

Differentiation of α 2,3 and α 2,6 Sialic Acid Linked Glycan Isomers using SelexION[®] Differential Mobility Spectrometry Technology

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Protein glycosylation is one of the most common and yet most complex post-translational modifications, displaying diversity both in site occupancy and in glycan structure. Sialic acids are typically found at the outermost ends of glycan chains, and have important function in many physiological and pathological processes, including pathogen binding and regulation of the immune response¹. In human cells, the linkage position of a sialic acid to a glycan side chain can be α 2,3 or α 2,6 to a galactose residue, α 2,6 to a N-acetylgalactosamine (GalNAc) residue, or α 2,8 to another sialic acid residue^{2,3}. The linkage configuration has important consequences for biological function, for example, upregulation of α 2,3 linked sialic acid is associated with metastasis progression in certain cancers^{4,5}. For the development of biopharmaceuticals, characterization of sialylation is essential for determination of function and efficacy⁶.

Differential mobility spectrometry (DMS) has been used for the separation of isomeric species in a number of recent studies⁷⁻⁹. The tuning of parameters such as separation voltage, compensation voltage, temperature and the presence of chemical modifiers, can allow the transmission of a particular species whilst others are lost to the electrode walls.

This study explores the selectivity of DMS for the resolution of isobaric glycan species containing α 2,3- and α 2,6-linked sialic acid¹⁷. The ability to separate glycans based on this linkage was demonstrated with both small glycans (Figure 3) and large glycans (Figure 1).

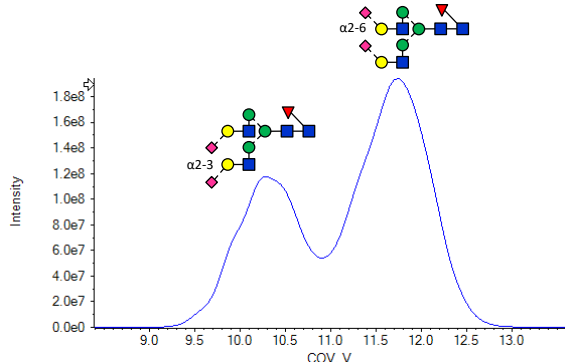


Figure 1. Separation of Larger Glycans with Different Sialic Acid Linkage. Isomeric separation of a doubly deprotonated disialylated biantennary glycan pair with DMS-MS, using methanol as the modifier. Separation voltage was set to 4300V.

Key Features of SelexION[®] Technology for Separation of Isomeric Glycans

- SelexION Technology is a planar differential mobility device (DMS) that separates molecules based on differences in their chemical properties, prior to entering the instrument orifice, thus providing an orthogonal level of selectivity.
- Use of chemical modifiers can provide enhanced separation of closely related species such as isomeric glycans with different sialic acid linkages.
- In combination with the QTRAP[®] System, acquisition of MS/MS spectra post separation provides the definitive identification of the glycan species.
- Separation of α 2,3- and α 2,6-linked sialic acid glycan pairs is an ongoing challenge in glycan analysis.

Experimental

Sample Preparation: Glycans were purchased from Dextra Laboratories (Reading, UK) and from TheraProteins (Barcarena, Portugal). Glycans were diluted to 0.5–25 $\mu\text{g}/\text{mL}$ in acetonitrile and water (20/80, v/v) containing 10 mM ammonium bicarbonate.

DMS-MS Conditions: The SelexION device (SCIEX) was mounted in the atmospheric region between the sampling orifice and IonDrive™ Turbo V source of a SCIEX QTRAP 6500 or 6500+ system (Figure 2). The temperature of the DMS cell was maintained at 150 °C, and the nitrogen curtain gas was operated at 30 psi. Methanol was added into the nitrogen curtain gas flow at 1.5% (mole ratio) as the chemical modifier. Separation voltage (SV) and compensation voltage (CoV) were scanned during continuous infusion of glycan solution at 5 $\mu\text{L}/\text{min}$. Either MRM or linear ion trap MS/MS data of the target glycan(s) was recorded at each increment of SV and CoV.

Separation of Glycans Based on Sialic Acid Linkage

DMS-MS was employed for the analysis of deprotonated molecular ions of $\alpha 2,3$ and $\alpha 2,6$ sialic acid linked isobaric glycan pairs, in the presence of methanol chemical modifier. The controlled addition of methanol vapour in the DMS cell induces different shifts in optimal CoV for the individual isomers, enhancing the separation of the isomeric glycans.

