, which are both known for their importance in monoclonal antibody effector functions.

2.2 Experimental Procedures

2.2.1 Materials

Standard monosaccharides, 8-aminopyrene-1,3,6-trisulfonic acid, boric acid, lithium hydroxide, trifluoroacetic acid, citric acid and sodium cyanoborohydride in 1 M tetrahydrofuran were all obtained from Sigma Aldrich (St. Louis, MO). 1 M sodium hydroxide solution was obtained from Agilent Technologies (Santa Clara, CA). High purity HPLC grade water was from J.T. Baker (Radnor, PA). The GlykoPrep digestion module was obtained from Prozyme (Hayward, CA). Bare fused silica capillaries were obtained from PolyMicro Technologies (Phoenix, AZ). PhyNexus normal phase pipette tips (200 μL volume with 20 μL bed volume) were purchased from PhyNexus (San Jose, CA).

2.2.2 Biotherapeutic Sample Preparation

Nine monoclonal antibody biotherapeutics (1 mL) were received from a biopharmaceutical company for monosaccharide analysis (Table 2.2).
Upon receipt of the samples, they were stored at -80°C until analyzed. The antibodies were deglycosylated (50 μg) in duplicate using a commercial kit from Prozyme (GlykoPrep™ Digestion Module) and the established company protocol. The kit allows for increased reaction times. A Beckman Coulter ProteomeLab SP centrifuge system with plate adapter was used for the centrifugation steps required in the deglycosylation protocol. Released glycans were completely dried using vacuum centrifugation.

Hydrolysis of the released N-glycans to corresponding monosaccharide pools was adapted from Reference 13. Briefly, 200 μL of 2 M trifluoroacetic acid (TFA) was added to the dried N-glycans released from the biotherapeutic samples, sealed, vortexed and centrifuged to ensure that the glycans were mixed into solution. Samples were then heated to 100° C for five hours. Halfway through the incubation, the samples were removed and quickly vortexed and centrifuged so that any condensate that had formed was brought back into the main solution.

After incubation was complete, the samples were removed and allowed to cool to room temperature. Once cooled, samples were dried completely by vacuum centrifugation. Once dry, 2.5 μL of 50 mM APTS in 1.2 M citric acid and 2.5 μL of sodium cyanoborohydride in 1 M tetrahydrofuran (THF) were added followed by incubation in a thermomixer at 55° C for one hour. 

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>24.6</td>
</tr>
<tr>
<td>Sample 2</td>
<td>24.8</td>
</tr>
<tr>
<td>Sample 3</td>
<td>25.1</td>
</tr>
<tr>
<td>Sample 4</td>
<td>21.9</td>
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<tr>
<td>Sample 5</td>
<td>24.6</td>
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<tr>
<td>Sample 6</td>
<td>24.5</td>
</tr>
<tr>
<td>Sample 7</td>
<td>23.6</td>
</tr>
<tr>
<td>Sample 8</td>
<td>24.3</td>
</tr>
<tr>
<td>Sample 9</td>
<td>24.5</td>
</tr>
</tbody>
</table>

Table 2.2: Received monoclonal antibody samples with their corresponding concentrations.
After incubation, the reaction was quenched by the addition of 50 μL of HPLC grade water. Samples were cleaned using PhyNexus normal phase pipette tips to remove excess APTS dye. Tips were first conditioned with 500 μL of 95% acetonitrile (ACN). Next, 475 μL of 100% ACN was added to 25 μL of labeled sample. The sample was then enriched on the tips by aspirating 15 times. After enrichment, the tips were washed four times with 500 μL of 95% ACN for the removal of unconjugated APTS. Labeled monosaccharides were then eluted using 50 μL of HPLC grade water into a clean Eppendorf tube. Cleaned samples were stored at -20°C until analyzed.

2.2.3 Standards Preparation

An internal standard, maltopentaose, was added to each sample to correct for run to run migration time variability. A solution of maltopentaose at a concentration of 6050 fmol/μL was prepared and labeled following the same procedure as previously described. The entire internal standard was cleaned using the PhyNexus normal phase pipette tips as previously described with the minor modification of an increased sample volume being cleaned. After cleaning, 1 μL was removed from this stock solution and further diluted with HPLC grade water to a total of 50 μL resulting in a concentration of 121 fmol/μL. 1 μL of this diluted internal standard was added to each of the samples per 50 μL final volume. The resulting concentration of the internal standard in each of the samples and standard monosaccharide panel was 2.42 fmol/μL.

In order to ensure that proper migration times were assigned to the biotherapeutic glycans, a panel of standards was also prepared. The standards that were included in this study were N-acetylglucosamine (GlcNAc), glucosamine (Gln), mannose (Man), fucose (Fuc), galactose (Gal) and glucose (Glc). Although glucose should not be present in the samples, it was included in case the biotherapeutic products did show glucose contamination. Glucose contamination could arise from incomplete biosynthesis of the N-glycans into mature (complex) glycan chains. Glucose contamination can also be attributed to contamination by dust from a
variety of sources such as cellulose wipes. Each of the monosaccharide standards was prepared at a concentration of 20 nmol/μL in HPLC grade water. 500 nmol was then removed from each stock and placed into separately labeled Eppendorf tubes. The solutions were vacuum centrifuged to dryness and then labeled with 2.5 μL of 50 mM APTS in 1.2 M citric acid and 2.5 μL of sodium cyanoborohydride in 1 M THF for one hour at 55 °C as previously described. After incubation, the samples were quenched with 50 μL of HPLC grade water and then cleaned with PhyNexus normal phase pipette tips as previously described, however one modification to the established protocol was that the entire labeled sample was cleaned. A standard monosaccharide panel was created by mixing 200 pmol of each individual monosaccharide to a final concentration of 2 pmol/μL and internal standard concentration of 2.42 fmol/μL. Individual monosaccharide solutions were run at a concentration of 200 pmol/μL with an internal standard concentration of 2.42 fmol/μL. The prepared solutions were stored at -20°C until analyzed.

2.2.4 Instrument Parameters

Instrumental analysis was performed using a Beckman Coulter PA 800 plus capillary electrophoresis (CE) system equipped with a laser induced fluorescence detector (Beckman Coulter, Brea, CA). The samples were run using capillary zone electrophoresis and a 50 μm i.d. (internal diameter), 360 μm o.d. (outer diameter) bare fused silica capillary. The effective length of the capillary (from inlet to window) was 50 cm with an overall length of 60 cm. Windows were manually created in the capillary by removing the protective polyimide coating using a precision window maker (MicroSolv) followed by cleaning with 100% isopropanol (IPA) to remove residual carbonaceous material. The CE was operated in normal polarity mode with the anode at the injection side and the cathode at the detection side. The background electrolyte used for this analysis was 120 mM lithium borate buffer at pH 10. The buffer was prepared by
first making a 120 mM boric acid solution that was adjusted to pH 10 by the addition of solid lithium hydroxide.

