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SEPARATION AND DETECTION OF PROTEIN POST-TRANSLATIONAL MODIFICATIONS BY LIQUID CHROMATOGRAPHY COUPLED WITH A NOVEL ION MOBILITY MASS SPECTROMETER

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OVERVIEW

Here we present the combination of IMS and oa-TOF mass spectrometry for the analysis of post translational modifications

Separation of differentially phosphorylated peptides, based upon ion mobility, has been demonstrated

Fragmentation before and after the IMS separation stage is demonstrated, providing additional specificity

INTRODUCTION

Post-translational modification (PTM) of proteins plays a fundamental role in cellular processes and their determination is one of the main goals of modern proteomics research. Among more than 200 known PTM's, phosphorylation, glycosylation and acetylation are the best characterized. However, the variety, diversity and heterogeneity of PTM's on proteins calls for novel analytical tools for qualitative and quantitative assessment of the structural and functional roles of these modifications.

For this purpose we have investigated the potential of a novel travelling wave ion mobility spectrometer for the separation, detection and mass determination of post-translationally modified proteins.

METHODS

IMS Enabled Mass Spectrometer The instrument used in these studies was an experimental hybrid Quadrupole/ TWIMS/oa-Tof mass spectrometer, **Figure 1**. Ions produced by an ESI source are sampled by a Z-Spray source and pass through a quadrupole that may be set to transmit a particular m/z or pass a substantial mass range. The ions then enter a novel three stage TWIMS device [1]. The first device (accumulation T-Wave) accumulates ions and releases them in a short pulse (200µs) into the next device (IMS T-Wave) in which the mobility separation is performed. The final device (transport T-Wave) is used to transport the separated ions into the oa-ToF for subsequent analysis. Fragmentation of ions may occur on entrance to the accumulation T-Wave and/or the transport T-Wave. The pressure in the accumulation and transport T-Wave regions was ~10^{-2} mbar of Ar and the pressure in the IMS-T-Wave was 0.5 mbar of N_2. In High Duty Cycle mode, the pusher is synchronised to the release of ions from the transport T-Wave device with a delay that is dependent upon the mobility separation such that the ion of interest is within the oa-extraction region when the oa-field is applied. This consequently leads to a signal increase over the entire mass range for a selected charge state.

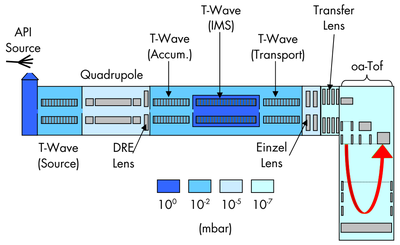


Figure 1. Schematic diagram of experimental instrumentation incorporating IMS and TOF MS.

Nanoscale Liquid Chromatography- mass spectrometry

Waters nanoACQUITY UPLC operated in trapping mode.

Trap column	180µm ID x 20mm long, Symmetry C18
Analytical col.	75µm ID x 100mm long, Atlantis dC18
Solvent A	Aqueous 0.1% formic acid
Solvent B	Acetonitrile + 0.1% formic acid
Injection mode	1µL Partial Loop.
Trapping	100% solvent A at 5µL/min for 5 mins.
Gradient	1—40% B in 30 minutes at 300nL/min.

A capillary voltage of 3.95kV was applied to the nanoESI probe with a nebulising gas flow of approximately 5psi. The source temperature was set to 70C.

Purification of phosphorylated peptides using TiO2 microcolumns

TiO2 microcolumns were packed in GELoader tips essentially as described previously [2]. Peptides originating from proteolytic digestion of the peptide mixture were diluted 1:5 in loading buffer (2,5-Dihydroxybenzoic acid (DHB) (300 mg/mL) in 80% acetonitrile/2% TFA) and loaded onto the TiO2 microcolumn. The column was washed with 10 µL loading buffer followed by 20-40µL 80% ACN/1% TFA.
Phosphorylated peptides were eluted using 20 µL NH_4OH, pH 10.5. The eluate was acidified and purified using Poros R3 reversed phase microcolumns. Phosphorylated peptides were eluted off the R3 column using 70 % ACN/0.1% TFA and lyophilized prior to LC-MS.
Samples were solubilised and then 25mM EDTA, 50mM Amm Phosphate added prior to analysis by LC-MS.
Phosphopeptide standards (Waters, Milford) were used for the infusion experiments.