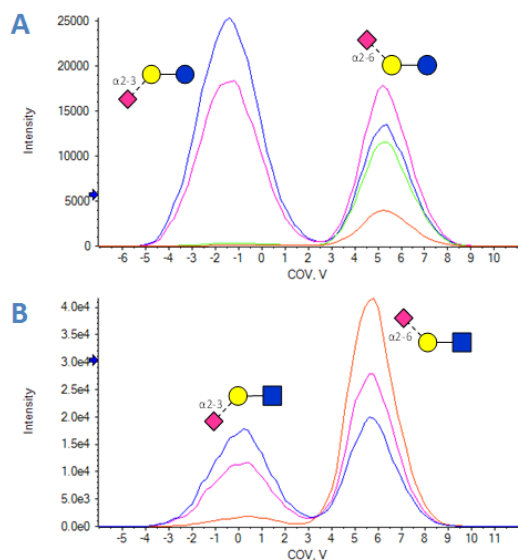


Figure 3. SelexION Technology Can Separate Isomeric Glycans Based on Sialic Acid Linkage. Isomeric separation of deprotonated sialylated glycans Neu5Ac $\alpha 2$ -3Gal $\beta 1$ -4Glc & Neu5Ac $\alpha 2$ -6Gal $\beta 1$ -4Glc (A), and Neu5Ac $\alpha 2$ -3Gal $\beta 1$ -4GlcNAc & Neu5Ac $\alpha 2$ -6Gal $\beta 1$ -4GlcNAc (B) with DMS-MS. Methanol was used as the chemical modifier for the DMS analysis; separation voltage was set to 4500V.

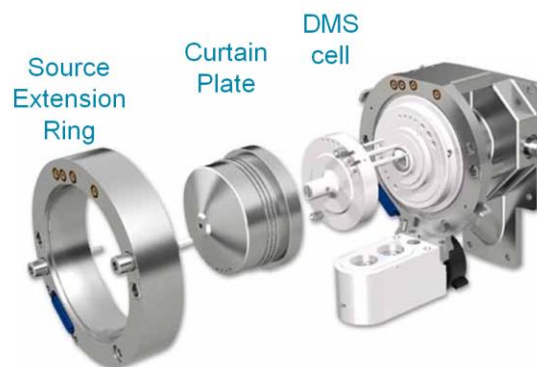


Figure 2. Configuration of the SelexION Differential Mobility Spectrometry Device on a QTRAP System. The SelexION cell is mounted in the atmospheric region between the source and sampling orifice, allowing it to be installed in minutes.

Figure 3 shows DMS separation of two sets of $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acid trisaccharide glycan pairs. While significant differences can be observed in the MS/MS spectra of the $\alpha 2,3$ and $\alpha 2,6$ isomers (Figure 4), it is the DMS separation that enhances these differences (e.g., an $\alpha 2,6$ isomer-specific fragment at m/z 306¹⁰). Without DMS, a convoluted MS/MS spectrum could complicate identification.

