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Phosphotyrosine biased enrichment of tryptic peptides from cancer cells by combining pY-MIP and TiO$_2$ affinity resins

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ABSTRACT: Protein phosphorylation at distinct tyrosine residues (pY) is essential for fast, specific and accurate signal transduction in cells. Enrichment of pY-containing peptides derived from phosphoproteins is commonly facilitated by use of immobilized anti-pY antibodies prior to phosphoproteomics analysis by mass spectrometry. We here report on an alternative approach for pY-peptide enrichment using inexpensive pY imprinted polymer (pY-MIP). We assessed by mass spectrometry the performance of pY-MIP for enrichment and sequencing of phosphopeptides obtained by tryptic digestion of protein extracts from HeLa cells. The combination of pY-MIP and TiO$_2$ based phosphopeptide enrichment provided more than 90% selectivity for phosphopeptides. Mass spectrometry signal intensities were enhanced for most pY-phosphopeptides (approximately 70%) when using the pY-MIP-TiO$_2$ combination as compared to TiO$_2$ alone. pY constituted up to 8% of the pY-MIP-TiO$_2$ enriched phosphopeptide fractions. The pY-MIP-TiO$_2$ and the TiO$_2$ protocols yielded comparable numbers of distinct phosphopeptides, 1693 and 1842 respectively, from microgram levels of peptide samples. Detailed analysis of physicochemical properties of pY-MIP-TiO$_2$ enriched phosphopeptides demonstrated that this protocol retrieved phosphopeptides that tend to be smaller (<24 residues), less acidic and almost exclusively monophosphorylated, as compared to TiO$_2$ alone. These unique properties render the pY-MIP based phosphopeptide enrichment technique an attractive alternative for applications in phosphoproteomics research.

Protein phosphorylation is a widespread and universal regulatory mechanism in prokaryote and eukaryote cell signaling and metabolism.¹⁻⁵ Highly sensitive mass spectrometry (MS) techniques now make phosphopeptide sequencing and quantitation feasible at a routine basis, giving rise to the rapidly growing field of phosphoproteomics.⁶⁻⁸

The development of chemical, biochemical and immunological methods for phosphoprotein and phosphopeptide isolation, enrichment and separation has received great attention in the context of phosphoproteomics. The most successful methods for phosphopeptide enrichment rely on the affinity of inorganic moieties towards the phosphate groups located on serine (pS), threonine (pT) and tyrosine (pY) residues in peptides and proteins. Fe$^{3+}$ and Ti$^{4+}$-immobilized metal ion affinity chromatography (IMAC) and titanium dioxide (TiO$_2$) are widely used methods for global phosphopeptide enrichment prior to MS.⁹⁻¹⁵ Despite their successful application in global phosphoproteome studies, affinity based methods such as IMAC and TiO$_2$ methods lack the capability to discriminate between the three common types of phosphorylated residues in eukaryotes, i.e. pS, pT and pY. In particular, phosphorylation at tyrosine residues is of immense interest due to its role in cell signaling and regulation of metabolism, cellular growth, development and differentiation.¹⁶⁻¹⁷ The event of pY is of low abundance (<1%) compared to pS (90%) and pT (10%) in most eukaryote cells.¹⁸ Many tyrosine kinases are membrane bound receptors of growth factors and hormones which initiate signal transduction.¹⁹⁻²¹ Aberrant phosphorylation of tyrosine residues on such receptors may lead to cellular transformation and cancer.¹⁸ The most efficient and specific bioanalytical method for enrichment of pY-peptides and proteins relies on anti-pY antibodies yielding thousands of pY-peptide identifications.²²⁻²⁴ Some drawbacks related to the use of anti-pY antibodies include the requirement of large amounts of starting material (up to hundreds of mg), variability of antibody quality and specificity/selectivity, and cost.²²⁻²⁵

