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CELINA WIERZBICKA

NEW FRACTIONATION TOOLS TARGETING ELUSIVE POST-TRANSLATIONAL MODIFICATIONS

MALMÖ UNIVERSITY

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Front illustration: Fishing the phosphotyrosine peptides out of a peptide mixture

by Katarzyna Czyżyńska-Gołos

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CONTENTS

ABBREVIATIONS	7
ABSTRACT	9
POPULÄRVETENSKAPLIG SAMMANFATTNING	11
LIST OF PUBLICATIONS	13
INTRODUCTION	15
Protein post-translational modifications	15
Protein phosphorylation	16
Protein histidine phosphorylation – an elusive PTM	17
The role of phosphorylation in disease pathogenesis	19
Limitations in phosphoproteomic studies	20
Enrichment strategies for phosphoproteomics	21
Enrichment techniques	21
Fractionation techniques	23
Combined methods	24
Molecularly imprinted polymers	25
MIPs for phosphoproteomics	26
Urea-based functional monomers	28
Imidazolium-based functional monomers	30
METHODS	32
Batch binding test	32
Molecularly imprinted solid phase extraction	35
High-performance liquid chromatography	36
Mass spectrometry	39
Nuclear magnetic resonance	40
Thermogravimetric analysis	43

RESULTS AND DISCUSSION	44
Paper I	44
Paper II	45
Paper III	47
Paper IV	49
Paper V	50
CONCLUDING REMARKS AND FUTURE OUTLOOK.....	52
ACKNOWLEDGEMENTS	54
REFERENCES	56
PAPERS I-V	65

ABBREVIATIONS

ACN	Acetonitrile
BSA	Bovine serum albumin
Da	Dalton
ESI	Electrospray ionization
EDMA	Ethylene glycol dimethacrylate
HPLC	High-performance liquid chromatography
His	Histidine
IMAC	Immobilized metal affinity chromatography
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
MS	Mass spectrometry
MALDI	Matrix assisted laser desorption/ionization
MOAC	Metal oxide affinity chromatography
MIP	Molecularly imprinted polymer
Fmoc	N-(9-fluorenylmethoxy)carbonyl
NIP	Non-imprinted polymer
PAC	Phosphoramidate chemistry
PETA	Pentaerythritol triacrylate
pHis	Phosphohistidine
pSer	Phosphoserine
pThr	Phosphothreonine
pTza	Phosphonotriazolylalanine
pTyr	Phosphotyrosine
PTM	Post-translational modification
SAX	Strong anion exchange
SCX	Strong cation exchange
Ser	Serine
Thr	Threonine
TEA	Triethylamine
TFA	Trifluoroacetic acid
Tyr	Tyrosine

ABSTRACT

Protein phosphorylation is a reversible post-translational modification (PTM) playing a central role in numerous biological events including disease pathogenesis. Thus, the analysis of phosphoproteome is crucial for understanding cellular regulation processes and can facilitate the development of new diagnostic and therapeutic tools.

Phosphoproteins are typically analyzed using liquid chromatography coupled with mass spectrometry (LC-MS) after proteolytic processing. However, phosphopeptides are notoriously difficult to analyze by LC-MS due their low abundance and transient nature. This creates a need for effective enrichment tools for phosphorylated proteins and peptides prior to mass spectrometry analysis.

The work presented in this thesis is focused on development and validation of methods and tools for enrichment of phosphopeptides with the use of molecular imprinting technology. In particular, the targeted PTMs include phosphorylation on tyrosine (pTyr) and histidine (pHis).

The key recognition element employed in developed synthetic receptors was 1,3-diaryl urea functional monomer FM1. This monomer is a potent hydrogen bond donor forming strong cyclic hydrogen bonds with oxyanions such as phosphates. The bias of the imprinted urea-based receptor towards different phosphorylated residues can be programmed by selection of the template. Thus, the N, C-protected phosphotyrosine and phosphonotriazolylalanine were used as templates to generate phosphotyrosine (pTyr MIP) and phosphohistidine (pHis MIP) selective molecularly imprinted polymers, respectively.

The application of previously reported pTyr MIP for phosphoproteomic studies was validated on complex biological samples of the mouse brain lysate digest spiked with standard peptides and HeLa cells digested proteins. Furthermore, the pTyr MIP was developed in the format of microspherical porous

beads characterized by uniformly sized and shaped particles with increased surface area and pore size as well as improved binding affinity and selectivity for larger pTyr peptides (2-3 kDa). This opens the way to generation of capture materials suitable for middle-down phosphoproteomics.

In response to the lack of adequate tools and methods for enrichment of acid-labile phosphohistidine peptides a pHis MIP-based approach is proposed as a solution. The method involving selective dephosphorylation of phosphoserine (pSer) peptide by alkali treatment of the sample, followed by extraction of base-stable pHis peptides with MIP was demonstrated on the sample of bovine serum albumin digest spiked with standard pSer and pHis peptides.

The last part of this thesis is focused on improving the recognition of phosphopeptides in aqueous media – the natural environment of biological samples. Guided by the principles of supramolecular chemistry, novel cationic host monomers were introduced for binding phosphates by ionic hydrogen bonds. These were used to synthesize MIPs showing enhanced binding of phosphopeptides in aqueous media.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Människokroppen är en komplex maskin baserad på tusentals biokemiska reaktioner varje sekund. En avgörande faktor för att säkerställa integriteten hos hela organismen är kommunikationen mellan enskilda celler. En av de metoder som används av levande organismer för detta ändamål är reversibel fosforylering av proteiner. Studien av kommunikationsvägar mellan celler är mycket viktigt för att förstå biologiska processer och sjukdomsförlopp.

Syftet med denna avhandling var att utveckla verktyg och metoder som underlättar studier av fosforylerade proteiner och peptider. Dagens analytiska metoder bygger på att proteinerna i ett biologiskt prov först bryts ned och att de resulterande peptiderna därefter anrikas följt av kvantitativ eller kvalitativ analys oftast med hjälp av mass spektrometri. Denna avhandling fokuserar på utveckling av syntetiska receptorer som kan diskriminera mellan fosforylerade och icke fosforylerade peptider. Dessa material framställdes med hjälp av molekylär imprinting teknik som gör det möjligt att skapa polymera material med en fördefinierad selektivitet för en speciell mål-molekyl eller molekyl-klass. Avtrycken görs genom polymerisering av syntetiska monomerer i närvaro av en mall (exempelvis en fosforylerad peptid eller dess fragment) vilken avlägsnas då polymeren har bildats. Detta ger upphov till hålrum med minne för mallen med avseende på dess form, storlek och funktionalitet. Likt en biologisk receptor kan polymeren därför känna igen molekyler som innehåller fragment med mallens struktur.

Detta enkla koncept användes för att generera syntetiska receptorer för molekylär igenkänning av peptider innehållande fosforylerade tyrosin och histidin-rester vilka idag är svåra att analysera. Metoder för anrikning av fosfopeptider under användning av molekylärt präglade polymerer validerades med av-

seende på existerande standardmetoder och enkla modelsystem samt på komplexa cell prov.

Fördelarna med materialen och metoderna som rapporteras i denna avhandling är först och främst att de i ett fall erbjuder en unik lösning (phosphohistidin) men generellt är fördelarna deras höga selektivitet kombinerat med hög stabilitet, reproducerbarhet, och enkla protokoll. Dessa erbjuder alltså både nya lösningar samt attraktiva alternativ till dagens etablerade anrikningstekniker.

LIST OF PUBLICATIONS

- I. Chen, J.; Shinde, S.; Subedi, P.; **Wierzbicka, C.**; Sellergren, B.; Helling, S.; Marcus, K. Validation of molecularly imprinted polymers for side chain selective phosphopeptide enrichment. *Journal of Chromatography A*, 1471 (2016) 45–50.
- II. Bllaci, L.; Torsetnes, S. B.; **Wierzbicka, C.**; Shinde, S.; Sellergren, B.; Rogowska-Wrzesinska, A.; Jensen, O. N. Phosphotyrosine biased enrichment of tryptic peptides from cancer cells exploiting combined pY MIP and TiO₂ affinity. Manuscript.
- III. **Wierzbicka, C.**; Torsetnes, S. B.; Jensen, O. N.; Shinde, S.; Sellergren, B. Hierarchically templated beads with tailored pore structure for phosphopeptide capture and phosphoproteomics. *RSC Advances*, Accepted (2017).
- IV. **Wierzbicka, C.**; Gajoch, K.; Jensen, O. N.; Sellergren, B. Selective enrichment of histidine phosphorylated peptides combining β -elimination and MIP-based pHis capture. Submitted for publication.
- V. **Wierzbicka, C.**; Liu, M.; Irgum, K.; Sellergren, B. Cationic pTyr/pSer imprinted polymers based on a bis-imidazolium host monomer: Phosphopeptide recognition in aqueous buffers demonstrated by μ -liquid chromatography and monolithic columns. *Journal of Materials Chemistry B*, 5 (2017) 953-960.

Contribution:

Paper I. Synthesis of templates, functional monomers and polymers.

Paper II. Synthesis of templates, functional monomers and polymers. Participation in manuscript preparation.

Paper III. Synthesis of all materials. Performance of experiments and characterizations (except SEM imaging, elemental analysis and nitrogen sorption), evaluation of data and writing the manuscript.

Paper IV. Synthesis of all materials. Performance of all experiments, evaluation of data and writing the manuscript.

Paper V. Synthesis of templates, functional monomers and polymers (except capillary monoliths). Performance of experiments (except characterization of capillary monoliths and micro-liquid chromatography), evaluation of data and writing the draft of the manuscript.

INTRODUCTION

Protein post-translational modifications

Biosynthesis of proteins is a complex multistep process involving transcription of information encoded in DNA to mRNA followed by translation step, where amino acids are assembled to form proteins. Post-translational modifications (PTMs) occur on proteins after the translation is complete and are responsible for change of structure and functions of proteins. These covalent modifications, including phosphorylation, acetylation, methylation, glycosylation and ubiquitination (Table 1) regulate nearly all biological processes in living organisms.¹⁻³

Table 1. Common post-translational modifications.

PTM	Function (examples)
Phosphorylation	Cell proliferation and differentiation, signal transduction
Acetylation	Protein stability
Methylation	Regulates protein–protein and protein–nucleic acid interactions
Glycosylation	Protein–ligand interactions, protein folding and conformation
Ubiquitination	Protein degradation

For example, reversible phosphorylation of proteins acts as a function on/off switch. Acetylation of lysine residues in histone is linked to transcription regulation.⁴ There is also a link between acetylation and cellular metabolism.^{5, 6} Protein methylation is known to be involved in cellular stress responses⁷ and

protein aging and repair.⁸ Glycosylation is an essential modification involved in fundamental processes such as inflammation, immune response, cell growth or protein folding and stability, just to name a few.^{9, 10} Proteolytic destruction of proteins, on the other hand, is initiated by protein modification with ubiquitin (ubiquitination).¹¹ The analysis of PTMs is therefore essential for understanding fundamental biological functions.

Protein phosphorylation

Phosphorylation is one of the most widely studied PTMs affecting basic biological processes such as cell growth, proliferation, differentiation, and apoptosis as well as cellular signal transduction.¹² It can occur on nine amino acids, i.e. serine, threonine, tyrosine, histidine, lysine, arginine, aspartic acid, glutamic acid, and cysteine. However, the phosphorylation of the former three amino acids capture most attention in phosphoproteomics research (Figure 1).

Figure 1. Structures of phosphoserine, phosphothreonine and phosphotyrosine.

This reversible modification is tightly regulated by the action of protein kinases and phosphatases being responsible for phosphorylation and dephosphorylation, respectively. Protein kinases catalyze the transfer of the γ -phosphate from adenosine triphosphate (ATP) to the specific residues in proteins (Figure 2).¹² Protein phosphatases, on the other hand, remove the phosphate group by enzymatic hydrolysis.

It is estimated that approximately 30% of proteins in eukaryotic organisms are phosphorylated at any time.¹³ In human, there are about 500 kinases and 100 phosphatases coded by ca. 2% of human genome.¹⁴ In eukaryotic organisms phosphorylation occurs predominantly on Ser, Thr and Tyr residues with

the ratio of 1800:200:1 (pSer:pThr:pTyr).¹⁵ Nevertheless, phosphorylation on histidine can also occur although it often remains undetected due to its acid-labile nature.

Figure 2. Protein phosphorylation cycle catalyzed by a kinase. Reproduced from Ubersax and Ferrell¹² with permission from Nature Publishing Group.

In recent years, however, an increasing interest in analysis of this elusive phosphorylation has been observed resulting in development of techniques for enrichment, detection and analysis of pHis peptides.¹⁶⁻²⁰

Protein histidine phosphorylation – an elusive PTM

Phosphorylation of histidine is well known to be involved in two component signaling pathways in prokaryotes and lower eukaryotes²¹⁻²³ and it is now becoming recognized in mammals and implicated in certain human disease states.²⁴⁻²⁷ It has been estimated that histidine phosphorylation in eukaryotes accounts for 6% of total protein phosphorylation,²⁸ thus it is significantly more abundant than pTyr modification. Nonetheless, little is known about the biological role of pHis as compared to phosphoester modifications. This is due to significant technical challenges in enrichment and detection of this PTM.

There are three possible isomers of phosphohistidine (pHis), i.e. π -pHis, τ -pHis and κ -pHis (Figure 3) of which the former two have been found *in*

vivo.¹⁶ Unlike the phosphoesters (pSer, pThr, pTyr), the phosphoryl group of pHis is attached to nitrogen atom making it a phosphoramidate. This high energy phosphoramidate bond is susceptible to hydrolysis in acidic environment and it defines unique stability profile of phosphohistidine.^{29, 30}

Figure 3. Structures of π -phosphohistidine, τ -phosphohistidine and π , τ -diphosphohistidine.

While O-phosphorylated amino acids, pSer and pThr, are stable in acidic media and labile in alkali environment, the phosphoramidates display reversed stability profile, i.e. they are base-stable and acid-labile. Most of the methods used in the phosphopeptide enrichment and detection involve acidic treatment, and thus fail to preserve pHis modification. Therefore, dedicated methods and tools are needed to study this PTM.

To date, several methods for detection and analysis of pHis have been reported.^{17, 29, 30} Generation of synthetic pHis proteins and peptides has clearly facilitated the development of such methods. Medzihradszky et al.³¹ used potassium phosphoramidate to synthesize histidine phosphorylated peptides. This method is highly selective for histidine and does not phosphorylate any other residues. The complex kinetics of the reaction prove that initially π -pHis is formed followed by π , τ -pHis and τ -pHis. Long reaction times (>12h) result in the disappearance of π -pHis yielding only the latter two isomers.³² This method was also successfully used for phosphorylation of proteins.^{17, 18, 33}

Although chemical phosphorylation gives access to pHis protein and peptide standards, it does not solve the problems related to high acid lability of this modification. For example, attempts to use pHis-based immunogens to produce antibodies failed due to facile dephosphorylation of pHis.^{34, 35} To overcome these issues, several stable analogues of pHis have been developed such as phosphonofurylalanine (1),³⁶ phosphonopyrrolylalanine (2)²⁹ and phsophonotriazolylalanine (3, 4)^{34, 37, 38} based analogues (Figure 4).

Figure 4. Examples of stable phosphohistidine analogues.

Kee et al.³⁴ successfully incorporated analog 3 in human histone H4 peptide sequence by solid-phase peptide synthesis (SPPS). This peptide was next used as an antigen to raise sequence specific antibody that could recognize histidine phosphorylated histone H4 but not the unmodified protein. After this first report on successful generation of pHis antibodies, the phosphoryltriazolyl-based analogues were used to produce pan-specific polyclonal pHis antibody³⁹ and monoclonal 1- and 3-pHis antibodies.⁴⁰

Despite a remarkable progress in analysis of pHis in recent years, this area of phosphoproteomics research is still in its infancy, especially in higher eukaryotes. Without a doubt, development of new mild enrichment techniques and advances in mass spectrometry analysis will have significant impact on elucidation of the biological role of pHis.

The role of phosphorylation in disease pathogenesis

Phosphorylation is responsible for regulation of many important processes such as cell proliferation, differentiation and apoptosis and plays a vital role in signal transduction. Disruptions in signaling pathways, regulated by kinases and phosphatases, are often recognized as a cause or a consequence of various diseases, including cancer,⁴¹⁻⁴³ neurodegenerative diseases,⁴⁴⁻⁴⁶ cardiovascular diseases,^{47, 48} inflammatory diseases,^{49, 50} and diabetes.⁵¹ This has led to an increasing effort to identify kinases and phosphorylated proteins that could serve as disease biomarkers or drug targets, especially in cancer.

More than 1200 proteins have been reported to be differentially expressed in human cancer,⁵² of which many belong to kinase family. These proteins are potential candidates for cancer biomarkers, however, due to technical limita-

tions in proteomic analysis of low abundant proteins as well as rigorous requirements for marker validation, only a limited number of these proteins has been approved by the US Food and Drug Administration (FDA) for clinical use. In fact, only three kinases were approved so far as cancer biomarkers, i.e. epidermal growth factor receptor for selection of therapy for colon cancer, KIT for diagnosis and selection of therapy of gastrointestinal stromal tumor and Her2 for prognosis, monitoring and selection of therapy for breast cancer.^{41, 53}

Kinases involved in cancer development can also serve as drug targets. In targeted cancer therapy two main approaches for kinase inhibition have emerged, i.e. based on monoclonal antibodies and small-molecule agents.⁵⁴ The prominent examples of trastuzumab (Herceptin®, Genentech) and imatinib (Gleevec®, Novartis) being the first FDA approved monoclonal antibody (1998) and small-molecule (2001) kinase inhibitors, respectively, paved the way for a new era of targeted cancer therapy. As many as 28 small-molecule kinase inhibitors have been approved by FDA by June 2015.^{55, 56} The fact that 19 of these drugs were approved since 2011, indicates a huge potential of such therapeutic approach.

Limitations in phosphoproteomic studies

It is evident that advances in phosphoproteomics had an enormous impact on the identification of novel disease biomarkers and drug targets. For a complete understanding of signaling pathways and its role in disease pathogenesis a comprehensive analysis of phosphoproteome is needed. This includes the identification of phosphoproteins and phosphopeptides, localization of the phosphorylation sites as well as quantitation of phosphorylation.¹³ Despite a tremendous advancement in MS-based techniques in recent years, the analysis of phosphoproteins and phosphopeptides is not a trivial task. Main challenges arise from low stoichiometry of phosphorylation, its transient nature and low abundance of signaling molecules. These issues are particularly evident when it comes to analysis of tyrosine phosphorylation. For example, in vertebrate cells, the tyrosine phosphorylation accounts for 0.05% of total protein phosphorylation.⁵⁷ Furthermore, the ionization of phosphopeptides in MS is often suppressed in presence of nonmodified peptides.¹³ For these reasons, it is a common practice to implement enrichment and/or fractionation steps prior to MS analysis.

Enrichment strategies for phosphoproteomics

Over the past years, many techniques to concentrate phosphoproteins and phosphopeptides from complex biological samples have been developed.⁵⁸⁻⁶⁰ Overall, these methods can be classified into two categories, i.e. more specific *enrichment techniques* (e.g. immunoaffinity-based methods, chemical derivatization and affinity chromatography, see Figure 5) and less specific *fractionation techniques* (HILIC, SAX, SCX). In a comprehensive phosphoproteomic studies enrichment and fractionation methods are often combined for better coverage of phosphoproteome.

Enrichment techniques

Immunoaffinity-based methods rely on selective capture of phosphoproteins by specific antibodies against phosphorylated serine, threonine and tyrosine residues.⁶¹⁻⁶³ In general, however, antibodies raised against pSer and pThr suffer from limited selectivity and therefore are not routinely used in phosphoproteomic studies.⁶⁴ Anti-phosphotyrosine antibodies, on the other hand, display high selectivity and are extensively used in the analysis of this low abundant modification at the level of both phosphoproteins⁶⁵⁻⁶⁷ and phosphopeptides.⁶⁸⁻⁷² Nevertheless, the immunoaffinity-based methods suffer from a number of drawbacks. For example, typically a large amount of protein sample (in mg scale) is required.⁷³ Furthermore, the high price of antibodies characterized by low stability and non-reusability along with laborious protocols call for more cost- and time-efficient alternative methods.

Immobilized metal affinity chromatography (IMAC) is based on interactions of negatively charged phosphopeptides with positively charged metal ions (Fe^{3+} , Al^{3+} , Ga^{3+} , Co^{2+} , Zr^{4+} and Ti^{4+}) chelated to nitrilotriacetic acid or iminodiacetic acid coated beads. This method is applicable for efficient extraction of pSer, pThr and pTyr proteins⁷⁴ and peptides.⁷⁵⁻⁷⁷ However, this technique suffers from significant non-specific binding of nonphosphorylated peptides bearing acidic residues. O-methyl esterification of carboxylic side chains was reported to significantly reduce this effect,⁷⁸ nevertheless, this derivatization method is not quantitative, thus it results in an increase of sample complexity.⁷⁹ Alternatively, loading the sample at low pH (50% ACN 0.1% TFA) on IMAC resin proved to reduce non-specific binding due to protonation of carboxyl groups of peptides.⁸⁰ Recently Zhou et al.^{81, 82} reported novel Ti^{4+} -IMAC resin showing superior selectivity and efficiency compared to Fe^{3+} -IMAC, Zr^{4+} -IMAC, TiO_2 and ZrO_2 in both semi-complex mixture of

tryptic peptides as well as digest of mouse liver lysate. The Cu^{2+} -IMAC, on the other hand, was applied for extraction of pHis peptide from histidine phosphorylated protein digest.⁸³

Figure 5. Analytical strategies for phosphoprotein and phosphopeptide enrichment. Reproduced from Thingholm et al.⁵⁸ with permission from John Wiley and Sons.

