ASSESSING ISOMERIC HETEROGENEITY OF CARBOHYDRATES
BY ION MOBILITY MASS SPECTROMETRY (IMMS)

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This work in its entirety is dedicated to my parents, my brothers and special gratitude is to my lovely husband Jia Pan. Your encouragement and unconditional support through my studies made this work possible.
ASSESSING ISOMERIC HETEROGENEITY OF CARBOHYDRATES

BY ION MOBILITY MASS SPECTROMETRY (IMMS)

Abstract

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May 2013

Chair: Herbert H. Hill

Ion mobility spectrometry (IMS) coupled to different types of mass spectrometry (MS), which create a powerful analytical tool called ion mobility mass spectrometry (IMMS), were employed to evaluate the isomeric heterogeneity of carbohydrates. Isomer separation was achieved on millisecond timescale for a wide variety of carbohydrate compounds including monosaccharide methyl glycosides, disaccharides, oligosaccharide-alditols isolated from glycoprotein and glycopeptides from glycoprotein digests using drift tube IMS or/and traveling wave IMS coupled to time of flight MS. With IMS coupled to tandem MS, fragmentation patterns for the individual resolved isomeric mobility peaks can be obtained, providing evidence for multiple isomeric precursors as mobility-separated species. The special design of Synapt G2 high definition MS, where a collision dissociation cell is located in front of and after traveling wave IMS, respectively, enables both mobility-resolved precursor ions to be fragmented and mobility-separated product ions to be dissociated. Many isomeric product ions were observed for parent disaccharides and higher oligosaccharide-alditols. And mobility-resolved product ions
from mass-selected oligosaccharide-alditol precursors yielded different characteristic mass spectra. A hybrid tandem IMMS instrument, IMMS-IMMS, dual gate drift tube IMS coupled to Synapt G2 MS was established. In addition to its capability in evaluating the structural complexity of precursor ions, ion mobility identity of product ions derived from individual mobility- and mass-selected precursor ions from a mixture of isomers were able to be measured for the first time. This provides a valuable tool to establish a direct precursor-product relationship between mobility-selected precursor ions and specific sets of product ions having unique mobilities. N-linked glycoprotein digests, without deglycosylation, were subjected to IMMS analysis directly. Mixtures of peptides and glycosylated peptides with different charge states were separated on different ion mobility-mass correlation trend lines. Tedious and time-consuming sample preparation was eliminated and the detection of low abundance ions was improved.

IMMS analysis is fast, sensitive, universal, is capable of resolving isomers and is able to provide unique mobility identities and valuable MS^n data on the basis of mobility resolved species including precursor and product ions. Ultimately, IMMS could serve as a novel tool for high throughput identification of glycoprotein.
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<td>AGP</td>
<td>Human α-1-acid-glycoprotein</td>
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<td>Human antithrombin III</td>
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<td>BSM</td>
<td>Bovine submaxillary mucin</td>
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<td>DTIMS</td>
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Dedication

This dissertation/thesis is dedicated to my husband (Jia Pan), my parents and my brothers.
CHAPTER ONE
INTRODUCTION

I. General Overview

1. Isomeric Heterogeneity of Carbohydrates

Glycans exist as different forms of glycoconjugates\textsuperscript{1,2} such as glycoproteins, glycosphingolipids, proteoglycans, glucosylphosphatidylinositol in biological system through glycosylation process. Glycosylation refers to the enzymatic process that attaches glycans to proteins, lipids, or other organic molecules.\textsuperscript{3, 4} It is one of the major protein post translational modifications, modulating and regulating protein functions, and it has been reported that more than half of human proteins are glycosylated.\textsuperscript{5} Glycans are often located in an environment of many proteins, they play important roles in a wide variety of biological processes\textsuperscript{6-10} and are involved in several physiological and pathological conditions, such as cell growth,\textsuperscript{11,12} tumor growth and metastasis,\textsuperscript{13, 14} anticoagulation,\textsuperscript{15, 16} immune recognition,\textsuperscript{8, 17} cell-cell communication\textsuperscript{18} and microbial pathogenesis.\textsuperscript{19, 20} Moreover, disease development and progression are usually associated with alternations on glycosylation on tissue and/or blood proteins. It is now well established that altered glycosylation varies significantly for cancer cells compared to normal cells such as prostate, breast, liver and ovarian cancers.\textsuperscript{9, 10, 21} Glycan structure change can serve as the implication of disease states, carbohydrate-based drugs, therapeutics and biomarkers are under development.

Unlike proteins and nucleic acids, glycans are indirect products of genes, none template-based biosynthesized through a series of enzymes\textsuperscript{22, 23} (> 50) including glycosyltransferases,
glycosidases and sugar nucleotide transporters. Different enzymes could compete with each other for the same acceptor oligosaccharides. As a result, glycans are mixtures of variants, generated inherently heterogeneity. Their structures are highly variable owing to differences in their anomeric configurations, monomer stereochemistry, inter-residue linkage positions and general branching features.\textsuperscript{24-28} In addition, with 80-90% carbons being chiral, carbohydrates exist as a large number of isomers in nature and represent an extremely complex sample group with high isomeric heterogeneity among molecules. For example, for an oligosaccharide composed of 10 aldohexose monomers, there are \(\sim 2.83 \times 10^{29}\) possible isomers of this compound, even not considering the stereochemistry of monosaccharide, there are still \(\sim 4.47 \times 10^{15}\) possible structures for one molecular formula. It has been shown that individual oligosaccharides /specific oligosaccharide isomers provide a number of potential glycan bio-markers for human health and diseases.\textsuperscript{6} The separation and identification of carbohydrate isomers are vitally important both for carbohydrate structural elucidation and ultimately for understanding the roles these molecules play in biological systems. Due to the structural complexity of carbohydrates, this poses a great challenge for current analytical techniques.

2. Current Analytical Methods Applied to Carbohydrate Structure Analysis

Over decades, mass spectrometry (MS) has become the most popular and sensitive technology for the analysis of glycans\textsuperscript{29-33} which normally provides accurate \(m/z\) identity of glycans. Among ionization sources of fast atom bombardment (FAB), matrix-assisted layer desolvation ionization (MALDI), electrospray (ESI) for sugar analysis, ESI is the most effective method for transferring those molecules from solution to gas phase ions. With the developments of high mass accuracy instruments such as ion cyclotron resonance (ICR), modern time of flight
(TOF) and orbitrap mass analyzer nowadays, molecular weight of glycans can be measured at ppb or even subppb errors. However, single MS lacks of the capability to distinguish various kinds of isomers having identical \( m/z \) values, it is not suitable to evaluate the isomeric heterogeneity of carbohydrates. Tandem MS usually provides more detailed structural information for oligosaccharides, however, tandem MS itself suffers from the blindness in the selection of isomeric precursor ions, the fragmentation spectra obtained can be resulted from the mixture of multiple structure variants, producing misleading structural information. Thus, the physical isolation of isomeric carbohydrates from a complex mixture is highly preferred for structural analysis using collision-induced dissociation (CID) experiments and tandem mass spectrometry. Nuclear magnetic resonance (NMR) spectroscopy can lead to full structure characterization of glycans, but also requires the isolation of single species prior to analysis. In addition, NMR is not a high throughput method and not feasible for biological samples with large mixtures of glycans.

Modern separation methods such as liquid chromatography (LC) and gas chromatography (GC) have been combined with MS for structural analysis of carbohydrates. LC remains the most accessible and commonly used technique for carbohydrate isomer separation; several columns including graphitized carbon chromatography, reversed ion pairing chromatography and normal phase chromatography have been able to differentiate isomeric oligosaccharides. However, the physical separation of glycan isomers by LC is typically time-consuming (on the scale of tens of minutes up to hours) in both establishing the method and performing the separation. In general, one specific column is only suitable for the separation of a certain class of glycan isomers and additionally, more than one LC separation in more than one orthogonal mode is often needed in order to purify structures to homogeneity as evaluated by
NMR. Column selection could be expensive and difficult especially for isomers with highly similar chemical and physical properties. Gunnar Hansson et al.\textsuperscript{43} were able to characterize a number of permethylated oligosaccharides using GC-MS where a special high temperature column was used. Similar to LC, this method was only suitable for a certain type of oligosaccharides, the separation time was ~30-60 mins and in addition, sample derivatization was required prior to analysis to enhance sample volatilization, which limited the structural variation. In all, both of these methods limit sample throughput, are complex with multiple analytical sample preparation steps and are non-reproducible among laboratories. Because stationary phases of chromatographic columns differ from batch to batch it is impossible to assign absolute retention times to specific oligosaccharide. The analytical techniques of LC and GC could serve as an initial separator for glycan mixtures, but not preferable for carbohydrate isomer differentiation. Rapid, sensitive and robust analytical methodologies are needed to efficiently and effectively evaluate the isomeric heterogeneity of carbohydrate compounds.

3. Ion Mobility Mass Spectrometry

(a) Drift Tube Ion Mobility Spectrometry (DTIMS)

Ion mobility spectrometry (IMS) separates gas phase ions according to their travel velocities under an electric field in a counter flow of neutral gas through an ion mobility drift tube.\textsuperscript{37-42} The separation is based on the size-to-charge ratio (Ω/z) where size is measured as the ion’s cross section, Ω. As with mass, cross section is an intrinsic property of an ion and the buffer gas through which the ion migrates, which can be measured reproducibly from time-to-time, instrument-to-instrument and laboratory-to-laboratory. Drift tube IMS employs a conventional stacked ring design.\textsuperscript{51} It is composed of conductive stainless steel drift rings
connected by resistors and separated by insulating ceramic rings. With the metal rods of drift rings staying out, all the drift and ceramic rings are enclosed in a ceramic tube which is covered by aluminum heating housing. The homogeneous electric field is created by applying a high potential to the beginning of the resistor chain and the potential is evenly decreased through the whole tube. A Bradbury-Nielsen ion gate is used to divide the IMS instrument into desolvation region where ions are desolvated and drift region where mobility separation occurs.

The mobility $K$ of an ion can be described by equation (1), where $v_d$ is the drift velocity in cm/s, $E$ is the electric field in V/cm, $L$ is the drift region length in cm, $t_d$ is the drift time of an ion in second (s), and $V$ (Volts) is the voltage across the drift tube. Units for $K$ are cm$^2$V$^{-1}$s$^{-1}$.

$$K = \frac{v_d}{E} = \frac{L^2}{t_dV} \quad (1)$$

For comparison of an ion’s mobility across different experimental systems, $K$ can be normalized to reduced mobility $K_o$ by gas pressure $P$ and temperature $T$ of measurement expressed in equation (2).

$$K_o = \frac{L^2}{t_dV} \times \frac{273.15}{T} \times \frac{P}{760} \quad (2)$$

The mobility separation results from differential ion-neutral interaction in ion mobility drift tube and their interaction provides a method to measure gas phase ion-neutral collision cross section, $\Omega$, using equation (3) where mobility is inversely related to $\Omega$. 

\[ \text{5} \]
\[ \Omega = \left[ \frac{3}{16N_o} \right] \left[ \frac{2\pi}{\mu kT} \right]^{1/2} \left[ \frac{ze}{K_o} \right] \] (3)

\( N_o \) is the standard number density of drift gas in molecules per cm\(^3\), \( \mu = \frac{mM}{m+M} \) is the reduced mass, \( m \) is the mass of the ion, \( M \) is the mass of the drift gas molecule; \( k \) is Boltzmann’s constant in J/K, \( z \) is the number of charges on the ion, and \( e \) is unit electronic charge in Coulombs.

(b) Traveling Wave Ion Mobility Spectrometry (TWIMS)

Synapt G2 high definition mass spectrometer (HDMS) is a hybrid quadrupole/traveling wave ion mobility/orthogonal time of flight MS\(^{52-56}\) and has been applied to a wide range of applications.\(^{57-62}\) This instrument employs a stacked ring ion guide technology which consists of a series of planar ring electrodes in sequential. Opposite phases of radio frequency (RF) voltages are applied to adjacent electrodes, providing a radial confining potential well to focus ions to the center. To propel ions moving forward, a DC voltage is superimposed on the RF voltages to one pair of electrodes and then moves to the next pair of electrodes in a repeating pattern at certain velocity. This creates a moving/nonhomogeneous electric field, which is called traveling wave electric field. With the introduction of a neutral gas, the ease with which an ion passes through the drift tube under the influence of the traveling wave electric field is dependent on its mobility. Ions with high mobility are more able to catch up with the wave, ions having low mobility roll over the wave-top more frequently and are left behind usually, thus mobility separation is achieved. Traveling wave ion mobility is operated under a reduced pressure of \( \sim3-4 \) mbar.
nitrogen. The design, electric field homogeneity and pressure are different from traditional drift tube IMS. Additionally, a trap and a transfer cell are located in front of and after traveling wave ion mobility separator respectively, where fragmentation can be induced in either or both cells by elevating collision energy (CE). This enables various types of ion mobility analyses become possible including mobility separation of product ions, dissociation of mobility separated precursor ions and fragmentation of mobility resolved product ions. Due to the non-uniform electric field in TWIMS, there is currently no method to calculate Ω values directly from experimental data. However, Ω values can be estimated based on the calibration methods detailed in the literature\textsuperscript{57, 61, 62} for TWIMS.

\textit{(c) Ion Mobility Mass Spectrometry (IMMS)}

In contrast to LC-MS and GC-MS, ion mobility spectrometer has been successfully interfaced to various types of mass spectrometers including quadrupole,\textsuperscript{63-65} quadrupole ion trap,\textsuperscript{66} time of flight (TOF)\textsuperscript{67-70} and fourier transform ion cyclotron resonance MS,\textsuperscript{71} which could be referred to ion mobility mass spectrometer (IMMS).\textsuperscript{72} Synapt G2 HDMS is a novel type of IMMS instrument. There are several advantages of IMMS over other analytical techniques, the benefits include increasing the peak capacity of a mass spectrometer,\textsuperscript{73} separating isomeric compounds\textsuperscript{74} having identical \textit{m/z} values, reducing chemical and random noise, measuring Ω/\textit{z} values, offering class identification by mass-mobility trend lines\textsuperscript{75-77} and charge states separation.\textsuperscript{78} The time required for ion mobility separation is in millisecond (ms) time range, which markedly reduces analysis time and increases sample throughput. Beside the applications for the detection of explosives,\textsuperscript{79-82} drugs,\textsuperscript{70, 83, 84} and chemical warfare reagents,\textsuperscript{85-87} IMMS has
proved particularly useful for the separation and identification of biomolecules in complex mixtures such as those encountered in metabolomics, proteomics and glycomics.

4. Current Applications of IMMS to Carbohydrate Structure Analysis

IMS is capable of separating isomeric compounds rapidly based on the ion’s collision cross section. Previous reports have demonstrated that IMMS can resolve several pairs of monosaccharide, disaccharide and oligosaccharide isomers. Isomeric mobility peaks have also been observed for specific glycans isolated from biological source in a few studies. By installing a second gate or selection gate in IMS and coupling the instrument to a tandem mass spectrometer, mobility selected fragmentation experiments are now feasible, providing evidence for multiple isomeric precursors as mobility-separated species. Due to the special design of Synpat G2, where a trap CID cell is located in front of traveling wave IMS, mobility-selected fragmentation experiments have also been achieved. Clemmer et al. initially showed the mobility of several product ions from individual oligosaccharide standards by fragmenting precursor ions with high injection energy prior to IMS, but no further investigation was performed. With the application of IMMS to biological samples, it has been demonstrated that measurements of glycan conformational and isomeric distributions by IMMS can give a more complete picture of the complexity of glycans accompanying disease states. Mclean et al. reported a separation of mixture of glycans and peptides in 2009 using IMMS, the differentiation of glycans and peptides with different charge states were achieved based on different ion mobility-mass correlation trend lines in 2-D ion mobility mass spectra. His analysis was based on a deglycosylated form of the enzymatically digested glycoprotein.
Currently, IMMS only showed its early conceptual and promising applications for carbohydrate isomer separation. For a complete assessment of isomeric heterogeneity of carbohydrates, further IMMS analysis can be performed in the following aspects: (1) The majority of glycan separations by IMMS have been performed on precursor ions using purified or commercially available standards. Mobility evaluation of isomeric heterogeneity of biological glycan mixtures such as O- and N-Linked glycans isolated from glycoproteins are not routine; fragmentation patterns from isomer mixtures can be difficult to interpret, leading to mis-identification of specific biological glycans. Acquisition of fragmentation patterns for mobility resolved individual isomeric precursors is critical to demonstrate the structural differences among isomeric species in a biological glycan mixture. (2) Compared to the numerous studies reported for positive ions in IMMS, only a limited number of studies have been carried out with anions. Different mobility separation can be achieved using anions or anion adducts and moreover, more isomeric species could be resolved for carbohydrate anions in comparison to cations in some cases. The mobility profiles of carbohydrate negative ions are also important for the identification of carbohydrate isomers. (3) More importantly, IMMS analyses that have been conducted have focused on isomeric carbohydrate precursor ions. Structural variation of isomeric product ions has not been investigated. Many of fragment ions can be isomers and their stereochemistry cannot be determined by MS. Knowing the cross section of fragment ions should aid in determining of their stereochemistry, leading to a more confident and accurate identification of the parent oligosaccharide. Mobility spectra of product ions derived from a specific precursor ion are not currently feasible for isomeric carbohydrate mixtures. The direct mobility precursor-product ion relationships could result in unambiguous identification of oligosaccharide isomer. (4) Current analytical techniques normally require the physical
separation (deglycosylation) of glycans and peptides for glycoprotein analyses, which are known as glycomics and proteomics, respectively, and often require tedious sample preparation. However, if the deglycosolation step can be eliminated, then site information for specific glycans can be preserved. The nature of each carbohydrate variant at each specific peptide site and their relative percentages is an essential one to be addressed to fully understand their biological roles. Thus, the development of novel strategies for high throughput identification of whole glycoprotein digests without deglycosylation is important.
II. Specific Aims

The primary goal of this project was to evaluate ion mobility mass spectrometry (IMMS) as a method for assessing the isomeric heterogeneity of carbohydrates.

The specific aims of this research were to:

1. Resolving a complete set of monosaccharide methyl glycoside structural isomers having subtle structure variations in gas phase by both DTIMS and TWIMS.
2. Application of ion mobility coupled to MS and tandem MS in determining isomeric heterogeneity of oligosaccharide-alditol mixture derived from O-linked glycoprotein by both positive and negative ESI.
3. Development of tandem ion mobility mass spectrometry, IMMS-IMMS, for fully characterizing the structure diversity of carbohydrate precursor and product ions using isomeric carbohydrate mixture.
4. Demonstrate ion mobility-mass correlation trend line separation of glycoprotein digests without deglycosylation.

The specific aims 1 and 2 were focused on the application of established IMMS methods for the separation of 20 monosaccharide isomers and the evaluation of stereo-structure differences of biological oligosaccharide mixture isolated from glycoprotein. A hybrid instrument was constructed in aim 3 for assessment of the complexity of isomeric precursor ion mixtures as well as for firmly establishing the mobilities of their specific sets of product ions. The advantages of IMMS in the analysis of glycoprotein digests were demonstrated in aim 4, where mixtures of peptides and glycopeptides with different charge states were subjected to IMMS analysis directly.
III. Attribution

Experiments fulfilling the specific aims of this thesis were designed and conducted by Li. Professor Herbert H. Hill Jr. provided advice and scientific direction for all aspects of this research. The analytes in this research were all supplied by Dr. Brad Bendiak at University of Colorado. Chapter 2 has been accepted and published in *Analytical Chemistry* (Li, H.; Giles, K.; Kaplan, K.; Siems, W. F.; Hill, H. H. Jr *Anal. Chem.* 2012, 84, 3231-3239). Chapter 3 will be submitted to *Rapid Communications in Mass Spectrometry*. The manuscript in Chapter 4 was written in the format necessary for submission to *International Journal of Mass Spectrometry*. Chapter 5 will be submitted to *Analytical Chemistry*. Chapter 6 has been revised based on the reviewers’ comments from *Analytical Chemistry*. Chapter 7 has been revised based on the reviewers’ comments from *International Journal for Ion Mobility Spectrometry*. The software control program for the two gate drift tube ion mobility spectrometer was updated using Labview 2009 by former graduate student Eric Davis at Washington State University. All the references were cited in the format required by *Analytical Chemistry*, except that the references in Chapter 7 were written according to the format by *International Journal for Ion Mobility Spectrometry*. 
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CHAPTER TWO

RESOLVING STRUCTURAL ISOMERS OF MONOSACCHARIDE METHYL GLYCOSIDES USING DRIFT TUBE AND TRAVELING WAVE ION MOBILITY MASS SPECTROMETRY

Abstract

Monosaccharide structural isomers including sixteen methyl-D-glycopyranosides and four methyl-N-acetylhexosamines were subjected to ion mobility measurements by electrospray ion mobility mass spectrometry. Two ion mobility-MS systems were employed: atmospheric pressure drift tube ion mobility time-of-flight mass spectrometry and a Synapt G2 HDMS system which incorporates a low pressure traveling wave ion mobility separator. All the compounds were investigated as [M+Na]+ ions in the positive mode. A majority of the monosaccharide structural isomers exhibited different mobility drift times in either system, depending on differences in their anomeric and stereochemical configurations. In general, drift time patterns (relative drift times of isomers) matched between the two instruments. Higher resolving power was observed using the atmospheric pressure drift tube. Collision cross section values of monosaccharide structural isomers were directly calculated from the atmospheric pressure ion mobility experiments and a collision cross section calibration curve was made for the traveling wave ion mobility instrument. Overall, it was demonstrated that ion mobility-mass spectrometry using either drift tube or traveling wave ion mobility is a valuable technique for resolving subtle variations in stereochemistry among the sodium adducts of monosaccharide methyl glycosides.
Introduction

Carbohydrates or glycans play important roles in a wide variety of biological processes\textsuperscript{1-5} and their structural elucidation is an essential prerequisite for understanding their many functions. They are highly variable in structure owing to differences in their monomer stereochemistries, inter-residue linkage positions, and general branching patterns.\textsuperscript{6-8} Moreover, their preparation from biological sources is frequently accompanied by complex mixtures, often isomeric mixtures, of molecules. NMR spectroscopy is useful for evaluation of isomeric heterogeneity, and for structural elucidation, but it is highly preferable to isolate single molecular species prior to determining the structures of unknowns using NMR.\textsuperscript{9-13} Typically, physical separation of the molecules is time consuming, usually involving more than one LC separation in more than one orthogonal LC mode.\textsuperscript{13-18} Mass spectrometric methods\textsuperscript{19-23} can analyze samples at far greater sensitivity, but have their own limitations. One critical issue is that mass spectrometry is not well suited for the evaluation of isomeric heterogeneity. With different variants of multi-stage mass spectrometry (MS\textsuperscript{n}) this applies not only to precursor ions having the same \textit{m/z} but also to many product or multi-stage product ions. Mass spectra at any stage of isolation/dissociation might result from dissociation of more than one isomeric precursor or product ions. We sought to answer the question as to whether small carbohydrate ions, in this case methyl glycosides varying in their stereochemistries, could be physically resolved in the gas phase as a requisite for evaluating the isomeric heterogeneity of small product ions derived from larger oligosaccharide precursors. The means employed here was ion mobility spectrometry (IMS), which is capable of physically separating isomeric ions based on their different drift velocities through an electric field in a counter flow of neutral gas.\textsuperscript{24-29}
IMS separates ions based on an ion’s collision cross section (Ω) to charge ratio. Ions of the same chemical formula but different sizes and configuration potentially can be resolved by IMS. When IMS is coupled to MS, it can provide valuable stereochemical information about analytes. Drift tube ion mobility mass spectrometry (DTIMMS) has been applied in the field of carbohydrate research and it has been reported that both isobaric carbohydrate standards and isomeric biological glycans could be unambiguously distinguished by IMS.\textsuperscript{30-35} Dwivedi et al.\textsuperscript{30} initially demonstrated that monosaccharide methyl glycosides could be resolved by atmospheric pressure ion mobility time-of-flight mass spectrometry. However, a complete set of methyl glycoside isomers were not investigated and systematic structural information associated with the mobility of isomers was not provided. Moreover, IMMS has also been demonstrated as a valuable tool to analyze complex glycan samples. Measurements of glycan conformational and isomeric distributions by IMMS can give a more complete picture of the complexity of glycans accompanying disease states.\textsuperscript{34-35} The mass-mobility correlation band occupied by carbohydrates\textsuperscript{34, 36-37} can provide a general metric for assigning unknown signals to particular molecular classes. Recently, traveling wave ion mobility spectrometry (TWIMS) has been developed\textsuperscript{38-40} and applied to a wide range of applications\textsuperscript{41-46} including glycomics.\textsuperscript{47-50} The electric field, pressure and design of the system\textsuperscript{38-40} are different from traditional DTIMS, and so conducting identical studies on both systems would be beneficial to further characterize the TWIMS separation.

Here we compare the performance of an atmospheric pressure drift tube IMS, employing a novel resistive glass tube design and a traveling wave IMS in a Waters Synapt G2 instrument, at low pressure, in differentiating 20 monosaccharide methyl glycoside structural isomers. Many anomeric and epimeric pairs of methyl glycosides were resolved in analyses performed on the
millisecond time scale on both systems. A detailed and direct comparison was made for the first time between the two different types of IMS systems based on the study of structural isomers of small, relatively rigid molecules.

**Experimental Section**

**Electrospray Ionization Ambient Pressure Resistive Glass Drift Tube Ion Mobility Time of Flight Mass Spectrometer (ESI-AP-DTIM-TOFMS)**

The instrument was described previously in detail by Kaplan et al. in 2010. This system will be simply referred to as the DTIMS in the following text. In this study, voltages applied to the ion gate and IMS exit were 9007 V and 773 V respectively, which resulted in a homogeneous drift field of 412 V/cm. The gate pulse width was 200 μs. A counter flow of nitrogen (1.5 L/min) was introduced at the exit end of the drift tube to provide both the drift gas and also to aid efficient desolvation of ions prior to the ion gate. The IMS tube was placed in a stainless steel cylindrical tube of the same length that did not touch the resistive glass, with an air gap of ~ 5 mm in between. The stainless steel tube was surrounded by heating jackets and heated. The buffer gas temperature was measured to be 92 °C.

TofDaqViewer software, developed by TOFWERKS AG, was used to view and collect all the data from the instrument. The data from each sample could either be completely or selectively exported based on the user-specified time range in the form of a 2D text file. IDL virtual machine software (www.exelisvis.com) was then used to generate 2D IMMS correlation spectra based the data exported from TofDaqViewer. The ESI was constructed at WSU. Detailed information on its construction and operation are given in the supporting information. The voltage applied to the ESI needle was 14.5 KV, producing a 3KV difference between the
ESI needle and the entrance of the ion mobility spectrometer. ESI solvent used in this study was a 50:50 (v/v) methanol: water mixture.

**Synapt G2 HDMS System-Traveling Wave Ion Mobility Spectrometry (TWIMS)**

The Synapt G2 HDMS (high definition mass spectrometry) (Waters Corp., Manchester, UK) is a hybrid quadrupole/IMS/orthogonal TOFMS instrument and has been previously described.\textsuperscript{38-40, 52, 53} The moving/non-uniform electric field in Synapt G2 is called the traveling wave or T-wave. In the IM cell of the Synapt G2, the ability of the ions to pass through the neutral gas under the influence of a traveling wave electric field is dependent on its mobility. Ions with high ion mobility are more able to keep up with the traveling wave and are overtaken by the pulses less often than the ions with low mobility.\textsuperscript{38, 39} This system will be simply referred to as TWIMS hereafter. In order to enhance the IM resolution, second-generation IM technology has been incorporated into the Synapt G2 instrument.\textsuperscript{52, 53} The resolving power of the TWIM cell in the Synapt G2 was increased 3~4 times compared to the traditional Synapt.

ESI voltage was at 3.0 KV and nitrogen was used as the drift gas at 90 mL/min. The TWIMS cell in this study was operated at nominally 3 mbar N\textsubscript{2} with a 40V, 900 m/s T-Wave. A complete set of instrumental parameters are included Table S-1 in the supporting information. Masslynx 4.1 (Waters Corporation, Milford, MA, USA) was used to collect and process all the data.

**Chemicals and Solvents**

All sugars used were the D-enantiomers. The α- and β-methyl glycopyranosides of galactose, glucose, mannose and 2-acetamido-2-deoxy-glucose were purchased from Sigma. The
α- and β-methyl glycopyranosides of 2-acetamido-2-deoxy-galactose were from Calbiochem. Those of allose, altrose, gulose, idose and talose were synthesized by Fischer-type glycosidation. The synthetic method, NMR spectra, chemical shifts and $J$-couplings are presented in the supporting information section.

For ion mobility studies, 20 μL of a 500 μM sample stock solution and 10 μL of a 1 mM NaCl stock solution were added to 1mL ESI solvent, resulting in a sample concentration of 10 μM and sugar/NaCl ratio of 1 : 1. Glycosides were examined to avoid any configurational interconversion or additional complexities observed for reducing sugars, which are typically present in aqueous solvent mixtures in multiple anomic configurations and ring forms. HPLC grade solvents (methanol, water) were used and purchased from J. T. Baker (Phillipsburgh, NJ).

**Reduced Mobility $K_0$, Collision Cross Section $\Omega$, Separation Factor $\alpha$ and Resolving Power $R_p$**

The mobility ($K$), reduced mobility ($K_0$) and collision cross section ($\Omega$) of an ion and their calculations have been widely described.\(^{24, 27, 30-34}\) Values of $\Omega$ can be directly determined from experimental data obtained using DTIMS. Due to the non-uniform electric field in TWIMS, there is currently no method to calculate $\Omega$ values directly from experimental data. However, $\Omega$ values can be estimated based on the calibration methods detailed previously in the literature\(^ {45, 46, 54-56}\) for TWIMS. Briefly, ions with known $\Omega$ values are used as calibrants and their drift times on TWIMS are measured. In general, a calibration curve by plotting $(\Omega \mu^{0.5})/(ze)^{45, 46, 54-56}$ values versus TWIMS corrected drift times, $t_d'$, is made. The $\Omega$ values for unknown compounds in TWIMS are thus determined using the calibration curve and drift time information obtained under exactly the same instrumental conditions. In this study we plot directly the $\Omega$ values obtained from the DTIMS versus $t_d'$ obtained using the TWIMS. Adjustment of $\Omega$ for reduced
mass and charge state is not essential here since the reduced mass is almost the same for all species studied and all are singly charged. The $t_d$ values have been corrected for $m/z$ dependent and independent offsets in the TWIMS as described elsewhere.\textsuperscript{45, 46} A power trend line of the form $\Omega = A \left( t_d \right)^B$ is used to fit the data, where $A$ is an incorporated correction factor for the electric field and other parameters on the TWIMS system; $B$ is dependent on many parameters such as T-Wave amplitude and velocity and accounts for the non-linear effects in TWIMS.

