Ultratrace Enrichment of Tyrosine Phosphorylated Peptides on an Imprinted Polymer

ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2011

Impact Factor: 5.64 · DOI: 10.1021/ac103086v · Source: PubMed

analytical chemistry

TECHNICAL NOTE

pubs.acs.org/ac

Ultratrace Enrichment of Tyrosine Phosphorylated Peptides on an Imprinted Polymer

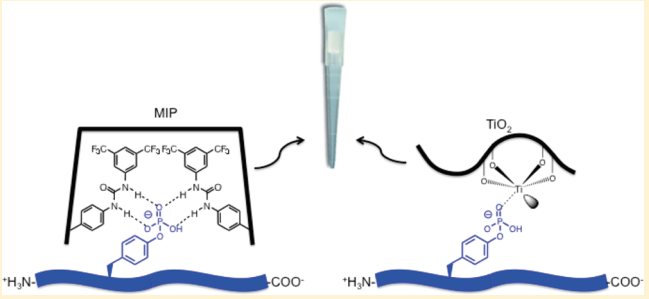
Stefan Helling,*[,†] Sudhirkumar Shinde,[§] Frederic Brosseron,[†] Anke Schnabel,[†] Thorsten Müller,[†] Helmut E. Meyer,[‡] Katrin Marcus,[†] and Börje Sellergren*[,§]

[†]Functional Proteomics and [‡]Medical Proteomics/Bioanalytics, Medizinisches Proteom-Center, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44801 Bochum, Germany

[§]INFU, Technische Universität Dortmund, Otto Hahn Strasse 6, D-44221 Dortmund, Germany

Supporting Information

ABSTRACT: Novel molecularly imprinted polymers (MIPs) designed to bind the side chain of phosphotyrosine can be used as artificial receptors for affinity-based enrichment of proteolytic peptides. In comparison with general enrichment methods for phosphorylated peptides such as TiO_2-based methods, the pTyr-imprinted polymers offered high selectivity for pTyr-containing peptides down to the low fmol level. This suggests MIPs as a new tool for affinity-based proteomics.



Enzyme catalyzed protein phosphorylation and dephosphorylation serves as a key regulatory mechanism of biological processes and is an established factor in numerous diseases.[1] Sensitive and selective methods capable of analyzing the phosphorylation state of proteins is, therefore, an urgent goal in drug discovery, proteomics, and diagnostics.[2,3] In humans, protein phosphorylation mainly involves the side chains of the amino acids Ser and Thr and to a lesser extent Tyr. Proteome wide quantitative analysis of these motifs at both protein and peptide level has proven very challenging.[4] One example is Tyr phosphorylation which is a substoichiometric modification often occurring in low-abundance proteins. Hence, this requires the use of highly selective enrichment strategies[5] with the most prominent involving pTyr specific antibodies,[6] chemoaffinity enrichment based on metal mediated chelation (immobilized metal ion affinity chromatography, IMAC),[7,8] Lewis acidic metal oxides (e.g., TiO_2),[9] strong cation-exchange chromatography (SCX),[10] or electrostatic repulsion hydrophilic interaction chromatography (ERLIC).[11] While antibodies show high selectivity and are extensively used for profiling tyrosine phosphorylation, they are costly and, as all proteins, succeptible to denaturation. On the other hand, the enrichment protocols based on chemoaffinity are not specific for a given phosphorylated amino acid residue, since they generally bind proteins or peptides containing pTyr, pSer, pThr, or other amino acids with an affinity for metal ions, e.g., cysteine, histidine. These phases have, therefore, found widespread use for the unbiased enrichment of phosphopeptides. This is not unproblematic given the bias commonly displayed by such phases with respect to the amino acid sequence or with respect to the number of phosphorylation sites.[2]

A method capable of overcoming these limitations, programmable to target phosphorylated amino acid residues or phosphopeptide sequences in a highly specific manner could, therefore, become an important asset in proteomics sample preparation. This can potentially be achieved by molecularly imprinted solid phase extraction relying on the use of robust polymer-based artificial receptors prepared by molecular imprinting.[12] In a first proof of principle, we show here that pTyr imprinted polymer receptors offer precisely such features. Their ability to provide selective enrichment of pTyr containing peptides clearly distinguishes the molecularly imprinted polymer (MIP)-based method from widespread applied protocols for general phosphopeptide enrichment (e.g., IMAC, TiO_2).

