Site-Specific Characterization of the O-linked Glycans at the Furin Cleavage Site of the Sars-CoV-2 Spike Protein using Cyclic IMS

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Introduction

- The global pandemic due to the SARS-CoV-2 virus has driven a rapid characterization efforts of the novel virion
- A furin cleavage site, a site critical to protein activation and cell entry,[1] is present in the linker region flanking the S1 and S2 subunits
- Three putative O-glycosylation sites were predicted along the region surrounding the furin cleavage site
- Site-specific, structure-specific characterization of the O-glycoforms along this region are demonstrated using the novel cyclic IMS system

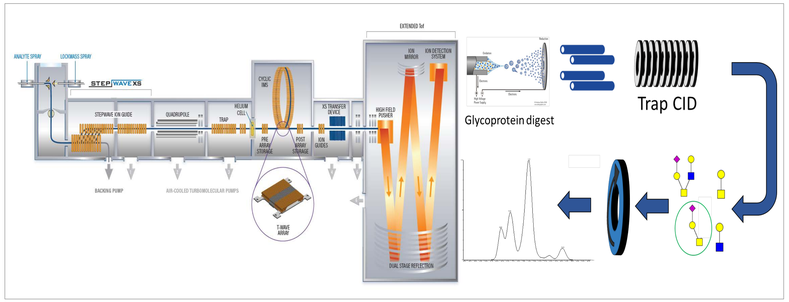

Figure 1) Left: Instrument diagram of the cyclic IMS. Right: MS workflow for targeted CID-IM experiments on the glycan fragments of O-glycopeptides.

Methods

- SARS-CoV-2 S protein was reduced and alkylated using 5 mM DTT and 15 mM iodoacetamide, respectively, prior to treatment with trypsin and PNGaseF
- Digested peptides were separated using a Waters nanoEase HSS T3 100 Å, 1.8 µm (75 µm x 15 cm) column in a trapping configuration with a nanoEase M/Z Symmetry C18 100 Å, 5 µm trap
- Mobile phases were 0.1% formic acid, 1ppm citric acid in water and acetonitrile, respectively
- Peptides were separated using over 60 minutes using a gradient from 10%B to 35%B prior to mass spectrometry
- Tryptic O-glycopeptides were quadrupole isolated prior to collisional activation in the trap region and ion mobility separation in the cyclic ion mobility cell, shown in Figure 1
- Data was collected using both 1 and 5 passes of the cyclic IM cell, optimized for trisaccharide fragments

Results: MS/MS

Targeted MS-IM-MS experiments were carried out on four O-glycoforms of tryptic peptide 56 (T56, A-D), they are summarized in Table 1. MS/MS spectra of glycopeptides A and C are shown Figure 2. Numerous oxonium ion fragments were observed from glycopeptide C, including fragments at *m/z* 528 and 819, which indicate the presence of an extended core 1 population. An ion at m/z 1022 was also observed, indicating a single pentasachharide glycoform .

Precursor Name	Glycopeptide ID	Observed Drift Time of NeuAc-Hex-HexNAc (ms)	Assigned Oxonium Ion Structures
T56-HexNAc(1)-Hex(1)-NeuAc(1)	A	73.8	α2-3, β1-3
T56-HexNAc(1)-Hex(1)-NeuAc(2)	B	68.7 70.4 73.8	β1-3, α2-3, α2-6 α2-3, β1-3
T56-HexNAc(2)-Hex(2)-NeuAc(1)	C	73.2 73.8 80.2	α2-6, β1-4 α2-3, β1-3 α2-3, β1-4
T56-HexNAc(2)-Hex(2)-NeuAc(2)	D	73.2 73.8 80.2	α2-6, β1-4 α2-3, β1-3 α2-3, β1-4

N-Acetylgalactosamine (GalNAc)
N-Acetylglucosamine (GlcNAc)
HexNAc (either GalNAc or GlcNAc)
Galactose (Gal)
N-Acetyleuraminic acid (NeuAc)

Table 1) Glycopeptides studied, the observed drift times of the HexNAc(1)-Hex(1)-NeuAc(1) oxonium ion fragment using five passes of the cyclic mobility cell, and the assigned structures of the oxonium ion fragment.

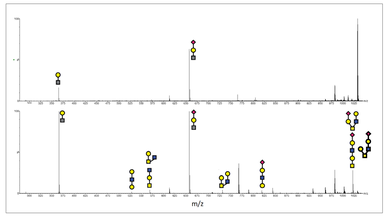

Figure 2) oxonium ions generated from o-glycopeptides A and C are shown as examples in the zoomed MS/MS spectra of O-glycopeptide, T56-HexNAc(1)-Hex(1)-NeuAc(1) (top) and T56-HexNAc(2)-Hex(2)-NeuAc(1), (bottom).