The applied electric field was 500 V/cm with a maximum current of 300 μA. The capillary was kept at a constant temperature of 25°C and the samples were kept at 10°C during analysis. Laser induced fluorescence detection was employed; excitation using a 488 nm argon laser with emission detection above 520 nm using a bandpass filter.

2.2.5 Analysis

Prior to sample analysis, the laser was recalibrated to ensure maximum response using the autocalibrate function of the instrument and LIF calibration mix (Beckman Coulter; Brea, CA).

The capillary was conditioned with 1 M sodium hydroxide (NaOH) for one hour using a pressure rinse at 60 psi followed by rinsing with 120 mM lithium borate buffer for 15 minutes at 60 psi. The 1 M NaOH rinse is very important because it deprotonates the silanol groups which in turn effectively maximizes the EOF in the capillary. This rinse ensures that the EOF is effectively the same during each of the runs. The EOF must be stable in order to accurately compare the migration times of various runs.

After the capillary was effectively conditioned, the samples were run in the following manner. The capillary was rinsed for 5 minutes at 60 psi with 1 M NaOH to ensure reproducible migration times. Following the reconditioning, the capillary was equilibrated by rinsing for 3 minutes at 60 psi with 120 mM lithium borate. The sample was then injected hydrodynamically using an applied pressure of 1 psi for 10 seconds. Separation voltage was then applied to the capillary for 25 minutes to separate the monosaccharides. Following each transition to new buffer vials, a water dip was included in the method to minimize any potential carryover thereby maximizing precision. New buffer vials were used after six injections were performed to ensure
reproducibility. In the case of the sample runs, new buffers were used after a complete run of one sample (duplicate samples with triplicate runs per duplicate).

The standards were run in a standard mix at a concentration of 2 pmol/μL per monosaccharide and a concentration of 2.42 fmol/μL of the internal standard. To ensure proper peak assignment, various standards were spiked into the mix and the intensified peak was recorded. This procedure was repeated until all of the standard peaks could be accurately assigned. In addition, prior to running the standard mix, the standard monosaccharides were run individually to assign approximate migration times and pattern of migration between the monosaccharides. The spiking experiment was a further confirmation of correct peak assignments.

Each of the duplicate samples was run in triplicate to provide 6 datasets for each sample to ensure enough data for statistical analysis. A variety of dilutions were performed on the samples ranging from 2 times to 10 times dilution with HPLC grade water to ensure a proper response from the detector (Table 2.3).

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Volume of Sample (μL)</th>
<th>Volume of Internal Standard (μL)</th>
<th>Concentration of Internal Standard (fmol/μL)</th>
<th>Volume of HPLC Quality Water (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1_1+2</td>
<td>10</td>
<td>1</td>
<td>2.42</td>
<td>39</td>
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<tr>
<td>Sample 2_1</td>
<td>49</td>
<td>1</td>
<td>2.42</td>
<td>0</td>
</tr>
<tr>
<td>Sample 2_2</td>
<td>5</td>
<td>1</td>
<td>2.42</td>
<td>44</td>
</tr>
<tr>
<td>Sample 3_1+2</td>
<td>10</td>
<td>1</td>
<td>2.42</td>
<td>39</td>
</tr>
<tr>
<td>Sample 4_1+2</td>
<td>10</td>
<td>1</td>
<td>2.42</td>
<td>39</td>
</tr>
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<td>Sample 5_1+2</td>
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<td>2.42</td>
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<td>Sample 6_1+2</td>
<td>10</td>
<td>1</td>
<td>2.42</td>
<td>39</td>
</tr>
<tr>
<td>Sample 7_1+2</td>
<td>49</td>
<td>1</td>
<td>2.42</td>
<td>0</td>
</tr>
<tr>
<td>Sample 8_1+2</td>
<td>5</td>
<td>1</td>
<td>2.42</td>
<td>44</td>
</tr>
<tr>
<td>Sample 9_1+2</td>
<td>10</td>
<td>1</td>
<td>2.42</td>
<td>39</td>
</tr>
</tbody>
</table>

*Table 2.3: Samples and their corresponding dilution for CE analysis.*

### 2.3 Results

All of the samples were diluted after cleaning to ensure a baseline that did not contain excessive noise. During preliminary sample analyses, reagent blanks were run and it was
discovered that due to the sensitivity of the laser, significant numbers of background peaks could be seen. This observation was further observed when looking at the preliminary sample runs. The resulting noise was minimized when the samples were cleaned with the PhyNexus normal phase pipette tips (Figure 2.4).

![Figure 2.4: The comparison of clean and unclean first replicate of Sample 1. A) The electropherogram obtained after cleaning the sample with PhyNexus normal phase pipette tips and diluting the sample 100 times with HPLC grade water. B) The electropherogram of the sample without any cleaning but 100 times dilution with HPLC grade water.](image)

The background noise seen in the baseline is drastically reduced after cleaning. The minimization of noise allows the peaks to be more clearly defined and differentiated.

To correctly assign the migration of the individual monosaccharides, the monosaccharides were run individually and then the resulting electropherograms were compared (Figure 2.5).
Figure 2.5: A comparison of the electropherograms relating to each individual monosaccharide in order to assign approximate migrations. The internal standard is around 7.5 minutes and the rest of the monosaccharides vary in their migrations from approximately 12 to 23 minutes.

The migration order of the monosaccharides was found to be glucosamine, GlcNAc, mannose, glucose, fucose and galactose, from earlier to later detection.

To verify the migration order in a sample, various monosaccharides were spiked into one of the samples during a series of runs to ensure that the proper peaks were being identified and selected for data analysis (Figure 2.6).
Figure 2.6: A comparison of the first replicate of Sample 7 and various spiked in monosaccharides to verify peak assignments. The internal standard is the unlabeled peak around 7 minutes.

Based on Figure 2.6, the migration time for the monosaccharide peaks in the sample were as follows: mannose ~18.5 minutes, glucose ~18.8 minutes, fucose ~20.5 minutes and galactose ~21 minutes. This migration order is also the same as was verified with the individual monosaccharides and standard panel. It is much more difficult to make definitive assignments for both glucosamine and GlcNAc due to contaminant peaks that are present at the same migration times. To conclusively assign these peaks, another experiment was performed where only glucosamine and GlcNAc were spiked into a sample separately (Figure 2.7).
Figure 2.7: A comparison of Sample 3 with either spiked in glucosamine or GlcNAc to verify the peak assignment. The internal standard peak is also seen around 6 minutes.