Advents in polymer chemistry and molecular mimicry²⁶ gave rise to the concept of using molecularly imprinted polymers (MIPs) for molecular recognition and separation of peptides and proteins.²⁷⁻³⁰ Recently, we introduced a new approach for phosphopeptide enrichment featuring neutral, urea-based imprinted phosphate receptors.³¹⁻³⁴ The resulting MIPs could in principle address some of the aforementioned deficiencies. In this context, phospho-
rosine imprinted polymers (pY-MIPs) were used to select-
ively enrich pY-peptides spiked at low levels into proteo-
ytic digests. However, this approach is yet to be demon-
strated on native biological samples. To investigate this,
we produced a pY-MIP based on a modified version of the
procedure reported by Engenbroich et al.36 and tested it
on simple and complex tryptic peptide mixtures, the lat-
ter derived from human cervical cancer cell (HeLa) pro-
tein extract. The method development involved assess-
ment of the properties of the pY-MIP alone and in c ombi-
nation with our established TiO$_2$ protocol for phospho-
peptide enrichment at the low microgram level. We find
that the sequential use of pY-MIP and TiO$_2$ enrichment
provide some distinct advantages for enhanced detection
of pY-peptides in phosphoproteomics.

**EXPERIMENTAL**

**Synthesis of pY-MIP.** Molecularly imprinted polymer
with pY affinity was prepared as described previously with
some modifications.33-35 (Supporting Information).

**Preparation of Semi-complex Peptide Mixture.** The
semi-complex peptide mixture, prepared as described
elsewhere,44 was brought to a final concentration of 1
pmol/µL per digested protein, and spiked with a mixture
containing four pY- and four pS-peptides (Supporting
Information).

**Cancer Cell Protein Extract.** Three 10 cm dishes of
human cervical cancer epithelial cells, HeLa, were cul-
tured and grown to 95% confluence. Stimulation with
sodium pervanadate (PVD)-supplemented media was
carried for a duration of 30 min. Cells were then washed
and scraped/transfered into 15 mL falcon tubes, and cen-
trífuged. The resulting protein lysate (approximately 1
mg) was reduced and alkylated, then digested with endo-
peptidase Lys-C for 3 h prior to overnight trypsin diges-
tion. See Supplementary Information for details. A vol-
ume of 2 µL tryptic peptide mixture was used for accurate
quantification of peptide concentration by amino acid
composition analysis (AAA).37

**Sample Desalting.** Peptide samples were desalted after
TiO$_2$ enrichment and prior to pY-MIP enrichment (see
Supplementary Information). Samples were then dried in
a vacuum concentrator and stored until further use.

**Enrichment Strategies.** The experimental work con-
sisted of four protocols: the reference enrichment TiO$_2$,
pY-MIP and two combined protocols: pY-MIP-TiO$_2$ and
TiO$_2$-pY-MIP. The workflow and experimental details for
analysis of the semi-complex peptide sample are provided
in Supporting Information, Figure S1.

**Direct pY-MIP.** The dry sample resulting from desalting
of 200 µg peptides (determined by AAA) was first dis-
solved in 2 µL of 10% TFA, then added 4 µL of Milli-Q wa-
ter and mixed thoroughly, before 194 µL MeCN was added
slowly drop-wise to give a final composition of 97%
MeCN in 0.1% TFA as the pY-MIP loading solution. pY-
MIP columns were prepared and operated similarly to the
desalting columns. They were manually packed in dispos-
able 200 µL pipette tips which were plugged with a layer
of C8 Empore$^\text{TM}$ extraction disk to retain pY-MIP parti-
cles. A suspension of pY-MIP in MeCN was added to the
plugged pipette tip until the length of the column reached
1.5 cm. Solvents and fractions were collected in 1.5 mL low-binding vials with perforated lids where a 200 µL tip could fit and be stable upon centrifugation. Passing
of liquid through the column was operated by centrifugal
forces spun at 1000 rpm for 10-15 min. Columns were first
activated with 200 µL of MeCN, and conditioned with 200
µL of 97% MeCN in 0.1% TFA. The reconstituted sample
described above was then added, and the column was
washed with 50 µL of 97% MeCN in 0.1% TFA. The collect-
ion vial was replaced by a new one and elution was per-
fomed in two steps using two solutions. First with 200 µL
95% MeOH in 0.1% TFA, followed by 200 µL of 50% MeOH in 1% TFA. Eluents were then pooled, dried using a vacuum concentrator and stored at -20°C until further
use.

**TiO$_2$ enrichment.** TiO$_2$ enrichment of 200 µg peptides
was performed as described by Palmisano et al.38 The de-
tailed experimental procedure is in the Supporting Infor-
mation.

