Differential Mobility Spectrometry Analysis of Glycans and Glycopeptides using SelexION® Technology

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Glycans and glycosylated biomolecules perform numerous roles through their interactions in cellular environments, with more than half of human proteins being glycosylated[1]. The characterization of glycosylation in biopharmaceuticals is essential for the determination of function and efficacy[2]. Thus, glycan analysis is gaining great interest. However, carbohydrates are highly variable in structure owing to differences in their anomeric configurations, monomer stereochemistry and inter-residue linkage positions. The separation of carbohydrates has been a great challenge for current analytical techniques[3-4]. Differential mobility spectrometry (DMS) is a technology that can separate gas-phase ions prior to analysis by mass spectrometry (MS)[5]. It has been used in the separation of various isomeric species, including stereoisomers[6], structural isomers[7], and tautomers[8]. In this study, DMS-MS was employed for the structural analysis of glycans and glycopeptides.

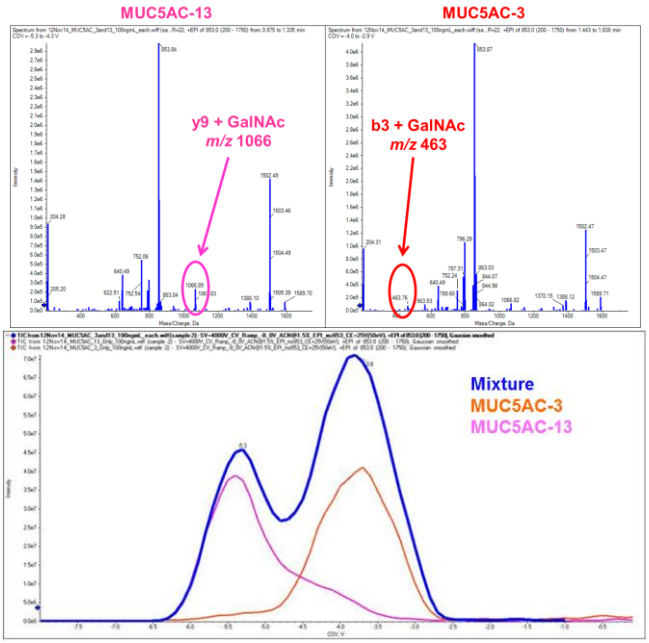


Figure 1. MS/MS and DMS Analysis of a Pair of Glycopeptide Isomers (MUC5AC-3, GTTPSPVPTTSTTSAP, and MUC5AC-13, GTTPSPVPTTSTTSAP). These two glycopeptides differ only in the location of the *O*-linked GalNAc modification; the red T in the peptide sequence represents the modified threonine residue. (Top) Monitoring diagnostic y or b-ions for the different sites of glycosylation allowed DMS separation of the $[M+2H]^{2+}$ ions of these glycopeptides to be monitored. With acetonitrile as the chemical modifier, the DMS afforded clear separation between the two peptide forms (bottom).

The separation of the isomeric glycan and glycopeptide species studied here was accomplished because of differences in the DMS behavior between each isomer[9]. The ions bear subtle structural differences that allow the DMS to separate them based on their different mobilities during the high- and low-field portions of the asymmetric waveform applied across the DMS cell (Separation Voltage, SV). As such, each isomer requires a different DC compensation voltage (CV) to bring their trajectories on-axis for successful sampling by the MS. Besides the differences between the isomers' structures, differences in how these species bind to added volatile chemical modifiers in the DMS cell makes their DMS behavior differences more prominent, yielding increased selectivity and peak capacity for the DMS experiments.

Key Features of SelexION® Technology for the Analysis of Glycans and Glycopeptides

- SCIEX SelexION® Technology provides an additional dimension of selectivity for structural elucidation of glycans and glycopeptides (Figure 1).
- The innovative planar design of the DMS cell uses an asymmetric RF waveform (SV) to separate ions based on differential mobility between the high and low fields.
- The compensation voltage (CV) is used to correct the trajectory of the ion of interest which traverses the cell and into the orifice while interferences are deflected into the cell walls.
- Allows use of chemical modifiers in differential mobility separations to dramatically increase resolving power and provide additional orthogonality to the separation.

