How to reduce sample complexity for MALDI analysis: Optimization of off-line LC MALDI analysis.

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Overview

- Here we describe the coupling of nanoACQUITY UPLC™ system to MALDI micro MX™ and MALDI Q-Tof™ instruments
- Evaluation of the system sensitivity and resolving power was performed
- The glycosylated recombinant protein Erythropoietin (EPO) was tryptically digested and analysed as an unseparated mixture and subsequently by LC-MALDI.

Introduction

Liquid chromatography coupled to mass spectrometry (MS) is a widely used approach for analyzing complex protein mixtures as it reduces the complexity of the mixture prior to the MS analysis.

Electrospray ionization is the preferred MS technique as it allows comprehensive on-line LC-MS/MS to be performed. However, there has been a growing interest in the use of MALDI ionization for analyzing complex tryptic digest mixtures that have been separated by off-line HPLC. This approach provides complementary information to that obtained by ESI, while deposition onto a MALDI target allows samples to be archived for further analysis.
In this work LC-MALDI has been performed using a nanoACQUITY UPLC system, a MALDI spotting device and MALDI analysis using both a conventional axial MALDI TOF, and a MALDI Q-Tof instrument. The advantages of the nanoACQUITY UPLC system stem from the ability to operate at higher pressures, allowing columns with smaller particle sizes (1.7µM) to be used resulting in either higher peak capacity separations, or reduced run times. Using a MALDI spotting device we have shown that we can couple this enhanced chromatographic platform to MALDI, with little additional chromatographic peak broadening observed compared to the on-line approach. As the nanoACQUITY is a direct flow LC system, providing flows down to 200 nL/min in gradient mode, the addition of matrix solution was greatly simplified. In a nanoLC system using a split-flow to get to nanolitre/min flow rates, the additional backpressure produced by mixing matrix solution and eluent has an adverse effect on the quality of the chromatography as the split ratio can vary.

Here we investigate the sensitivity of the analytical system using a serial dilution of a tryptic digest from alcohol dehydrogenase (ADH). Performance with a more complex mixture was evaluated, using a mixture of four digested proteins. Finally we show the application of this technique in the analysis of the recombinant glycosylated protein, Erythropoietin (EPO).

Methods

Sample Preparation

- *ADH protein digest*:
A dilution series of the commercially available ADH (www.waters.com) was carried out in aqueous 0.1% TFA. The concentration range tested was from 5fmol to 200fmol loaded on-column.

- *Four protein mixture:*
Bovine serum albumin (BSA), Enolase, ADH and Phosphorylase B were reduced, alkylated and then tryptically digested. The four individual protein digests were mixed in equimolar amounts, with 50 fmol of the mixture injected on column.

- *Erythropoietin:*
Recombinant human erythropoietin (EPO) was treated with PNGase F to remove the N-linked glycans, leaving only O-linked glycans. Peptides were produced by digestion with trypsin. The sample was diluted in aqueous 0.1% TFA to 500 fmol/µL.

Mass Spectrometry

- *MALDI micro MX™*
Acquisition and operation of the MALDI micro MX were as previously described [1]. In summary positive ion reflectron mode was used with 200 shots acquired per spot. The instrument was calibrated using a multi-point poly(ethylene glycol) calibration over the m/z range 800-3000.

- *MALDI Q-Tof Premier™*
Acquisition in positive ion mode, in V-mode. Instrument multi-point externally calibrated using poly(ethylene glycol) over the m/z range 50-3000.

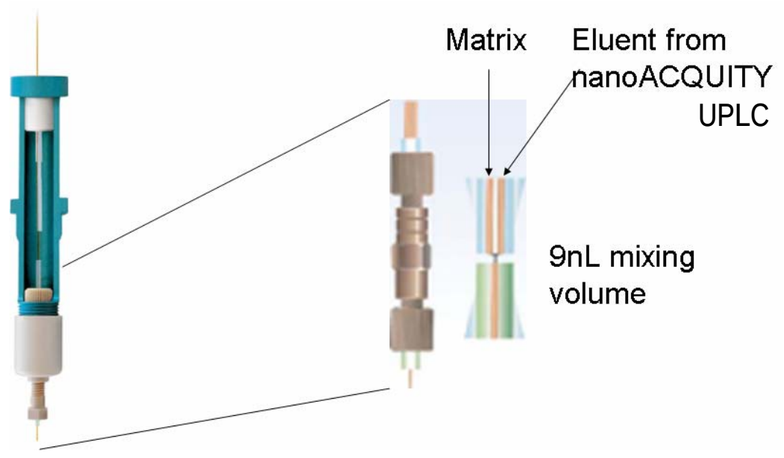

Figure 1: Close-up of the spotting tool that is on the CTC MALDI spotting device.