The MIP was prepared using urea monomer 1 in a 2:1 stoichiometric ratio to the template Fmoc-pTyrOEt (2). As previously described in detail,[13] each urea group establishes cyclic hydrogen bonds with the phosphate anion, leading post-polymerization to a neutral but exceptionally tight binding site for the pTyr side chain. A nonimprinted polymer (NIP) was prepared identically to the imprinted polymers but omitting the template. Imprinting effects were first assessed by chromatography using the crushed polymer monoliths as stationary phases. Thus, Fmoc amino acid derivatives were injected onto the columns in an acetonitrile rich mobile phase buffered with TFA. Table S-1 (Supporting Information) shows that the MIP exhibited a strong affinity for the low molecular pTyr analyte,

Received: November 23, 2010
Accepted: January 16, 2011
Published: February 09, 2011

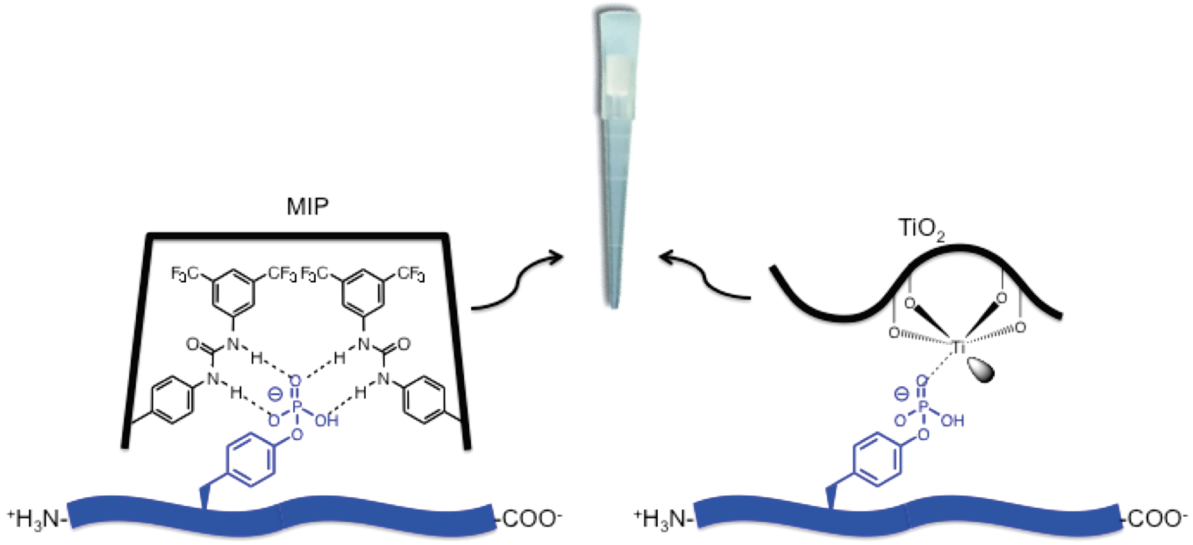


Figure 1. MIP and TiO_2 affinity sorbents compared in the pipet tip SPE format and anticipated principle binding interactions with a pTyr peptide.

considerably lower retentivity for pSer, and that the nonphosphorylated counterpart was only weakly retained. The NIP displayed weak retentivity for all analytes.

Solid phase extraction was performed using small columns in the form of disposable pipet tips packed up to the same bed height with stationary phase using C8 plugs to retain the material (Figure 1). Prior to packing, the sorbents were subjected to extensive conditioning (Supporting Information). For selectivity tests, samples were analyzed by electrospray ionization (ESI)-MS and the efficiency of the enrichment was calculated by comparing the peak intensities of pTyr containing peptide ion masses to their nonphosphorylated counterparts.

