

# Advancing Effective Glycan Analysis

Melissa Sherman\* PhD, CEO,  
MOBILion Systems Inc, Chadds Ford, Pennsylvania, USA

Lance Wells PhD,  
Georgia Research Alliance Distinguished Investigator, Professor of Biochemistry and Molecular Biology, Director of integrated life sciences, and co-director of the Thermo Fisher appointed Center of Excellence in Glycoproteomics at the CCRC at the University of Georgia, Athens, Georgia, USA

Mike Tiemeyer PhD,  
Distinguished Research Professor of Biochemistry and Molecular Biology, Co-director of the Thermo Fisher appointed Center of Excellence in Glycoproteomics at the University of Georgia, and Co-Director of the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, USA  
Corresponding author\*

## Abstract

Researchers are paying increasing attention to a critically important class of biomolecules: glycans. Although less studied than other major building blocks of life - proteins, lipids, and nucleic acids - complex and diverse glycans are essential to life and are omnipresent in organisms from archaea to humans [1].

Recent advances in glycoscience have shone new light on glycans and their role as key metabolic, structural, and physical components in biological structures. The potential of glycoengineering has been established, perhaps most significantly, in therapeutic antibody development.

This article outlines examples of the role glycans play clinically, the challenges of glycan analysis, summarises the tools available to elucidate their intricate structures, and presents data comparing traditional LC-MS workflows with the emerging technique of high-resolution ion mobility (HRIM) spectroscopy.

## Glycans, Glycoengineering and Drug Development

With knowledge of glycobiology expanding, the tremendous importance of the function of glycans in many branches of life science [2] including vital fields such as oncology [3], immunology [4], and infectious disease [5] is being elucidated. Moreover, developments in glycan analysis have coincided with an increased capacity to control glycosylation of specific biomolecules. Known as glycoengineering, this process involves manipulating glycosylation patterns, either by genetic modulation of specific glycosyltransferases or through chemical manipulation of glycoconjugates after biosynthesis [6].

Differences in glycosylation have been shown to affect therapeutic monoclonal antibody (mAb) activity in a number of