The chromatographic separation system was attached to a MALDI modified autosampler (CTC, Basel, CH).

A gradient over 45 min (5 to 40% MeCN) was used on a 75 µm x100mm column,1.7 µm BEH C18, (Waters, Milford, MA) at a flow rate of 300 nL/min. Eluent fractions were spotted directly onto a MALDI target using the MALDI spotter. Matrix solution and eluent were mixed prior to spotting as shown in Figure 1. The backpressure generated by the addition of the matrix flow at 1.7 µL/min did affect the chromatographic performance of the system. Post column dead-volumes were kept to a minimum using a short transfer line (ca. 0.3 m 25 µm i.d. fused silica capillary) from the column exit to the spotting device. Deposition times between 10 and 45 seconds per spot were used.

Figure 2: nano ACQUITY UPLC linked to the MALDI spotting device in the laboratory.

Results

Sensitivity

The ADH tryptic digest was injected onto the column at loadings of 5 fmol to 250 fmol. The eluent deposition was optimised to 30 seconds per spot to have the maximum sensitivity and separation of components.

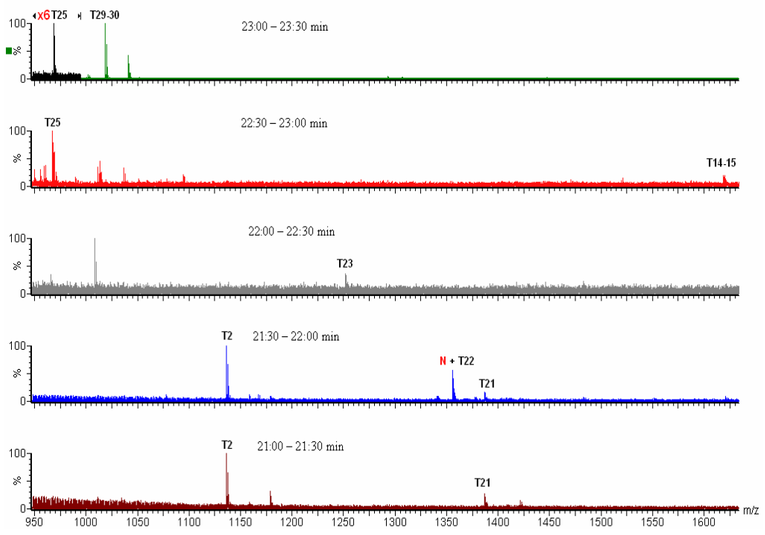

Figure 3: MS spectra from 5 spots of the ADH digest analyzed at the 5 fmol level on column (retention time 21:00 – 23:30 min).

It is clear from this data that at the 5 fmol level, ADH peptides were detected with good signal to noise.

Separation efficiency

To test the separation efficiency of the system a more complex sample was used. A mixture of Bovine serum albumin (BSA), Enolase, ADH and Phosphorylase B was injected on column at 50 fmol and analyzed as detailed above.
For comparison the same solution was analyzed under the same chromatographic conditions by LC-ESI-MS. In the ESI experiment chromatographic resolution of *ca* 20 seconds at base was observed for BSA peptide T6 (1163.63 Da).

