APPLICATION OF CYCLIC ION MOBILITY MASS SPECTROMETRY FOR HIGH PEAK CAPACITY SEPARATIONS OF NATIVE AND DEUTERATED PEPTIDE MIXTURES

Martin Palmer[1], Dale A. Cooper-Shepherd[1], Malcolm Anderson[1], James I. Langridge[1], Robert Tonge[1], John R. Engen[2]
[1]Waters Corporation, Wilmslow, Cheshire, UK, SK9 4AX; [2]Northeastern University, Boston, MA 02115

OVERVIEW

- **Enhanced peak capacity separations are enabled by the SELECT SERIES Cyclic IMS**
- **Infusion-only experiments are comparable to rapid LC-MS runs**
- **Multi-pass IMS mode allows separation of congested deuterated peptide spectra**
- **Critical amide deuteration is maintained during high mobility resolution experiments**
- **Gas phase back-exchange could reveal hidden structures of intact proteins**

INTRODUCTION

A major time bottleneck for the analysis of peptides originating from protein digests is the requirement for long shallow chromatographic separations. The introduction of hybrid ion mobility-TOF mass spectrometers has proven advantageous in retaining peak capacity when chromatographic times are shortened, due to the extra dimension of ion mobility (IM) separation. Advances in travelling-wave IM instrumentation have led to the development of cyclic IM (cIM) technology that provides high/ variable IM separation by increasing the number of passes, or cycles, before MS detection.

Here we investigated the utility of cIM for peptide separation under fast chromatographic conditions and by direct infusion. In addition, we monitored the retention of deuterium by exchanged peptides and proteins within the cIM device.

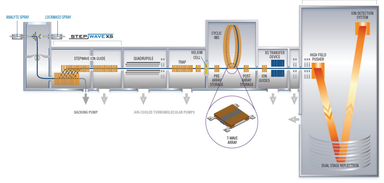

Figure 1. Schematic of the Cyclic IMS instrument

METHODS

An enolase tryptic digest (Waters part no. 186002325), peptide P1 (HHHHHHIIKIIK) and myoglobin (Sigma-Aldrich) were used as model systems in this study. Data were acquired in both infusion and LC-MS modes. Fully deuterated P1, enolase and myoglobin were prepared by reconstitution in 100% D_2O at pD 2 for at least 24 hours.

LC-MS analysis

Enolase digest (1 pmole) was injected onto an ACQUITY BEH 2.1x50 mm 1.7 µm C18 column at 45 °C. Mobile phases were water + 0.1 % formic acid (A) and acetonitrile + 0.1 % formic acid (B). The flow rate was set to 0.6 mL/min and the proportion of B was ramped from 1—90 % over 3 minutes.

Peptide infusion experiments

Enolase digest (1 pmol/µL) and P1 were administered using a 250 µL Hamilton syringe at 3 µL/min. For deuterated samples the syringe and solutions were kept cold before infusion.

Deuterated samples were either infused directly from acidified D_2O solution (0.2 % formic acid) to maintain sidechain deuteration or quenched to remove sidechain deuteriums by diluting 10 x in H_2O + 0.2 % formic acid.

Experiments were performed on a SELECT SERIES Cyclic IMS-enabled quadrupole time-of-flight (Q-cIM-ToF) mass spectrometer (Figure 1). The cIM device and array (Figure 2) provide a 98 cm circular path at 90˚ to the instrument axis. By controlling the array region ions can be sent on multiple passes around the device, 98 cm per pass.

Increasing numbers of passes between 1 and 10 were performed, resulting in a resolution range of ~65 to 200 (Ω/ΔΩ). Nitrogen was used as both the mobility buffer gas, and collision gas in the trap cell and transfer segmented quadrupole. Helium was employed in the cIM entrance cell.

Data were acquired using MassLynx v4.2 and processed using DriftScope software.

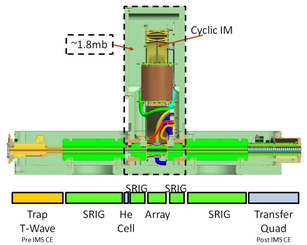

Figure 2. Schematic of the cyclic IMS region

RESULTS

The separating power of the Cyclic IMS was tested by subjecting an enolase tryptic digest to a rapid LC gradient (3 minutes) with the instrument mode in single pass HDMS^E. 34 peptides were identified giving a sequence coverage of 82%, which is typical of much longer LC runs with this sample (Figure 3). For comparison we acquired the same sample by infusion. Strikingly, all but one of the peptides from the LC experiment were detected in the infusion experiment with unique *m/z* and arrival time distributions.