It can be seen that GlcNAc is only a small peak next to the glucosamine peak. Glucosamine is due to the deacetylation of GlcNAc during acid hydrolysis. The migration times of the GlcNAc and glucosamine peak also correlate with the standard mix. This experiment helped to confirm the identity of the GlcNAc peak. Although most samples did contain the contaminant peaks, as were mentioned earlier, it was still possible to accurately assign the correct monosaccharide to the peaks migrating between 11 and 12 minutes.

Reproducibility of resulting electropherograms was also high as shown in Figure 2.8.
Monosaccharide peaks present in the electropherogram of each replicate were integrated to find their respective peak areas using the software's manual peak integration function. The resulting peak areas were then normalized against the internal standard. To find the peak area for N-acetylglucosamine, the sum of the degradation product glucosamine and N-acetylglucosamine was used. The percentage of monosaccharide in each of the replicates was then evaluated by dividing the normalized peak area of the monosaccharide against the total sum of the peak areas for that run. The data was then averaged across the six replicate runs per sample and the standard deviation was evaluated for statistical purposes. The resulting data is shown in the following tables.
<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
<td>GlcNAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>3.79</td>
<td>12.01</td>
</tr>
<tr>
<td>2</td>
<td>3.58</td>
<td>11.50</td>
</tr>
<tr>
<td>3</td>
<td>3.89</td>
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<tr>
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</tr>
<tr>
<td>Average</td>
<td>4.56</td>
<td>12.84</td>
</tr>
<tr>
<td>SD</td>
<td>0.89</td>
<td>1.34</td>
</tr>
</tbody>
</table>

*Table 2.4: Monosaccharide distribution in Sample 1 based on six replicate runs.*

<table>
<thead>
<tr>
<th>Sample 2</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
<td>GlcNAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>2.57</td>
<td>3.99</td>
</tr>
<tr>
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<tr>
<td>Average</td>
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<tr>
<td>SD</td>
<td>1.23</td>
<td>4.52</td>
</tr>
</tbody>
</table>

*Table 2.5: Monosaccharide distribution in Sample 2 based on six replicate runs.*

<table>
<thead>
<tr>
<th>Sample 3</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
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<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>1.66</td>
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<tr>
<td>SD</td>
<td>0.33</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Table 2.6: Monosaccharide distribution in Sample 3 based on six replicate runs.*
### Table 2.7: Monosaccharide distribution in Sample 4 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 4</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GlnAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>run #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.67</td>
<td>8.43</td>
</tr>
<tr>
<td>2</td>
<td>6.38</td>
<td>8.16</td>
</tr>
<tr>
<td>3</td>
<td>6.23</td>
<td>8.09</td>
</tr>
<tr>
<td>4</td>
<td>5.84</td>
<td>12.33</td>
</tr>
<tr>
<td>5</td>
<td>5.90</td>
<td>12.79</td>
</tr>
<tr>
<td>6</td>
<td>5.56</td>
<td>12.31</td>
</tr>
<tr>
<td>Average</td>
<td>6.10</td>
<td>10.35</td>
</tr>
<tr>
<td>SD</td>
<td>0.41</td>
<td>2.34</td>
</tr>
</tbody>
</table>

### Table 2.8: Monosaccharide distribution in Sample 5 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 5</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GlnAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>run #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.02</td>
<td>9.42</td>
</tr>
<tr>
<td>2</td>
<td>3.61</td>
<td>9.20</td>
</tr>
<tr>
<td>3</td>
<td>3.51</td>
<td>9.06</td>
</tr>
<tr>
<td>4</td>
<td>4.74</td>
<td>11.49</td>
</tr>
<tr>
<td>5</td>
<td>4.56</td>
<td>11.26</td>
</tr>
<tr>
<td>6</td>
<td>3.92</td>
<td>11.30</td>
</tr>
<tr>
<td>Average</td>
<td>3.89</td>
<td>10.29</td>
</tr>
<tr>
<td>SD</td>
<td>0.66</td>
<td>1.17</td>
</tr>
</tbody>
</table>

### Table 2.9: Monosaccharide distribution in Sample 6 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 6</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GlnAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>run #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.45</td>
<td>6.43</td>
</tr>
<tr>
<td>2</td>
<td>3.34</td>
<td>6.21</td>
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<tr>
<td>3</td>
<td>3.16</td>
<td>6.02</td>
</tr>
<tr>
<td>4</td>
<td>3.40</td>
<td>8.73</td>
</tr>
<tr>
<td>5</td>
<td>3.16</td>
<td>8.62</td>
</tr>
<tr>
<td>6</td>
<td>2.71</td>
<td>8.57</td>
</tr>
<tr>
<td>Average</td>
<td>3.04</td>
<td>7.43</td>
</tr>
<tr>
<td>SD</td>
<td>0.38</td>
<td>1.33</td>
</tr>
</tbody>
</table>
### Table 2.10: Monosaccharide distribution in Sample 7 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 7</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
<td>GlcNAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>4.20</td>
<td>11.53</td>
</tr>
<tr>
<td>2</td>
<td>5.22</td>
<td>11.10</td>
</tr>
<tr>
<td>3</td>
<td>5.12</td>
<td>11.32</td>
</tr>
<tr>
<td>4</td>
<td>3.24</td>
<td>14.95</td>
</tr>
<tr>
<td>5</td>
<td>2.89</td>
<td>15.25</td>
</tr>
<tr>
<td>Average</td>
<td>4.28</td>
<td>13.16</td>
</tr>
<tr>
<td>SD</td>
<td>1.01</td>
<td>2.03</td>
</tr>
</tbody>
</table>

### Table 2.11: Monosaccharide distribution in Sample 8 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 8</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
<td>GlcNAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>6.70</td>
<td>15.98</td>
</tr>
<tr>
<td>2</td>
<td>5.94</td>
<td>15.56</td>
</tr>
<tr>
<td>3</td>
<td>6.18</td>
<td>15.69</td>
</tr>
<tr>
<td>4</td>
<td>4.36</td>
<td>13.43</td>
</tr>
<tr>
<td>5</td>
<td>4.53</td>
<td>13.51</td>
</tr>
<tr>
<td>Average</td>
<td>5.35</td>
<td>14.59</td>
</tr>
<tr>
<td>SD</td>
<td>1.04</td>
<td>1.27</td>
</tr>
</tbody>
</table>

### Table 2.12: Monosaccharide distribution in Sample 9 based on six replicate runs.

<table>
<thead>
<tr>
<th>Sample 9</th>
<th>Internal Standard Normalized Peak Areas</th>
<th>% Monosaccharide Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>run #</td>
<td>GlcNAc</td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>4.70</td>
<td>15.76</td>
</tr>
<tr>
<td>3</td>
<td>4.82</td>
<td>15.54</td>
</tr>
<tr>
<td>4</td>
<td>3.76</td>
<td>9.77</td>
</tr>
<tr>
<td>5</td>
<td>3.49</td>
<td>9.71</td>
</tr>
<tr>
<td>6</td>
<td>3.17</td>
<td>9.54</td>
</tr>
<tr>
<td>Average</td>
<td>4.08</td>
<td>12.61</td>
</tr>
<tr>
<td>SD</td>
<td>0.69</td>
<td>3.22</td>
</tr>
</tbody>
</table>

Table 2.10: Monosaccharide distribution in Sample 7 based on six replicate runs.