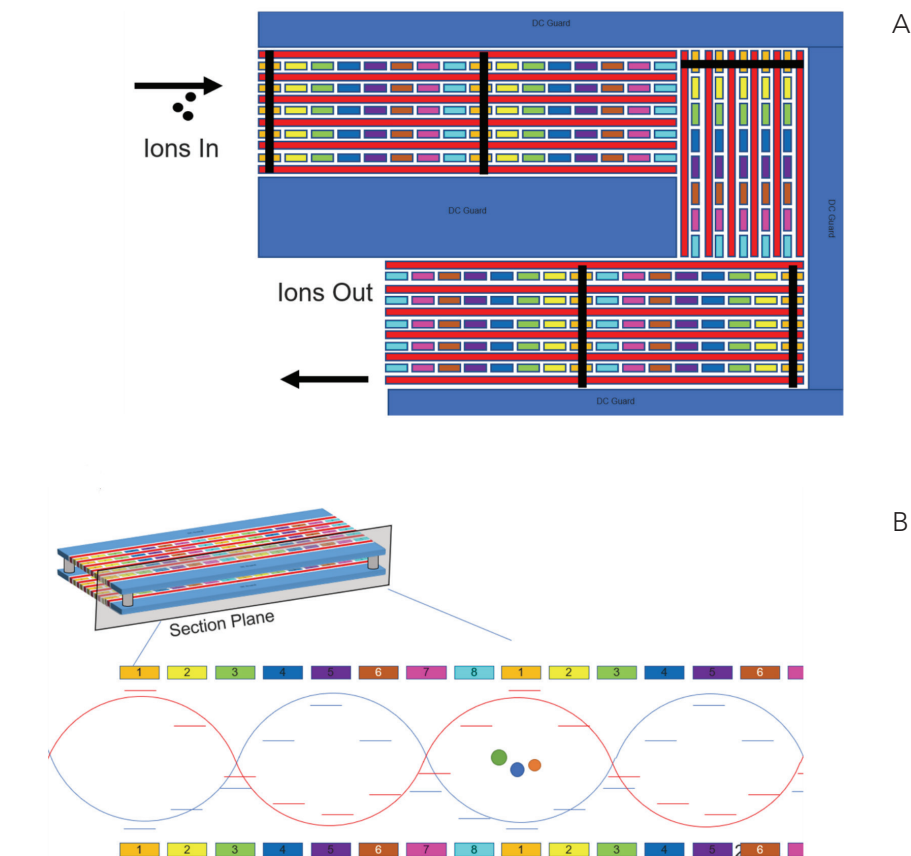


Figure 1: A) Arrangement of interspersed RF and DC electrodes on PCBs which enable trapping and manipulation of ions around corners along a serpentine path. B) Trapped ions traverse with the traveling wave created by the electrodes and separate based on size and shape.

ways [7,8]. As a key example, reduction or elimination of fucose monosaccharides from Fc domain glycans (also known as defucosylation) has been repeatedly shown to increase antibody-dependent cellular cytotoxicity (ADCC). Defucosylating antibody constructs leads to higher

activation of natural killer (NK) as well as other immune cells. This, in turn, causes greater ADCC where the therapeutic mAb binds, including cancer cells [12,13]. Genentech (South San Francisco, California, USA) applied this strategy to develop Gazyva (obinutuzumab) to succeed its

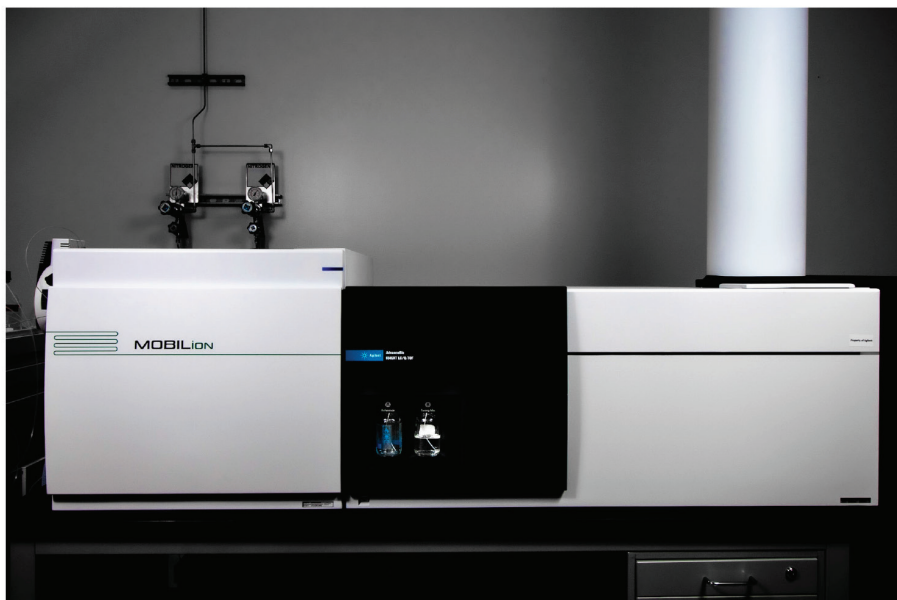


Figure 2: MOBILion's High Resolution Ion Mobility Instrument coupled to an Agilent Q-ToF.

\$7 billion blockbuster CD20-targeting mAb, Rituxan (rituximab). Patients receiving Gazyva showed greater depth of remission and increased progression-free survival compared to those who received Rituxan [9].

Additionally, glycosylation variation in patients can correlate with treatment outcomes. In order to optimise treatments and clinical trial outcomes (e.g. patient stratification), it is critically important to have the ability to identify glycosylation profiles in a high throughput manner.

With respect to vaccine development, another example of the clinical impact of glycans includes the HIV-1 envelope proteins, which is richly decorated with glycan structures that help the virus evade recognition by the immune system. This so-called 'glycan shield' is an active area of research and serves as a potential target for vaccine development and broadly neutralising antibody production [10]. Similarly, the COVID-19 pandemic has brought attention to the role the SARS-CoV-2 spike protein glycosylation plays throughout viral infection and in therapeutic development [11,12]. Building on research into broadly neutralising antibodies against HIV [13], researchers are now also looking to apply a similar approach to coronaviruses [14].

