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DISSERTATION

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„Preparation, implementation and evaluation of affinity chromatographic tools for targeted proteome identification by liquid chromatography – tandem mass spectrometry“

Verfasser

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Manuscript 1: A chemically cleavable linker for immobilizing bait compounds in a target protein pull-down concept. Submitted to Bioconjugate Chemistry.

Lebenslauf

Summary

In the course of this work two main projects comprising the development and implementation of analytical tools for proteomic applications were realized. Both projects included methodology development and methodology optimization of specific enrichment methods to facilitate target proteome identification by liquid chromatography (LC) – tandem mass spectrometry (MS/MS). Optimized protocols were then used for biological applications.

In the first part tin dioxide (SnO_2) was examined for the use as enrichment material for selective phosphopeptide enrichment. Tin dioxide was provided in the form of porous microspheres, which were manufactured by the nanocast process [Smått, 2007]. Initially, a prescreening of different surface treated tin dioxide materials was performed. The most suitable material was chosen for method optimization, which comprised optimization of the loading and elution buffer composition, including variations of the pH, the ion strength and the percentage of organic solvent, as well as the evaluation of possible buffer additives to increase selectivity and phosphopeptide recovery rates. The applicability of the optimized protocol was demonstrated by the LC-MS/MS analysis of a tryptic digest of a mix of phosphorylated and nonphosphorylated model proteins [see Appendix Publication no. 1]. Beneficial features of the new method were a simple loading buffer composition and good practicability. Furthermore, different nanocast TiO_2 materials, with variations in diameter, pore size, and surface treatment were examined for material dependent parameter optimization. Whereas variations in pore size yielded comparable results, the largest differences in terms of selectivity and recovery rates were achieved by surface treatment with acid (HF or HCl) or base (NaOH) [see Appendix Publication no. 2]. Finally the phosphoproteome of HeLa cells was analyzed using nanocast tin dioxide and titanium dioxide (TiO_2) and a commercial TiO_2 material (Sachtopore) For the enrichment with the titania materials an enrichment protocol of Sugiyama et al. [Sugiyama, 2007] was used. In sum 1595 unique phosphopeptide were identified, combining the results from all 3 methods. Using the new stannia material 619 unique phosphopeptides were found and 140 peptides were exclusively identified using this material. By combining both nanocast materials in one experiment 1137 identified phosphopeptides could be identified, providing a complementary coverage of the given proteome [see Appendix Publication no. 3]. The highest rate of unspecific binding was observed using the stannia material, but the simple loading buffer composition (no additives in high concentrations) makes this material ideal for a 2-dimensional setup (e.g. strong cation exchange fractionation followed by SnO_2 enrichment). In this case the lower selectivity would be less of a concern. The second project included the conception, synthesis and biological application of a chemically cleavable linker for targeted

protein pull down experiments. Parts of this project were carried out in cooperation with the Center of Molecular Medicine (CeMM) of the medical university of Vienna. Based on previous work of the group [Foettinger, 2007], an indolylacetic acid – malondialdehyde derivative was synthesized, which could be selectively cleaved at the indolyl nitrogen by treatment with pyrrolidine or hydrazine. The linker construct was further attached to hydrazide functionalized agarose- or acrylamide beads. For a biological application the kinase inhibitor bosutinib, a drug used for the therapy of chronic myeloid leukemia (CML), was coupled onto beads via this linker system for enrichment of known and potential interaction partners. The protein pull down experiment was realized at the CeMM using a K562 cell lysate for affinity purification. The successful application of the concept could be demonstrated by immunoblot and LC-MS/MS protein analysis [see Appendix Manuscript no. 1].

Zusammenfassung

Die Doktorarbeit umfasste die wissenschaftliche Bearbeitung von 2 Hauptprojekten auf dem Gebiet der Proteomanalytik. Beide Projekte beinhalteten im wesentlichen Methodenentwicklung und Methodenoptimierung von spezifischen Anreicherungsverfahren zur Erleichterung der jeweiligen Ziel-Proteomidentifizierung (Proteomics) mittels Flüssigkeitschromatographie (LC) – Tandem-Massenspektrometrie (MS/MS). Im weiteren Verlauf der Arbeit wurde, basierend auf den jeweils optimierten Protokollen, die biologische Anwendbarkeit untersucht.