The first test of the pTyr−MIP was performed using phosphorylated angiotensin II (pAng II) (DRVpYIHPF) and its nonphosphorylated form (Ang II) as model peptides using a high load of peptide (400 pmol in a 200 μL sample volume) in order to facilitate detection (Figures 2A and S-1, Supporting Information). The highest peptide ion intensities were detected in the washing step, and only minor amounts were detected in the flow through. This reflects a good general binding capacity of the sorbent. Moreover, the relative MS intensities of Ang II and pAng II in the eluates confirmed that the MIP-based enrichment was selective. Compared to Ang II, the tyrosine-phosphorylated peptide was significantly enriched (t test, $p = 9.6 \times 10^{-6}$), in mean with 20-fold higher intensities corresponding to up to a 35-fold enrichment in single experiments. Meanwhile, using a control NIP as sorbent, the recovery was low (6.2%) and the enrichment of phosphopeptides only 5.5-fold.

In view of these promising results, the selectivity of the material was checked with a sample of higher complexity containing an equimolar mixture of Ang II, pAng II, the triply tyrosine-phosphorylated peptide pInsR and its nonphosphorylated form InsR, the serine-phosphorylated β-casein peptide (pCas), and the threonine-phosphorylated lipase peptide (pLipase), 400 pmol each. The peptide recovery in each fraction is shown in Figure 2B. First, we note that the 3-fold enrichment of pAng II over Ang II is lower by almost a factor of 10 compared to the results from the single component analysis. Moreover, the measured ion intensities were low, and only 1% of the total pAng II intensity was detected in the eluate (washing step, 98%). Instead, pronounced enrichment was observed for multiply phosphorylated peptides. Hence, with a 36% recovery in the eluate, the triply tyrosine-phosphorylated pInsR showed 12-fold enrichment compared to its nonphosphorylated form (t test, $p \leq 0.015$). Considering the poor ionization behavior of the pInsR peptide, the real enrichment factor for this peptide was higher, and by referencing with respect to the ion intensities in each fraction instead of the total intensities, the enrichment of the pInsR peptide was 33-fold compared to the InsR peptide. This difference correlated with the initially observed poor ionization characteristics in untreated peptide mixtures.

In order to evaluate the specificity of pTyr MIPs, we compared the method with a widely used technique for general phosphopeptide enrichment. For this, we performed a TiO_2-based affinity enrichment of the same model peptide mixture according to a well established protocol.[9] In this experiment, flow through and washing fractions were collected in the same vials and compared with the elution fractions with respect to the presence of phosphorylated and nonphosphorylated peptides (Figure 2C). The high signal intensities in the flow through fractions and low intensities in the elution fraction show that the nonphosphorylated peptides were not enriched. This contrasted with the enrichment of the singly phosphorylated peptides at serine, threonine, and tyrosine residues which were significantly enriched in the eluates. For instance, pAng II was 18-fold enriched with reference to Ang II (t test, $p \leq 0.0017$). These were expected results in agreement with previous literature reports.[9] However, the triply phosphorylated InsR peptide displayed only minor intensities in both fractions, and no significant enrichment was observed.

The extraction results shown in Figure 2 demonstrate a bias of the MIP for a pTyr containing peptide. This contrasts with TiO_2 where monophosphorylated pTyr, pSer, and pThr peptides are enriched to a roughly equal extent. Thus, the MIP displays a preference for phosphorylation motifs in accordance with the choice of template, illustrating a fundamental difference from the less discriminative behavior of TiO_2 sorbents. As previously noted by Thingholm and co-workers,[9] multiply phosphorylated peptides exhibit stronger affinity to the TiO_2 material than singly

