Analysis of Phosphopeptides using the cHiPLC® System

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Phosphorylation is a ubiquitous protein post-translational modification that plays a critical role in signal transduction and cell to cell communication. It is a reversible process controlled by kinases and phosphatases and dis-regulation of phosphorylation is strongly related to several human diseases. This makes it important to understand phosphorylation at a systemic level.

NanoLC-MS based phosphoproteomics is a powerful tool for the study of post-translational modifications as it provides sensitive peptide analysis, which is essential to detect low expression levels of phosphopeptides and phosphoproteins. Eksigent's cHiPLC® system is a chip-based platform that, in combination with the Eksigent NanoLC-Ultra® system, delivers superior sensitivity, column-to-column reproducibility, and exceptional ease of operation[1]. The cHiPLC® system is a "docking station" for up to three microfluidic chips. The system's flexible design and built-in 10-port nano valve allow for easy switching between different types of experiments such as direct injection and trap-loading.

In this application note, we describe use of the cHiPLC® platform to analyze standard phosphopeptides as well as investigate the mouse endothelial cell phosphoproteome.

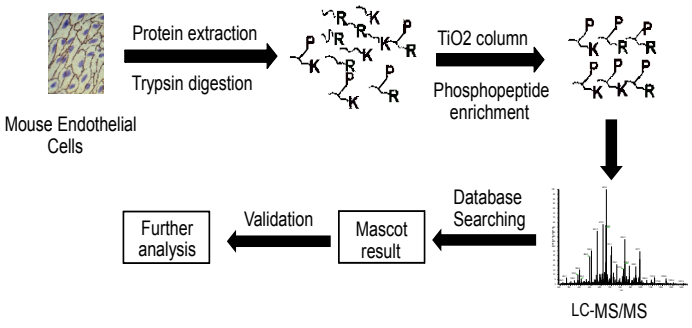

Figure 1. Workflow of the Phosphoproteomic Analysis of Mouse Endothelial Cells. Mouse endothelial cells were cultured and the extracted proteins were digested into tryptic peptides. The phosphorylated peptides were further enriched off-line using TiO2 column and then analyzed with on-line chip based LC-MS/MS method. Peak lists were searched by Mascot as well as manual interpretation.

Key Features of the cHiPLC® System

- Plug & Play simplicity
  - Expert results for non-experts
  - Cost and time savings
  - Switch workflows easily in multi-user labs
- Workflow flexibility
  - Direct injection
  - Trap and Elute – dirty samples
  - Dual column multiplexing – increasing throughput[2]
  - Serial 2-column – increasing peak capacity and depth of proteome coverage[3]
- Any lab, any person, any MS
  - A new level of reproducibility
  - Improved robustness for increased uptime
  - Multi-lab projects now a reality

Experimental

Sample Preparation: Standard phosphopeptides consisting of mono-, di-, tri-, and tetra-phosphorylated synthetic peptides were obtained from Sigma-Aldrich (St. Louis, USA). Mouse endothelial cells were cultured and the extracted proteins were denatured, reduced and carbamidomethylated before being subjected to tryptic digestion. In order to enrich phosphopeptides, the digested peptide mixtures were loaded onto a titanium dioxide (TiO2) column (Titansphere PHOS-TiO Kit, Centrifuge / Pipette Tip, 3mg / 200uL, 24/pkg, GL Sciences, Inc, USA) and the phosphopeptides were eluted using 5% ammonium hydroxide solution. All samples were dissolved in water with 0.1% formic acid for LC-MS analysis.

LC-MS/MS: An Eksigent NanoLC-Ultra® 1Dplus system (Eksigent, part of AB SCIEX Dublin, CA, USA) was used in combination with a cHiPLC® system (Eksigent) in direct inject mode. The mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A linear gradient from 3-40% B in 15 min was used for phosphopeptide standards and 2-50% B in 60 min was used for mouse endothelial cell digest. All separations were run at 300 nL/min. The chip column was packed with ChromXP™ (Eksigent) C18 3 µm particles and had a dimension of 75 µm i.d. x 15 cm. MS data was acquired using an LTQ ion trap mass spectrometer (ThermoFisher, USA). Peptide assignments were performed searching peak lists using Mascot. Phosphopeptides identified with a score of more than 25 were considered to be potentially significant and were then manually interpreted to confirm the presence of a phosphopeptide.