The separation factor ($\alpha$) is by $\alpha = \frac{t_d (2)}{t_d (1)}$, where $t_d (2)$ is the drift time of the slower drifting ion and $t_d (1)$ is the drift time of the faster drifting ion. The experimental resolving power ($R_p$) is traditionally defined by the drift time $t_d$ divided by the peak full width at half maximum (FWHM): $R_p = \frac{t_d}{\Delta t_d}$. The $R_p$ of TWIMS was theoretically studied by Shvartsburg and Smith in 2008\textsuperscript{40} where they showed that due to the non-linear relationship between mobility and drift time on the TWIMS, the mobility resolving power is approximately twice the temporal resolving power, $R_p$. Zhong et al.\textsuperscript{53} have experimentally characterized the ion mobility resolution of the second generation TWIMS as a function of different experimental parameters. Both theory and experiment show that the resolution is dependent on wave height, wave velocity, IM cell length, pressure and other parameters.

**Results and Discussion**

**Structures of 20 Monosaccharide Methyl Glycoside Isomers and MS spectra**

Monosaccharides are the basic units of larger carbohydrate molecules. For the methyl-D-pyranosides of hexoses, where the chirality at C-5 by definition is invariant, four additional chiral carbons are present that may vary in stereochemistry (C-1 to C-4), giving rise to 16 isomeric forms with the same exact mass and chemical formula. In addition, 4 isomers of the
methyl glycosides of two important N-acetylhexosamines: N-acetylgalactosamine and N-acetylglucosamine, were also included in this study. The structures and nomenclature for all 16 methyl-D-glycopyranosides and the 4 methyl-N-acetyl-D-hexosamines are shown in Figure 1a. Representative mass spectra for the sodiated adduct of α-Me-glucopyranoside at m/z 217 and α-Me-N-acetylglucosamine at m/z 258 are shown in Fig. 1b and Fig. 1c. Other methyl-glycopyranosides and methyl-N-acetylhexosamines had identical mass spectra to those shown in Figure 1b and Figure 1c, respectively. Therefore using MS alone, it is impossible to differentiate those isomers that have identical m/z values. Carbohydrates have an especially large number of stereo-isomeric variants differing at anomeric and/or epimeric positions. Anomers are cyclic structures that only differ in the configuration at the acetal carbon for glycosides (carbon 1) which is called the anomeric carbon. For the \(^4\text{C}_1\) chair conformations as drawn in Figure 1a, α-glycosides have the -OCH\(_3\) in the axial position, and β-glycosides have the -OCH\(_3\) in the equatorial position. Epimers are diastereomers that differ at only one asymmetric carbon. For example, α-methyl-talopyranoside and α-methyl-galactopyranoside are epimers differing only at carbon two (Figure 1a). Overall, all the structures are categorized into β and α configurations in Figure 1a with their corresponding names shown in the right column. It is evident that all the isomers only vary in subtle structural differences.

**Overall Mobility Separation Patterns of Monosaccharide Structural Isomers by DTIMS and TWIMS**

The overall ion mobility spectra of the 16 structural isomers of the methyl-D-glycosides obtained on the DTIMS and TWIMS instruments are displayed in Figure 2a and Figure 2b, respectively. Corresponding abbreviations are listed on the right. Each DTIMS mobility
spectrum was acquired for 5 minutes with 3 μL/min ESI sample flow rate. Each dataset shown in Figure 2b from TWIMS was acquired for two minutes with a 5 μL/min infusion of the sample. The results showed that the majority of monosaccharide structural isomers exhibited unique mobility drift times, even though not all of them were fully resolved. Many drift time orders matched between these two sets of data, but not all. Based on the traditional definition, $R_p$ for DTIMS was ~75 with FWHM of 0.3 ms; for TWIMS the temporal $R_p$ was ~15-20 with FWHM of around 0.16 ms, which translates to a mobility resolution of 30-40 (see above), which is in keeping with results presented previously for singly charged species.\(^{52}\) It should be noted that the experimental conditions for DTIMS in this study were for high sensitivity (operating conditions for the DTIMS were set to approximate the sensitivity of the TWIMS), not optimum resolving power; the measured resolving power for the DTIMS was ~83 % of its optimum resolving power under the same operating conditions.\(^{57}\)

In all of the spectra, the predominant carbohydrate ions produced by ESI were found to be the Na\(^+\) adduct of the saccharide. The formation of metal ion-saccharide adducts are common in electrospray ionization. [M+H]\(^+\) ions were also detected at low abundance for certain monosaccharide structural isomers, however, the mobility separation between isomers was less than for the sodiated ions (see Table S-2 in supporting information). Cerda and Wesdemiotis\(^{58}\) using ab initio calculations demonstrated that Na\(^+\) interacts with sugars through multidentate coordination with oxygen lone electron pairs. The favored [monosaccharide + Na]\(^+\) structures contained pyranose rings in the chair or boat conformation that permits tri or tetradebate coordination of Na\(^+\). Thus the resolution observed among structural isomers of monosaccharide methyl glycosides is attributed not only to their different stereochemistries but also to the conformational changes induced by the Na\(^+\) metal ion. Clearly, different methyl glycosides vary
in the compactness of their Na\textsuperscript{+} adducts which is reflected in their overall cross-sectional areas and drift times. With both instruments, the α-Tal stereochemical arrangement showed the shortest drift time and α-Glc had the longest drift time. The major differences observed between mobility profiles in comparing the two instruments were between the α-, β-Ido, and α-, β-Gul configurations. With DTIMS, very similar drift times were observed, and reduced mobility differences (Table 1) were within the expected experimental variation and could be considered not separated. While TWIMS exhibited better separation, the α-Gul (2.89 ms) and β-Gul (2.81 ms) anomers had longer drift times than the α- and β-Ido anomers (2.70 ms). The TWIMS and DTIMS techniques, while similar, are different in pressure, electric field homogeneity and temperature, thus some differences in resolution and relative order of separations empirically might have been expected.

The mobility spectra of four methyl-N-acetylhexosamines that are commonly found in mammalian oligosaccharides are shown in Figure 3. While separation between sodium adducts of the epimers was observed, no separation was seen between anomers. Interestingly, the α- and β-Me-GalNAc anomers drifted faster (higher mobility) than the α- and β-Me-GlcNAc anomers (lower mobility). This suggests, at least with sodium adducts, that the methyl-GalNAc species adopt more compact overall structures than those of the methyl-GlcNAc anomers. In addition, the drift time profile of these four isomers matched exactly between the two IMS systems. Overall, monosaccharides having different stereochemistries coordinate the sodium ion differently, depending on the electron donor groups available and their relative 3-dimensional spatial orientations. This clearly results in different overall shapes and compactness for the coordination complexes of different methyl glycosides, thereby resulting in different ion mobilities.
Separation between Anomers and Epimers

All the monosaccharide structural isomers in this study can be categorized as 10 pairs of anomers based on the orientation of the \(-\text{OCH}_3\) group in either the axial (α) or equatorial positions (β) as shown in Fig. 1a. Six pairs of anomers including the α and β-Tal, Man, Glc, Gal, Alt and All were baseline or fully separated in DTIMS. Separation for the same six pairs of anomers was also observed on TWIMS, however, with lower resolution. However, as mentioned previously, the anomer pair of α and β-Gul was partially separated in TWIMS, while no separation was observed in DTIMS. Examples of the separation profiles of 4 pairs of anomers on the two different systems are shown in Figure 4. Two-dimensional IMMS plots overlaid from individual IMS spectra obtained from DTIMS are shown with \(m/z\) along the x axis and mobility drift time along the y axis having units in μs. For each anomeric pair, the equivalent 1D overlaid TWIMS spectra are also displayed.

It is worthy of note that in some cases (Figure 4, panels a and d) the α anomer showed a shorter drift time than the β, but in other cases (Figure 4, panels b and c), the reverse was true. It is important to point out that ab initio calculations\(^{58}\) have indicated that some sugars coordinate with preferred conformations that can be either \(^4\text{C}_1\) or \(^1\text{C}_4\) chair forms, or boat forms, and more generally for all stereoisomeric variants, other forms such as the skew, half-chair or sofa forms would need to be seriously considered in theoretical calculations. Depending on the stereochemistry and relative orientation of hydroxyl groups, different methyl glycosides could participate in different multidentate coordination complexes having different shapes and compactness and with different positioning of the central \(\text{Na}^+\) either above or below a plane drawn between C-1, C-3 and C-5, for example. While the detailed coordination complexes are beyond the scope and intent of this paper, it is evident that solely modifying the stereochemistry
at C-1 can dramatically affect the ion mobility of sodium complexes, and their overall cross-sectional areas are experimentally significantly different as evaluated by ion mobility spectrometry.

In the same way, all the 20 monosaccharide methyl glycosides can be classified as 26 pairs of epimers depending on the asymmetry at carbons 2, 3 and 4. For each α and β anomic configuration, C-2 epimers include the pairs of Tal and Gal, Man and Glc, Ido and Gul and Alt and All; C-3 epimers include the pairs of Tal and Ido, Man and Alt, Gul and Gal and Glc and All; C-4 epimers include pairs of Tal and Man, Ido and Alt, Glc and Gal, Gul and All and GalNAc and GlcNAc. Six out of eight pairs of C-2 epimers, five out of eight pairs of C-3 epimers and eight out of ten pairs of C-4 epimers were baseline/fully separated using DTIMS. For TWIMS, five pairs of C-2 epimers demonstrated good separation and eight pairs of C-4 epimers were baseline or partially differentiated, however, only α-Glc and α-All showed significant separation for C-3 epimers. Figure 5 shows the overlaid 2D IMMS plots and overlaid 1D IMS plots for 4 representative epimeric pairs including (a) C-2 epimers of β-Glc and β-Man; (b) C-3 epimers of α-Gal and α-Gul; (c) C-4-epimers of β-All and β-Gul; (d) C-4 epimers of α-GlcNAc and α-GalNAc. Specific epimeric carbons are highlighted by blue dots. These four epimeric pairs having the same m/z were all fully separated on the mobility scale in the DTIMS system. In the TWIMS system, separation between epimeric pairs of β-Glc and β-Man, β-All and β-Gul, and α-GlcNAc and α-GalNAc was observed, although peaks were partially overlapping. There was a little separation between α-Gal and α-Gul in TWIMS. The higher separation degree and resolution for these 4 epimeric pairs in the DTIMS compared to the TWIMS system is apparent. Clear separation was achieved when glycosides varied solely in the stereochemistry at one carbon as shown in Figure 5, for many epimeric pairs. It can therefore be concluded that
metal ion coordination of carbohydrates as characterized by ion mobility measurements in the
gas phase is exquisitely sensitive to changes in the saccharide stereochemistry. The
stereochemistry at positions C-1, C-2, C-3 and C-4 all influence complexation with the metal ion
as measured by ion mobility and evidently contribute to the shapes and overall conformations of
the sugar complexes.

Separation of these compounds as mixtures was also considered an important point to
demonstrate. Selected mixtures of different isomers having equal concentrations of 10 μM were
examined and it was demonstrated that the same mobilities were observed in the mixture as seen
with the compounds run individually (see Figures S-1 and S-2 in supporting information).

\[K_o, \Omega \text{ and } \alpha \text{ Calculations}\]

The parameters of drift time \((t_d \text{ and } t'_d)\), reduced mobility \((K_o)\), collision cross section \((\Omega)\)
and separation factor \((\alpha)\) for the anomeric pairs of all 20 monosaccharides are displayed in Table
1. Reproducibility was checked with selected individual samples (see Figures S-3 and S-4 in
supporting information), indicating variations in \(K_o\) of ±0.01 (cm\(^2\) V\(^{-1}\) S\(^{-1}\)) on the DTIMS system;
Drift time variation is ± 0.03 ms on the TWIMS system. The reported \(K_o\) and \(\Omega\) values were
determined directly from the experimental data obtained from the DTIMS. Ambient pressure in
Pullman, Washington ranged from 690 Torr to 700 Torr during this study. As listed, \(K_o\) values
ranged from 1.53 to 1.28 cm\(^2\) V\(^{-1}\) S\(^{-1}\) with corresponding \(\Omega\) values ranging from 127 to 152 Å\(^2\)
for all 20 methyl glycoside structural isomers. Values of \(\alpha^{(a)}\) were calculated by using the ratio
of \(K_o(2)/K_o(1)\) (where 2 is the ion having the higher \(K_o\), and 1 is the ion having the lower \(K_o\)). This
ratio accounts for any pressure and temperature influence on the variation of drift time values.
Values of \(\alpha^{(b)}\) were obtained from the ratio of \(t_d'\) values obtained using the TWIMS system. Even
though, as shown in Figure 4, some isomeric mobility peaks were better separated in the DTIMS system as compared to TWIMS, the separation factors from the two systems for the same anomeric pairs generally matched with little variation. TWIMS had similar α values in most of the cases or even higher α values in some cases (such as between α- and β-Gul and α- and β-Glc) compared to DTIMS. In the same way, α between any pairs of monosaccharide structural isomers could also be calculated and compared. However, the data reported in Table 1 were determined using one set of experimental conditions on each system and the variation of α values between the two instruments could be observed under a range of different experimental conditions.

A plot of Ω vs. $t_d'$ is shown in Figure 6 by using the data in Table 1. Two points overlapped; therefore, only 18 points are observable in the figure. Fitting the data using the power law expression given earlier, a curve with the relationship $\Omega = 73.06(t_d')^{0.59}$ and $R^2 = 0.91$ is found. Good correlations have been observed previously for TWIMS calibration curves of this type in a wide range of applications. However, in reality the isomeric monosaccharide methyl glycosides do not serve as particularly good calibrants since they all share $m/z$ values that were too close and as shown in Figure 1(b) and 1(c), very similar drift times hence only cover an extremely small drift time region (mainly distributed between 2.6-3.0 ms), which could contribute to the relatively low $R^2$ value. In addition, it was observed that some isomers were distributed in vertical or horizontal patterns in Figure 6, which means that some specific isomers were separable in one mobility system (different Ω values or $t_d'$ values), while not in the other (same $t_d'$ values or Ω values). The TWIMS and DTIMS techniques, while similar, have fundamental differences in the way they separate ions such as electric field homogeneity, temperature and pressure, thus some differences in resolution is not unexpected.
Conclusions

This study demonstrated the separation of 20 structural isomers of monosaccharide methyl glycosides using two different ion mobility instruments: ESI-AP-DTIM-TOFMS (DTIMS) and the Synapt G2 (TWIMS). It was shown that stereoisomers of methyl glycopyranosides exhibited different mobilities and although some of them were well resolved, some isomeric pairs showed overlapping peaks. Using only one separation gas with each instrument and only one alkaline earth metal, sodium, it was possible to baseline separate a number of isomeric compounds having only subtle structural differences. Coordination strength, -OH and -OCH$_3$ group configurations and coordination geometry induced by Na$^+$ adduction all influenced ion mobility drift times of the different sugar stereoisomers. Different drift gases and metal ions$^{30,59}$ may be needed to resolve other pairs of isomers in future studies. As expected, the DTIMS system provided higher resolving powers than the TWIMS system but the separation factors for anomeric pairs between the two instruments were similar. Even though there were many similarities between the separations using DTIMS and TWIMS, some differences were observed.

Acknowledgements

This work was supported in part by the National Institutes of Health with Grant # 5R33RR020046. Hilary Major (Waters, Manchester) is thanked for help in obtaining the Synapt G2 data.
References


Table 1. Drift times ($t_d$ and $t_d'$), reduced mobilities ($K_o$), collision cross sections ($\Omega$) and separation factors ($\alpha$) for the sodiated adducts of all 16 structural isomers of Me-D-glycopyranosides and 4 structural isomers of methyl-N-acetylhexosamines employing ion mobility mass spectrometry.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_d$ (a) (ms)</th>
<th>$t'_d$ (b) (ms)</th>
<th>$K_o$ (a)</th>
<th>$\alpha$ (a)</th>
<th>$\alpha$ (b)</th>
<th>$\Omega$ (a) ($\text{Å}^2$)</th>
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<tbody>
<tr>
<td>$\beta$-Tal</td>
<td>22.24</td>
<td>2.70</td>
<td>1.48</td>
<td>1.03</td>
<td>1.02</td>
<td>131.34</td>
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<tr>
<td>$\alpha$-Tal</td>
<td>21.40</td>
<td>2.66</td>
<td>1.53</td>
<td>1.03</td>
<td>1.02</td>
<td>126.98</td>
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<tr>
<td>$\beta$-Man</td>
<td>22.81</td>
<td>2.77</td>
<td>1.45</td>
<td>1.02</td>
<td>1.01</td>
<td>134.01</td>
</tr>
<tr>
<td>$\alpha$-Man</td>
<td>23.34</td>
<td>2.81</td>
<td>1.42</td>
<td>1.02</td>
<td>1.01</td>
<td>136.80</td>
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<tr>
<td>$\beta$-Ido</td>
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<td>1.47</td>
<td>1.01</td>
<td>1.00</td>
<td>132.19</td>
</tr>
<tr>
<td>$\alpha$-Ido</td>
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<td>22.33</td>
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<td>1.00</td>
<td>1.03</td>
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<td>1.03</td>
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<td>2.96</td>
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<td>1.04</td>
<td>139.83</td>
</tr>
<tr>
<td>$\alpha$-Glc</td>
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<td>1.37</td>
<td>1.02</td>
<td>1.04</td>
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<tr>
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<td>1.04</td>
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</tr>
<tr>
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<td>1.46</td>
<td>1.05</td>
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<td>1.33</td>
<td>1.01</td>
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<td>145.05</td>
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<tr>
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<td>1.28</td>
<td>1.00</td>
<td>1.00</td>
<td>151.84</td>
</tr>
<tr>
<td>$\alpha$-GlcNAc</td>
<td>25.61</td>
<td>3.47</td>
<td>1.28</td>
<td>1.00</td>
<td>1.00</td>
<td>151.84</td>
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</table>

Note: a: data obtained or derived from ESI-DTIM-TOFMS (DTIMS).
  b: data obtained or derived from ESI-TWIM-TOFMS (TWIMS).
Figure 1. (a) Structures and nomenclature for the 16 methyl-D-glycopyranosides and 4 methyl-N-acetylhexosamines used in this study. (b) The mass spectrum of α-Me-glucopyranoside as a [M+Na]^+ adduct at m/z 217. The other 15 methyl-D-glycopyranosides showed essentially the same mass spectrum. (c) The mass spectrum of α-Me-N-acetyl glucosamine as a [M+ Na]^+ adduct at m/z 258. The other 3 methyl-N-acetylhexosamines showed essentially the same mass spectrum. Sugar carbons are numbered as illustrated.
**Figure 2.** (a) Overall mobility spectra of 16 structural isomers of methyl-D-glycopyranosides obtained on the AP-DTIM-TOFMS instrument. (b) Overall mobility spectra of the same 16 structural isomers collected using the Synapt G2 TWIMS instrument. All mobility spectra were extracted for sodiated ions [M+Na]^+ having m/z 217.
**Figure 3.** (a) Overall mobility spectra of four structural isomers: α- and β-methyl-N-acetyl glucosamine and α- and β-methyl-N-acetylgalactosamine obtained on the AP-DTIM-TOFMS. (b) Overall mobility spectra of the same four structural isomers collected using the WATERS Synapt G2 instrument. All mobility spectra were extracted for sodiated ions [M+Na]$^+$ having $m/z$ 258.
**Figure 4.** Overlaid two-dimensional IMMS plots (top of each panel, from DTIMS) and IMS plots (1-D data from TWIMS, bottom of each panel) of selected pairs of anomeric methyl glycopyranosides: (a) Overlaid α- and β-methyl-talopyranosides; (b) Overlaid α- and β-methyl-mannopyranosides; (c) Overlaid α- and β-methyl-altropyranosides; (d) Overlaid α- and β-methyl-allopyranosides. All the β configurations are in red color.
Figure 5. Overlaid two-dimensional IMMS plots (top of each panel, from DTIMS) and IMS plot (1-D data from TWIMS, bottom of each panel) of selected pairs of epimeric methyl glycopyranosides: (a) Overlaid C-2 epimers: β-methyl-glucopyranoside and β-methyl-mannopyranoside; (b) Overlaid C-3 epimers: α-methyl-galactopyranoside and α-methyl-gulopyranoside; (c) Overlaid C-4 epimers: β-methyl-allopyranoside and β-methyl-gulopyranoside; (d) Overlaid 4-epimers of methyl-N-acetylhexosamines: α-methyl-N-acetylglucomamine and α-methyl-N-acetylgalactosamine. All the slower drifting (longer drift time) epimeric ions are shown in red.
Figure 6. Plot of $\Omega$ vs. $t_d'$ for TWIMS using $\Omega$ values of 20 monosaccharide methyl glycosides structural isomers calculated from DTIMS (y axis) and their corrected drift time values derived from TWIMS (x axis).
Supporting Information for Chapter Two

House Built ESI

The ESI solvent was introduced with a Chemyx Fusion 200 syringe pump (Chemyx Inc. Stafford, TX) into a 20 cm long, 100 μm i.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary was then connected to another 7 cm long, 100 μm i.d. silica capillary tube via a zero dead volume stainless steel union (Valco Instruments Co. Inc., Houston, TX) where a 14.5 KV ESI voltage was applied (3.5 KV greater than the voltage on the first ring of the IMS). The other end of the 7 cm electro-spray capillary tube was positioned on the central transmission axis of the DTIMS tube.

Fisher-Type Glycosidation

Hexoses (100 mg) were dissolved in 10 mL dry methanol by heating to about 90 ºC for 5-15 min. The sample was cooled to room temperature, trifluoroacetic acid (Aldrich) was added to 1 M, and the mixture was heated in a Teflon-capped tube under argon to 105 ºC for 26 h. The sample was concentrated under high vacuum and taken up in 0.5 mL water followed by 5 mL of acetonitrile. Methyl glycosides of all four anomic configurations and ring forms were separated by normal phase HPLC. First, separation was carried out for all samples in multiple batches of about 2-3 mg/batch, on a column of Glyco-PAK N (0.78 x 30 cm, Waters), eluted with 95/5 v/v acetonitrile (Mallinckrodt, ChromAR grade)/water at 1.0 mL/min. Detection was with UV at 200 nm using a Waters 486 variable wavelength detector. Each fraction was concentrated and analyzed by NMR at 500 MHz (described below). Two methyl glycosides (the β-pyranosides of
altrose and talose) contained isomeric contaminants and were further fractionated by normal
phase separation on a Shodex DC-613 column (0.6 x 15 cm) that had been converted to the Cs+
form, eluted with 92/8 v/v acetonitrile/water at 0.6 mL/min, detecting at 200 nm. All fractions
were concentrated and analyzed by NMR using a Varian Inova instrument located at the
University of Colorado Health Sciences Center. Spectra were acquired in D$_2$O at 25ºC with a
trace of acetone as an internal chemical shift standard at $\delta = 2.225$ ppm and are reproducible to
within 0.002 ppm. $^1$H-$^1$H $J$-couplings are accurate to within 0.2 Hz. Assignments were made
through decoupling experiments in one dimension or though 2D COSY correlations. In all cases
except for the $\beta$-methyl idopyranoside where the anomeric H-1 nearly overlapped with the HOD
peak near $\delta = 4.77$ ppm, a presaturation pulse was applied to the HOD peak to diminish its
intensity.
Table S-1. Synapt G2 (TWIMS) instrumental parameters

<table>
<thead>
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<th>Gas flow rate (mL/min)</th>
<th>Triwave</th>
<th>Triwave DC</th>
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</thead>
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<tr>
<td>Helium (He) 140</td>
<td>Trap</td>
<td>Entrance 3</td>
</tr>
<tr>
<td>IMS (N₂) 90</td>
<td>Wave velocity 311 m/s</td>
<td>Trap DC (V)</td>
</tr>
<tr>
<td>Trap gas (Ar) 2.0</td>
<td>Wave height 6 V</td>
<td>Bias 45</td>
</tr>
<tr>
<td>IMS</td>
<td>Wave velocity 900 m/s</td>
<td>Exit 0</td>
</tr>
<tr>
<td>Wave height 40 V</td>
<td>IMS</td>
<td>Entrance 25</td>
</tr>
<tr>
<td>Wave height 4 V</td>
<td>DC (V)</td>
<td>Helium cell 35</td>
</tr>
<tr>
<td>Mobility separation delay after trap release was enabled and set at 450 μs.</td>
<td>Transfer DC (V)</td>
<td>Helium exit -5</td>
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<tr>
<td></td>
<td>Entrance</td>
<td>Bias 3</td>
</tr>
<tr>
<td></td>
<td>Exit 15</td>
<td></td>
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</table>

Table S-2. Drift times \( t_d \) of [M+H]⁺ ions of certain monosaccharide structural isomers

<table>
<thead>
<tr>
<th>Compound</th>
<th>( t_d ) (ms) of [M+H]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Me-Man</td>
<td>22</td>
</tr>
<tr>
<td>α-Me-Man</td>
<td>22</td>
</tr>
<tr>
<td>α-Me-Ido</td>
<td>21.3</td>
</tr>
<tr>
<td>β-Me-Gul</td>
<td>21.3</td>
</tr>
<tr>
<td>α-Me-Gul</td>
<td>21.3</td>
</tr>
<tr>
<td>β-Me-Glc</td>
<td>21.7</td>
</tr>
<tr>
<td>α-Me-Glc</td>
<td>22</td>
</tr>
<tr>
<td>β-Me-Gal</td>
<td>22</td>
</tr>
<tr>
<td>α-Me-Gal</td>
<td>22</td>
</tr>
<tr>
<td>β-Me-All</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Note: The data were observed on DTIMS, no reasonable mobility peaks were detected for the rest of samples. In addition, for the data acquired on TWIMS, the predominant ions observed were [M+Na]⁺ ions, [M+H]⁺ ions were all at extremely low abundance.
Figure S-1. 2D Mass mobility spectra of selected mixtures obtained from DTIMS. Data shown include 2 anomer pairs α- & β-Me-Alt and α- & β-Me-Allo; 2 epimer pairs α-Me-Allo & -Alt and α-Me-GalNAc & -GlcNAc; 2 pairs of random mixtures β-Me-Glc & -Tal and β-Me-Man & α-Me-Gal.
**Figure S-2.** 2D Mass mobility spectra of selected mixtures obtained from TWIMS. Data include one epimer pair β-Me-GalNAc & -GlcNAc; one random mixture β-Me-Man & α-Me-Glc.

**Figure S-3.** 2-D Overlaid mass mobility spectra of selected compounds showing the data reproducibility on DTIMS. The examples include β-Me-Tal, β-Me-Ido, α-Me-Gul, β-Me-Gal, α-Me-GlcNAc and α-Me-GalNAc. (Black traces are the data reported in the paper, red traces are the reproduced data).
Figure S-4. 2-D Overlaid mass mobility spectra of selected compounds showing the data reproducibility on TWIMS. Individual samples include β-Me-Man, β-Me-Ido, α-Me-Allo, β-Me-Gal and α-Me-GalNAc (Black traces are the data reported in the paper, red traces are the reproduced data).
CHAPTER THREE
ION MOBILITY MASS SPECTROMETRY ANALYSIS OF ISOMERIC DISACCHARIDE PRECURSOR, PRODUCT AND CLUSTER IONS

Abstract

The isomeric heterogeneity of disaccharide precursor and product ions was evaluated using traveling wave ion mobility mass spectrometry in both positive and negative ion modes. Separation was observed but not fully resolved for all the disaccharide isomers based on the mobility profiles of the precursor ions. 2-, 4- and 6-linked disaccharides dissociated to give the structurally informative product ions previously shown to be monosaccharide-glycolaldehydes. The mobilities of these product ions derived from different disaccharide isomers were measured and compared. Multiple mobility peaks were observed for both monosaccharide-glycolaldehyde cations and anions, which indicated additional stereoisomers as verified by NMR. More importantly, the mobility patterns for isomeric monosaccharide-glycolaldehyde product ions were different, which enabled identification and separation of their respective disaccharide precursor ions. Representative synthetic monosaccharide-glycolaldehyde standards were subjected to traveling wave ion mobility examination as well, where their drift time values matched with the corresponding product ions derived from disaccharides. Additionally, disaccharide cluster ions were observed in abundance. They were the [2M+Na]+ ion in the positive mode and [2M-H]- and [3M-H]- ions in the negative mode. The mobility profiles of these cluster ions could also serve as an additional identifier for individual carbohydrate isomers. Results showed that a majority of isomeric cluster ions had different drift times and moreover,
more than one mobility peak was detected for a number of specific cluster ions. Anion effects, using chloride adducts as examples, on the influence of mobility separation was investigated for disaccharide precursor as well as the corresponding chloride adducted cluster ions detected at the same time.

**Introduction**

Carbohydrates are involved in numerous biological activities through their interaction with proteins. More than half of human proteins are glycosylated. Unlike proteins and nucleic acids, carbohydrates are indirect products of gene expression and are biosynthesized through a consecutive series of enzymes. Sequencing methods developed for the proteome and genome cannot be applied for studies of carbohydrate structure. Their structural diversity and heterogeneity arise from the large number of isomers possible, varying in anomeric configuration, and differing in linkage positions and branching. Differentiation of isomeric carbohydrate species poses a grand challenge for analytical techniques.

While mass spectrometry (MS) is extensively used for carbohydrate structure analysis, it lacks the ability to separate ions having identical m/z values. Even with multiple isolation/dissociation steps carried out on tandem MS instruments, fragmentation spectra resulting from isomeric precursor mixtures complicate product ion patterns and limit absolute structure identification of unknown saccharides. Liquid and gas chromatography are the most common separation methods employed for isomer differentiation prior to mass spectral analysis, but both of these methods limit sample throughput, are complex with multiple analytical sample preparation steps and can be difficult to reproduce with precision among laboratories. Because stationary phases of chromatographic columns differ from batch to
batch it is not possible to assign absolute retention times to specific saccharides; even relative retention times vary from column to column. Moreover, sample derivatization, especially for gas chromatography, is often needed to enhance volatilization, but can decrease resolution in complex mixtures and is limited in molecular size. One approach to decrease analysis time, increase resolving power and improve reproducibility has been to incorporate ion mobility spectrometry (IMS) as the separation process prior to mass spectrometry. Rather than differences in equilibrium between two phases, IMS separation is based on the physical size of the ion providing hard experimental data related to the structure of the ion. Similar to mass spectrometry where ion separation is based on the mass-to-charge ratio \( (m/z) \) of the ion, in IMS separation is based on the size-to-charge ratio \( (\Omega/z) \) where size is measured as the ion’s cross section, \( \Omega \). An ion’s cross sections is characteristic of an ion’s size and can be measured reproducibly from time-to-time, instrument-to-instrument and laboratory-to-laboratory. As with mass, cross section is an intrinsic property of an ion and the buffer gas through which the ion migrates. When the separation capability of IMS is combined with mass identification, stereostructural differences of carbohydrate compounds can be resolved by IMMS. IMMS has been applied to the separation and detection of various complex biological samples in the areas now delineated as metabolomics, proteomics and glycomics.