## The Challenge of Glycan Analysis

To leverage the potential of glycoengineering and enhanced knowledge of glycosylation patterns, researchers are increasingly interested in utilising better

tools and methods for quickly and accurately determining glycan structures. However, despite impressive advances, glycan analysis still remains challenging, largely due to the nature of glycans themselves.

Arguably the principal structural challenge is that glycans are one of the most structurally diverse biomolecule families [2]. Though most mammalian glycans are built from around nine monosaccharide units, these monomers can be attached to one another through many different glycosidic linkages, including in a branched fashion [15]. Furthermore, glycans may range from one monosaccharide to many. Collectively, the number of possible glycan structures quickly expands with each additional monomer and branch. It is estimated that for a hexasaccharide there are, in total,  $\sim 10^{12}$  possible glycan structures, though they may not all occur in nature [16].

In addition, glycan assembly is not template-driven like DNA, RNA, and protein biosynthesis. Instead, glycans are made through interlaced networks of glycosyltransferases acting in the endoplasmic reticulum and Golgi. As a result, different glycan structures can occur at the same glycosylation position, generating biomolecules that are identical except for the glycans they carry (i.e. glycoforms). The distributions of glycoforms for a given glycoconjugate are also subject to change as glycosyltransferase expression changes.

Importantly, many of the monosaccharides that make up glycans are isomers of one another - glucose, galactose, and mannose being prime examples. This means that

many different glycan structures can have the same mass, charge, and physical properties, but vary widely in function and recognition. Separation and identification of these complex isomeric materials can be exceedingly time-consuming and resource intensive.

## The State of Glycan Analysis Workflows

As described above, glycan structures are inherently complex and heterogeneous. The go-to technique for glycan analysis has long been mass spectrometry (MS) - it offers the resolving power and sensitivity needed to elucidate specific glycan structures and glycoforms isolated from biological materials [17,18]. However, to achieve meaningful resolution between highly similar glycans within the same sample, separation techniques are needed ahead of MS analysis.

On a practical level, LC separation of highly similar biomolecules - like many glycans are - often requires extended run times, creating a potential bottleneck in analytical workflows. Even with long run times, some glycans behave so similarly that LC coelution cannot be avoided, which complicates or even prevents full structural assignment [19].

So, while LC-MS remains the standard workflow for glycan analysis, there is a pressing need for faster techniques with better resolving power.

More recently, High Resolution Ion Mobility Mass Spectrometry (HRIM-MS) based on Structures for Lossless Ion Manipulation (SLIM) has emerged as a promising separation strategy in glycan analysis [20]. Unlike liquid chromatography, MOBILion's HRIM system separates ionised molecules in the gas phase. The system uses printed circuit boards (PCBs), see Figure 1, held in a chamber maintained at constant pressure (2-4 torr). The PCBs have a series of radio frequency (RF), direct current (DC) and traveling wave electrodes printed on them that provide an ion conduit through which the analytes traverse along an exceptionally long serpentine path. The electric fields that propel the ions also prevent them from striking surfaces while moving, therefore preventing any losses along their way. Depending upon the speed of the traveling wave, ions either 'surf' and are not separated, or they undergo enough collisions with the gas molecules present that they roll over the traveling wave peaks, and separation occurs.