Experimental

Sample Preparation: Glycans were purchased from Sigma-Aldrich (Oakville ON, Canada) and from Dextra Laboratories (Reading, UK). For work in positive ionization mode, glycans were diluted to 1 µg/mL in methanol and water (50/50, v/v) containing 50 µM sodium chloride. For negative mode work, glycans were diluted to 1-5 µg/mL with acetonitrile and water (20/80, v/v) containing 10 mM ammonium bicarbonate. Two glycopeptides, supplied by Anaspec (Fremont, CA), were diluted to 10 µg/mL with acetonitrile and water (50/50, v/v) containing 0.1% formic acid.

DMS-MS Conditions: The SelexION® device was mounted in the atmospheric region between the SCIEX QTRAP® 5500 or 6500 system's sampling orifice and a Turbo V™ source (Figure 2). The temperature of the DMS cell was maintained at 150 ºC, and the nitrogen curtain gas was operated at 30 psi. Chemical modifiers (water, methanol or acetonitrile) were added into the nitrogen curtain gas flow at 1.5% (mole ratio). The fundamentals of the DMS device have been described elsewhere[5]. In this study, both separation voltage (SV) and compensation voltage (CV) were scanned using Analyst® Software 1.6. As the SV was stepped from 0 to 4000 V, CV was scanned from -40 V to +20 V. These data were plotted as dispersion plots, with SV as the x-axis, and the optimal CV for ion transmission as the y-axis.

Data Analysis: Data was analyzed using PeakView® Software.

Separation of Isomeric O-Linked Glycopeptides

Finally, the separation of a pair of isomeric glycopeptides (MUC5AC-3 and MUC5AC-13) was demonstrated (Figure 1). These two glycopeptides are different only in the site of the *O*-linked GalNAc modification. Diagnostic y and b ions, that retained the *O*-linked GalNAc modification, were observed in the MS/MS spectra of the separated glycopeptides, and allowed confirmation of DMS separation.

Separating Disaccharides and Trisaccharides

SelexION® technology was employed to analyze the sodiated or deprotonated molecular ions of disaccharides and trisaccharides. Maltose and melibiose have the same mass, however differ structurally based on the sugar linkage. To assess the ability of DMS to separate these very similar sugars, the compensation voltage (CV) was ramped across a range of separation voltages (SV) and the CV optimum was determined at each step. These CV curves were then plotted for each sugar to visualize separation potential (Figure 3).

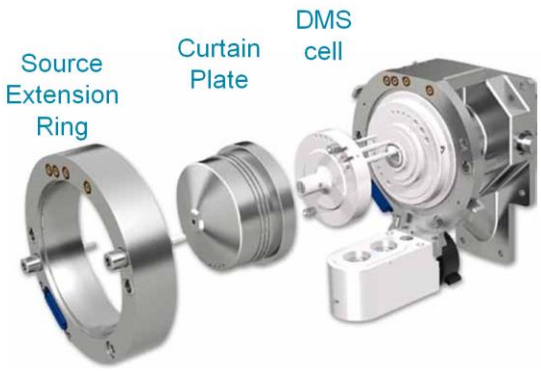


Figure 2. The SelexION® Differential Mobility Spectrometry Device. The DMS interface is directly coupled to the orifice plate. A modified curtain plate accommodates the DMS cell, which can be easily installed and removed without the use of any tools and without venting the system. The source extension ring enables use of the standard SCIEX QTRAP® system sources.