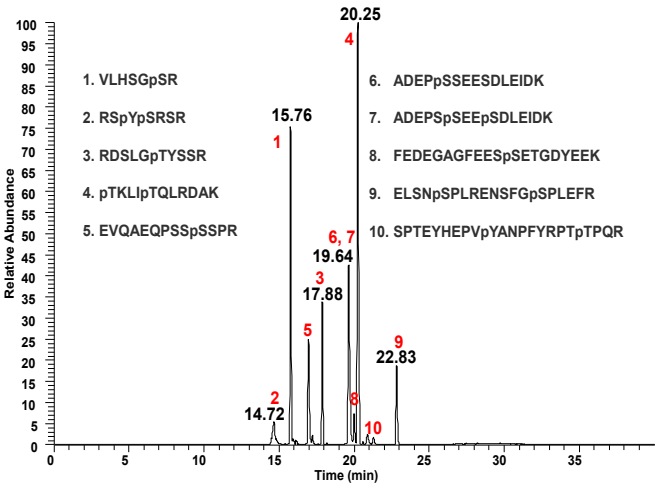

Figure 2. Extracted Ion Chromatogram of a 10 Standard Phosphopeptide Mix. A mixture of mono- and di- phosphorylated peptides were analyzed using the cHiPLC® system with a reverse phase C18 chip column. Good separation was obtained on the 10 standard peptides using a 15 minute linear gradient.

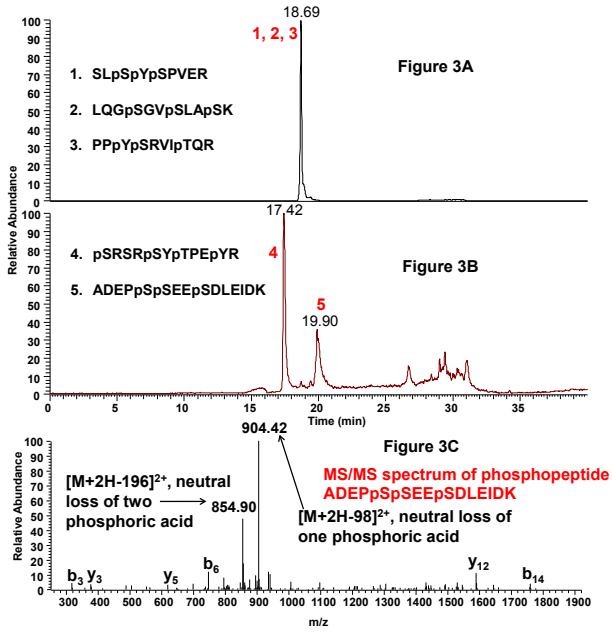

Figure 3. Extracted Ion Chromatogram of a 5 Standard Phosphopeptide Mix (Tri- and Tetra- Phosphorylated Peptides). Phosphopeptide 1, 2 and 3 have similar hydrophobicity and they were co-eluted as shown (A). Phosphopeptide 4 and 5 were separated from other components; however their signal was low compared to 1, 2 and 3 as seen by the extracted ion chromatograms (B). The evidence of the multi-phosphorylated peptides' identification (ADEPpSpSEEpSDLEIDK) was proved through the MS/MS spectrum (C). A series of y ions and b ions were observed and the most two abundant fragment ions result from neutral loss of phosphoric acid.

High Quality Phosphopeptide Separations

Identification of phosphopeptides is greatly enhanced by high quality chromatographic separations. Figure 2 demonstrates the separation and identification of a 10 standard phosphopeptide mix (mono- and di- phosphorylated peptides). As shown, all of the phosphorylated components in this sample were well separated under a 15 min gradient, with a total run time of 45 minutes. The injection volume used was 1 µL and the total injection amount was 400 fmole.

In addition to the mono- and di-phosphorylated peptides, an investigation of tri- and tetra-phosphorylated peptides was also performed. As presented in Figure 3, five tri- and tetra-phosphopeptides (1 pmole) were separated and identified by reverse phase LC-MS using the cHiPLC® system. Phosphopeptides 1, 2 and 3 have very similar hydrophobicity and eluted together on the reverse phase ChromXP™ column (Figure 3A). Phosphopeptides 4 and 5 were separated from the other components. However, their signals are very low when viewed relative to phosphopeptides 1, 2 and 3. The extracted ion chromatogram for only phosphopeptides 4 and 5 (Figure 3B) shows clear detection of these two multi-phosphorylated peptides—as determined by the m/z of the parent ions as well as

neutral loss of 49 or 98 Da (one or two phosphoric acid loss of doubly charged ions) and some fragment ions upon MS/MS (Figure 3C).