Table 2.11: Monosaccharide distribution in Sample 8 based on six replicate runs.

Table 2.12: Monosaccharide distribution in Sample 9 based on six replicate runs.
The resulting data is further compared between the samples in Figure 2.9.

![Bar chart showing the average percentage of each respective monosaccharide in each of the nine investigated samples.]

**Figure 2.9:** A plot of the average percentage of each respective monosaccharide in each of the nine investigated samples.

### 2.4 Discussion

Although slight shifts were seen between the internal standards in each of the replicate runs, this variation in the migration behavior of the internal standard is attributed to varying electrolyte concentrations resulting from electrolysis and depletion during electrophoresis. However, the analyte peaks in the samples align according to the individual and mixed standards used for identification. In all of the samples, small peaks correlating to glucose were observed; however, as previously mentioned, this is most likely due to environmental contaminants during sample preparation or from the formulation buffer that the samples were originally prepared in, and not indicative of incomplete biosynthesis. The presence of small contaminant peaks were also observed but are most likely attributed to incomplete hydrolysis of
GlCNAc residues. Since the effector function monosaccharides are of interest, these peaks were not further investigated as they did not interfere with subsequent analyses.

Since the method used is not optimized for acetylated sugars (deacetylation is seen during acid hydrolysis), the resulting percentage of GlCNAc is lower than theoretical, considering that typical glycan chains on monoclonal antibodies contain four GlcNAc units. This percentage change affects the other percentages; however, for the purpose of this study, it was more important to compare the percentages of fucose to galactose since these glycans can have an impact on effector functions.

All of the samples studied showed a greater percentage of fucose than galactose. Sample 3, Sample 4, Sample 5, Sample 6 and Sample 7 all showed a 1.3 to 1.4 times greater percentage of fucose than galactose. Sample 1 and Sample 2 showed a 1.5 to 1.6 times greater percentage of fucose than galactose. Finally, Sample 8 and Sample 9 showed a 1.7 to 1.8 times greater percentage of fucose than galactose. These changes in the level of fucose are important in effector functions. As previously mentioned, it is desirable that biotherapeutics do not contain fucose because a greater ADCC response is seen in the absence of fucosylation. However, all of the samples in this study showed appreciable levels of fucose. This glycan design could decrease any extra side effects seen with non-fucosylated glycans on mAb, in addition to effectively activating other ADCC mechanisms. All of the samples also showed a percentage of galactose present, albeit in lower quantities than the fucose. The lower levels of galactose seen could indicate a stronger response to the CDC pathway. The glycan chains present on the mAb indicate the ability to activate both the ADCC and CDC for effective apoptosis of the target cell such as a cancerous cell.

Based on the percentages of monosaccharides present, the identity of the starting glycan chains can be presumed. In each possible glycan chain there will be four GlcNAc residues and three mannose residues. As can be seen in Figure 2.9, the mannose percentages are high
compared to the GlcNAc percentages since the hydrolysis was not optimized for analysis of GlcNAc residues. Disregarding these percentages, it can be seen that the fucose is higher in all of the samples but in different ratios, as seen in Figure 2.9. Considering that only one fucose is added, it can be theorized that all of the glycan chains did contain fucose. Based on the presence of galactose in all samples, all glycan chains did contain varying levels of galactose. These variations would indicate mixtures of G0, G1 and G2. Due to the low levels of galactose compared to fucose, G2 would probably be a minor structure in the glycans. The ratios of fucose to galactose further show the variations in levels of G0 to G1 in each sample. These inferences should be further validated by intact glycan analysis profiling.

2.5 Conclusions

Capillary zone electrophoresis can be effectively applied to hydrolyzed glycan chains released from biotherapeutic products. The resulting data can indicate which structures predominate and shows a comparison of the monosaccharides important in effector functions. The nine samples analyzed showed glycan chains that were largely fucosylated and mostly contained either none or one galactose residues. These modifications can help to increase the activation of both the ADCC and CDC response pathways in targeting cancerous or other diseased cells. In addition, the methodology can be applied throughout manufacturing to monitor the glycosylation of the product. This work shows the importance of experimental design to control for potential variables that could impact the analytical results.
2.6 References

CHAPTER THREE

CHARGE NEUTRALIZATION OF SIALIC ACIDS ON N-LINKED GLYCANS FOR IMPROVED LC-MS DETECTION

3.1 Introduction

Changes in glycosylation are a hallmark of diseases such as cancer.\textsuperscript{1} It has been widely shown that increases in branching and sialylation and alterations in the levels of core and antennary fucose occur with increasing disease pathology. Therefore, the ability to accurately elucidate the structure of complex glycans and uncover how the structures change with disease has become increasingly important. Although many analysis routes based on analytical separations, mass spectrometry and ancillary techniques such as exoglycosidase digestion have been employed for oligosaccharide analysis, an increasingly popular method for deciphering the structures of glycans involves the use of LC-MS.\textsuperscript{2}