Metal oxide affinity chromatography (MOAC) is another technique for extraction of phosphopeptides. Although a number of different metal oxides, including zirconium, aluminum, iron, and tin were applied for phosphopeptide enrichment⁸⁴ the titanium dioxide (TiO_2) is most widely used.^{85, 86} The nature of metal oxide and phosphate group interaction is characterized by Lewis acid/base interaction with bidentate mode^{84, 87} meaning that phosphopeptides are well retained in acidic conditions. Thus, typically loading of the sample is performed at pH 2-3 while elution occurs in strongly alkaline conditions. In general, TiO_2 enrichment is considered to be more specific for binding of phosphopeptides compared to IMAC,⁶⁰ although it is not free from unspecific

binding of nonphosphorylated peptides. This issue was however largely diminished by optimization of the protocol, e.g. by using additives in the loading step such as 2,5-dihydroxybenzoic acid,⁸⁶ lactic acid,⁸⁸ glycerol⁸⁹ or glycolic acid⁹⁰ that compete with nonphosphorylated peptides for binding to low affinity sites. The TiO₂-based phosphopeptide enrichment has been employed in several large scale phosphoproteomic studies.⁹¹⁻⁹⁴

Chemical derivatization methods are based on exploiting unique properties of phosphorylated residues that can be selectively modified prior to enrichment step. For example, pSer and pThr undergo facile dephosphorylation *via* β -elimination in alkali conditions resulting in dehydroalanine and β -methyldehydroalanine residues, respectively. A subsequent Michael addition reaction with ethanedithiol allows for modification with biotin tag and consequent enrichment with immobilized avidin⁹⁵ or direct affinity purification on activated thiol resin.⁹⁶ These methods are however specific only for enrichment of pSer and pThr since pTyr does not undergo β -elimination. Additionally, a co-elution of O-glycosylated proteins/peptides, that are susceptible to β -elimination, can occur. Another method, applicable for enrichment of pSer, pThr and pTyr peptides is based on phosphoramidate chemistry (PAC).⁹⁷ Phosphopeptides are immobilized on a solid support in a multistep process *via* phosphoramidate bond and subsequently isolated by hydrolysis in acidic media. In general, chemical derivatization methods are not routinely used in phosphoproteomic studies due to laborious protocols and non-quantitative character of reactions that leads to increase of sample complexity.

Fractionation techniques

Fractionation techniques are usually employed in a form of column liquid chromatography allowing for a gradual separation of a sample based on properties such as charge or hydrophilicity. These methods are not as efficient and specific as enrichment techniques, nevertheless, they are often employed in phosphoproteomics workflows, especially in combination with enrichment techniques.

Ion exchange chromatography allows to separate phosphopeptides from nonphosphorylated peptides due to their different charge states. In strong cation exchange (SCX) negatively charged phosphopeptides interact poorly with anionic stationary phase and therefore elute early from the column. This contrasts with the strong anion exchange (SAX) where phosphopeptides are strongly retained on the cationic stationary phase.⁹⁸ However, the separation is not always straightforward since the charge state of peptides is affected by the

number of present acidic and basic residues in the sequence. Both SCX and SAX have been used for prefractionation of phosphopeptides prior to enrichment, e.g. with IMAC or TiO_2 .^{69, 75, 79, 99, 100}

In hydrophilic interaction chromatography (HILIC) the neutral hydrophilic stationary phase is used.¹⁰¹ Typically the elution is performed in the acetonitrile-water system starting with high organic mobile phase. Thus, peptides are separated according to their hydrophilicity/hydrophobicity with more polar peptides (for example phosphopeptides) eluting later. HILIC was shown to be an efficient prefractionation method prior to specific phosphopeptide enrichment.¹⁰²

Combined methods

Due to partial complementarity of described methods, it is a common practice to combine two or more techniques in a multidimensional enrichment workflow. Typically, a prefractionation method (e.g. SCX, SAX, HILIC) is applied before specific enrichment step (e.g. IMAC or MOAC) although combination in reverse order has also been reported.¹⁰³ In another approach, the so-called SIMAC (sequential elution from IMAC),¹⁰⁴ the multi-phosphorylated peptides are bound to IMAC resin while the unbound fraction containing mono-phosphorylated peptides is loaded on the TiO_2 . Thus, the elution fractions from IMAC and TiO_2 are enriched with multi- and mono-phosphorylated peptides, respectively.

Overall, each of the enrichment and fractionation methods has advantages and limitations and none of these techniques can provide full coverage of phosphoproteome. Due to significant differences in reported protocols such as sample type and amount, instrument setup, data processing, etc. it is very difficult to make a direct comparison of available enrichment methods. Bodenmiller et al.¹⁰⁵ made an attempt to compare and assess reproducibility and specificity of commonly used methods, i.e. Fe^{3+} -IMAC, PAC and TiO_2 using tryptic digest of the cytosolic fraction of *Drosophila melanogaster* Kc167 cells. It was shown that all the investigated methods are highly reproducible, however, IMAC and PAC gave the best result allowing for identification of >500 phosphopeptides and insignificant contamination with nonphosphorylated peptides (9 and 34, respectively). Importantly, the overlap of identified phosphopeptides was only 33-35% indicating differences in selectivity of investigated methods. This confirms that a combination of several methods can increase the coverage of studied phosphoproteome.

Molecularly imprinted polymers

Molecular imprinting is an attractive approach to form synthetic materials with molecular recognition properties.¹⁰⁶⁻¹⁰⁸ This technique is based on copolymerization of functional monomer(s) and crosslinker in presence of a template, which is removed subsequently. Thus, a molecularly imprinted polymer (MIP) possess cavities that are complementary in size, shape and functionality to the template allowing for selective rebinding of the template and its close analogues (Figure 6). This simple concept is applicable to a variety of target molecules ranging from ions and small molecules to macromolecules (e.g. proteins) and microorganisms. Likewise, many different approaches to form strong interactions between template and functional monomer(s) are available, e.g. covalent and non-covalent interactions as well as ligand exchange (Figure 6).

As a result, the molecular imprinting technology (MIT) has found a number of applications including purification and separation,^{109, 110} sensing^{111, 112} and drug delivery¹¹³ owing to unique recognition properties of MIPs characterized by high affinity and specificity for target molecule.¹¹⁴ Furthermore, MIPs are robust materials with high stability in a wide range of temperature and solvents. The low cost of production and possibility of multiple reuse of the material are additional attractive features of MIPs.

Figure 6. Schematic representation of the molecular imprinting process showing possible interactions between template and functional monomer, including covalent and non-covalent interactions as well as ligand exchange. Reproduced from Alexander et al.¹¹⁵ with permission from John Wiley and Sons.

Nevertheless, the molecular imprinting technology is not free from limitations. Most commonly used non-covalent imprinting approach¹¹⁶ suffers from a number of technical challenges owing to the non-covalent nature of interactions between template and functional monomer(s), e.g. hydrogen bonding, van der Waals interactions or hydrophobic interactions. The dynamic nature of prepolymerization self-assembled complex being in equilibrium with free monomers and template leads to formation of heterogeneous binding sites with different affinity for the target analyte. An excess of functional monomer can be used to shift the equilibrium towards monomer-template complex, however, the polymerization of remaining free functional monomer often leads to high degree of non-specific binding. Instead, a stoichiometric imprinting can be used.^{117, 118} In this approach the functional monomer interacts very strongly with the template providing a high degree of template complexation, thus it can be used in stoichiometric ratio resulting in more uniform binding sites and low non-specific binding.

Another commonly postulated difficulty in MIT is the imprinting of water soluble biological macromolecules, for example proteins.^{119, 120} This is due to large size and complexity of such targets resulting in slow mass transfer to the binding site. Two main approaches to overcome these difficulties were developed, namely epitope imprinting and surface imprinting. The epitope imprinting approach is based on using a small fragment of protein or peptide as a template.^{121, 122} The binding sites formed in this way are able to recognize not only template but also larger molecules bearing the imprinted fragment. In the surface imprinting approach, the immobilization of template on the supporting material (e.g. silica micro- and nanoparticles, magnetic nanoparticles or nanowires/nanotubes) enables creation of imprinted sites on the surface of the polymer.^{123, 124} As a result the highly accessible binding sites located on the surface of the imprinted material are formed.

MIPs for phosphoproteomics

Several approaches to develop phosphopeptide selective molecularly imprinted polymers have been reported to date.¹²⁵⁻¹³³ The first attempt to apply molecular imprinting technology for phosphoproteomics was reported by Sellaergren et al.¹²⁵ The neutral urea-based receptor was synthesized using N, C-protected phosphotyrosine as a template and ethylene glycol dimethacrylate (EDMA) as a crosslinker. This crushed monolith material

showed bias for pTyr peptides in presence of pSer and Tyr peptides. Further optimization and validation of this system resulted in successful extraction of pTyr peptide spiked at a femtomol level in bovine fetuin digest.¹³¹ Later on, Chen et al.¹³² showed a complementary selectivity of pTyr and pSer imprinted polymers in a mixture of twelve peptides and incorporation of pSer MIP enrichment in combination with SCX in proteomics workflow. The MIP-based method showed superior performance compared to TiO₂-based enrichment. Recently, the pTyr MIP-based phosphopeptide enrichment was validated in a comparative study with state of the art phosphopeptide enrichment techniques, i.e. immunoaffinity- and TiO₂-based enrichments (Paper I). It was shown that pTyr MIP is an attractive alternative for established phosphoenrichment methods allowing for a controlled bias of material towards specific phosphorylation site.

In another approach, the monodisperse 4-vinylpyridine-based MIPs targeting organophosphates were shown to non-selectively trap phosphorylated peptides (pSer, pThr, pTyr) and phosphopeptides from a tryptic digest of α -casein.¹²⁶ Ren et al.^{127, 128} have used charged metal ion-based functional monomers (Zn²⁺ and Ti⁴⁺) for imprinting of phenylphosphonic acid (PPA) as an epitope template. Obtained materials displayed the affinity for phosphorylated peptides in aqueous solution with a preference for tyrosine phosphorylated peptides as contrasted with unbiased enrichment with TiO₂.

Surface imprinting approach has also been employed for development of phosphopeptide selective MIPs.^{130, 133} For example, Li et al.¹³³ reported on surface imprinted fluorescent receptor based on CdTe quantum dots. The receptor gave linear fluorescence enhancement response to pTyr peptide which could also be detected in a spiked sample of tryptic digest of skim milk. However, the concentration of pTyr peptide tested in this work was in μ M range which is well above the range found in biological samples. Recently Chen et al.¹³⁰ reported on phosphate imprinted mesoporous silica nanoparticles using dual-template docking oriented molecular imprinting approach. Obtained imprinted nanoparticles displayed an excellent affinity for template analogue, i.e. adenosine monophosphate (AMP) and were able to extract pSer peptides from β -casein digest as well as from nonfat milk sample. However, the affinity for peptides phosphorylated on Thr and Tyr residues was not studied.

Urea-based functional monomers

The core of this thesis relies on the use of urea-based functional monomer FM1 (Figure 8A) as a recognition unit in MIPs targeting phosphorylated peptides reported in Papers I-IV.

1,3-Disubstituted ureas have long been exploited as neutral receptors for anions such as carboxylates, phosphates and sulfonates.¹³⁴⁻¹³⁶ This type of hosts are potent hydrogen bond donors capable of forming two-fold cyclic hydrogen bonds with anion guest (Figure 7). The strength of interaction increases with the acidity of urea protons and basicity of complexed anion. Thiourea is more acidic than urea ($pK_a = 21.1$ and 26.9 , respectively in DMSO),¹³⁶ thus thioureas should form stronger hydrogen bonds with guest compared to ureas.¹³⁷

Figure 7. Schematic representation of the interaction between urea ($\text{X}=\text{O}$) or thiourea ($\text{X}=\text{S}$) receptors and carboxylate anion.

Another factor influencing the strength of interaction is the type of solvent in which the recognition occurs. Neutral receptors such as ureas and thioureas, which rely solely on hydrogen bond interactions are highly susceptible to competition with polar protic solvents for anion binding. Thus, this type of receptors are most effective in aprotic organic solvents.¹³⁸

Functional monomers FM1-FM4 (Figure 8A) were evaluated by NMR titration experiments with tetrabutylammonium hydrogen-1-naphtyl phosphate (TBAHNP) guest in DMSO- d_6 (Figure 8B). The data points obtained for each monomer were fitted to 1:1 interaction model to determine values of association constant (K_a) and maximum complexation induced shift (CIS_{max}) (Table 2).

The 1,3-diaryl monomers (FM1 and FM2) interacted more strongly with guest compared to alkyl-aryl counterparts (FM3 and FM4). Furthermore, thiourea monomer FM4 interacted more strongly with guest compared to urea analogue FM3 as expected based on the difference in acidity of ureas and thioureas (*vide supra*).

Table 2. Values of association constants (K_a) and maximum complexation induced shifts (CIS_{max}) for FM1-FM4 titrated with TBAHNP in DMSO-d₆.

Monomer	K_a (M^{-1})	CIS_{max} (ppm)
FM1	1489±7	2.14
FM2	743±10	1.96
FM3	266±14	1.91
FM4	439±5	2.00

However, this relationship did not hold true for FM1 and FM2 pair of monomers. This unexpected finding can be ascribed to previously reported preference of diaryl thioureas for (Z, E) conformation as opposed to preferential (Z, Z) conformation of diaryl ureas¹³⁹ and it is in agreement with the previous study on FM1 and FM2.¹²⁵

Figure 8. (A) Structures of functional monomers FM1-FM4 and (B) plot of complexation induced shift (CIS) *vs.* free concentration of guest (C_{free}) fitted to 1:1 interaction model for ¹H MNR titrations of FM1-FM4 with tetrabutylammonium hydrogen-1-naphthyl phosphate (TBAHNP) in DMSO-d₆ at 25 °C.

Based on the above results, the FM1 was selected as a functional monomer for synthesis of molecularly imprinted polymers targeting pTyr and pHis peptides (Papers I-IV). Schematic representation of interactions between tyrosine phosphorylated peptide and binding site in the imprinted polymer is shown in Figure 9.

Figure 9. Schematic representation of interactions between tyrosine phosphorylated peptide and phosphotyrosine imprinted polymer (pTyr MIP).

Imidazolium-based functional monomers

Recognition of anions in water is essential for biological and environmental applications, however, it remains a great challenge in modern supramolecular chemistry.¹⁴⁰ Neutral receptors bearing urea, amide or pyrrole functionality were extensively studied for recognition of anions,^{141, 142} nevertheless, their compatibility with aqueous media is limited. Water is a polar solvent with the ability of hydrogen bond donation and acceptance, thus it effectively competes with neutral receptors for binding anions. Charged receptors, on the other hand, can take advantage of the electrostatic effect, and thus compete more effectively with polar protic solvent.

Commonly used charged receptors for anions based on guanidinium or ammonium employ $^+N-H\cdots X^-$ type of ionic hydrogen bond. In contrast, imidazolium-based receptors can interact with anions through $(C-H)^+\cdots X^-$ type of ionic hydrogen bond (Figure 10).¹⁴³

Figure 10. Schematic representation of the ionic hydrogen bond interaction between imidazolium receptor and anion X^- .

A number of such receptors was reported before in the literature and reviewed elsewhere.¹⁴⁴⁻¹⁴⁷ For example, Kwon et al.¹⁴⁸ reported water-soluble imidazolium anthracene derivative (Figure 11) which acts as a potential fluorescent receptor for guanosine 5'-triphosphate (GTP) in 100% aqueous solution. The value of K_a derived from the fluorescence titration with GTP in 10 mM HEPES buffer (pH = 7) was 87000 M^{-1} .

Figure 11. Fluorescent receptor for GTP.

In Paper V a set of imidazolium-based functional monomers was used to synthesize MIPs. Materials prepared in capillary monolith format were able to strongly retain phosphopeptides in highly aqueous mobile phase as opposed to nonphosphorylated ones.

METHODS

Analytical methods for identification, purification, separation and quantification of chemical compounds as well as material characterization techniques were extensively used in this thesis. This section explains the basic principles of the most important methods used during the course of this work. Details about instrumentation and conditions of all analyses are given in Experimental Sections of Papers I-V.

Batch binding test

One of the most basic and simple tests for characterization of binding properties of MIPs is the batch binding test.¹⁴⁹ It relies on equilibration of a known amount of the polymer (m , in g) with the solution of a known amount of the analyte (n_0 , in moles) until the equilibrium is reached. The polymer is then separated from the solution and the amount of unbound analyte (n_{free} , in moles) in the solution is measured. The amount of bound analyte per unit mass of the polymer (B , in mol g⁻¹) can be then calculated from equation (1):

$$(1) \quad B = (n_0 - n_{free})/m$$

The experiment is typically done in parallel for both MIP and NIP under identical conditions (Figure 12). The difference between materials originating from the imprinting phenomenon can be expressed as imprinting factor (IF) and it can be calculated from equation (2):

$$(2) \quad IF = \frac{D_{MIP}}{D_{NIP}} = \frac{B_{MIP}/C_{free\ MIP}}{B_{NIP}/C_{free\ NIP}}$$

Where D (L g⁻¹) is the distribution ratio and C_{free} (mol L⁻¹) is the concentration of the unbound analyte.

Figure 12. Results of the batch binding test of Fmoc-pTyr-OH for microspherical phosphotyrosine imprinted polymer (MIP) and the corresponding non-imprinted polymer (NIP). The test was performed using 0.5 mL of 0.5 mM solution of the analyte in 95% ACN and 2 h equilibration with 10 mg of the polymer (Paper III). The imprinting factor (IF) was calculated using equation (2).

However, the imprinting factor is dependent on the amount of the analyte (n_0), the amount of the polymer (m) and the volume of the sample (V). Thus, it is recommended to do batch binding test for an array of different n_0 while keeping m and V constant. The obtained data points can be represented on the graph of B vs. C_{free} , the so-called binding isotherm (Figure 13).

Figure 13. Binding isotherm for the same polymers and conditions as described in Figure 12. The data points were recorded for 0.05 – 1.5 mM concentration of the analyte. The values of B_{max} and K_a ($K_a = 1/K_d$) were obtained from data fit to mono-Langmuir model (Paper III).

The experimental data can be fitted to a theoretical model using a graph fitting software, for example GraphPad Prism (GraphPad Software, La Jolla, CA, USA) or OriginPro (Originlab Corporation, Wellesley Hills, MA, USA). A number of such theoretical binding isotherm models can be used to characterize binding sites of the imprinted polymer.¹⁵⁰ The simplest mono-Langmuir model assumes that there is only one type of homogeneous binding sites (Table 3). It allows to calculate values of the maximum amount of analyte bound to the polymer particles at saturation (B_{max}) and the dissociation constant (K_d) which is a reverse of the association constant (K_a).

Table 3. Adsorption isotherm models. Reproduced from Ansell¹⁴⁹ with permission from Springer.

Model	Equation	Description of binding sites
Mono-Langmuir	$B = \frac{B_{max} \cdot C_{free}}{K_d + C_{free}}$	One type homogeneous
Bis-Langmuir	$B = \frac{B_{max1} \cdot C_{free}}{K_{d1} + C_{free}} + \frac{B_{max2} \cdot C_{free}}{K_{d2} + C_{free}}$	Two classes of binding sites
Freundlich ^a	$B = A \cdot C_{free}^m$	Continuous distribution of binding sites. Infinite number of weak sites decaying to few strong ones
Langmuir-Freundlich ^a	$B = \frac{B_{max} \cdot a \cdot C_{free}^m}{1 + a \cdot C_{free}^m}$	Gaussian distribution of binding sites

^a A , a and m are empirical constants.

The mono-Langmuir model is a great simplification given the heterogeneous nature of the binding sites typically observed in MIPs. Thus, the data should be fitted to several models and the one giving the best fit (the best description of binding sites) should be used. The most common models used to fit MIP binding isotherms are listed in Table 3.¹⁴⁹

Molecularly imprinted solid phase extraction

Another type of experiment used in this work to probe binding properties of polymers is the molecularly imprinted solid phase extraction (MISPE) test which is a variation of classical SPE test.^{151, 152} The sample is loaded on the conditioned polymer packed in the SPE cartridge (Figure 14). The flow-through fraction contains all unbound analytes. The washing step is performed to remove all non-specifically bound analytes (washing fraction) and it is followed by the elution step.

Figure 14. Schematic representation of the solid phase extraction (SPE) experiment with (A) loading, (B) washing and (C) elution steps.

The amount of each analyte in collected fractions can be measured and expressed as a percentage of initial sample amount (Figure 15).

Figure 15. Results of the SPE test for (A) bulk pTyr MIP and (B) the corresponding NIP showing % of initial amount of each analyte in the flow-through, washing and elution fractions. Conditions: loading 100% ACN, washing 95% ACN, elution 80% MeOH + 1% TFA.

In contrast to batch binding test, the binding in SPE test does not necessarily occur under equilibrium conditions. Thus, data cannot be fitted to theoretical models and information about B_{max} and K_a cannot be obtained. Nevertheless, such test is a useful source of information about binding properties of MIP.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a separation method that allows to purify, identify and quantify compounds and can be used both in analytical and preparative scale.¹⁵³ The liquid solvent (mobile phase) containing sample is passed through the column filled with the sorbent (stationary phase) at high pressure with the aid of a pump. The separation of individual components of the sample is based on a difference in their affinity to the stationary phase. Eluted compounds can be detected, for example, by measurement of ultra-violet (UV) absorbance, refractive index or by mass spectrometry analysis.

Polymers developed in this work were characterized by batch binding and SPE tests with a mixture of compounds (e.g. Fmoc-amino acids or peptides). The concentration of analytes in samples was measured by HPLC-UV analysis. This required development of methods for separation of mixtures of three or four compounds. Parameters for developed methods for separation of Fmoc-amino acids and peptides are given in Table 4 and examples of chromatograms are shown in Figure 16.

Figure 16. HPLC-UV chromatograms of separated mixtures of (A) Fmoc-pSer-OH [1], Fmoc-pTyr-OH [2] and Fmoc-Tyr-OH [3]; (B) VpSI [1], VpYI [2] and VYI [3]; (C) VpSI [1], VpHI [2], VEI [3] and VHI [4]; (D) DRVpSIHPF [1], DRVpYIHPF [2] and DRVYIHPF [3].

Table 4. HPLC methods for separation of Fmoc-amino acids (Method 1, Figure 16A), tripeptides (Method 2, Figure 16B) (Method 3, Figure 16C) and octapeptides (Method 4, Figure 16D).