Results: CID-IM

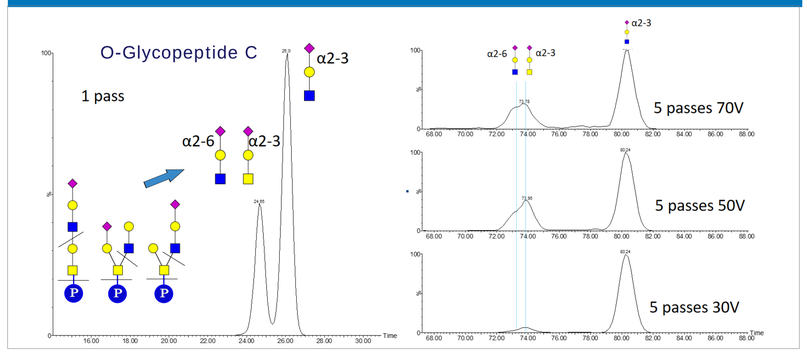

Figure 3) Left: Single Pass MS-CID-IM-MS of T56-HexNAc(2)-Hex(2)-NeuAc(1). Right: Five Pass MS-CID-IM-MS of T56-HexNAc(2)-Hex(2)-NeuAc(1) using 70V, 50V, and 30V to activate the precursor.

Results: CID-IM

Two mobility populations are observable using a single pass of the ion mobility cell to separate the *m/z* 657 trisaccharide fragment of glycopeptide C, shown in Figure 3, left. Using five passes, the ions with shorter drift time is separated into two peaks (Fig 3. right). Increasing the activation energy in the trap results in an increased abundance of the more compact of the two, likely due to the known increased stability of α2-6 linkages relative to α2-3. In combination with comparison to literature, the arrival time distribution (ATD) centered at 73.2 ms is assigned to a NeuAcα2-6Galβ1-4GlcNAc, a fragment of an extended core 1 structure.[2] The adjacent ATD measured at 73.8 ms was the only oxonium ion observed for glycopeptide A and is assigned to a NeuAcα2-3Galβ1-3GalNAc, consistent with exoglycosidase work (not shown) and the known linkages of a core 1 base. The oxonium ion with the longest drift time, at 80.24 ms, is consistent with literature values for a NeuAcα2-3Galβ1-4GlcNAc and supports a second linkage isomer for the extended core 1 structure.[2]

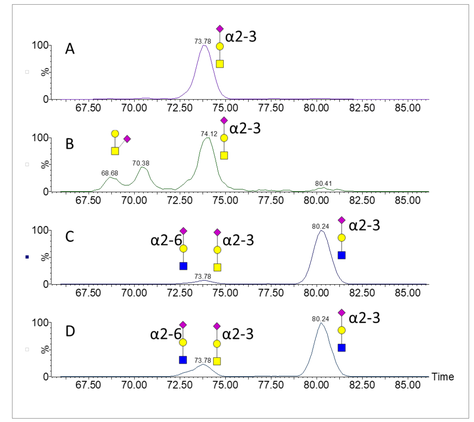

Figure 4) Arrival time distributions of the m/z 657 ion fragment of glycopeptides A-D using five passes of the cyclic ion mobility cell. The penta- and hexasaccharide glycoforms produce remarkably similar distributions, suggesting a high degree of similarity of the precursor glycoform distributions. Structures resulting from a core GalNAc-NeuAc linkare are only observed for glycopeptide B.

Conclusions

- Site-specific and structure-specific characterization of four O-glycoforms adjacent to the furin cleavage site of the SARS-CoV-2 S protein is demonstrated using cyclic IMS to separate the HexNAc-Hex-NeuAc fragment
- Five passes of the cyclic IMS resolves a NeuAcα2-3Galβ1-3GalNAc fragment from a NeuAcα2-6Galβ1-4GlcNAc fragment
- These results unambiguously confirm the presence of core 1, core 2, and extended core 1 structures

References

[1] Hoffmann M, Kleine-Weber H, and Pöhlmann S. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. Mol Cell. 2020 78(4):779-784
[2] Guttman M and Lee KK. Site-Specific Mapping of Sialic Acid Linkage Isomers by Ion Mobility Spectrometry. Anal. Chem. 2016; 88(10):5212–5217.

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