Separation of Asp/IsoAsp Isobaric Peptides using Trapped Ion Mobility Spectrometry (TIMS)

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Introduction

- Monoclonal antibodies are proteins that provide unparalleled specificity and potency in the realm of biotherapeutics directed towards malignancies such as cancer.
- One of the barriers to the commercialization of these molecules is the risk of degradation via the isomerization of aspartate (Asp) to isoaspartic acid (isoAsp) in the complementarity-determining regions of the light chains. Asp/isoAsp are isobaric, making their characterization extremely challenging via traditional LC-MS/MS techniques.
- Here, we describe the utility of trapped ion mobility on the timsTOF Pro QTOF, to separate isobaric peptides containing Asp/isoAsp residues. (Fig. 1)

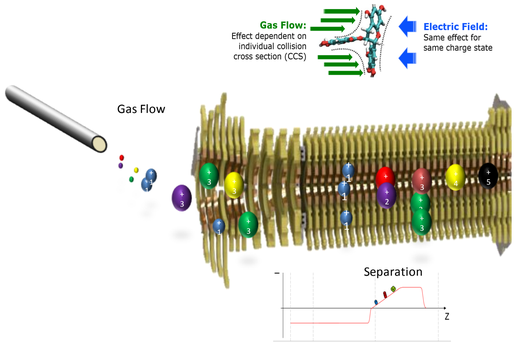

Fig. 1 Trapped Ion Mobility (tims) – Separation
The source gas propels ions into the tims device while a simultaneous opposing electric gradient field is also introduced in the analyzer which separates the ions as function of their collisional cross sectional area or CCS. In the dual TIMS analyzer, the first TIMS section accumulates the ions, and the second resolves them by mobility. Once a sufficient number of ions are trapped and separated, lowering the electrical potential releases time-resolved ions from the TIMS device into the downstream mass analyzer.

Methods

- The standard compounds were purchased from Biomatik (Wilmington, DE). Nine mixtures of isobaric peptides were prepared at Pfizer (St. Louis, MO).
- Direct infusion experiments were carried out on the timsTOF Pro QTOF at a flow rate of 3µl/min.
- Chromatographic separations were performed on the Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA) using a Waters XSelect CSH C18 2.5 um 2.1 x 150mm C18 column (Waters, Milford, MA) at 300 µl/min and a column oven temperature of 50°C with direct loading and a 20 min gradient.
- LC-TIMS MS/MS data were obtained on a timsTOF Pro instrument operated in PASEF mode, enabling the selection of 12 precursors within 12 ms. Data were analyzed using DataAnalysis software (Bruker Daltonics).

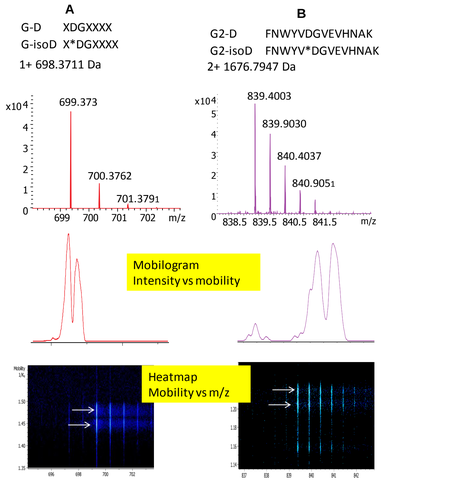

Fig. 2 Trapped Ion Mobility (tims) separation of a singly charged (A) and doubly charged (B) peptide pair containing Asp/isoAsp. The mobilogram shows good resolution of the two Asp/isoAsp peptide pairs and this is reflected in the heatmap of mobility vs m/z as well.

