CONFIDENT O-GLYCOSYLATION SITE IDENTIFICATION USING A CYCLIC ION MOBILITY– MASS SPECTROMETER EQUIPPED WITH ECD FUNCTIONALITY

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OVERVIEW

- Site specific analysis of O-glycosylation using ECD (Electron Capture Dissociation) enables the retention of the glycan moieties on the peptide backbone and allows the glycosylation site(s) to confidently assigned using the resulting fragment ions.
- In this study, we demonstrate the utility of ECD on a cyclic IMS-enabled instrument for unambiguous assignment of glycosylation site locations for O-glycopeptide species from Enbrel (etanercept).
- Explore the advantages of the ExD cell placed post-IMS on the SELECT SERIES™ Cyclic™ IMS mass spectrometer.
- Use multi-pass IMS to resolve precursor ions by ion mobility prior to ECD fragmentation.
- Isolate O-glycopeptides with IMS^n (slicing) followed by multi-pass IMS to determine if multiple glycoforms are present.

SELECT SERIES™ CYCLIC™ IMS

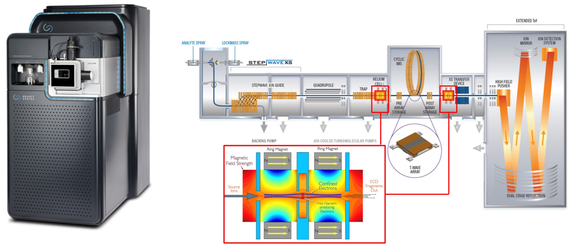

Figure 1. SELECT SERIES CYCLIC IMS instrument schematic with eMSion ExD cell. Note that the ExD cell can be installed either pre- OR post-IMS.

METHODS

- **Sample:**
  Infusion: 4.0 µM solution of reduced and alkylated Enbrel (etanercept) trypsin digest was used for static nanospray infusion experiments.
  LC-MS: 2.0 µM solution of Enbrel trypsin digest was used for targeted LC-HDMS/MS ECD experiments.[1]
- **Mass Spectrometer:** SELECT SERIES Cyclic IMS mass spectrometer equipped with post IMS ExD cell (eMSion)
  Infusion: targeted HDMS/MS ECD with multi-pass IMS and IMS^n (slicing) followed by multi-pass IMS with ECD fragmentation
  LC-MS: targeted HDMS/MS ECD on Enbrel O-glycopeptides
- **Inlet:** ACQUITY™ I-Class UPLC™ System
  Column: ACQUITY Premier CSH C_{18} Column 130Å, 1.7 µm, 2.1 x 100 mm
  Mobile Phases: MPA 0.1% Formic Acid in Water, MPB 0.1% Formic Acid in Acetonitrile
  Gradient: 1.0 - 35.0%B over 25 minutes
  Column Temperature: 60°C
- **Data Processing:** ECD MS/MS data were interpreted using a semi-automated mass spectral annotation using ExDViewer BETA v4.2.7 (eMSion).

RESULTS & DISCUSSION

INFUSION EXPERIMENTS

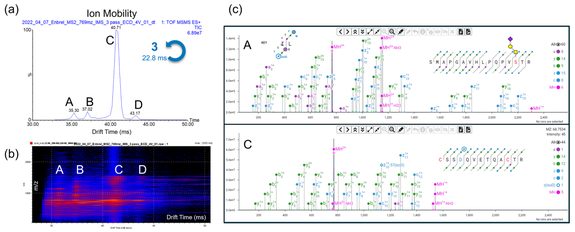

Figure 2. Panel (a) ion mobility profile from a quadrupole isolated precursor ion at 769 m/z with 3 pass (22.8 ms) IMS separation prior to ECD fragmentation. A total of four precursor ions (peaks A-D) are separated with 3 pass IMS prior to ECD fragmentation. Panel (b) 2D plot of m/z vs. Drift Time (ms) for the drift aligned ECD fragment ion spectra for peaks A-D. Panel (c) corresponding ECD mass spectra for peaks A and C highlighting precursor ions of different relative abundance can resolved by ion mobility prior to ECD fragmentation. The minor component Peak A will be further analyzed by IMS^n separation.

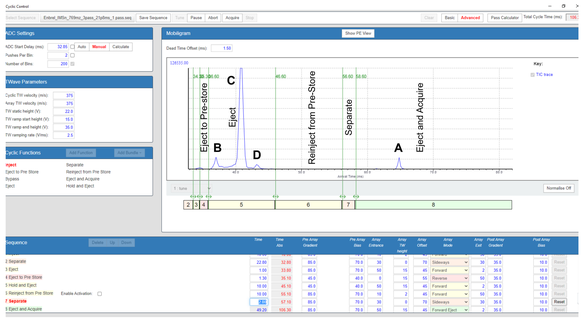

Figure 3. Cyclic Sequence for IMS^n slicing experiment with Peak A (768.70 m/z, 35.3 ms). The initial IMS separation is 3 passes (22.8 ms) with Peak A ejected to the pre-store array and Peaks B-D are ejected from the cyclic IMS. Peak A is re-injected into the cyclic IMS racetrack and separated with 2 ms (1 pass).