Mass Spectrometer Acquisition Parameters

The mass spectrometer was set to acquire data from m/z 350-1600 in 1.5 seconds with an 0.1 second inter-scan delay.

High Duty Cycle Mode Calibration

Calibration of the relationship between the pusher and transport T-Wave was achieved by infusion of a tryptic digest of Yeast Enolase (Waters Corporation), and the centre of the drift time window for doubly charged species over the mass range recorded.

RESULTS

Separation of phosphorylated species by IMS

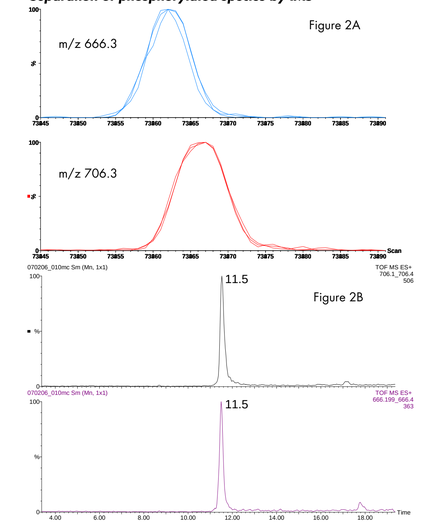


Figure 2. A) Multiple drift time profiles overlaid for the IMS separation of the ion at m/z 706.3 corresponding to the di-phosphorylated peptide EQLSTSEENSK and the ion at m/z 666.3 corresponding to the mono-phosphopeptide species; B) The two phosphopeptides co-elute by HPLC as shown in the selected ion mass chromatograms.

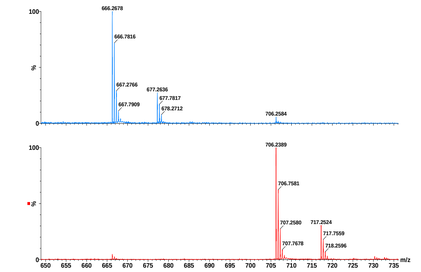
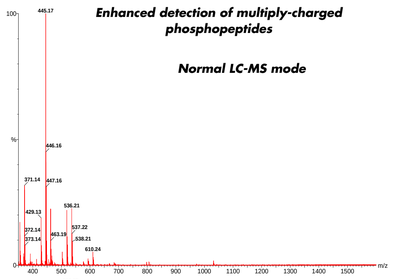


Figure 3. Mass spectra acquired at a retention time of 11.5 minutes, but at different IMS drift times; 3.3-3.6msec and 4-4.4msec respectively. Separate mass spectra showing the di-phosphorylated peptide and mono-phosphorylated peptide can easily be obtained.

Enhanced detection of multiply-charged phosphopeptides

Normal LC-MS mode



LC-IMS MS mode (multiply charged region selected)

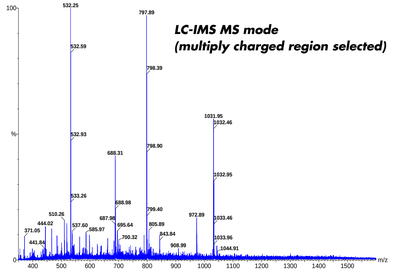


Figure 5. Mass spectrum acquired in the normal LC-MS mode of operation (top) compared to the LC-IMS MS mode of operation (bottom), at an LC retention time of 18.4 minutes. The bottom spectrum is produced by selecting only the portion of the data where multiply charged ions are present. This removes singly charged species, improving the signal-to-noise ratio and allowing the detection of peptides that may have been initially obscured by background ions.

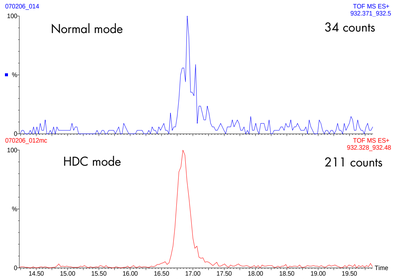


Figure 6. Mass chromatograms for m/z 932.4 acquired in the high duty cycle (HDC) mode of operation (bottom) compared to the spectrum acquired in the Tof MS mode of operation, top.