MS analysis can be performed in the positive ion mode with the result of pseudomolecular, [M+H]\textsuperscript{+}, or adduct, [M+Na]\textsuperscript{+}, ions.\textsuperscript{2} Once these ions undergo fragmentation with collision induced dissociation (CID), the result is predominantly B- and Y- type glycosidic cleavages.\textsuperscript{2} The resulting fragment ions provide information on the composition of the glycan but the resulting fragments are unable to identify linkage or positional isomer information.\textsuperscript{2} Some isomer separation and associated identification is possible depending on the LC stationary phase used, such as porous graphitized carbon (PGC).\textsuperscript{2} Monosaccharide rearrangements for positive ionization have also been documented but have been observed much less frequently for negative ionization thus far.\textsuperscript{3} Due to the limitations of positive ionization, our lab has previously developed a negative ionization method that utilizes ammonium fluoride in the chromatographic mobile phase when performing separations on a PGC microfluidic LC chip with quadrupole time-of-flight (Q-TOF) mass spectrometric detection.\textsuperscript{2}
Microfluidic chips are gaining popularity due to their ease of operation and high reproducibility.\textsuperscript{2} A commercialized chip was introduced by Agilent Technologies in the mid 2000’s.\textsuperscript{4} The microfluidic chip is microfabricated using polyimide which is thermally stable in addition to being insoluble in the majority of organic media.\textsuperscript{4} The channels are laser ablated and following assembly, the chip is then slurry packed with the stationary phase chemistry of choice.\textsuperscript{4} One advantage of the chip system is that it integrates two columns into one small chip.\textsuperscript{4} The chip contains first an enrichment column for sample loading and desalting online followed by an analytical column for subsequent separation (Figure 3.1).\textsuperscript{4} The chip also contains an integrated ESI emitter tip for direct ionization into the MS system.\textsuperscript{4} All of the commercial microfluidic chips have a radio frequency (RF) tag that encodes product information that is read by the computer, which allows for accurate tracking of the hours on the chip, LC packed material and number of injections.\textsuperscript{4}

\textbf{Figure 3.1:} A commercial Agilent HPLC chip highlighting the various features: nano LC column, enrichment column, ESI tip emitter and radio frequency tag. Reprinted with permission from John Wiley and Sons [Journal of Separation Science], 2007.\textsuperscript{4}
The chip runs at nanoliter flow rates, allowing for much greater sensitivity due to the formation of smaller droplets resulting in more efficient ionization. The microfluidic chip format has been readily applied by Lebrilla’s group to analyze N-glycans in both human serum and cancers, predominantly using positive ion mode. Using the technology, almost 200 glycan structures were identified from human serum based upon an informatics combinatorial approach to annotate all possible structures to a particular m/z value detected.

PGC has gained popularity as a stationary phase due to its unique retention capabilities. Despite the hydrophobicity of the stationary phase, PGC is able to effectively retain very polar compounds due to both hydrophobic, polar interactions and inducible dipole-dipole interactions with glycans. Since PGC is a hydrophobic material, traditional reversed phase solvents may be used for elution. One of the main advantages of using PGC as a stationary phase is its ability to separate both positional and linkage isomers in addition to anomers. The presence of anomers can be eliminated by reduction of the glycan to its corresponding alditol. However, our lab has found that the MS and MS/MS were more sensitive when the glycan was not reduced. PGC is also able to separate both neutral and sialylated glycans in one run allowing for a simpler methodology.

Negative ion polarity was selected for the developed method because CID of oligosaccharide ions produces C-type glycosidic cleavages and A-type cross ring cleavages (Figure 3.2).
Cross-ring cleavages can provide information on the linkage positions between the monosaccharides in the glycan chain. C-type glycosidic cleavages can give information on the composition of the glycan. In addition, negative ion fragmentation also can lead to D- and E-type ions which can give information on the composition of the arms of the glycan. The D-ion is formed from a C- and Z-cleavage which will show the composition of the 6-antenna (Figure 3.3). This ion is also accompanied by an ion relating to the D-ion with a loss of water. The E-ion is caused by two C-type cleavages resulting in information on the 3-antenna of the glycan.
In order to produce negatively charged ions for subsequent analysis, in work from our lab, fluoride was used in the mobile phase. Fluoride is in the anionic form at a pH of 7.0 which was important due to the upper pH limit of 8.0 on the microfluidic chip. Fluoride forms a hydrogen bond with hydroxyl groups on the monosaccharide; however, due to the high gas phase basicity of the fluoride anion, it rapidly extracts a proton from the monosaccharide ring resulting in the generation of the pseudomolecular ion \([M-H]^-\), accompanied by the neutral loss of HF. The fluoride can also form stable, negatively charged adducts with the glycan, \([M+F^-]\); however, these adducts are usually present in very low levels due to the high gas phase basicity of fluoride. The fluoride also aids in complete recovery of sialylated glycans from the PGC stationary phase, presumably due to its ability to interrupt and displace glycans retained through electrostatic or dipole-dipole interactions. The developed method has previously been applied to N-glycans released from murine and human IgG. It is also important to further develop the methodology for sialylated N-glycans.

Sialic acids are a family of nine carbon monosaccharides that contain an acidic carboxylic acid group in addition to the possibility of various modifications at other hydroxyl groups (Figure 3.4).
Sialic acids are involved in cell-surface interactions, protecting cellular proteins from proteolysis, aiding cell adhesion and influencing the half-lives of circulating glycoproteins. Sialic acids can be linked to the underlying glycan chain through either an α(2,3) or α(2,6) linkage. Recent research has uncovered a role for sialylated N-glycans in diseases such as cancer. It has been found that there is an upregulation of the enzyme responsible for adding sialic acid in an α(2,6) linkage. This finding was correlated with cancer progression, metastatic spread and poor prognosis. An overrepresentation of sialic acids on the surface of glycoproteins has also been seen in cancer cells. All of these factors necessitate the need to be able to accurately compare structural changes seen in diseased and normal sialylated N-glycans.
The glycosidic bond between the sialic acid and the underlying glycan chain is labile due to the presence of the carboxylic acid proton. During MS analysis, the sialic acid is preferentially lost resulting in intense B1 type ions that dominate the MS² spectrum, reducing the abundance of diagnostic C-type ions.

Our lab has previously applied the fluoride-mediated negative ion nanoLC-MS with microfluidic chip method to highly sialylated glycans released from a standard plasma protein, alpha-1 acid glycoprotein. Alpha-1 acid glycoprotein is a plasma protein that has five N-linked glycosylation sites that contain highly sialylated complex N-glycans. The N-glycans contribute to 45% of the protein’s molecular weight. It has been found that alpha-1 acid glycoprotein concentration increases during acute phase syndromes and diseases such as cancer and rheumatoid arthritis. Changes in the glycan structure have also been shown to occur in the degree of branching, sialylation and fucosylation.