Results
Direct Infusion

- Direct infusion experiments on the timsTOF Pro QTOF demonstrated the ability to differentiate a single change between isoAsp and Asp pairs of peptides. Of the nine isobaric peptide mixtures, seven peptide pairs showed good separation and the remaining two were partially separated. (Fig. 2)
- Shorter peptides with up to 15 amino acids showed better resolution in the mobility dimension. This suggests that the impact on mobility of a single site change from Asp to isoAsp is reduced in longer peptides as compared to shorter peptides. (Fig. 3)
- In addition, we were able to elucidate primary sequence isoAsp peptide positions that mitigated the impact on the mobility. Peptides containing multiple aspartic acid residues changed to isoAsp at different positions were not distinguishable by trapped ion mobility. (Fig. 4)
- Of the seven peptide mixtures that showed good mobility separation, all peptides with Asp show a smaller mobility than peptides that contain isoAsp. This is likely due to the fact that isoAsp can generate a kink in the peptide backbone thereby introducing a larger gas phase conformation.

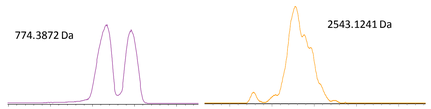

Fig. 3 The smaller peptide pair containing Asp/isoAsp on the left shows better mobility resolution as compared to the longer peptide pair to the right.

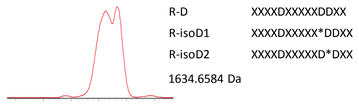

Fig. 4 Direct infusion of a mixture of 3 peptides with Asp/isoAsp forms and isoAsp at different positions. There is partial separation of the Asp/isoAsp peptide but the different positions of isoAsp are not distinguishable by mobility.

LCMS

- In combination with LC-MS, further resolution could be achieved with trapped ion mobility on some of the peptide mixtures that were not resolved in the mobility dimension using direct infusion experiments. This could be attributed to varying degrees of chromatographic separation which in turn contributed to better mobility resolution. (Fig. 5)
- LC-MS/MS experiments with PASEF scans using CID fragmentation were performed; however we did not obtain characteristic a, b or y fragment ions to be able to distinguish between the Asp and isoAsp containing peptides.

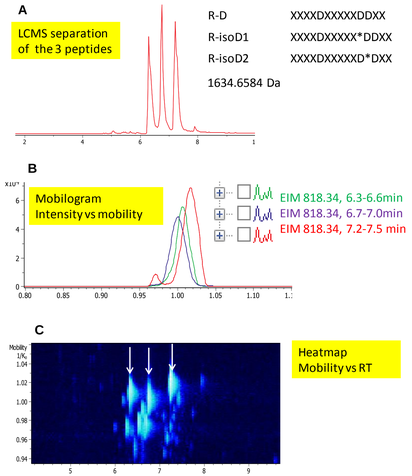

Fig. 5 LC-MS helped to achieve better resolution on the mixture of 3 peptides with Asp/isoAsp forms and isoAsp at different positions. **A** shows the chromatographic separation of the 3 peptides. **B** Extracted ion mobilograms are plotted for the 3 peptides and show partial mobility resolution of the 3 species. **C** The mobility resolution of the 3 peptides is shown in the heatmap of mobility vs RT.

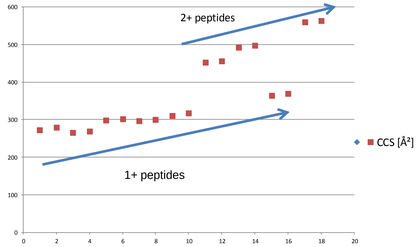

Fig. 6 Collisional Cross Section (CCS) values for the 9 peptide mixtures are plotted. There is a clear trend of increasing CCS values with increasing peptide lengths. Charge state of the peptide has an effect on the CCS value; doubly charged peptides are distinctly separated from the singly charged peptides.

Conclusions

- We demonstrate the capability of the timsTOF Pro to separate isobaric peptides mixtures containing asp/isoAsp residues..
- Utilizing a combination of direct infusion and PASEF (Parallel Accumulation Serial Fragmentation) LC-MS/MS experiments, we are able to separate upwards of 20 isobaric peptides species without the need of specialized and complex chromatography. of trapped ion mobility on the timsTOF Pro QTOF
- Collisional Cross Section values are unique and add a layer of confirmatory evidence for peptide identification
- Future experiments include quantitation of Asp vs isoAsp peptides in mixtures.

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