**TiO$_2$-pY-MIP.** Following TiO$_2$ enrichment (Supplemen-
tary Information) samples were dried in a vacuum con-
centrator, re-dissolved and applied for pY-MIP (vide su-
pra). Eluents were dried by a vacuum concentrator and
stored at -20°C.

**pY-MIP-TiO$_2$.** Samples were first processed with pY-MIP
(vide supra) above. The resulting eluents were dried, re-
dissolved in TiO$_2$-loading solution and incubated with
TiO$_2$ beads (TiO$_2$ enrichment described in Supplementary
Information). The amount of TiO$_2$ beads was determined
based on the amount of peptides in the pY-MIP eluate,
thus one additional replicate was prepared for AAA analy-
sis. The peptide amount was found to be 2 µg and 0.3 mg
TiO$_2$ beads were used for enrichment. Washing and elu-
tion were performed as described previously, however,
Volumes were reduced to 100 µL and 50 µL for washing
and elution, respectively. Eluents were desalted as de-
scribed below and, finally, dried by a vacuum concen-
trator and stored at -20°C.

**MALDI MS.** MALDI MS was performed using an Ultra-
flextreme mass spectrometer and processed with FlexA-
lysis software (Bruker Daltonics, Bremen, Germany).

**Reversed-phase nanoLC-ESI-MS/MS.** Samples were
chromatographically separated by an EASY-nLC system
(Thermo Scientific, Odense, Denmark) coupled online to a
LTQ-Orbitrap Velos mass spectrometer (Thermo Scien-
tific, San Jose CA). Peptides were separated on a 20 cm
long fused silica capillary column (75 µm ID), packed in-
house with ReproSil-Pur C18 AQ, 3 µm reversed-phase material (Dr. Maisch, Ammerbuch-Entringen, Germany).
Peptides were eluted following a 130 min gradient of mo-
bile phase A (0.1% FA) to 34% mobile phase B (95% MeCN in 0.1% FA), flow rate of 250 nL/min. The LTQ-Orbitrap
Velos was operated in positive ion mode with data-
dependent acquisition. The full scan was acquired in the Orbitrap with an automatic gain control (AGC) target value of $1 \times 10^6$ ions and a maximum fill time of 500 ms. Full-MS scans were acquired with resolution of 60,000 FWHM followed by 10 MS/MS scans of the most intense ions, also acquired with a mass resolution at 15000 (HCD normalized collision energy = 35; activation time 10 ms). Raw data were viewed in Xcalibur v2.0.7 (Thermo Fisher Scientific, USA).

**Data Analysis.** Data analysis of the raw data was performed with Proteome Discoverer v1.4 (Thermo Scientific) and searched against the Swiss-Prot human v3.53 database (20,199 entries) using an in-house Mascot server (v2.3, Matrix Science Ltd, London, UK). Database searches were performed as follows: precursor mass tolerance of 10 ppm, product ion mass tolerance of 0.05 Da, Cys carbamidomethylation as fixed modification, oxidation of Met and phosphorylation on S/T/Y as variable modifications and two missed cleavages of trypsin were allowed. Only peptides with q-value of 0.01 (Percolator), search engine 1, Mascot rank 1 and cut-off value of Mascot score $\geq 22$ were considered for further analysis. Phosphopeptides were filtered for pRS values $\geq 95$.

Relative quantification of phosphopeptides was achieved by using the Precursor Ion Area (PIA) feature of Proteome Discoverer. Normalization of data for pairwise comparison of two sets of triplicate data, e.g. pY-MIP-TiO$_2$ vs TiO$_2$, was performed in GraphPad Prism v7 using a scaling factor. The 0% level was set to the lowest value among the minima in the six intergroups and the 100% level was set to the highest value among the maxima in the six intergroups. Comparative analysis of phosphopeptide intensities between protocols was performed using the average across the three replicates of each protocol. Paired t-tests were performed in GraphPad Prism v7.

**Phosphorylation Motif Analysis.** Sequence logos were visualized with IceLogo 1.2. Aligned sequences for IceLogo analysis were extracted using the motif-X algorithm. Subtractive IceLogo analysis was performed by using the TiO$_2$ dataset as background and comparing it to pY-MIP-TiO$_2$, TiO$_2$-pY-MIP and pY-MIP datasets using a p-value setting of 0.05.