Im Zuge des ersten Projekts wurde Zinnoxid (SnO_2) im Berich der Metalloxid – Affinitätschromatographie als Material zur Phosphopeptidanreicherung untersucht. Zinnoxid wurde dabei in Form von porösen Mikropartikeln, welche nach dem Nanocasting-Verfahren hergestellt wurden [Smått, 2007], verwendet. Anhand einer Mischung aus phosphorylierten und nicht phosphorylierten Peptiden wurde ein Vorabscreen von unterschiedlich oberflächenbehandelten Zinnoxidmaterialien durchgeführt. Mit dem bestgeeigneten Material wurde eine Methodenoptimierung vorgenommen. Dies beinhaltete die Optimierung der Lade- und Elutionspufferzusammensetzung durch Variation des pH-Werts, der Ionenstärke und des Anteils an organischem Lösungsmittel, sowie die Evaluierung von etwaigen Pufferzusätzen zur Verbesserung der Selektivität und der Phosphopeptid-Wiederfindungsrate.

Anhand eines tryptischen Verdaus von Modellproteinen wurde die Anwendbarkeit des optimierten Protokolls gezeigt [siehe Appendix Publikation Nr. 1]. Das optimierte Protokoll zeichnete sich insbesondere durch eine einfache Ladepufferzusammensetzung und einfache Durchführbarkeit aus. Weiterführend wurden Zinnoxidmaterialien unterschiedlichen Durchmessers, unterschiedlicher Porengrösse und unterschiedlicher Oberflächen-behandlung untersucht, um eine materialseitige Parameteroptimierung zu erhalten. Während unterschiedliche Porengrössen vergleichbare Ergebnisse lieferten, hatte die Oberflächenbehandlung mit Säure (HF, HCl) oder Base (NaOH) den größten Einfluß auf Selektivität und Wiederfindungsrate von Phosphopeptiden [siehe Appendix Publikation Nr. 2]. Zum Abschluss des Projekts wurde ein Phosphoproteomikexperiment eines HeLa-Zelllysatverdaus mit Nanocast-Zinnoxid (optimiertes Protokoll), Nanocast-Titanoxid (TiO_2) und einem kommerziellen TiO_2-Material (Sachtopore) durchgeführt. Die Phosphopeptidanreicherung mit Titanoxid wurde nach einem Protokoll von Sugiyama et al. durchgeführt [Sugiyama, 2007]. Insgesamt konnten 1595 unterschiedliche Phosphopeptide mit Hilfe aller drei Anreicherungsmaterialien identifiziert werden. Allein 619 Phosphopeptide wurden durch SnO_2 identifiziert und davon 140 exklusiv durch SnO_2 nachgewiesen [siehe

Appendix Publikation Nr. 3]. Durch Kombination beider Nanocast-Materialien in einem Experiment wurden 1137 Phosphopeptide identifiziert, was eine komplementäre Abdeckung des HeLa-Zellproteoms ermöglichte. Zwar war der Anteil an unspezifischer Bindung bei Zinnoxid am höchsten, die einfache Ladepufferzusammensetzung (keine hochkonzentrierten Additive) erlaubt jedoch eine einfache Durchführung eines 2-dimensionalen Anreicherungsverfahrens (Fraktionierung mittels starkem Kationenaustauscher gefolgt von einer Anreicherung mit SnO_2), wobei die geringere Spezifität eine untergeordnete Rolle spielen würde.