Collision-induced dissociation of mass-selected glycan ions is often used to aid in the identification of the parent ion. During CID processes, dissociation at glycosidic bonds and/or cross ring cleavages generate substructures such as monosaccharides, the most basic unit, and disaccharides, the smallest substructure still containing a linkage between two sugars. Many of these fragment ions are isomers and their stereochemistry cannot be distinguished by mass spectrometry. Knowing the cross section of these fragment ions will aid in determining their
stereochemistry, leading to the overall identification of the parent oligosaccharide. It has been demonstrated that isomeric monosaccharide methyl glycosides with subtle structure differences can be distinguished using IMMS. Ion mobility differentiation of disaccharide isomers have also been shown. However, current applications of IMMS to the separation of small carbohydrate structural isomers have been mainly focused on precursor ions. Ion mobility methods for identifying isomeric carbohydrate product ions derived from isomeric precursor ions have not been investigated.

Bendiak and colleagues have reported that 2-, 4- and 6-linked disaccharides dissociated to generate product ions of monosaccharide-glycolaldehydes. According to their findings, the fragmentation spectra of monosaccharide-glycolaldehyde anions were dependent on their stereochemistries, which enabled the anomeric configurations and stereochemistries of 20 isomeric variants to be assigned (all hexopyranoses and common N-acetylhexosamines). However, should these ions be derived from two or more branches in a larger branched oligosaccharide, then two or more monosaccharide-glycolaldehyde isomers might well be present as an isomeric mixture, which would confound their assignments solely based on dissociation patterns. Furthermore, these compounds were found to have up to three structural variants in aqueous solution as assessed by NMR, an open-chain form and two hemiacetals with cyclization of the aldehyde with the OH-2. However, to date, no information has been presented as to whether more than one structure of these molecules exists in the gas phase, either for negative or positive ions. In this study, we use 2-, 4- and 6-linked disaccharides as representative examples and employ traveling wave ion mobility mass spectrometry to evaluate the isomeric heterogeneity of their precursor ions as well as the structurally informative product ions, monosaccharide-glycolaldehydes, in both positive and negative mode.
Experimental Section

Chemicals and Solvents

Disaccharide isomers investigated in this study include D-Glc-\(\alpha\)-(1-2)-D-Glc, D-Glc-\(\beta\)-(1-2)-D-Glc, D-Glc-\(\alpha\)-(1-4)-D-Glc, D-Glc-\(\beta\)-(1-4)-D-Glc, D-Gal-\(\beta\)-(1-4)-D-Glc, D-Gal-\(\alpha\)-(1-6)-D-Man, D-Gal-\(\alpha\)-(1-6)-D-Glc and D-Gal-\(\beta\)-(1-6)-D-Gal (Glc denotes glucose, Gal denotes galactose and Man denotes mannose). Their structures and nomenclature are shown in Table 1. All the disaccharides are reducing sugars (having an -OH group at the anomeric carbon in more than one stereochemical configuration or ring form) and had either 2-, 4- or 6-linkages. Also contained in Table 1 are eight monosaccharide-glycolaldehyde synthetic standards. There are four isomeric Hex-glycolaldehydes (M. W. 222) including \(\alpha\)-D-glucopyranosyl-2-glycoaldehyde (\(\alpha\)-D-Glc-GA), \(\beta\)-D-glucopyranosyl-2-glycoaldehyde (\(\beta\)-D-Glc-GA), \(\alpha\)-D-galactopyranosyl-2-glycoaldehyde (\(\alpha\)-D-Gal-GA) and \(\beta\)-D-galactopyranosyl-2-glycoaldehyde (\(\beta\)-D-Gal-GA). The counterparts to these ions containing the 2-acetamido-2-deoxy group were the HexNAc-glycoaldehydes (M. W. 263) including (2-acetamido-2-deoxy-\(\alpha\)-D-glucopyranosyl)-2-glycoaldehyde (\(\alpha\)-D-GlcNAc-GA), (2-acetamido-2-deoxy-\(\beta\)-D-glucopyranosyl)-2-glycoaldehyde (\(\beta\)-D-GlcNAc-GA), (2-acetamido-2-deoxy-\(\alpha\)-D-galactopyranosyl)-2-glycoaldehyde (\(\alpha\)-D-GalNAc-GA) and (2-acetamido-2-deoxy-\(\beta\)-D-galactopyranosyl)-2-glycoaldehyde (\(\beta\)-D-GalNAc-GA) were examined by IMMS as well. Dissociation of 2-, 4- and 6-linked disaccharides yield product ions of monosaccharide-glycolaldehydes. A dissociation scheme having D-Glc-\(\alpha\)-(1-2)-D-Glc as a precursor ion and \(\alpha\)-Glc-GA as a product ion is shown at the bottom of Table 1. It was demonstrated that this type of product ion is composed of an intact non-reducing sugar and a two carbon aglycon derived from the reducing sugar, for 2-, 4- and 6-linked disaccharides. All the disaccharides and NH\(_4\)Cl were purchased from Sigma Chemical Co. (St. Louis, Missouri)
and used without modification. Monosaccharide-glycoaldehyde standards were synthesized as described previously.\(^9,50,51\) LC-MS grade solvents of methanol and water were purchased from Thermo Fisher Scientific Inc. and equal volume mixture of methanol and water was used as electrospray ionization (ESI) solvent. A final concentration of 10 \(\mu\)M was prepared for all the sugar samples using ESI solvent for the positive mode study and the concentration was increased to 30 \(\mu\)M for negative mode measurements. In order to investigate the effects of chloride adduction on the influence of ion mobility separation for disaccharides, 4 \(\mu\)L of a stock solution of \(\text{NH}_4\text{Cl}\) (10 mM in ESI solvent) was added to 1 mL of a 20 \(\mu\)M disaccharide solution to enhance the formation of chloride adducts, resulting in a molar ratio of salt: sugar of 2:1 in each sample. Due to the potential reaction between ammonia and aldehyde group of reducing sugars to form Schiff base, the samples containing \(\text{NH}_4\text{Cl}\) were prepared freshly prior to analysis.

**Ion Mobility Mass Spectrometry Measurements**

All the experiments were performed on a Synapt G2 high definition mass spectrometer (HDMS) (Waters Corp., Manchester, UK). It is a hybrid quadrupole/ion mobility/orthogonal time of flight MS. The traveling wave ion mobility device that is incorporated into the instrument employs dynamic inhomogeneous electric fields under reduced pressures. The effects on mobility of these unique instrumental parameters have been thoroughly described.\(^{52-54}\) A trap and a transfer cell are located in front of and after the ion mobility separator, respectively. Collision induced dissociation (CID) can be initiated in either or both cells by elevating the collision energy (CE). A wave height of 40 V and a wave velocity of 650 m/s were utilized for ion mobility separation in both positive and negative modes. Nitrogen was used as a drift gas at a flow rate of 90 mL/min, resulting in a pressure of \(~3.5\) mbar in the ion mobility device. Two
types of experiments were performed in this study. (1) To obtain mobility profiles of precursor and the corresponding cluster ions formed, no CE were used in the trap and transfer cells. All the ions pass through the trap cell and were evaluated by traveling wave IMS directly and then transferred to the TOF mass analyzer. (2) To investigate the mobility of product ions derived from disaccharides, a specific disaccharide precursor ion was first selected by the quadrupole, and appropriate CE was applied in the trap cell to induce fragmentation. Then, mobility separation of all the product ions occurred sequentially in the traveling wave ion mobility unit of the instrument and all the ions then travelled through the transfer cell and were measured by the TOF analyzer. ESI voltages were 3.2 KV and 2.25 KV for the positive and negative ion modes, respectively. Nitrogen was used as the desolvation gas at 200°C with a flow rate of 600 L/hr. Samples were injected using a syringe pump (Chemyx Inc., Stafford, TX) at a flow rate of 3 μL/min and the data was acquired for 3 min for all the analytes. Masslynx V4.1 (Waters Corp., Manchester, UK) was used to collect and process the data. More instrumental parameters are summarized in Table S-1 in supporting information.

Results and Discussion

Separation of Isomeric Monosaccharide-glycolaldehyde Standards

Monosaccharide-glycolaldehyde product ions are structurally informative and their fragmentation spectra can be used to assign/determine the stereochemistries of 20 structural variants. Fig. 1 displays the traveling wave ion mobility spectra of the monosaccharide-glycolaldehyde standards used in this study. The inserted window accompanying each compound represents the spectrum with the intensity scale magnified as labeled. Fig. 1a shows the mobility profiles of sodiated adducts of α-Glc-GA, β-Glc-GA, α-Gal-GA and β-Gal-GA at m/z 245.1 in
the positive mode. The major mobility peak for these isomers showed identical drift times at 2.45 ms. However, multiple mobility peaks were detected for all individual monosaccharide-glycolaldehydes, for example, three mobility peaks were observed for β-Glc-GA and β-Gal-GA with drift time values of 2.45 ms, 2.6 ms and 2.8 ms, and two mobility peaks were seen for α-Glc-GA and α-Gal-GA at 2.45 ms and 2.8 ms. This indicates that there are more than one gas-phase stereo-structures for each Hex-glycolaldehyde. As mentioned, monosaccharide-glycolaldehydes can exist in multiple isomeric acyclic / cyclic forms in the gas phase through internal hemiacetal formation between the glycolaldehyde carbonyl group and the sugar 2-hydroxyl group, which is supported by NMR spectroscopy of the compounds in aqueous solution. The mobility spectra of Hex-glycolaldehyde anions at m/z 221.1 are shown in Fig. 1b. There was no obvious resolution among isomeric Hex-glycolaldehyde anions and more than one mobility peak was found for all standards. Fig. 1c displays the ion mobility spectra of [M+Na]+ ion of HexNAc-glycoaldehydes at m/z 286.1. Partial separation was achieved for epimer pairs such as α-GlcNAc-GA and α-GalNAc-GA, but no differentiation was detected for anomer pairs of α-β-GlcNAc-GA and α-β-GalNAc-GA. Again, more than one mobility peak was detected for single compounds; two baseline separated mobility peaks were observed for α-β-GlcNAc-GA (2.9 & 3.2 ms), two partially resolved peaks were found for α-β-GalNAc-GA (2.8 & 3.0 ms). Mobility evaluation of HexNAc-glycolaldehydes as [M-H]- ions at m/z 262.1 is presented in Fig. 1d. Beside the predominant mobility peak, an additional ion mobility peak having extremely low intensity was partially resolved from the major peak for specific m/z 262.1 ions. For HexNAc-glycolaldehydes, better ion mobility separation and sensitivity especially for the low abundance structural variants were obtained using cations in comparison to anions. Overall, ion mobility showed its unique separation capability to evaluate the isomeric heterogeneity of these
monosaccharide-glycolaldehyde ions in the gas phase; multiple structural configurations were resolved on the millisecond timescale. This is impossible to assess by typical mass spectrometric techniques, although other gas-phase spectroscopy techniques may be able to provide some information about isomeric components.

*Isomeric Disaccharide Precursor and Product Ion Separations*

Fig. 2a displays the traveling wave ion mobility spectra of isomeric disaccharide precursor ions of D-Glc-α-(1-2)-D-Glc, D-Glc-β-(1-4)-D-Glc, D-Gal-α-(1-6)-D-Glc and D-Gal-β-(1-4)-D-Man, respectively in the positive mode as [M+Na]⁺ ions at m/z 365.1. Identical drift times (3.5 ms) were observed for D-Glc-α-(1-2)-D-Glc, D-Glc-β-1-4-D-Glc and D-Gal-α-(1-6)-D-Glc precursor ions; their collision cross section variations were too small to be differentiated by IMS. A drift time value of 3.4 ms was obtained for D-Gal-β-(1-4)-D-Man, making it partially resolved from other isomers. The traveling wave ion mobility resolving power obtained was ~30-40 in this study and the separation can be improved by using IMS with higher resolving power. Mobility separation of the monosaccharide-glycolaldehyde product ions of α-D-Glc-GA, β-D-Glc-GA, α-D-Gal-GA and β-D-Gal-GA, derived from disaccharide isomers in Fig. 2a, are shown in Fig. 2b. This was achieved by selecting specific precursor ions using the quadrupole and applying 30V collision energy (CE) in the trap cell prior to the traveling wave ion mobility measurement. More than one mobility peak was observed for the majority of sodiated monosaccharide-glycolaldehyde product ions at m/z 245.1 and the drift time values matched with their respective synthetic standards shown in Fig. 1a. However, some mobility peaks of low abundance were not observed for m/z 245.1 product ions in comparison to the mobility spectra acquired from standards, for example, isomeric mobility peaks at 2.8 ms were missing for
product ions of $\alpha$-Gal-GA and $\beta$-Gal-GA. This was probably due to overall low intensity (~thousand counts) obtained for them, making the structural variants with even lower abundance not detectable. It is also possible that the ratio of stereoisomers of monosaccharide-glycolaldehydes obtained directly after dissociation of a disaccharide in the gas phase may be different than that resulting from electrospray of the standards already equilibrated in aqueous solution, for a number of reasons. Although little separation was achieved for the major peak of different isomeric monosaccharide-glycolaldehyde product ions, the mobility patterns of this specific product ion, derived from different disaccharide isomers, were different, which enabled the separation of these isomeric product ions. For instance $\alpha$-D-Glc-GA and $\beta$-D-Glc-GA (Fig 2b) showed distinct patterns, one with a more abundant secondary peak near 2.8 ms and one with a secondary peak near 2.6 ms. It is worthy of note that only four selected disaccharides were shown in all the figures through the manuscript and corresponding data for other disaccharide compounds are included in the supporting information.

Isomeric disaccharides were also examined by traveling wave IMMS in the negative ion mode. The mobility spectra of D-Glc-\(\alpha\)-(1-2)-D-Glc, D-Glc-\(\beta\)-(1-4)-D-Glc, D-Gal-\(\alpha\)-(1-6)-D-Glc and D-Gal-\(\beta\)-(1-4)-D-Man as [M-H]$^-\$ precursor ions at m/z 341.1 are shown in Fig. 3a. The four disaccharide isomers were not fully differentiated, however, two partially resolved mobility peaks were observed for D-Glc-\(\alpha\)-(1-2)-D-Glc and D-Gal-\(\beta\)-(1-4)-D-Man. This could be attributed to the reducing end of disaccharides, where the -OH group at the anomeric carbon could exist in either the $\alpha$ or $\beta$ pyranose configuration or even the aldehyde open chain form, resulting in multiple isomeric forms for a single molecule to be resolved by IMS. In this study, Hex-glycolaldehyde product anions were observed in abundance along with the precursor ions without any CE applied (see mass spectrum in Fig. 4b below). This auto-fragmentation was
observed for all the disaccharide isomers and matched with previous findings that disaccharides having 2-, 4-, and 6-linkages yield monosaccharide-glycolaldehyde product anions frequently. These product anions evidently resulted from in-source fragmentation. Fig. 3b displays the mobility spectra of [M-H]− product ions of α-D-Glc-GA, β-D-Glc-GA, α-D-Gal-GA and β-D-Gal-GA at m/z 221.1 derived from their corresponding precursor ions in Fig. 3a. Two isomeric mobility peaks were detected for all four product anions and the mobility values were consistent with the respective standards in Fig. 1b. The relative abundance between the two configurational states of m/z 221.1 varied among the product ions having different stereochemistries, for example, the two peaks were of approximately equal intensity for β-D-Glc-GA, while the isomer having faster mobility was the predominant peak for the other product ions. Additionally, the drift time was extended to 3.6 ms for product ions of α-D-Glc-GA and α-D-Gal-GA by expanding the intensity scale where this number (3.6 ms) reached the limit of the mobility value of their precursor ions of D-Glc-α-(1-2)-D-Glc and D-Gal-α-(1-6)-D-Glc. This indicates that disaccharide precursor ions dissociated to give monosaccharide-glycolaldehyde product ions spontaneously in the ion mobility drift tube. It also suggests that ion mobility could serve as a potential tool for real time reaction monitoring. Only 2- and 6-linked disaccharides showed the phenomenon of auto-dissociation in the ion mobility drift tube and there was no observable mobility tailing detected for m/z 221.1 ions produced from 4-linked disaccharides. The experimental conditions used were the same, thus besides linkage position, other structural differences among disaccharide isomers may also contribute to the results that were observed. In all, negative ions of isomeric monosaccharide-glycolaldehydes derived from isomeric disaccharide precursor ions showed different mobility patterns, which yielded unique information about their precursor ions.
that is complementary to the direct mass spectral dissociation patterns of the \( m/z \) 221 product ions themselves.\(^9\)

**Mobility Separation of Isomeric Disaccharide Cluster Ions**

In addition to the single molecule precursor ions, disaccharide cluster ions were typically observed at the same time. The mobilities of isomeric disaccharide cluster cations and anions were examined and reported for the first time in this investigation. Fig. 4 shows the mass spectra of the disaccharides D-Glc-\( \alpha \)-(1-2)-D-Glc in the positive mode (Fig. 4a) and D-Gal-\( \alpha \)-(1-6)-D-Glc in the negative mode (Fig. 4b). Beside the major \([M+Na]^+\) ion at \( m/z \) 365.1, a sodiated cluster ion having two disaccharide molecules \([2M+Na]^+\) at \( m/z \) 707.2 was observed. In the negative mode, four related mass peaks were found; they were the precursor ion \([M-H]^−\) at \( m/z \) 341.1, a cluster ion \([2M-H]^−\) at \( m/z \) 683.2 and a cluster ion \([3M-H]^−\) at \( m/z \) 1025.3. All other disaccharides showed the same ion clusters in both the positive and negative ion modes but the relative intensities of different ion species varied. A cluster ion at \( m/z \) 683.2 was detected at higher abundance than the single disaccharide anion at \( m/z \) 341.1; this may be caused in part by preferential in-source fragmentation of \( m/z \) 341.1 ions to give product ions such as the \( m/z \) 221.1 ion as shown in the mass spectrum (Fig. 4b). Sonoda et al.\(^6\) studied the carbohydrate clustering effect in aqueous solution using fructose as an example. They found that carbohydrates tend to form hydrogen bonded clusters on increasing concentration. The concentration of disaccharides in this investigation (10 \( \mu \)M positive mode or 30 \( \mu \)M negative mode) was much smaller than the numbers (1-5 M) reported in Sonada’s study.\(^6\) The formation of disaccharide clusters perhaps happens during the ionization (desolvation) process where the solvents start to evaporate and solutes transit from scattered “isolated” molecules to H-bonded clusters in less diluted solutions.
Moreover, the multiple –OH groups on disaccharides could enhance the formation of cooperative hydrogen-bonded networks as compared to fructose. Cluster ions having up to three of disaccharide molecules were found in the negative mode but not in the positive mode, which could in part be due to the relatively higher concentrations that were used for electrospray of the anions. The observation of cluster ions may be eliminated by decreasing sample concentrations. The mobilities of isomeric disaccharide cluster ions were acquired and are reported here for the first time.

Traveling wave ion mobility spectra of cluster cations and anions, using D-Glc-α-(1-2)-D-Glc, D-Glc-α-(1-4)-D-Glc, D-Glc-β-(1-2)-D-Glc and D-Gal-β-(1-4)-D-Man as examples, are shown in Fig. 5. For the [2M+Na]+ ion cluster at m/z 707.2, central drift time values obtained were 6.4 ms, 6.4 ms, 6.8 ms and 5.7 ms for the different disaccharide isomers displayed in Fig. 5a. Mobility measurements of cluster ions could aid in the differentiation of disaccharide isomers. For example, D-Glc-α-(1-2)-D-Glc and D-Gal-β-(1-4)-D-Man were only slightly separated as sodium adducts based on mobility spectra of single molecule precursor ions (Fig. 2a), but were baseline resolved as their respective cluster cations (6.4 and 5.7 ms, Fig. 5a). In addition, two partially resolved mobility peaks were observed for the dimeric clusters of Gal-β-1-4-Man, indicating that there are at least two isomeric forms / structure variants / stereochemical isomers. In the negative ion mode, based on the mobility spectra of the dimeric [2M-H]− ions at m/z 683.2 (Fig. 5b), D-Gal-β-(1-4)-D-Man having a drift time of 5.9 ms was resolved from the other isomers. Two mobility peaks were detected for D-Glc-β-(1-2)-D-Glc. The mobility profiles of [3M-H]− ions at m/z 1025.3 are shown in Fig 5c, where multiple mobility peaks were frequently observed for specific cluster ions: three clearly separated peaks for D-Glc-α-(1-2)-D-Glc, three barely resolved peaks for D-Glc-α-1-4-D-Glc and two partially distinguished peaks for
D-Glc-β-1-2-D-Glc. This is the first time that the structural heterogeneity of sugar cluster ions, using reducing disaccharides as examples, has been investigated by ion mobility. Multiple mobility peaks for a single cluster ion may result from differences (α, β) at the reducing end of disaccharides as discussed above or different clustering interactions, which refers to the geometry disaccharides adopt to form the clusters through various intermolecular interactions.

In general, more mobility peaks were resolved for disaccharide cluster ions in comparison to the single molecule precursor ions. This may be explained in part by more widely differing sizes and shapes of clusters due to stereochemically-dependent interactions that enable them to be more easily separated by IMS. Mobility data of cluster ions could also serve as a further analytical property for identification of sugar stereo- and linkage isomers.

**Mobility Separation of Disaccharides as Chloride Adducts and Chloride Adduct Clusters**

Disaccharide chloride adducts were observed upon adding an NH₄Cl solution to each sample. The ions observed were the [M+Cl]⁻ ion at m/z 377.1, the [2M+Cl]⁻ ion at m/z 719.1 and the [3M+Cl]⁻ ion at m/z 1061.1. Their representative mass spectra are included in supporting information. The mobility spectra of disaccharide chloride adducts for D-Glc-α-(1-2)-D-Glc, D-Glc-α-(1-4)-D-Glc, D-Glc-β-(1-2)-D-Glc and D-Gal-β-(1-4)-D-Man are displayed in Fig. 6. Partial mobility separation was achieved for [M+Cl]⁻ ions among these isomers as shown in Fig. 6a. An additional mobility peak having faster mobility was detected with very low abundance for all the disaccharides except D-Gal-β-(1-4)-D-Man. In Fig. 6b is shown the ion mobility separation of isomeric [2M+Cl]⁻ ions, where three mobility peaks were partially resolved for both D-Glc-α-(1-4)-D-Glc and D-Glc-β-(1-2)-D-Glc. Compared to the [2M-H]⁻ cluster ions in Fig. 5b, more isomeric forms were differentiated as chloride adduct clusters. However, this scenario was
reversed for [3M+Cl]− ions as shown in Fig. 6c, where one peak was found for all the disaccharides, having drift time values of 8.8 ms, 8.7 ms, 9.0 ms and 8.8 ms, respectively; fewer mobility peaks were differentiated for specific [3M+Cl]− ions in comparison to their respective [3M-H]− ions (Fig.5c). The structural configuration(s) of disaccharide cluster ions induced by the addition of chloride is expected to be different from the cluster ions having lost one proton. The collision cross section area differences among multiple isomeric forms of a single cluster species may become either larger or smaller upon adduction of a Cl− ion as demonstrated by IMS (compare Fig. 5b and c to Fig. 6b and c). In addition, it is worth noting that no separation method has infinite resolution; there is always the possibility that some isomeric structural variants could coincidentally co-elute as one ion mobility peak, such as the broad peak detected for the [3M+Cl]− ion of D-Glc-β-(1-2)-D-Glc (Fig. 6c). The results shown in Fig. 6 indicate that different anions can influence the ion mobility separation of carbohydrate structural isomers, both for single molecules and for their corresponding cluster ions. However, no specific rules can be concluded as the structures of specific clusters are unknown. Further investigations need to be performed on more negative ion forms to more fully understand the mobility characteristics observed here.

**Conclusions**

The isomeric heterogeneity of disaccharides was evaluated as precursor, product and cluster ions using traveling wave ion mobility mass spectrometry in both the positive and negative ion modes. Partial mobility separation was achieved for isomeric precursor ions having the same m/z values but differing in anomeric configurations, linkage positions (2-, 4- and 6-linked), or monomer components. The mobilities of isomeric product ions derived from
disaccharides, the monosaccharide-glycolaldehydes, were measured and compared. More than one isomeric mobility peak was obtained for specific monosaccharide-glycolaldehyde product ions derived from disaccharides, which appear to correspond to multiple structural isomers reported in previous studies.\textsuperscript{9,50,51} Moreover, the mobility distributions of this product ion were different among disaccharide isomers, which enables the separation of isomeric product ions and provides another physical property for characterization of isomeric disaccharides. Both 2- and 6-linked reducing disaccharides were found to partially dissociate to yield Hex-glycolaldehyde anions within the ion mobility drift tube as demonstrated by the mobility spectra. Differentiation of isomeric cluster ions was observed by IMS as well; a majority of cluster ions showed multiple mobility peaks, which may due either to alternate (α, β) reducing end structures of the disaccharides and/or the three-dimensional geometry of their interactions. Furthermore, mobility separation of carbohydrate isomers including precursor and cluster ions could be altered using different anions, such as [M-H]\textsuperscript{−} and [M+Cl]\textsuperscript{−} ions in this study.

With a number of natural non-reducing sugars examined by IMS to date,\textsuperscript{34, 35, 46, 48, 57, 58} they all yielded single IMS peaks. Multiple mobility peaks for a specific saccharide solely happened to a portion of reducing sugars\textsuperscript{34, 46, 58-61} investigated so far, which correspond to multiple stereoisomers caused by the reducing end as confirmed by NMR\textsuperscript{62} or other spectroscopy.\textsuperscript{55} And we have observed that reduction of reducing sugars to their alditols\textsuperscript{34,35,58} and conversion of reducing end to methylated group,\textsuperscript{46,47} where the structures were verified by NMR, have always resulted in a single IMS peak. Thus, it was demonstrated that IMMS is an advantageous method to assess the isomeric heterogeneity, not conformational heterogeneity, of carbohydrate compounds. The analysis is fast, sensitive and convenient, able to resolve isomers and differentiate multiple structural variants within single species.
Acknowledgements

This work was supported in part by the National Institutes of Health with grant # 5R33RR020046.
References


Table 1. Structures and nomenclature for isomeric disaccharides and monosaccharide-glycolaldehydes. The dissociation scheme from D-Glc-α-(1-2)-D-Glc to α-D-Glc-GA is shown at the bottom.

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<th>Disaccharide M. W. 342</th>
<th>Disaccharide M. W. 342</th>
<th>Hex-glycolaldehyde M. W. 222</th>
<th>HexNAc-glycolaldehyde M. W. 263</th>
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Note: Hex denotes hexose, HexNAc denotes N-acetyl hexosamine, Glc denotes Glucose, Gal denotes Galactose and Man denotes mannose.
Figure 1. (a) Traveling wave ion mobility separation (TWIMS) of Hex-glycolaldehyde standards in the positive mode as [M+Na]$^+$ ions at m/z 245.1. (b) TWIMS of Hex-glycolaldehyde standards in the negative mode as [M-H]$^-$ ions at m/z 221.1. (c) TWIMS of HexNAc-glycolaldehyde standards in the positive mode as [M+Na]$^+$ ions at m/z 286.1. (d) TWIMS of HexNAc-glycolaldehyde standards in the negative mode as [M-H]$^-$ ions at m/z 262.1.
Figure 2. (a) TWIMS of isomeric disaccharide precursor ions in positive mode as [M+Na]+ ions at m/z 365.1. The disaccharides displayed were D-Glc-α-(1-2)-D-Glc, D-Glc-β-(1-4)-D-Glc, D-Gal-α-(1-6)-D-Glc, and D-Gal-β-(1-4)-D-Man. (b) TWIMS of Hex-glycolaldehyde product ions derived from disaccharides shown in (a) in positive mode as [M+Na]+ ions at m/z 245.1. The corresponding product ions generated were α-D-Glc-GA, β-D-Glc-GA, α-D-Gal-GA, and β-D-Gal-GA.
Figure 3. (a) TWIMS of isomeric disaccharide precursor ions in negative mode as [M-H]- ions at m/z 341.1. The disaccharides displayed were D-Glc-α-(1-2)-D-Glc, D-Glc-β-(1-4)-D-Glc, D-Gal-α-(1-6)-D-Glc and D-Gal-β-(1-4)-D-Man. (b) TWIMS of Hex-glycolaldehyde product ions derived from disaccharides shown in (a) in the negative mode as [M-H]- ions at m/z 221.1. The corresponding product ions generated were α-D-Glc-GA, β-D-Glc-GA to α-D-Gal-GA and β-D-Gal-GA.
Figure 4. (a) The mass spectrum of D-Glc-α-(1-2)-D-Glc in the positive mode as [M+Na]^+ ions at m/z 365.1 and as [2M+Na]^+ ions at m/z 707.2. Other disaccharides had essentially the same observed ions. (b) The mass spectrum of D-Gal-α-(1-6)-D-Glc in the negative mode as [M-H]^− ions at m/z 341.1, [2M-H]^− ions at m/z 683.2 and [3M-H]^− ions at m/z 1025.3. The ion having m/z 221.1 represented a product ion derived from spontaneous in-source dissociation of the disaccharide in the negative mode.
Figure 5. Traveling wave ion mobility separation (TWIMS) of isomeric disaccharide cluster ions of (a) [2M+Na]^+ ions at m/z 707.2 (b) [2M-H]^- ions at m/z 683.2 and (c) [3M-H]^+ ions at m/z 1025.3. The disaccharides used are labeled at the left in the same order from top to bottom for all three panels.
Figure 6. (a) TWIMS of the selected disaccharides for the [M+Cl]$^-$ ion at m/z 377.1; (b) for the [2M+Cl]$^-$ ion at m/z 719.1 and (c) for the [3M+Cl]$^-$ ion at m/z 1061.1. The isomeric disaccharides shown at the left are in the same order from top to the bottom for all three panels.
Supporting Information for Chapter Three

Table S-1. Additional instrumental parameters of Synapt G2 HDMS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Positive mode</th>
<th>Negative mode</th>
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<td>Desolvation Gas</td>
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<tr>
<td>Argon Gas (Trap &amp; Transfer cell)</td>
<td>2 mL/min</td>
<td>2 mL/min</td>
</tr>
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</table>

Figure S-1. (a) TWIMS of isomeric disaccharide precursor ions in positive mode as [M+Na]$^+$ ions at m/z 365.1. The disaccharides displayed were D-Glc-α-(1-4)-D-Glc, D-Glc-β-(1-2)-D-Glc, D-Gal-β-(1-4)-D-Glc and D-Gal-β-(1-6)-D-Gal. (b) TWIMS of Hex-glycolaldehyde product ions derived from disaccharides shown in (a) in positive mode as [M+Na]$^+$ ions at m/z 245.1. The corresponding product ions generated were α-D-Glc-GA, β-D-Glc-GA, β-D-Gal-GA and β-D-Gal-GA.
**Figure S-2.** (a) TWIMS of isomeric disaccharide precursor ions in positive mode as [M-H]− ions at m/z 341.1. The disaccharides displayed were D-Glc-α-(1-4)-D-Glc, D-Glc-β-(1-2)-D-Glc, D-Gal-β-(1-4)-D-Glc and D-Gal-β-(1-6)-D-Gal. (b) TWIMS of Hex-glycolaldehyde product ions derived from disaccharides shown in (a) in positive mode as [M-H]− ions at m/z 221.1. The corresponding product ions generated were α-D-Glc-GA, β-D-Glc-GA, β-D-Gal-GA and β-D-Gal-GA.
Figure S-3. Traveling wave ion mobility separation (TWIMS) of isomeric disaccharide cluster ions of (a) \([2M+Na]^+\) ions at \(m/z\) 707.2 (b) \([2M-H]^-\) ions at \(m/z\) 683.2 and (c) \([3M-H]^-\) ions at \(m/z\) 1025.4. The disaccharides used are labeled at the left in the same order from top to bottom for all three panels.
**Figure S-4.** (a) The mass spectra of disaccharides as chloride adducts of [M+Cl]\(^{-}\) ion at m/z 377.1, [2M+Cl]\(^{-}\) ion at m/z 719.1 and [3M+Cl]\(^{-}\) ion at m/z 1061.2 in negative mode. The representative examples are D-Gal-\(\alpha\)-(1-6)-D-Glc, D-Glc-\(\beta\)-(1-4)-D-Glc and D-Gal-\(\beta\)-(1-4)-D-Man from top to bottom.