Sample	Method 1	Method 2	Method 3	Method 4
	Fmoc-pSer-OH, Fmoc-pTyr-OH and Fmoc-Tyr-OH	VpSI, VpYI and VYI	VpSI, VpHI, VEI and VHI	DRVpSIHFP, DRVpYIHFP and DRVYIHFP
Column	Synergi Polar RP 75 x 2 mm	Prodigy ODS 150 x 4.6 mm	XBridge C18 5µm 50 x 4.6 mm	Prodigy ODS 150 x 4.6 mm
Mobile phase A	H ₂ O + 0.1% TFA	H ₂ O + 0.1% TFA	10mM NH ₄ HCO ₃	H ₂ O + 0.1% TFA
Mobile phase B	MeOH + 0.1% TFA	ACN + 0.1% TFA	ACN/10mM NH ₄ HCO ₃ (9:1)	ACN + 0.1% TFA
Gradient	40% B to 65% B in 10 min	15% B to 20% B in 10 min	0% B to 35% B in 60 min	20% B to 30% B in 10 min
Flow rate (mL/min)	0.6	1.5	0.5	1.5
UV detection (nm)	265	210	210	210
Run time (min)	12	6	18	6

Mass spectrometry

Mass spectrometry (MS) is an analytical technique where ionized compounds are separated based on their mass to charge (m/z) ratio. Regardless of technical differences, typically mass spectrometer is composed of three parts, i.e. ion source, mass analyzer and detector (Figure 17).¹⁵⁴ In a typical MS analysis sample is introduced through the inlet system into the ion source where compounds are ionized. Subsequently, the ions are separated according to their m/z ratio in the mass analyzer and detected in the detector.

Figure 17. Components of the mass spectrometer.

Mass spectrometry is widely used in proteomic studies and a number of different setups of the instrumentation are available.¹⁵⁵ Two most commonly used techniques to vaporize and ionize the sample are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).

The mechanism of ESI is based on the formation of gas phase ions from sprayed droplets of the sample passed through a capillary maintained at high voltage (2-5 kV).¹⁵⁶ The ions are transferred to gas phase due to evaporation of the solvent from charged droplets of the sample. This happens with the aid of elevated temperature of the ion source and the flow of nitrogen. An advantage of ESI is the compatibility with chromatographic separation module (HPLC).

In MALDI the sample is deposited on a metal plate along with the matrix compound in a dry, crystalline form. The pulsed laser irradiation leads to the sublimation and ionization of the sample. The matrix compound is responsible for absorption of the laser beam and converting it to heat energy, thus facilitating sample desorption and ionization.¹⁵⁷ Both ESI and MALDI are soft ionization methods and do not lead to the fragmentation of analyzed compounds.

The ionized sample is introduced to the mass analyzer which is the key element of the mass spectrometer, determining sensitivity, resolution and mass accuracy of the method. Four most commonly used in proteomics mass analyzers are time-of-flight (TOF), ion trap, quadrupole and Fourier transform

ion cyclotron resonance (FT-ICR).¹⁵⁵ In the work presented in this thesis, mass spectrometers with the former three types of mass analyzers were used.

The TOF mass analyzer is usually coupled with MALDI. Ions are accelerated by an electric field and separated along the flight tube as a result of different velocities. Ions with a lower m/z have higher velocity, and thus reach detector faster than ions with a higher m/z value.¹⁵⁸ MALDI-TOF/TOF-MS was used in Papers I-III.

Orbitrap is one of the most recently invented mass analyzers. It is a type of ion trap analyzer where ions are trapped around central spindle electrode. The m/z values of ions are derived from their oscillation frequencies.¹⁵⁹ The nanoLC-ESI-MS/MS with Orbitrap mass analyzer was used in Paper II.

The quadrupole mass filter is composed of four parallel rods. Depending on the electric field applied to quadrupole rods only ions of specified m/z are allowed to pass through the analyzer.¹⁶⁰ LC-ESI-MS with quadrupole mass analyzer was used in Paper IV.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a technique used to study the structure of compounds and interactions between various molecules.¹⁶¹ When nuclei with a non-zero spin (e.g. ^1H , ^{13}C , ^{19}F or ^{31}P have spin $I=1/2$) is placed in a strong magnetic field B_0 , spins of the nuclei are aligned with it. Delivery of radiofrequency (RF) pulses generates a temporary magnetic field B_1 which twists the spins out of the alignment with B_0 . As spins return to alignment with B_0 the energy gained upon RF pulse is released and recorded as a function of signal intensity *vs.* time which is called the free induction decay (FID). This is then converted to spectrum using the Fourier Transformation.

The NMR spectroscopy was extensively used in this thesis for confirmation of the structure and purity check of synthesized compounds including templates and functional monomers (Figure 18).

Figure 18. Zoomed portion (4.6 – 9.8 ppm) of the ^1H NMR spectra of functional monomer FM1 recorded in DMSO- d_6 at 25 °C using an Agilent (Varian) Mercury 400 MHz instrument.

NMR titration experiments were used to study interactions between functional monomers and templates or template analogs. Typically, the functional monomer (host) was titrated with an increasing amount of the template (guest). The change of NMR shielding, and thus chemical shift is observed upon formation of host-guest complexes (Figure 19A). The obtained data plotted as complexation induced shift *vs.* free concentration of guest can be fitted to one of the binding models to derive the value of association constant (K_a).¹⁶² Titration data of functional monomer FM1 with tetrabutylammonium salt of phenylphosphonic acid (TBAHPPA) was fitted to 1:1 interaction model and the calculated K_a was $7350 \pm 320 \text{ M}^{-1}$ (Paper V, Figure 19).

Figure 19. (A) Zoomed portion (8.5 – 12.5 ppm) of the 1H NMR spectra of functional monomer FM1 (host) titrated with an increasing amount of guest (0 – 10 equivalents) recorded in DMSO- d_6 at 25 °C using an Agilent (Varian) Mercury 400 MHz instrument. (B) Plot of the average complexation induced shift (CIS) of protons H7 and H8 *vs.* free concentration of guest (C_{free}) fitted to 1:1 interaction model and calculated values of maximum CIS at saturation (CIS_{max}) and association constant (K_a). (C) Structures of host and guest.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) is a type of thermal analysis where the amount and the rate of change in the mass of the sample is measured as a function of temperature or time.¹⁶³ This method is most often used to determine thermal stability and compositional properties of materials.

Figure 20. TGA curves obtained for silica beads used for the preparation of microspherical pTyr polymer (red trace, 3% weight loss), pTyr MIP polymer-silica composite (green trace, 30% weight loss) and pTyr MIP-M after etching of silica (blue trace, 94% weight loss). Samples were heated from 50 to 900 °C with a heating rate of 20 °C/min, under the nitrogen atmosphere (Paper III).

In Paper III, microspherical polymer beads were synthesized using porous silica particles as a vessel for polymerization. TGA analysis was used to evaluate removal of silica after polymer synthesis. Silica particles do not decompose during TGA analysis (Figure 20, red trace), thus weight loss is insignificant. Polymer, on the other hand, made of organic building blocks, undergoes thermal degradation resulting in significant weight loss of the sample (Figure 20, blue trace). The amount of removed silica was determined by comparison of the weight loss of samples before (Figure 20, green trace) and after etching of the silica.

RESULTS AND DISCUSSION

PAPER I

Validation of molecularly imprinted polymers for side chain selective phosphopeptide enrichment.

It was previously shown that a phosphotyrosine molecularly imprinted polymer (pTyr MIP) utilizing urea-based functional monomer FM1 (Figure 21) can be used for biased enrichment of tyrosine phosphorylated (pTyr) peptides.¹²⁵ In Paper I the pTyr MIP-based protocol for selective extraction of pTyr peptides was further developed and optimized. The method was validated on both simple mixture of thirteen peptides (four pTyr, five pSer and four non-phosphorylated) and complex sample of the digested mouse brain lysate spiked with these peptides. Moreover, the method was compared with commonly used phosphoenrichment techniques, i.e. TiO_2 affinity chromatography and immunoaffinity.

Figure 21. Structure of the functional monomer FM1.

Enrichment of phosphopeptides from model peptide mixture confirmed that the pTyr MIP preferentially binds to pTyr peptides (four pTyr and three pSer

peptides were found in the elution fraction). This contrasted with TiO_2 method which extracted eight out of nine phosphopeptides (except doubly tyrosine phosphorylated peptide) which is in agreement with previous reports showing unbiased affinity of TiO_2 to phosphopeptides.¹⁶⁴ Each of three commonly used anti-phosphotyrosine antibodies extracted only one pTyr peptide from the same sample. This was likely due to a multistep protocol that increases the risk of sample loss, as the higher amount of sample (200 pmol compared to 1 pmol) used for enrichment resulted in the identification of all pTyr peptides. The selectivity of investigated phosphoenrichment methods was also reflected in a test with a complex sample of digested mouse brain lysate spiked with model peptide mixture where similar results were obtained.

The study reported in Paper I shows that the pTyr MIP-based method is an attractive alternative to established phosphopeptide enrichment techniques allowing for biased enrichment of low abundant pTyr peptides. Furthermore, it offers the advantage of simple protocol that minimizes sample handling, and thus reduces the risk of sample loss.

Although results presented in Paper I are promising it was crucial to demonstrate that pTyr MIP can be used for extraction of endogenous, low abundant pTyr peptides from a biological sample.

PAPER II

Phosphotyrosine biased enrichment of tryptic peptides from cancer cells exploiting combined pY MIP and TiO_2 affinity.

In Paper II, the implementation of pTyr MIP in phosphoproteomic workflows using tryptic digest of HeLa cells lysate is reported. Four methods including pTyr MIP, TiO_2 and combinations thereof (pTyr MIP – TiO_2 and TiO_2 – pTyr MIP) were evaluated and compared in terms of selectivity towards pTyr, pSer and pThr phosphorylation sites, number and signal intensity of identified phosphopeptides and their physicochemical properties.

The complex biological sample posed a great challenge for pTyr MIP alone as the number of identified phosphopeptides and pTyr peptides was significantly lower compared to other methods involved in this study (Table 5). The presence of a large number of nonphosphorylated peptides in the elution fraction from pTyr MIP indicates a high degree of non-specific binding. This is most likely driven by the hydrophobic interactions of peptides with the poly-

mer surface which is an important limitation of this method. It was previously reported for phosphoserine molecularly imprinted polymer (pSer MIP) featuring the same FM1 functional monomer (Figure 21) that the combination of the pSer MIP-based enrichment with SCX prefractionation of the sample significantly increased the number of phosphopeptides extracted from a tryptic digest of HEK 293T cell lysate.¹³² In Paper II, the pTyr MIP enrichment was combined with TiO₂-based method which is known to have an unbiased affinity for phosphopeptides and it was expected to improve sample cleanup. Thus, TiO₂ was implemented both before and after sample treatment with pTyr MIP (TiO₂ – pTyr MIP and pTyr MIP – TiO₂ methods, respectively). The combined methods provided a slightly lower number of identified phosphopeptides compared to TiO₂ alone (Table 5) which is likely due to more sample handling steps resulting in sample loss. A closer look at the ratios of identified pSer, pThr and pTyr peptides (Table 5) revealed that TiO₂ following pTyr MIP (pTyr MIP – TiO₂) increased the relative number of identified pTyr peptides compared to the reference TiO₂ method.

Table 5. Number of phosphopeptides identified with four enrichment methods studied in Paper II and the ratio of peptides with different phosphorylated residues (pSer, pThr and pTyr).

Method	Number of phosphopeptides ^a	pSer (%) ^b	pThr (%) ^b	pTyr (%) ^b
pTyr MIP	412 (258)	91.9	5.8	4.6
TiO ₂	1843 (1209)	88.1	7.9	8.5
TiO ₂ – pTyr MIP	1328 (909)	88.8	5.7	6.9
pTyr MIP – TiO ₂	1695 (1081)	84.2	7.4	9.5

^a Accumulated number of phosphopeptides identified in three replicates. Numbers in parentheses refer to peptides identified in minimum two replicates. ^b Calculated considering peptides identified in minimum two replicates.

Enhanced selectivity of pTyr MIP – TiO₂ method towards pTyr peptides was also reflected in the increased intensity of 70% of pTyr peptides commonly identified with the TiO₂ method. This was not observed when the sample was treated by the two enrichment methods in reversed order (TiO₂ – pTyr MIP). It indicates that pTyr MIP indeed can enrich phosphopeptides with a bias towards pTyr peptides, however, it requires additional sample processing to remove non-specifically bound peptides which otherwise disturb MS-based analysis of phosphopeptides.

The analysis of physicochemical properties of phosphopeptides enriched by the four studied methods revealed that pTyr MIP and pTyr MIP-TiO₂ methods extracted less acidic and more basic phosphopeptides when compared with TiO₂ and TiO₂-pTyr MIP methods. This implies that in case of combined methods, the method used as a first in the sequence predetermines the properties of enriched phosphopeptides. The bias of TiO₂ method towards acidic phosphopeptides is in agreement with previous reports.¹³² Furthermore, three methods based on pTyr MIP displayed a preference for mono-phosphorylated and relatively short peptides (6-24 residues). In contrast, TiO₂ enriched significantly more multi-phosphorylated and larger (≥ 25 residues) phosphopeptides. As it was concluded in Paper III the preference of pTyr MIP for shorter peptides is related to the broad pore size distribution of the material with a significant number of pores in the micro- to low mesoporous regime (1-20 nm). This size bias can be changed using pore templating approaches.

Interestingly, the overlap of phosphopeptides identified by TiO₂ and pTyr MIP – TiO₂ or TiO₂ – pTyr MIP was only 50 and 45%, respectively. This indicates that implementation of pTyr MIP in phosphoproteomic workflows can provide access to previously unknown phosphorylation sites.

PAPER III

Hierarchically templated beads with tailored pore structure for phosphopeptide capture and phosphoproteomics.

Paper III describes the design, synthesis and evaluation of pTyr MIP in the format of porous microspherical beads. The classical bulk polymerization *via* free radical polymerization is by far the most commonly used method to synthesize MIPs. The simple protocol and compatibility with many functional groups determined the utility of this technique for generation of synthetic MIP receptors. Although commonly used, this approach is not free from limitations. Firstly, the polymer prepared in bulk format requires tedious and time-consuming processing by crushing and sieving to obtain particles in desired size range. This often leads to significant loss of the material, thus making the process cost-ineffective especially when used template or monomers are expensive. Secondly, the obtained particles are irregularly shaped and sized which limits their application, for example in chromatography. Furthermore, the binding sites can be destroyed by grinding, thus reducing the capacity of the

polymer. For these reasons alternative MIP formats have been developed, for example micro- and nanobeads, membranes or monoliths.¹⁶⁵⁻¹⁶⁷

There are even more drawbacks of crushed bulk MIPs when it comes to the analysis of large molecules, for example peptides or proteins. The diffusion of such bulky entities to the binding pocket is slow and it can be hampered by insufficient porosity of the material.

Phosphoproteomic studies are most commonly based on a bottom-up approach where digested protein fragments with the mass up to roughly 3 kDa are analyzed. In recent years, however, an increasing interest in top-down (analysis of whole proteins) and middle-down (analysis of large protein fragments, typically 3-20 kDa) approaches has been observed.¹⁶⁸ The invaluable piece of information gained thanks to these techniques is the combinatorial nature of the PTMs located in distant parts of the protein which is lost in the bottom-up approach.¹⁶⁹ Certainly, enrichment of peptides and large protein fragments requires dedicated tools compatible with the size of these molecules.

In an attempt to enhance the compatibility of previously reported crushed bulk pTyr MIP (Papers I and II) with the size of tryptic phosphopeptides, the polymer was prepared in the format of porous microspherical beads (Paper III). Mesoporous silica particles (30 μm average particle size) were used as vessels for polymerization in two variants, i.e. with template either dissolved in monomer cocktail (pTyr MIP-M) or immobilized on the silica surface (pTyr MIP-H). Both new materials were characterized by increased surface area and average pore size compared to the crushed bulk pTyr MIP. After the initial evaluation of the materials on simple ternary mixtures of amino acids and peptides, polymers were challenged with the sample of twelve digested proteins (including three phosphorylated on serine residues) spiked with standard pTyr, pSer and Tyr peptides. The samples were analyzed using MALDI-MS. The study of the normalized signal intensities of spiked peptides in elution fractions from MIPs and in the sample before enrichment revealed that all polymers reduced sample background and enriched phosphorylated peptides. The pTyr MIP-M proved to give highest values of pTyr/pSer and pTyr/Tyr ratios after enrichment. It was also more effective in the enrichment of larger peptides compared to the bulk pTyr MIP which is ascribed to increased porosity and surface area of the microspherical polymer.

In summary, silica templated MIP synthesis is a useful approach to bias phosphopeptide enrichment to larger peptide size. Moreover, it allows to control size and shape of obtained polymer beads and results in high yield of material as contrasted with classical bulk polymerization.

PAPER IV

Selective enrichment of histidine phosphorylated peptides combining β -elimination and MIP-based pHis capture.

For many years modifications localized on serine, threonine and tyrosine residues were in the spotlight of phosphoproteomics research. The blossom of this area would not be possible without all the effort to develop tools for detection and analysis of these modifications. Phosphohistidine, one of the elusive cousins of phosphohydroxy amino acids, was overlooked for a long time due to its unique acid-labile nature and the consequent lack of adequate tools for its analysis.

In Paper IV the spotlight is turned to pHis modification. Currently, the limited choice of techniques for the enrichment of pHis peptides is one of the main factors hampering the study of this modification. Methods commonly used for extraction of O-phosphorylated peptides (for example MOAC and IMAC) are not appropriate for pHis peptides since they involve acidic conditions. Thus, mild enrichment methods preserving pHis modification, ideally avoiding acidic treatment of the sample are needed.

Paper IV describes a method for enrichment of pHis peptides with the use of a molecularly imprinted polymer. The pHis MIP was synthesized using a stable analogue of phosphohistidine (Fmoc-pTza-OEt) as a template and functional monomer FM1 (Figure 21). The polymer displayed affinity towards model pHis peptide in basic conditions (95% ACN + 0.1% TEA) even in the complex sample of digested bovine serum albumin (BSA). These conditions, however, despite the compatibility with pHis modification did not provide sufficient selectivity of the polymer in presence of other phosphopeptides. The interfering affinity for pSer modification, being the most abundant phosphorylation in biological samples, was eliminated by treatment of the sample with a strong base capable of selective dephosphorylation of pSer (*via* β -elimination) in presence of pHis. This approach was proven successful on the sample of BSA tryptic digest spiked with model pHis and pSer peptides. Importantly, the proposed workflow did not involve any acidic treatment, thus the labile pHis modification could be preserved.

In conclusion, the MIP-based protocol reported in Paper IV is an attractive alternative to currently available techniques for the enrichment of pHis peptides. New enrichment methods compatible with pHis modification will certainly facilitate the progress in research on this PTM.

PAPER V

Cationic pTyr/pSer imprinted polymers based on a bis-imidazolium host monomer: Phosphopeptide recognition in aqueous buffers demonstrated by μ -liquid chromatography and monolithic columns.

As shown in Papers I-IV, the urea-based imprinted polymers can effectively capture phosphorylated peptides even from complex samples. However, the recognition of phosphopeptides with this type of material is most effective in high organic media such as 95% ACN. This is very different from natural, aqueous environment of proteins. Such a dramatic change of the solvent system from aqueous to highly organic can result in problems with solubility of the sample.

In Paper V an attempt to generate pTyr selective MIPs compatible with protic and aqueous media is reported. A series of functional monomers (FM5-FM7, Figure 22) bearing imidazolium group were studied and compared with the reference urea-based monomer FM1 (Figure 21).

Figure 22. Structures of functional monomers FM5-FM7.

NMR titration study revealed that FM1 interacted strongly with mono-TBA salt of phenylphosphonic acid (PPA·TBA) in DMSO- d_6 ($K_a = 7350 \pm 320 \text{ M}^{-1}$) while imidazolium-based monomers FM5-FM7 displayed significantly weaker association constants in the same conditions ($K_a < 350 \text{ M}^{-1}$). This proves domination of hydrogen bond interactions in aprotic media. When solvent was

changed to CD₃OD no complexation induced shift (CIS) was observed for FM1 and FM5 while FM6 showed a rather weak response (CIS = 0.015 ppm) which nevertheless confirms the contribution of electrostatic interactions. Thus, FM6 was titrated with PPA-2TBA in CD₃OD to reveal significantly stronger interactions with $K_a = 1680 \pm 121 \text{ M}^{-1}$.

Binding properties of crushed bulk polymers featuring monomers FM1, FM5-FM7 were evaluated in methanol using template analogue Fmoc-pTyr-OH. As expected, the polymer prepared with FM1 displayed lowest values of K_a and maximum capacity (B_{max}). This was ascribed to the effect of protic polar solvent that competes with imprinted cavities for binding the target analyte. Polymers featuring imidazolium-based monomers FM5-FM7 showed improved binding properties in these conditions as a result of combined electrostatic interactions and ionic hydrogen bonds. The most promising functional monomer FM6 was then used for preparation of pTyr and pSer imprinted and non-imprinted capillary monoliths (250 μm i.d.). These monolith columns were evaluated by micro-liquid chromatography using a mixture of Fmoc-protected amino acids and octapeptides of general sequence Fmoc-X-OH and DRVXIHPF, respectively, where X stands for pTyr, pSer, Tyr or Ser residue. It was found that for both types of sample, imprinted capillaries displayed high retention of phosphorylated entities with the preference for the residue that was used for imprinting (i.e. pTyr imprinted capillary retained more strongly pTyr analyte than pSer analyte and *vice versa*). In contrast, the non-imprinted capillary did not retain any of these analytes. Importantly, this behavior of materials was observed in both high organic (95% ACN) and low organic (10% ACN – 90% HEPES buffer pH 7.4) mobile phases.

The promising results reported in Paper V prove that receptors combining electrostatic interactions and ionic hydrogen bonds can effectively compete for binding anions in polar protic solvents. Thus, imidazolium-based functional monomers employed in MIPs improve recognition of phosphorylated peptides in aqueous media.

CONCLUDING REMARKS AND FUTURE OUTLOOK

The work presented in this thesis is focused on development and validation of enrichment tools and methods for phosphoproteomics with the use of molecular imprinting technology.