**Figure 1. MALDI MS spectra of a simple peptide mixture analyzed before and after phosphopeptide enrichment.**

(A) Control, untreated peptide mixture, (B) Eluate obtained by pY-MIP processing of the simple mixture, (C) superimposed spectra obtained by TiO$_2$, pY-MIP-TiO$_2$ and TiO$_2$-pY-MIP enrichment of the simple peptide mixture shown in blue, orange and green, respectively. Five spectra panels in (D) show four pY-peptides, DRVpYIHPF ($m/z$: 1126.51), GADDSTpYTAAR ($m/z$: 1198.44), GADDPpYPYAR ($m/z$: 1278.41), TRDIpYETDPYRK ($m/z$: 1862.68) and one detected pS-peptide AVPSPPPAPSPR ($m/z$: 1154.55). Phosphopeptides in the spectra are marked with their $m/z$ value, pY-peptides are labelled by red asterisks.
RESULTS

Phosphopeptide Enrichment and MS analysis. We initially tested the pY-MIP performance for phosphopeptide enrichment by using a simple tryptic peptide mixture containing regular (non-phosphorylated) peptides and a series of phosphorylated peptides, four of which were spiked-in synthetic pY-peptides (see the experimental workflow and synthetic peptides in Figure S1 and Table Si, Supporting Information). Untreated control sample and the pY-MIP processed samples were analyzed by MALDI MS (Figure 1, A-B). The pY-MIP sample processing protocol reduced the complexity of the MALDI mass spectrum, i.e. fewer ion signals were observed as compared to the control sample. Several pY-peptide signals were observed as indicated by asterisks in Figure 1B. Several other peptide ion signals were also observed. Based on these initial observations and additional pY-MIP assessment by LC-MS/MS experiments (see below) we concluded that the pY-MIP alone did not have sufficient capacity and specificity to allow for enrichment of phosphopeptides from very complex peptide mixtures, such as those derived from human cell lysate.

Next, we hypothesized that the combination of pY-MIP and TiO$_2$ enrichment could provide the capacity, specificity and selectivity for enrichment of pY-peptides in phosphoproteomics experiments.

We investigated the sequential use of pY-MIP and TiO$_2$ (pY-MIP-TiO$_2$ protocol) or TiO$_2$ and pY-MIP (TiO$_2$-pY-MIP protocol) and compared their performance to the TiO$_2$ protocol used as benchmark. Initial tests using MALDI MS (Figure 1C) were encouraging as they demonstrated enhanced selectivity for pY-peptides and reduced sample complexity, although at reduced signal-to-background levels for the pY-peptides. However, the comparison of superimposed spectra (insets in Figure 1D) exhibit a higher relative signal from most phosphopeptides for pY-MIP-TiO$_2$ than the two other methods (TiO$_2$ and TiO$_2$-pY-MIP).

Assessment of pY-MIP by LC-MS/MS. Next, we applied LC-MS/MS to assess the performance of pY-MIP based protocols for phosphopeptide enrichment from a complex tryptic peptide mixture derived from HeLa cell protein extract. We treated the HeLa cells with the phosphatase inhibitor sodium pervanadate to enhance protein tyrosine phosphorylation levels. We tested pY-MIP, pY-MIP-TiO$_2$, and TiO$_2$-pY-MIP protocols and compared them to our standard TiO$_2$ enrichment protocol (Figure 2).

The combined pY-MIP-TiO$_2$ protocol identified 1693 phosphopeptides, whereas 136 were phosphorylated only on tyrosine (Table 1). The standard TiO$_2$ protocol retrieved 1842 phosphopeptides, 142 thereof phosphorylated exclusively on tyrosine. Thus, serial use of pY-MIP prior to TiO$_2$ performed as well as TiO$_2$ alone, both protocols identifying on the order of 1700-1800 phosphopeptides (pRS $\approx$95%).