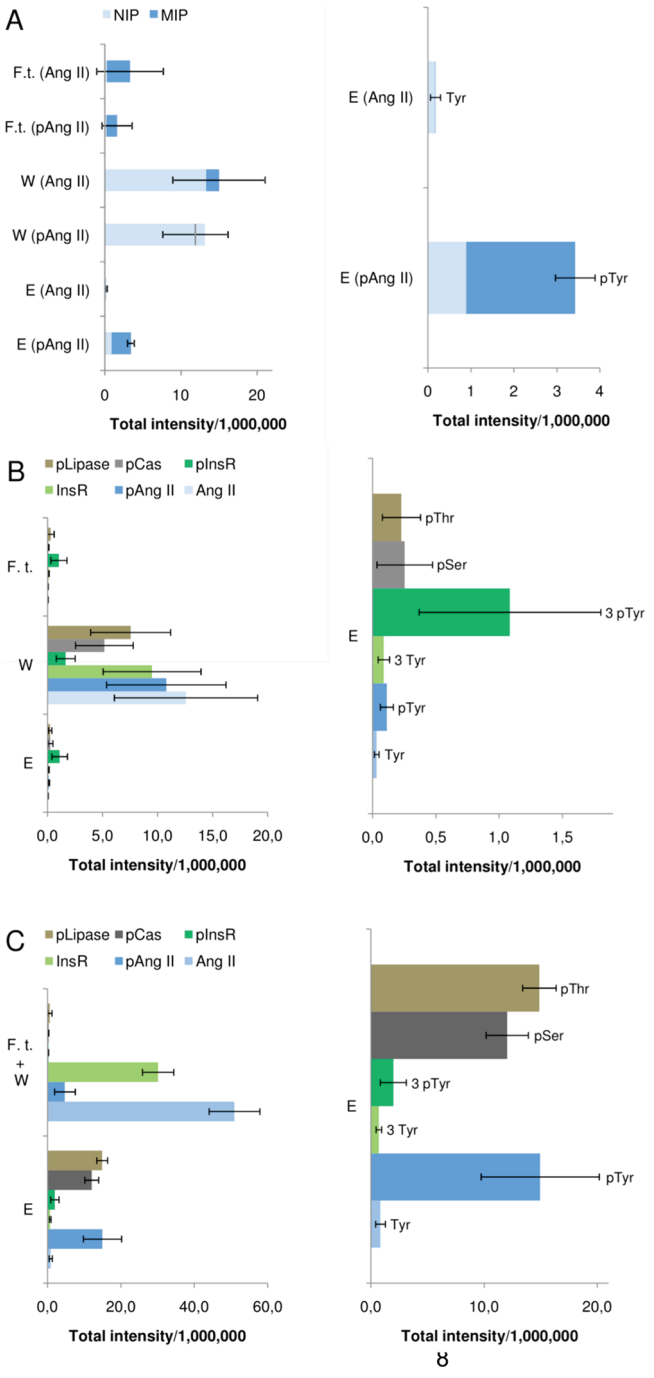


Figure 2. Ion intensities of standard peptide fractions analyzed by ESI-MS after pTyr−MIP, NIP, and TiO_2 enrichments. (A) Ion intensities of Ang II and pAng II enriched by pTyr−MIP material and the non-imprinted material (NIP) as control, considering the flow through (F.t.), washing step (W), and the eluate (E), and on the right, an enlarged view of the eluate fraction data. The intensity of the pAngII signal in the wash fraction after the MIP-based enrichment is denoted by a gray line. (B and C) Enrichment of Ang II, pAng II, InsR, pInsR, pCas, and pLipase with (B) a pTyr−MIP and (C) a TiO_2 column. The phosphorylated amino acid residue and the number of phosphorylations have been indicated.

phosphorylated species and are, therefore, hard to displace from the sorbent once bound. This is a plausible explanation for the poor recovery of the triply phosphorylated pInsR peptide. Additionally, for the MIP, the high enrichment of the triply tyrosinephosphorylated peptide at the expense of the monophosphorylated peptide indicates a competitive behavior for binding to a limited number of sites. Apart from size exclusion effects arising from the meso- and microporosity of the materials (Table S-3, Supporting Information), the capacity is limited by the heterogeneous binding site distribution with only a limited number of high energy binding sites.[13] In the TFA modified mobile phase, the capacity of the high affinity sites are estimated to be less than 10 pmol/mg MIP which in view of the sample load of 400 pmol total peptide would suggest that the sorbent is heavily overloaded in these experiments. This situation is different in the case of TiO_2 enrichment which displays a high capacity directly scaling with the accessible surface area of the sorbent.[9] In the case of pTyr enrichment, the limited capacity is less problematic since the concentration of such peptides typically is much lower. We, therefore, decided in a subsequent experiment to check the effect of sample loading on enrichment and recovery.