**Figure S-5.** (a) TWIMS of the selected disaccharides for [M+Cl]\(^{-}\) ion at m/z 377.1. (b) TWIMS of the same selected disaccharides for [2M+Cl]\(^{-}\) ion at m/z 719.1 (c) TWIMS of the same selected disaccharides [3M+Cl]\(^{-}\) ion at m/z 1061.2. The isomeric disaccharides are in the order of D-Gal-\(\alpha\)-(1-6)-D-Glc, D-Gal-\(\beta\)-(1-6)-D-Gal, D-Glc-\(\beta\)-(1-4)-D-Glc and D-Gal-\(\beta\)-(1-4)-D-Glc from top to the bottom for all three panels.
CHAPTER FOUR
EVALUATION OF ION MOBILITY MASS SPECTROMETRY FOR
DETERMINING THE ISOMERIC HETEROGENEITY OF
OLIGOSACCHARIDE-ALDITOLS DERIVED FROM BSM
(POSITIVE MODE)

Abstract

Rapid separation and independent analysis of isomeric species is needed for the structural characterization of carbohydrates in glycomics research. Ion mobility-mass spectrometry techniques were used to examine a series of isomeric neutral oligosaccharide-alditols derived from bovine submaxillary mucin. Several analytical techniques were employed: (1) Off line separation of the oligosaccharide-alditol mixture by HPLC; (2) Direct and rapid evaluation of isomeric heterogeneity of oligosaccharides by electrospray ionization-ion mobility-time of flight mass spectrometry; and (3) Mobility-selected MS$^2$ and MS$^3$ to evaluate isomeric mobility peaks by dual gate ion mobility-tandem mass spectrometry. Multiple isomeric ion mobility peaks were observed for the majority of oligosaccharide-alditols, which was achieved on the millisecond time scale after LC separation. In complex mixtures, the limitations of LC-MS in assessing isomeric heterogeneity of oligosaccharides using one column were evident. Fragmentation spectra obtained from the collision-induced dissociation of isomeric precursor ions could be essentially identical, or dramatically different for a given precursor $m/z$ using the dual-gate ion mobility quadrupole ion trap mass spectrometer. This further confirmed the need for rapid physical resolution of isomeric precursor species prior to their tandem mass spectral analysis.
Introduction

Carbohydrate isomers result from different stereochemical configurations of the hydroxyl groups at each carbon in their sugar rings, different linkage positions, or/and different numbers of branching points.\(^1\)\(^-\)\(^3\) Isomers are ubiquitously present and are usually seen in biological glycan samples.\(^4\)\(^-\)\(^8\) Glycans coordinate with glycosyltransferase expression and carbohydrate isomers are frequently related through common core structures in some cases.\(^9\)\(^-\)\(^13\) Their analysis still faces a central and nontrivial problem: how to identify and quantitate the number of starting species. Separation of carbohydrate isomers is vitally important both for their structural elucidation and ultimately for understanding the roles these molecules play in biological systems.

One common analytical approach used is MS.\(^14\)\(^-\)\(^18\) However, frequently many sets of isomers in carbohydrate samples derived from natural sources can be difficult to be assessed by MS alone, particularly in cases where precursor ions generate product ions all having the same \(m/z\), even after higher stages of tandem mass analysis (MS\(^n\)). Liquid chromatography (LC) has been used to separate complex glycan mixtures\(^19\)\(^-\)\(^23\) prior to MS, but this alone is not effective for detecting similar isomers that might happen to co-migrate. A second column that separates according to some different physical property is then required to evaluate fractions. This requires that multiple fractions be concentrated and then chromatographed a second time, which can be time consuming. Furthermore, there is no guarantee that two columns are adequate for resolving all isomers, in which case the process needs to be repeated using a third column (and the time magnified with even more fractions) to evaluate isomeric heterogeneity. If isomeric precursor ions yield identical product ions, knowledge of whether or not they are present in a mixture is easy to miss. NMR typically requires glycans to be in a purified state for structure characterization,\(^24\)\(^-\)\(^27\) which is not feasible for biological samples with large numbers of isomeric
mixtures. High resolution separation techniques that can rapidly assess a wide variety of separation conditions that resolve isomeric structures are needed.

Ion mobility spectrometry (IMS), separating ions based on their ion-neutral collision cross sections, serves as a robust and fast gas phase separation technique in many fields including glycomics research. It has been demonstrated that IMS can resolve many monosaccharide methyl glycoside isomers with subtle structural differences and is also capable of distinguishing various oligosaccharide isomers. Moreover, with the establishment of a dual gate ion mobility-tandem MS system in our lab, mobility selected fragmentation experiments are now feasible, providing evidence for multiple isomeric precursors as mobility-separated species. A number of studies of carbohydrate isomerism using ion mobility-mass spectrometry (IMMS) in the literature have been performed on purified or commercially available standards. With the application of IMMS to biological samples, isomeric ion mobility peaks have been observed. However, fragmentation patterns for the individual resolved isomeric mobility peaks were rarely reported. Yet this information is critical in assessing the isomeric composition of carbohydrates within a set of mobility-selected peaks at a given m/z value.

Here we use neutral oligosaccharide-alditols derived from bovine submaxillary mucin (BSM) as an example to demonstrate the value of IMMS techniques to evaluate the isomeric heterogeneity of precursor ions having selected m/z values. BSM neutral O-linked oligosaccharides were chosen because they have been shown to contain a large number of isomers, which posed a challenge to multi-step LC/MS or LC/MS/MS analysis. Ambient pressure IMS coupled to mass spectrometry and tandem mass spectrometry were found to provide advantages for structural glycomics described herein. As the upfront IMS separation can
be performed rapidly (millisecond time frame) and subsequent MS\textsuperscript{n} analysis can still be performed on precursor ions, IMMS gives added value to classical MS\textsuperscript{n} analyses of isolated m/z values. Herein we report on the value of ion mobility separations to show that multiple isomers can be observed in mixtures after HPLC separation prior to their independent MS\textsuperscript{n} analysis.

**Experimental Section**

**Chemicals and Samples**

BSM, NaBH\textsubscript{4} and NaCl were purchased from Sigma-Aldrich (St. Louis, MO). All solvents (methanol and water) used were HPLC grade and supplied from J. T. Baker Inc. (Philipsburg, N.J., USA). A mixture of equal volume methanol and water was used as an electrospray ionization (ESI) solvent. Martensson et al.\textsuperscript{4} described the procedure for the preparation and separation of neutral oligosaccharide-alditols from BSM, but the procedure was scaled down 10-fold in this preparation. Briefly, 1.0 g BSM was treated with 0.05M NaOH/1.0 M NaBH\textsubscript{4} to release acidic and neutral oligosaccharide-alditols (only the neutral oligosaccharides were used in current study). The neutral oligosaccharide-alditol mixture was concentrated to dryness and then re-dissolved in 10 ml CH\textsubscript{3}CN/H\textsubscript{2}O (74:26), and injected in 0.5-ml batches onto a semipreparative Glycopak N\textsuperscript{43} normal-phase chromatography column (Millipore Corp., Bedford, MA). The column was operated using acetonitrile/water in the range of 85/15 to 70/30 as a mobile phase at a flow rate of 5.0 ml/min. A set of fractions were collected based on UV absorbance at 200 nm. Fractions from 1 to 10 were collected and rotary evaporated to dryness. Each fraction contains several oligosaccharide-alditols including isomers.

ESI solvent (1 mL) was added to each fraction to prepare stock solutions. Different dilution ratios were used for the different fractions, resulting in final solutions with a
concentration of ~0.01 mg/ml for ion mobility-time of flight MS and ~0.1 mg/ml for ion
mobility-tandem MS. Sodiated adducts were exclusively observed for all the oligosaccharide-
arditol species in this study. To enhance their formation, either 2 μL or 10 μL of a 5 mM NaCl
solution was added to each 1 ml sample to give a final concentration of 10 μM NaCl for ion
mobility-time of flight MS and 50 μM NaCl for ion mobility-quadrupole ion trap MS,
respectively.

**Ambient Pressure Resistive Glass Drift Tube Ion Mobility Time of Flight Mass Spectrometer**

This system was fully described previously by Kaplan et al. in 2010 and will be simply
referred as IM-TOFMS in the following text. Voltages used in this study were: 12.5 KV for ESI,
9.0 KV on the entrance of the resistive glass IMS tube, 7300 V on the ion gate and 795 V on the
end of the IMS, resulting in a homogeneous electric field of 325 V/cm. A gate pulse width of 0.1
ms was utilized in order to obtain higher resolving power and better evaluate isomeric mixtures.
Nitrogen was used as the drift gas at a flow rate of 1.5 L/min.

TofDaqViewer software (TOFWERKS AG) was used to collect all the data from IM-
TOFMS. The data from each sample could either be completely or selectively exported based on
the user-specified time range in the format of a 2-dimensional (2D) text file. IDL virtual machine
software ([www.exelisvis.com](http://www.exelisvis.com)) was then used to generate 2D IMMS correlation spectra based on
the exported text file data.

**Ambient Pressure Dual Gate Ion Mobility Quadrupole Ion Trap Mass Spectrometer**

The system was reported by Clowers et al. previously in detail and has been applied to
several studies. The voltages applied were as follows: 13 KV for the ESI, 10 KV on the 1st
ring of the IMS, 9.28 KV and 1.28 KV for the 1st and 2nd gates of the IMS and 342 V on the last ring. The IMS system was coupled to an LCQ Deca QITMS (Thermo Electron, San Jose, CA) through the extended post-drift region. Nitrogen drift gas was introduced at the low-voltage end of the drift tube at a flow rate of 1 L/min. With both gates open, the instrument serves as a quadrupole ion trap mass spectrometer. The two gate ion mobility control software was rewritten using the Labview 2009 version instead of Labview 6.1 reported previously. There are two operation modes basically: selected mobility monitoring (SMM) mode which allows a specific drift time window of ions to be transmitted to the ion trap and dual gate scanning mode (DGS) which determines ions’ drift times through a successive series of stepped ion gate pulsing experiments. However, the DGS mode is a rather slow process for overall mobility range scanning particularly for complicated samples. Mobility-selected fragmentation was performed in the SMM mode using the MS^n function in the quadrupole ion trap with appropriate timing of gates. The gate pulse width of the 1st gate was at 0.3 ms for all the SMM experiments in this study. For adjacent peaks, the second gate cursor was set in the range of 0.2 or 0.3 ms of the central drift time determined on the valley side. The other cursor of the second gate window was at the corresponding peak edge on the baseline side. The mobility selected MS^2 data was acquired for 10 min and MS^3 spectrum was collected for 15 min on the IM-QITMS in this study.

**Electrospray Ionization (ESI) Source**

All samples were introduced and ionized by ESI in the positive mode. The ESI emitter was prepared in-house using 360 μm o. d. × 100 μm i. d. silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA). It (~ 5 cm) was connected to the sample transfer line (~ 15 cm) through a zero dead volume stainless steel union (Valco Instruments Co. Inc., Houston, TX)
where ESI voltage was applied. The end of the capillary tube from the ESI emitter was positioned at the center of the IMS tube for IM-TOFMS and with an upward angle of 45° for the IM-QITMS. Samples were injected at a flow rate of 5 μl/min by a Chemyx syringe pump model 110 or an on-board syringe pump.

**IMS Theory**

IMS has been discussed in detail elsewhere.\textsuperscript{28,46-48} It is a gas phase separation technique based on the drift velocity of ions \((v_d, \text{cm/s})\) under a homogeneous electric field \((E, \text{V/cm})\) in a counter flow of neutral drift gas. The relationship is expressed by equation (1), where \(K\) [in \((\text{cm}^2/(\text{V} \times \text{S}))\)] is termed the mobility coefficient and is related to measureable parameters of the instrument through the following equation,

\[
K = \frac{v_d}{E} = \frac{L^2}{t_d V} \quad \text{Equation (1)}
\]

where \(L\) is the drift tube length in cm, \(V\) is the voltage applied across the drift region in Volts, and \(t_d\) is the drift time in seconds. \(K\) can be standardized by temperature \(T\) (Kelvin) and pressure \(P\) (Torr) to a reduced mobility parameter \(K_o\) based on equation (2).

\[
K_o = \frac{L^2}{t_d V} \times \frac{273.15}{T} \times \frac{P}{760} \quad \text{Equation (2)}
\]

The two IMMS instruments utilized in this study were in the same location (Pullman, WA) and both kept at 200°C. The drift tube length and voltage applied through the drift region on the IM-QITMS instrument were different from that of the IM-TOFMS, which results in different drift time values for the same compound between the two instruments. However, \(K_o\) values are constant for a given compound in a given buffer gas at one temperature (add refs). Thus, the drift
times of oligosaccharide-alditols on the IM-QITMS instrument were determined using equation (2) based the values obtained from the IM-TOFMS in this study. As defined by the parameters presented above, the relation of \( t_{d(IM-QIT)} = 1.32 \times t_{d(IM-TOF)} \) was determined, and this was validated by the instrumental standard 2, 6-Di-tert-butylpyridine and disaccharide-alditol standard α-D-GalNAc-(1-3)-GalNAc-ol (see Supporting Information).

Results and Discussion

Fractionation of Neutral Oligosaccharide-alditols and Evaluation of Their Mass-to-Charge Constituents by LC-MS

LC-MS is commonly used to separate and analyze oligosaccharides and different columns have been applied for glycomics research.\(^{19,21-23}\) In this study, a semi-preparative column, Glycopak N, was used as it has been shown to resolve neutral oligosaccharide isomers as confirmed by MS/MS and \(^1\)H-NMR.\(^4\) The HPLC elution profile of a neutral oligosaccharide-alditol mixture from BSM with UV detection at 200 nm is shown in Figure 1a.\(^{39}\) Mass spectra of the entire starting mixture and individual HPLC fractions acquired on the QITMS are displayed in Figure 1b. In total, 10 fractions were obtained through the collection of peaks and shoulders shown in Figure 1a that are known to contain isomeric oligosaccharide-alditols.\(^4\) Fraction 1 was not examined as it is virtually entirely the reduced monosaccharide-alditol D-GalNAc-ol as shown by NMR.\(^4\) It is worth pointing out that the early fractions had more sample, with the latter fractions having progressively less from fraction to fraction. Ions of \( m/z \) 449, 554, 611, 652, 757, 773, 814, 919, 960 and 1065 were the major ions observed and were distributed in different LC fractions, with a general progression from smaller structures in initial fractions to larger
structures in later fractions. All the m/z values in this paper were attributable to sodium adducts of the oligosaccharide-alditols. LC fractionation followed by concentration of fractions increased the concentration of low abundance m/z species which enabled many of them to be studied that were not feasible with the entire mixture, particularly in the later fractions that have less overall abundance. Ions having m/z 449 and 554 were mainly observed in fractions 2 and 3, respectively; those at m/z 611 and 652 were mainly found in fractions 4-7; those at m/z 757 were found in fractions 5-7; those at m/z 773 were present in fractions 6-8; those at m/z 919 and m/z 960 mainly appeared in fractions 8-10; and those at m/z 1065 were found in fractions 9 and 10.

Three points are worthy of note regarding oligosaccharide-alditol mixtures as complex as these. First, in collecting peaks largely from valley to valley as shown in Figure 1a, it is feasible that some structures may be found solely within one peak but some may span parts of both peaks because the valleys do not always come down to baseline. Therefore the same actual compound can be (and sometimes is) found within adjacent peaks. Second, isomers having the same m/z can be found in one or more LC peaks (Figure 1b and 4). Third, some isomers dissociate to yield identical product ions, sometimes in very similar ratios. For example, we know from previous studies that there are 5 isomers in this sample that have a m/z of 449 (D-GlcNAc-β-1-3-D-GalNAc-ol, D-GalNAc-α-1-3-D-GalNAc-ol, D-GalNAc-β-1-3-D-GalNAc-ol, D-GlcNAc-β-1-6-D-GalNAc-ol, and D-GalNAc-α-1-6-D-GalNAc-ol). The first three dissociate to give the same product ions in nearly equal abundance, as do the latter two. This prompts three questions. (1) how would one know whether different isomers are present in two adjacent LC fractions, as some isomers give identical product ions in nearly the same ratios? (2) how would one know whether the same compound is present in two adjacent LC fractions (i.e. spanning both sides of the valley between fractions), as these dissociation patterns are from the same
compound hence would be identical? (3) how would one know whether different isomers are present in the same LC fraction when they give essentially identical dissociation patterns? Clearly, some technique is required such as physical separation of isomers or ion spectroscopy to discriminate species that yield near-identical dissociation spectra.

*Rapid Evaluation of Isomeric Heterogeneity of Neutral O-linked Oligosaccharide-alditols using IM-TOFMS*

Individual HPLC fractions, were analyzed by IMMS to evaluate whether they could be resolved into two or more isomeric mobility peaks. The complete mixture of oligosaccharide-alditols from BSM and individual HPLC fractions were analyzed using the IM-TOFMS system and data was acquired for 5 mins per sample. Unfortunately, for 2D mass-mobility correlation spectra using the entire mixture of oligosaccharide-alditols, only low mass ions were observed with high intensity. Most higher mass ions were found at much lower intensities, which is probably due to (1) overall greater quantities of the lower mass components (2) charge competition and (3) low duty cycle of the IMS (a narrow gate width of 0.1 ms used).

Figure 2 shows the overlaid 2D mass-mobility correlation spectra and the additional 1D overlaid mobility profiles for selected $m/z$ components in different fractions. Since only two 2D spectra can be overlaid at the same time using IDL software, in order to compare mobility profiles for a specific $m/z$ in multiple LC fractions, additional overlaid 1D mobility spectra are aligned on the right with the same drift time scale as the 2D spectrum. Different fractions are denoted with different colors as labeled for individual plots. For example, Figure 2a displays the overlaid 2D mass-mobility profiles for $m/z$ 611 from fractions 4 (blue) and 5 (black). Shown on the right are overlaid 1D mobility spectra for $m/z$ 611 from fractions 5 (black) and 6 (red). It was
observed that the m/z 611 precursor ions in fractions 4 and 6 had different drift times, they are isomers. As studied previously, three isomers of m/z 611 was reported with the relative amounts of 4.0: 2.2: 0.27,\(^4\) thus the minor mobility peak for m/z 611 in fraction 5 which had the same drift time as m/z 611 from fraction 4 may represent a different minor isomer. The m/z 611 ion was a sodium adduct ion with the proposed composition of Hex\(_1\)HexNAc\(_1\)HexNAc-ol. Other examples in Figure 2 include sodiated trisaccharide-alitols with composition HexNAc\(_2\)HexNAc-ol at m/z 652 (Figure 2b) from fractions 4, 5 and 6, sodiated tetrasacchride-alitols with composition DeoxyHex\(_1\)Hex\(_1\)HexNAc\(_1\)HexNAc-ol at m/z 757 from fractions 5, 6 and 7 (Figure 2c), Hex\(_2\)HexNAc\(_1\)HexNAc-ol at m/z 773 from fractions 6, 7 and 8 (Figure 2d), Hex\(_1\)HexNAc\(_2\)HexNAc-ol at m/z 814 from fraction 7 (Figure 2e); and hexasaccharide-alitol DeoxyHex\(_2\)Hex\(_2\)HexNAc\(_1\)HexNAc-ol at m/z 1065 (Figure 2f) from fraction 9. The composition information was validated by the m/z values and fragmentation patterns. For fraction 10, the signal accumulated on the IM-TOFMS instrument was extremely low which was primarily due to the relatively small amount of sample gathered from the LC and hence no data is presented here. For each individual plot in Figure 2, only a narrow m/z range is selectively displayed where multiple peaks on the m/z axis represent the main ion along with its corresponding (mainly \(^{13}\)C) isotopomers. It is clear that all the ions have more than one mobility peak detected along the ion mobility (vertical) axis which indicates multiple isomeric forms. Low abundance mobility peaks were confirmed by the reproducibility of multiple runs of the same sample. Colored cross peaks show visually the relative intensities of isomer distributions and their ion mobilities. For low abundance isomers, depending on the intensity threshold selected, cross peaks were sometimes not observable yet could be observed in the 1D mobility plots along the vertical axis for selected m/z values.
Overall, 2 isomeric peaks were detected for \( m/z \) 611, \( m/z \) 652 and \( m/z \) 814; 4 different drift times distributions including shoulder were observed for \( m/z \) 757 and \( m/z \) 773; 3 partially separated mobility peaks were detected for \( m/z \) 1065. The results show that (1) IMS can resolve isomeric oligosaccharides in the millisecond time frame that co-migrated in LC peaks, (2) the technique is orthogonal to the physical interactions governing many LC separations and (3) can be performed without any prior knowledge of the nature of isomeric structures or their spectroscopy such as specific UV/Vis or IR absorption wavelengths. Of course, no separation technique has infinite resolving power therefore coincidental co-migration of some compounds, even after HPLC and ion mobility separation, is not unexpected.

**Mobility Selected Fragmentation by Dual Gate IM-QITMS**

While multiple isomers may be observed by IMS how can one confirm or validate the isomeric information obtained by IM-TOFMS? First, oligosaccharide standards might be used to evaluate the ion mobility times. However, in cases where samples are derived from unknown origins or even in cases where they may be known, isolation of the standards for ion mobility comparisons may be time consuming. Moreover, they may not be only one isomeric component in a single ion mobility peak. The conventional method, widely accepted by the mass spectrometry community, is to acquire MS\(^n\) spectra of individual isomers that may be used for the confirmation of isomeric species. For multiple isomers having a specific \( m/z \) found in single LC fractions, ion mobility information combined with mobility selected fragmentation information can be used to discriminate between many isomeric components. It should be pointed out that in Figures 3-6 the second gate drift time windows used to select isomeric mobility peaks on the IM-QITMS instrument are different from their corresponding drift times.
acquired on the IM-TOFMS instrument as discussed previously, but they are in agreement with reduced mobility values.

Figure 3a displays the 2D IMMS spectra of m/z 449 from HPLC fraction 2 with three isomeric mobility peaks labeled. A barely separated shoulder was detected on the side of peak 1 having a longer drift time. By comparing the overlaid individual mobility profiles of available disaccharide-alditol standards isolated from BSM (supporting information), it was found that α-D-GalNAc-(1-3)-D-GalNAc-ol and β-D-GlcNAc-(1-3)-D-GalNAc-ol co-eluted as peak 1, α-D-GalNAc-(1-6)-D-GalNAc-ol co-eluted with the barely separated shoulder of peak 1, Peak 2 matched with β-D-GlcNAc-(1-6)-D-GalNAc-ol. The exact structure of the mobility peak (peak 3) with the longest drift time remains unknown but may be the additional structure β-D-GalNAc-(1-3)-D-GalNAc-ol which was not available or possibly another HexNAc-HexNAc-ol, as there were still additional LC peaks reported in reference that were not fully characterized. Therefore, in complex mixtures, it is feasible and actually expected that some isomeric forms of oligosaccharides may happen to co-migrate when one specific adduct or gas is used. Figure 3b and 3c show the mobility selected fragmentation spectra for mobility peaks 1 and 2, respectively as labeled in Figure 3a, by setting the second gate windows at 42.2-43.1 ms or 44.1-44.6 ms on the dual gate IM-QITMS. They shared the major fragments such as m/z 431, m/z 389, m/z 286 and m/z 246, but with different intensity ratios. According to the MS/MS spectra of the available disaccharide-alditol m/z 449 standards acquired (supporting information), the fragmentation pattern in Figure 3c (mobility peak 2) matched with the tandem spectrum of β-D-GlcNAc-1-6-D-GalNAc-ol which was consistent with the data described above, while Figure 3b (mobility peak 1) was a combination of the spectra of other standards that co-migrated in this mobility window. In addition, MS/MS spectrum of isomeric peak 3 was not obtained due to sensitivity limitation.
Figure 4a shows the 2D mass mobility correlation plot for the ion at \( m/z \) 757 from HPLC fraction 6, acquired on the IM-TOFMS instrument. Two peaks are labeled on the IMS plot shown along the right vertical axis. In Figures 4b and 4c the tandem spectra are presented for peaks 1 and 2 acquired on the IM-QITMS instrument by setting the second gate drift time windows between 57.4-58.1 ms and 59.1-60.0 ms respectively. Since mobility-selected MS\(^2\) spectra of the \( m/z \) 757 ion for isomeric mobility peaks 1 and 2 showed essentially very similar spectra (supporting information), the mobility selected tandem spectra shown here are MS\(^3\) spectra of the major product ion \( m/z \) 611 derived from precursor ion \( m/z \) 757. Product ions of \( m/z \) 388, 408, 431, 449, 491, 551 and 593 were observed in both spectra, but the relative intensities were different. The \( m/z \) 388 ion was the major product ion for isomeric mobility peak 2, while \( m/z \) 408 and 449 were of much higher intensity for isomeric mobility peak 1. As illustrated by Zhu et al.,\(^3\) \( m/z \) 757 isomers having the HexNAc-ol substituted at different positions can have either \( m/z \) 388 or \( m/z \) 408/449 as the characteristic product ions. The fragmentation pathways for isolated standards were also included in his study.\(^3\) Different tandem spectra obtained in this example validated the isomeric mobility peaks detected and also may provide evidence for potential structure elucidation.

The analysis of isomeric precursor ions of \( m/z \) 919 (sodium adduct) having the composition of [DeoxyHex\(_1\)Hex\(_2\)HexNAC\(_1\)HexNAC-ol] is presented in Figure 5. Shown in Figure 5a is the overlaid 2D IMMS spectra of \( m/z \) 919 in fractions 8 and 9 and in total 3 isomeric mobility peaks labeled as 1, 2 and 3 were detected. Two partially separated isomeric mobility peaks (peaks 2 and 3) were detected for \( m/z \) 919 in fraction 9 (red trace, Figure 5a). Note that the barely separated faster mobility shoulder in fraction 8 labeled as mobility peak 1 (black trace, Figure 5a) indicates another isomeric form for the \( m/z \) 919 precursor ion. Due to the sensitivity
limitation of the instrument and the low resolution between isomeric peaks 1 and 2 in fraction 8, it was not able to acquire the mobility-separated fragmentation data for \( m/z \) 919 in fraction 8. Figures 5b and 5c display the mobility selected MS/MS spectra of isomeric mobility peaks 2 and 3 of \( m/z \) 919 in fraction 9 using second gate drift time windows of 65.0-65.8 ms and 66.4-66.9 ms on the dual gate IM-QITMS, respectively. The two spectra were very similar and both had \( m/z \) 773 as a predominant product ion through the neutral loss of a DeoxyHex residue. However, minor difference was observed having \( m/z \) 593 detected only in Figure 5b and \( m/z \) 534 only appeared in Figure 5c. An additional isolation/dissociation step (MS\(^3\)) was then further performed for the isomeric peaks 2 and 3 in HPLC fraction 9 using \( m/z \) 773 as the second-stage precursor ion derived from the \( m/z \) 919 ion, as shown in Figures 5d and 5e. It was observed that the \( m/z \) 611 ion derived from the apparent neutral loss of a Hex residue was the major fragment derived from \( m/z \) 773 in both spectra. Fragments of \( m/z \) 388 (neutral loss of HexNAc-ol from \( m/z \) 593), 408 (neutral loss of HexNAc from \( m/z \) 611) and 755 were also shared by isomeric mobility peaks 2 and 3. However, there were differences in dissociation of the \( m/z \) 773 product ion. Fragments of \( m/z \) 449 (neutral loss of Hex from \( m/z \) 611), 551, 593 and 714 were only found upon dissociation of the \( m/z \) 773 ion derived from isomeric mobility peak 2 (Figure 5d), while product ions of \( m/z \) 422, 557 were uniquely present for the \( m/z \) 773 ion derived from isomeric mobility peak 3 (Figure 5e). Moreover, the relative intensities of \( m/z \) 388 and \( m/z \) 408 in Figures 5d and 5e were different. Further fragmentation of \( m/z \) 611 might give more structural evidence to differentiate isomeric mobility peaks 2 and 3, but this was beyond the sensitivity capability of the current instrument.