It was shown that the pTyr MIP is capable of biased enrichment of pTyr peptides from a complex biological sample. The overlap of phosphopeptides identified by pTyr MIP-based method and TiO_2 reaching only about 50% suggests that the pTyr MIP can access distinct parts of phosphoproteome, possibly revealing new previously unknown phosphorylation sites. Currently, the greatest limitation of the pTyr MIP in the analysis of complex biological samples is the high level of non-specific binding. This effect can be reduced by coupling pTyr MIP with other phosphoenrichment and/or fractionation techniques. Nevertheless, further optimization of the polymer design and composition to reduce non-specific interactions with nonphosphorylated peptides is recommended. This was achieved to some extent by changing the format of the pTyr MIP to microspherical beads. This newly developed material displayed improved selectivity for phosphorylated peptides as compared to a classical crushed bulk polymer.

The molecular imprinting technology was also used to develop a novel method for enrichment of pHis peptides. It was demonstrated that pHis MIP can extract pHis peptide from BSA protein digest. These promising results are the first step to place MIPs on the map of enrichment techniques for elusive phosphorylations such as pHis. Further optimization and validation of the method can provide an efficient tool facilitating research on this important PTM.

Moreover, relying on the principles of supramolecular chemistry, it was shown that an improved recognition of phosphopeptides in aqueous media can be achieved with the use of imidazolium-based functional monomers.

Successful application of MIPs in the field of phosphoproteomics is not a trivial task. However, given the unique advantages offered by MIPs such as affinity and selectivity, robustness and low production cost it is worth the effort to make it happen.

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PAPERS I–V

I

Validation of molecularly imprinted polymers for side chain selective phosphopeptide enrichment

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ABSTRACT

Selective enrichment techniques are essential for mapping of protein posttranslational modifications (PTMs). Phosphorylation is one of the PTMs which continues to be associated with significant analytical challenges. Particularly problematic are tyrosine-phosphorylated peptides (pY-peptides) resulting from tryptic digestion which commonly escape current chemo- or immuno- affinity enrichments and hence remain undetected. We here report on significant improvements in this regard using pY selective molecularly imprinted polymers (pY-MIPs). The pY-MIP was compared with titanium dioxide (TiO₂) affinity based enrichment and immunoprecipitation (IP) with respect to selective enrichment from a mixture of 13 standard peptides at different sample loads. At a low sample load (1 pmol of each peptide), IP resulted in enrichment of only a triply phosphorylated peptide whereas TiO₂ enriched phosphopeptides irrespective of the amino acid side chain. However, with increased sample complexity, TiO₂ failed to enrich the doubly phosphorylated peptides. This contrasted with the pY-MIP showing enrichment of all four tyrosine phosphorylated peptides at 1 pmol sample load of each peptide with a few other peptides binding unselectively. At an increased sample complexity consisting of the standard peptides spiked into mouse brain digest, the MIP showed clear enrichment of all four pY-peptides.

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1. Introduction

Phosphorylation is an important post-translational modification affecting the biological properties of carbohydrates, proteins, and lipids. Approximately 30% of the proteins encoded by the human genome are phosphorylated, and abnormal phosphorylation is now recognized as a cause or a consequence of many human diseases [1,2]. The characterization of phosphorylated proteins is a challenging analytical task since many of the proteins targeted for phosphorylation are low abundant and phosphorylation is typically substoichiometric. A small fraction of phosphorylated proteins is modified at tyrosines and are less abundant (0.05%) than phosphoserine/phosphothreonine (pS90%/pT9.95%) proteins [2]. Therefore, high throughput analysis using mass spectrometry to elucidate the tyrosine-phosphorylated proteome requires sensitive and efficient enrichment procedures before or after an enzymatic digestion.

In view of the plethora of alternative approaches being proposed, current methods have multiple limitations, as outlined below. (1) There is a notable lack of robust nonimmune-based methods featuring side chain selectivity, for instance, allowing fractionation of pY- and pS-peptides [3]. (2) Current methods require access to a large amount of sample, typically in the milligram range [4]. (3) Current methods notably based on TiO₂ affinity display pronounced sequence bias, typically in favor of peptides rich in acidic residues. A large portion of the phosphoproteome may therefore remain invisible using these techniques. (4) Current methods are both complicated and time consuming.

In principle, it is now possible to perform affinity purification experiments using anti-pY-antibodies where tyrosine-phosphorylated proteome is enriched and its dynamic changes are monitored [5–7]. However, for an efficient affinity purification of tyrosine-phosphorylated peptides, a large amount of expensive antibodies and sample material are required [3]. Here we wish to report on a successful site selective phosphopeptide enrichment from small sample quantities of model peptide mixtures and biological samples using phosphotyrosine imprinted polymers [8,9]. We reveal benefits of this fractionation tool in a comparative study

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Fig. 1. Workflows for MIP-, TiO_2 - and antibody-based enrichment of tyrosine-phosphorylated peptides followed by mass spectrometry based identification and quantification.

(Fig. 1) with reference methods relying on titanium dioxide (TiO_2) [10,11] and anti pY-antibodies [12,13] based enrichments. In this report, we compare three phosphoenrichment techniques i.e. antibodies, TiO_2 , and pY-MIP. Previous reports have not focused on this aspect [8,9].

2. Experimental section

2.1. MIP washing and conditioning

20 mg pY-MIP in a 1.5 mL Eppendorf tube was washed at room temperature with methanol (MeOH) (0.1% TFA) in a Thermomixer (Eppendorf) for 5 min, 2 h and overnight agitation (1200 rpm) sequentially. The sample was centrifuged at $16,000 \times g$ sedimentation and supernatant discarded after each wash. The conditioning of the MIPs was carried out 2 times for 10 min and 2 h in acetonitrile (MeCN) (0.1% TFA) sequentially agitated in a thermomixer at room temperature. For every washing and conditioning step, the slurry was centrifuged and the supernatant discarded.

2.2. Preparation of pY MIP pipette tips and solid phase extraction

A 200 μL pipette tip (Eppendorf) was filled with a C8 membrane plug (3 M EmporeTM extraction disc, IVA Analysentechnik, Meerbusch) at the bottom. The conditioned MIP material in 500 μL (MeCN) (0.1% TFA) was vortexed, of which 15 μL of the slurry containing ca. 600 μg MIP was quickly pipetted into the pipette tip. After 3 min of sedimentation, the remaining solvent was removed by a 20 mL syringe (Terumo, Leuven, Belgium) equipped with a Chromabond[®] adapter (Adapter PP-columns, Machery-Nagel). The sample was firstly loaded with loading solvent (95% MeCN, 0.1% TFA) and eluted with (E1) 90% MeOH, 0.1% TFA and water; (E2) 50% MeOH, 0.1% TFA and water. The solvent was removed by a micro-centrifuge (Eppendorf 5415R) at low speed from 200 $\times g$ to 600 $\times g$ accordingly. The resulting fractions were dried and redissolved in 0.1% TFA and subsequently analyzed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (Fig. 1) for fast peptide detection.

2.3. Preparation of digested mouse brain tissue and peptide spiking

For studying the SPE performance for a complex sample, pY, pS and non phosphorylated peptides were spiked in a trypsin digested mouse brain lysate. Mouse brain tissue was lysed with a Dounce homogeniser and a tight pestle stroking 30 times up and down in lysis buffer containing 7 M urea, 30 mM tris(hydroxymethyl)aminomethane and 2 M thiourea in a glass tube on ice. After homogenization, the lysate was sonicated six times for 10 s with 10 s intervals to avoid sample heating. Centrifugation was carried out at $16,000 \times g$ for 15 min and the supernatant was collected. The lysis process was repeated once and two supernatant fractions were pooled in one Eppendorf tube. Protein concentration was determined by Bradford protein assay (Bio-Rad).

Afterwards a short-gel enrichment was performed to remove contaminants from the sample. Hereto, 25 μg lysate was mixed with lithium dodecyl sulphate (LDS), heated at 95°C for 5 min then loaded on an acrylamide gel. Acrylamide gel was polymerized previously for 20 min in a 10 mm thick gel chamber (XCell-SureLockTMMiniCell). Gel solution contained 12.5% acrylamide, 2.5 M bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (pH 6.8) and polymerization was started after addition of 50 μL of 40% (w/v) ammonium persulfate and 10 μL of tetramethylethylenediamine. Electrophoresis was performed with 50 V for only 15 min in order to avoid full protein separation. The gel was stained with coomassie blue imperialTM Protein Stain (Thermo) for 10 min then destained with water overnight.

The band containing enriched brain proteins was then cut into small pieces and washed with buffer A (10 mM ammonium hydrogen carbonate) for 10 min and buffer B (buffer A: acetonitrile 50:50) for 10 min alternating for 6 times until the dye was removed. The gel pieces were dried for 1 h in the rotational vacuum concentrator. Trypsin was added to the gel pieces (trypsin:protein: 1:20) and gel pieces were incubated at 37°C for 16 h. The digestion was stopped by adding (MeCN/ H_2O :50/50 (0.1% TFA)) and using ultrasonic bath for 15 min twice. The supernatant was collected into a glass vial then dried in the rotational vacuum concentrator, and finally redissolved in 20 μL water containing 0.1% TFA. The peptide concentration was determined by amino acid analysis via an ultra high performance liquid chromatography (UHPLC) (Acquity UPLC,

Waters). The peptide mixture (0.016 µg) was spiked into the mouse brain digest (3.4 µg) before the pY-MIP SPE experiment.

2.4. Immunoprecipitation (IP) of phosphopeptides with 4G10, PY20, PY100 antibodies

A peptide mixture sample was reconstituted in IP buffer containing 73 mM sodium chloride, 2.7 mM potassium chloride, 0.02% Tween20, 25 mM -tris[hydroxymethyl]aminomethane and the pH was then adjusted to 7.4 using hydrochloric acid. Magnetic beads conjugated with M-280 sheep anti-mouse secondary antibody (Dynabeads, Life Technologies) were incubated with anti-pY-immunoglobulin G (IgG) primary antibody for 3 h under constant rotation at 4 °C and washed 3 times followed by crosslinking with 0.2 M dimethyl pimelimidatedihydrochloride in triethanolamine buffer (pH 8.2, 20 °C) for 30 min to avoid co-elution of pY-antibody with the peptides. The crosslinking reaction was stopped by adding 1 mL 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (pH 7.5, adjusted by HCl), washed 3 times with IP buffer then incubated for 15 min under strong agitation (crosslinking protocol of IgG and magnetic beads by Dynabeads, Life Technologies). The conjugated IgG and magnetic beads (prewashed three times with IP buffer) were added to the sample and incubated overnight at 4 °C under constant rotation [9]. After incubation, the beads were washed five times with 1 mL IP buffer (without Tween20) followed by washing twice with 1 mL water. Peptides were eluted twice with 0.15% TFA at 20 °C on a Thermomixer (Eppendorf) with strong agitation for 30 min, dried in rotational vacuum concentrator, then redissolved in 5 µL of water containing 0.1% TFA mixed with 5 µL MALDI matrix solution.

2.5. Phosphopeptide enrichment with TiO₂ beads

0.6 mg TiO₂ beads were washed twice with loading buffer composed of 80% acetonitrile, 5% TFA and 1 M glycolic acid then packed in a tip column. Sample was loaded twice onto the TiO₂ beads, incubated for 3 min statically and supernatant removed by short centrifugation. The tip column was then washed twice with loading solution, twice with 80% MeCN, 1% TFA and then twice with 10% MeCN, 0.1% TFA. Three eluting steps were carried out using 50 µL elution solvents as follows: elution 1 (E1): 250 mM NH₄HCO₃ and NH₄OH pH 9.1; elution 2 (E2): 125 mM NH₄HCO₃ and NH₄OH pH 10.5; elution 3 (E3): NH₄OH pH 11.3.

After the three elution steps, the tip column was washed with 2 µL 30% MeCN. The elution fractions were pooled and then acidified with 15 µL formic acid. All washing and elution procedures were accompanied by centrifugation at 1500 × g for 2 min. After desalting of the samples with C18 self-made tips (Oligo R3™ Bulk Media), they were dried in a rotational vacuum concentrator, redissolved in 5 µL of water containing 0.1% TFA and were mixed with 5 µL MALDI matrix solution.

2.6. MALDI TOF/TOF mass spectrometry

Mass spectrometric measurement of the fractions collected during analysis was performed with Flex Control software (Bruker Daltonics) using a MALDI reflector time of flight mass spectrometer (Ultraflex II MALDI TOF/TOF; Bruker Daltonics). The MALDI plate used was Anchor Chip target plate with Transponder Technology (Bruker Daltonics). Data collection, in terms of the scanning conditions and number of the scans, was performed identically for all samples unless otherwise noted. The spectra was collected by accumulating 400 laser shots under reflector mode (relative laser focus: 55%) and further analyzed with the Flexanalysis 3.0 software (Bruker Daltonics).

Table 1

Tyrosine and serine containing model peptides used to probe the phosphoselectivity of the P1, P2, TiO₂, and anti-phosphotyrosine antibodies.

Peptide Sequence	Abbreviation	[M+H] ⁺
AVPSPPAPSP-amide	T-SpS	998,609
VILGPSPAPHR	CpS	1029,5241
DRVYIHFP	Y	1046,5418
DRVpSIHFP	pS	1050,4768
GADDSYYTAR	YY	1118,475
DRVpYIHFP	pY	1126,5081
AVPSPPAPSPR	SpS	1155,5558
GADDSYpYTAR	YpY	1198,4412
GADDSYpYTAR	pYpY	1278,4076
WWGSGPSCGGSGGGK	4S	1420,624
WWGSGPpSCGGSpSGGGK	2S2pS	1580,5567
TRDIVETDYRK	3Y	1622,7809
TRDipYETDpYpYRK	3pY	1862,6799

The sample preparation prior to the MALDI measurements was performed as follows: All fractions were collected in glass vials (HPLC glass insert vial) separately and dried in a rotational vacuum concentrator. After 10 min of drying, the two elution fractions were collected in one vial. The dried samples were dissolved in 5 µL of water (0.1% TFA) and mixed with 5 µL of MALDI matrix with additional ultrasonication for 10 min. 2 µL of this mixture was deposited on the MALDI target plate and dried at room temperature. MALDI matrix solution was prepared by dissolving 40 mg DHB (2, 5-dihydroxybenzoic acid), in MeCN:Water (1:1 v/v, 1 mL) with 1% phosphoric acid and 0.1% TFA.

3. Results and discussion

Three experiments comprising phosphopeptide enrichment using either MIPs, TiO₂ or pY-antibodies were carried out in replicate and representative results are provided for each of the enrichment strategies.

3.1. Comparison of P1 and P2 pY-MIPs

The pY-MIPs described in our previous report [14] are methacrylic (P1) or acrylic (P2) based highly crosslinked polymers featuring a hydrophobic (P1) or hydrophilic (P2) polymer scaffold and high affinity binding sites for the phosphorylated amino acid side chain. The binding sites incorporate 1,3-disubstituted urea groups acting as quadruple hydrogen bond donors for chelating the phosphate guest acceptor. In contrast to TiO₂ or IMAC based enrichments, recognition is here driven by hydrogen bonding involving no charged host species, hence the stationary phase is neutral. This feature we anticipated would reduce any charge dependent sequence bias commonly experienced with the current affinity phases.

Solid phase extraction (SPE) was performed using micro columns in form of disposable pipette tips (Fig. 1) packed with <1 mg imprinted polymer (P1 or P2); 50 µg TiO₂ beads, or 20 pmol of IgG anti-pY- antibodies (see Supporting information) incubated in a reaction tube. Selectivity was probed on a mixture of 13 peptides (1 pmol each) (Table 1) containing mono and multiply phosphorylated peptides at either serine or tyrosine in addition to their nonphosphorylated counterparts.

After loading of the mixture the resulting flow through fractions were compared with elution fractions with respect to the presence of tyrosine-, serine- and non-phosphorylated peptides.

P2 (Table S1) prepared using the hydrophilic crosslinker pentaerythritoltriacylate (PETA) was first compared with P1 (Fig. S1) made using the more hydrophobic crosslinker ethylene glycol dimethacrylate (EGDMA). As expected from our previous report [14], P2 demonstrated the most promising performance. Hence, although P1 clearly enriched mono-phosphorylated and

Fig. 2. MALDI-TOF/TOF-MS analysis of phosphorylated peptides from solid phase extraction of a model peptide mixture (1 pmol of each peptide); (A) flow-through from P1 pY-MIP; (B) elution from P1; (C) flow-through from P2 pY-MIP; (D) elution from P2.

Fig. 3. MALDI-TOF/TOF-MS spectra of SPE fractions after enrichment of a model peptide mixture (1 pmol of each peptide) (A) elution from pY-MIP (P2) material; (B) elution from TiO_2 column; (C) elution fraction after immunoprecipitation from phosphotyrosine antibody 4G10 (3 μg , Merck); (D) elution fraction after immunoprecipitation from phosphotyrosine antibody PY20 (3 μg , Biozol); (E) elution fraction after immunoprecipitation from phosphotyrosine antibody PY100 (3 μg , Cell Signaling).

multi-phosphorylated peptides, no clear discrimination between serine- and tyrosine-phosphorylated peptides was observed. This contrasted with the behaviour of P2. The flow-through fractions contained in this case most of the non-phosphorylated and serine-phosphorylated peptides while all tyrosine-phosphorylated

peptides could be detected in the elution fraction (Fig. 2). Based on these findings, we continued to compare the performance of the MIP SPE with two established phospho-enrichment techniques based on TiO_2 and pY-antibodies.

Fig. 4. MALDI-TOF/MS spectra of SPE fractions, after enrichment of spiked peptides (1 pmol of each peptide) in a digested mouse brain lysate; (A) elution from pY-MIP P2 material; (B) elution from TiO_2 column; (C) elution fraction after immunoprecipitation using the phosphotyrosine antibody 4G10 (3 μg , Merck); (D) elution fraction after immunoprecipitation using the phosphotyrosine antibody PY20 (3 μg , Biozol); (E) elution fraction after immunoprecipitation using the phosphotyrosine antibody PY100 (3 μg , Cell Signaling).

3.2. Performance of TiO_2 approach

First we studied TiO_2 -based affinity enrichment of the model peptide mixture using a well established protocol [15]. TiO_2 beads reportedly bind all phosphorylated peptides without discrimination from phosphorylation of serine, tyrosine and threonine and indeed, we found that 8 out of 9 phosphorylated peptides were enriched with TiO_2 (Fig. 3). Notably however, the doubly tyrosine-phosphorylated peptide pYpY could only be detected at higher sample loads (100 pmol, Fig. S2) presumably due to the excessive affinity and slow displacement of pYpY from TiO_2 columns. This contrasted with the MIP SPE where pYpY was detectable at fmol sample loads and showed that MIP SPE allows a more sensitive detection of tyrosine-phosphorylated peptides.

3.3. Study of immunoprecipitation approach using 3 anti-pY antibodies

We thereafter assessed the performance of different pY-antibodies. Three generic anti pY-antibodies 4G10 (Merck), pY100 (Cell Signalling) and pY20 (Biozol) widely used in the field of phosphotyrosine research [12,13,16–18] were used to carry out immunoprecipitation (IP) with the 13 peptide mixture. Only the triply tyrosine-phosphorylated peptide (3pY) was enriched by pY-antibodies (Fig. 3; Figs. S3–S5) when low peptide amount was applied, while P2 was able to enrich all four tyrosine-phosphorylated peptides (pY, YpY, pYpY, 3pY) at low sample amounts corresponding to 1 pmol of each peptide. To better under-

stand the selectivity and sensitivity of the antibodies, an excessive sample of 200 pmol of each peptide was loaded. All tyrosine-phosphorylated peptides (pY, YpY, 2pY, 3pY) could now be detected by MALDI-MS regardless of the antibody used (Fig. S6). A reason for this high sample load could be that IP involves many washing steps which can lead to a loss of the bound targeted peptides from the antibodies. The large incubation volume (1.5 mL Eppendorf tube) and resulting sample dilution further reduces detection sensitivity in the IP procedure.

A combination of 4G10 and PY100 is recommended [13] for the identification or the affinity purification of a larger number of tyrosine-phosphorylated peptides. Our study indicated that this combination leads to an increased unspecific binding (Table S2 and Fig. S7). In contrast to antibodies, all tyrosine-phosphorylated peptides were enriched using P2 independent of the sample load (1 pmol or 200 pmol) (Fig. 2).

Encouraged by the above results, the SPE performance of the pY-MIP was further investigated for a complex sample and was again benchmarked against TiO_2 and anti-pY-antibodies. The peptide mixture (1 pmol of each peptide; ca. 15 ng sample) was therefore spiked in digested mouse brain lysate. The mouse brain lysate was analyzed before spike-in experiment and 44 phosphopeptides out of 10,400 total peptides were identified from 200 ng mouse brain digest (Table S2). Unexpectedly, the MALDI-MS approach could be used to detect the target peptides in a simple and unequivocal way (Fig. 4). Nevertheless, for lower spiking levels (0.5 pmol), a loss of 2S2pS, pYpY and YpY was observed when the TiO_2 method was used (Fig. S8), whereas the P2 method still led to the enrich-

ment of all tyrosine-phosphorylated peptides (Fig. S9). This result indicates that P2 can be used to extract both mono and multiply tyrosine-phosphorylated peptides in complex samples. In addition, the method relying on P2 displayed the highest sensitivity of all methods as reflected in the enrichment of all four tyrosine-phosphorylated peptides from complex mouse brain lysate (Fig. S9). In contrast, antibodies (Fig.S9-S12) and TiO_2 (Fig.S8) failed at the lower sample loads; the latter as described in our previous work [19].

4. Conclusion

The focus of this work has been to compare three phosphopeptide enrichment procedures using otherwise identical experimental conditions (type and amount of loaded sample) concerning their efficiency to capture targets in a spiked brain lysate digest. The results show that imprinted polymers can complement and potentially even substitute well established bioanalytical tools in protein PTM research. Their high affinity and programmable selectivity combined with their compatibility with typical LC–MS hydro-organic mobile phases allow the use of streamlined enrichment protocols which is both time and sample saving. The excessive spiking levels we justify by the use of MALDI-TOF/TOF-MS as the common detection technique as well as the insensitivity of the immunoenrichment protocol to lower spiking levels. Ongoing work will demonstrate the applicability of the method for pY selective enrichment from real samples [20].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchroma.2016.10.018>.