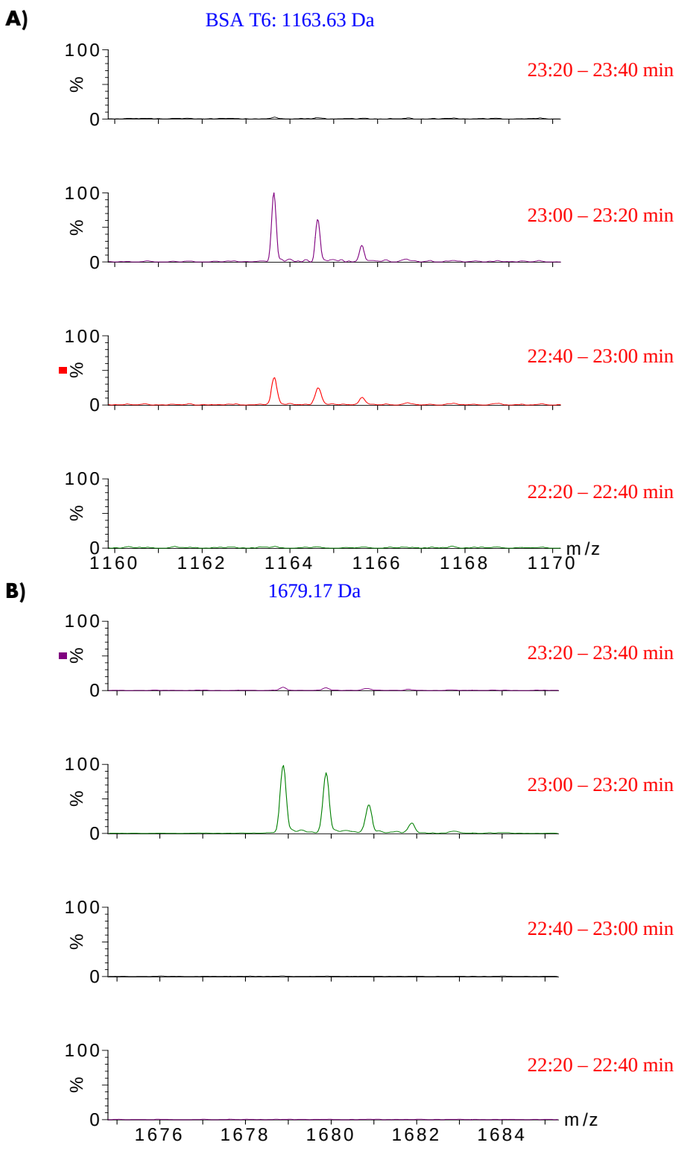

Figure 4: A small time region from the separation of the 4 protein digests mixture, showing adjacent MALDI spots.

Figure 4a, shows that the peptide at m/z 1163.63 is observed in two consecutive two spots, between retention time 22:40 min to 23:20 min. Figure 4b, shows a peptide of m/z 1679.19 was detected in just one spot. It is not possible to determine exactly what the resolving power of the combined MALDI spotter/ nanoACQUITY UPLC set-up is, as the resolution of the time axis is limited by the deposition time. From the data we acquired, it can be seen that it is less than or equal to 20 seconds at base, as it was possible to capture all of one peptide in one 20 second fraction. It is always possible that the signal from a particular peptide is split between two fractions.
The estimated resolution of the LC MALDI spotting experiment is consistent with the resolution observed in the on-line ESI MS experiment. This indicates that little or no loss of chromatographic resolution is observed when adding the MALDI spotting step.

Recombinant EPO sample

Recombinant human erythropoietin (EPO) was analyzed with and without chromatographic separation using mass spectrometry.
This protein contained only O-linked glycans as the N-linked glycans were removed prior to analysis using PNGase F.
There were two expected O-linked glycopeptides present in the peptide mixture. Figure 5 shows the sequence and the glycan structures of the two O-linked glycosylated tryptic peptides, both occurring at the same site on the amino acid backbone. The upper structure has a monoisotopic mass 2120.9849 Da. The lower structure has a monoisotopic mass 2412.0803 Da.

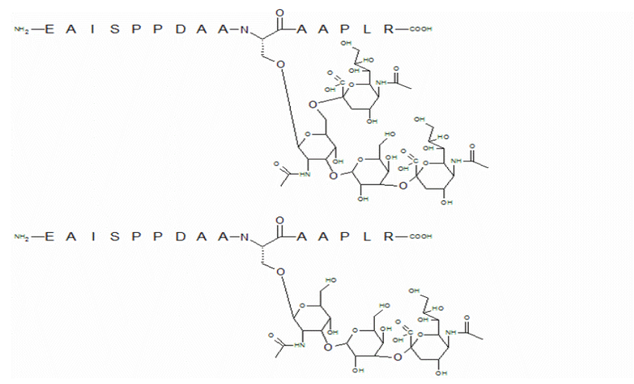

Figure 5: Sequence and glycan structure of the two O-linked glycopeptides, present in the EPO protein digest.

MALDI MS analysis without any prior separation was performed on1 µL of the EPO digest.
This sample produced a MALDI MS spectrum (top spectrum Figure 6) of EPO containing 29 peaks from which MS/MS spectra could be obtained. In the first experiment, the two O-linked glycopeptides were not observed.
In a second experiment a 1 µL aliquot was chromatographically separated prior to MALDI MS and MS/MS analysis. The LC separation produced 49 spots containing EPO peptides.
A reconstituted de-isotoped MS spectrum (bottom spectrum Figure 6) was produced from the 49 separate MS spectra. This contained 91 peaks from which MS/MS data could be obtained In this case the two O-linked glycopeptides were observed and the peptides interrogated by MALDI MS/MS.