Having determined that the serial use of pY-MIP prior to TiO$_2$ exhibited rather good characteristics for phosphopeptide recovery, we set out to further study the recovery of pY-, pS- and pT-peptides by using a quantitative approach. We compared the phosphopeptide ion intensities of the pY (n=100), pS (n=998) and pT (n=56) peptides that were detected and identified by both the pY-MIP-
TiO$_2$ protocol and the TiO$_2$ protocol. For this analysis, only peptides with one type of phosphorylated residue, on one or more sites, were considered (Figure 3).

Table 1. Phosphopeptide identification by affinity enrichment using four protocols (three technical replicates each).

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<tr>
<td>Non-phosphopeptides (np)</td>
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<td>588</td>
<td>68</td>
<td>71</td>
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<tr>
<td>Phosphopeptides (5%)</td>
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<td>1703</td>
<td>2213</td>
<td>1703</td>
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<tr>
<td>Phosphopeptides (10%)</td>
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<td>374</td>
<td>371</td>
<td>42</td>
</tr>
<tr>
<td>Phosphopeptides (15%)</td>
<td>1663</td>
<td>1328</td>
<td>1842</td>
<td>412</td>
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</tbody>
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Note. Method selectivity values, in the lower panel, were calculated as follows: $pp$ selectivity = $100 \times pp/(pp + np)$.

For each individual phosphopeptide, we determined the ratio of normalized ion intensities observed by using the two protocols (Figure 3). If there was no discrimination or differences in binding affinity/avidity between the TiO$_2$ protocol and pY-MIP-TiO$_2$ protocol for these phosphopeptides, then the phosphopeptide ratios should distribute evenly in the logarithmic diagram, with 50% being between 0 and 1 and 50% being above 1. Figure 3 shows that this indeed is the case for pS-peptides. The distribution of pY-peptides, on the other hand, is biased towards higher absolute recovery, i.e. higher phosphopeptide ion intensities for 72% of the pY-peptides when using the pY-MIP-TiO$_2$ protocol as compared to the TiO$_2$ protocol. This implies that pY-MIP-TiO$_2$ has a higher affinity for a majority of the pY-peptides than does TiO$_2$. Interestingly, the common set of pT-peptides, although a small number, also exhibited higher ion signal intensities when using pY-MIP-TiO$_2$ rather than TiO$_2$ alone. Differences in ion intensities between pY-MIP-TiO$_2$ and TiO$_2$ were significant ($P<0.0001$) for pY and pT peptides (for pY: $M=3.7$ and $SD = 6.5$, for pT: $M = 7.9$ and $SD = 10.8$), but not for pS- ($P=0.7$, $M = 0.1$, $SD = 10.1$). The TiO$_2$-pY-MIP protocol did not provide any advantages with regard to quantitative enrichment of phosphopeptides (Figure S3 and additional statistics in Supporting Information).

In summary, pY-MIP has interesting properties as a first-line enrichment method for complex peptide samples from a human cancer cell line, when followed by TiO$_2$ enrichment. The total number of phosphopeptides and the number of pY-peptides are comparable to those obtained by TiO$_2$ alone, despite added sample handling steps. Importantly, ion signal intensities were enhanced for a majority of pY-peptides as compared to TiO$_2$ enrichment alone. This was also observed for some pT-peptides, but not for pS-peptides indicating that pY-MIP resin selects bulkier phosphoamino acid residue side chains such as pY and pT.


Next, we studied the physicochemical properties of the phosphopeptides that were enriched by TiO$_2$, pY-MIP, pY-MIP-TiO$_2$ and TiO$_2$-pY-MIP protocols. Previous studies suggest a slight preference of TiO$_2$ towards acidic phosphopeptides in contrast to IMAC which was reported to enrich predominantly multiphosphorylated peptides. For this analysis we considered only phosphopeptides that were detected in at least two technical replicates, and were unique to TiO$_2$, pY-MIP-TiO$_2$ and TiO$_2$-pY-MIP (Figure S4). Given the limited number of phosphopeptides detected by pY-MIP, we considered all the identified phosphopeptides (258 phosphopeptides, minimally detected in two replicates).