In order to test the sensitivity of the MIP-based enrichment, the pTyr peptide pAng II was spiked in amounts of 1 pmol down to 1 fmol to a tryptic digest of 10 pmol of bovine fetuin (Figures 3 and Figure S-2, Supporting Information). Given that the fetuin digest is abundant in doubly serine-phosphorylated peptides which could interfere with the efficiency of the pTyr−MIP enrichment (Figure S-3, Table S-2, Supporting Information), this would offer a stringent test for the potential of the MIP-based enrichment. Interestingly, regardless of the amount of spiked peptide, the pAng II peptide ion could be unequivocally identified in the eluates by its fragment ion pattern. Moreover, the doubly serine-phosphorylated fetuin peptide HTFSGVApSVESSpSGEAFHVGK was absent in these fractions. The protein identification with Mascot resulted in the identification of no protein or in single experiments of just one protein in the eluate fractions, the α-2-HS-glycoprotein precursor with only one peptide (TPIVGQPSIPGGPVR; see Supporting Information for more details). Instead, the bulk of the protein was recovered in the flow through and washing fractions. Comparable experiments with TiO_2 material displayed higher limits of detection and lacked the pTyr selectivity observed with the MIP. The phosphorylated peptides pAng II and pLipase could here be detected down to the 50 fmol level.

CONCLUSIONS

As we have demonstrated here, phosphopeptide enrichment using a pTyr imprinted polymer results in a high selectivity and affinity for peptides containing the imprinted amino acid. A pTyr−MIP could, hence, be used to capture pTyr peptides at the femtomol level in the presence of 4 orders of magnitude higher concentrations of proteolytic peptide fragments additionally containing serine-phosphorylated peptides. The same MIP exhibited an interesting preference for a triply phosphorylated over a monophosphorylated peptide, contrasting with the opposite preference exhibited by the TiO_2 affinity enrichment. At the same time, we note that the presently used MIP represents a first generation material and that this proof of concept is not limited to phosphotyrosine enrichments. MIPs for other motifs (pSer, pThr) perform as well as the pTyr MIP in this report, and we believe that these receptors collectively will satisfy new needs in affinity enrichment not met by current generation antibodies.