The same criteria were applied to the isomeric pentasaccharide-alditols of DeoxyHex\(_1\)Hex\(_1\)HexNAc\(_2\)HexNAc-ol having \( m/z \) 960 (HPLC fractions 8 and 9) as shown in
Figure 6. Figure 6a displays the overlaid 2D IMMS spectra of the \( m/z \) 960 precursor ion from fractions 8 and 9. Four different drift time distributions were observed in all: two isomeric mobility peaks 1 and 3 were observed for \( m/z \) 960 in fraction 9 and another two isomeric mobility peaks 2 and 4 were detected for \( m/z \) 960 in fraction 8. In addition, the valley between isomeric mobility peaks 1 and 3 may represent another isomeric form which was overlapped with isomeric mobility peak 2 in fraction 9. Mobility-selected fragmentation was only performed for isomeric mobility peaks 1 and 3 in fraction 8, not for low abundance or barely separated mobility peaks in fraction 9 in this example. Shown in Figures 6b and 6c are MS\(^2\) spectra of the \( m/z \) 960 precursor ion corresponding to isomeric peaks 1 and 3 in fraction 8 by selectively transferring them to the QITMS using second gate drift time windows of 66-66.6 ms and 67.4-68.4 ms, respectively. The spectra are very similar and shared the predominant fragment \( m/z \) 814 (loss of DeoxyHex from \( m/z \) 960) and fragments \( m/z \) 757 (loss of HexNAc from \( m/z \) 960) and 652 (loss of DeoxyHex-Hex from \( m/z \) 960) which was consistent with previous structural evidence.\(^4\) Considering the monosaccharide makeup and biosynthetic mechanisms for generating O-linked oligosaccharides, it is not unusual to observe similar or even identical CID pathways for closely structurally-related oligosaccharide-alditols in BSM.\(^3\)\(^9\) Changing the stereochemistry of one sugar at one position may not have much of an effect on their MS and tandem mass spectra, especially when they fragment in the positive mode where the main fragments frequently result from cleavages at glycosidic bonds. Thus IMS becomes even more relevant and important in the evaluation of the isomeric heterogeneity of oligosaccharides in cases where similar or even identical fragmentation spectra may be obtained. Further fragmentation of isomeric mobility peaks 1 and 3 was carried out to MS\(^3\) by selecting \( m/z \) 814 as the second-stage precursor ion as shown in Figures 6d and 6e. Major product ions at \( m/z \) 611 and 449 were observed for isomers
from both ion mobility-selected peaks. However, different fragments were found in each spectrum, for example, m/z 754, 635, 593 in Figure 6d and m/z 797, 772, 694, 575, 516, 507 in Figure 6e. Higher order MS\textsuperscript{n} of product ions such as m/z 611 may be needed in order to further distinguish the structural differences between isomeric peaks 1 and 3. In practice, this is again limited by the sensitivity of the dual gate IMMS instruments and the diminishing amounts of successive product ions available in multi-stage ion trap dissociation experiments.

As shown in above examples, valuable information regarding the isomeric heterogeneity of oligosaccharide-alditols was obtained by combining mobility differentiation and mobility selected MS\textsuperscript{n} data, which clearly showed that isomers were present for virtually all precursor ions and also demonstrated the advantages of IMMS for exploring the complexity of carbohydrate structures. However, the exact structure(s) for each isomeric mobility peak in this study could not be elucidated since the tandem spectra collected from a single mobility peak could still be potentially attributed to an isomeric mixture of oligosaccharides.

**Conclusions**

Using neutral oligosaccharide-alditols isolated from BSM as a model for O-linked glycoprotein structures, it was demonstrated that ion mobility-mass spectrometry has the capability to rapidly evaluate the isomeric heterogeneity of oligosaccharide mixtures without any prior knowledge required. Even after a single HPLC column, isomeric structures that were present in fractions could frequently be resolved as independent peaks using IMMS. This was demonstrated for structures ranging from disaccharide-alditols to hexasaccharide-alditols. Isomeric precursor ions that would normally be grouped into the same m/z pool were thereby separated into isolable drift time windows that enabled independent MS/MS spectra and in some
cases MS$^3$ spectra to differentiate isomeric structures or isomer pools. While it is still possible that many ion mobility peaks contain isomeric precursors, ion mobility coupled to tandem stages of mass spectrometry enabled different sets of product ions to be assigned to different isomeric precursors within a given m/z pool in some cases. This is clearly an advantage in analysis of complex mixtures of glycans derived from biological sources. A single LC column (even more than one LC column)$^4$ is frequently inadequate to resolve isomeric species present in complex oligosaccharide samples. While MS$^n$ information is valuable for the structure identification of oligosaccharides, it is desirable to resolve as many isomeric species as possible prior to MS$^n$, and it is valuable simply to know how many isomeric precursor ions may be contributing to MS$^n$ spectra. Clearly, more orthogonal analytical separation steps may be needed to fully resolve isomeric species existing in nature, and as many separation steps as possible that can be routinely concatenated for rapid analyses is a sought-after goal.

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References


Figure 1. (a) HPLC elution profile of neutral oligosaccharide-alditols isolated from bovine submaxillary mucin (BSM) and (b) mass spectra of the mixture before separation and of the concentrated individual HPLC fractions 2-10 from panel a. (Note: Figure 1a was reprinted from reference 39 Anal. Bioanal. Chem. 2009, 394, 1853-1867.)
Figure 2. Evaluation of isomeric heterogeneity of oligosaccharide-alditols released from BSM by IM-TOFMS. Shown are overlaid mobility profiles for representative oligosaccharide-alditol ions having selected \( m/z \) values from different HPLC fractions. (a) Overlaid 2-D mass-mobility spectra for \( m/z 611 \) from fractions 4 (blue) and 5 (black). Overlaid 1-D mobility spectra for \( m/z \) 611 from fraction 5 (black) and 6 (red) are aligned on the right; (b) Overlaid 2-D mass-mobility spectra for \( m/z 652 \) from fractions 4 (blue) and 5 (black). Overlaid 1-D mobility spectra for \( m/z \) 652 from fractions 5 (black) and 6 (red) are shown on the right. (c) Overlaid 2-D mass-mobility spectra for \( m/z 757 \) from fractions 5 (blue) and 6 (black). Included on the right are overlaid 1-D mobility spectra for \( m/z \) 757 from fractions 6 (black) and 7 (red). (d) Overlaid 2-D mass-mobility spectra for \( m/z 773 \) from fractions 6 (blue) and 7 (black). Displayed on the right are overlaid 1-D mobility spectra for \( m/z \) 773 from fraction 7 (black) and 8 (red). (e) 2-D IMMS plot of \( m/z \) 814 from fraction 7; (f) 2-D IMMS plot of \( m/z \) 1065 from fraction 9. (Note that the overlaid 1-D mobility spectra share the same drift time scale with the corresponding 2-D spectra).
Figure 3. (a) 2-D IMMS spectra of m/z 449 from HPLC fraction 2; (b) MS/MS spectrum of m/z 449 in LC fraction 2 from the 42.2-43.1 ms mobility window (peak 1) on the dual gate IM-QITMS; (c): MS/MS spectrum of m/z 449 in LC fraction 2 from the 44.1 -44.6 ms (peak 2) mobility window on the dual gate IM-QITMS.
Figure 4. (a) 2-D IMMS plot of m/z 757 from HPLC fraction 6 acquired on IM-TOFMS. (b) MS$^3$ spectrum of the m/z 611 product ion derived from m/z 757 in fraction 6 of the 57.4-58.1 ms (peak 1) mobility window on dual gate IM-QITMS. (c) MS$^3$ spectrum of the m/z 611 product ion derived from m/z 757 in fraction 6 of the 59.1-60.0 ms (peak 2) mobility window on dual gate IM-QITMS.
Figure 5. (a) Overlaid 2-D IMMS spectra of the m/z 919 precursor ion from HPLC fractions 8 (black trace) and 9 (red trace). (b) MS/MS spectrum of the m/z 919 precursor ion in HPLC fraction 9 of the 65.0-65.8 ms mobility window (peak 2) on the dual gate IM-QITMS; (c) MS/MS spectrum of the m/z 919 precursor ion in HPLC fraction 9 of the 66.4-66.9 ms (peak 3) mobility window on the dual gate IM-QITMS; (d) MS^3 spectrum of the m/z 773 product ion derived from m/z 919 in HPLC fraction 9 of the 65.0-65.8 ms (isomer 2) mobility window on dual gate IM-QITMS. (e) MS^3 spectrum of m/z 773 product ion derived from m/z 919 in LC fraction 9 of the 66.4-66.9 ms (isomer 3) mobility window on IM-QITMS.
Figure 6. (a) Overlaid 2-D IMMS spectra of m/z 960 from HPLC fractions 8 (black trace) and 9 (red trace). (b) The MS/MS spectrum of m/z 960 in HPLC fraction 8 of the 66-66.6 ms (peak 1) mobility window on the dual gate IM-QITMS; (c) MS/MS spectrum of m/z 960 in fraction 8 of the 67.4-68.4 ms (peak 3) window on dual gate IM-QITMS; (d) MS³ spectrum of the m/z 814 product ion derived from the m/z 960 precursor ion in fraction 8 of the 66-66.6 ms (peak 1) mobility window on the dual gate IM-QITMS; (e) MS³ spectrum of the m/z 814 product ion derived from the m/z 960 precursor ion in fraction 8 of 67.4-68.4 ms (peak 3) mobility window on the dual gate IM-QITMS.
**Supporting Information for Chapter Four**

![Figure S-1](image1.png)

**Figure S-1.** (a) Ion mobility spectra (IMS) of 2, 6-di-tert-butylpyridine acquired on IM-TOFMS. (b) IMS of 2, 6-Di-tert-butylpyridine acquired on dual gate IM-QITMS. (c) IMS of α-D-GalNAc-(1-3)-GalNAc-ol acquired on IM-TOFMS. (d) IMS of α-D-GalNAc-(1-3)-GalNAc-ol acquired on dual gate IM-QITMS. This figure demonstrated the relation of $t_d(\text{IM-QIT}) = 1.32 \times t_d(\text{IM-TOF})$ by using an instrument standard and a disaccharide-alditol standard.

![Figure S-2](image2.png)

**Figure S-2.** Overlaid mobility spectra of individual disaccharide-alditol standards isolated from BSM. alpha 13 denotes α-D-GalNAc-(1-3)-D-GalNAc-ol, alpha16 denotes α-D-GalNAc-(1-6)-D-GalNAc-ol, beta 16 denotes β-D-GlcNAc-(1-6)-D-GalNAc-ol, beta 13 denotes β-D-GlcNAc-(1-3)-D-GalNAc-ol. The standards were obtained as described previously (reference 4 in the paper). The spectra were obtained on an ion mobility-quadrupole mass spectrometer using single ion monitoring (SIM) from Excellims Inc. (20 Main Street, Acton, MA, Tel. 978.264.1980, Fax 978.264.1981)
Figure S-3. MS/MS spectra for disaccharide-alditol standards isolated from BSM. a. MS/MS spectrum of α-D-GalNAc-(1-3)-D-GalNAc-ol. b. MS/MS spectrum of α-D-GalNAc-(1-6)-D-GalNAc-ol. c. MS/MS spectrum of β-D-GlcNAc-(1-6)-D-GalNAc-ol (reduced mobility=0.98) d. MS/MS spectrum of β-D-GlcNAc-(1-3)-D-GalNAc-ol. Note: the spectra shown below were acquired on dual gate IM-QITMS, both gates were open and ion mobility spectrometry was only served as an ion transfer device in this case, the fragmentation occurred in the trap cell of the mass spectrometer. 32V Collision Energy and 30 ms activation time were applied for all compounds.
Figure S-4. Mobility-selected MS$^2$ spectra of m/z 757 for isomeric mobility peaks 1 and 2 in HPLC fraction 6. (Supporting information for Figure 4 in chapter 4)
CHAPTER FIVE
APPLICATION OF TRAVELING WAVE ION MOBILITY MASS SPECTROMETRY FOR DETERMINING THE ISOMERIC HETEROGENEITY OF OLIGOSACCHARIDE-ALDITOLS OF BSM BY NEGATIVE MODE ELECTROSPRAY IONIZATION

Abstract

Traveling wave ion mobility mass spectrometry on a Synapt G2 instrument was utilized to evaluate the isomeric heterogeneity of neutral oligosaccharide-alditols isolated from bovine submaxillary mucin (BSM) by negative electrospray ionization. The oligosaccharide-alditol mixture was pre-separated on an off line HPLC column and the structural homogeneity of individual LC fractions were investigated. Multiple ion mobility peaks due to structural isomers were observed for a number of oligosaccharide-alditols from single LC fractions. The separation by ion mobility occurs on the millisecond scale. The collision-induced dissociation cells located in front of and after the ion mobility separation device in the Synapt G2 enabled oligosaccharide precursor or product ions to be separated by ion mobility and independent fragmentation spectra to be acquired for isomeric carbohydrate precursor or product ions. It was shown that more than one mobility peak could be resolved for specific product ions as well; the mobility distributions of product ions provided additional structural information in differentiating the isomeric structures of their precursor ions. Different or similar MS/MS spectra were obtained for independent mobility peaks at a single m/z, representing structural variants or sterochemical isomers for one molecular formula. This was observed both for oligosaccharide precursor and
product ions. In addition, the mobilities of both \([M-H]^-\) and \([M+Cl]^-\) ions, formed by adding either \(\text{NH}_3\text{OH}\) or \(\text{NH}_4\text{Cl}\) to the electrospray solvent, were examined and compared for selected oligosaccharide-alditols. Better separation among structural isomers appeared to be achieved for some \([M+Cl]^-\) anions.

**Introduction**

As one of the most predominant post-translational modifications in biological systems, glycosylation contributes to the vast functional diversity\(^1\)\(^-\)\(^6\) in nature including protein folding, protein-protein interaction, cell-cell communication, cancer metastasis,\(^7\)\(^-\)\(^9\) and immune system coordination.\(^\)\(^10\) Carbohydrate compounds, with 80~90% of carbons being chiral in addition to having positional linkage isomers, often exist as a complex population displaying considerable isomeric heterogeneity among molecules.\(^6\) The capability to rapidly separate various oligosaccharide isomers from a mixture is an advance towards structural analysis of independent carbohydrate precursor isomers and furthers development of glycomics research.

Over decades, mass spectrometry (MS) has become a sensitive and popular tool for the determination and identification of oligosaccharides isolated from glycoproteins.\(^11\)\(^-\)\(^16\) However, MS analysis of carbohydrate compounds has been challenging due to the presence, quite frequently, of large numbers of oligosaccharide isomers having identical \(m/z\) values. MS\(^n\) information is unreliable without separation of isomeric mixtures, and establishing unambiguous precursor-product ion relationships for specific structures becomes tenuous with sets of more than one precursor isomer. Thus, the physical isolation of isomeric carbohydrates from a complex mixture is highly preferred for structural analysis using collision-induced dissociation (CID) experiments and tandem mass spectrometry. With liquid chromatography (LC) coupled to
several columns including graphitized carbon chromatography, reversed ion pairing chromatography and normal phase chromatography have been able to differentiate isobaric oligosaccharides. However, LC-MS can be time consuming, both in establishing the method and performing the separation; sample derivatization is sometimes required prior to analysis. In general, one specific column is only suitable for the separation of a certain class of glycan isomers and in addition, multi-dimensional orthogonal LC methods are often needed in order to purify structures to homogeneity as evaluated by NMR. Other techniques like gas chromatography (GC), or capillary electrophoresis (CE) typically show higher resolution separations in general, nonetheless closely-related isomers have some probability of co-migration in any system; there is currently no universal single-separation method for resolution of carbohydrate isomers. Rapid, sensitive, efficient and robust analytical methodologies are needed to address the analytical challenge of structural identification of isomers in carbohydrate analysis. Preferably, such methods should employ multiple orthogonal types of separations of high resolution, each with the capability of independently resolving many isomeric species.

Ion mobility mass spectrometry (IMMS) is capable of rapidly differentiating isomers using carbohydrate standards and mixtures of glycans from biological sources. The separation is dependent on the drift velocity of gas phase ions under an electric field in a counter flow of drift gas through an ion mobility spectrometry (IMS) drift tube prior to MS. The majority of separations of glycans by IMS have been performed for the precursor ions in the positive mode. Only a limited number of studies have been reported using the negative mode. More importantly, the isomeric heterogeneity of oligosaccharide product ions derived from biological mixtures has not been evaluated in the negative ion mode. Clemmer et al. studied the mobility of negative product ions derived from simple oligosaccharide standards in
1997 by fragmenting precursor ions with high injection energy. Recently, a Synapt G2 high definition mass spectrometer (HDMS),\textsuperscript{44, 45} utilizing a traveling wave ion mobility instrument coupled to mass spectrometry,\textsuperscript{46-50} has been used in a wide range of applications including glycomics.\textsuperscript{51-56} The G2 HDMS is a hybrid quadrupole/ traveling wave ion mobility /orthogonal time of flight (TOF) MS. Additionally, a trap cell is located in front of the traveling wave ion mobility separator and a transfer cell behind it, whereby ions can be dissociated at either location. This special design enables both mobility-resolved precursor ions to be fragmented and mobility-separated product ions to be dissociated. Martensson et. al. previously reported\textsuperscript{23} that neutral O-linked oligosaccharide-alditols isolated from bovine submaxillary mucin (BSM) contained a large number of isomers thus BSM serves as a good paradigm for glycomics of O-linked oligosaccharides. The separation of isomeric oligosaccharide-alitol species and the evaluation of the stereochemistry of their structures still pose a great challenge for multi-step LC/MS or LC/MS/MS analysis.

Here we report a negative mode study using a traveling wave IMMS to evaluate the isomeric heterogeneity of neutral oligosaccharide-alditols of BSM. The unique capabilities of IMMS for isomer differentiation and its advantages in assessing stereo-structural differences of glycans within biological carbohydrate mixtures were demonstrated. Individual LC fractions from the separation of an oligosaccharide-alditol mixture were used as target analytes; carbohydrate isomeric heterogeneity within single LC fractions was investigated. The analysis of oligosaccharide-alditols included isomer separation and acquisition of mobility-resolved fragmentation spectra for both precursor and product ions using multiple functions of the Synapt G2 instrument.
Experimental Section

Materials

All the solvents (methanol, water and acetonitrile) were optima LC/MS grade and supplied by Fisher Chemical (Thermo Fisher Scientific Inc.). All the chemicals (NaOH, NaBH₄, NH₄OH and NH₄Cl) plus the glycoprotein of bovine submaxillary mucin (BSM) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. An equal volume mixture of methanol and water was used to prepare the electrospray ionization (ESI) solvent.

Martensson et al.²³ described the procedure for the preparation and separation of a neutral oligosaccharide-alditol mixture from BSM. Briefly, 1.0 g BSM was treated with 0.01M NaOH/1.0 M NaBH₄ to release acidic and neutral oligosaccharide alditols (only the neutral oligosaccharides were used in current study). The neutral oligosaccharide alditol mixture was then separated on a semipreparative Glycopak N normal phase HPLC column²³ (Millipore Corp., Bedford, MA) using acetonitrile/water as mobile phase in the range of 85/15 to 70/30 at flow rate of 5.0 ml/min. Totally 10 HPLC fractions were collected based on UV absorbance at 200 nm and concentrated to dryness through rotary evaporation. The unseparated mixture and individual HPLC fractions were then analyzed by traveling wave ion mobility mass spectrometry.

1 ml ESI solvent was added to each HPLC fraction (dried sample) to prepare stock solutions. According to the separation chromatogram (see Fig. 1 below), the sample amount decreased as the LC fraction increased, thus later LC fractions contained lower quantities of oligosaccharide-alditols. Different dilution ratios were used for different fractions, resulting in a final solution having concentration of ~0.03 mg/mL carbohydrate for each fraction. To promote the formation of deprotonated and chloride adducted anions in the negative ion mode, 25 μL of each of NH₄OH and NH₄Cl stock solutions (1mM in ESI solvent) was added to 1 ml of
individual diluted oligosaccharide-alditol LC fractions, resulting in concentrations of 25 μM NH₄OH and 25 μM NH₄Cl in the solution.

**Traveling Wave Ion Mobility Mass Spectrometry Measurements**

All experiments were performed on the Synapt G2 high definition mass spectrometry (HDMS) (Waters-Micromass Corp., Milford, MA, USA). A full description of a standard Synapt HDMS system is presented elsewhere⁴⁶-⁵⁰ and the improvements made in the Synapt G2⁴³,⁴⁴ have also been described previously. The Synapt G2 instrument is composed of a quadrupole, a trap cell, a traveling wave ion mobility separator, a transfer cell and a TOFMS in sequential order. The quadrupole can be used to filter a specific precursor ion; fragmentation can be carried out in either or both the trap cell and the transfer cell by applying appropriate collision energy (CE). Traveling wave IMS suffers from low resolution in the standard Synapt, but the resolution is increased 3-4 times in the Synapt G2.

Several operation modes were employed in this investigation: 1) MS mode: the system was used only as a mass analyzer to acquire the mass values of neutral oligosaccharide-alditols in the different LC fractions; 2) Ion Mobility MS mode: the ion mobility separation was utilized to evaluate the isomeric heterogeneity of oligosaccharide-alditol precursor ions with knowledge of precursor m/z values; 3) Ion mobility-MS/MS mode: the quadrupole was set to allow precursor ions having a specific m/z to pass through the trap into the traveling wave for ion mobility separation. Finally the mobility separated precursor ions were collision dissociated in the transfer cell to obtain the fragmentation spectra in the TOFMS; 4) MS/MS-ion mobility mode: precursor ions were filtered by the quadrupole and fragmented in the trap cell prior to IMS analysis. Isomeric product ions were evaluated by traveling wave IMS; 5) MS/MS-ion mobility-
MS/MS mode: In addition to the process described in method 4, fragmentation of the mobility-resolved product ions were obtained in the transfer cell; this is referred to as time aligned parallel (TAP) fragmentation. The ion trap and the ion transfer cell were converted to collision induced dissociation (CID) cells by increasing the collision energy (CE) in each cell. Through all the experiments, the TOFMS was operated in the “V” mode, which is the most sensitive mode. The mass resolution obtained for the target ions was > 10,000 and the mass accuracy were within ± 0.1 Da in this study. A wave height of 30 V and a wave velocity of 300 m/s were used to perform traveling wave ion mobility separation. N₂ was used as the drift gas at a flow rate of 90 mL/min, resulting in a pressure of ~ 3.5 mbar in the ion mobility device. Ions were accumulated in the trap cell and released into the IM cell over a period of 200 μs in this study. Other parameters were as follows: ESI voltage 2.5 KV; desolvation gas (200°C) 600 L/hr; source temperature 200°C; argon gas (in trap and transfer cells) 2 mL/min; helium gas (introduced in front of the IMS cell) 180 mL/min. Samples were injected at a flow rate of 5 μL/min using a syringe pump (Chemyx Inc. Stafford, TX). Masslynx™ 4.1 (Waters-Micromass Corp., Milford, MA, USA) was used to acquire and process all the data.

Results and Discussion

LC and MS Analysis of BSM Neutral Oligosaccharide-alditols

Fig. 1 shows the HPLC elution profile of a neutral oligosaccharide-alditol mixture isolated from BSM with UV detection at 200nm; this semi-preparative column separation took place in 4 hours. The quantity of glycans in the longer eluting fractions was found to decrease according to the LC separation chromatogram. The initial mixture and all the fractions were subjected to mass measurements on the Synapt G2 in the MS only mode and the results are
summarized in Table 1. Both deprotonated and chloride adducts were observed for all the oligosaccharide-alditols. [M-H]• ions were more intense than [M+Cl]• ions which could be due to better initial formation of deprotonated ions during electrospray or possibly due to the decomposition of [M+Cl]• ions to [M-H]• ions with the neutral loss of HCl. Fraction 1 contained GalNAc-ol and was not examined. Two observations are worthy of note: (1) A number of oligosaccharide-alditols were detected in the sample mixture prior to LC, however, large oligosaccharide-alditols, found in fractions 8, 9 and 10, were either not detected or were present at very low intensity in the mass spectra of the mixture. This may in part be due to the extremely low quantity of higher mass compounds found in the mixture relative to the lower mass components and in part due to charge competition with the more abundant ions. LC separation increased the amounts of low abundant oligosaccharide-alditols in individual LC fractions, enabling them to be analyzed by traveling wave IMS. (2) There were multiple oligosaccharide-alditols detected in the majority of individual HPLC fractions with the later fractions having larger molecules. Additionally, molecules having specific m/z values were observed in multiple HPLC fractions, e.g. using [M-H]• ions as examples, m/z 425.2 was detected in fractions 2 and 3; m/z 587.2 and 628.3 appeared in fractions 4, 5 and 6; m/z 733.3 was seen in fractions 5, 6 and 7; m/z 895.3 and 936.3 were in both fractions 8 and 9, and m/z 1041.4 was observed in fractions 9 and 10. The same BSM neutral oligosaccharide-alditol mixture and individual HPLC fractions have been subjected to drift tube IMMS studies by the authors using positive mode ESI. The major oligosaccharides observed herein were the same as those identified in the positive mode. The complicated mass distribution prompts the following questions: (1) How would one know whether compounds having the same m/z in different fractions are isomers or not? (2) Is an oligosaccharide having a specific m/z from a single HPLC fraction one pure isomer? (3) How
can the isomeric nature of oligosaccharide-alditols for both precursor and product ions be best evaluated?

**Evaluation of Oligosaccharide-alditol Precursor Ions**

All individual HPLC fractions from fractions 2 to 10 were subjected to traveling wave ion mobility measurements in the ion mobility MS mode. The data was acquired in 3 min for each fraction. Fig. 2 shows overlaid mobility profiles for the same oligosaccharide-alditol precursor ions found in different HPLC fractions. For example, in Fig. 2a are overlaid mobility spectra for the m/z 623.2 ions that were observed in fractions 4, 5 and 6. This is a [M+Cl]⁻ ion having the composition of Hex₁HexNAc₁HexNAc-ol. Two mobility peaks of m/z 623.2 were clearly observed with fractions 5 and 6 having identical mobility profiles. The predominant mobility peak (drift time 4.4 ms) in fractions 5 and 6 showed the same mobility as the minor peak in fraction 4, and *vice versa* for the peaks observed from fraction 4. Fig. 2b displays overlaid mobility profiles of the [M-H]⁻ ion at m/z 628.2 from fractions 4, 5 and 6 having the proposed molecular formulae of HexNAc₂HexNAc-ol. The mobility profiles had similar characteristics as observed in Fig. 2a but had slightly longer drift times of 4.8 ms and 5.1 ms. Mobility profiles of chloride-adducted tetrasaccharide-alditols having the molecular formulae of deoxyHex₁Hex₁HexNAc₁HexNAc-ol at m/z 769.3 are shown in Fig. 2c. Fractions 5 and 6 shared the same drift time distributions having two partially separated mobility peaks (drift time 5.3 ms and 5.7 ms) detected at different intensities. The single mobility peak observed (drift time 5.7 ms) in fraction 7 was partially overlapped with the less abundant mobility peaks observed in fractions 5 and 6 for m/z 769.3. In Fig. 2d, another example for the [M-H]⁻ precursor ion at m/z 895.4 from HPLC fractions 8 and 9 is presented, having the proposed sugar composition of
deoxyHex$_1$Hex$_2$HexNAc$_1$HexNAc-ol. Two isomeric mobility peaks were detected in fraction 9; the peak having a shorter drift time (6.3 ms) was overlapped with the single peak observed in fraction 8. There were in total 5 different drift time values for chloride-adducted pentasaccharide-alditols, deoxyHex$_1$Hex$_1$HexNAc$_2$HexNAc-ol, at m/z 972.4 overlaid from fractions 8 and 9 in Fig. 2e. Three separated mobility peaks were detected in fraction 9, and 3 partially separated mobility peaks were detected in fraction 8. For the deprotonated hexasaccharide-alditols having the composition deoxyHex$_2$Hex$_2$HexNAc$_1$HexNAc-ol at m/z 1041.4 from fractions 9 and 10 shown in Fig. 2f, only one mobility peak was detected in fraction 9, while two were observed in fraction 10. Sugar compositions reported in this study were derived from the fragmentation spectra and validated by the m/z values.

Several conclusions can be made based on these results: First, isomeric oligosaccharide-alditols can be resolved by traveling wave IMS, sometimes fully, in the negative ion mode. Second, IMS is a truly orthogonal separation technique as compared to LC. For example, as shown for precursor ions having m/z 623.2 and 628.2 (Fig. 2a and b), isomers that eluted earlier by LC actually drifted more slowly using IMS. The separation principles, of course, are entirely different. Third, the majority of LC peaks contained isomers as assessed by IMS; thus one should expect that every LC fraction of oligosaccharides derived from glycoproteins may harbor isomeric species. This has been well-established with glycoproteins where enough sample is available to analyze LC fractions by NMR.$^{23}$ Fourth, LC separations are typically on the order of many minutes (sometimes hours, as shown in Fig. 1 for this semi-preparative separation), whereas TWIMS can evaluate isomeric heterogeneity in less than 10 ms. Fifth, the resolving power of TWIMS peaks in this study was measured with a FWHM of ~0.2-0.3ms, thus coincidental co-migration of some compounds, even after HPLC and ion mobility separation, is
not unexpected. Hence the number of IMS peaks detected for one \( m/z \) only denotes the minimum number of carbohydrate isomers that can be differentiated by IMS under one defined set of experimental conditions. Finally, a specific oligosaccharide-alditol can sometimes be found solely within a single LC peak, but it is possible to find it within two of them. When collecting peaks from valley to valley as shown in Fig. 1, some structures may span parts of both peaks as the valleys do not always reach baseline.

**Fragmentation Spectra of Mobility Separated Precursor Ions**

Mobility-resolved MS/MS spectra for isomeric oligosaccharide-alditol precursor ions were obtained by operating the Synapt G2 in the ion mobility-MS/MS mode as described in the experimental section. Fig. 3a displays the overlaid mobility spectra of pentasaccharide-alditols having the composition deoxyHex\(_1\)Hex\(_1\)HexNAc\(_2\)HexNAc-ol as \([M-H]\) ions at \( m/z \) 936.4 in fractions 8 and 9. Two isomeric mobility peaks were observed for fraction 9 while three isomeric mobility peaks were detected for fraction 8, labeled as 1, 2 and 3. The corresponding fragmentation spectra for mobility peaks 1, 2 and 3 in fraction 8 are displayed in Fig. 3b, 3c and 3d, respectively; 45 V CE was applied in the transfer cell after ion mobility separation. It was apparent that all three tandem spectra were different: for mobility peak 1 (Fig 3b) the base peak was the parent ion with a strong fragment ion of 715.3. For peak 2 (Fig. 3c), the base peak was the 247.1 fragment ion with a second strong fragment ion at 407.2. Finally, peak 3 (Fig. 3d) also had a base peak at 247.1 but the second strongest fragment ion was at \( m/z \) 570.2. The fragments in Fig. 3 resulted from dissociation at glycosidic linkages with losses of deoxyHex, Hex, HexNAc and HexNAc-ol as well as cross-ring cleavages. Some fragments were shared by all three mobility peaks, albeit with different relative intensities; some fragments were unique.
These data indicated that the three mobility peaks separated for m/z 936.4 in fraction 8 carry different stereo-structure or linkage information as validated by their different MS/MS spectra. This is important to point out because without the IMS separation any mass spectra of the selected precursor ion(s) does (do) not enable a direct relationship to be established between a specific combination of product ions and individual isomers of precursor ions.