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III

Hierarchically templated beads with tailored pore structure for phosphopeptide capture and phosphoproteomics

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ABSTRACT: Two templating approaches to produce imprinted phosphotyrosine capture beads with controllable pore structure are reported and compared with respect to their ability to enrich phosphopeptides from tryptic peptide mixture. The beads were prepared by polymerization of urea-based host monomer and crosslinker inside the pores of macroporous silica beads with both free and immobilized template. In the final step the silica was removed by fluoride etching resulting in mesoporous polymer replicas with narrow pore size distributions, pore diameters ≈ 10 nm and surface area > 260 m²/g. The beads displayed pronounced phosphotyrosine affinity and selectivity in binding tests using model peptides in acetonitrile rich solutions with a performance surpassing solution polymerized bulk imprinted materials. Tests of the beads for the enrichment of phosphopeptides from tryptic digests of twelve proteins revealed both pY/pS and pY/Y selectivity. This was reflected in a nearly 6-fold increase in the enrichment factor of a 23-mer pY peptide and pY/pS normalized intensity ratios up to 1.5, when comparing the template mesoporous beads with the bulk materials.

Introduction

Protein phosphorylation is a reversible post-translational modification (PTM) playing a central role in numerous biological events, several of which are directly linked to disease pathogenesis.¹ Phosphoproteomics, i.e. the dynamic mapping of the occurrence of this PTM, has become an essential activity in the development of new drugs and diagnostic methods.^{2,7} Difficulties in the analysis of phosphorylated proteins arise from their low abundance (e.g. phosphotyrosine) and transient nature. These challenges were addressed in functional proteomics using phosphopeptide specific affinity enrichment techniques and mass spectrometry, recently in parallel high throughput formats.^{4,7} Several chemoaffinity-based techniques to concentrate phosphopeptides from complex biological samples have been developed to date, each with their benefits and limitations, the latter generally referring to a lack of side chain selectivity, i.e. recognition of pY, pS and pT respectively, a bias towards certain amino acids or sequence motifs and incompatibility with high throughput formats.³ Another limitation is inherent in the bottom up proteomics approach per se.⁸ Since this relies on the digestion of proteins to small peptides (<3 kDa) by trypsin followed by enrichment and LC-MS based readout of the peptide amino acid sequences, it is difficult to pinpoint multiple modifications (e.g. histone acetylation, methylation, phosphorylation) that are far apart from each other. Top down or middle down proteomics analyzing intact proteins or partially digested proteins (3–20 kDa) respectively address these issues.⁸ Enrichment techniques adapted to larger phosphopeptide targets are therefore needed.

Molecular epitope imprinting has in the above contexts emerged as a possible alternative affinity technique.⁹ Several approaches to develop phosphopeptide selective molecularly imprinted polymers (MIPs) have been reported to date.^{9–14} In our first report, we introduced an epitope imprinting approach relying on the templating of minimal fragments of the targeted peptides.⁹ Hence, imprinting of N, C-protected phosphoamino acids such as pY and pS produced binding sites complementary to pY and pS peptides, respectively. The success of this approach depended on the combined use of lipophilic phosphoamino acid salts and urea-based host monomer in a 1:2 stoichiometry. Incorporation of the resulting ternary complexes in the polymer scaffold followed by template removal left behind binding sites capable of binding the phosphoamino acid guest by quadrupole hydrogen bond interactions.

Having addressed the molecular recognition properties of these materials we have recently turned our attention to their morphological properties. The first generation materials were prepared as porous monoliths following the classical approach to produce macroporous polymers.¹⁵ This results in materials displaying a wide distribution of pore sizes with a significant number of pores in the micro- and low mesoporous range. As a result, the inner pores of such materials show a size dependent binding preference and are likely to be poorly accessible to larger peptides. This will compromise their use for unbiased phosphopeptide enrichment and especially for middle down phosphoproteomics. In order to address this weakness, approaches to control the pore sizes and their distribution are required.

Grafting of thin polymer films or templated synthesis using inorganic supports of known morphology are very promising in this regard.^{16–18} A prominent example is porous silica which is commonly used as a sacrificial template for the synthesis of mesoporous organic polymer networks with defined particle size, shape and porosity. Hierarchical imprinting takes advantage of this morphology control to obtain imprinted polymer beads exhibiting highly accessible binding sites.¹⁹ The molecular template can either be immobilized on the walls of the mold¹⁹ or simply dissolved in the monomer mixture.²⁰ This gives rise to polymer beads featuring enhanced binding site accessibility and faster mass transfer in chromatography. We have here assessed the latter two approaches as means to reduce the size bias in pY selective phosphopeptide enrichments. Micron sized mesoporous polymer beads were thus prepared and compared with a bulk-imprinted benchmark in a mass spectrometry assay of the enrichment of phosphopeptides from tryptic peptide mixtures. We hypothesized that hierarchical imprinting can generate efficient affinity capture for phosphopeptides, particularly of larger phosphopeptides surpassing 3 kDa that are of interest in studies of PTM crosstalk. Our results demonstrate the feasibility of this approach to boost both overall phosphopeptide and pY selectivity.

Experimental

Materials

Pentaerythritol triacrylate (PETA) was from Polysciences (Warrington, PA, USA). 2,2'-azobis(2,4-dimethyl)valeronitrile (ABDV) was from Wako Chemicals GmbH (Neuss, Germany); 1-Hydroxybenzotriazole (HOBt), 1,2,2,6,6-pentamethylpiperidine (PMP), triethylammonium bicarbonate (TEAB) buffer (1 M), acetic anhydride (Ac₂O) and formic acid (FA) were from Fluka (Deisenhofen, Germany). Trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (MeOH) were from VWR chemicals. Dry dichloromethane (dry DCM), triethylamine (TEA), ammonium hydrogen difluoride (NH₄HF₂), 2,5-dihydroxybenzoic acid (DHB), piperidine, ninhydrin, acrylamide, iodoacetamide (IAA) and 1,4-dithiothreitol (DTT) were from Sigma-Aldrich (Milwaukee, USA). Dimethylformamide (DMF), dry acetonitrile (dry ACN) were from Acros Organics. Fmoc-pTyr-OH, Fmoc-pSer-OH, Fmoc-Tyr-OH and Fmoc-Gly-OH were from Bachem GmbH (Weil am Rhein, Germany). Fmoc-Tyr(PO(NMe₂)₂)-OH, N-ethyl-diisopropylamine (DIPEA), (Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyBOP) and dichloromethane (DCM) were from Merck KGaA (Darmstadt, Germany). Empore C8 extraction disk was from 3M Bioanalytical Technologies (St. Paul, MN, USA). Trypsin was from Promega (Madison, WI, USA).

Proteins and peptides. The twelve proteins used for protein digestion were: Carbonic anhydrase (bovine), Ribonuclease B (bovine pancreas), Serum albumin (bovine), Lactoglobulin (bovine), α -Casein (bovine), β -Casein (bovine), Ovalbumin (chicken), Lysozyme (chicken), Alcohol dehydrogenase (Baker's yeast), Myoglobin (whale skeletal muscle), α -Amylase (Bacillus species) and Transferrin (human). Transferrin was from ACE Biosciences A/S (Hileroed, Denmark), other proteins were from Sigma-Aldrich (Milwaukee, USA). Phosphopeptides YSSDPTGALT-EDSIDDTLFPVPEpYINQSVPK, GSHQISLDNPDpYQQDFFPK, MHLPSPTDSNFPpYR, RPAGSVQNPVpYHNQPLNPAPSRD, and GSTAENAEpYLR were custom synthesized by Genic-Bio (Shanghai, China). GADDSpYpYTAR, GADDSYpYTAR, GADDSYYTAR, DRVpYIHPF, DRVpSIHPF, VpYI, VpSI and VYI were custom synthesized by LifeTein LLC (Hillsborough, NJ, USA). DRVYIHPF was from Fluka (Deisenhofen, Germany).

Amino-functionalized macroporous silica beads (NH₂@Si) with an average particle size of 30 μ m, a surface area (S) of 45 m²/g, average pore diameter (D_p) of 47.5 nm, and a pore volume (V_p) of 0.81 mL/g were purchased from Fuji Silysia Chemical Ltd. (Kozoji-cho, Kasugai Aichi, Japan). N-(9-Fluorenylmethoxycarbonyl)-O'-phosphonotyrosine ethyl ester (Fmoc-pTyr-OEt, **2**)⁹ and N-3,5-bis(trifluoromethyl)-phenyl-N'-4-vinylphenylurea (**1**)²¹ were synthesized as reported elsewhere.

Apparatus and methods

High-Performance Liquid Chromatography (HPLC). HPLC measurements were carried out on Alliance 2795 instrument equipped with 2996 PDA detector (Waters, Milford, MA, USA).

Scanning Electron Microscopy (SEM). The particle morphology and size were determined using Zeiss EVO LS 10 (E)SEM (Carl Zeiss AG, Oberkochen, Germany) at T = 25 °C, EHT = 15 kV, WD = 4.5 mm.

Optical microscopy. Optical micrographs were acquired using Nikon Optiphot Epi-Fluorescence microscope equipped with polarizing filters, phase contrast and a DS-U1 digital camera.

Thermogravimetric Analysis (TGA). TGA was carried out using a TGAQ500 (TA Instruments). The sample (5–10 mg) was placed in a platinum pan, which is suspended in a sensitive balance together with the reference pan. The sample was then heated in a furnace with a heating rate of 20 °C/min, under N₂ atmosphere.

Elemental Analysis. Carbon, nitrogen, and hydrogen contents were determined by elemental analysis at the Department of Organic Chemistry, Johannes Gutenberg Universität Mainz using a Heraeus CHN-rapid analyzer (Hannau, Germany).

FT-IR Spectroscopy. Infrared spectra were recorded using a Thermo Nicolet Nexus 6700 instrument (Thermo Scientific, Waltham, MA, USA).

UV absorbance measurements were performed on a Sa-fire plate reader (Tecan Group Ltd., Männedorf, Switzerland) using a quartz 96-well microplate (Hellma GmbH, Müllheim, Germany).

MALDI-TOF/TOF-MS All mass spectra were obtained using MALDI reflector time of flight mass spectrometer (ultrafleXtreme MALDI-TOF/TOF-MS/MS; Bruker Daltonics, Bremen, Germany) controlled by flexControl software (version 2.4, Bruker Daltonics, Bremen, Germany). The system was operated in positive ion reflector mode only recording MS1 spectra in the m/z range of 700–4000. Dihydroxybenzoic acid (DHB, 40 mg/mL) in 50% ACN and 1% phosphoric acid was used as matrix.²² Relative laser fluence was set at 55%. Signals from 1000 laser shots (10×100 shots at 10 different positions) were averaged. Data collection, in terms of the scanning conditions and number of the scans, was performed identically for all samples unless otherwise noted. Mass spectrometric data analysis was performed using FlexAnalysis 3.4 software (Bruker Daltonik GmbH, Bremen, Germany). The signal intensity threshold for identification of peptides in the sample was arbitrary set at 9000 a. u.

For the peptide YSSDPTGALTEDSIDDTFLPVPEpYINQSVPK threshold value was set at 3000 a. u.

Nitrogen sorption Nitrogen sorption measurements were performed on the ASAP2020 Sorption Analyzer (Micrometrics, Norcross, GA, USA). Prior to the measurements, samples (100–150 mg) were heated at 105 °C under high vacuum (10^{-5} Pa) for 8 h. The specific surface areas S were evaluated by using the Brunauer–Emmett–Teller (BET) method, the specific pore volumes V_p and the average pore diameter D_p by using the Barrett-Joiner-Halenda (BJH) theory applied to the desorption branch of the isotherm.

Measurement of swelling NMR tubes were filled with dry polymer particles (100 mg) and the height of dry polymer bed was measured. Next ACN (1 mL) was added and the particles allowed to soak in the solvent for 24 h. The particles were then allowed to settle and the bed height of the swollen particles was measured. The swelling factor was calculated as the ratio of the bed height of the swollen particles to the bed height of the dry particles.

Silica surface modification

$\text{NH}_2\text{@Si}$ (20 g) was suspended in DMF (100 mL) in a 250 mL round bottomed flask equipped with an overhead stirrer. Next, acetic anhydride was added (20 mL) and the suspension was stirred at room temperature overnight. Thereafter the silica was filtered off, washed with DMF (3×50 mL) and MeOH (3×50 mL) and dried under vacuum overnight to yield *N*-acetylated silica (AcNH@Si). Negative ninhydrin test confirmed complete protection of the surface amino groups. The degree of acetylation was confirmed by TGA analysis.

Immobilization of pY-template on silica

The pY template was immobilized on the silica surface in a three step process (Figure S1).

Coupling: $\text{NH}_2\text{@Si}$ (3.0 g) was suspended in DMF/DCM (50/50 v/v; 30 mL) under nitrogen in a two neck 100 mL round bottomed flask equipped with an overhead stirrer. Next, Fmoc-Tyr($\text{PO}(\text{NMe}_2)_2$)-OH (1.0 eq), PyBOP (1.1 eq), HOBT (1.1 eq) and DIPEA (2.2 eq) were added according to desired amount of template loading on silica surface (see Table S1 for the specific amounts used in each case). The reaction mixture was stirred under nitrogen atmosphere overnight. The silica was then filtered off and washed on a glass funnel with DMF (3×10 mL) and DCM (3×10 mL) and dried in vacuo.

Endcapping: The modified silica from the previous step (2.5 g) was suspended in DMF (30 mL) in a 100 mL round bottomed flask equipped with an overhead stirrer. After addition of acetic anhydride (2 mL) the reaction mixture was stirred at room temperature for 4 h. Next, the silica was filtered off and washed with DMF (3×10 mL) and MeOH (3×10 mL) and finally dried in vacuo to give Fmoc-p* Tyr@Si . Ninhydrin tests indicated that the protection of the amino groups was complete.

Deprotection: The silica from the previous step (2.0 g) was suspended in TFA/ H_2O (9:1 v/v; 5 mL) and shaken in a sealed 20 mL scintillation glass vial overnight. The solid was thereafter filtered off, washed with water (3×10 mL) and MeOH (3×10 mL) and dried in vacuo to yield silica with immobilized template Fmoc-pTyr@Si (Fmoc-pTyr@Si-A - Fmoc-pTyr@Si-E, Table S1).

Quantification of the immobilized template: 10 mg of silica containing immobilized template (Fmoc-pTyr@Si-A - Fmoc-pTyr@Si-E, Table S1) was mixed with 1 mL of 20% piperidine in DMF and shaken for 1 h. Next, the sample was centrifuged and the supernatant analyzed by UV absorbance measurements using a microplate reader ($\lambda = 301$ nm). The amount of immobilized template was calculated using Fmoc-Gly-OH as an external standard.

Preparation of pY- imprinted polymers

Crushed monoliths (MIP-B, NIP-B). Fmoc-pTyr-OEt template (**2**) (100 mg, 0.20 mmol) was dissolved in dry ACN (2.4 mL) in a 20 mL scintillation glass vial. Next, PMP (71 μL , 0.40 mmol), functional monomer **1** (146 mg, 0.40 mmol), acrylamide (28 mg, 0.40 mmol), PETA (1.55 g, 5.20 mmol) and ABDV (1% w/w of total monomers) were added to the solution. A sample of this pre-polymerization mixture (750 μL) was saved for subsequent preparation of the polymer beads. The remaining mixture was cooled on ice bath while bubbled with N_2 for 10 min and transferred to a 50 mL Schlenk tube under continuous N_2 flow. Next, tube was closed with glass stopper and placed for 24 h in a water bath heated to 50 °C. The obtained polymer monolith (MIP-B) was removed from the tube, lightly crushed and washed with

MeOH/1 M HCl (80:20 v/v) (3 x 30 mL) followed by solvent extraction with MeOH in a Soxhlet apparatus for 24 h. The coarse particles were then crushed and sieved. The fraction of particles with the size range 25–50 µm was used in all experiments. Non-imprinted polymer (NIP-B) used as a control was prepared in the same way but with the omission of template.

Polymer microspheres (MIP-M, NIP-M). Samples of AcNH@Si (1.0 g) were first deaerated in 50 mL Schlenk tubes (three cycles vacuum-N₂) and then allowed to soak in MIP-B or NIP-B prepolymerization mixtures (750 µL, *vide supra*) under continuous N₂ flow while stirring with a spatula until the particles were freely flowing (indicating completion of the pore filling). Next, the tubes were closed with glass stopper and the polymerization was thereafter initiated by placing the tubes in a water bath heated to 50 °C. After 24 h the resulting composite beads were transferred to 50 mL polypropylene centrifugation tubes followed by addition of the etching solution (3 M NH₄HF₂ in water). The tubes were subsequently shaken on a rocking table for 24 h. Thereafter, the polymers were washed with MeOH/1 M HCl (80:20 v/v) (3 x 40 mL) and solvent extracted in a Soxhlet apparatus with MeOH for 24 h. The resulting polymer beads (MIP-M, NIP-M) were dried in vacuo overnight.

Hierarchically imprinted microspheres (MIP-H, NIP-H). Fmoc-pTyr@Si-(A-E) (1.0 g each) were each soaked in dry ACN (2 mL) in 50 mL Schlenk tubes. Next, PMP and functional monomer **1** (both 2 eq with respect to template loading) were added and the solvent thereafter removed under vacuum. Next, the silica samples were deaerated (three cycles vacuum-N₂) and kept under continuous N₂ flow. A monomer mixture consisting of acrylamide (28 mg, 0.39 mmol), PETA (1.55 g, 5.20 mmol) and ABDV (1% w/w based on total monomers) dissolved in acetonitrile (2.4 mL) was prepared. The silica samples were thereafter soaked in 750 µL of the monomer mixture. For the non-imprinted material the monomer mixture (750 µL) was mixed with urea monomer (the same amount as for corresponding MIP) prior to addition to the silica. The tubes were closed with glass stoppers and placed in a water bath heated to 50 °C for 24 h. Next, the materials were submitted to etching, washing and solvent extraction as in the case of MIP-M and NIP-M described above. Five hierarchically imprinted MIPs (MIP-EA – MIP-HE) and corresponding NIPs (NIP-EA – NIP-HE) were obtained (Table S2).

Solid phase extraction

Polymer particles (20 mg) were packed in single fritted SPE cartridges (ISOLUTE, Biotage) and protected with a frit on top. The polymers were first conditioned with 95% ACN + 0.1% FA (2 x 1 mL) followed by loading of an equimolar mixture of Fmoc-pTyr-OH, Fmoc-pSer-OH and Fmoc-Tyr-OH (1 mL, each at 100 µM concentration) in 95% ACN + 0.1% FA. The washing step was performed

with 80% ACN + 0.1% FA (1 mL) and it was followed by elution with 80% MeOH + 1% TFA (1 mL). Flow-through (FT), washing (W) and elution (E) fractions were analyzed by reversed phase HPLC to determine the unbound amount of each compound. The column was Synergi 4 µm POLAR-RP 80 Å (Phenomenex, 75 x 2 mm). Mobile phases were (A) H₂O + 0.1% TFA and (B) MeOH + 0.1% TFA. A linear gradient method of 40% B to 80% B in 15 min at a flow rate of 0.6 mL/min was used. The injection volume was 20 µL and the detection was performed by UV absorbance measurement at 265 nm. The resulting peak areas were used to calculate the amount of bound analytes (*B*) on the polymer according to equation (1). The results are averages of three independent experiments.

$$(1) \quad B = (C_0 - c)v/m$$

Where *C*₀ is the initial solute concentration, *c* is the final free solute concentration in the supernatant, *v* is the total volume of the adsorption mixture, and *m* is the mass of polymer in each vial.

Binding isotherms

The polymers (10 mg each) were separately mixed with 0.5 mL of Fmoc-pTyr-OH at 0.05, 0.1, 0.25, 0.50, 1.0 and 1.5 mM concentration in 95% ACN and shaken vigorously for 2 h. Next, the samples were centrifuged and the supernatant analyzed by reversed phase HPLC using method described above to determine concentration of unbound Fmoc-pTyr-OH. The amount of bound amino acid per unit mass of polymer (*B*) was calculated according to equation (1). Each experiment was performed three times. Binding curves were constructed by plotting *B* against free concentration *c* and were subsequently fitted by non-linear regression in the GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) to a Langmuir mono-site model:

$$(2) \quad B = B_{max} \cdot c / (K_d + c)$$

where *B*_{max} is the maximum amount of solute bound by the polymer particles at saturation. The association constants *K*_a were calculated as the inverse of the dissociation constants (*K*_d).

Equilibrium binding tests

The polymers (10 mg each) were suspended in 0.5 mL of a mixture of VpYI, VpSI and VYI (each 20 µM) in 95% ACN + 0.1% FA and shaken vigorously for 2 h. Next, the samples were centrifuged and the supernatant (400 µL) was dried (Genevac EZ-2 evaporator), dissolved in 15% ACN (200 µL) and analyzed by reversed phase HPLC. The column was Prodigy 5 µm ODS-3 100 Å (Phenomenex, 150 x 4.6 mm). Mobile phases were (A) H₂O + 0.1% TFA and (B) ACN + 0.1% TFA. A linear gradient method of 15% B to 20% B in 10 min at a flow rate of 1.5 mL/min was used.

The injection volume was 100 μL and the detection was performed by UV absorbance measurement at 210 nm. The amount of bound peptide per unit mass of the polymer (B) was calculated according to equation (1). Each experiment was performed three times. The test was repeated for octapeptides, using instead a mixture of DRVpYIHPPF, DRVpSIHPPF and DRVYIHPPF (each 20 μM) in 95% ACN + 0.1% TFA. The method for HPLC analysis was a linear gradient of 20% B to 30% B in 10 min with other parameters remaining unchanged.

Extraction of phosphopeptides from protein digest

Protein digestion

The twelve proteins, three thereof phosphorylated on serine residues (α -Casein, β -Casein and Ovalbumin), were separately dissolved in TEAB (50 mM) to concentrations of 20 pmol/ μL . Proteins were reduced with 10 mM DTT at 56 $^{\circ}\text{C}$ for 30 min. The proteins were subsequently alkylated with 40 mM IAA at room temperature for 30 min kept in dark. The proteins were digested with trypsin (1% w/w) at 37 $^{\circ}\text{C}$ for 12 h. The protein digest sample was prepared by mixing the peptides originating from the proteins in equimolar ratio and dilution with 0.1% TFA to reach a final concentration of 1 pmol/ μL . The mixture was stored at -20°C until further use.