The peptides were first grouped as either acidic, basic, neutral or hydrophobic (Figure 4, upper panel).
Peptides were assessed as hydrophobic when constituted by 25% or more hydrophobic residues. The relative prevalence of peptides within these groups is represented by relative frequencies (RF) obtained by normalizing to the total number of peptides for each method. The chemical differences between pY-MIP-methods and reference method (TiO₂) were then statistically tested through their RFs by one-way Analysis of Variance (ANOVA, p <0.05) followed by Dunnett’s test for multiple comparisons. The relative enrichment of acidic peptides (Figure 4, upper panel) was lower than the reference (TiO₂) for all the three pY-MIP methods. More specifically, the differences between pY-MIP and TiO₂ (30±3 vs. 49±1%) and pY-MIP-TiO₂ vs. TiO₂ (27±3 vs. 49±1%) were statistically determined as extremely significantly different (P<0.001), whilst TiO₂-pY-MIP vs. TiO₂ (42±2 vs. 49±1%) were significantly different (P<0.05). The relative enrichment of basic peptides (Figure 4, upper panel) was higher for two of the three pY-MIP-methods, i.e. stand-alone pY-MIP and pY-MIP-TiO₂ (Table S2 and Supporting Information).

The relative enrichment of neutral peptides (Figure 4, upper panel) was characterized by significantly higher RFs for the two combined methods compared to the reference (TiO₂-pY-MIP vs. TiO₂: 21±1 vs. 13±1%, P<0.001, pY-MIP-TiO₂ vs. TiO₂: 21±12 vs. 13±1%, P<0.001) whilst no significant difference was found for TiO₂ and pY-MIP (13±1 vs. 12±1%, P>0.05). Similarly, when hydrophobicity was considered, the combined methods pY-MIP-TiO₂ and TiO₂-pY-MIP yield significantly more hydrophobic peptides compared to TiO₂ and pY-MIP: (41±2 vs. 19±1%, P<0.001), and (25±2 vs. 19±1%, P<0.01) (Figure 4, upper panel). Also, the two stand-alone methods, pY-MIP and TiO₂ differ from each other, but with a lower statistical significance (15±1 vs. 19±1, P<0.05).
The analysis of charge distribution in phosphopeptides originating from the four methods suggests that pY-MIP and pY-MIP-TiO$_2$ unlike TiO$_2$, are not biased towards acidic peptides. This agrees with our previous report describing pS-MIP based phosphopeptide enrichments. The two combined methods pY-MIP-TiO$_2$ and TiO$_2$-pY-MIP recover more uncharged and hydrophobic residues than the two stand-alone methods (pY-MIP and TiO$_2$).

Next, we investigated the phosphopeptides to determine the degree of phosphorylation and length of peptides for each of the four enrichment protocols (Figure 4, lower panel).

Phosphorylated peptides were divided into mono- or multiphosphorylated groups and into peptides of length 6-24 residues and peptides of length ≥25 residues. The RFs for these groups were then statistically tested with Dunnett’s method for multiple comparisons in ANOVA (one-way, p<0.05) similarly to previous section. We observed that pY-MIP-based methods have a distinct preference for monophosphorylated and relatively short phosphopeptides (Figure 4, lower panel and Supporting Information, Table S2).

We conclude that pY-MIP may act as size-filter for tryptic phosphopeptides retrieved from a biological sample. As we have concluded in our recent report, this is related to the broad pore size distribution of the material with a significant number of pores in the low meso- to microporous regime (1-20 nm). The result of the filtering effect in this case however, is that the three pY-MIP-based methods demonstrate similar size profiles, with a shorter average peptide length compared to those derived from TiO$_2$. Also, small tryptic peptides have higher chances of bearing a singular phosphorylated site resulting in a higher probability of pY-MIP-based methods recovering monophosphorylated peptides. Such complementarity of pY-MIP may be advantageous, allowing researchers to tailor enrichment strategies for particular subsets of phosphopeptides.

Motif Analysis. Protein kinases bind to their substrates by recognizing certain amino acid motifs (consensus sequences) surrounding a phosphorylation site (specificity determinants) and are broadly classified as proline-directed, acidicophilic and basophilic. To assess whether the observed chemical differences of phosphopeptides derived by our four analytical methods would be reflected in the sequence motifs and classes of kinase substrates, we analyzed the amino acid sequences with IceLogo. We performed a subtractive sequence logo analysis using the TiO$_2$ dataset as the reference and comparing to the pY-MIP, TiO$_2$-pY-MIP, and pY-MIP-TiO$_2$ datasets.

pS and pT motifs were enriched for proline (P) in the position next to the phosphorylated site (+1) and depleted for acidic amino acid residues (E,D) at that position (Figure 5A-C). This agrees with the bias observed in our previous report on pS-MIP based phosphopeptide enrichments and suggests that a proline residue next to the phosphorylation site may promote binding to the pY-MIP groove, possibly by introducing a kink in the peptide chain.