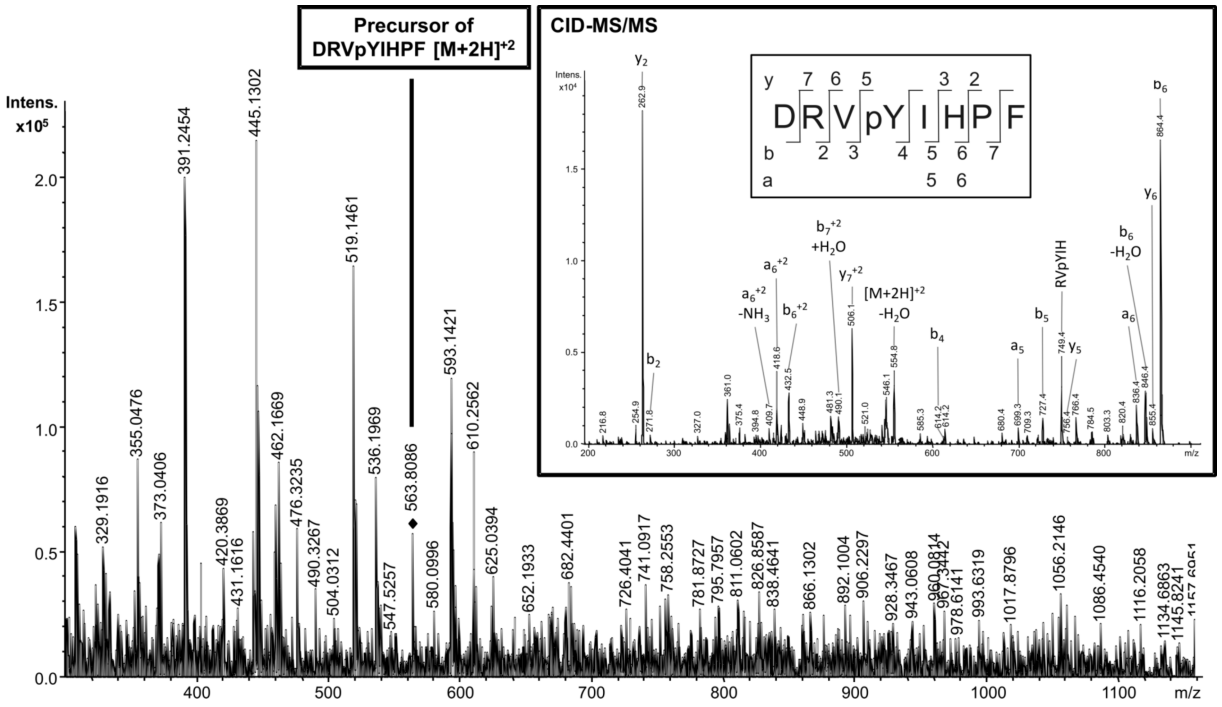


Figure 3. NanoLC-ESI-MS/MS analysis of the MIP-enriched pAng II peptide (1 fmol) spiked into a tryptic digest of bovine fetuin (10 pmol). The spectra represent the MS detection of HPLC-eluted pAng II. The doubly charged precursor ion, detected in the MS scan (marked ion), was automatically chosen for a collision-induced dissociation (CID)-fragmentation experiment, unequivocally explaining the peptide sequence by the detected fragment ions.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, including details of the polymer production, HPLC evaluation, peptide enrichment, and peptide identification. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: stefan.helling@rub.de (S.H.) and B.Sellergren@infu.uni-dortmund.de (B.S.).

■ ACKNOWLEDGMENT

K.M. and B.S. contributed equally to this work. This project was financially supported by the Deutsche Forschungsgemeinschaft (DFG) under Project Numbers Se 777/9-1 and MA 3257/2-1 and by the BMBF (Grant 01 GS 08143), as well as by Forum (Forschungsförderung Ruhr-Universität Bochum Medizinische Fakultät; F656-2009).

■ REFERENCES

(1) Johnson, S. A.; Hunter, T. *Nat. Methods* **2005**, *2*, 17–24.
(2) Bodenmiller, B.; Mueller, L. N.; Mueller, M.; Domon, B.; Aebersold, R. *Nat. Methods* **2006**, *4*, 231–237.
(3) Sickmann, A.; Meyer, H. E. *Proteomics* **2001**, *1*, 200–206.
(4) Piggee, C. *Anal. Chem.* **2009**, *81*, 2418–2420.
(5) Rush, J.; Moritz, A.; Lee, K. A.; Guo, A.; Goss, V. L.; Spek, E. J.; Zhang, H. X.; Zha, X.-M.; Polakiewicz, R. D.; Comb, M. J. *Nat. Biotechnol.* **2005**, *23*, 94–101.
(6) Lemeer, S.; Ruijtenbeek, R.; Pinkse, M. W.; Jopling, C.; Heck, A. J.; den Hertog, J.; Slijper, M. *Mol. Cell. Proteomics* **2007**, *6*, 2088–2099.
(7) Anderson, L.; Porath, J. *Anal. Biochem.* **1986**, *154*, 250–254.
(8) Li, S.; Dass, C. *Anal. Biochem.* **1999**, *270*, 9–14.
(9) Thingholm, T. E.; Jorgensen, T. J.; Jensen, O. N.; Larsen, M. R. *Nat. Protoc.* **2006**, 1929–1935.
(10) Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villén, J.; Li, J.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P. *Proc. Natl. Acad. Sci.* **2004**, *101*, 12130–12135.
(11) Alpert, A. J. *Anal. Chem.* **2008**, *80*, 62–76.
(12) Alexander, C.; Andersson, H. S.; Andersson, L. I.; Ansell, R. J.; Kirsch, N.; Nicholls, I. A.; O'Mahony, J.; Whitcombe, M. J. *J. Mol. Recognit.* **2006**, *19*, 106–180.
(13) Emgenbroich, M.; Borrelli, C.; Shinde, S.; Lazraq, I.; Vilela, F.; Hall, A. J.; Oxelbark, J.; De Lorenzi, E.; Courtois, J.; Simanova, A.; Verhage, J.; Irgum, K.; Karim, K.; Sellergren, B. *Chem.—Eur. J.* **2008**, *14*, 9516–9529.