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IMPROVED CHROMATOGRAPHIC AND MASS SPECTROMETRIC PROPERTIES OF SMALL PHOSPHOPEPTIDES BY N-TERMINAL DERIVATISATION

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OVERVIEW

- This poster details a preliminary evaluation of N-terminal derivatization, using TMPP-Ac-OSu, for the enhanced analysis of phosphopeptides
- We have studied the effect of TMPP derivatization on the HPLC performance for small hydrophilic phosphopeptides
- This approach has been coupled with nanoscale HPLC on-line to an ESI Q-Tof Premier
- In addition, off-line HPLC coupled with MS/MS on a MALDI Q-Tof mass spectrometer and PSD MX on a MALDI micro MX mass spectrometerhas been used

INTRODUCTION

Protein phosphorylation is one of the most common post translational modifications and is especially important in a number of key biological processes, such as intracellular signaling.
Numerous strategies for studying phosphorylation have been described, with several incorporating trypsin digestion with subsequent analysis by LC-MS and MS/MS. However, analysis of small hydrophilic phosphopeptides by LC-MS/MS techniques is particularly challenging due to their poor retention characteristics on reverse phase media. During the HPLC experiment they are often 'lost', either by not binding to the trapping column during the loading step often employed in nano LC or by eluting in the void volume (direct loading LC experiment). In addition the analysis of these species by either ESI or MALDI MS is complicated by their low molecular weight.
In this work we describe the N-terminal derivatization of small phosphopetides using N-Tris (2,4,6-trimethoxyphenyl) phosphonium-acetic acid N-hydroxysuccinimide ester (TMPP-ac-OSu). This derivatization improves the retention on reverse phase material, increases the mass of the peptide by 572 Da and enhances the fragmentation in MS/MS mode for singly charged peptides [1,2].

METHODS

Sample preparation

Four synthetic phosphopeptides (Southampton University) were used. These had the following sequences: -

-**NDRSpE**	(MW 699.2225 Da)
-**DSpT**	(MW 401.0835 Da)
-**GHNSpLK**	(MW 734.3113 Da)
-**SNEDYpR**	(MW 862.2858 Da)

- Phosphopeptides were solubilized and subsequently diluted to 100 pmol/µL in 0.5M 4-Methylmorpholine buffer / 80% acetonitrile (MeCN).
- TMPP derivatization solution (Waters, Milford, MA) was diluted in acetonitrile and added to phosphopeptide solutions at a molar ratio of 33:1.
- Samples were incubated at room temperature for 30 minutes followed by a 100 fold dilution with 25% Acetonitrile/0.1% trifluoroacetic acid (TFA) solution.
- The matrix used for MALDI experiments was alpha-cyano-4-hydroxycinnamic acid matrix (CHCA) (Waters, Milford, MA), 2.5 mg/mL (1/1 v/v MeCN/0.1 % aqueous TFA).

LC Conditions

LC conditions used in the nano electrospray and LC-MALDI experiments are summarized in ***Table 1***.

	Nano Scale LC ESI MS	Nano Scale LC MALDI MS
Pump	Waters® CapLC™	Waters CapLC
Sample Loading	125 fmol of each phosphopeptide	200 fmol of each phosphopeptide
Column	NanoEase™ Atlantis® dc18 (100 mm x 75 mm)	NanoEase Atlantis dc18 (150 mm x 75 mm)
Gradient	5 % → 85 % MeCN over 45 min	5 % → 85 % MeCN over 45 min
Flowrate	200 nL/min	200 nL/min
Mobile Phase	A: MeCN B: 1 % formic acid	A: MeCN B: 0.1 % TFA
MALDI spotter	N/A	2700 MS
Spotting time	N/A	30 sec per spot
Matrix addition	N/A	2.5 mg/mL CHCA added at 1.8mL/min

Table 1. *LC conditions*

MS Conditions

LC Q-Tof-MS

- Samples were analyzed on a Q-Tof™ Premier (Waters, Manchester, UK) using nanoscale LC-MS (see Table 1).
- All data were acquired in continuum mode over the m/z range 50-1990 in W-Optics™ mode.
- Instrument resolution was better than 17,500 FWHM and typical instrument source conditions were as follows: Capillary Voltage 4.25 kV, Cone Voltage 28 V, Extraction Cone Voltage 2 V, Source Pressure 1.65 mbar.

MALDI Q-Tof MS and MS/MS

- Data were acquired in positive ion mode on a MALDI Q-Tof (Waters, Manchester, UK).
- Precursor ions were selected using a quadrupole and fragmented by collision induced dissociation (CID) to provide MS/MS data.
- Polyethylene Glycol (PEG) was used for an external multi-point calibration.

Axial MALDI Tof MS and PSD MX

- Both MS and parallel PSD (PSD MX™) data were acquired on a MALDI micro MX™ (Waters, Manchester, UK).
- Data were acquired in positive ion mode using automated software control.
- In MS mode, a tryptic digest of alcohol dehydrogenase was used to generate a multi-point external calibration.
- In PSD MX mode, PSD data were acquired in parallel[3] and calibrated using PSD fragments generated from $P_{14}R$ (Sigma, St Louis, MO).
- Six PSD segments, at two reflectron voltages each were automatically acquired per sample.