The subtractive sequence motif analysis confirms that the pY-MIP based methods recover pS/pT-phosphopeptides that are less acidic than those recovered by TiO$_2$ alone (Figure 5A-C). Subtractive sequence motif analysis for pY-phosphopeptides provided a different result (Figure 5 D-F). Small hydrophobic amino acid resi-
dues (A,V) were enriched in the -1 and +2 positions. Interestingly, K was enriched in the -1 position for the pY-MIP-TiO2 method, whereas T was enriched in the +1 position (Figure 5E). Although this sequence motif analysis was based on a rather small number of pY-peptides it suggests that hydrophobic interactions play a role in substrate binding of the pY-MIP.

We conclude that phosphopeptides obtained by the four enrichment protocols derived primarily from the activity of proline-directed kinases. The incorporation of pY-MIP in the combinatorial methods is associated with a reduction on the representation of motifs recognized by acidic-kinases compared to the reference TiO2; a result consistent with the analysis of chemical properties revealing depletion of acidic phosphopeptides when pY-MIP was incorporated in the workflows. However, IceLogo did not reveal any observable difference in regulation of basic residues in +1 position, which is usually a signature of basophilic kinase sites. Interestingly, the apparent enrichment of T-residues in proximity to pS/pT (position +2) and adjacent to pY (position +1) by pY-MIP-TiO2 could be of interest in targeting dual specificity kinases.47

Figure 5. Sequence logos of phosphorylation sites obtained by subtractive sequence motif analysis against the TiO2 dataset. IceLogos centered on pS and pT residues were determined using default values of probability (p<0.05). A nine and five amino acid residue window was used for pS/pT (A-C) and pY (D-F) sequences respectively.

DISCUSSION

We thoroughly analyzed the performance of pY-MIP-based phosphopeptide enrichment by using a complex cancer cell (HeLa) protein extract. The potential of using pY-MIP for the enrichment of pY-peptides was previously investigated for simple peptide mixtures and brain lysates spiked with synthetic peptides.33 Yet, to the best of our knowledge there is no report describing the use of pY-MIP for enrichment of endogenous, low abundant pY-peptides, directly from a biological sample. Our present work using HeLa cell lysates resulted in the development of three pY-MIP-based workflows: direct (stand-alone) pY-MIP, pY-MIP-TiO2, and TiO2-pY-MIP. Their performance for enrichment of pY-, pS-, and pT-peptides was compared to TiO2 including assessment of enrichment efficiency, physicochemical properties of recovered peptides, and kinase substrate motifs.

The main finding of this work is that pY-MIP quantitatively enrich pY-peptides when succeeded by TiO2, in the form of a combined method pY-MIP-TiO2. Nearly 70% of pY-peptides which were detected simultaneously by pY-MIP-TiO2 and the reference TiO2 method, had higher signal intensities than their TiO2 counterpart (Figure 3). The pY-MIP-mediated enrichment of pT-peptides is a novel finding of high interest, since pT is 9-10-fold less common than pS-sites.9 This cross reactivity can have different causes. The intrinsic side chain selectivity of the pY-MIP has been extensively proven at the amino acid level and is ascribed to a tight neutral binding site, binding the phosphate group in a cleft-like manner. The interactions with peptides however also involve non-specific binding, notably hydrophobic effects.35 This results in a preference for hydrophobic sequences, e.g. proline rich sequences, that is known to be more abundant in cancer-related pT motifs.40 Finally, pY-MIP-TiO2 did not show superiority over TiO2 for the quantitative enrichment of pS-peptides. Both methods showed comparable representation of the common pS-peptides (Figure 3).