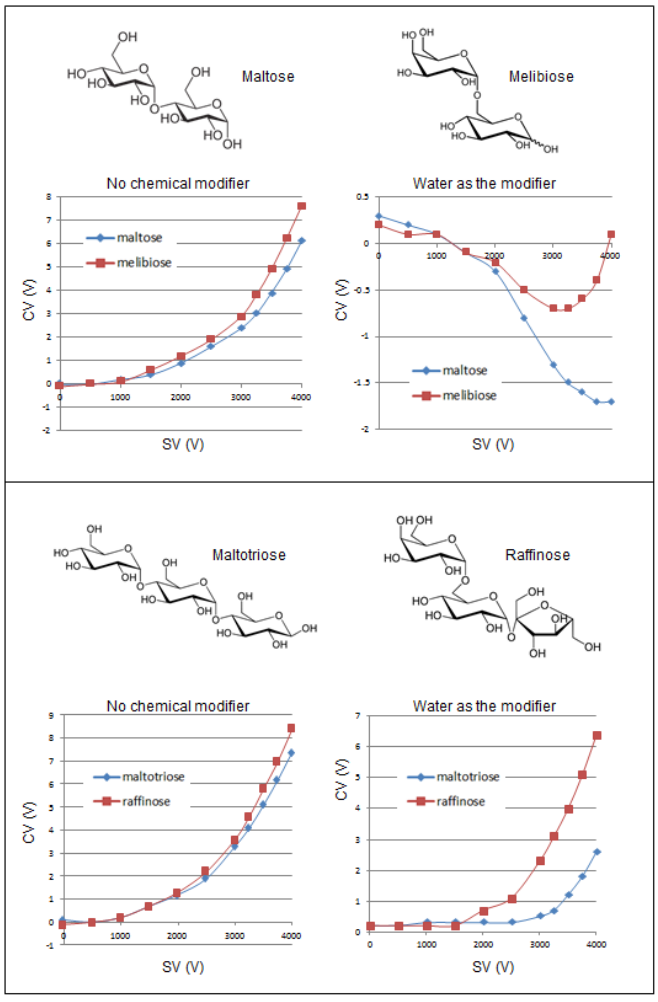


Figure 3. Separation of Isomeric Glycans with DMS-MS. (Top) Two sodiated disaccharides were analyzed using DMS and were found to fully separate with the addition of water as a chemical modifier; note the clear separation of the red and blue trend lines above 3000 V SV. Similar observation was found for the two sodiated trisaccharides (bottom).

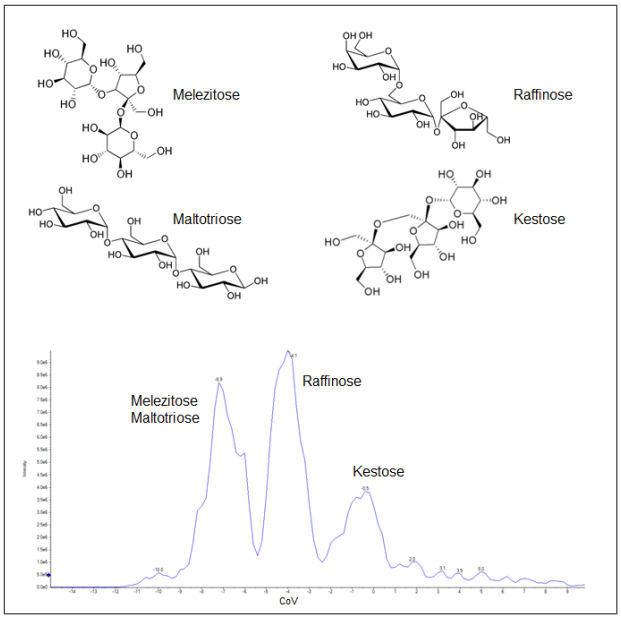


Figure 4. DMS Separation of Four Sodiated Trisaccharide Isomers. Using methanol as a chemical modifier enabled the separation of 3 out of 4 sugar isomers, as seen in the ionogram (bottom). Separation voltage was set to 4000 V.

When these carbohydrates were analyzed without chemical modifiers, molecular ions could not be fully separated (Figure 3, left). The controlled addition of water or methanol vapor in the DMS cell induced different shifts in optimal CVs at the higher separation voltages, enhancing the separation of isomeric carbohydrates. The ability to add different chemical modifiers is a unique feature of the planar differential mobility cell and provides an additional orthogonal level of separation that can be explored for different molecule types.