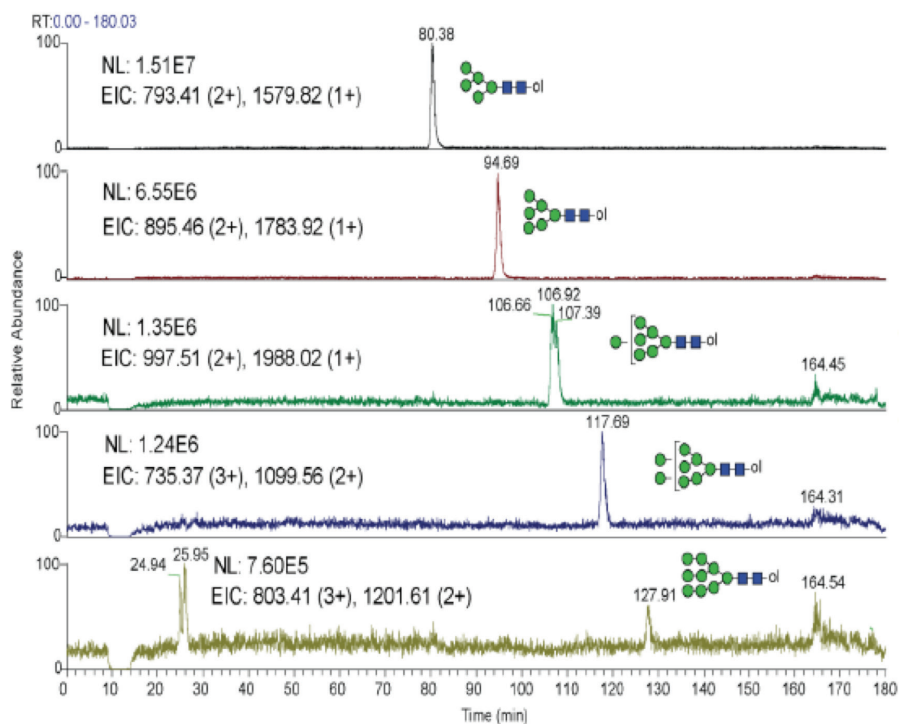


Figure 3A: Extracted high mannose reduced permethylated N-linked glycans separated by 180-minute LC workflow.

In HRIM, as with other ion mobility technologies, an ion's migration time is determined by its mass-to-charge ratio and its size and shape. As ions are driven along the separation path; the collision with an inert buffer gas slows them down to a degree proportional to their size. The length of the ion separation path is crucial to achieving the high degree of separation required to gain adequate resolution of glycans with challenging structural diversity. MOBILion's first HRIM product has an ion path of 13 meters (42 feet). The unique serpentine path design of the printed circuit boards allows for the 13m ion path to fit into a device the size of a briefcase allowing for unparalleled separation of isomeric structures.

HRIM set-up is straightforward because it occurs in the gas phase under nearly ideal conditions. There is no need to optimise columns, flow rates, or liquid components as with LC. In addition, HRIM achieves rapid separations with data collection in the order of milliseconds to seconds. Much has been written about how the most advanced HRIM approaches boost resolving power by expanding the pathlength [21] and when combined with MS (HRIM-MS, Figure 2) offers tremendous potential for seamless separation of glycans followed by structural determination in biomedical and clinical research [22,23].

## HRIM-MS in Practice

Recent work at the Complex Carbohydrates Research Center (CCRC) at the University of Georgia, Athens, Georgia, USA, has demonstrated the performance of HRIM-MS. Experiments compared this next-generation ion mobility technology, with an optimised LC-MS protocol [24].

The experimental set-up at CCRC used the MOBILion HRIM instrument coupled to an Agilent 6545 Quadrupole Time of Flight (Q-ToF). Permethylated N-glycans released from Fetuin and RNaseB were analysed by 180-minute reverse phase nanoflow LC-MS/MS, typical of a traditional LC N-glycan analysis workflow. The same glycan species were analysed using HRIM-MS. In 2 minutes, all glycans identified using the 180-minute LC separation were separated and identified using the HRIM-MS workflow. This 90-fold time savings allows, for example, a three-month LC analysis to be compressed to a one-day analysis. Moreover, the HRIM-MS analysis resolved the structural isomers of a biantennary, disialylated complex glycan that could not be resolved with LC (Figure 3B, G5).

Being able to rapidly run hundreds of samples enables the establishment of a 'normal range' of glycan heterogeneity. That advantage allows greater insight into the behaviour of glycans in a wide range of illnesses, in a time frame compatible with the fast pace of biopharmaceutical development.