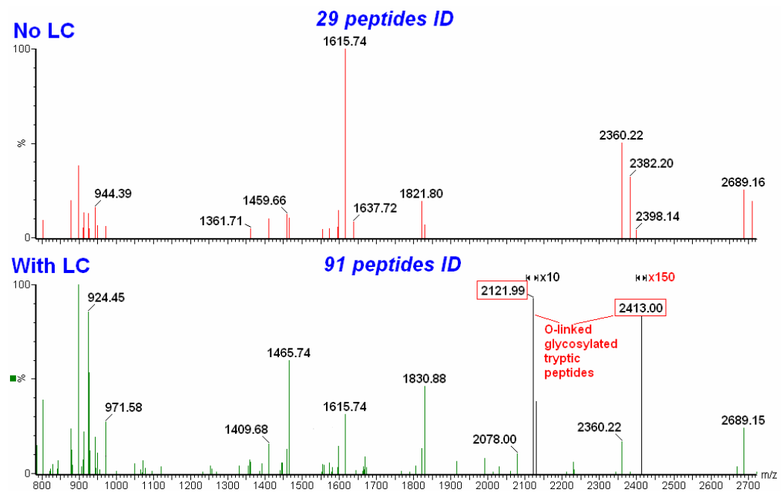

Figure 6: MS spectra of EPO protein digest. The top spectrum shows the de-isotoped spectrum after direct analysis (no separation). The bottom spectrum is a composite of the 49 MS spectra produced by the LC-MALDI method.

This example highlights the advantage that an LC separation brings to a MALDI experiment, in that the overall resolving power of the analytical system is greatly improved. As the peptides of interest are distributed over numerous spots suppression of lower intensity peaks is minimized, as is chemical noise. This effect is especially important in the case of post translationally modified peptides, such as glycopeptides or phosphopeptides, which exhibit lower ionization efficiency than their unmodified counterparts.

Figure 7 shows the MS/MS spectra of the two O-linked glycopeptides from the LC separated EPO digest. The fragmentation of the glycan can clearly be seen in both cases, as well as several peptide related fragment ions.

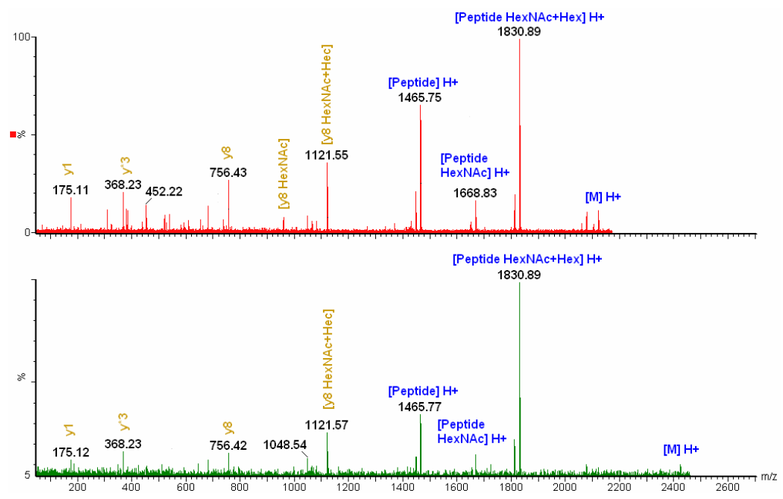

Figure 7: MS/MS spectra of the two O-linked glycopeptides from EPO. The top spectrum shows the MS/MS spectrum of the peak at m/z 2120.98 Da, whilst the bottom spectrum shows the MS/MS spectrum of the m/z 2412.08 glycopeptide.

Conclusions

- A new combination of nanoACQUITY UPLC with MALDI spotting and MALDI analysis (axial MALDI TOF and MALDI Q-TOF) was evaluated.
- Sensitivity: 5 fmol of ADH digest on column was successfully separated and analysed.
- Chromatographic resolution: Similar chromatographic resolution *vs* on-line LC-MS was observed, (*ca* 20 sec peak width at base).
- LC-MALDI identified 62 additional peptides from an EPO tryptic digest.
- Two O-linked glycopeptides from two the different glycoforms of EPO were analyzed by LC-MALDI MS/MS.

Acknowledgements

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[1]: E. Claude, M. Snel, T. Franz, A. Bathke, T. MckKenna, J. Langridge; *Parallel post source decay versus peptide mass fingerprinting—Enhanced protein identification on a MALDI-TOF-MS*, ASMS poster 2005.

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