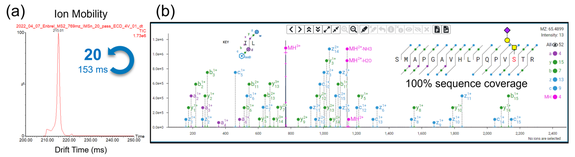

Figure 4. IMS^n-ECD experiment with 768.70 m/z precursor (Peak A). Panel (a) Ion mobility profile for IMS^n slicing experiment w/ 20 pass (153 ms) ion mobility separation prior to ECD fragmentation. Panel (b) Corresponding IMS^n resolved ECD fragment ion spectra of T19 peptide (SMAPGAVHLPQPVS$_{199}$TR) glycosylated with HexNAc-Hex-NeuAc at the serine 199 site. A single S_{199} glycoform is observed after IMS^n slicing followed by 20 passes across the cyclic IMS.

LC-MS EXPERIMENTS

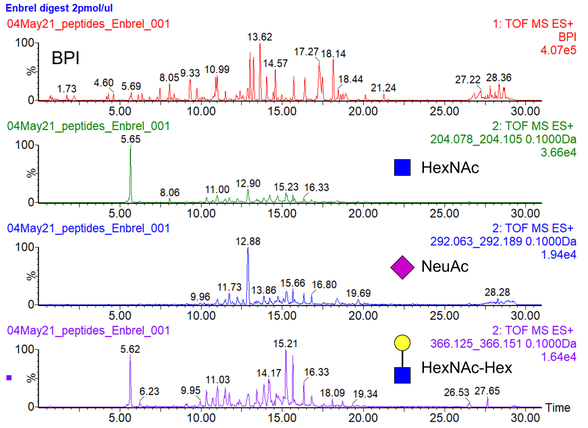

Figure 5. BPI chromatogram for the LC-HDMS^E (single pass IMS) analysis of the Enbrel (etanercept) tryptic digest. Extracted ion chromatograms for the characteristic oxonium ions: HexNAc (204.08 m/z), NeuAc (292.10 m/z), and HexNAc-Hex (366.14 m/z) are shown for the elevated energy function.

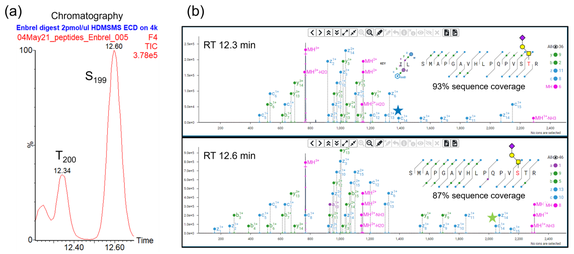

Figure 6. Panel (a) TIC chromatogram for LC-HDMS/MS ECD experiment for m/z 768.70 (+3) with chromatographic peaks at RT 12.3 and 12.6 min, respectively. Panel (b) corresponding ECD MS/MS spectra for the T19 tryptic peptide (SMAPGAVHLPQPVSTR) glycosylated with a single HexNAc-Hex-NeuAc at position T_{200} (12.3 min) and S_{199} (12.6 min). Note that the sequence coverage may be under-estimated since ECD does not cleave before proline residues.

RT (min)	Peptide	O-glycan(s)	Position(s)
14.7	T1: LPAQVAFT$_8$PYAPEPGS$_{16}$T$_{17}$CR		T$_8$
15.3			T$_8$
10.9	T19: S$_{186}$MAPGAVHLPQPVS$_{199}$T$_{200}$R	&	S$_{199}$ & T$_{200}$
11.7		&	S$_{199}$ & T$_{200}$
11.9			S$_{199}$ & T$_{200}$
12.2		&	S$_{199}$ & T$_{200}$
12.3			T$_{200}$
12.6			S$_{199}$
12.8		&	S$_{199}$ & T$_{200}$
18.3	T22-23: T$_{243}$HT$_{245}$CPPCPAPELLGGPS$_{259}$VFLFPPKPK		T$_{245}$
19.6			T$_{245}$

Table 1. List of ENBREL (etanercept) O-glycopeptides and glycosylation sites identified by targeted ECD.

CONCLUSIONS

- The utility of electron capture dissociation on the SELECT SERIES Cyclic IMS was investigated for the sequencing of peptides with labile post-translational modifications, namely O-glycans.
- The infusion IMS-ECD experiments with quadrupole selected precursor ions highlight the ability of multi-pass IMS to resolve multiple precursor ions before ECD fragmentation.
- IMS^n (slicing) combined with multi-pass IMS enables gas-phase isolation based on ion mobility prior to ECD fragmentation.
- A total of eleven O-glycans, including positional isomers, were identified and located from the biotherapeutic Enbrel.

REFERENCES

1. S. Ippoliti, D. Cooper-Shepherd, . Q. Yu, J. I. Langridge, Confident O-glycosylation Site Identification for ENBREL (etanercept) Using the ECD Functionality of SELECT SERIES Cyclic IMS System, Application Note, 720007458EN

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