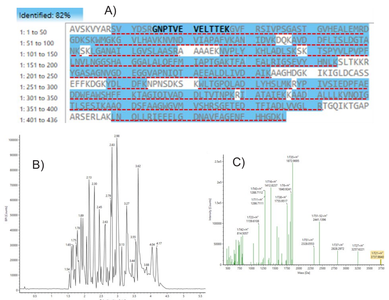

Figure 3. A) Coverage map of enolase showing 82 % coverage obtained from the LC-MS experiment. Blue highlighted regions indicate identified peptides from the LC-MS experiment, red underlined regions indicated peptides detected in the infusion-only experiment. B) BPI chromatogram of enolase tryptic digest on a rapid LC timescale. C) UNIFI mass scale component plot showing detected peptides from the LC-MS run.

The geometry of the cIM instrument provides the capability to increase the mobility resolution by increasing the number of passes around the device. Therefore for complex peptide mixtures and rapid LC timescales there is an opportunity to increase peak capacity. This is an important factor for HDX-MS experiments, where fast separations are required in order to minimise loss of labelling information through back-exchange processes.

Complex spectra are disentangled at high mobility resolution

To test the separating power of the Cyclic IMS we conducted an infusion-only experiment on an undeuterated and deuterated (quenched) enolase tryptic digest (Figure 4). To illustrate the separation we focussed on a congested region of the mass spectrum, around *m/z* 700.

When increasing the number of passes around the cIM from 1 to 5 we saw a concomitant increase in the number of features in the arrival time distribution for this *m/z* range (Figure 4A).

When the IM domain is collapsed the undeuterated spectrum of enolase digest is crowded and dominated by two isotope distributions (Figure 4B top). The spectral congestion is increased significantly, as expected, in the deuterated sample as the broadened isotope distributions now overlap.

By employing the mobility separation after 5 passes, we can fully disentangle 4 overlapping species (Figure 4) and interpret their extents of deuterium uptake for the purpose of protein dynamics studies.

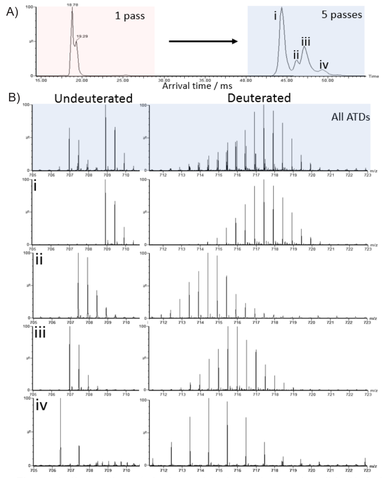

Figure 4. Separation of deuterated isotope clusters by cIM. A) Arrival time distributions of a group of similar m/z peptides after 1 pass (left) and 5 passes (right). B) Top: m/z spectra for all arrival times in the 5 passes data for undeuterated (left) and deuterated (right) enolase digest. i-iv) m/z spectra of arrival time segments from A (right) showing the mobility separation of highly overlapping isotope clusters after 5 passes around the cIM device.

Critical backbone amide NH deuteration is retained

The increase in gas phase residence time with increasing numbers of passes raises the question as to whether deuterium and valuable labelling information is maintained.

To test this we took the model peptide P1, which is commonly used to assess back-exchange and scrambling effects. Infusion-only experiments were performed on undeuterated, deuterated and deuterated/quenched P1, and extent of uptake at 1 and 10 Cyclic IMS passes recorded.

The undeuterated sample gives a simple, natural isotope distribution at *m/z* 775, as expected (Figure 5 A).

The deuterated sample sprayed from D_2O-containing solution showed a broadened isotope distribution centred on *m/z* 788.7 after 1 pass (~15 msec separation). After 10 passes (~150 msec separation) we observed a distribution centred on *m/z* 787.7 *m/z* indicating that some gas phase back exchange has occurred (Figure 5 B-C). This is unsurprising, as when analysing from a D_2O-containing solution labile sidechain deuteriums will be available for facile gas phase back-exchange with OH^- and H_2O in the surrounding buffer gas.

