A HIGHLY VERSATILE CYCLIC ION MOBILITY – MASS SPECTROMETER FOR ROUTINE TO IN-DEPTH BIOPHARMACEUTICAL CHARACTERIZATION

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OVERVIEW

- The structural diversity of monoclonal antibodies (mAb) results from the variety of protein modifications that can occur at different stages of the production process.
- The combination of ion mobility (IM) and HRMS provides additional higher-order structural information (*e.g.,* native gas-phase collision cross section (CCS), collision induced unfolding (CIU) and protein-ligand binding) that is typically not afforded by HRMS.
- A novel, highly versatile MS platform, the SELECT SERIES™ Cyclic™ IMS mass spectrometer was developed to help address these complex biophysical challenges.
- Herein, we plan to characterize mAb samples produced during cell line development using routine LC-MS biopharmaceutical workflows (subunit analysis, peptide mapping, and released glycan) and in-depth characterization focusing on low level PTMs and native mAb collision induced unfolding (CIU).

SELECT SERIES CYCLIC IMS

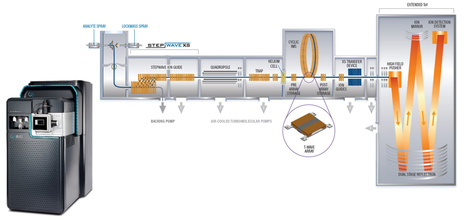

Figure 1. Instrument schematic and image of the SELECT SERIES™ Cyclic™ Ion Mobility Q-TOF mass spectrometer

METHODS

- Three IgG1 monoclonal antibodies produced during cell line development were used for this study.
- The mAb samples were reduced with dithiothreitol (DTT) and deglycosylated with PNGase F prior to subunit analysis.
- Each sample was denatured with 3M guanidine HCl, reduced with DTT and alkylated with iodoacetic acid prior to Trypsin/LysC (Promega) digestion for peptide mapping.
- The N-linked glycans were released and derivatized using a RapiFluorMS (RFMS) released glycan protocol.
- Prior to native CIU each mAb was buffer exchanged into 100 mM ammonium acetate using a micro Bio-Spin P-6 column (BioRad). The native mAb collision induced unfolding (CIU) experiments were processed in CIUSuite 2 software [1].
- The resulting data were processed in UNIFI™ for subunit, peptide mapping, and released glycan workflows.

SUBUNIT ANALYSIS

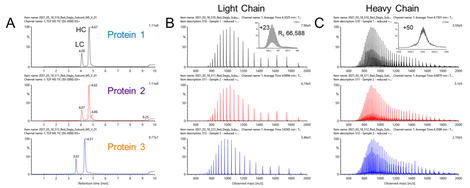

Figure 2. Reduced mAb LC-MS analysis of reduced + deglycosylated mAb samples. Panel (A) TIC chromatogram of each mAb sample: **Protein 1**, **Protein 2**, and **Protein 3**, (B) light chain raw mass spectrum with the inset figure highlighting the mass spectral resolution of 66,588 for the +23 charge state ion, and (C) heavy chain mass spectrum with the inset figure showing an expanded view of the +50 charge state.

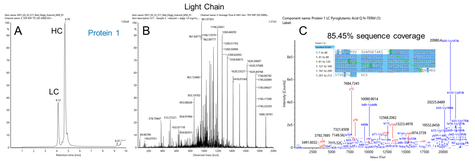

Figure 3. Subunit level topdown MS^E (CID) analysis of reduced + deglycosylated **Protein 1** mAb sample. Panel (A) Elevated Energy (CE Ramp: Trap TWIG 25-40V) TIC chromatogram, (B) light chain (R_t = 4.12 min) elevated energy mass spectrum, and (C) CID (b/y) fragment ion annotated BayeSpray deconvoluted mass spectrum with the corresponding sequence coverage map with 85.45% fragment ion coverage. Fragment ions are identified within ± 10 ppm.

PEPTIDE MAPPING

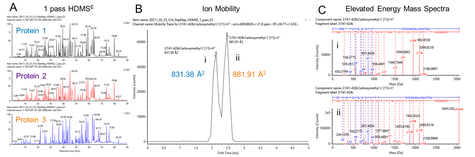

Figure 4. Peptide mapping LC-HDMS^E (single pass) analysis of trypsin digested samples. Panel (A): TIC Chromatogram of each sample. Panel (B): extracted ion mobility arrival time distribution for the $[M+5H]^{+5}$ charge state precursor ion at (609.88 m/z). (C) Elevated energy mass spectra for each ion mobility peak i and ii, corresponding to heavy chain T41-42 SRWQQGNVFSCSVMHEALHNHYTQK.

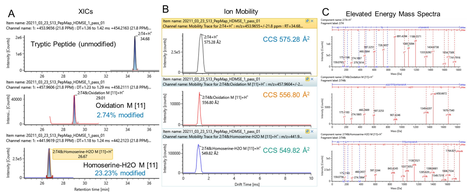

Figure 5. Panel (A): XIC's for the heavy chain T4 peptide (ASGYTFTGHYMHWVR) with the unmodified peptide $[M+4H]^{+4}$ (m/z 453.9655), oxidized methionine (M) (m/z 457.9604) modification, and the homoserine – H_2O (M) modification at m/z 441.9617. Panel (B) shows extracted ion mobility arrival time distribution for the $[M+4H]^{+4}$ charge state precursor ion with the measured CCS (A^2) annotated. Panel (C) corresponding elevated energy mass spectra for each modified peptide form.