As shown in Figure 4, excellent reproducibility is achieved for the phosphopeptide separation using Eksigent nanoLC and cHiPLC® system. This is very important for quantitative applications requiring reproducible retention time such as MS/MS^{ALL} with SWATH™ Acquisition or time-scheduled MRM studies for targeted quantitation.

Analysis of Complex Phosphoproteomic Samples

Additionally, analysis of a mouse endothelial cell phosphoproteome was performed in order to identify the maximum number of phosphoproteins. Compared to the general proteome, the analysis of a phosphoproteome is much more difficult since phosphoproteins typically exist at relatively low abundance compared to non-phosphorylated peptides. Because of these issues, enrichment for the phosphopeptides in the mouse phosphoproteome was performed using titanium dioxide (TiO2) packing material prior to LC-MS analysis. The phosphoproteomic analysis workflow is shown in Figure 1.

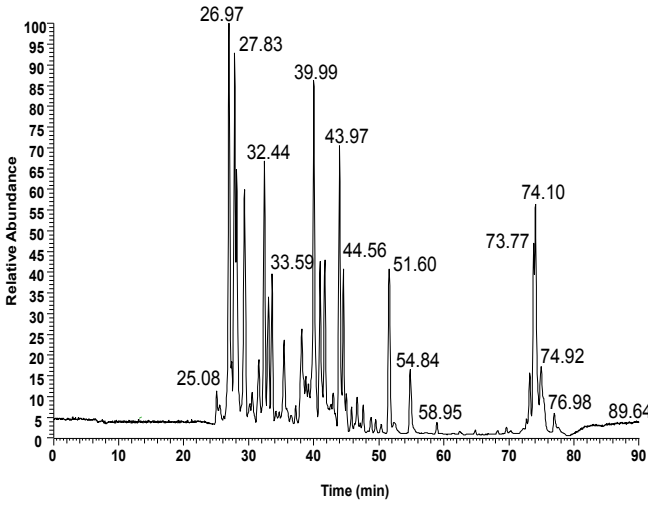

Figure 5. Base Peak Chromatogram of Mouse Endothelial Cell Protein Digest containing Enriched Phosphopeptides. The TiO2 column off-line enriched phosphopeptides resulted in a significant reduction in sample complexity and the chromatogram shows a high quality separation.

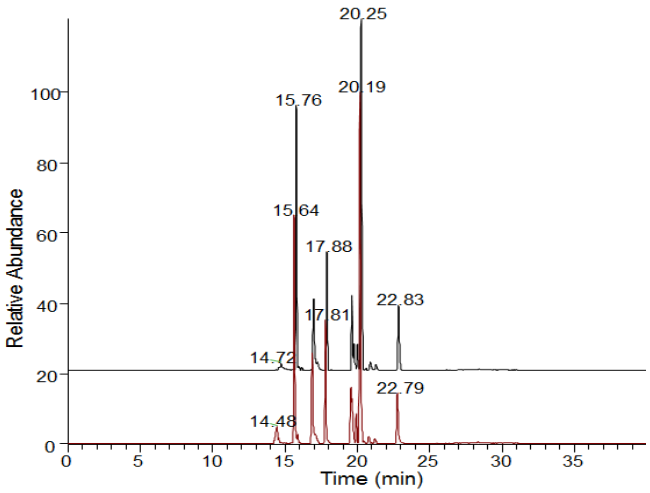

Figure 4. Reproducibility of Phophospeptide Separations. Excellent reproducibility has been achieved using the Eksigent nanoLC and cHiPLC® system for the phosphopeptide separations.

The base peak chromatogram of the mouse endothelial cell protein digest from a single unfractionated 5% ammonium hydroxide elution after phosphopeptide enrichment shows a significant reduction in sample complexity and high quality separation (Figure 5).

The top 10 proteins (ranked by Mascot scores) identified from the mouse endothelial cell protein digest were listed in Table 1. The phosphopeptides matching to these proteins were indicated as well. A total of 98 proteins (152 peptides) were identified from this single LC-MS/MS analysis. Within the total identification, 88% of all the peptides were phosphorylated peptides and 63 out of 98 proteins are phosphorylated proteins, showing good enrichment from the TiO2 step. This enrichment is further illustrated in Table 1; only 3 peptides detected for the top 10 proteins were non-phosphorylated proteins.