N-glycans with one or two sialic acids were accurately identified based on fragmentation spectra as presented in Dr. Wenqin Ni’s dissertation (Table 3.1). However, when glycans with three or more sialic acids were analyzed, the major fragment ions that were observed were from the loss of sialic acid residues caused by the lability of the glycosidic bond in addition to negative charge repulsion mechanisms. The aim of the research presented here was to evaluate various charge neutralization strategies such as methyl esterification and methyl amidation of the sialic acids in order to reduce the overall charge of the oligosaccharide anions formed, thereby facilitating fluoride-mediated ionization and the generation of informative negative ion CID MS/MS fragmentation spectra for deep oligosaccharide structural analysis. The resulting data showed that methyl amidation with (7-azabenzotriazol-1 yloxy)trispyrriolidinophosphonium hexafluorophosphate (PyAOP) proved to be a promising approach for the charge neutralization of sialic acids and subsequent application of our fluoride mediated negative ion method.
Table 3.1: Identified released N-glycans from alpha-1 acid glycoprotein using the fluoride mediated negative ion chip-LC-MS/MS method. Figure reprinted with permission from Dr. Wenqin Ni.\textsuperscript{13}

### 3.2 Experimental Procedures

#### 3.2.1 Materials

Methyl iodide, sodium fluoride, dimethyl sulfoxide, sodium chloride, 3-methyl-1-(p-tolyl)triazene, (7-azabenzotriazol-1-yloxy)trispyrroliadinophosphonium hexafluorophosphate, methylamine hydrochloride, N-methylmorpholine and ammonium fluoride were obtained from Sigma Aldrich (St. Louis, MO). High purity HPLC grade water, acetonitrile and methanol were
purchased from J.T. Baker (Radnor, PA). The glycan standards (A2, A3 and DSLNT) were all obtained from Prozyme (Hayward, CA) (Figure 3.5). The PGC HPLC-Chip II (part number: G4240-64010) was purchased from Agilent Technologies (Waldbronn, Germany). BondElut OMIX, SCX 100 μL pipette tips were from Agilent Technologies (Santa Clara, CA). Thermo Scientific Pierce C18 tips were obtained from Thermo Fisher Scientific Inc. (Pittsburgh, PA). PhyNexus Normal Phase pipette tips (1000 μL volume with a 10 μL bed volume) were purchased from PhyNexus (San Jose, CA).

Figure 3.5: Glycan structures of the three standard glycans: A3 (trisialylated, galactosylated, triantennary glycan), A2 (disialylated, galactosylated, biantennary glycan) and DSLNT (disialyllacto-N-tetraose). Missing linkage information denotes possibility of linkage isomer species. A3 may have the Gal linked with β(1-3/4) to the GlcNAc and NeuAc linked α(2-3/6) to the Gal. A2 may have the NeuAc linked α(2-3/6) to the Gal.
3.2.2 Methyl Esterification Using Methyl Iodide and Sodiated Glycans

The methyl esterification procedure performed using methyl iodide (MeI) is based on Reference 18 with minor modification for the sodiation process.18 A3 (trisialylated, gactosylated, triantennary glycan) was reconstituted to a concentration of 100 μM. 1.6 nmols of the stock solution were brought up to 100 μL volume with water. Strong cation exchange (SCX) tips were first conditioned with 1 M sodium fluoride (NaF) four times to ensure proper coating of the resin with sodium. The glycan solution was then slowly aspirated through the preconditioned cation exchange tips ten times to promote sodiation of the glycans. After aspiration, the glycan was dried completely using vacuum centrifugation. Once dry, the glycan was dissolved in 10 μL of dimethyl sulfoxide (DMSO). Then 10 μL of methyl iodide was added and the sample was vortexed and centrifuged. The reaction was allowed to stand at room temperature with gentle shaking for two hours. After incubation, the methyl iodide was removed with a stream of nitrogen. 500 μL of methanol was added to the solution before the sample underwent further vacuum centrifugation to remove the DMSO. The sample was then reconstituted with 8 μL of water to a final concentration of 200 μM (200 pmol/μL) before mass spectrometric analysis.

3.2.3 Methyl Esterification Using Methyl Iodide and a Salt Solution

The methyl esterification procedure using methyl iodide is based on Reference 18 with minor modification for the sodiation process.18 A2 (disialylated, galactosylated biantennary glycan) was reconstituted to a final concentration of 100 μM. 1.6 nmols from the A2 stock were dried completely using vacuum centrifugation. To the dried glycan, 5 μL of a 1 mM sodium chloride solution was added (5 times excess molar equivalence to glycan). Then 10 μL of DMSO and 10 μL of methyl iodide were added and the sample was vortexed and centrifuged. The solution was incubated at room temperature with shaking for four hours. After incubation, the methyl iodide was removed by a stream of nitrogen. 500 μL of methanol was added and the
solution was dried completely using vacuum centrifugation. Once dry, the sample was reconstituted with 8 μL of water before MS analysis.

3.2.4 Methyl Esterification Using 3-Methyl-1-(p-tolyl)triazene

The methyl esterification procedure using 3-methyl-1-(p-tolyl)triazene (MTT) is based on Reference 17 with minor modification for the sample clean-up procedure.17 A2 was reconstituted to a final concentration of 100 μM. 1.6 nmols from the A2 stock were dried completely using vacuum centrifugation. 20 μL of a 0.1 M MTT solution in 50:50 acetonitrile:DMSO (v/v) was added to the sample. The samples were vortexed and centrifuged before incubation at 60°C for one hour. After incubation, 500 μL of methanol was added and the sample was dried completely. Once dry, the sample was reconstituted with 100 μL of 5% acetonitrile in water for subsequent clean-up with C18 packed pipette tips. The sample was also centrifuged at 12,000 rpm for ten minutes to pellet any residual MTT. The tips were previously washed twice with 50% acetonitrile in water and then equilibrated twice with 5% acetonitrile in water. The sample was then aspirated five times to remove the leftover MTT with the glycans being unretained and remaining in the liquid phase under the clean-up conditions used. The tips were further washed with 20% acetonitrile in water to elute any methylated sialic acids that did bind to the C18 resin. The two fractions were combined and dried completely. Once dry, the sample was reconstituted with 8 μL of water before MS analysis.

3.2.5 Methyl Amidation Using (7-Azabenzotriazol-1-yloxy)trispyrrilidinophosphonium hexafluorophosphate and Methylamine

The methyl amidation procedure using (7-azabenzotriazol-1-yloxy)trispyrrilidinophosphonium hexafluorophosphate (PyAOP) and methylamine is based on Reference 16 with minor modification in the sample clean-up procedure.16 Disialyllacto-N-tetraose (DSLNT) was reconstituted to a final concentration of 200 μM. 2 nmols from the stock
were dried completely using vacuum centrifugation. 25 μL of 1 M methylamine hydrochloride with 0.5 M N-methylmorpholine in DMSO were added to the glycan and the solution was vortexed. Then 25 μL of 50 mM PyAOP in DMSO was added and again the solution was vortexed and centrifuged before incubation. The reaction was incubated at room temperature for thirty minutes. After incubation, 50 μL of water and 900 μL of acetonitrile were added to the sample for subsequent normal phase tip clean-up. The tips were previously conditioned twice with 95% acetonitrile in water. The sample was then aspirated 10 times to promote glycan binding to the resin. The tips were then washed four times with 95% acetonitrile in water. After washing, the glycans were eluted five times with 200 μL of water. The eluted solution was then dried completely using vacuum centrifugation. After vacuum centrifugation, the sample was reconstituted with 10 μL of water for MS analysis. The methyl amidation procedure was also subsequently applied to 2 nmols of A2 following the previously described protocol.