Phosphopeptide MS-IMS-MS experiments

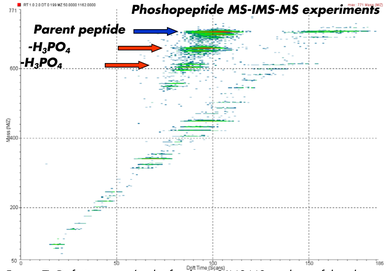


Figure 7. Drift time vs m/z plot for the MS-IMS-MS analysis of the phosphopeptide VNQIGTLSESIK; m/z 724.8 (2+). In this experiment the ion was first mass selected with the quadrupole, limited fragmentation induced prior to IMS separation and followed by mass analysis with the oa-TOF. The sequential neutral loss of H_3PO_4 can be clearly observed, separated from the intact peptide both by m/z and drift time.

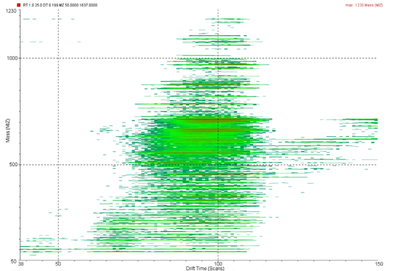


Figure 8. Drift time vs m/z plot from the MS-IMS-MS analysis of the phosphopeptide m/z 724 (2+). Similar to Figure 7 except that fragmentation was induced both pre and post IMS separation. In this case separate fragmentation spectra can be acquired for the parent phosphopeptide and neutral loss species in parallel.

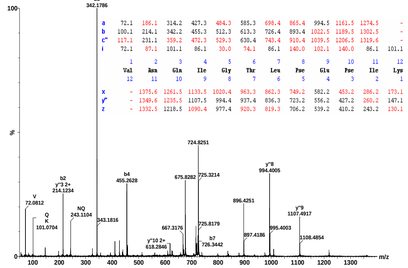


Figure 9: Fragment ion spectrum from the MS-IMS-MS experiment in Figure 8.

Tyrosine phosphorylation

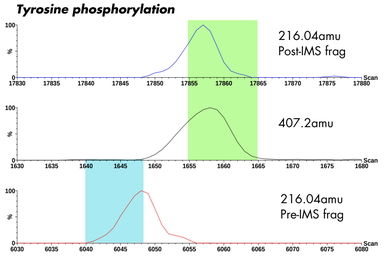


Figure 11. IMS drift time separation of the intact phosphopeptide NVPLYK and comparison to the IMS drift time of the fragment ion m/z 216.04 produced before & after IMS separation. The associated drift time of the peptide and immonium ion is identical when fragmentation occurs post IMS, whereas a difference in drift time can be observed when the fragmentation occurs prior to IMS separation.

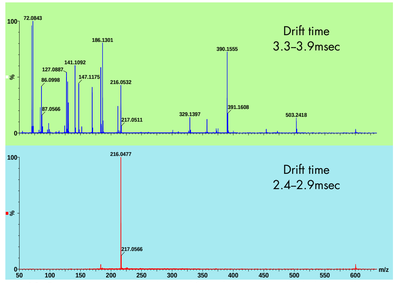


Figure 12. Fragment ion spectra obtained from the separate shaded areas shown in Figure 11, for the tyrosine phosphopeptide. A) Fragmentation occurring post IMS where a full fragment ion spectrum of the precursor is obtained and B) Fragmentation performed prior to IMS separation. Here, fragments are separated based upon their mobility and the increased specificity that IMS separation provides can be seen.

CONCLUSION

- **The use of ion mobility combined with oa-TOF mass spectrometry for the analysis of phophopeptides has been presented.**
- **Separation of mono and di-phosphorylated peptides using IMS has been shown.**
- **The high duty cycle (HDC) mode of operation provides improved sensitivity for the detection of modified peptides.**
- **The use of MS-IMS-MS allows fragmentation patterns to be obtained from both the intact phosphopeptide and peptide neutral loss species in parallel.**
- **The coupling of IMS and TOF MS shows great potential for the analysis of complex phosphopeptide mixtures**

References

[1] "Travelling Wave Ion Propulsion in Collision Cells" K. Giles, S.Pringle, K. Worthington and R. Bateman— Presented at the 51st ASMS Conference, Montreal, Canada 2003. The travelling wave device described here is similar to that described by Kirchner in US Patent 5,206,506 (1993).

[2] Larsen MR et al *Mol Cell Proteomics* 2005, 4(7), 873-86

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