## Looking Ahead

Glycan analysis, or glycomics, in which scientists elucidate the specific glycosylation patterns of biomolecules, has become an essential aspect of biomedical research, drug development, and biopharma quality control. Compared to traditional methods, HRIM excels in ease of use, generalisability of one method to multiple analyte classes, resolution, and speed of analysis. These attributes permit researchers to tackle the complexity of glycan structures and their heterogeneity on timescales that enable high-throughput analysis. The HRIM analyses discussed here are nearly 100-fold faster than LC analyses, but are only the beginning. More rapid sampling - a subject of ongoing development - could speed analysis times by additional orders of magnitude.

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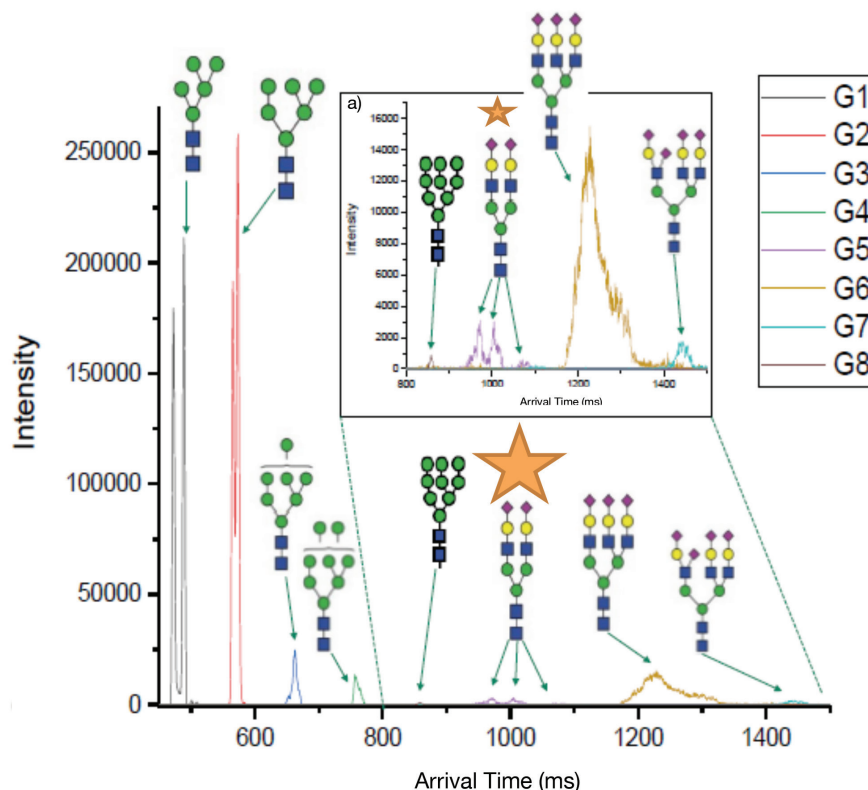


Figure 3B: Overlaid extracted MOBILigram of released permethylated non-reduced N-glycans from the HRIM analysis in 2 minutes with the added information of the resolution of G5 isomers (starred) achievable only by HRIM. a) Zoom in on arrival times 800-1500ms showing resolution of G5 isomers.

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\*Lance Wells is a Georgia Research Alliance Distinguished Investigator, Professor of Biochemistry and Molecular Biology, Director of integrated life sciences, and co-director of the Thermo Fisher appointed Center of Excellence in Glycoproteomics at the CCRC at the University of Georgia. He can be reached at [lwells@ccrc.uga.edu](mailto:lwells@ccrc.uga.edu).

\*\* Michael Tiemeyer is a Distinguished Research Professor of Biochemistry and Molecular Biology, Co-director of the Thermo Fisher appointed Center of Excellence in Glycoproteomics at the University of Georgia, and Co-Director of the

Complex Carbohydrate Research Center, University of Georgia. He can be reached at [mtiemeyer@ccrc.uga.edu](mailto:mtiemeyer@ccrc.uga.edu).

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