The alternative TiO2-pY-MIP method was inferior to TiO2 both by qualitative and quantitative performance. All resulting phosphopeptides exhibited lower ion intensities as compared to their counterpart enriched by TiO2, (Figure S3). This reduction of intensities can be related to peptide losses during successive steps of sample preparation.

Our attempts to develop a one-step stand-alone pY-MIP enrichment method did not succeed at this time; pY-MIP bound excessively to non-phosphorylated peptides, possibly due to interactions between pY-MIP and hydrophobic regions and oxygen groups in the peptides. The unspecific peptide binding of pY-MIP when presented to complex peptide mixture, without prior preparation or fractionation, is in agreement with the results of Chen et al.,30 who used a chemically similar MIP (imprinted for pS). From our results, we conclude that pY-MIP-TiO2 enrich pY-peptides, based on pY-MIP-affinity, and it is comparable to the stand-alone TiO2 protocol, despite a few additional sample handling steps. Importantly, the combined method provides an access to distinct parts of phosphoproteome since the overlap of phosphopeptides enriched by these two approaches (pY-MIP-TiO2 and TiO2) was only about 50%. This new pY-MIP-TiO2 strategy is applicable to quantitative analysis of differentially phosphorylated proteins in biological studies, e.g. to study aberrant cell signaling in health and disease.

Chemical biases of phosphopeptide enrichment methods are widely reported and constitute an ongoing topic of research in phosphoproteomics.50 Diverse chemical interactions between resin and amino acid residues and sequences lead to preferential binding of certain subsets of phosphopeptides. Since pY-MIP and TiO2 exploit dif-
ferent chemical properties, we anticipated physicochemical variability among enriched phosphopeptides. Our results suggest that pY-MIP and pY-MIP-TiO₂ perform similarly to each other and differently from TiO₂. The most pronounced differences relate to the frequency of charged peptides, peptide length and level of phosphorylation. The pY-MIP features charge neutral binding sites where phosphate recognition occurs through complementary multidentate hydrogen bonding. Thus, unlike the acidophilic nature of TiO₂, the pY-MIP binds to target phosphopeptides independent of their charge state—a property reflected in our results by roughly twofold underrepresentation of acidic phosphopeptides by pY-MIP and pY-MIP-TiO₂ as compared to TiO₂ and TiO₂-pY-MIP.

Monophosphorylated and short phosphopeptides were 20–30% more frequent among the three pY-MIP-methods than in TiO₂ (Figure 4, lower panel). This is in agreement with previous results showing that the pY-MIP binding energy is sufficient for interaction with short peptides. In a pool of tryptic peptides with various lengths and levels of phosphorylation, the pY-MIP bias towards short and less phosphorylated peptides becomes prominent, potentially hampering the MS detection of multiphosphorylated peptides in the pY-MIP-based workflows.

Our detailed assessment of phosphopeptide recovery led to the identification of motifs recognized predominantly by proline-directed and acidophilic kinases—a commonly reported result. The identification of basophilic kinase substrates is known to be more difficult. This issue has been overcome by e.g. SAX-mediated depletion of acidic phosphopeptides. Yet, in our study, the higher representation of basic phosphopeptides by pY-MIP and pY-MIP-TiO₂ (compared to TiO₂ and TiO₂-pY-MIP) was not associated with increased identification of basophilic kinase motifs. Subtractive sequence motif analysis revealed a slight bias towards pY-phosphopeptides containing hydrophobic residues near the phosphorylation site.

CONCLUSIONS

We present the first thorough assessment of pY-MIP as an enrichment tool in phosphoproteomics using limited amounts of biological sample. We found that sequential phosphopeptide enrichment by pY-MIP and TiO₂ recovers pY- and also pT- (but not pS-) peptides in a more quantitative manner than by TiO₂ alone. This pY-MIP-TiO₂ protocol preferably recovers short, monophosphorylated peptides, with less acidic and more basic residues as compared to TiO₂. These findings demonstrate that pY-MIP-TiO₂ has analytical advantages and is particularly applicable to phosphoproteome analysis aimed at studies of low abundant pY- and pT-peptides.

ASSOCIATED CONTENT

Supporting Information

-Supplementary figures, tables and experimental details (pdf)
-List of detected phosphopeptides and peptides (Excel).

The Supporting Information is available free of charge on the ACS Publications website.

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