The same analysis was performed for the tetrasaccharide-alditols having the sugar composition deoxyHex\textsubscript{1}Hex\textsubscript{1}HexNAc\textsubscript{1}HexNAc-ol; the results are shown in Fig. 4. In Fig. 4a are displayed the overlaid mobility profiles for the [M-H]\textsuperscript{-} ions at m/z 733.3 in fractions 5 and 6. Two mobility peaks were detected in both fractions labeled as 1 and 2. The fragmentation spectra for peaks 1 and 2 in fraction 6 were obtained by applying 30 V CE in the transfer cell and are shown in Fig. 4b and 4c, respectively. The m/z 407.2 ion was the predominant product ion in Fig. 4b, which was formed by a neutral loss of deoxyHex\textsubscript{1}Hex\textsubscript{1} from the precursor ion. A dramatically different fragmentation spectrum was observed for the isomeric peak 2 in Fig. 4c, having m/z 247.1 as the base mass peak. The mobility separated fragmentation spectra for the two isomeric mobility peaks of m/z 733.3 were clearly different and the data indicate that m/z 733.3 precursor ions contain different structures varying in the linkage or branching patterns. However, the information from MS/MS alone is not adequate to derive unambiguous structures. Overall, mobility-resolved fragmentation spectra shown in both Fig. 3 and Fig. 4 verified that the multiple isomeric peaks detected by TWIMS were actually different molecular precursors, further validating the unique separation capability of ion mobility and its utility for discriminating between isomeric compounds.
Evaluation of Oligosaccharide-alditol Product Ions

In comparison to the evaluation of isomeric carbohydrate precursor ions using ion mobility-mass spectrometry, its use for separating potential isomeric product ions is still in its infancy. Here we report the resolution of isomeric product ions generated from a specific oligosaccharide-alditol precursor ion using the MS/MS-ion mobility operation mode where a CID experiment was performed prior to the traveling wave ion mobility separation. Fig. 5 shows the result of an experiment whereby a selected deprotonated tetrasaccharide-alditol, Hex1HexNAc2HexNAc-ol from LC fraction 7 (precursor ion m/z 790.3) was dissociated to its corresponding product ions prior to separation by traveling wave IMS. The fragmentation spectrum of m/z 790.3 is shown in Fig. 5a using a 35 V CE in the trap cell; the product ions observed were m/z 389.1, 424.1, 521.2, 569.2, 587.2, 610.2 and 749.3. The extracted ion mobility spectra for the precursor ion of m/z 790.3, and for product ions of m/z 749.3 and 569.2 are presented in Fig. 5b. Three isomeric mobility peaks were resolved for the precursor ion m/z 790.3 with mobility peaks at 5.48 ms and 5.63 ms being barely resolved. Two mobility peaks were fully separated for the product ion m/z 749.3 at drift times of 5.21 and 5.9 ms and partially resolved for the product ion m/z 569.2 at drift times 4.35 and 4.83 ms; the mobility shoulder at 4.35 ms of low abundance was shown in the inserted window by expanding the intensity scale and confirmed by checking its reproducibility. Fig. 5 demonstrates an example of isomeric precursor ions giving rise to isomeric product ions derived from a biologically relevant mixture of oligosaccharide-alditols that are not experimentally atypical. This is not surprising, since the structures of many carbohydrate product ions derived from isomeric precursor ions varying in their molecular structures would be expected to be different in many cases. It is also entirely
possible that isomeric product ions may be derived from the same precursor ion, depending on its branching pattern or even linear structure.

**Fragmentation Spectra of Mobility Separated Product Ions**

To further demonstrate the isomeric heterogeneity of oligosaccharide product ions, fragmentation spectra of individual isomeric product ions were obtained. This was achieved by operating the Synapt G2 HDMS in MS/MS-ion mobility-MS/MS mode as explained above. A representative example is shown here in the analysis of isomeric product ions generated from the precursor ion of \( m/z \) 1041.4 in HPLC fraction 9; the spectra are displayed in Fig. 6. Fig. 6a shows the MS/MS spectrum of \( m/z \) 1041.4; the product ions yielded were \( m/z \) 877.4, 715.3, 551.2, 510.2, 409.1 and 367.1. Fragmentation was carried out in the trap cell using 50V CE and the isomeric heterogeneity of all the ions were evaluated by traveling wave IMS. Fig. 6b displays the mass-extracted mobility spectra for the precursor ion(s) of \( m/z \) 1041.4 and selected isomeric product ions of \( m/z \) 715.3 and 551.2. Interestingly, only one mobility peak was detected for \( m/z \) 1041.4 at 7.32 ms while two isomeric peaks were observed for its corresponding fragment ions at \( m/z \) 715.3 (4.88 and 5.48 ms) and 551.2 (4.01 and 4.50 ms). As mentioned previously, a single IMS precursor peak could still be an isomeric mixture, but it is also possible that more than one isomeric product ion may be derived from the same precursor. The separation that occurs in the ion mobility drift tube is based on an ion’s collision cross section. It is entirely feasible for isomeric precursor ions where their overall geometry and differences in structural configuration are small, that they may coincidentally co-migrate by IMS. However, when such oligosaccharide precursor ions break down to different substructures, differences in the stereochemistry and branching of product ions may give rise to cross-sectional areas that are very different. This can
result in separable drift times for product ions that may, in combination with their dissociation spectra, more effectively differentiate two precursor ions that happen to co-migrate by IMS. These additional criteria might be especially powerful when combined with the information obtained from pure standards, where precursor mobility, precursor dissociation patterns, product mobility patterns and mobility-selected product ion dissociation patterns could all be rapidly recorded and stored as unique database criteria for individual compounds.

To demonstrate the utility of the Synapt G2 instrument to obtain mobility-selected product ion dissociation patterns, also called time aligned parallel (TAP) fragmentation, the most intense product ion (m/z 715.3) was dissociated. m/z 715.3 is derived from the neutral loss of deoxyHex₁Hex₁ from the precursor ion m/z 1041.4, the resulting composition is deoxyHex₁Hex₁HexNAc₁HexNAc-ol. In this mode fragmentation of a product ion can be carried out in the transfer cell in addition to fragmentation of a selected precursor ion in the trap cell. In this experiment, 36 V CE was applied in the transfer. Fig. 6c and Fig. 6d display the MS/MS spectra of m/z 715.3 corresponding to the two isomeric mobility peaks at 4.88 ms and 5.48 ms, respectively. The two spectra were similar but some features were quite different; the abundance of product ion m/z 205.0 was much higher in Fig. 6c in comparison to Fig. 6d with the same CE used; product ions of m/z 247.0, 367.1, 389.2, 551.3 and 673.4 appeared in both spectra; additionally, ions of m/z 655.4, 512.3 and 409.1 were only observed in Fig.6c, ions of m/z 510.2 and 407.2 were present uniquely in Fig.6d. The data unambiguously indicates that the two product ion mobility peaks of m/z 715.3 represent at least two stereochemical and/or branch isomers possibly differing in anomeric configurations. This is the first report of individual fragmentation patterns for ion mobility-separated oligosaccharide product ions, further
demonstrating the value of IMS through its capability of discriminating between isomeric substructures derived from (potentially) isomeric precursor ions.

**Anion Effects on the Mobility Separation**

Oligosaccharides can form various metal adducts such as Na⁺, K⁺, Ag⁺, Co²⁺, Cu²⁺, and Ca²⁺ in the positive mode and anion adducts like F⁻, Cl⁻, Br⁻, NO₃⁻, CH₃COO⁻ in the negative mode. Using electrospray ionization. Dwevidi et al. reported that the nature of cations did markedly affect the mobility separation of carbohydrate isomers using monosaccharide methyl glycoside as candidates; no anion effect on the separation of oligosaccharide isomers by ion mobility has been reported. Herein, the mobilities of chloride adducts of oligosaccharide-alditols from BSM were measured as well and compared with corresponding [M-H]⁻ ions. Fig. 7 displays the IMS spectra of both [M-H]⁻ and [M+Cl]⁻ ions for four different oligosaccharide-alditols from different HPLC fractions. They are (a) trisaccharide-alditols of sugar composition Hex₁HexNAc₁HexNAc-ol in fraction 4; (b) tetrasaccharide-alditols of composition deoxyHex₁Hex₁HexNAc₁HexNAc-ol in fraction 6; (c) tetrascarhide-alditols of composition Hex₁HexNAc₂HexNAc-ol in fraction 7 and (d): pentascarhide-alditols of composition deoxyHex₁Hex₁HexNAc₂HexNAc-ol in fraction 9, respectively. In Fig. 7a, no separation of isomers was observed based on the mobility profile of the [M-H]⁻ ion at m/z 587.2, while the [M+Cl]⁻ ion at m/z 623.2 showed two mobility peaks for the same species. Two isomeric mobility peaks were observed for both anions at m/z 733.3 and m/z 769.3 in Fig. 7b. The [M-H]⁻ ion showed slightly higher resolution between two isomers as compared to their [M+Cl]⁻ counterparts. There were in total three mobility peaks detected for both [M-H]⁻ and [M+Cl]⁻ ions of the oligosaccharide-alditols shown in Fig. 7c with similar resolution between
them. However, the mobility profiles were dramatically different. Among the three isomeric mobility peaks (Fig. 7c), the first two partially separated isomeric mobility peaks having respective drift times of 5.4 and 5.7 ms were both at high abundance for [M-H]⁻ ion at m/z 790.3 but only the first isomeric mobility peak at 5.8 ms was present as the major peak for [M+Cl]⁻ ions at m/z 826.3. Apparently some carbohydrates preferentially loose a proton while others preferentially adduct an anion. Fig. 7d displays the ion mobility separation of isomeric pentasaccharide-aliditol species. Three peaks were observed and better resolution was achieved for the [M+Cl]⁻ ions at m/z 972.4 in comparison to the [M-H]⁻ ions at m/z 936.4. Similar to Fig. 7c, the relative intensities for the isomeric mobility peaks were not consistent between [M-H]⁻ and [M+Cl]⁻ anions. In general, [M+Cl]⁻ ions had slightly longer drift times than [M-H]⁻ ions in traveling wave IMS and in addition it appeared that more isomeric species were able to be resolved with slightly better resolution using [M+Cl]⁻ ions. However, no specific rules of separation by mobility could be concluded. More experiments in the negative ion mode are needed to fully understand how different anions influence the mobility of carbohydrate isomer ions.

**Conclusions**

Using O-linked oligosaccharide-aliditols isolated from bovine submaxillary mucin as a model mixture of isomeric carbohydrate molecules in the negative ion mode, this study demonstrated the value of traveling wave ion mobility mass spectrometry in differentiating carbohydrate stereo- and/or branch isomers. Many isomeric precursor ions were observed when individual HPLC fractions were analyzed by traveling wave ion mobility spectrometry. Isomeric
mobility peaks were observed for the majority of oligosaccharide-alditols ranging from disaccharide-alditols to hexasaccharide-alditols. Furthermore, it was demonstrated that many isomeric product ions could be separated by traveling wave ion mobility and that either \( m/z \) and mobility-selected precursor ions or \( m/z \) and mobility-selected product ions could be dissociated to yield mass spectra that differed between isomeric species. Specific product ions derived from a single selected oligosaccharide-alditol were shown to be resolved into more than one independent mobility peak. And, for the first time, that mobility-resolved product ions yielded different characteristic mass spectra. It is worth noting that some mobility peaks were partially or barely resolved, thus higher resolving power and possibly orthogonal gas-phase separations may be needed in order to completely separate all the structural variants within a single oligosaccharide mixture. However, with appropriate carbohydrate standards the possibility now exists to define more exacting criteria for databases: (1) The precursor ion mobility drift time under defined conditions (2) The dissociation pattern of the precursor ion (3) The mobility drift time pattern of all product ions derived from a precursor ion and (4) the product ion dissociation patterns of all product ions derived from a given precursor, with mobility selection of those product ions when they resolve from other isomeric product ions. Together, these significantly increase the probability for identification of a structure provided these patterns can be reproduced from lab to lab.

Acknowledgements

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Table 1. Mass-to-charge values of oligosaccharide-alditols detected in the sample mixture and in individual HPLC fractions in the negative ion mode.

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<th>m/z [M+Cl]</th>
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<th>m/z [M+Cl]</th>
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<td>623.2; 664.2; 769.3</td>
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<td>461.1</td>
<td>7</td>
<td>733.3; 790.3</td>
<td>769.3; 826.3</td>
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Figure 1. HPLC elution profile of neutral oligosaccharide-alditols isolated from bovine submaxillary mucin (BSM). (This figure was reprinted from reference 35 Anal. Bioanal. Chem. 2009, 394, 1853-1867.)
**Figure 2.** Overlaid mobility profiles of individual oligosaccharides in multiple LC fractions. (a): Overlaid mobility profile of the \( m/z \) 623.2 precursor ions in LC fractions 4, 5 and 6; (b): Overlaid mobility profiles of the \( m/z \) 628.2 precursor ions in LC fractions 4, 5 and 6; (c) Overlaid mobility profile of the \( m/z \) 769.3 precursor ions in LC fractions 5, 6 and 7; (d): Overlaid mobility profiles of the \( m/z \) 895.4 precursor ions in LC fractions 8 and 9; (e): Overlaid mobility profiles of the \( m/z \) 972.4 precursor ions in LC fractions 8 and 9; (f): Overlaid mobility profiles of the \( m/z \) 1041.4 precursor ions in LC fractions 9 and 10.
Figure 3. (a): Overlaid mobility profiles of the $m/z$ 936.4 precursor ions in BSM HPLC fractions 8 and 9; further dissociation spectra are shown for valley-to-valley selected peaks 1, 2, and 3 as indicated for the blue profile of fraction 8. (b): fragmentation pattern of the $m/z$ 936.4 ion for mobility peak 1 in panel a; (c): fragmentation pattern of the $m/z$ 936.4 ion for mobility peak 2 in panel a; (d): fragmentation pattern of the $m/z$ 936.4 ion for mobility peak 3 in panel a.
Figure 4. (a): Overlaid mobility profiles of precursor ions having $m/z$ 733.3 in BSM HPLC fractions 5 (blue trace) and 6 (red trace); further dissociation spectra are shown for valley-to-valley selected peaks 1 and 2 as indicated for the red profile of fraction 6. (b): fragmentation pattern of the $m/z$ 733.3 ion for mobility peak 1 in panel a; (c): fragmentation pattern of the $m/z$ 733.3 ion for mobility peak 2 in panel a.
Figure 5. Isomeric analysis of product ions derived from precursor ions having m/z 790.3 in BSM HPLC fraction 7. (a): fragmentation pattern of the precursor ion m/z 790.3 in HPLC fraction 7; (b): TWIMS spectra of the precursor ions having m/z 790.3 and product ions of m/z 749.3 and m/z 569.2 from HPLC fraction 7.
Figure 6. Analysis of isomeric product ions generated from precursor ions of m/z 1041.4 in BSM HPLC fraction 9. (a) Fragmentation pattern of the m/z 1041.4 precursor ion in fraction 9; (b) TWIMS spectra of the precursor ion of m/z 1041.4 and the product ions of m/z 715.3 and m/z 551.2 from HPLC fraction 9; (c) Fragmentation pattern of product ion m/z 715.5 having a drift time of 4.88 ms derived from precursor ion m/z 1041.4; (d) Fragmentation pattern of product ion m/z 715.5 having a drift time of 5.48 ms derived from the precursor ion m/z 1041.4. (Note that the m/z 715.3 and m/z 715.4 refer to the same product ion, the mass difference is within the mass accuracy ± 0.1 dalton as reported in the experimental section).
Figure 7. IMS spectra of both deprotonated and chloride adduct ions of oligosaccharide-alditols having specific m/z values from BSM illustrating the effect of different anions on isomer separation. (a) HexHexNAc1HexNAc-ol in HPLC fraction 4; (b) DeoxyHex1Hex1HexNAc1HexNAc-ol in HPLC fraction 6; (c) Hex1HexNAc2HexNAc-ol in HPLC fraction 7; (d) DeoxyHex1Hex1HexNAc2HexNAc-ol in HPLC fraction 9.
CHAPTER SIX
CARBOHYDRATE STRUCTURE CHARACTERIZATION
BY TANDEM ION MOBILITY MASS SPECTROMETRY (IMMS)²

Abstract

A high resolution ion mobility spectrometer was interfaced to a Synapt G2 high
definition mass spectrometer (HDMS) to produce IMMS-IMMS analysis. The hybrid instrument
contained an electro-spray ionization source, two ion gates, an ambient pressure linear ion
mobility drift tube, a quadrupole mass filter, a traveling wave ion mobility spectrometer
(TWIMS) and a time of flight mass spectrometer. The dual gate drift tube ion mobility
spectrometer (DTIMS) could be used to acquire traditional IMS spectra, but also could
selectively transfer specific mobility selected precursor ions to the Synapt G2 HDMS for mass
filtration (quadrupole). The mobility and mass selected ions could then be introduced into a
collision cell for fragmentation followed by mobility separation of the fragment ions with the
traveling wave ion mobility spectrometer. These mobility separated fragment ions are finally
mass analyzed using a time-of-flight mass spectrometer. This results in an IMMS-IMMS
analysis and provides a method to evaluate the isomeric heterogeneity of precursor ions by both
DTIMS and TWIMS, to acquire a mobility-selected and mass-filtered fragmentation pattern and
to additionally obtain traveling wave ion mobility spectra of the corresponding product ions. This
new IMMS² instrument enables the structural diversity of carbohydrates to be studied in greater
detail. The physical separation of isomeric oligosaccharide mixtures was achieved by both
DTIMS and TWIMS, with DTIMS demonstrating higher resolving power (70–80) than TWIMS
(30–40). Mobility selected MS/MS spectra were obtained, and TWIMS evaluation of product ions showed that isomeric forms of fragment ions existed for identical m/z values.

**Introduction**

Since the first discussion of ion mobility spectrometry (IMS) or plasma chromatography in the 1970’s,1–4 IMS has been applied as an analytical separation and detection tool for explosives,5–8 drugs,9–11 chemical warfare reagents12–14 and biological compounds.15–17 IMS is a unique gas phase ion separation technique based on the ion’s collision cross section (Ω),18,19 which makes it an ideal candidate for differentiation of isomers having identical m/z values but different structures or configurations. When coupled with mass spectrometry (MS),20–23 IMS becomes a powerful analytical tool called ion mobility mass spectrometry (IMMS)24 in which the mass-to-size ratio (m/Ω) provides a measure of an ion’s cross section m/A² density. The benefits of IMMS separation include increasing the peak capacity of a mass spectrometer, separating compounds with the same m/z values,25 reducing chemical and random noise,26 measuring Ω/z values,27 offering class identification by mobility-mass correlation lines28–30 and charge state separation.31 IMMS has proved particularly useful for the separation and identification of biomolecules in complex mixtures such as those encountered in metabolomics,26,30,32,33 glycomics31,34,35 and proteomics.36–38 Conventionally, IMMS instruments utilize IMS either at atmospheric pressure or reduced pressure as a separator for gas phase ions followed by a mass analyzer to provide m/z information. Recently, a traveling wave IMMS,39–41 the Synapt G2 high definition mass spectrometry (HDMS),42,43 was developed and has been widely used.44–49 It is a hybrid quadrupole/ion mobility separator/orthogonal-TOF instrument. The design, electric field homogeneity and pressure are different from traditional drift tube IMS. Moreover, an additional
trap and transfer cells were installed in the front and after the traveling wave ion mobility spectrometer (TWIMS) and could be used to fragment ions before and/or after traveling wave mobility separations.

The primary structures of carbohydrates are extremely complicated compared to nucleic acids and proteins, normally existing in numerous isomeric forms due to differences in the stereochemistry of their monosaccharides, branching of the structures and alternate linkage locations between sugar units. It has been shown that isomeric oligosaccharide species, either simple standards or mixtures prepared from biological sources, can be resolved on the mobility scale. By installing a second gate or selection gate in IMS and connecting the instrument to a tandem mass spectrometer, mobility-selected fragmentation spectra can be collected to provide evidence for differences in structures between mobility-separated isomeric carbohydrate precursor ions. Compared to LC, the most commonly employed separation method, IMS provides advantages of speed (μs or ms scale) and sensitivity, with comparable or higher resolving power for the separation of isomers. Currently, although a number of studies have focused on characterization of isomeric carbohydrate precursor ions employing IMS, structural variation of isomeric product ions has not been extensively investigated. Clemmer et al. showed the mobility of product ions of simple oligosaccharides by fragmenting precursor ions with high injection energy prior to IMS. In the Synapt G2 instrument, a trap cell is installed in front of a TWIMS, which enables the isomeric heterogeneity of product ions to be evaluated. However, determining the mobilities of both precursor and product ions, where product ions are derived from precursor ions within a specific mobility window, is not currently feasible for isomeric carbohydrate mixtures. Consequently, the mobility spectra of product ions generated from a
mixture of isomeric precursor ions results in ambiguous precursor-product ion relationships. It is impossible to assign the mobility peaks of product ions to specific precursor ions in these cases.

In this study, we describe a new ion mobility mass spectrometry instrument, a dual gate drift tube IMS (DTIMS) interfaced to a Synapt G2 HDMS, which provides a novel analytical approach of IMMS-IMMS. It is capable of carrying out an additional MS function compared to the IMS-IMS instrument reported previously. This is also the first instrument that incorporates DTIMS, TWIMS, mass selection, mobility selection and tandem MS together. Mixtures of isomeric oligosaccharides were used to evaluate multiple capabilities of this hybrid instrument. Results include mobility separation of isomeric mixtures of precursor ions using both DTIMS and TWIMS, mobility-selected tandem spectra and traveling wave ion mobility evaluation of product ions derived from mobility and mass selected precursor ions.

**Experimental Section**

**Chemicals and Solvents**

NaCl, D-Gal-α-(1-4)-D-Gal (4α-galactobiose), D-Glc-β-(1-6)-D-Glc (gentiobiose), the trisaccharides raffinose and maltotriose, and the pentasaccharides cellopentaose and maltopentaose were purchased from Sigma Chemical Co., St. Louis, Missouri. The pentasaccharide [D-Man-α-1-6-[D-Man-α-1-3]-D-Man-α-1-6]-[D-Man-α-1-3]-D-Man (branched (Man)₅ is used in the following text) was purchased from V-labs, Covington, Louisiana. LC-MS grade solvents of methanol and water were purchased from Thermo Fisher Scientific Inc.. Disaccharide-alditols D-Gal-α-1-4-D-Gal-ol and D-Glc-β-1-6-D-Glc-ol were prepared from 4α-galactobiose and gentiobiose (See supporting information for reduction method).
Sample Preparation

200 μM stock solutions of disaccharide-aldehydes with 200 μM NaCl in each sample were initially prepared using electro-spray (ESI) solvent (50:50 v:v methanol: water) and were diluted in 1:1 ESI solvent for individual analysis (100 μM each). They were mixed unequally resulting in a mixture of 100 μM D-Gal-α-1-4-D-Gal-ol and 50 μM D-Glc-β-1-6-D-Glc-ol. Raffinose and maltotriose were prepared in the same way; raffinose at 50 μM, maltotriose at 100 μM in the mixture. Unequal concentrations of samples in the mixture were used to decrease ion suppression effects. 300 μM stock solutions of pentasaccharides with 300 μM NaCl in each sample were prepared and were mixed in equal volumes for mixture analysis (100 μM each). They were then diluted 1:2 in ESI solvent for separate analyses of individual compounds (100 μM each).

Electrospray Ionization (ESI)

The details of the construction of the lab-built ESI source are included in the supporting information.

Dual Gate Drift Tube Ion Mobility Spectrometer (DTIMS)

Two Bradbury-Nielson ion gates separated the IMS tube into a desolvation region (7.5 cm length), drift region (21.0 cm length) and post drift region (1.0 cm length). Nitrogen was used as the drift gas at a flow rate of 2 L/min and the tube was maintained at 180°C. Voltages at the first ring, first ion gate, second ion gate and last ring of IMS tube were 10.0 KV, 9.01 KV, 743 V and 427 V, respectively, resulting in a homogeneous electric field of 394 V/cm. The control software and operations of the dual gate IMS system were the same as described in previous
publications, except that the software was updated with the 2009 version of Labview (National Instruments, Austin, TX). Briefly, there are two operation modes: dual gate scanning (DGS) which determines the drift time of an ion by a series of successive stepped ion gate pulsing experiments and selected mobility monitoring (SMM) which allows ions of specific drift time window widths to be transferred to the mass analyzer. For the results shown in this study, the parameters used for the DGS mode were as follows: the gate pulse widths of both ion gates were 0.3 ms, the step resolution (the increment by which the second gate delay was sequentially increased) was at 0.1 ms, pulses/step (number of ion gate pulses before the 2\(^{nd}\) gate is moved to the next delay) was set at 300, and the scan window was normally within 3 or 4 ms as defined from the second gate delay. For the SMM mode, the first ion gate was kept at 0.3 ms, and the second gate was open for a certain drift time range (specified for different compounds) to select the target analyte. The pulsing of the first ion gate of the DTIMS was synchronized with the data acquisition of the MS in both modes.

**Dual Gate DTIMS Interfaced to Synapt G2 HDMS**

The Synapt G2 HDMS instrument has been described elsewhere. The TWIMS is used under reduced pressure and employs traveling wave ion guide technology with a non-uniform electric field. The overall schematics of the ESI, ambient pressure, dual gate DTIMS, Synapt G2 HDMS instrument and the detailed interface connection (inset) are displayed in Figure 1. To make the connection, the original sample cone was replaced with a modified sample cone which is almost identical to the original one except that an extended threads section (i. d. 1/8\)” was added to the orifice (the pictures of original and modified sample cones are shown in Figure S-1 in supporting information). The end of the DTIMS tube was sealed with a metal plate having an
extended threaded orifice (i. d. 1/8") in the center and was electrically isolated from the last ring of the DTIMS by ceramic rings. A hollow stainless steel tube (o. d. 1/8", i. d. 1/24") having a 90 degree bend with unequal lengths on either side of the bend (4 cm and 2 cm), was used to connect the orifice on the metal plate of the DTIMS with the modified sample cone on the Synapt G2. The voltages on the metal plate and 90 degree tube were the same to the voltage as applied on the sample cone which was -68 V when Synapt G2 was in the TOF mode and 87 V when the Synapt G2 was in the mobility TOF mode (positive ion mode). The TWIMS was operated with 40 V wave height, 650 m/s wave velocity at a pressure of ~ 3.5 mbar. Nitrogen was used as the drift gas at a flow rate of 90 mL/min, He was introduced at 180 mL/min to the helium cell installed in front of the ion mobility separator. Argon was used in the trap and transfer cells at a flow rate of 2 mL/min. The trap release time was 200 μs, and the mobility separation delay after the trap release was enabled and set at 450 μs. Masslynx 4.1 (Waters Corporation, Milford, MA, USA) was used to collect and process the data.

**Operation Modes of the Hybrid DTIMS-Synapt G2 Instrument**

This hybrid instrument was operated in three modes in this study: (1) with both gates open, the DTIMS serves as an ion transmission device, and the system is in essence a Synapt G2 HDMS only; (2) with the dual gate IMS operating in the DGS mode and the Synapt G2 working only as a TOF mass analyzer, the system is an atmospheric pressure DTIMS-TOFMS, whereby traditional drift tube ion mobility spectra can be obtained; (3) with the dual gate IMS operating in SMM mode, compounds within a selected drift time range on the DTIMS can be transferred to the Synapt G2 for further analysis including mass selection by the quadrupole, fragmentation in the trap cell and TWIMS evaluation of product ions in sequential. The mobility selected
fragmentation spectra and traveling wave ion mobility spectra for product ions derived from specific isomeric saccharide from a mixture were obtained using this mode.

Results and Discussion

Disaccharide-alditols: D-Gal-α-1-4-D-Gal-ol and D-Glc-β-1-6-D-Glc-ol

With both DTIMS and TWIMS incorporated into one system, it is convenient to compare the separation capabilities of the two types of IMS. The isomeric disaccharide-alditols D-Gal-α-1-4-D-Gal-ol and D-Glc-β-1-6-D-Glc-ol were first investigated. It should be noted that all the m/z values reported for oligosaccharides in this study were sodiated adducts in the positive ion mode. Figures 2a and 2b show the mobility spectra of the disaccharide-alditol mixture by TWIMS and DTIMS, respectively. The data was acquired for 2 mins using the TWIMS and for 4 mins using the DTIMS. It is apparent that DTIMS demonstrated higher resolving power and better resolution between isomers. According to the traditional definition of resolving power ($R_p$) which is drift time divided by the peak width at half maximum, the experimental $R_p$ for DTIMS in this study was 75 ± 5 and the temporal $R_p$ for TWIMS was ~20 which corresponds to a mobility resolution of ~40. By comparing the spectra of the mixture (Figures 2a and 2b) with the overlaid corresponding individual spectra displayed in Figures 2c and 2d, D-Glc-β-1-6-D-Glc-ol (peak 2) had a slower mobility than D-Gal-α-1-4-D-Gal-ol (peak 1). A strong ion suppression effect was observed in the mixture, with D-Gal-α-1-4-D-Gal-ol (100 μM) detected at lower intensity than D-Glc-β-1-6-D-Glc-ol (50 μM). As discussed by Wesdemiotis and Bowers, Na$^+$ ions tend to coordinate with oxygen sites in carbohydrates through multidentate interactions, which can distort the normal structures of the neutral molecules. This results in
experimentally preferred conformations for Na\(^{+}\) adducts of sugar stereoisomers with differences in their overall shapes. Hence, the separation of isomers in IMS results from both the structural differences and also the conformational changes induced by Na\(^{+}\) complexation.

Once the drift times of the carbohydrates were determined, selected mobility mode experiments were conducted for 10 mins for all the samples in this study to acquire mobility selected MS/MS data and also TWIMS spectra of fragment ions. Figures 2e and 2f display the mobility selected fragmentation spectra for D-Gal-\(\alpha\)-1-4-D-Gal-ol and D-Glc-\(\beta\)-1-6-D-Glc-ol respectively by transferring mobility peaks 1 and 2 (Figure 2b) to the trap cell of the Synapt G2 separately. The 2\(^{nd}\) gate windows on the DTIMS of 24.5-25.2 ms and 25.5-26.5 ms were used, respectively. The corresponding structures and proposed fragmentation pathways of the isomers are shown on the right. A major product ion of \(m/z\) 205 resulted from glycosidic cleavage of the precursor ion (\(m/z\) 367) was observed for both disaccharide-alditols, but in very different abundance. Even with a higher collision energy (CE) of 40V applied to D-Glc-\(\beta\)-1-6-D-Glc-ol compared to that of 35 V applied to D-Gal-\(\alpha\)-1-4-Gal-ol, the intensity of \(m/z\) 205 was still lower for D-Glc-\(\beta\)-1-6-D-Glc-ol which makes the two spectra distinguishable. The subsequent traveling wave mobility of fragment \(m/z\) 205 was almost the same for the two disaccharide-alditols. Their TWIMS spectra as well as the fragmentation spectra acquired from individual standards are displayed in Figures S-2 and S-3 in the supporting information. It is worthy to note that the percent error for different mass ions observed in this study was ~±3%.