The protein digest (20 μL , 1 pmol/ μL) and peptide mixture (5 μL , 1 pmol/ μL of each peptide, see Table 2) were diluted to 1 mL with loading solvent (95% ACN + 1% FA) (diluted digest). Polymers (2 mg each) were mixed with 100 μL of diluted digest and agitated in a Thermomixer at 25 $^{\circ}\text{C}$ (1300 rpm) for 30 min. The polymer suspension was transferred to a pipette tip microcolumn (200 μL) protected with a C8 plug and passed through the column with the aid of a syringe and the flow-through fraction was collected. Thereafter washing was performed with 95% ACN + 1% FA (100 μL). The flow-through and washing fractions were pooled. Finally elution was performed with 80% MeOH + 0.1% TFA (100 μL) and the elution fraction saved. The combined flow-through and washing (FTW) and elution (E) fractions as well as a fraction of the sample before MIP treatment were dried in a speedvac and redissolved in 0.1% TFA (5 μL). Each sample (0.5 μL) was spotted together with the DHB matrix (0.5 μL) on the MALDI target plate, dried and analyzed by MALDI-TOF/TOF mass spectrometry.

Results and discussion

Preparation of materials

The purpose of the study was to investigate the influence of porosity parameters in phosphotyrosine (pY) imprinted materials on their static and dynamic peptide binding properties. We therefore decided to compare the performance of our previously reported pY-MIP (used as a benchmark) featuring a broad pore size distribution,

with controlled pore size formats based on silica templating (Figure 1).

The benchmark polymer was prepared using the functional monomer **1** and the bis-PMP salt of Fmoc-pTyr-OEt dissolved in ACN in a 2:1 stoichiometric ratio. Acrylamide was added as a supplementary monomer to provide additional hydrogen bond stabilization and pentaerythritoltriacylate (PETA) was used as a crosslinking monomer. Conventional azo-initiated thermal polymerization at 50 $^{\circ}\text{C}$ subsequently afforded the imprinted and non-imprinted polymers which were subsequently crushed and sieved to a 25–36 μm particle size fraction. The particles were subjected to template removal by washing with acidified MeOH, followed by extraction with MeOH and drying.

Figure 1. (A) Structures of the urea-based functional monomer (**1**) and Fmoc-pTyr-OEt template (**2**); Schematic representation of the preparation of (B) MIP-M and (C) hierarchically imprinted MIPs (MIP-HA – MIP-HE).

The silica templated polymers were subsequently prepared following two alternative approaches (Figure 1). In both approaches we used mesoporous ($D_p \approx 50$ nm) spherical silica microparticles as vessels for polymerization in two variants, i.e. with free or immobilized template. In the former approach (Figure 1B) endcapped silica was allowed to soak in the prepolymerization mixture (identical to the one used to prepare the benchmark polymer, i.e. containing pY template, monomers and initiator) whereafter the particles were thermally cured at 50 $^{\circ}\text{C}$. This contrasted with the latter approach (Figure 1C) where the template had been pre-immobilized on the surface of the silica pore walls (Figure S1). After 24 h curing the silica mold was dissolved by treatment with an aqueous solution of NH_4HF_2 leaving behind organic polymer beads with a size and morphology reflecting those of the original silica (Figures S3 and S4).

A crucial parameter in the latter approach is the surface coverage with immobilized template. A high coverage

may lead to template clustering which precludes the formation of separate binding sites whereas a low coverage leads to materials featuring insufficient binding capacity. We therefore studied this parameter in more detail and compared polymers prepared from five different silica templates with increasing template coverages (Table S1). The polymers were characterized by elemental analysis (Table S3), optical and scanning electron microscopy (SEM) (Figure S3 and Figure S4), IR-spectroscopy (Figure S6), swelling measurements and nitrogen sorption analysis (Table 1, Figure S5). This confirmed their identity and near identical chemical compositions. The SEM images confirmed that the silica templated materials retained the spherical shape and size of the silica scaffold after etching. This indicates that the residual beads originated from the polymer formed in the silica pore system. Otherwise only the nitrogen sorption technique, which provides information concerning the porous properties of the materials, and associated swelling tests gave evidence for differences between the polymers (Table 1, Figure S5).

Table 1. Physical properties of imprinted and non-imprinted polymers.^a

Polymer	S (m ² /g)	V _p (mL/g)	D _p (nm)	Swelling (mL/mL)
MIP-B	72	0.06	3.4	2.3
NIP-B	248	0.48	8.5	1.4
MIP-M	264	0.84	12.4	2.1
NIP-M	325	1.22	15.1	1.7
MIP-HE	326	0.94	10.4	1.9
NIP-HE	201	0.89	17.0	1.9

^a The BET specific surface area (S), specific pore volume (V_p) and average pore diameter (D_p) were calculated from the nitrogen adsorption isotherms whereas the swelling was determined by soaking 100 mg of packed bed of polymer particles in ACN as described in the experimental section.

Thus, all polymers except MIP-B exhibited a mesoporous morphology with surface areas larger than 200 m²/g and relatively narrow pore size distributions (Figure S5) with average pore diameters exceeding 10 nm. This contrasted with MIP-B, featuring nearly no dry state porosity but significant swelling in acetonitrile. In view of the contrasting MIP/NIP properties in this case, the origin of this effect is template related. It has previously been attributed to a template induced delay in the phase separation.⁹

We gratefully noted that the silica-templated materials featured significantly increased surface areas and average pore sizes. It remained to be investigated how this morphology difference translated into the peptide binding properties of the materials.

Binding isotherms and imprinting efficiency

In order to compare the affinity and capacity of the materials we recorded the binding isotherms of all materials for the template analogue Fmoc-pTyr-OH in 95% ACN

(Figure 2A and Figure S2). This solvent was also used in the polymerization step and will hence promote polymer chain conformers present during imprinting, this in turn enhancing imprinting efficiency. Generally, binding to the imprinted polymers exceeded by far that to the corresponding non-imprinted polymers, the latter displaying binding capacities not exceeding 8 μmol/g. Comparing different isotherm models²³ it was clear from the correlation coefficients that the data for all imprinted polymers fitted well to a 1:1 binding model (Table S4). Hence, the imprinted sites appear uniform in this concentration interval.²⁴ This is in agreement with imprinting stemming from a near stoichiometric monomer-template complex formation. Indeed, ¹H NMR titrations revealed strong interactions between the two species with *K_a* exceeding 10000 M⁻¹.

Figure 2. (A) Binding isotherms of Fmoc-pTyr-OH (concentration range 0.05–1.5 mM) for MIP-B (blue circles), NIP-B (red squares), MIP-M (green triangles), NIP-M (purple triangles). The results are averages of three replicates with error bars representing standard deviation. (B) Nominal (*B_{max}^{nom}*) versus measured (*B_{max}^{meas}*) capacity of MIPs. (C) Association constants (*K_a*, blue bars) and binding capacities (*B_{max}*, red squares) for Fmoc-pTyr-OH interacting with imprinted polymers in 95% ACN. The binding parameters were obtained by fitting of the binding data in Figure 2A and Figure S2 to a Langmuir mono-site binding model. (D) Imprinting efficiency (IE) of MIPs.

Figure 2C shows the binding constants and capacity estimated by non-linear curve fitting of the data in Table S4 assuming a Langmuir mono-site binding model. The silica-templated polymer MIP-M prepared using free template displayed the most promising performance after ranking of the polymers in terms of both binding constant and saturation capacity (Figure 2C). This is likely to be the result of a more open pore system (Table 1) offering improved accessibility for the guest to the imprinted sites. However, a direct comparison of saturation capacities may be misleading given the different template loads used when synthesizing the materials. Instead, it is interesting to compare the imprinting efficiency which relates the experimentally determined capacity to the nominal value based on the actual load of the template. The silica

templated materials (e.g. MIP-M, MIP-HA) show the highest values approaching 35% imprinting efficiency, to be compared with an efficiency of 23% for the benchmark material MIP-B (Figure 2D, Table S4). Turning to the hierarchically imprinted materials two striking features should be noted. First of all, the template immobilization seems to compromise the binding affinity as seen in the ca. three times lower binding constants for these materials compared to the benchmark MIP-B and MIP-M. Immobilization prevents imprinting memory with respect to the C-terminal substituent of the template. Nevertheless, the absence of a clear trend when comparing MIP-H(A-E) indicates that the quality of the binding sites is similar for these materials, also in agreement with the strong monomer template interactions (*vide supra*). The second striking feature is the apparent correlation between the measured capacity and the nominal value (Figure 2B). Also this observation is in agreement with the presence of stable monomer template complexes. It is interesting to note that the efficiency decreases with template coverage. This we attribute to template clustering and site coalescence as a result thereof.

Investigation of binding selectivity

In order to probe the binding selectivity of the materials a competitive solid-phase extraction (SPE) experiment was performed. An equimolar mixture of three amino acids (Fmoc-pTyr-OH, Fmoc-pSer-OH and Fmoc-Tyr-OH) was loaded onto each material packed in SPE cartridges (see experimental part) followed by a washing and an elution step. The amount of each analyte in each fraction was quantified by reversed phase HPLC analysis (Figure 3 and Figure S7).

Non-imprinted materials showed an overall weak retention of the analytes, all of which were quantitatively recovered in the load and wash fractions (Figure S7). Nevertheless, as can be seen in the wash fraction recoveries, a weak preference for the phosphorylated analytes, notably pY, was present. This effect was clearest for the materials prepared in presence of free template (MIP-B, MIP-M). When considering the imprinted materials all of them showed a pronounced selectivity for phosphorylated amino acids with a clear preference for phosphotyrosine. MIP-B, MIP-M and MIP-H(C-E) retained phosphotyrosine very strongly while phosphoserine was mainly found in the wash fractions. The retention of phosphotyrosine on the hierarchically imprinted materials increased with the order of increasing template load, a result corroborated by the measured saturation capacities.

To gain insight into the potential phosphopeptide preference of the materials we designed a model system based on the peptide hormone angiotensin II (DRVYIHPF) including different phosphorylated analogues and truncated sequences. The ability of the receptors to discrimi-

nate pY and pS in the same sequence context and in peptides of different sizes was thus investigated by comparing the retention of the phosphorylated peptides DRVpYIHPF, DRVpSIHPF, VpYI, VpSI, with the nonphosphorylated peptides DRVYIHPF and VYI.

Figure 3. Results of SPE test with Fmoc-pTyr-OH (A) and Fmoc-pSer-OH (B) showing % of each analyte in the flow-through (FT), washing (W) and elution (E) fractions. The following conditions were applied: loading 95% ACN + 0.1% FA, washing 80% ACN + 0.1% FA, elution 80% MeOH + 1% TFA. The bars show an average of three replicas and the error bars represent standard deviation.

We first investigated binding of the shorter sequences VpYI, VpSI and VYI. A ternary mixture of these peptides was thus equilibrated with each of the materials followed by measurement of unbound fractions. The uptakes calculated for the imprinted materials showed a pronounced phosphopeptide preference, in the case of MIP-B and MIP-M accompanied by a notable pY selectivity (Figure 4). An interesting reversal of the binding preference was observed for the non-imprinted materials which all exhibited a strong pS selectivity. We believe this to be related to the different basicities of the alkyl versus aryl phosphate groups (pY: $pK_2 = 5.80$; pS: $pK_2 = 6.19$).

Figure 4. Results of binding equilibrium test with an equimolar mixture of VpYI, VpSI and VYI in 95% ACN + 0.1% FA for (A) MIPs and (B) NIPs. The bars show an average of three replicas and the error bars represent standard deviation.

Turning to the native octapeptides (DRVpYIHPF, DRVpSIHPF and DRVYIHPF) we narrowed the investigation to include MIP-B, MIP-M and MIP-HE. Equilibrium binding tests using an equimolar mixture of the three peptides led to the binding results shown in Figure 5. Considering first the uptakes measured for the pY peptide we note that these are significantly lower on the imprinted materials prepared using free template (MIP-B and MIP-M: ca. 22 and 18% respectively) compared to the uptakes displayed by the hierarchically surface imprinted

material (MIP-HE: 50%). Moreover, the uptakes shown by the former materials are lower than those measured for the tripeptides.

Figure 5. Results of binding equilibrium test of an equimolar ternary mixture of DRVpYIHPF, DRVpSIHPF and DRVYIHPF in 95% ACN + 0.1% TFA. The bars show an average of three replicas and the error bars represent standard deviation.

All in all, this indicates that the binding sites in these materials are more buried and therefore less accessible. This also explains the high binding specificity which is reflected in the lack of uptake of the pS peptide. On the other hand, MIP-HE, featuring the surface imprinted sites, did bind this peptide, albeit to a lower extent than the pY peptide. The non-imprinted materials NIP-M and NIP-HE showed either no side chain selectivity or a preference for pS (NIP-B).

Phosphopeptide enrichment from complex peptide mixtures

To validate the ability of the controlled pore size materials to enrich phosphopeptides from complex peptide mixtures we turned to protein digests. Hence, a sample originating from the tryptic digestion of 12 proteins (of which three contain a high abundance of serine-phosphorylated residues, i.e. ovalbumin, α -casein and β -casein)²⁵ was spiked with 8 pY peptides one pS peptide and two Y peptides (Table 2).

The size of the peptides ranged from 8 to 31 amino acids with charges spanning from -5 to +0.1 and GRAVY index from -1.27 to -0.26 (for the parent nonphosphorylated peptides). Phosphotyrosine peptides represented less than 1% (by mass) of all peptides in the sample. The polymers (2 mg) were equilibrated with the spiked protein digest sample in the loading buffer for 30 min. Thereafter, the particle suspensions were transferred with the pipette to tip columns and the packing then protected with a C8 plug. The flow-through (FT) fraction was collected and pooled with the fraction collected in the washing step (W). Thereafter elution was performed and the resulting fraction (E) collected and saved. All fractions were dried, redissolved and analyzed by MALDI-MS with respect to the presence of pY, pS and nonphosphorylated peptides.

As can be seen in Figure 6A, five out of the eight spiked pY peptides along with four pS peptides from the protein digest and the spiked pS peptide were identified in the crude sample before enrichment (Figure 6A, Table S5). The sample clean-up performed with the MIPs allowed the reduction of ion signals stemming from nonphosphorylated peptides to a great extent (Figure 6B-D, Table S5) for improved detection of phosphopeptides. MIP-B was able to extract seven out of the eight spiked pY peptides whereas spherical MIPs (MIP-M and MIP-HE) extracted all spiked pY peptides. The combined flow-through and washing fractions from the MIPs, on the other hand, contained four (MIP-B and MIP-M) or two (MIP-HE) pS peptides and only two (MIP-B, MIP-M) or three (MIP-HE) of the eight pY peptides (Table S5, Figure S8).

Table 2. Model peptides spiked in the twelve protein tryptic digest.

Peptide sequence	Size ^a	[M+H] ⁺	Net charge ^b	GRAVY index ^c
DRVYIHPF	8	1046.54	0.1	-0.32
DRVpSIHPF	8	1050.48	0.1	-0.26
GADDSYYTAR	10	1118.47	-1	-1.24
DRVpYIHPF	8	1126.51	0.1	-0.32
GADDSYpYTAR	10	1198.44	-1	-1.24
GADDSpYpYTAR	10	1278.41	-1	-1.24
GSTAENAEPYLR	11	1290.54	-1	-0.98
MHLPSPTDSNFPYR	13	1644.68	+1	-1.00
GSHQISLDNPDpYQQDFPK	19	2316.00	-1	-1.27
RPAGSVQNPVpY-HNQPLNPAPSRD	23	2594.23	+2	-1.25
YSSDPTGALTED-SIDDTFLPVPEpYINQSVPK	31	3478.58	-5	-0.55

^a Number of amino acids; ^b Net charge at pH 7; ^c GRAVY index for the parent nonphosphorylated peptide.

Peptide ion signal intensities in MALDI-MS are dependent on the ionization efficiency of each of the analytes and the solvent/matrix composition.²⁶ This precludes its use for direct quantitative analysis unless special precautions are taken and carefully optimized internal standards are used. Changes in the relative abundance of a given compound can, however, be determined by comparing normalized signal intensities.²⁶ Hence, we calculated the relative signal intensities of the pY, pS and Y peptides respectively to the total intensity of all selected peptides in a given fraction (Table S6, Figure 7). The increase in the relative signal intensities of the pY peptides together with a decrease of the nonphosphorylated peptide signals show that the MIPs in principle work as expected and confirm our previous observations.¹⁰

A more refined analysis of the data allows further conclusions concerning the relative performance of the materials. Hence, we compared the normalized signal intensities collectively for all pY and pS peptides (Figure 8A) and of all pY and Y peptides (Figure 8B).

Figure 6. MALDI mass spectra obtained for peptide mixture before enrichment (A), elution fraction from MIP-B (B), MIP-M (C) and MIP-HE (D). Marked are spiked phosphotyrosine peptides (asterisk), phosphoserine peptides (square) and spiked phosphoserine and nonphosphorylated peptides (circle).

This showed that the overall pY/pS ratio increased significantly after enrichment. On the other hand, the highest phosphopeptide specific enrichment factor (pY/Y) was obtained using the templated materials MIP-M and MIP-HE. Hence, the nonphosphorylated peptides were depleted from the sample treated with these MIPs. MIP-M here proved to be the most successful in removing the Y peptide (angiotensin II).

Figure 7. Normalized signal intensities for the pY, pS and Y peptides found in the elution fractions given as a percentage of accumulated intensity of selected ions in a given fraction.

purpose, we compared groups of peptides (one pY, one pS and one nonphosphorylated peptide) of similar size with the pY being of size 8, 14, 19 and 24 amino acids.

Figure 8. Ratios of normalized signal intensities of the pY, pS and Y peptides for nontreated and treated samples (A, B) and with respect to peptide size for the treated samples (C, D).

Given the aim of enriching larger phosphopeptide fragments, we were interested in how the selectivity and enrichment responded to the size of the peptide. For this

The octapeptides corresponding to the angiotensin II model system (*vide supra*) offer the most stringent test for the pY/pS selectivity since it features peptides of identical sequences at the C and N terminus. In spite of the

sequence similarity, the MIPs showed a marked pY selectivity ($pY/pS \approx 1.5$) also in the more complex digest sample. Turning to the size dependent phosphospecific enrichment (Figures 8C and 8D) we observed clear differences between the materials.

Whereas the benchmark material MIP-B showed the lowest pY/Y ratio and only a minor increase of the ratio with peptide size, both the mesoporous materials MIP-M and MIP-HE showed a clear trend of increasing enrichment efficiency with increasing size of the peptide. This we attribute to the larger average pore diameter of these materials and a relative increase in the number of sites associated with such pores. In addition to size exclusion effects, it is important to consider the surface chemistry of the materials. The silica template defines not only the average size and distribution of pores in the polymer replica but also the surface chemistry of these pores. Hence, as we have previously shown by the Engelhardt test,¹⁸ surface hydrophilicity is affected by the type of silica surface modification, e.g. TMS endcapped silica leads to a more hydrophobic material than a material synthesized using a rehydroxylated silica template. The acetamide endcapped silicas used in the current study may have resulted in a more hydrophilic surface compared to that present in the benchmark material. Such material will display a lower degree of non-specific peptide binding.

Conclusions

In conclusion, we have shown that silica templated MIP synthesis and surface imprinting are useful approaches to bias the phosphopeptide preferences to larger peptide sizes (> 2 kDa) as well as to suppress non-specific binding of nonphosphorylated peptides. Further optimization of the synthesis parameters is in progress to adapt the materials to middle-down and top-down phosphoproteomics workflows.

Associated content

Supporting information

Binding isotherms, SEM images and optical micrographs, nitrogen sorption, TGA and elemental analysis, ATR-FTIR and MALDI spectra, MALDI peak intensity analysis.

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Supporting Information

Hierarchically templated beads with tailored pore structure for phosphopeptide capture and phosphoproteomics

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Table of contents

Figure S1. Template immobilization on silica surface.....	S2
Figure S2. Binding isotherms	S3
Figure S3. SEM images	S4
Figure S4. Optical micrographs	S5
Figure S5. The BJH differential pore volume.....	S6
Figure S6. ATR-FTIR spectra.....	S7
Figure S7. SPE test results	S8
Figure S8. MALDI mass spectra.....	S8
Table S1. Characterization of silica with immobilized template	S9
Table S2. Characterization of polymeric materials.....	S9
Table S3. Elemental composition of imprinted and non-imprinted polymers.	S10
Table S4. Binding constants (K_a) maximum binding capacity (B_{max}) and imprinting efficiency (IE).....	S10
Table S5. Peptides found in the sample before enrichment and in combined flow-through and washing (FTW) and elution (E) fractions.....	S11
Table S6. Peak intensity analysis for samples before and after MIP enrichment.	S12

Figure S1. Template immobilization on silica surface, (a) PyBOP, HOBT, DIEA, Fmoc-Tyr(PO(NMe₂)₂)-OH, DMF, room temperature, 24 h; (b) Ac₂O, DMF, 2 h; (c) TFA/H₂O 9/1 (v/v), room temperature, overnight. See experimental part for details.

Figure S2. Binding isotherms of Fmoc-pTyr-OH (concentration range 0.05 mM–1.5 mM) for surface imprinted polymers and corresponding non-imprinted polymers. (A) MIP-HA/NIP-HA, (B) MIP-HB/NIP-HB, (C) MIP-HC/NIP-HC, (D) MIP-HD/NIP-HD, (E) MIP-HE/NIP-HE. Points show an average of three replicas with the error bars representing standard deviation.

Figure S3. SEM images of (A) MIP-B, (B) NIP-B, (C) MIP-M, (D) NIP-M, (E) MIP-HE, and (F) NIP-HE at different magnifications.

Figure S4. Optical micrographs of synthesized polymers and silica microparticles.

Figure S5. The BJH differential pore volume ($dV/d\log(w)$) distribution plots against pore width for (A) MIP-B, MIP-M, MIP-HE and (B) NIP-B, NIP-M, NIP-HE.

Figure S6. ATR-FTIR spectra of imprinted and non-imprinted polymers (A) MIP-B/NIP-B, (B) MIP-M/NIP-M, (C) MIP-HA/NIP-HA, (D) MIP-HB/NIP-HB, (E) MIP-HC/NIP-HC, (F) MIP-HD/NIP-HD, (G) MIP-HE/NIP-HE.