Figure 6 shows an example of an MS/MS spectrum of the doubly charged phosphopeptide SApSPDDDLGSSNWEAADLGNEER from a small acidic protein (Sequence ID: IPI00127941) in this mouse endothelial cell protein digest. In addition to a series of matched y-ions, the most abundant fragment ion at m/z 1208.70 is from the parent ion neutral loss of one phosphoric acid, strongly confirming this phosphopeptide sequence.

Sequence ID	Sequence Name	Identified Phosphopeptides
IPI00320208	*Elongation factor 1-beta*	*DDDDIDLFGpSDDEEESEEAK* *DDDDIDLFGpSDDEEESEEAKK* *YGPSSVEDTTGSGAADAKDDD* *DIDLFGpSDDEEESEEAK*
IPI00554929	*Heat shock protein HSP 90-beta*	*EQVANpSAFVER* *IEDVGpSDEEDDSGK* *EKEIpSDDEAEEEK*
IPI00944009	*Isoform 3 of Elongation factor 1-delta*	*DIDLFGpSDEEEEDKEAAR* *GATPAEDDEDKDIDLFGpSDEEE EDK*
IPI00223443	*Isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2*	*EPEEGEDDRDpSANGEDDS* *MESEAGADDpSAEEGDLLDD DDNEDR* *MESEAGADDpSAEEGDLLDD DDNEDRGDDQLELK*
IPI00127989	*Prostaglandin E synthase 3*	*DWEDDpSDEDMSNFDR*
IPI00130343	*Uncharacterized protein*	*No phosphopeptides identified*
IPI00849626	*nucleophosmin-like*	*CGSGPVHISGQHLVAVEED* *AEpSEDEDEEDVK*
IPI00648014	*Uncharacterized protein*	*No phosphopeptides identified*
IPI00649841	*Uncharacterized protein*	*No phosphopeptides identified*
IPI00127941	*Small acidic protein*	*SApSPDDDLGSSNWEAADLGNE ER*

Table 1. Top 10 Proteins and the Corresponding Phosphopeptides Identified from a Mouse Endothelial Cell Protein Digest.

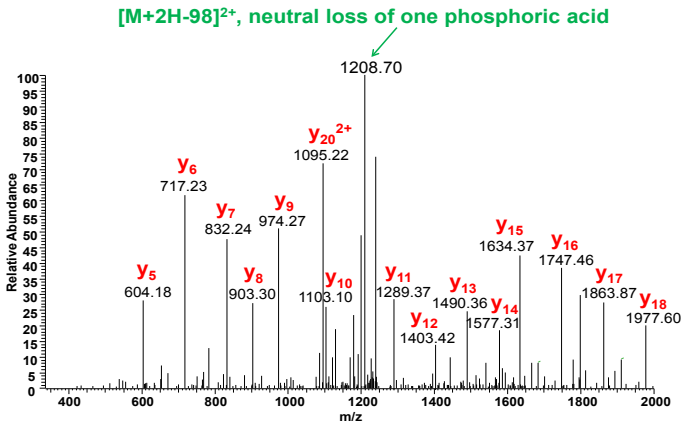

Figure 6. MS/MS Spectrum of One Phosphopeptide from Mouse Endothelial Cell Protein Digest. A doubly charged peptide with sequence SApSPDDDLGSSNWEAADLGNEER was identified from the small acidic protein. Nice MS/MS fragmentation pattern provided a confident peptide ID and confirmed the presense of a phosphoserine residue.

Conclusions

A simple, plug and play microfluidic platform (cHiPLC® system) was coupled with a high performance nanoLC system (nanoLC-Ultra® system) for the analysis of phosphopeptides in complex samples. Both standard samples and phosphopeptides from complex cell lysates were shown to be well separated and easily analyzed using the microfluidic columns. Ions with excellent signal to noise were observed even for the most hydrophilic phosphopeptides, including di-, tri-, and tetra-phosphorylated peptides.

References

1. cHiPLC®-nanoflex System: Making nanoLC/MS Easier and More Reproducible. Eksigent Product Note 0910-1000
2. Increasing Throughput of nanoLC using Two-Column Switching Workflows. AB SCIEX Technical Note 1870411-01
3. Increasing Depth of Coverage using Serial Two-Column Workflows. AB SCIEX Technical Note 1870211-02

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