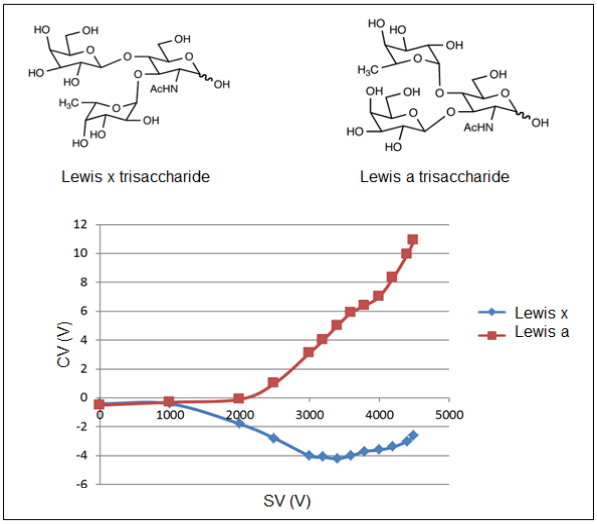
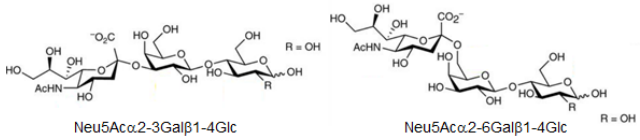


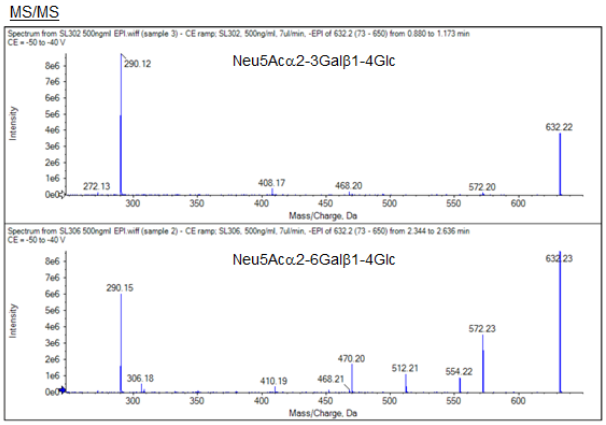
Figure 5. DMS Separation of Deprotonated Lewis a and Lewis x Trisaccharide Isomers. For these sugars, using methanol as a chemical modifier provided clear separation at separation voltages above 3000V.

In addition to maltotriose and raffinose, two additional isomeric trisaccharides were studied (Figure 4). As well as using water as the chemical modifier, the separation was explored using methanol. In this case, good separation was obtained for 3 out of 4 sugars (Figure 4). The final set of sugars tested were deprotonated Lewis x and Lewis a trisaccharides. In this case, methanol provided the best separation by DMS (Figure 5).

Differentiation by Sialic Acid Linkage

Another structural feature of glycans that makes them challenging to study is the attachment of sialic acid residues to the ends of the glycan chains. Understanding this linkage is important as it impacts biological function.





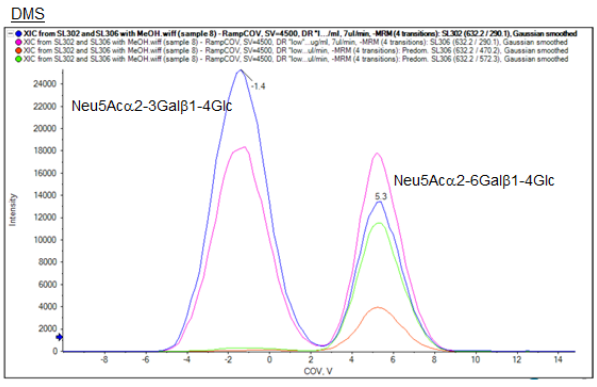


Figure 6. Separation of Deprotonated Glycan Isomers with Differing Sialic Acid Linkages. (Top) MS/MS of pair of deprotonated isomers (Neu5Acα2-3Galβ1-4Glc and Neu5Acα2-6Galβ1-4Glc) provided diagnostic fragment ions for monitoring DMS separation between the sugars. (Bottom) Using methanol as the chemical modifier and a separation voltage of 4500V, baseline separation of the two isomers, differing only by their sialic acid linkage, was achieved.