Figure S7. Results of SPE test with Fmoc-pTyr-OH (A), and Fmoc-pSer-OH (B) and Fmoc-Tyr-OH (C, D) showing % of each analyte in flow-through (FT), washing (W) and elution (E) fractions. The following conditions were applied: loading 95% ACN + 0.1% FA, washing 80% ACN + 0.1% FA, elution 80% MeOH + 1% TFA. The bars show the average of three replicas and the error bars represent standard deviation.

Figure S8. MALDI mass spectra obtained for combined flow-through and washing fraction (FTW) from (A) MIP-B, (B) MIP-M and (C) MIP-HE. Marked are spiked phosphotyrosine peptides (asterisk), phosphoserine peptides (square) and spiked phosphoserine and nonphosphorylated peptides (circle).

Table S1. Characterization of silica with immobilized template.

Sample	Nominal template coverage		Real template coverage ^a		Mass loss (%) ^b
	($\mu\text{mol}/\text{m}^2$)	($\mu\text{mol}/\text{g}$)	($\mu\text{mol}/\text{m}^2$)	($\mu\text{mol}/\text{g}$)	
Fmoc-pTyr@Si-A	0.44	20	0.23	10	3.1
Fmoc-pTyr@Si-B	0.60	2	0.40	18	3.5
Fmoc-pTyr@Si-C	0.76	34	0.73	33	3.8
Fmoc-pTyr@Si-D	0.90	40	0.86	39	4.1
Fmoc-pTyr@Si-E	1.20	54	1.22	55	4.5

^a Calculated by Fmoc-cleavage test; ^b Determined by thermogravimetric analysis.

Table S2. Characterization of polymeric materials.^a

Sample	Silica used for polymerization	Mass loss (%) ^a
MIP-B	-	94.3
NIP-B	-	94.4
MIP-M	AcNH@Si	93.9
NIP-M	AcNH@Si	93.1
MIP-HA	Fmoc-pTyr@Si-A	94.9
NIP-HA	AcNH@Si	94.7
MIP-HB	Fmoc-pTyr@Si-B	94.2
NIP-HB	AcNH@Si	94.9
MIP-HC	Fmoc-pTyr@Si-C	93.2
NIP-HC	AcNH@Si	94.4
MIP-HD	Fmoc-pTyr@Si-D	93.5
NIP-HD	AcNH@Si	95.6
MIP-HE	Fmoc-pTyr@Si-E	95.2
NIP-HE	AcNH@Si	97.3

^a The mass loss was determined by thermogravimetric analysis (TGA). The mass loss of the composites before etching was roughly 30%.

Table S3. Elemental composition of imprinted and non-imprinted polymers.^a

Polymer	Theoretical			Experimental		
	%C	%H	%N	% C	% H	% N
MIP-B	56.30	5.91	1.06	56.36	5.80	0.98
NIP-B	56.30	5.91	1.06	55.88	6.94	0.96
MIP-M	56.30	5.91	1.06	56.28	6.89	0.99
NIP-M	56.30	5.91	1.06	55.78	6.28	0.92
MIP-HA	56.42	6.09	0.62	56.24	6.94	0.50
NIP-HA	56.42	6.09	0.62	56.08	6.82	0.49
MIP-HB	56.39	6.04	0.74	56.16	6.13	0.65
NIP-HB	56.39	6.04	0.74	56.05	5.81	0.69
MIP-HC	56.33	5.95	0.95	56.22	6.88	0.92
NIP-HC	56.33	5.95	0.95	56.08	6.80	0.82
MIP-HD	56.31	5.92	1.03	56.14	5.75	0.96
NIP-HD	56.31	5.92	1.03	55.78	5.77	0.96
MIP-HE	56.25	5.84	1.24	56.13	5.69	1.16
NIP-HE	56.25	5.84	1.24	56.20	5.53	1.14

^a The theoretical nitrogen content for polymers prepared in absence of urea monomer **1** was 0.47%.

Table S4. Binding constants (K_a), maximum binding capacity (B_{max}) and imprinting efficiency (IE) for MIPs and NIPs.

Polymer	$K_a \times 10^3 (M^{-1})$	$B_{max} (\mu mol/g)$	IE (%)
MIP-B	16.5 ± 2.6	25.0 ± 1.0	23
MIP-M	16.7 ± 2.3	36.9 ± 1.3	33
MIP-HA	5.9 ± 2.6	11.4 ± 1.4	36
MIP-HB	3.0 ± 0.9	18.7 ± 2.0	33
MIP-HC	7.8 ± 1.9	23.8 ± 1.6	23
MIP-HD	5.5 ± 1.4	27.3 ± 2.2	22
MIP-HE	4.8 ± 1.3	31.2 ± 2.8	18
NIP-B	2.1 ± 0.5	7.8 ± 0.7	-
NIP-M	1.8 ± 0.5	6.5 ± 0.7	-
NIP-HA	1.2 ± 0.3	4.2 ± 0.6	-
NIP-HB	17.4 ± 49	1.4 ± 0.8	-
NIP-HC	2.2 ± 0.5	3.2 ± 0.2	-
NIP-HD	13.7 ± 4.7	1.8 ± 0.1	-
NIP-HE	8.2 ± 6.9	2.3 ± 0.5	-

Table S5. Peptides found in the sample before enrichment and in combined flow-through and washing (FTW) and elution (E) fractions.^a

Peptide sequence	[M+H] ⁺ (Da)	Origin	Sample	MIP-B		MIP-M		MIP-HE	
				FTW	E	FTW	E	FTW	E
DRVYIHPF	1046.54	Spiked	+	+	+	+	+	+	+
DRVSIHPF	1050.48	Spiked	+	-	+	-	+	-	+
GADDSYYTAR	1118.48	Spiked	+	+	+/-	+	+	+	+
DRVpYIHPF	1126.51	Spiked	+	+	+	+	+	+	+
GADDSYpYTAR	1198.44	Spiked	-	-	+	-	+	-	+
GADDSpYpYTAR	1278.41	Spiked	-	-	+/-	-	+	-	+
GSTAENAEpYLR	1290.54	Spiked	-	-	+	-	+	-	+
TVDMEpSTEVEFTK	1466.61	CASA2 ^b	+	+	+	+	+	+	+
MHLPSPTDSNFpYR	1644.69	Spiked	+	+	+	+	+	+	+
VPQLEIVPNpSAEER	1660.79	CASA1 ^c	+	+	+	+	+	+	+
DIGpSEpSTEDQAMEDIK	1927.69	CASA1 ^c	-	-	+	-	+	-	+
YKVPQLEIVPNpSAEER	1951.95	CASA1 ^c	+	+	+	+	+	+/-	+
FQpSEEQQTDELDQDK	2061.83	CASB ^d	+	+	+	+	+	+/-	+
GSHQISLDNPpYQQDFPK	2315.99	Spiked	+	+/-	+	+/-	+	+	+
RPAGSVQNPVpYHNQPLNPAPSRD	2594.22	Spiked	+	-	+	-	+	-	+
YSSDPTGALTEDSIDDTFLPVPEpYINQSVPK ^e	3478.58	Spiked	+	-	+	-	+	+/-	+

^a ++ = peptide signal found in the sample; - = peptide signal not found in the sample; +/- = peptide signal intensity below 9000 a. u. threshold limit;

^b α -S2-Casein; ^c α -S1-Casein; ^d β -Casein; ^e intensity threshold was 3000 a. u.

Table S6. Peak intensity analysis for samples before and after MIP enrichment.

Peptide sequence	[M+H] ⁺ (Da)	Origin	Intensity (a. u.)			
			Sample	MIP-B	MIP-M	MIP-HE
DRVYIHPF	1046.54	Spiked	363213	43437	35139	31570
DRVpSIHPF	1050.48	Spiked	144086	43153	100226	95112
GADDSYYTAR	1118.48	Spiked	42264	5691	11240	13426
DRVpYIHPF	1126.51	Spiked	224855	99533	213266	223734
GADDSYpYTAR	1198.44	Spiked	0	11759	371113	54946
GADDSpYpYTAR	1278.41	Spiked	0	7799	21441	21148
GSTAENAEpYLR	1290.54	Spiked	0	24775	79378	97943
TVDMEpSTEVFTK	1466.61	CASA2 ^a	125076	12472	19664	29531
MHLPSPTDSNFpYR	1644.69	Spiked	207047	137941	281338	199752
VPQLEIVPNpSAEER	1660.79	CASA1 ^b	66293	47317	114561	124710
DIGpSEpSTEDQAMEDIK	1927.69	CASA1 ^b	0	9353	24299	24762
YKVPQLEIVPNpSAEER	1951.95	CASA1 ^b	169969	109210	267326	202206
FQpSEEQQTDELQDK	2061.83	CASB ^c	71904	60667	167517	123085
GSHQISLDNPDpYQQDFPK	2315.99	Spiked	51887	34352	110974	65043
RPAGSVQNPVpYHNQPLNPASRD	2594.22	Spiked	35786	27254	99005	60164
YSSDPTGALTEDSIDDTFLPVPEpYINQSVPK	3478.58	Spiked	3982	3078	8240	3641
Total intensity of all peptides			1506362	677791	1924727	1370773
Total intensity of pY peptides			523557	346491	1184755	726371
Total intensity of pS peptides			577328	282172	693593	599406
Total intensity of Y peptides			405477	49128	46379	44996
% of pY peptides intensity			34.76	51.12	61.55	52.99
% of pS peptides intensity			38.33	41.63	36.04	43.73
% of Y peptides intensity			26.92	7.25	2.41	3.28
Normalized pY/pS ratio			0.91	1.35	1.88	1.34
Normalized pY/Y ratio			1.29	5.46	19.78	12.50

^a α -S2-Casein; ^b α -S1-Casein; ^c β -Casein.

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Cationic pTyr/pSer imprinted polymers based on a bis-imidazolium host monomer: phosphopeptide recognition in aqueous buffers demonstrated by μ -liquid chromatography and monolithic columns†

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We report on the design and characterization of imprinted cationic host polymers for selective trapping of phosphoserine and phosphotyrosine peptides. A series of imidazolium host monomers were synthesized and characterized with respect to binding affinity and stoichiometry of interaction with salts of phenylphosphonic acid. The strongest binders were subsequently used for the preparation of imprinted polymers in the form of crushed monoliths, using Fmoc-phosphotyrosine-ethyl ester or Fmoc-phosphoserine-ethyl ester as templates in combination with a hydrophilic crosslinking monomer. The polymers were compared with respect to binding and its dependence on solvent, and whether charged or uncharged host monomers were used. The recipes were subsequently implemented in the capillary monolith format for evaluation by micro-liquid chromatography in both buffered and organic media. Results from both tested formats reveal that the cationic host polymers displayed enhanced recognition in polar and buffered media, in contrast to neutral urea-based hosts which showed best results in acetonitrile rich mobile phases.

Introduction

Host–guest chemistry directed toward anion recognition has made significant progress during the last years.^{1–4} These efforts have resulted in low molecular weight hosts capable of selective complexation of oxyanions in water.¹ It is difficult to engineer such hosts for the recognition of guests of higher complexity or of larger size. In this context the concept of molecularly imprinted polymers (MIPs) has been very successful.^{5–10} Formation of MIPs relies on a proper choice of monomers capable of interacting with complementary functional groups of a template molecule. Upon polymerization, the functional group arrangement is carried into the polymer while the template molecule can be removed *e.g.* by a simple solvent extraction step. Properly implemented,

this leaves behind binding sites in the resulting polymer that are capable of rebinding the template with high fidelity. This “top down” approach to receptor design can be used to construct hosts for rather complex guests without prior knowledge of their structure. The main downside is that it requires preparative quantities of templates.

A number of imprinting strategies targeting small anions have been reported in the literature.^{11,12} Design of such polymers drawing inspiration from anion recognition by low molecular weight receptors has been especially rewarding. These can be divided into charged hosts where recognition is driven by ion pairing (*e.g.* Brønsted acid–base interactions)^{13–15} and neutral hosts binding the anions primarily by hydrogen bonding.^{16–20} These categories of hosts typically display different solvent dependencies, *i.e.*, the ionic interactions which operate over a longer range are less susceptible to the presence of water than hydrogen bonding receptors where water can dramatically alter or even suppress binding.¹

We have recently reported on a range of imprinted anion receptors for carboxylates,^{16,17,21} phosphates¹⁸ and sulfates²² featuring 1,3-diaryl ureas (Fig. 1A) as the common host monomer. We have used this concept to construct receptors for structural features characterizing protein post-translational modifications (phosphorylation, sulfation, and glycosylation),

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† Electronic supplementary information (ESI) available: Titration data containing ¹H-NMR CIS curves and Jobs plots for host-monomer guest interactions. Binding curves, ATR-FTIR spectra for the imprinted and nonimprinted polymers. Field effect scanning electron microscopy (FESEM) images for the TRIM monolith substrate, the NIP monolith, and the pS and pY monolithic MIPs at five different magnifications ranging from 520 to 125 000 $\times$. See DOI: 10.1039/c6tb02864k

Fig. 1 Monomers and binding ability. Theoretical hydrogen bonding interactions with the target phosphate group and (a) 1,3-diaryliurea host monomers or (b) bis-imidazolium host monomers.

the former mainly compatible with less competitive organic solvent systems. Imprinted receptors for phosphorylated peptides have been particularly powerful in extracting very low levels of phosphorylated biomarkers from tryptic digests.²³ In order to extend the solvent compatibility of such receptors to water and thereby be able to target proteins in their natural environment we decided to assess the use of monomers based on water compatible host motifs.

The use of imidazolium derivatives as anion receptors has in this context been extensively reported in the literature.^{4,24} The success of these receptors is to a large degree ascribed to the ability of the imidazolium group to engage in strong [C–H]⁺ ionic hydrogen bonding interactions which prevail also in polar protic media. The recognition of phosphate guests in water (e.g., ATP, DNA) has been amply demonstrated.⁴ For example, Kim *et al.* reported a novel water-soluble imidazolium anthracene derivative, acting as a potential fluorescent chemosensor for GTP in 100% aqueous solution (pH = 7.4).²⁵

We have here evaluated mono- and bis-imidazolium monomers (Fig. 1B) as cationic host monomers predesigned to bind divalent oxyanions by combined ionic and hydrogen bonding, possibly aided by π – π stacking interactions. We report on their interactions with salts of phenylphosphonic acid in protic media and on polymers prepared by stoichiometric imprinting using the most potent monomers. Finally their potential use as receptors for selective enrichment of phosphopeptides from aqueous media has been demonstrated.

Experimental

Trimethylolpropane trimethacrylate (TRIM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tetrabutylammonium hydroxide (TBAOH; 1 M in methanol), methanol (MeOH), phosphorus (V) oxychloride (POCl₃), dry dichloromethane (DCM), magnesium sulfate, acrylamide (AAM) and phenylphosphonic acid (PPA) were purchased from Sigma-Aldrich (Steinheim, Germany). Pentaerythritol triacrylate (PETA) was from Polysciences (Warrington, PA, USA) and 1-vinylimidazole was from Alfa Aesar (Karlsruhe, Germany). *N,N'*-Azo-bis(2,4-dimethyl)valeronitrile (ABDV) from Wako Chemicals GmbH (Neuss, Germany); DMSO-*d*₆, methanol-*d*₄ (CD₃OD), sodium hydrogen carbonate (NaHCO₃), acetone and ethyl acetate were obtained from VWR (Radnor, PA, USA). Trifluoroacetic acid (TFA), 2,2,4-trimethylpentane, *N*-ethyl-diisopropylamine (DIPEA), *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), and diethyl ether were from Merck (Darmstadt, Germany) and benzoin methyl

ether (BME), 1,2,2,6,6-pentamethylpiperidine (PMP) and 3-[(methacryloyl)oxypropyl]trimethoxysilane (γ -MAPS) were obtained from Fluka (Buchs, Switzerland). The acetonitrile (ACN) was from J. T. Baker (Phillipsburg, NJ, USA). Sulfur and dry toluene were from Acros Organics (NJ, USA).

The test probes Fmoc-SerOH, Fmoc-TyrOH, Fmoc-pSerOH and Fmoc-pTyrOH [Fmoc = *N*-(9-fluorenylmethoxy)carbonyl] and *L*-tyrosine ethyl ester hydrochloride and *L*-serine ethyl ester hydrochloride were purchased from Bachem GmbH (Bubendorf, Switzerland) and the angiotensin peptides and their phosphorylated counterparts [DRVSIHPF, DRVYIHPF, DRVPSIHPF, and DRVpYIHPF] were purchased from LifeTein, LLC (Hillsborough, NJ, USA). The functional monomers *N*-3,5-bis(trifluoromethyl)-phenyl-*N'*-4-vinylphenylurea (FM1),¹⁶ 3-benzyl-1-vinyl-1*H*-imidazolium bromide (FM2)²⁶ and 1,1'-[2,6-pyridinediylbis(methylene)]-bis[3-vinyl]-1*H*-imidazolium dibromide (FM3)²⁷ were synthesized as reported before. The reagent *N*-(3,5-bis(trifluoromethyl)phenyl)-2-chloroacetamide was synthesized as reported elsewhere.³⁴ The templates Fmoc-pTyrOEt, Fmoc-pSerOEt and the monomer 1-[(3,5-Bis-trifluoromethyl-phenylcarbonyl)-methyl]-3-vinyl-1*H*-imidazolium chloride (FM4) were synthesised as described in the ESI†

The mono- and ditetrabutylammonium salts of PPA (PPA-TBA and PPA-2TBA) were prepared as follows: PPA (0.2 g; 1.26 mmol) was dissolved in methanol (2 mL) followed by addition of 1 or 2 equivalents of TBAOH (1 M in methanol), respectively. Next the solvents were removed on a rotary evaporator and the residues dried *in vacuo* overnight.

Apparatus and methods

All NMR spectra were recorded at 25 °C using an Agilent (Varian) Mercury 400 MHz instrument. Purification of templates was performed on preparative HPLC Waters 600 HPLC instrument (Waters, Milford MA, USA) equipped with in-line degasser, a Waters 2487 dual absorbance detector, a Waters 2700 sample manager and a Waters Fraction collector II. A Waters Alliance 2695 analytical HPLC equipped with Photodiode Array Detector 2996 was used for quantification of Fmoc-pTyrOH in binding isotherm experiments. A micro-HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-10 ADVP pump and an SPD-10 AVP UV-detector fitted with a 35 nL capillary cell from LC Packings (Amsterdam, The Netherlands) was used in the chromatographic evaluation of capillary monoliths. Infrared spectra were recorded using a Thermo Nicolet Nexus 6700 instrument (Thermo Scientific, Waltham, MA, USA). Mass spectra were recorded on Waters ZQ 2000 LCMS System in positive ion mode.

Preparation of molecularly imprinted polymers in the bulk format

The compositions of the bulk imprinted and nonimprinted polymers are given in Table 1. The polymers were prepared by dissolving template (0.5 mmol; T), functional monomer (0.5 or 1 mmol; FM), acrylamide (1 mmol), and PETA (3.96 g; 13.3 mmol) in porogen (5.6 mL). The initiator ABDV (1% w/w of total monomer mass) was added to the solution. The solution was

Table 1 Composition of imprinted (P1–P5) and nonimprinted (PN1–PN5) polymers

Polymer	Funct. mon. (FM)	Template (T)	Molar ratio T/FM/PETA	Porogen ^a	Swelling ^b (mL mL ⁻¹)
P1	FM1	Fmoc-pTyrOEt-2PMP	1/2/26	ACN	1.6
PN1	FM1	—	—/2/26	ACN	1.3
P2	FM2	Fmoc-pTyrOEt-2TBA	1/2/26	MeOH/Tol.	1.3
PN2	FM2	—	—/2/26	MeOH/Tol.	1.4
P3	FM3	Fmoc-pTyrOEt-2TBA	1/1/26	MeOH	1.1
PN3	FM3	—	—/1/26	MeOH	1.2
P4	FM3	Fmoc-pTyrOEt-2TBA	1/1/26	MeOH/Tol.	1.4
PN4	FM3	—	—/1/26	MeOH/Tol.	1.3
P5	FM4	Fmoc-pTyrOEt-2TBA	1/2/26	MeOH/Tol.	1.3
PN5	FM4	—	—/2/26	MeOH/Tol.	1.4

^a Porogens were pure acetonitrile (ACN) or methanol (MeOH), or a 1 : 1 (v/v) mixture of methanol and toluene. ^b Swelling factor measured as the bed volume of swollen polymer after sedimentation in methanol divided by the bed volume of dry polymer.

transferred to a glass ampoule, cooled to 0 °C, and purged with a flow of dry nitrogen for 10 min. The tubes were then flame-sealed while the contents were still being cooled, and the polymerization initiated by placing the tubes in a water bath heated to 50 °C. After 24 h the tubes were broken and the polymers crushed using the minimal force required. The recovered polymers were thereafter washed with MeOH/0.1 M HCl (1 : 1 v/v), extracted in a Soxhlet apparatus with methanol for 24 h, and dried under vacuum. The dried polymers were finally subjected to a grinding and dry sieving step whereby the fraction between 25 and 36 µm was used to evaluate the binding properties. Non-imprinted polymers were prepared as described above, but with omission of the template molecule from the pre-polymerization solution.

¹H NMR spectroscopic titrations and estimation of binding affinities and stoichiometries

The complex stoichiometry was first assessed using the Job method of continuous variation. Stock solutions of the host monomer and guest (10 mM and 25 mM in DMSO-d₆ or CD₃OD, respectively) were combined in NMR tubes, thereby resulting in the following molar ratios: 0 : 10, 2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2, 10 : 0. Total concentration of host and guest was 2 mM. ¹H NMR spectra were thereafter recorded and the proton signals, which could be monitored for eight of the mixing ratios, were used for the evaluation of the complex stoichiometry from plots of the product of the mole fraction of host (*f_M*) and the complexation induced shift (CIS) versus *f_M*.