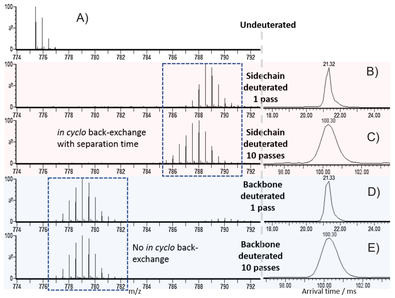

Figure 5. Amide deuteration is maintained. A) Mass spectrum of undeuterated P1. B) Mass and 1 pass arrival time data for P1 electrosprayed from D_2O solution. C) Mass and 10 pass arrival time data for P1 from D_2O solution. Back-exchange is observed and attributed to sidechains. D) Mass and 1 pass arrival time data for deuterated P1 electrosprayed from a H_2O quench solution. E) Mass and 10 pass arrival time data for P1 from H_2O quench solution. No back-exchange is observed.

We conducted the same experiment after diluting the deuterated P1 peptide 10 x into H_2O-containing solution, therefore forcing the loss of the labile sidechain deuteriums prior to ionisation (this approach is more representative of an HDX-MS experiment). We then observed an isotope distribution centred on *m/z* 779 after 1 pass. After 10 passes we observed no change in the centroid position, confirming that no amide back-exchange was occurring as a function of time spent *in cyclo*, (Figure 5 D-E).

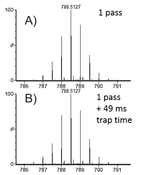

Figure 6. Sidechain back-exchange occurs in the cIM device. A) The isotope distribution of the P1 peptide after electrospraying from D_2O solution analogous to that in Figure 5B. The peptide has undergone a single cIM pass. B) The isotope distribution of the P1 peptide after a single pass and an additional 49 ms trapping time. The similarity of the two spectra indicates that the sidechain back-exchange is not occurring in the trap cell.

To determine the origin of the sidechain back-exchange we conducted an experiment in which the trapping time was varied independently of the cIM separation time. We found that there was no difference in isotope centroid position between a single cIM pass and a single pass with an additional 50 ms of trapping time (Figure 6.). This result supports the theory that the sidechain back-exchange is occurring in the Cyclic IMS device and not in the trap (Figure 2). This feature of the instrument might be exploited to conduct gas phase ion-neutral chemistries.

Revealing hidden conformations of intact proteins?

ESI of intact myoglobin (Figure 7) from a D_2O-containing solution showed the same trend as for the P1 peptide; extent of back-exchange depends on the IMS separation time.

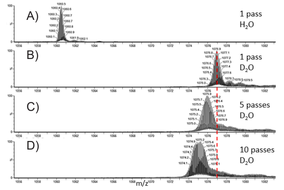

Figure 7. Sidechain back-exchange on myoglobin.

A) 16+ charge state from H_2O solution. B-D) 16+ charge state in D_2O with a varying number of passes as indicated in the figure.

Sidechain back-exchange is greatest after ten passes, as expected. The arrival time distribution (ATD) for the ten pass data exhibited more than five conformational families, with only one observed after a single pass (not shown).

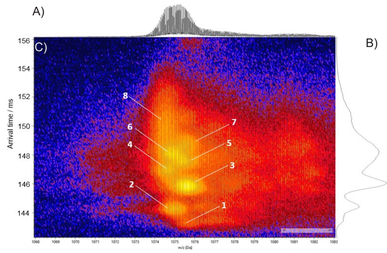

Figure 8. Revealing hidden protein conformations. A) Myoglobin from D_2O after ten passes. B) ATD after ten passes showing at least 5 conformations. C) Viewing arrival time and m/z as a 2D plot reveals further conformations with different deuterium uptakes and ATDs.

Interestingly, deuterium back-exchange reveals additional conformations for the myoglobin 16+ charge state, bringing the total to eight (Figure 8). The deuterium uptake of these species is not directly correlated to arrival time, thus reporting on different gas phase structures with different back-exchanging residues exposed.

CONCLUSION

- **Cyclic IMS in single pass mode aids rapid peptide separations**
- **Multi-pass mode provides higher ion mobility resolution further separating complex MS data**
- **Critical backbone amide deuteriums are retained *in cyclo*, whereas fast-exchanging labile sidechain deuteriums may back-exchange**
- **Gas phase back-exchange combined with cIM can reveal hidden protein conformations**

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