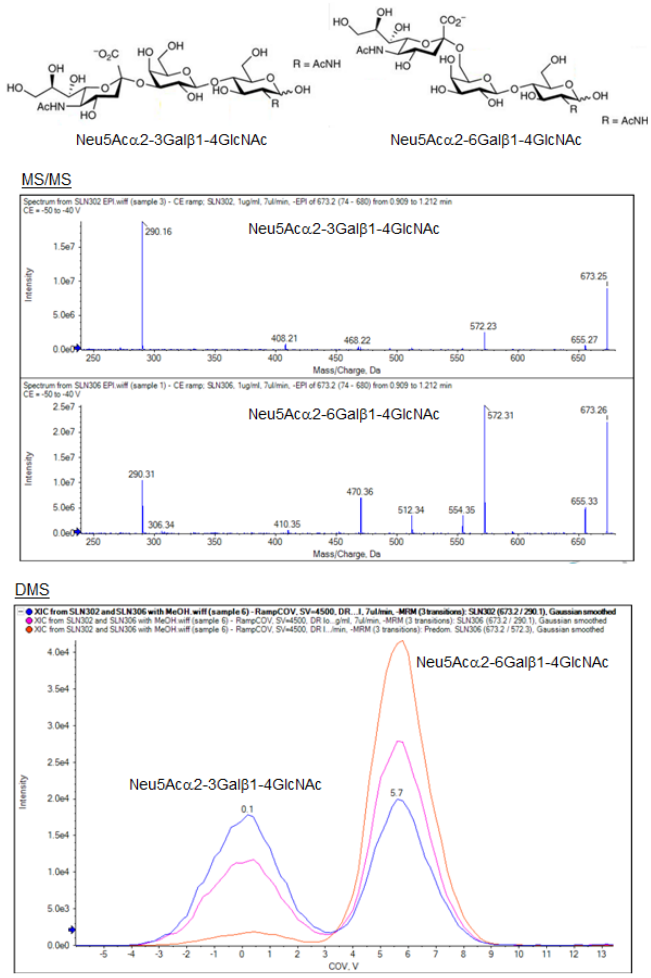


Figure 7. Separation of Deprotonated Glycan Isomers with Differing Sialic Acid Linkages. (Top) Structure and MS/MS of a pair of deprotonated isomers (Neu5Acα2-3Galβ1-4GlcNAc and Neu5Acα2-6Galβ1- 4GlcNAC). (Bottom) Using methanol as the chemical modifier and a separation voltage of 4500V, baseline separation of the two isomers, differing only by their sialic acid linkage, was achieved.

Here, two pairs of glycans with α2-3- and α2-6-linked sialic acid were studied to determine whether DMS could resolve this type of structural difference. As shown in Figure 6 and 7, in the presence of methanol as a chemical modifier, baseline separation of these isomers could be achieved.

Conclusions

In this study, SelexION® Technology was used to analyze biologically relevant glycan and glycopeptide isomers, to determine whether differential mobility separation would be useful in the study of glycan and glycopeptide structural isomers. With the addition of gas-phase chemical modifiers to the DMS, various isomeric species were successfully separated including:

- Sodiated and deprotonated glycans,
- Deprotonated glycans containing α2-3 or α2-6 linked sialic acid,
- Protonated glycopeptides.

Coupled with QTRAP® system technology, high sensitivity MS/MS data with CID fragmentation can also be collected, to identify diagnostic fragments for monitoring separation (such as the GalNAc modification fragments for the *O*-linked glycopeptide, Figure 1) or to confirm identity of glycans after separation. SelexION® Technology coupled to QTRAP® systems provides a fast and convenient approach for isomeric glycan and glycopeptide analyses.

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