¹H NMR spectroscopic titrations were performed in dry deuterated solvents. The dissociation (*K_d*) and association (*K_a*) constants for the interaction between the hosts and guests were determined by titrating an increasing amount of guest (PPA-TBA or PPA-2TBA) into a constant amount of functional monomer (FM1–FM4). The initial concentration of the functional monomer was 2 mM and the amount of added guest was 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, and 10.0 equivalents. The CISs of relevant protons were followed and titration curves were constructed by plotting CIS vs. free guest concentration (*c*). The raw titration data were fitted to a 1 : 1 or to a cooperative binding site model²⁵ by nonlinear regression using the built-in functions of GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA), from which the association constants were calculated.

For a 1 : 1 stoichiometry of interaction a Langmuir mono-site model was used according to eqn (1):

$$\text{CIS} = \text{CIS}_{\text{max}} c / (K_d + c) \quad (1)$$

where CIS_{max} is the maximum CIS value at saturation and *K_d* is the dissociation constant.

In the case of the cooperative binding behavior the curves were fitted using the empirical Hill equation according to eqn (2):

$$\text{CIS} = \text{CIS}_{\text{max}} c^h / (K_d^h + c^h) \quad (2)$$

where *h* is the Hill slope and *K_d* is the dissociation constant. The association constants *K_a* were calculated as the inverse of the dissociation constants.

Binding tests using imprinted and nonimprinted polymers

Polymers (P1–P5 and PN1–PN5; 2 mg each) were separately mixed with 200 µL of Fmoc-pTyrOH at 0.25, 0.5, 1.0, 1.3 and 2.0 mM concentration in methanol and shaken vigorously for 2 h. Next the samples were centrifuged and the supernatants were analyzed by HPLC to determine the concentration of unbound Fmoc-pTyrOH. The experiments were performed in two parallel replicas. The specific amount of solute bound by the polymer particles (*B*) was determined by the following formula:

$$B = (C_0 - c)v/m \quad (3)$$

where *C₀* is the initial solute concentration, *c* is the final free solute concentration in the supernatant, *v* is the total volume of the adsorption mixture, and *m* is the mass of polymer in each vial. Binding curves were constructed by plotting *B* against free concentration *c* and were subsequently fitted by non-linear regression in the Graphpad Prism 7 curve fitting software to a Langmuir mono-site model:

$$B = B_{\text{max}} c / (K_d + c) \quad (4)$$

where *B_{max}* is the maximum amount of solute bound by the polymer particles at saturation. The association constants *K_a* were calculated as the inverse of the dissociation constants (*K_d*).

Preparation of molecularly imprinted and non-imprinted monolithic capillary columns

Capillary pre-treatment procedure. UV-transparent fused silica capillaries (250 μm i.d. $\times$ 360 μm o.d.) obtained from Polymicro Technologies (Phoenix, AZ, USA) were treated as described elsewhere,²⁹ with the procedure being selected on the basis of a previous study.³⁰ Briefly, the capillaries were washed with acetone and deionized water, then 1 M NaOH was flushed through the capillary for approx. 5 min, where after the capillary was sealed in the filled state. It was heated at 100 $^{\circ}\text{C}$ for 2 h, then cooled to room temperature and washed first with deionized water and then acetone (15 min each), and finally dried by a flow of dry nitrogen for at least 1 h. A silanization reaction to introduce methacrylic anchoring groups on the surface was carried out by filling the capillary with a degassed mixture of 10% γ -MAPS in toluene, sealing it in the filled state and keeping it at room temperature for 2 h. Finally, the capillary was washed with toluene and dried with a flow of dry nitrogen for at least 1 h.

Capillary monolithic core supports synthesis. Monolith substrates were prepared following literature procedure³¹ from a precursor solution consisting of 40% (w/w) TRIM in a mixture of 2,2,4-trimethylpentane/toluene 4:1 (w/w) as porogen, with BME added at 1% of the TRIM monomer weight as photopolymerization initiator. The γ -MAPS activated capillary was cut into 70 mm long pieces that were filled with the monolith precursor solution by piercing both ends through the septa of two GC vials which had been partially filled with the monolith precursor solution and applying pressure in one of the vials using a syringe. When the capillary was filled, the pressure was released and both ends were carefully kept below the surfaces of the respective vials. The capillary/vial assembly was then subjected to photopolymerization in a UV-Spectrolinker XL-1500 from Spectronics Corporation (Westbury, NY, USA) at 365 nm with irradiation intensity in the polymerization zone at 3.2 mW cm^{-2} , as determined by an International Light Model IL1400 radiometer (Newburyport, MA, USA) fitted with a model XRL140B probe. After the polymerization was completed for 1 h, one millimeter was cut from each end of the capillaries, which were thereafter flushed with methanol for at least 1 h at 4 $\mu\text{L min}^{-1}$, making a note of the back pressure of each capillary.

Grafting of the imprinted and non-imprinted polymer to capillary monolithic core supports. The MIP prepolymerization cocktail was prepared in the manner optimized in our previous work.²² The templates Fmoc-pSerOEt (3.4 mg; 7.8 μmol) targeting phosphorylated serine (pS MIP) or Fmoc-pTyrOEt (4.0 mg; 7.8 μmol) targeting phosphorylated tyrosine (pY MIP) were separately mixed with two equivalents of the strong base TBAOH to form *in situ* the corresponding bis-TBA phosphate salts. Two cocktails were then prepared by mixing the above-mentioned individual templates with monomer FM3 (3.6 mg; 7.8 μmol), acrylamide (0.9 mg; 12.7 μmol), and PETA (59 mg; 197.8 μmol) in dry toluene (478 μL) and dry methanol (478 μL) to make up the MIP grafting solutions. BME (1% w/w with respect to the combined monomer weight) was added as the initiator, and the grafting solutions were finally given a 5 min purge with

nitrogen to remove dissolved oxygen. The nonimprinted polymer (NIP) was prepared in the same way, but with the template bis-TBA salt omitted from the grafting mixture. At least ten column volumes of these grafting solutions were introduced into each TRIM core monolith capillary using syringe pump. Both capillary ends were then sealed with GC rubber septa and frozen at -70°C by clamping between two blocks of solid carbon dioxide for 1 h. The capillary was thereafter rapidly transferred to the surface of a KP 282 cooling-plate (FRYKA-Kältetechnik GmbH, Esslingen, Germany) set at -15°C and the photopolymerization was carried out under a JD-230 Gel Curing UV Lamp (Yayuo Jiadi Electrical Factory, Zhejiang, China) with four 9 W fluorescent tubes having emission maximum around 370 nm to establish the imprinted polymer layer. Prior to use, the UV lamp had been warmed up around 3 h to ascertain constant temperature and intensity during the course of the 60 min photo-polymerization period. The light intensity in the polymerization zone was determined to be 2.8 mW cm^{-2} , as described above. After the polymerization was completed, a 1 mm piece was trimmed from each capillary end, and the finished NIP and MIP capillaries were thereafter individually flushed by methanol containing 0.1% TFA at 10 $\mu\text{L min}^{-1}$ for at least 1 h before further evaluation. The back pressure was measured again during the washing step, and columns that did not show an increase in the back pressure of 0.4 ± 0.1 MPa compared to the back-pressures before grafting were discarded.

Field emission scanning electron microscopy (FESEM). Samples with fresh rupture surfaces prepared by snapping in liquid nitrogen were positioned on adhesive carbon foils attached to standard aluminum sample stubs and mechanically secured to the holders by using conductive glue (Ted Pella Inc., Redding, CA, USA). All samples were thereafter coated with a sequence of 10 nm of carbon by evaporation in an Edwards E14 vacuum coating unit integrating an automatic tilt and rotate device (Edwards High Vacuum Ltd, Crawley, UK) and 5 nm of iridium in a Quorum Q150TS sputter coater (Quorum Technologies, Ringmer, UK). The coated samples were analyzed in a Zeiss Merlin field emission scanning electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) operated at 4 kV. Images were captured at standardized magnifications from randomly chosen areas.

Results and discussion

Monomer synthesis and characterization

The imidazolium based receptors interact with oxyanions through combined $[\text{C-H}]^+$ anion hydrogen bonding and ionic attraction, the relative importance of which depends on the basicity of the guest and the polarity of the medium.¹⁸ Given the enhanced water compatibility of such receptors our goal was to find out whether polymerizable hosts can be used in imprinting of phosphorylated amino acids and how they compare with the previously reported neutral urea-based receptors.

As a first step in our evaluation of imidazolium based hosts we thus decided to compare the urea host monomer FM1 with the mono- and bis-imidazolium monomers FM2–FM4 shown in Fig. 2.

Fig. 2 Functional monomers used to prepare phosphotyrosine and phosphoserine imprinted polymers.

The monomers FM2–FM4 were synthesized as we have reported previously in one (FM1–FM3) or two (FM4) steps by nucleophilic substitution of corresponding alkylhalides with 1-vinyl-imidazole. Tetrabutylammonium hydrogenphenylphosphonate (PPA-TBA) and bis-tetrabutylammonium phenylphosphonate (PPA-2TBA) were chosen as mono- and di-anionic guests respectively, mimicking the phenylphosphate substituent of the template, Fmoc-pTyrOEt. The receptor monomer solutions (2 mM in DMSO- d_6 or CD $_3$ OD) were titrated with a standard solution of the anion guest up to a ten-fold molar excess. Table 2 shows the maximum complexation induced NMR shifts (CIS) of the protons used to calculate the given association constants (K_a) and the complex stoichiometries, the latter determined by Jobs method of continuous variation (Fig. S1–S6, ESI†).²⁸

The titrations performed in DMSO- d_6 were accompanied by pronounced downfield shifts of the urea protons (FM1) or imidazolium (FM2–FM4) ring protons, indicative of hydrogen bonding dominating in the former case (FM1) or contributing in the latter (FM2–FM4) to the host guest interactions. These signals were absent when performing the titrations in CD $_3$ OD which indicates enhanced exchange of these protons or host deprotonation in this solvent. The reported CIS values refer in this case to the FM1 aryl or the FM2–FM4 vinyl protons. The signals that could be monitored throughout the titration were used to calculate free and bound concentrations and the association constants from the resulting binding curve fitted to a 1 : 1 or a cooperative binding model using the Langmuir (1) or Hill (2) equations, respectively.

Considering first the relative complex stabilities involving the monofunctional monomers FM1 and FM2, the mono-tetrabutylammonium salt (PPA-TBA) was used as a monoanion guest in order to promote formation of 1 : 1 complexes. We were interested to see how protic *versus* nonprotic solvents of different polarity influenced the interactions. After having confirmed the 1 : 1 stoichiometry from the Job's plots in DMSO, the 1 : 1 binding model was used in order to determine the respective association constants. As seen in Table 2 the urea monomer FM1 formed the more stable complexes ($K_a = 7350 \text{ M}^{-1}$ for FM1 *versus* $K_a = 82 \text{ M}^{-1}$ for FM2) in DMSO- d_6 , whereas no interactions were observed for any of the monofunctional host monomers when probing binding in the protic solvent CD $_3$ OD. This speaks in favor of hydrogen bonding as the dominating interaction and agrees with previous literature reports on urea and imidazolium receptors.²

A different behavior was observed for receptor FM3 which features two cationic sites and, like FM1, two hydrogen bond donor sites. Relative to FM1, also this monomer interacted more weakly with the monoanion guest (PPA-TBA) in DMSO- d_6 ($K_a = 266 \text{ M}^{-1}$) whereas, in contrast to FM2, binding of the guest could in this case be detected in CD $_3$ OD (CIS = 0.015 ppm). This agrees with the presence of a strong electrostatic contribution to the binding. Being designed to complex the dianion in a presumed 1 : 1 stoichiometry we tested their ability to complex PPA-2TBA. The divalent anion interacted strongly with FM3, as viewed by the steep CIS plots (Fig. S4, ESI†). The sigmoidal shape of the curve and the Jobs plots reveal that the binding was cooperative with an apparent association constant of $K_a = 1680 \text{ M}^{-1}$ and a preferred 1 : 2 host-guest stoichiometry. Apparently, binding of the first guest equivalent induces a host conformation complementary to a second guest equivalent. This unusual stoichiometry has also been observed for bis-imidazolium hosts interacting with singly charged phosphate ions and has either been attributed to binding-induced conformational changes³² in the host or left without explanation.³³ Attempts to gain more detailed structural information from 2-dimensional NMR or crystal structures proved fruitless. Nevertheless, given the lack of interaction between the mono-imidazolium monomer FM2 and the phosphonate guest in methanol, the structure of the termolecular complex between FM3 and PPA-2TBA must

Table 2 Association constants, stoichiometries, complexation induced shifts and Hill slopes for complexes formed between functional monomers FM1–FM4 and tetrabutylammonium salts of phenylphosphonic acid (PPA) guests in DMSO- d_6 or CD $_3$ OD

Host	Guest	Solvent	Proton	K_a^a (M^{-1})	Cplx. (H : G)	CIS ^a (ppm)	h^a
FM1	PPA-TBA	d_6 -DMSO	NH (15, 21)	7350 ± 320	1 : 1	2.90	1.0
FM1	PPA-TBA	CD $_3$ OD	CH (2, 4)	n/a ^b	n/a ^b	n/a ^b	n/a
FM2	PPA-TBA	d_6 -DMSO	CH (8)	82 ± 5	1 : 1	1.07	1.0
FM2	PPA-TBA	CD $_3$ OD	CH (9)	n/a ^b	n/a ^b	n/a ^b	n/a ^b
FM3	PPA-TBA	d_6 -DMSO	CH (7, 8)	266 ± 17	1 : 1	0.23	1.0
FM3	PPA-TBA	CD $_3$ OD	CH (2, 22)	n/a ^c	1 : 1	0.015 ^d	n/a
FM3	PPA-2TBA	CD $_3$ OD	CH (2, 22)	1680 ± 121	1 : 2	0.15	1.9
FM4	PPA-TBA	d_6 -DMSO	CH (8)	332 ± 16	1 : 1/1 : 2	0.82	1.1

^a Association constants (K_a), complexation induced shifts (CIS) and Hill slope (h) based on the shift values of the resonance signals indicated.

^b Titration and Job's experiment did not result in any observable CIS. ^c Determination of K_a was not possible due to lack of curvature. ^d The value represents CIS of sample with 10 equivalents of guest.

involve both imidazolium groups simultaneously coordinating the two phosphonate guests. Assuming both imidazolium moieties in an *anti* conformation with respect to the pyridine ring plane, it is likely that binding of the first guest equivalent is a slow process due to the rearrangements involved. This may however open up a tight site allowing a second guest molecule to bind from the back side of the pyridine moiety.

Further studies are under way to elucidate the nature of these interactions.

Polymer preparation and characterization

Having established the potency of the bis-imidazolium tweezer monomer FM3 to complex phosphonates in methanol we turned our attention to the polymer preparation. We wanted first to see whether the binding behavior in solution would be reflected in the ability of the corresponding MIPs to recognize and bind phosphorylated amino acid guests. Hence a series of polymers were prepared using Fmoc-pTyrOEt as template according to the description in Table 1.

The polymers were prepared using the functional monomers in a 2:1 (P1, P2, P5) or 1:1 (P3, P4) stoichiometric ratios to template (Table 1). With P3 and P4 we wanted to investigate the influence of porogen polarity on polymer properties. Nonimprinted polymers were prepared identically to the imprinted polymers but omitting the template. Acrylamide was added as a supplementary monomer to provide additional hydrogen bond stabilization and pentaerythritoltriacylate (PETA) was used as crosslinking monomer in the indicated solvents. Conventional azo-initiated thermal polymerization at 50 °C subsequently afforded the imprinted and nonimprinted polymers. The polymers were crushed and sieved to a 25–36 μm particle size fraction obtained after crushing and sieving the polymers were subjected to template removal by washing with acidic methanol, followed by extraction with methanol using a Soxhlet apparatus. The polymers were characterized by IR-spectroscopy (Fig. S8, ESI†) and swelling measurements (see Table 1) which confirmed their identity and verified that they were comparable, in terms of morphology and composition.

Template rebinding tests

Simple binding tests were conducted in order to compare the polymers with respect to their ability to recognize and bind the template in the protic solvent methanol. Fig. 3 shows the binding constants and capacity estimated by non-linear curve fitting of the data in Fig. S7 (ESI†) assuming a Langmuir monosite binding model. The ranking of the polymers in terms of binding constants agrees overall with the relative host affinities for PPA obtained from the NMR titrations in solution. Thus P1 prepared using the neutral urea host failed as expected to bind the template in MeOH. On the contrary, P2 and P5 prepared from the cationic host FM2 and the hybrid design FM5 both showed imprinting and enhanced binding capacity in methanol. All imidazolium-based imprinted polymers displayed capacities approaching the nominal capacity expected based on the amount of added template (dashed line in Fig. 3) indicating that the imprinting was nearly stoichiometric.

Fig. 3 Association constants (K_a ; blue bars) and binding capacities (B_{max} ; black squares) for Fmoc-pTyrOH interacting with imprinted (P1–P5) and non-imprinted (PN2–PN5) polymers in methanol. The binding parameters were obtained by fitting of the binding data in Fig. S7 (ESI†) to a Langmuir monosite binding model. The dashed line represents a B_{max} corresponding to the nominal capacity of the materials assuming quantitative template incorporation.

Also in agreement with the solution binding data, the bis-imidazolium tweezers of MIPs P3 and P4 displayed the highest affinity for the template with P4 being superior to P3 presumably since it was prepared in a less competitive solvent system.

Microliquid chromatography (μ -LC)

Microliquid chromatography using the capillary column format is an attractive technique for separation problems requiring low sample loads and mass spectrometry interfacing. These needs apply in the proteomics area where sample volumes are strongly limited but where the demand for detection sensitivity and selectivity is high. In order to shrink the imprinted polymers to match the μ -LC format we made use of our previously reported technique based on grafting of imprinted polymer layers onto flow-through poly-TRIM monoliths utilizing unreacted surface double bonds as anchor points. Thus, imprinted and non-imprinted capillaries for both phosphotyrosine and phosphoserine using the FM3 bis-imidazolium based host monomer were prepared for use in the μ -LC-mode. The morphological structure investigated by field emission scanning electron microscopy (FESEM, Fig. 4 and Fig. S9, ESI†) showed the high porosity and good homogeneity, which is essential to ensure fast mass transport and low back-pressure – factors advantageous to the enhancement of separation performance. As is seen, the three-dimensional skeletal structure was retained after the photo-grafting step and a significant loss of pores could not be seen up to the maximum magnification of 125k.

Fig. 5 shows the elution profiles of a mixture of two nonphosphorylated Fmoc amino acids and their two phosphorylated counterparts injected on the nonimprinted (NIP), pS-imprinted (pS-MIP) and pY-imprinted (pY-MIP) capillary monoliths using as eluent high (95% ACN) and low organic (10% ACN) mobile phases. It is obvious that the NIP had no recognition capability for any of these four amino acids and exhibited minimal non-specific interactions, since all the amino acids eluted very close to the void volume with both the organic rich and the mainly aqueous mobile phases. On the contrary, the phosphorylated

Fig. 4 Scanning electron micrographs of TRIM capillary (upper left) and grafted nonimprinted polymer (NIP) (upper right), pS-imprinted (pS-MIP) (lower left) and pY-imprinted (pY-MIP) capillary monolith (lower right).

Fig. 5 μ -LC chromatograms acquired after injection of amino acids mixture on NIP and MIP capillary monoliths. Amino acids peaks: Fmoc-SerOH (1); Fmoc-TyrOH (2); Fmoc-pTyrOH (3); Fmoc-pSerOH (4). μ -LC conditions: mobile phase, left: ACN/H₂O (0.1% TFA) (95/5), right: ACN/HEPES buffer (100 mM, pH 7.4) (10/90); flow rate, 4 μ L min⁻¹; monitoring, UV at 254 nm; injection volume, 35 nL.

Fmoc amino acids (Fmoc-pTyrOH and Fmoc-pSerOH) had high retention on the imprinted monoliths. The selectivity also matched the imprinted epitope, *i.e.*, Fmoc-pSerOH had higher retention than Fmoc-pTyrOH on the pS MIP and *vice versa*. Some retention was also seen for the unphosphorylated Fmoc amino acids on both the imprinted monoliths, albeit substantially lower than the retention shown by their phosphorylated counterparts. It is thereby evident that the capillary monoliths imprinted by surface grafting retained a strong and specific retention for the phosphorylated amino acids under these radically different solvent conditions.

Likewise, when a mixture of four model human Angiotensin II peptide homologues consisting of two nonphosphorylated (DRVSIHPF and DRVYIHPF) and the corresponding phosphorylated (DRVpSIHPF and DRVpYIHPF) octapeptides were injected (Fig. 6) the chromatographic behavior showed similarly strong selectivities and absence of non-specific interactions.

Again, distinct high affinities were observed for the phosphorylated peptides on the pS and pY imprinted capillary monoliths compared to their NIP counterparts. Accordingly, the

Fig. 6 μ -LC chromatograms acquired after injection of a mixture of human Angiotensin II octapeptides on NIP and MIP capillary monoliths. Peptides peaks: DRVSIHPF (1); DRVYIHPF (2); DRVpSIHPF (3); DRVpYIHPF (4). μ -LC conditions: mobile phase, left: ACN/H₂O (0.1% TFA) (95/5), right: ACN/HEPES buffer (100 mM, pH 7.4) (10/90); flow rate, 4 μ L min⁻¹; monitoring, UV at 210 nm; injection volume, 35 nL.

pS/pY imprinted monolith displayed corresponding preference for the pS/pY peptide, respectively. It is particularly noteworthy that both the relative retentivity and resolution prevailed in buffered media with the pY-MIP showing somewhat higher selectivity and better discrimination performance compared to the pS-MIP. These results prove the successful application of epitope imprinting using single amino acid as a template to create PTM specific sites also for oligopeptides, even when the phosphorylation sites are located well away from the terminals.

Conclusions

Imprinted receptors for phosphorylated proteins have been proven to be particularly powerful in extracting very low levels of phosphorylated biomarkers from tryptic digests.^{2,3} In order to extend the solvent compatibility of such receptors to water and thereby be able to target proteins in their natural environment we have shown that a bis-imidazolium monomer as cationic host predesigned to bind divalent oxyanions by combined ionic and hydrogen bonding interactions can in principle be used for this purpose. These receptors can be used for selective enrichments and display a remarkable crossreactivity with phosphorylated peptides in buffered media. The programmable selectivity allows a completely inverted elution order of a phosphoserine and a phosphotyrosine model peptides, having an otherwise identical amino acid sequence, with the exception of the phosphosite.

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