RELEASED GLYCAN

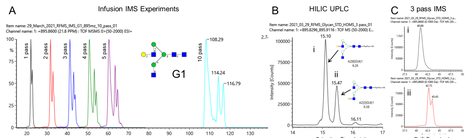

Figure 6. Panel (A): direct infusion experiment with the RFMS released glycan standard where the A2G1 glycan precursor ($[M+2H]^{+2}$, 895.86 m/z) was quadrupole isolated prior to single and multi-pass ion mobility. Panel (B): 895.96 m/z XIC of HILIC UPLC separated RFMS released glycan standard where A2G1 separates into two chromatographic peaks (peaks i / ii) for each glycoform. Panel (C): extracted ion mobility arrival time distribution for the 3 pass IMS experiment of each glycoform (i / ii).

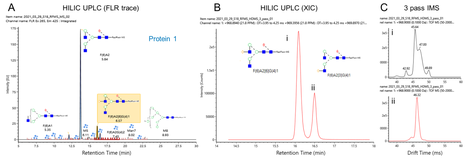

Figure 7. Panel (A): RFMS released glycan fluorescence (FLR) chromatogram from the released glycans from **Protein 1**. Panel (B): 969.89 m/z XIC where FA2G1 separates into two chromatographic peaks (peaks i / ii) for each glycoform. (C) extracted ion mobility arrival time distribution for the 3 pass IMS experiment of each glycoform (i / ii) to illustrate that multiple conformational isomers of peak (i) are present.

COLLISION INDUCED UNFOLDING

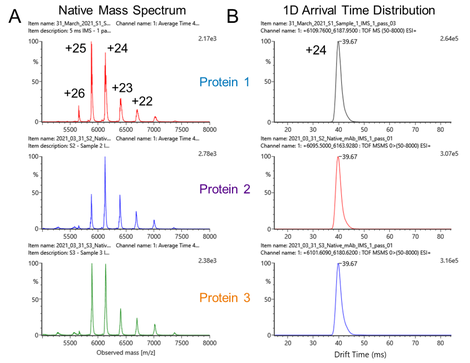

Figure 8. Panel (A): static nanospray native mass spectra for **Protein 1**, **Protein 2**, and **Protein 3**. Panel (B): single pass ion mobility experiment with the +24-charge state extracted arrival time distribution (ATD) shown. Note that the ion mobility ATD profiles are identical (39.67 ms) for each mAb sample.

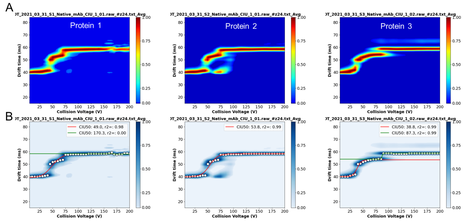

Figure 9. Panel (A) contains the collision induced unfolding (CIU) fingerprints for the +24-charge state of **Protein 1**, **Protein 2**, and **Protein 3**. Panel (B) shows the corresponding CIU50 plot, which provides both feature detection and determination of the transition midpoint between two unfolded states. Different unfolding patterns are observed between **Protein 1/2** and **Protein 3**, which have different amino acid sequences and disulfide bonding patterns.

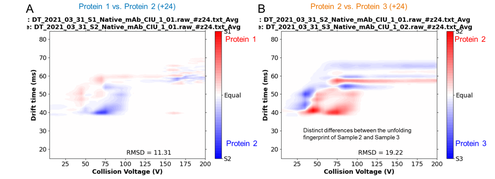

Figure 10. Panel (A) represents the CIU comparison difference plot for the +24-charge state of **Protein 1** (red) vs. **Protein 2** (blue). Panel (B) shows the CIU comparison difference plot for the +24-charge state of **Protein 2** (red) vs. **Protein 3** (blue). The blue / red heat map helps visualize the regions of the CIU fingerprint that have significant differences.

CONCLUSIONS

- Herein, we have demonstrated the versatility of the SELECT SERIES™ Cyclic™ IMS Q-TOF mass spectrometer to perform routine LC-MS biopharmaceutical characterization (subunit analysis, peptide mapping, and released glycan) and provide an in-depth biophysical characterization using collisional induced unfolding of native mAbs.
- **SUBUNIT ANALYSIS:** reduced mAb analysis of light and heavy chain illustrates high resolution of ~66,000 is achieved for the light chain +23 charge state ion. Topdown MS^E of the light chain results in ~85% sequence coverage by fragmenting the entire charge state envelope.
- **PEPTIDE MAPPING:** Single pass HDMS^E results in 95-97% sequence coverage for **Protein 1** and **Protein 3**. The peptide precursor gas-phase collision cross section (CCS) are beneficial to curate modified peptide forms with multiple chromatographic retention times found within the three mAb samples.
- **RELEASED GLYCAN:** multi-pass ion mobility combined with HILIC chromatography has shown beneficial to further resolve conformational isomers from the RFMS labeled released glycans.
- **COLLISON INDUCED UNFOLDING:** CIU helps resolve different unfolding patterns that are observed between Protein 1/2 and Protein 3. In future studies we plan to utilize CIU to monitor mAb stability as a function of stress (*e.g.,* formulation conditions, temperature, pH, freeze/thaw cycles, etc.).

References

1. Daniel A. Polasky, Sugyan M. Dixit, Sarah M. Fantin, and Brandon T. Ruotolo. "CIUSuite 2: Next-Generation Software for the Analysis of Gas-phase Protein Unfolding Data." *Anal. Chem.* 2019. DOI: 10.1021/acs.analchem.8b05762

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