3.2.6 LC-MS Analysis

Data acquisition was performed using Agilent MassHunter Workstation Acquisition software, version B.02.00. The liquid chromatography portion of the instrument was an Agilent 1200 series liquid chromatography module with autosampler, cap pump and nano pump. The LC module flows to the Agilent HPLC Chip Cube which was coupled to an Agilent 6520 Accurate Mass Q-TOF MS system. Mobile phase A was composed of 10 mM ammonium fluoride (NH₄F) in 97% water:3% acetonitrile and mobile phase B of 10 mM NH₄F in 90% acetonitrile and 10% water. The microfluidic PGC chip contained a 40 nL enrichment column followed by a 75 μm x 43 mm separation column packed with 5 μm graphitized carbon material containing pores of 250 Å.

Samples were isocratically loaded onto the column using 100% mobile phase A at a flow rate of 4 μL/min. The samples were then separated on the column using the following linear gradient: 0 to 2.5 minutes, 0% B; 2.5-20 minutes, 0-16% B; 20-30 minutes, 16-44% B; 30-35
minutes, 44-100%B; and an isocratic hold for twenty minutes at 0%B to re-equilibrate the column. MS analysis was run in negative ion polarity with the fragmentor voltage set to 160 V and the skimmer set to 65 V. When CID was performed, the collision energy was set to 30 V. The mass range for MS analysis was from 400-3000 m/z while MS/MS analysis had a mass range from 100-3000 m/z. The voltage on the capillary was variable depending on the age of the chip and mainly ranged from 1950-2150 V.

3.2.7 Data Analysis

Data analysis was performed on Agilent MassHunter Qualitative analysis software, version B.02.00. Ions were extracted using a 50 ppm mass accuracy window. In addition, GlycoWorkBench (GWB) was used to predict m/z values for the glycan samples. When MS² experiments were performed, GWB was used semi-automatically to search the data against theoretical fragments in addition to manual checks and searching.

3.3 Results and Discussion

The first charge neutralization strategy performed was methyl esterification of the sialic acids using methyl iodide. This procedure has been in use for many years in the literature as a way to reduce the charge on sialic acids. It was previously found that the glycans reacted more rapidly when they were first converted into their sodium salts, most likely due to sodium’s low ionization energy. Normally this procedure is performed using a column containing cation exchange resin. In order to simplify the sodiation procedure, the use of strong cation exchange tips was attempted. The resulting MS analysis of 200 pmol of the reacted glycan showed an incomplete reaction (Figure 3.6)
Figure 3.6: The extracted ion chromatograms (EIC’s) of methyl esterified sialic acids on A3 and unreacted A3 from the sample that underwent reaction with methyl iodide.

As seen in Figure 3.6, the intensity of the unreacted A3 structure is two-fold higher than the glycan m/z that relates to the methyl esterified sialic acids on A3. The presence of distinct peaks in the chromatogram is the result of separation of the anomeric forms of the glycan in addition to linkage isomers. It is important to note that sialic acids will produce a much higher intensity response than their corresponding neutral glycans at the same concentration in negative ion mode, due to the inherent negative charge on the sialic acids. In addition, the spectra that resulted from the A3 glycan standard, which is isolated from the bovine fetuin N-glycan pool, were very complicated due to the presence of NeuGc and possible assignments for sodiated or fluorinated structures (Figure 3.7).
The ions relating to \( m/z 972.667 \) could either relate to the methyl esterified sialic acids on A3 or an A3 molecule that is both sodiated and fluorinated. Due to the presence of the \( m/z \) that relates to the fluorinated methyl esterified sialic acids of A3, the ions at 972.667 most likely relate to the methyl esterified sialic acids. However, to be sure of this assignment, the MS\(^2\) spectrum would need to be further investigated. Nevertheless, the spectra show an incomplete conversion to methyl esterified sialic acids on A3. The incomplete reaction could be caused by multiple reasons. The most likely explanation is in the reaction time and the inability to completely sodiate the glycans. The reaction was performed for two hours based upon the original report so the investigation of a longer time might be warranted. In addition, the glycans were sodiated using the SCX tips and therefore it is assumed that the kinetic rate of sodiation is sufficiently fast to result in complete sodium salt formation during the rate of passivation flow through the packed pipette tip. Due to the aforementioned factors, another methyl esterification reaction was performed with methyl iodide using a salt solution in the reaction conditions to promote glycan sodiation. In order to also simplify the spectra obtained, A2 (isolated from human fibrinogen) was used which should not contain appreciable levels of NeuGc. A salt
solution of sodium chloride was used in the reaction milieu to promote in-situ sodium salt formation of the oligosaccharides present in the DMSO/methyl iodide environment thereby removing potential variability associated with the solid phase based sodiation. Any traces of chloride that were left in the solution after incubation, would not interfere with the MS analysis since fluoride has a higher gas phase basicity and will thus replace any paired chlorides on the glycan. Analyzing 200 pmol of the reacted glycan showed that using a salt solution, unfortunately, also did not produce a complete derivatization of the sialic acids (Figure 3.8).

Figure 3.8: EIC’s from the A2 glycan that underwent methyl esterification. A) EIC of the methyl esterified sialic acids on A2. B) EIC of A2 with only one sialic acid methyl esterified. C) EIC of unmodified A2 left in the sample. D) MS spectrum relating to A. E) MS spectrum relating to B. F) MS spectrum relating to C.
The majority of the sample was found to contain only one sialic acid with a methyl group or no modifications were seen. These results again indicate incomplete derivatization through reaction with methyl iodide. Due to the issues associated with these methyl iodide based reactions, a different approach was investigated for methyl esterification by employing the reagent MTT.

MTT was shown to be a fast reaction in the literature with subsequent minimal sample clean-up required, thereby making the reaction attractive for investigation. The reaction was again initially investigated using the A2 standard to yield potentially simpler spectra using the methodology presented in the published protocol. After injecting 500 pmol, the reaction yielded mainly a mix of both sialic acids being methyl esterified and only one sialic acid being modified with little unreacted A2 seen (Figure 3.9).