**Isomeric Trisaccharides: Raffinose and Maltotriose**

Figures 3a and 3b display the mobility separation of a mixture of structural isomers of raffinose (D-Gal-\(\alpha\)-1-6-D-Glc-\(\beta\)-1-2-D-Fru) and maltotriose (D-Glc-\(\alpha\)-1-4-D-Glc-\(\alpha\)-1-4-D-Glc)
using TWIMS and DTIMS respectively. There was little or no separation in TWIMS but partial separation was observed in DTIMS labeled as peaks 1 and 2. Ion suppression was also evident with raffinose having higher ionization efficiency in the mixture even at comparatively lower concentration (50 μM) than maltotriose (100 μM). In comparison with mobility profiles acquired from their individual standards (Figures 3c and 3d), raffinose drifted faster than maltotriose. In addition, another small mobility shoulder peak was observed for maltotriose in both TWIMS and DTIMS by expanding the intensity scale as shown in the inserted windows of Figures 3c and 3d. Higher resolving power and resolution were again demonstrated using DTIMS. Raffinose is termed a non-reducing oligosaccharide as both the Glc and Fru sugar units are glycosidically linked to each other at their anomeric positions, which locks each of these sugars in a single cyclic configuration. However, maltotriose is a reducing trisaccharide (see structure at the bottom right of Figure 3) having an –OH group at the reducing end in either the α or β pyranose configuration or even the aldehyde open chain form, which can result in more than one isomeric form for the molecule as examined by IMS. The direct evidence of the ring opening of monosaccharide anions in the gas phase has been demonstrated by Brown et al. in 2011.63 Therefore, different Na⁺ complexed maltotriose ions could be formed with different collision cross-sectional areas and it is not unexpected to have more than one mobility peak detected. Figures 3e and 3f are the corresponding MS/MS spectra for mobility peaks 1 and 2 in Figure 3b by selecting the compounds with drift time windows of 31-31.5 ms and 32-32.5 ms on DTIMS respectively and applying the same CE (53 V) in the trap cell of the Synapt G2. The structures and proposed fragmentation pathways for peak 1 which is raffinose and peak 2 which is maltotriose are shown on the right. Both compounds with precursor ions of m/z 527 gave rise to predominant product ions of m/z 365 and product ions of m/z 347 and 203 as well. However,
more fragments were observed for maltotriose such as \( m/z \) 185, 305, 407, 467 and 509 where \( m/z \) 509, 347 and 185 were derived from the \( \text{H}_2\text{O} \) loss from product ions of \( m/z \) 527, 365 and 203. These differences in fragmentation spectra demonstrated that the compounds were isomers and provided evidence for the mobility-separated precursor ions within the mixture. Moreover, all the fragments were reproducible using individual standards and the relative abundance was kept consistent for all the major fragments as well (see Figure S-4 in supporting information).

All the ions that resulted from the collision induced dissociation (CID) in the trap portion of the Synapt G2 shown in Figures 3e and 3f were then evaluated by the TWIMS. Figures 4a and 4b are the TWIMS spectra of the product ions corresponding to selected peak 1 (raffinose) and peak 2 (maltotriose) from the mixture, respectively and the corresponding \( m/z \) values for the major resolved mobility peaks are indicated. The same spectra acquired by injecting raffinose and maltotriose standards under identical experimental conditions are shown in Figures 4c and d, which unambiguously reproduced the data acquired from mobility selection experiments (Figures 4a and 4b). It was observed that product ions with smaller \( m/z \) values were distributed with faster drift times. The drift time values for the most abundant product ion \( m/z \) 365 were 3.47 and 3.53 ms for raffinose and maltotriose respectively, and they were barely resolved. More mobility peaks were detected in Figure 4b which corresponded to other unique fragments of maltotriose as shown in Figure 3f. Mobility identities of product ions derived from individual mobility-selected precursor ions from a mixture of isomers were able to be measured for the first time. In addition, the overall mobility profiles of product ions were obviously different for dissociated raffinose and maltotriose which could serve as further identities to differentiate the isomeric compounds. It is important to note that a direct precursor-product relationship can be established for individual product ions at 3.47 and 4.83 ms, for the first isomer, raffinose and at
2.28, 2.98, 3.53, 4.34 and 5.05 ms, for the second isomer, maltotriose, with each product ion mobility having a measurable \( m/z \). This direct relationship is not possible to establish unambiguously from a mixture of isomeric precursor ions only selected by \( m/z \), i.e. where a mixture of isomeric precursors are simultaneously dissociated using CID. This should provide a valuable tool for assessment of the complexity of isomeric precursor ion mixtures and for firmly establishing their specific sets of product ions.

**Isomeric Pentasaccharides: Cellopentaose, Maltopentaose and Branchehd (Man)\textsubscript{5}**

To further demonstrate the capability of the hybrid IMS-Synapt G2, isomeric oligosaccharides of higher molecular weight were investigated: cellopentaose \([\text{D-Glc-}\alpha-1-4-(\text{D-Glc-}\alpha-1-4)\textsubscript{3}-\text{D-Glc}]\), maltopentaose \([\text{D-Glc-}\beta-1-4-(\text{D-Glc-}\beta-1-4)\textsubscript{3}-\text{D-Glc}]\) and branched (Man)\textsubscript{5}. Cellopentaose and maltopentaose are both composed of five glucose monomers but one is in \( \alpha-1-4 \) linkage and the other in \( \beta-1-4 \) linkage. The mixture of these three isomeric pentasaccharides was resolved into two partially separated peaks using TWIMS as shown in Figure 5a. Interestingly, they were separated into four mobility peaks on DTIMS as shown in Figure 5b where three abundant peaks were labeled as peaks 1, 2 and 3. The traveling wave ion mobility spectrum of the mixture was acquired for 3 mins, and the time taken to scan the 4 ms range of the mixture by dual gate DTIMS was \( \sim \) 8 mins. Figures 5c and 5d display the overlaid mobility spectra of the individual pentasaccharides using TWIMS and DTIMS respectively. It was observed that cellopentaose had the fastest mobility, branched (Man)\textsubscript{5} had the slowest mobility, and maltopentaose was in the middle. In addition, two peaks were observed for cellopentaose using DTIMS. All the compounds had essentially identical drift time values within the mixture as compared to running them individually and the relative elution order of the compounds was
the same using both types of IMS. The three pentasaccharides presented here are all reducing oligosaccharides (see structures on the right of Figure 6), having a reducing monosaccharide that exists in solution in at least two predominant (α and β pyranose) configurations, as discussed earlier. The low abundant mobility peak of cellopentaose detected by DTIMS is therefore not surprising, which may denote the α/β or open chain configuration. The relatively low resolving power of TWIMS makes this corresponding peak undetectable. Even though only one mobility peak was observed for maltopentaose and branched (Man)₅, it is still possible that more than one reducing-end configuration could exist for them and the Ω differences may simply be small enough where they co-migrate as one peak.

Based on the drift time values determined, second gate drift time windows of 38-38.7 ms, 39.5-40.3 ms and 40.6-41.5 ms on the DTIMS were used. These correspond to mobility peaks 1, 2 and 3 as shown in Figure 5 and were used to acquire the mobility selected MS/MS spectra for cellopentaose, maltopentaose and branched (Man)₅, respectively, from the mixture. The spectra are displayed in Figures 6a, 6b and 6c, respectively. A trap CE of 80 V was used for all three peaks and the corresponding structures and fragmentation pathways are also included (Figure 6). Varying solely in their anomeric configurations, the major product ions observed for cellopentaose and maltopentaose were almost identical but were present in different abundance. Those include ions of \( m/z \) 689, 527, 365, and 203 that resulted from glycosidic bond cleavage, X type ions of \( m/z \) 731, 569, and 407 and A type ions of \( m/z \) 791, 629, 467, and 305 according to the nomenclature of Domon and Costello.⁶¹ In addition, the loss of \( \text{H}_2\text{O} \) was also widely observed among product ions such as \( m/z \) 833 (\( \text{H}_2\text{O} \) loss from \( m/z \) 851), 671 (\( \text{H}_2\text{O} \) loss from \( m/z \) 689), 509 (\( \text{H}_2\text{O} \) loss from \( m/z \) 527) and 347 (\( \text{H}_2\text{O} \) loss from \( m/z \) 365). The \( m/z \) 509 product ion appeared as the base peak for cellopentaose, while \( m/z \) 347 was detected having the maximum
intensity for maltopentaose. Moreover, the relative intensities of product ion pairs of \(m/z\) 509/527 and \(m/z\) 671/689 were different for the two isomers as shown in Figures 6a and b. Branched \((\text{Man})_5\) dissociated to yield all the major product ions observed for cellopentaose and maltopentaose, but in a dramatically different intensity distribution having \(m/z\) 527 and 689 as major fragments. Moreover, three unique product ions of \(m/z\) 275, 437 and 599, which are derived from cross ring cleavage as proposed on the structure shown in Figure 6 were observed only for branched \((\text{Man})_5\). The mobility-selected tandem spectra validated the experimental mobility and dissociation data of individual precursor ions, demonstrating that IMS is a valuable tool to separate compounds with subtle structure differences which can be difficult and time consuming for LC or which involves derivatization for GC, and is impossible with MS or MS\(^n\) alone.

To study the isomeric heterogeneity of product ions, pentasaccharide precursor ions were dissociated in the trap and TWIMS spectra of their product ions were collected and compared. Figure 7 displays the overlaid traveling wave ion mobility spectra for product ions of \(m/z\) 509, 527, 671 and 689 generated from cellopentaose, maltopentaose and of branched \((\text{Man})_5\). First, more than one mobility peak was frequently detected for specific product ions derived from one isomeric compound. For example, two mobility peaks were found for the \(m/z\) 527 product ion(s) derived from maltopentaose as shown in Figure 7b. More obviously, two partially or baseline separated isomeric mobility peaks were observed for all four product ions of cellopentaose (blue traces in Figures 7a-d). This suggests that multiple structural configurations exist for each specific product ion. This could be the result of structural differences at the free reducing end – OH included in the fragment ion, or could represent completely different fragmentation pathways giving rise to very different isomeric species. Either types of product ions may arise in
different proportions from the corresponding multiple isomeric forms of reducing precursor ions. Second, by comparing the mobility distributions of single product ions derived from the three isomeric precursors, it was noticed that they had different drift times even though not fully resolved. This is reasonable and not unexpected. As these isomeric precursor ions had different stereochemistries and/or anomeric configurations, the product ions generated from them should be structurally unique, thus resulting in different mobilities. As shown in Figure 7a, m/z 509 of branched (Man)$_5$ drifted faster than m/z 509 from maltopentaose, and overlapped with one of the two mobility peaks of m/z 509 derived from cellopentaose. For product ions of m/z 671 and 689 displayed in Fig. 7c and d, maltopentaose and branched (Man)$_5$ showed similar mobilities and overlapped with the slower drifting mobility peak of cellopentaose. Another phenomenon worthy of note was that the mobility elution order of product ions can be similar or different from the mobility distribution orders of their corresponding precursor ions. For example, in Figure 7d, the m/z 689 product ion of cellopentaose drifted fastest, that of maltopentaose was of intermediate mobility, and that of branched (Man)$_5$ drifted slowest which matched with the relative orders of the mobilities of their precursor ions (Figure 5). However, Figure 7b demonstrated the opposite scenario where, for the product ion of m/z 527, branched (Man)$_5$ had smallest drift time as compared to maltopentaose and cellopentaose, while its precursor ion had the largest drift time as displayed in Figure 5. Again, this is not unexpected as isomeric product ions may have product ions that need not correlate in the magnitude of their overall cross-sectional areas with any relationship between their isomeric precursor ions. In all, the mobility of an oligosaccharide depends on its overall structural configuration when coordinated to a Na$^+$ ion. In addition, due to the limited resolving power of TWIMS, isomeric ions with completely different structures could happen to coincidentally co-migrate, thus each independent peak may represent more than one
ion structure. Overall, the data presented herein provides direct evidence for the isomeric heterogeneity of carbohydrate product ions for the first time. (The MS/MS spectra and TWIMS spectra of product ions acquired from individual standards are included in Figures S-5 and S-6 in supporting information).

**Conclusions**

A lab built atmospheric pressure dual gate IMS was successfully coupled to a Synapt G2 HDMS instrument, resulting in a hybrid IMMS-IMMS instrument. Isomeric mixtures of oligosaccharides including disaccharide-alditols, trisaccharides and pentasaccharides were differentiated in the positive mode as sodiated adducts. The mobility elution order of compounds was the same for DTIMS and TWIMS, but the higher resolving power of DTIMS enabled better separation among isomers. For mobility-selected fragmentation patterns, isomers having subtle structural differences generated identical product ions with different relative intensities; unique product ions were observed for isomers having larger structural variations. The concepts of carbohydrate isomer separation and mobility-selected fragmentation were demonstrated previously,\textsuperscript{54-57} however, the addition of TWIMS after the collision cell in this hybrid instrument offers a method to evaluate the isomeric heterogeneity of product ions for mass- and mobility-selected precursor ions. Different mobilities were observed for product ions having identical \textit{m/z} values. This information is valuable as it establishes a direct precursor-product relationship between mobility-selected precursor ions and specific sets of product ions having unique mobilities in addition to specific \textit{m/z} values. These data should serve as further useful information to uniquely identity individual carbohydrate isomers, allowing unambiguous assignments of different oligosaccharide isomers within mixtures. This hybrid instrument
demonstrated here can provide valuable information not obtainable with typical CID of \( m/z \)-selected precursor ions with added value for structural characterization of isomeric carbohydrates.

Additionally, interfacing a linear drift tube IMS to Synapt G2 directly enables simple, accurate and straightforward calibration\(^{48}\) of traveling wave mobility cell for future studies. Furthermore, the concept of IMMS-IMMS system demonstrated in this study could be extended to other types of ion mobility devices, such as differential mobility spectrometry, which can serve as a mobility filter and may be capable to provide additional advantages. The development of alternative IMS and Synapt G2 combinations is possible, which would promote greater in-depth studies of gas phase ions than are possible with current instruments.

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References


Figure 1. Schematics of the instrument, showing the electrospray ionization (ESI) source, the ambient pressure, dual gate ion mobility drift tube, and the Synapt G2 high definition mass spectrometry unit that includes a quadrupole, a traveling wave ion mobility region and a dual stage reflectron time-of-flight mass spectrometer. The inserted window shows the detailed interface connection.
Figure 2. (a) TWIMS separation of a disaccharide-alditol mixture (precursor \(m/z\) \([M + Na^+] = 367\)). (b) DTIMS separation of the same disaccharide-alditol mixture. (c) Overlaid individual spectra of D-Gal-\(\alpha\)-1-4-D-Gal-ol (blue) and D-Glc-\(\beta\)-1-6-D-Glc-ol (red) obtained on TWIMS. (d) Overlaid individual spectra of D-Gal-\(\alpha\)-1-4-D-Gal-ol (blue) and D-Glc-\(\beta\)-1-6-D-Glc-ol (red) obtained on DTIMS. (e) Mobility selected MS/MS spectrum of mobility peak 1 (from panel b) using a 24.5-25.2 ms window on the dual gate DTIMS. The structure and fragmentation pathway for D-Gal-\(\alpha\)-1-4-D-Gal-ol are shown on the right. (f) Mobility selected MS/MS spectrum of mobility peak 2 using a 25.5-26.5 ms window on the dual gate DTIMS. The structure and fragmentation pathway for D-Glc-\(\beta\)-1-6-D-Glc-ol are shown on the right.
Figure 3. (a) TWIMS separation of a trisaccharide mixture (precursor m/z [M + Na\(^+\)] = 527). (b) DTIMS separation of the same mixture. (c) Overlaid individual spectra of raffinose (blue) and maltotriose (red) obtained on TWIMS. (d) Overlaid individual spectra of raffinose (blue) and maltotriose (red) obtained on DTIMS. (e) Mobility selected MS/MS spectrum of mobility peak 1 using the drift time window of 31-31.5 ms on DTIMS (panel b). The structure and fragmentation pathway for raffinose are shown on the right. (f) Mobility selected MS/MS spectrum of mobility peak 2 using the drift time window of 32-32.5 ms on DTIMS (panel b). The structure and fragmentation pathway for maltotriose are shown on the right.
Figure 4. (a) TWIMS separation of product ions derived from mobility selected peak 1 (raffinose) shown in Figure 3, panel b, fragmentation in panel e. (b) TWIMS separation of product ions for mobility selected peak 2 (maltotriose) shown in Figure 3, panel b, fragmentation in panel f. (c) TWIMS separation of raffinose and its product ions acquired from the standard run individually. (d) TWIMS separation of maltotriose and its product ions acquired from the standard run individually. The corresponded m/z values for the major resolved product ion mobility peaks were labeled in Figures 4a and 4b.
Figure 5. (a) TWIMS separation of a pentasaccharide mixture (precursor \( m/z \ [M + Na^+] = 851 \)). (b) DTIMS separation of the same pentasaccharide mixture. (c) Overlaid individual spectra of cellopentaose (blue), maltopentaose (red) and branched (Man)_5 (green) obtained on TWIMS. (d) Overlaid individual spectra of cellopentaose (blue), maltopentaose (red) and branched (Man)_5 (green) obtained on DTIMS.
Figure 6. Mobility-selected MS/MS spectra of pentasaccharides separated on the drift tube ion mobility region of the instrument. (a) Mobility-selected MS/MS spectrum of peak 1 (Figure 5b) using the drift time window of 38-38.7 ms on DTIMS. The structure and proposed fragmentation pathway of cellopentaose are shown on the right. (b) Mobility-selected MS/MS spectrum of peak 2 (Figure 5b) using the drift time window of 39.5-40.3 ms on DTIMS. The structure of maltopentaose is shown on the right. The fragmentation pathway to yield all the major product ions was identical to cellopentaose as labeled in a and is not shown here. (c) Mobility-selected MS/MS spectrum for peak 3 (Figure 5b) using the drift time window of 40.6-41.5 ms on DTIMS. The structure and proposed fragmentation pathway of branched (Man)$_5$ are displayed on the right.
**Figure 7.** Overlaid TWIMS separation of product ions derived from isomeric pentasaccharide precursor ions at m/z 851. (a) Overlaid TWIMS separation of product ion m/z 509 for cellopentaose (blue), maltopentaose (red) and branched (Man)$_5$ (green). (b) Overlaid TWIMS separation of product ion m/z 527 for cellopentaose (blue), maltopentaose (red) and branched (Man)$_5$ (green). (c) Overlaid TWIMS separation of product ion m/z 671 for cellopentaose (blue), maltopentaose (red) and branched (Man)$_5$ (green). (d) Overlaid TWIMS separation of product ion m/z 689 for cellopentaose (blue), maltopentaose (red) and branched (Man)$_5$ (green).
Supporting Information for Chapter Six

**Reduction Method of Disaccharide-alditols: D-Gal-α-1-4-D-Gal-ol and D-Glc-β-1-6-D-Glc-ol**

D-Gal-α-(1-4)-D-Gal (4α-galactobiose), D-Glc-α-(1-6)-D-Glc (gentiobiose) were purchased from Sigma Chemical Co., St. Louis, Missouri. The disaccharides (10 mg) were reduced with sodium borohydride (1 M aqueous, 2 mL) at room temperature for 48 h. Following neutralization with acetic acid and diluting 5-fold, the sodium was removed by passage through a small column containing a 10 molar equivalent (H⁺/Na⁺ of original sodium borohydride) of the H⁺ form of well-washed Dowex AG-50W-X8, 100-200 mesh resin (Bio-Rad Laboratories, Hercules, California), which converted the sodium borate to boric acid. The boric acid was removed as the volatile trimethylborate with 5 rotary evaporations from methanol (about 10 mL) containing 1% acetic acid, followed by 3 rotary evaporations with methanol alone. This yielded, essentially quantitatively, the reduced disaccharide now having the respective alditol at the at the former reducing end of the disaccharide.

**House Built ESI**

The sample was introduced by a F200 Chemyx Syringe pump (Chemyx Inc.) at a flow rate of 3 μL/min through a 20 cm long, 360 μm o.d. × 150 μm i.d. fused silica capillary tubing. This tubing was connected to another 10 cm long capillary tube via a zero dead volume union where a 13.0 KV ESI voltage was applied. The other end of the shorter capillary tubing was pointed toward the center of the target screen of the ion mobility tube with an upward angle of 45 degrees.
a. Original sample cone  

b. Modified sample cone

Figure S-1. Pictures of original sample cone (on the left) and modified sample cone (on the right)

Drift Time (ms)

Figure S-2. (a) Total traveling wave ion mobility spectra of both precursor and product ions shown in Fig. 2e for mobility-selected D-Gal-α-1-4-D-Gal-ol. (a’) Total traveling wave ion mobility spectra of D-Gal-α-1-4-D-Gal-ol precursor ion and its product ions acquired from the standard. (b) Total traveling wave ion mobility spectra of both precursor and product ions shown in Fig. 2f for mobility selected D-Glc-β-1-6-D-Glc-ol. (b’) Total traveling wave ion mobility spectra of D-Glc-β-1-6-D-Glc-ol precursor ion and its product ions acquired from the standard. The corresponding m/z values for the major mobility peaks were labeled. (note: the peak at 4.25ms in Figure S-2b was an artifact and not reproducible).
**Figure S-3.** Fragmentation spectra for D-Gal-α-1-4-D-Gal-ol (CE 35 V) and D-Glc-β-1-6-D-Glc-ol (CE 40 V) acquired from the individual standards (supporting information corresponding to Fig. 2e and 2f in Chapter 6).

**Figure S-4.** Fragmentation Spectra for raffinose and maltotriose acquired from individual standards (supporting information corresponding to Fig. 3e and 3f in Chapter 6). Note: the CE used for maltotriose was 57 V for the standard, which was different from the CE (53V) applied while it was in the mixture in the paper.
**Figure S-5.** Fragmentation spectra of cellopentaose, maltopentaose and [D-Man-α-1-6-[D-Man-α-1-3]-D-Man-α-1-6]-[D-Man-α-1-3]-D-Man acquired from individual standards (supporting information corresponding to Fig. 6a, 6b and 6c in chapter 6).
Figure S-6. TWIMS of product ions of pentasaccharides acquired from specific standards cellopentaose (blue), Maltopentaose (red), [D-Man-α-1-6-[D-Man-α-1-3]-D-Man-α-1-6]-[D-Man-α-1-3]-D-Man (green), respectively (Supporting corresponded to Fig.7 in chapter 6).
CHAPTER SEVEN

ION MOBILITY-MASS CORRELATION TREND LINE SEPARATION OF GLYCOPROTEIN DIGESTS WITHOUT DEGLYCOSYLATION

Abstract

A high-throughput ion mobility mass spectrometer (IMMS) was used to rapidly separate and analyze peptides and glycopeptides derived from glycoproteins. Two glycoproteins, human α-1-acid glycoprotein and antithrombin III were digested with trypsin and subjected to electrospray traveling wave IMMS analysis. No deglycosylation steps were performed; samples were complex mixtures of peptides and glycopeptides. Peptides and glycosylated peptides with different charge states (up to 4 charges) were observed and fell on distinguishable trend lines in 2-D IMMS spectra in both positive and negative modes. The trend line separation patterns matched between both modes. Peptide sequence was identified based on the corresponding extracted mass spectra and collision induced dissociated (CID) experiments were performed for selected compounds to prove class identification. The signal-to-noise ratio of the glycopeptides was increased dramatically with ion mobility trend line separation compared to non-trend line separation, primarily due to selection of precursor ion subsets within specific mobility windows. In addition, isomeric mobility peaks were detected for specific glycopeptides. IMMS demonstrated unique capabilities and advantages for investigating and separating glycoprotein digests in this study and suggests a novel strategy for rapid glycoproteomics studies in the future.
Introduction

Ion mobility spectrometry (IMS) is a gas phase ion separation technique according to ions’ traveling velocities through an electric field in a counter flow drift gas. IMS has been demonstrated as a powerful detection tool for drugs, explosives and chemical warfare agents [8, 11, 23]. When IMS is combined with mass spectrometry (MS), a novel analytical method called ion mobility mass spectrometry (IMMS) is created [22]. The application of IMMS to complex biological samples has become one of the most rapidly growing areas in the MS field. High throughput separation capability demonstrated by IMMS has found great utility in life science research, such as metabolomics [7, 24], glycomics [6, 20, 41] and proteomics [10, 37, 40]. The unique benefits include charge states and molecule class separation (glycans, peptides and lipids etc.) [12, 31] in the form of different trend lines on the basis of 2-D IMMS spectra as well as separation of isomeric precursor ions of biological origin [38].

Glycoproteomics identifies and characterizes proteins having carbohydrates as post-translational modifications [28]. Aberrant protein glycosylation is related to various diseases [2] including cancer and immune system deficiencies [33]. Current analytical techniques normally require the physical separation of glycans and peptides for analyses [27], which are known as glycomics and proteomics, respectively, and often require tedious sample preparation. Direct analysis of protease digested glycoproteins employs liquid chromatography (LC) separation followed by MS or MS² measurements [21, 25]. Among the numerous LC peaks detected in such analyses, it is difficult to assign peak identities (such as specific peptides and unique structures of glycopeptides). Moreover, no specific patterns have been observed, making analysis considerably more difficult. Compared to the applications of IMMS in the analysis of glycans and peptides, limited studies have been demonstrated for analysis of glycopeptides by IMMS.
Mclean et al. [13] reported a related study in 2009, however, the analysis was based on a deglycosylated form of the enzymatically digested glycoprotein. The analyte was a mixture of glycans and peptides and in fact, IMMS separation of glycans and peptides was demonstrated. Olivova et al. [29] separated light and heavy chains of a reduced antibody in the gas phase using IMMS. Additionally, they determined the glycosylation site as well as the glycan sequence using the unique dual-collision-cell design of the instrument. Both studies were performed using traveling wave ion mobility mass spectrometry (TWIMMS) [16]. The TWIMMS instrument is a hybrid quadrupole/ion mobility/orthogonal time of flight MS and has been widely described in the literature [32]. The mobility separation employs traveling wave ion guide technology, having a non-homogeneous electric field under reduced pressure.

In the analysis of glycopeptides derived from glycoproteins, there are three important questions to be addressed. (1) What is the peptide sequence containing the glycosylation site(s), both for N- and O-linked glycosylation and for clustered sites? (2) Where is/are the glycosylation site/sites on a peptide? (3) What are the structures and structural heterogeneity, including isomeric heterogeneity, of the carbohydrate components? Information about which components are glycopeptides, and partial information for the nature of the carbohydrate components can be obtained through collision-induced dissociation (CID), which preferentially cleaves glycopeptides at glycosidic linkages. Partial information for the peptide sequence can be obtained currently using electron transfer dissociation (ETD), both for O- and N-linked glycopeptides, hence the use of both methods in combination typically yields more information about the nature of glycopeptides [1, 3, 19, 39]. A key remaining issue, however, is the isomeric heterogeneity at glycopeptide sites. The presence of a peptide component linked to individual carbohydrate isomers makes the problem of their separation and independent analysis even more
difficult than resolution of the carbohydrate isomers themselves. Yet the question as to the specific nature of each carbohydrate variant at each specific peptide site and their relative percentages is an important one to be addressed to fully understand their biological roles. Here, we report an application of IMMS to glycoproteomics in which proteolytic digested glycoproteins were investigated directly, without deglycosylation, by traveling wave ion mobility mass spectrometry. The hypothesis was that glycans attached to peptides would have different ion densities and form identifiable trend lines in the IMMS spectra. To test this hypothesis, two common glycoproteins were digested with trypsin and the resultant entire mixture of peptides/glycopeptides was evaluated by IMMS.

**Experimental Section**

**Chemicals and Materials**

Formic acid (FA), methanol and water (LC-MS grade) were purchased from Thermo Fisher Scientific Inc. An equal volume mixture of methanol and water was used as the electrospray ionization (ESI) solvent, and 0.1% FA was added to the ESI solvent for positive mode studies. Two glycoproteins, human α-1-acid glycoprotein (AGP) and human antithrombin III (ANT III), were investigated. Both glycoproteins were obtained from the Red Cross. The AGP was desialylated by treatment with 0.1 N H₂SO₄ for 1 h (the original hydrolysis conditions described by Spiro [35]), followed by neutralization with sodium bicarbonate and thorough dialysis against water, then freeze-dried. The ANT III was used without removal of sialic acid. The glycoproteins were digested at 1 mg/mL with trypsin (two additions, 24 h apart, 1/20 mg/mg relative to the glycoproteins) for 48 h at pH 7.4 in 20 mM ammonium bicarbonate containing 20% acetonitrile, and then frozen and freeze-dried. A concentration of 0.2 mg/mL AGP digest
and 0.4 mg/mL ANT III digest were subjected to ion mobility analysis in both positive and negative modes. Acetonitrile and all the chemicals used in the processes of desialylation and trypsin digestion were purchased from Sigma-Aldrich (St. Louis, MO) and used directly.

**Instrument**

IMMS experiments were performed on a Synapt G2 High Definition Mass Spectrometer (HDMS) (Waters Corp., Manchester, UK) in both positive and negative modes. It is a hybrid quadrupole/IM/orthogonal high resolution TOF MS and has been thoroughly described [17, 34]. For traveling wave ion mobility separations, a wave height of 40 V and a wave velocity of 650 m/s were employed. Trap release time was 200 μs and the separation delay after trap release was 450 μs. Nitrogen with a flow rate of 90 mL/min was used as drift gas, resulting in a pressure of ~3.5 mbar for ion mobility separation. According to Td (Townsend) = E / N, where E is the electric field in V/cm, N is the number density in cm$^{-3}$, the current experimental settings resulted in a maximum Td value of 46.2 V⋅cm$^2$. For IMMS experiments of glycoproteins, the data acquisition time was 3 min for the positive mode and 5 min for the negative mode studies. Selected fragmentation experiments occurred in the trap cell located in front of the ion mobility separator by elevating the collision energy (CE). Samples were injected into the ESI source directly using a syringe pump (Chemyx Inc., Stafford, TX) at a flow rate of 3 μL/min. The program Masslynx V4.1 (Waters Corp., Manchester, UK) was used to collect and analyze the MS data. The 2-dimensional IMMS spectra were generated using the program Driftscope V2.2 (Waters Corp., Manchester, UK). Other instrumental parameters are included in Table S1 in the supporting information.
Results and Discussion

Human α-1-acid-glycoprotein in Positive IMMS Mode

Human α-1-acid glycoprotein (AGP) is a single polypeptide chain of 183 amino acids with ~45% carbohydrate content at five different N-linked glycosylation sites [11]. The AGP gene encodes two major variants, AGP1 and AGP2, having a 22 amino acid difference. AGP is involved in a number of activities of potential physiological significance, where an immunomodulatory function as well as binding activities have been shown to be dependent on the carbohydrate moiety [15]. The glycosylation patterns of AGP have been extensively characterized [14, 21, 25, 36], expressing di-, tri- and tetra-antennary glycan structures, with many isomeric carbohydrate structures known to be present [5, 14, 18, 35]. Each of the glycosylation sites (Asn-15, -38, -54, -75, -85) can be occupied by branched oligosaccharides of various structures composed of hexose (Hex), N-acetylhexosamine (HexNAc) and fucose (Fuc), resulting in a carbohydrate-rich complex protein. Note that AGP was desialylated in this study, thus no sialic acid was found in the carbohydrate structures.