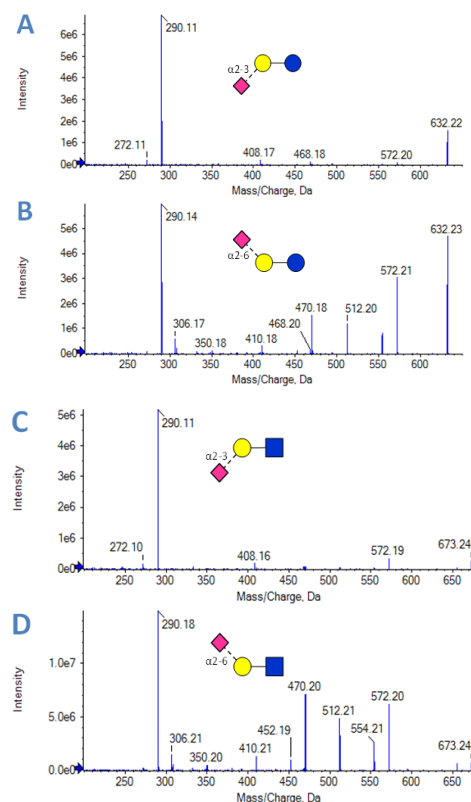


Figure 4. MS/MS Differences Enable Definitive Identification of the Sialic Acid Linkage. Linear ion trap MS/MS spectra of the $[\text{M}-\text{H}]^-$ ions of Neu5Ac $\alpha 2$ -3Gal $\beta 1$ -4Glc & Neu5Ac $\alpha 2$ -6Gal $\beta 1$ -4Glc (A, B), parent m/z 632.2, and Neu5Ac $\alpha 2$ -3Gal $\beta 1$ -4GlcNAc & Neu5Ac $\alpha 2$ -6Gal $\beta 1$ -4GlcNAc (C, D) parent m/z 673.2. Spectra were acquired with collision energy ramp -45V to -49V (A, B) or -53V to -57V (C, D).

Following successful differentiation of sialic acid linkage in two small glycan pairs, the behaviour of a pair of much larger di-sialylated biantennary glycans was attempted (Figure 1). Corresponding MS/MS data are shown in Figure 5. In addition to the $^{0,4}A_2$ -CO₂ fragment ion at m/z 306, the prominent B₃ ion at m/z 655 in the MS/MS spectrum of the α 2,6 glycan is also thought to be diagnostic for this linkage¹⁰.

For all three glycan pairs, a more negative CoV was observed for the α 2,3 isomer than for the α 2,6 isomer, suggesting that the α 2,3 isomers bind more strongly with the methanol vapor than the α 2,6 isomers¹¹⁻¹³.

Conclusions

In this study, differential mobility spectrometry (DMS) was used to analyze pairs of mono- and di-sialylated glycan isomers. With the addition of methanol chemical modifier to the DMS, the α 2,3 sialylated isomer was successfully separated from the α 2,6 form in all three isomer pairs studied, in spite of the varying sizes of the glycans¹⁷. In addition, a more negative CoV was consistently observed for the α 2,3 form than for the α 2,6 form. Recent studies have examined the use of other ion mobility technology for differentiation of α 2,3 from α 2,6 sialylation¹⁴⁻¹⁶. Separation of the trisaccharide isomer pairs shown in Figures 1 and 3 was demonstrated by Guttman and Lee¹⁴ and Hinneburg *et al*¹⁵; Barroso *et al* achieved only partial separation of the non-fucosylated version of the isomer pair shown in Figures 1 and 5¹⁶. However, other ion mobility technology was not shown to distinguish the large fucosylated glycan pair that was successfully separated here using differential mobility spectrometry (e.g., it was unable to resolve the analogous Neu5Gc glycan pair)¹⁶. Given the promise shown by DMS for this application, the use of SelexION Technology for the separation of a large range of differentially sialylated glycan forms warrants further investigation. Such a method would be invaluable in research into the biological function of these important glycan components, as well as in the characterization of biopharmaceuticals.

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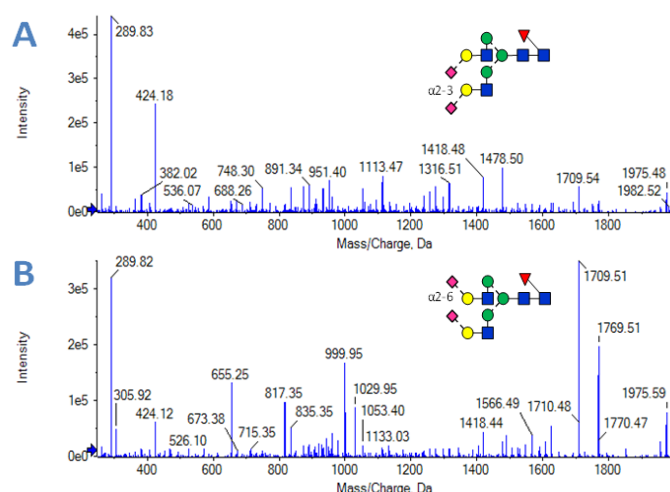


Figure 5. MS/MS of Larger Glycans After DMS Separation. Linear ion trap MS/MS spectra of the $[M-2H]^{2-}$ ions of a pair of di-sialylated biantennary glycans after separation by DMS-MS (parent m/z 1183.5) shows additional diagnostic ions for the α 2,6 glycan. MS/MS spectra were taken at CoV values of 10.3V (A) and 11.7V (B) during the DMS analysis shown in Figure 1. MS/MS spectra were acquired with collision energy -74V.

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