RESULTS

Evaluation of phosphopeptide trapping & separation using HPLC

- Both unmodified and TMPP modified phosphopeptides were analyzed by LC-ESI-MS. The unmodified species were not bound to the trapping column and therefore were not detected by the mass spectrometer. However, in the case of the TMPP modified sample, peptides were retained on the reverse phase trapping column and subsequently separated on the reverse phase column. Chromatograms are shown in ***Figure 1***.
- It can be seen from these data that **TMPP-NDRSpE** and **TMPP-SNEDYpR** have co-eluted at a chromatographic retention time of 29.72 minutes. **TMPP-GHNSpLK** is resolved from the other two phosphopeptides and elutes at 30.63 minutes.
- **TMPP-DSpT** was not well resolved under the HPLC conditions used and gave a relatively broad chromatographic peak at approximately 30 minutes.

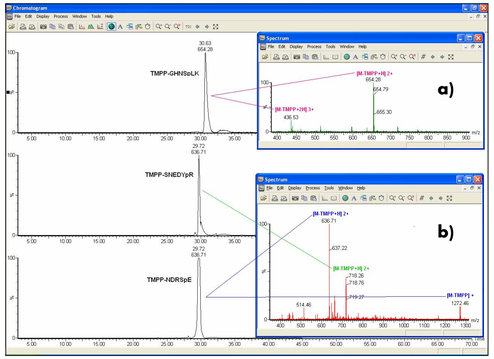

Figure 1. *Reconstructed mass chromatograms of **TMPP-GHNSpLK**, **TMPP-SNEDYpR** and **TMPP-NDRSpE**. a) MS spectrum at 30.63 mins. b) MS spectrum at 29.72 mins.*

- Modified phosphopeptides were also analyzed using off-line HPLC coupled to MALDI-MS and MALDI-MS/MS. Figure 2 shows the MS spectra recorded from fractions collected at different retention times, all four TMPP modified phosphopeptides were detected.
- The order of elution is slightly different between the LC-ESI and LC-MALDI experiments, although the retention time differences between the various phosphopeptides was similar in both experiments.
- One of the reasons for the differences in retention times between the on-line LC-ESI and the off-line LC-MALDI experiments is that a different chromatographic set-up was used in each case (*cf.* ***Table 1***)

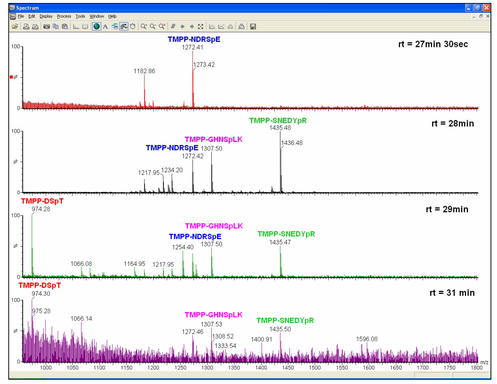

Figure 2. *The MALDI MS spectra recorded from fractions collected at different retention times, all four TMPP modified phosphopeptides were detected.*

Effect of TMPP derivatization on MALDI MS/MS

To study the effect of TMPP derivatization on MALDI MS/MS fragmentation patterns, the derivatized peptides **TMPP-NDRSpE** and **TMPP-GHNSpLK** were analyzed by CID (MALDI Q-Tof) and PSD MX. The fragmentation data for these peptides are shown in ***Figures 3*** and ***4*** respectively.

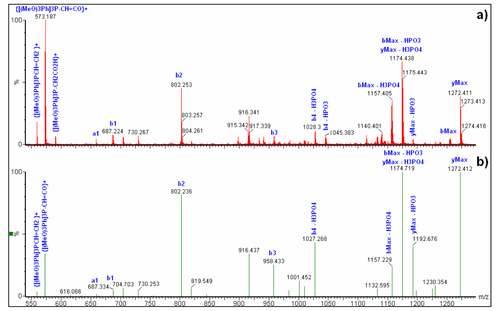

Figure 3. *MS/MS spectra of **TMPP-NDRSpE** a) MALDI Q-Tof b) MALDI PSD-MX*

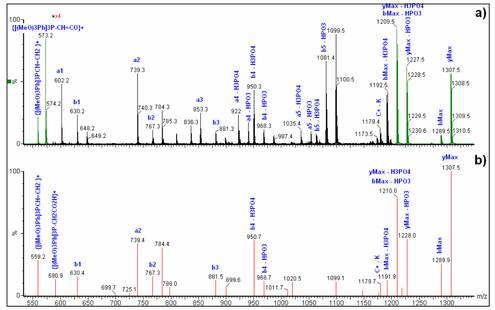

Figure 4. *MS/MS spectra of **TMPP-GHNSpLK** a) MALDI Q-Tof b) MALDI PSD-MX*

- The fragment ions observed from peptides with N-terminal TMPP derivatization are exclusively $*a_n$ or $*b_n$ type ions. The fragmentation is directed to these ions, due to the charge localization at the N-terminus, by the TMPP group.
- In the MS/MS spectra of TMPP modified peptides, peptide fragment ions are observed between 573.1898 Da and the precursor ion mass. The 573.1898 Da ion is the M^+ of the TMPP tag.
- As internal rearrangement is minimized, chemical noise in product ion spectra of TMPP-derivatized peptides is extremely low[2].

CONCLUSIONS

- The derivatization of peptides and phoshopeptides using TMPP-Ac-OSu modification is a quick and simple reaction
- Low molecular weight hydrophilic phosphopeptides did not bind to reverse phase trapping columns, which precluded their analysis by nanoscale LC-MS
- Derivatization of phosphopeptides with TMPP greatly improved the retention of these small hydrophilic peptides on trapping columns, and thus enabled nanoscale LC-MS studies
- Analysis and interpretation of MALDI MS/MS spectra from singly charged ions was improved with the TMPP modified peptides
- Future studies will look at enhancing the sensitivity of this analytical strategy and the analysis of endogenous phospho-peptides from biological sources

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