Fig. 1 displays the 2-D IMMS plot of the AGP trypsin digest with drift time (ms) on the x axis and m/z on the y axis; five separate trend lines were observed. They were identified as trend line I: +1 charged peptides, trend line II: +2 charged peptides, trend line III: +2 charged glycopeptides, trend line IV: +3 charged glycopeptides and trend line V: +4 charged glycopeptides. The class identification was based on the extracted mass spectra (m/z values) from each region and on further fragmentation experiments with a detailed analysis as shown in subsequent figures. Thus in a single analysis by IMMS, peptides and glycopeptides were able to be distinguished in different 2-D regions. Clear charge state separation was also achieved, with up to 2+ peptides and 4+ glycopeptides. In comparison to MS analysis alone, the added
separation capability of ion mobility enables the complex biological mixture to be rapidly organized into specific 2-D patterns based on their structural similarities. Bio-molecular structural separation by IMMS has been shown previously, however, this is the first example of the trend line separation of peptides and glycopeptides with different charge states, which is difficult or impossible to achieve with other analytical separation techniques such as GC and LC. Mass spectra extracted from trend lines I (+1 charged peptides) and II (+2 charged peptides) are displayed in Fig. 2a and 2b, respectively. A number of peaks (labeled with asterisks) were identified as tryptic peptides of AGP according to their m/z values. Corresponding peptide sequences and additional identified m/z values of low abundance (not labeled in Fig. 2) are summarized in Table S2 in the supporting information. Additionally, peptide fragments were observed using ESI in this study, presumably resulting from in-source fragmentation. The overall peptide data results in a ~40% coverage of the amino acid sequence for AGP1 and ~35% for AGP2. The relatively low peptide sequence recovery rate may arise in part from an inefficient enzyme digestion, since large glycan moieties at multiple sites may hinder proteolysis and the possibility of missed cleavages increased for the sites close to glycosylated asparagines. Further investigations will be required to identify the source of this low peptide recovery rate. No protein reduction and alkylation were performed prior to trypsin digestion in this study, which may also affect the results. Unidentified peaks in Fig. 2 could result from trypsin autodigestion, peptide fragments generated during electrospray, impurities in the sample or any other potential peptide modifications.

Fig. 3 shows extracted mass spectra for trend lines III (+2 glycopeptides), IV (+3 glycopeptides) and V (+4 charged glycopeptides), respectively. The inset on the right in each spectrum demonstrates the isotopic patterns characteristic of +2, +3 and +4 charged ion.
mixtures, glycopeptides usually have much lower ionization efficiency and sensitivity than peptides, making their analysis more difficult. In direct analysis of glycopeptide digests, peptide components normally tend to dominate the abundance of precursor ions isolated for the mass analyzer, hence ion statistics of glycopeptides selected for MS/MS are often poor. With the added ion mobility trend line separation, the signal (S) to noise (N) ratio of glycopeptides has been greatly improved, mostly as the result of removal of many of the unglycosylated peptides within selected ion mobility time windows. For example, as shown in Fig. 3c-1, the S/N ratio was 2 for m/z 1343.4 with MS only (top spectrum), whereas the ratio increased 10-fold using IMMS separation (bottom spectrum). It is common that carbohydrates tend to form sodium adducts and peptides normally adopt up to two protons in the positive mode. Charge competition between Na\(^+\) and H\(^+\) was observed for several glycopeptides, since both glycan and peptide are contained in a single compound. Correlated m/z peaks with a mass difference of 22 (m/z difference of 11) were observed for the majority of +2 charged glycopeptides. This indicated one H\(^+\) was replaced by a Na\(^+\), such as pairs of m/z of 1379.6/1390.6, 1562.2/1573.2 and 1699.4/1710.4, while ions containing Na\(^+\) were at much lower intensity. Charge competition was more pronounced for +3 charged glycopeptides as demonstrated by the dash circled peaks in Fig. 3b. The peaks having one or more Na\(^+\) (such as m/z 1140.5 and 1264.6) were at higher abundance in this case. Improved sensitivity may be explained in that glycopeptides coordinate with Na\(^+\) ions more easily as the carbohydrate portion becomes larger. This scenario was not observed for +4 charged glycopeptides, where the charge distribution appeared to involve two Na\(^+\) ions adducted to the glycan and two H\(^+\) ions on the peptide chain. Trend line separation provided additional information that was not available with MS analysis alone. To further validate the trend line identities, fragmentation experiments were performed for selected m/z
values and representative examples are shown in Fig. 4. Fig. 4a shows the MS/MS spectrum for the singly charged peptide $m/z$ 994.5 having the sequence TEDTIFLR. The b and y product ions that were observed matched with the precursor ion peptide sequence. The MS/MS spectra of +2 ($m/z$ 1885.4) and +3 ($m/z$ 1264.6) charged glycopeptides from trend lines III and IV are displayed in Fig. 4b and 4c, respectively. They both showed sequential carbohydrate unit losses of Hex, HexNAc or Fuc as displayed in spectra. Thus, fragmentation analysis further supported the trend line assignments in Fig. 1. More fragmentation examples of glycopeptides were demonstrated and the spectra are included in Fig. S1 and Fig. S2 in supporting information where primarily sugar monomer unit losses were observed as well. AGP is a highly glycosylated protein, where +4 charged glycopeptides, especially for glycoforms with tri or tetra-antennary glycans, are expected as has been reported previously [15, 19, 29]. Even though no direct fragmentation evidence was obtained for the +4 charged ions due to their low abundance in the sample, trend line V was exclusively assigned to +4 charged glycopeptides.

**Human Antithrombin III in Positive IMMS Mode**

Human antithrombin III (ANT III) is a glycoprotein composed of 464 amino acids having 4 N-linked glycosylation sites (Asn-96, -135, -155 and -192). The carbohydrate chain is primarily of the bi-antennary complex type composed of hexose (Hex), N-acetylhexosamine (HexNAc), fucose (Fuc) and sialic acid (SA) [4, 30]. It functions as a plasma protease inhibitor with physiological anticoagulant activity, circulating in the blood and becoming active when associated with glycosaminoglycans [26]. Fig. 5 displays the IMMS spectrum of ANT III digested with trypsin in the positive mode. The ions were distributed on trend lines I, II and III with class identification of +1 charged peptides, +2 charged peptides and +3 charged
glycopeptides, respectively. Their corresponding mass spectra and evidence from dissociation are discussed below. More peptides were observed than glycopeptides for ANT III compared to AGP. This is not surprising since ANT III is a much larger protein and has lower carbohydrate content than AGP. It was observed that the separation between trend lines II and III was slightly obscured in this example. This could presumably be improved by using higher resolution IMS. The traveling wave ion mobility resolving power [34] in this study was ~30-40. It is worth noting that trend line IV denotes a class of compounds with systematic mass differences that may result from the matrix used during the sample preparation or purification steps and is not identified here. A mass spectrum of this trend line (Fig. S3) and an expansion are contained in the supporting information, indicating the presence of some highly regular polymer or polymers as contaminants of the electrosprayed sample. In addition, the region between trend lines III and IV contained high noise levels according to the extracted mass spectrum. IMMS was able to separate the chemical noise from the analytes of interest and was also able to provide information regarding the sample purity. Fig. 6 displays the extracted mass spectra for corresponding trend lines of ANT III. The majority of peaks (labeled with asterisks) in Fig. 6a were identified as singly charged peptides [M+H]+ derived from ANT III. Doubly charged peptides (asterisks labeled) were found in trend line II mainly having the formula [M+2H]2+, while corresponding sodium coordinated peaks were also observed for certain species, for example, m/z 400.7 and 411.7 were the [M+2H]2+ and [M+H+Na]2+ ions for the peptide sequence IPEATNR. Peptide fragments were detected as well; detailed analyses are summarized in Table S3 of the supporting information. A 40% coverage of the amino acid sequence of ANT III was obtained. This relatively low value could be due to inefficient trypsin digestion as discussed above. Fig. 6c is the mass spectrum for trend line III containing triply charged
glycopeptides. A series of peaks with mass differences of 22 were observed as shown in the inserted window. However, the situation was different from the charge competition discussed for AGP above. Where all the H\(^+\) ion adducts are replaced by Na\(^+\) ions stepwise for +3 charged glycopeptides, only four correlated peaks (having mass differences of 22) should be observed which did not match with the experimental spectrum displayed in Fig. 6c. This indicates that an additional –OH has been converted to –ONa, this is likely due to the many hydroxyl groups available on the carbohydrate portion in this specific example. Moreover, it also reflects that a relatively high amount of sodium salt was present in the sample. Further fragmentation was performed for selected compounds within different trend lines from ANT III in Fig. 7. The selected peptide precursor ions were [LVSANR]H\(^+\) at m/z 659.4 and [LPGIVAEGR]2H\(^+\) at m/z 456.3. Their corresponding a, b, c, y, and z product ions were observed as shown in Fig. 7a and b, respectively. Fig. 7c shows the MS/MS spectrum of a triply charged glycopeptide of m/z 1270.3 from trend line III and the inset illustrates its isotopic pattern. Neutral losses of Fuc, Hex, HexNAc, SA and the ion of [Hex+HexNAc]H\(^+\) were observed as expected, which further supported the class identification of trend line III.

**AGP and ANT III in Negative IMMS Mode**

We also evaluated the behavior of glycopeptide ions in negative mode operation of the IMMS instrument. Fig. 8 displays the 2-D IMMS plot of a trypsin digest of AGP in negative mode. Five trend lines were observed including -1 and -2 charged peptides and -2, -3 and -4 charged glycopeptides. The trend line separation obtained was consistent with the results obtained in the positive mode. Fewer ions were observed in the negative mode as compared to the positive and this is attributed mainly to the low ionization efficiency of compounds in the
negative mode. Data analysis was performed as described above. The extracted mass spectra for
trend lines I to V (Fig. S4) and the fragmentation spectra for selected glycopeptides (Fig. S5 and
Fig. S6) are included in the supporting information. The negative mode 2-D IMMS plot of the
ANT III tryptic digest is shown in Fig. 9. Four trend lines were assigned: they were -1 and -2
charged peptides, -3 charged glycopeptides and unidentified compounds with systematic mass
differences, which matched with the positive mode results well as shown in Fig. 5. The region at
the bottom of the spectrum was identified as noise according to the extracted mass spectrum
(Fig. S7 in supporting information). The same respective region was only slightly visible in the
positive mode. The difference may be due to the lower relative sensitivity and S/N ratio in the
negative mode as compared to the positive and could be improved by increasing the sample
concentration and the data acquisition time. The extracted mass spectra for individual trend lines
and MS/MS spectra for selected glycopeptides are displayed in Fig. S8 and Fig. S9 in the
supporting information, respectively. The negative mode trend line separation for glycoprotein
digests further confirmed and demonstrated the unique separation capability of IMMS, where
compounds with structural similarities were able to be grouped into specific 2-D IMMS trend
patterns when derived from a complex biological mixture.

**Glycopeptide Isomer Differentiation**

IMS is capable of separating isomeric compounds rapidly [6, 31, 38, 40, 41] based on the
ion’s collision cross section. Fig. 10 displays the traveling wave ion mobility spectra of four
selected glycopeptides (a) $m/z$ 737.7 with three positive charges from ANT III; (b) $m/z$ 927.0
with three positive charges from AGP; (c) $m/z$ 1208.0 with two negative charges from AGP; (d)
$m/z$ 1333.6 with two positive charges from AGP. More than one mobility peak was observed for
specific glycopeptides, representing different stereochemical isomers at a single m/z. There were two fully resolved mobility peaks for m/z 737.7; two partially differentiated mobility peaks for m/z 927.0; two isomeric mobility peaks for m/z 1208.0 and a board mobility peak with barely resolved shoulders for m/z 1333.6. This probably resulted from structure heterogeneity of the carbohydrate components contained in glycopeptides, which may indicate that specific peptides are linked to different carbohydrate isomers. However, the mobility separation shown in Fig. 10 is more complex than the resolution of carbohydrate stereo- or branch isomers themselves. The peptide portion and the interaction between peptide and glycan also affect the overall structural configuration of a glycopeptide, which would influence the mobility resolution. Beside the representative examples in Fig. 10, the majority of glycopeptides had a broad mobility peak detected similar to Fig. 10d, demonstrating that multiple structure variants may co-elute. IMS systems with higher resolving power would be needed for complete and better separation among glycopeptide isomers.

Conclusions

In this study, we demonstrate the use of ion mobility mass spectrometry to separate and identify peptides and glycopeptides (from trypsin-digested glycoproteins) having different charge states, using both positive and negative ESI. Only standard proteolytic digestion was needed for IMMS analysis, where glycopeptide components separated from peptides in unique 2-D trend regions. This enabled glycopeptides or peptides to be isolated in individual mobility windows in the millisecond time frame with far fewer peptide components, which would typically require LC separations to achieve, sometimes over the course of hours [5]. Overall, the advantages provided by IMMS include structural trend line and charge state separation,
millisecond timescales, the capability to assess isomeric heterogeneity of ionic species and reduced chemical noise for the improved detection of low abundance ions. These results may lead to the development of novel strategies for high throughput identification of complex glycoproteins.

Acknowledgements

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35. Spiro RG (1966) Analysis of sugars found in glycoproteins. Meth Enzymol 8:3-26


Figure 1. 2-D IMMS plot of trypsin digested human α-1-acid glycoprotein in positive mode. Trend line I: 2-D region containing +1 charged peptides; Trend line II: 2-D region containing +2 charged peptides; Trend line III: 2-D region containing +2 charged glycopeptides; Trend line IV: 2-D region containing +3 charged glycopeptides; Trend line V: 2-D region containing +4 charged glycopeptides.
Figure 2. (a) An extracted mass spectrum corresponding to +1 charged peptides (trend line I in Fig. 1). (b) An extracted mass spectrum corresponding to +2 charged peptides (trend line II in Fig. 1). These m/z values represent precursor ions extracted from a line running through the 2-dimensional trend zones shown in Fig. 1. Asterisks indicate major identified m/z values corresponding to peptide sequences derived from human α-1-acid glycoprotein. For the detailed sequence information and additional non-labeled identified m/z values (low abundance), see Table S2 in the supporting information.
Figure 3. (a) An extracted mass spectrum corresponding to +2 charged glycopeptides (trend line III in Fig. 1). (b) An extracted mass spectrum corresponding to +3 charged glycopeptides (trend line IV in Fig. 1). The m/z values show the extracted precursor ions from a line running through each trend region. The mass difference for circled peaks in (b) is $\Delta m = 22$. (c) An extracted mass spectrum corresponding to +4 charged glycopeptides (trend line V in Fig. 1). c-1 shows the expanded mass spectra and S/N ratios for the m/z 1343.4 precursor ion region derived from the mixture (top) and following IMMS, derived from the trend line (bottom). The inset in each spectrum on the right illustrates the isotopic patterns for a +2, +3 and +4 charged glycopeptide, respectively.
Figure 4. (a) MS/MS spectrum for the +1 charged peptide of the precursor ion having $m/z$ 994.5 from trend line I in Fig. 1 using a CE of 53 V. (b) MS/MS spectrum for a +2 charged glycopeptide of $m/z$ 1885.4 from trend line III in Fig. 1 using 48 V CE. (c) MS/MS spectrum for a +3 charged glycopeptide of $m/z$ 1264.6 from trend line IV in Fig. 1 using a CE of 33V. Underlined peaks are precursor ions.
Figure 5. 2-D IMMS plot of trypsin digested human antithrombin III in positive mode. Trend line I: 2-D region showing separation of +1 charged peptides; Trend line II: 2-D region showing separation of +2 charged peptides; Trend III: 2-D region showing separation of +3 charged glycopeptides; Trend line IV: Unidentified compounds with systematic mass differences.
Figure 6. (a) An extracted mass spectrum corresponding to +1 charged peptides (trend line I in Fig. 5). (b) An extracted mass spectrum corresponding to +2 charged peptides (trend line II in Fig. 5.) (c) An extracted mass spectrum corresponding to +3 charged glycopeptides (trend line III in Fig. 5). Asterisks indicate major identified m/z values corresponding to peptide sequences derived from human antithrombin III. For the detailed sequence information and additional non-labeled identified m/z values (low abundance), please see Table S3 in the supporting information.
**Figure 7.** (a) MS/MS spectrum for a +1 charged peptide of $m/z$ 659.4 from trend line I in Fig. 5 using a CE of 36 V. (b) MS/MS spectrum for a +2 charged peptide of $m/z$ 456.3 from trend line II in Fig. 5 using a CE of 25 V. (c) MS/MS spectrum for +3 charged glycopeptide of $m/z$ 1270.3 from trend line III in Fig. 5 using a CE of 50V. The inset illustrates the isotopic pattern for the +3 charged ions. Underlined peaks are precursor ions.
Figure 8. 2-D IMMS plot of trypsin-digested human α-1-acid glycoprotein in negative mode. Trend line I: 2-D region for separation of -1 charged peptides; Trend line II: 2-D region for separation of -2 charged peptides; Trend line III: 2-D region for separation of -2 charged glycopeptides; Trend line IV: 2-D region for separation of -3 charged glycopeptides; Trend line V: 2-D region for separation of -4 charged glycopeptides.
**Figure 9.** 2-D IMMS plot of trypsin digested human antithrombin III in negative mode. Trend line I: 2-D region for separation of -1 charged peptides; Trend line II: 2-D region for separation of -2 charged peptides; Trend line III: 2-D region for separation of -3 charged glycopeptides; Trend line IV: Unidentified compounds with systematic mass differences.
**Figure 10.** Traveling wave ion mobility spectra of selected glycopeptides (a) +3 charged glycopeptide \(m/z\) 737.7 from ANT III (in trend line III in Fig. 5); (b) +3 charged glycopeptide \(m/z\) 927.0 from AGP (in trend line IV in Fig. 1); (c) -2 charged glycopeptide \(m/z\) 1208.0 from AGP (in trend line III in Fig. 8); (d) +2 charged glycopeptide \(m/z\) 1333.6 from AGP (in trend line III in Fig.1). Note: the \(m/z\) values labeled are monoisotopic masses.
Supporting Information for Chapter Seven

Table S-1. Additional instrumental parameters of Synapt G2 HDMS

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Table S-2. Identified singly and doubly charged peptides corresponding to human α-1-acid glycoprotein

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Table S-3. Identified singly and doubly charged peptides corresponding to ANT III

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<th>m/z (+1)</th>
<th>Peptide Sequence</th>
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Figure S-1. MS/MS spectra of +2 charged glycopeptides from trend line III in Figure 1 in chapter 7.
The collision energy of 50V, 42V and 33V were used for the precursor ions from top to the bottom, respectively. Note: underlined peaks are precursor ions.

Spectrum rough analysis for a:
Neutral loss of Hexose (Hex) includes 366.1-204.1; 528.2-366.1; 690.2-528.2; 893.3-731.1; 1055.4-893.3; 1334.1-1253.1 and 1516.7-1435.7.
Neutral loss of N-acetylhexosamine (HexNAc) includes 731.1-528.2; 893.3-690.2 and 1815.0-1611.9.

Spectrum rough analysis for b:
Neutral loss of Hexose (Hex) includes 366.1-204.1; 528.2-366.1; 690.2-528.2; 893.3-731.1; 1055.4-893.3 and 1197.0-1116.0.
Neutral loss of N-acetylhexosamine (HexNAc) includes 731.1-528.2; 893.3-690.2; 1175.6-972.4 and 1298.5-1197.0.

Spectrum rough analysis for c:
Neutral loss of Hexose (Hex) includes 366.1-204.1; 528.2-366.1; 690.2-528.2; 893.3-731.1; 1055.4-893.3 and 1197.0-1116.0.
Neutral loss of N-acetylhexosamine (HexNAc) includes 731.1-528.2; 893.3-690.2; 1175.6-972.4 and 1298.5-1197.0.
**Figure S-2.** MS/MS spectra of +3 charged glycopeptides from trend line IV in Figure 1 in the paper.

The collision energy of 38V and 40V were used for the ions from top to the bottom, respectively. Note: underlined peaks are precursor ions.

Spectrum rough analysis for a:
Neutral loss of Hexose (Hex) includes 366.1-204.1; 528.2-366.1 and 996.8-942.5.

Spectrum rough analysis for b:
Neutral loss of Hexose (Hex) includes 366.1-204.1; 528.2-366.1; 2072.4-1990.9 and 2255.0-2174.0.
Neutral loss of N-acetylhexosamine (HexNAc) includes 731.1-528.2 and 2174.0-2072.4.
Figure S-3. (a) Extracted mass spectrum of trend line IV in Figure 5 for human antithrombin II. (b) Mass spectrum with expanded region from $m/z$ 550 to $m/z$ 750.
Figure S-4. Extracted mass spectra corresponding to different trend lines in Figure 8 for human α-1-acid glycoprotein in chapter 7. The inset on the right in each spectrum illustrates the isotopic patterns of -2, -3 and -4 charged ions.
Figure S-5. MS/MS spectra of -2 charged glycopeptides from trend line III in Figure 8 for human α-1-acid-glycoprotein in chapter 7. The collision energy of 68V, 57V and 50V were used for the precursor ions from top to the bottom (Fig. a-c), respectively. Note: underlined peaks are precursor ions.

Spectrum rough analysis:

a. Neutral loss of Hexose (Hex) includes 1640.6-1478.5 and 1843.7-1663.6.
   Neutral loss of N-acetylhexosamine (HexNAc) includes 1681.6-1478.5 and 1843.7-1640.6.

b. Neutral loss of Fucose (Fuc) includes 877.4-731.3; 1391.0-1318.0 and 1382.5-1390.5.
   Neutral loss of Hexose (Hex) includes 1640.6-1478.5; 1663.6-1501.5; 1783.6-1621.6 and 1843.7-1681.6.
   Neutral loss of N-Acetylhexosamine (HexNAc) includes 1478.5-1275.4 and 1843.7-1640.6.

c. Neutral loss of Fucose (Fuc) includes 877.4-731.3; 1199.9-1126.4; 1208.5-1135.4 and 1864.7-1718.6.
   Neutral loss of Hexose (Hex) includes 731.3-551.2; 1095.4-933.3; 1113.4-951.3; 1418.5-1256.5 and 1718.6-1556.5.
   Neutral loss of N-acetylhexosamine (HexNAc) includes 1113.4-951.5 and 1478.5-1275.4.
**Figure S-6.** MS/MS spectra of -3 charged glycopeptide from trend line IV in Figure 8 for human α-1-acid-glycoprotein in chapter 7. The collision energy of 50V was used. Note: underlined peaks are precursor ions.

Spectrum rough analysis:
Neutral loss of Fucose (Fuc) includes 1847.8-1774.7, 1831.2-1858.2 and 959.5-795.4
Neutral loss of Hexose (Hex) includes 844.4-682.3
Neutral loss of N-acetylhexosamine (HexNAc) includes 2208.8-2005.8

**Figure S-7.** Mass spectrum of the noise region labeled in Figure 9 for human antithrombin III.
Figure S-8. Extracted mass spectra corresponding to different trend lines in Figure 9 for human antithrombin III in chapter 7.
**Figure S-9.** MS/MS spectrum of -3 charged glycopeptide from trend line III in Figure 9 for human antithrom III in chapter 7. Underlined peak is precursor ion.

Spectrum rough analysis:
Neutral loss of Hexose (Hex) includes 695.3-533.3; 1060.4-979.4; 1326.3-1164.4 and 1928.3-1906.3;
Neutral loss of N-acetylhexosamine (HexNAc) includes 736.4-533.3 and 823.4-602.3.
CHAPTER EIGHT

CONCLUSIONS

I. Overall Conclusion

The overall conclusion from this study is that IMMS provides an advantageous analytical tool to assess the isomeric heterogeneity of carbohydrates. The analysis is fast, sensitive, convenient and universal. This was demonstrated by analyzing various samples including monosaccharide methyl glycosides, disaccharides, oligosaccharide-aliditol mixture isolated from O-linked glycoprotein and human glycoprotein digests. The carbohydrate structural isomerism covered contained anomeric configuration, monosaccharide stereochemistry, linkage positions and branching events. Using IMS coupled to different types of MS and tandem MS instruments, the isomeric heterogeneity of both carbohydrate precursor and product ions were evaluated. The unique advantages and data provided by IMMS analysis include: (1) capability to resolve saccharide isomers on millisecond timeframe; (2) measurement of ion’s collision cross section (Ω) value; (3) mobility separation of isomeric product ions derived from a given precursor ion; (4) obtainment of mobility selected fragmentation pattern for isomeric precursor ions and isomeric product ions; (5) alteration of mobility separation using different ion adductions; (6) charge states and molecular classes differentiation by ion mobility-mass correlation trend lines; Together, all the information from IMMS should significantly increase the probability for identification of individual carbohydrate isomers, allowing unambiguous assignments of different oligosaccharide isomers within mixtures.
II. Specific Conclusions

The experiments and results demonstrated in Chapter 2 showed that ion mobility-mass spectrometry using either drift tube or travelling wave ion mobility were capable of resolving subtle variations in the stereochemistries among isomeric monosaccharide methyl glycosides. A number of structural isomers exhibited different drift times. Higher resolving power was observed using the drift tube IMS. In general, drift time pattern matched between the two systems, but some differences were observed, which may due to the different electric fields (homogeneous electric field for DTIMS and non-homogeneous moving electric field for TWIMS) and pressures (atmospheric pressure for DTIMS and reduced pressure for TWIMS) employed by two instruments. Stereo-structure differences and coordination geometry induced by Na$^+$ adduction both influenced ion mobility resolution for the monosaccharide isomers.

Using reducing 2-, 4- or 6-linked disaccharides as representative examples, the isomeric heterogeneity of disaccharide precursor and product ions was evaluated using traveling wave IMMS in both positive and negative modes in Chapter 3. Separation using TWIMS was observed but not fully achieved for all the disaccharide isomers based on the mobility profiles of the precursor ions. The mobility patterns of the structurally informative product ion, monosaccharide-glycoaldehyde, derived from different disaccharide isomers, were different, which enabled the separation of isomeric product ions and provided further identity for their precursor ions. Multiple mobility peaks were observed for specific monosaccharide-glycoaldehyde cations and anions, which indicated additional stereoisomers as verified by NMR.$^9$ Additionally, disaccharide cluster ions including [2M+Na]$^+$, [2M-H]$^-$ and [3M-H]$^-$ ions were observed in abundance for all the disaccharides. Partial separation was achieved among isomeric cluster ions by TWIMS; a majority of cluster ions showed multiple mobility peaks which may
result from the structure characteristic of reducing sugars. Mobility data of cluster ions could also serve as a further analytical property for identification of sugar stereo- and linkage isomers. Anion effects, using chloride adducts as examples, on the influence of mobility separation was demonstrated for both disaccharide precursor as well as the corresponded chloride adducted cluster ions detected at the same time.

In Chapter 4, individual HPLC fractions from the separation of O-linked oligosaccharide-alditol mixture, isolated from bovine submaxillary mucin, were investigated by ion mobility coupled to TOFMS and quadrupole ion trap MS in positive mode. All the oligosaccharides were ionized as sodiated adducts. Multiple isomeric ion mobility peaks were observed for the majority of oligosaccharide-alditols, ranging from disaccharide-alditols to hexasaccharide-alditols, in single LC fractions on millisecond scale. The limitations of LC-MS in assessing isomeric heterogeneity of oligosaccharides using one column were evident. Ion mobility coupled to tandem stages of MS enabled different sets of product ions to be assigned to different isomeric precursor ions for a given m/z. However, the fragmentation spectra obtained from the CID could be essentially identical for isomeric precursor ions. This further confirmed the need for rapid physical resolution of isomeric species prior to their tandem mass spectral analysis.

In contrast to chapter 4, a negative mode evaluation of the isomeric heterogeneity of oligosaccharide-alditol HPLC fractions was performed using traveling wave IMS in Chapter 5. The collision-induced dissociation cells located in front of and after the ion mobility separation device in Synapt G2 HDMS enabled oligosaccharide precursor or product ions to be separated by ion mobility and independent fragmentation spectra to be acquired for isomeric carbohydrate precursor or product ions. Multiple isomeric mobility peaks were differentiated for a majority of oligosaccharide-alditol anions within individual HPLC fractions. It was also demonstrated that
many isomeric product ions could be separated by traveling wave ion mobility. Mobility-selected precursor ions were dissociated to yield mass spectra that differed between isomeric species. And, for the first time, mobility-resolved product ions yielded different characteristic mass spectra. Additionally, the mobilities of both [M-H]$^-$ and [M+Cl]$^-$ ions were examined and compared for selected oligosaccharide-al ditols. Better separation among structural isomers appeared to be achieved for some [M+Cl]$^-$ anions.

Chapter 6 demonstrated the development of a novel instrument of IMMS-IMMS by interfacing a dual gate drift tube ion mobility spectrometer to a Synapt G2 HDMS. This provides a method to evaluate the isomeric heterogeneity of precursor ions by both DTIMS and TWIMS, to acquire a mobility-selected and mass-filtered fragmentation pattern and to additionally obtain traveling wave ion mobility spectra of the corresponding product ions. Mobility identities of product ions derived from mobility-selected precursor ion from an isomeric oligosaccharide mixture were able to be measured for the first time. This information is valuable as it establishes a direct precursor-product relationship between mobility-selected precursor ions and specific sets of product ions having unique mobilities in addition to specific $m/z$ values. This new IMMS$^2$ instrument enables the structural diversity of carbohydrates to be studied in greater detail: (1) for assessment of the complexity of isomeric precursor ion mixture; (2) for firmly establishing the mobilities of their specific sets of product ions.

Glycoprotein digests without deglycosylation, resulting sample was mixture of peptides and glycopeptides, were analyzed by IMMS directly in Chapter 7. Peptides and glycopeptides with different charge states (up to 4 charges) were separated on distinguishable ion mobility-mass correlation trend lines on the basis of 2-D IMMS spectra in both positive and negative modes. The trend line separation patterns matched between two modes. Conventional tedious
and time-consuming sample preparation protocol for glycopeptide analysis was eliminated, only standard proteolytic digestion was needed for IMMS analysis. Chemical noise was also reduced using IMMS analysis, which further improved the detection of low abundance ions. In addition, isomeric mobility peaks were observed for specific glycopeptides, this probably resulted from the isomeric heterogeneity of carbohydrate component contained in glycopeptides, but is more complex than the resolution of carbohydrate isomers themselves. These results may lead to the development of novel strategies for high throughput identification of complex glycoproteins in the future.