Figure 3.9: EIC 's from the sample that underwent methyl esterification with MTT. A) EIC of A2 with two methyl esterified sialic acids. B) EIC of A2 with one methyl esterified sialic acid. C) EIC of unmodified A2.
The levels of unmodified A2 are lower than with previous methyl esterification approaches, based on intensity; however, there is still incomplete derivatization. The levels of methylation of one and both sialic acids modified seem to be very similar. In addition, when the EIC of two modified sialic acids is further investigated, the appearance of a third methyl group addition to the glycan can be seen (Figure 3.10).

![Figure 3.10: The top panel contains the EIC of A2 with both sialic acids methyl esterified. The earlier retention on the chromatogram shows A2 with two modified sialic acids as seen on the bottom left. The later retention on the chromatogram shows further methylation of the glycan as seen on the bottom right.](image-url)
The additional methyl group is most likely being added to the amide on the GlcNAc residues based on the appearance of only one methyl and not multiple methyls seen which would indicate modification to the hydroxyl groups. Typically, methylation of hydroxyl groups is performed at a highly alkaline pH as in permethylation approaches. The procedure utilized here is not at a high enough pH for this modification to proceed. The original publication did not report the further modification as observed in this instance; however, the protocol was applied to glycans that were blotted onto resins through the reducing end. It can be theorized that the blotting introduced steric hindrance and the MTT molecule would not be able to get close enough to the GlcNAc residues for further reaction. In our reaction, the glycans were in free solution, and therefore, steric hindrance would not be an issue.

In order to reduce the further methylation seen on the glycan, two optimization approaches were attempted. The first was a short time study in which the reaction was run for a reduced time, in order to try and identify reaction conditions where the methylation of the carboxylic groups were selectively derivatized. 200 pmol of each time point were injected on the Q-TOF for further analysis. In both time points, only unmodified A2 was observed suggesting that reduction of the reaction time was not viable. The next approach that was attempted was to run the reaction with a lower equivalence of MTT to evaluate if reduced molar equivalents of the MTT reagent could specifically target the carboxylic acids over the amide groups. Instead of 0.1 M MTT, 30 mM MTT (one third of the original equivalence) was reacted with the same time and temperature conditions. 400 pmol was injected onto the Q-TOF for analysis. The resulting spectrum showed only unmodified A2 implying that the lower molar ratio of MTT was not sufficient for the reaction to proceed to methyl esterified sialic acids on A2.

Since both methyl esterification protocols did not produce the desired modified sialic acids, a further approach based upon methyl amidation was undertaken. PyAOP is often used as a condensing reagent in peptide synthesis and is able to effectively overcome steric hindrance. In addition, methylamine is a stronger nucleophile than ammonium chloride which has also
been used in methyl amidation reactions, such as with 4-(4,6-Dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride (DMT-MM).\textsuperscript{16,22} A general reaction mechanism for phosphonium salts is shown in Figure 3.11.\textsuperscript{23}

\begin{figure}
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\caption{A general reaction scheme for the methyl amidation of carboxylic acids using phosphonium salts. The phosphonium salt depicted is benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP). The notation OBt refers to the benzotriazole group lost during phosphonium coupling to the carboxylic acid. Reprinted from Tetrahedron, 61, Christian A.G.N Montalbetti and Virginie Falque, Amide bond formation and peptide coupling, 10827-10852, 2005, with permission from Elsevier.\textsuperscript{23}}
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The reaction with PyAOP was first applied using DSLNT which contains both an $\alpha(2,3)$ and $\alpha(2,6)$ linked sialic acid, thereby allowing for the investigation of both reaction efficiency and the absence of linkage specificity or bias. 400 pmol of the reacted DSLNT was injected onto the Q-TOF for subsequent analysis. Only completely modified DSLNT was seen (Figure 3.12).
Figure 3.12: The EIC from DSLNT with both sialic acids methyl amidated. The corresponding MS spectrum is shown in the upper corner along with the structure of DSLNT. The presence of multiple peaks arises from anomeric separation of the milk sugar.

Since the reaction proved promising with DSLNT, it was further applied to A2 to verify that the reaction was complete with complex glycan structures. 400 pmol of reacted A2 was injected into the Q-TOF for subsequent analysis. The resulting spectra showed a mixture of two sialic acids converted to methyl amides and one sialic acid with a methyl amide (Figure 3.13).
Figure 3.13: EIC’s from A2 that underwent methyl amidation. A) EIC showing both sialic acids are modified, with the MS spectrum to the right. B) EIC showing that only one sialic acid is modified, with the MS spectrum to the right.

When A2 underwent methyl amidation of the sialic acids, no unreacted A2 was seen however there was a large proportion of A2 that contained only one sialic acid with a methyl amide, suggesting an incomplete reaction. For our reaction, a much larger amount of glycan was reacted than the original protocol had used. The reaction conditions therefore require further optimization to identify the optimal molar equivalence when large amounts of glycan are used. In addition, further optimization may be investigated such as an increase in reaction time or instrumentation optimization. The presence of partially methyl amidated sialic acids might require further optimization of instrument parameters such as the fragmentor voltage or capillary voltage to minimize any loss of the functional group during ionization. The initial reaction results show promise and further optimization will only strengthen the methodology.
3.4 Conclusions and Future Directions

The overall aim of this research was to identify derivatization chemistry capable of charge reduction of sialylated glycans to facilitate fluoride-mediated negative ion LC-MS/MS based characterization of complex oligosaccharides. Two methyl esterification procedures using methyl iodide and MTT failed to result in the complete conversion to methyl esterified sialic acids under the conditions used. On the other hand, PyAOP and methylamine showed complete conversion with simple glycans such as the milk sugar DSLNT. When applied to the A2 glycan standard, a mix of complete and partially derivatized glycan structures were observed using Q-TOF MS indicating that the initial investigations presented herein, although promising, require further optimization. The difference in conversion might be attributable to steric hindrance introduced by the glycan chain monosaccharides in A2 requiring longer reaction times. Proposed optimization steps will include the identification of optimal glycan to reactant molar equivalents, reaction times and the source and instrument parameters. In addition, the newly optimized method should also be tested on the A3 standard glycan to ensure that higher antennary species can undergo complete conversion. Further optimization might also be warranted for the collision energy. The MS² spectra were not analyzed during the previously described experiments as the research focus was on chemistry identification to ensure complete conversion. However, once the optimized charge neutralization strategy is developed, the tandem MS parameters will require optimization to ensure proper fragmentation that will allow for deep N-glycan analysis.

Once the method has been fully optimized, the negative ESI method will be applied to the characterization of the triantennary and tetraantennary glycans species released from alpha-1 acid glycoprotein isolated from both diseased, such as cancer, and normal samples to investigate subtle structural differential expression present on the glycosylation on this abundant acute phase protein.
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