Das zweite Projekt umfasste die Konzeption, Synthese und praktische Anwendung eines chemisch spaltbaren Linkers für Protein-„pull-down“-Experimente. Bereiche dieses Projekts wurden in Kooperation mit dem Center of Molecular Medicine (CeMM) der Medizinischen Universität Wien durchgeführt. Basierend auf Arbeiten aus unserer Arbeitsgruppe [Foettinger 2007] wurde ein Indolessigsäure-Malondialdehydderivat synthetisiert, welches selektiv durch Pyrrolidin oder Hydrazin am Indolstickstoff gespalten werden kann. Dieses Derivat wurde an Hydrazid-funktionalisierte Agarose- oder Acrylamidbeads gekoppelt. Zur biologischen Evaluierung dieses Systems wurde der Kinaseinhibitor Bosutinib, ein Wirkstoff zur Therapie der chronischen myeloiden Leukämie, an den Linker gekoppelt, um potentielle Interaktionspartner des Wirkstoffs selektiv für die Identifizierung mittels -Gelelektrophorese und LC-MS/MS anzureichern. Der Pulldown wurde an einem K562-Zelllysat am CeMM durchgeführt. Die erfolgreiche Durchführung konnte mit Immunoblotanalyse und LC-MS/MS-Proteomanalyse gezeigt werden [siehe Appendix Manuscript Nr. 1].

1 Aim

Analysing the proteome is a challenging task due to the plethora of possible analytes. Mass spectrometry is the technique of choice when analyzing the (human) proteome. Although capable of determining thousands of analytes in a single run, certain analytes are present in substoichiometric amounts and may not be detected without specific purification steps. Both topics of my thesis were based on the development and implementation of specific affinity purification tools to enrich for a certain class of proteins to facilitate their mass spectrometric identification.

Accordingly a review about purification tools for phosphoproteomics was chosen as the introduction part of the thesis. Therein the theoretical background of one dissertation project is fully described and separation tools and mass spectrometric technologies for proteomic purposes are discussed in detail.

2 Tools for analysing the phosphoproteome and other phosphorylated biocompounds

The enrichment, separation and analysis of phosphate group containing biomolecules plays an ever increasing role in recent separation science. Starting from the preparative enrichment of phospholipids for biotechnological purposes, the separation and purification of pDNA or mRNA, to the specific preconcentration of phosphoproteins and peptides and carbohydrates to ease their later identification by mass spectrometry, many new methods and materials were therefore developed. Most improvements in this field were triggered by the need of phosphopeptide enrichment technology for the analysis of cellular protein phosphorylation in the proteomics field with the help of liquid chromatography - mass spectrometry instrumentation.

The high sensitivity and the possibility to combine mass spectrometry with different liquid chromatography separation techniques with the accessibility for high throughput online analysis make mass spectrometry to the instrument of choice for proteome analysis. Suppression effects and low quality fragment spectra of phosphopeptides in mass spectrometry interfere with the identification of phosphoproteins. Recent developments in phosphopeptide enrichment and fragmentation technologies successfully help to overcome these limitations.

1 Introduction

Phosphate groups are a very common functional group in a variety of different biological compounds. They form the hydrophilic backbone of DNA or RNA poly- and oligomers and represent the hydrophilic group in many amphiphilic membrane lipids. Reversible protein phosphorylation works as a molecular switch which allows the regulation of metabolism and signal transduction in cells [1]. Phosphorylation marks the activation of sugars for metabolism, and the triphosphate nucleotide ATP serves as the main energy carrier and phosphate group donor in all organisms. The phosphate group is capable to carry two negative charges depending on the pH of the solvent. Typical pKa values are 2.2 for the singly charged and 7.2 for the doubly charged free phosphate [2]. These values can vary depending on the specific chemical environment. Phosphate groups are basically tetrahedral, with a symmetry depending on the number of substituents on the O-atoms [3].

Most enrichment methods take advantage of the ionic and Lewis base character of the phosphate group for interaction. Therefore, methods based on ion exchange mechanisms are generally suitable for the analysis of phosphates. Additionally the Lewis base properties allow coordinative binding to positively charged iron or gallium central atoms in a chelating matrix, a concept called immobilized metal affinity chromatography (IMAC), and recently new metal oxide materials like TiO2, ZrO2 or SnO2 which are said to possess Lewis acid and ion exchange properties are frequently used for selective phosphopeptide enrichment [4]. In addition to the methods mentioned above, there are different chemical phosphosite tagging techniques, and of course phosphosite- and/or phosphoamino acid-specific antibodies are available and are widely used.

The majority of enrichment and separation strategies were developed as a result of the need for the selective enrichment of phosphopeptides to facilitate their identification by mass spectrometry.

Reversible protein phosphorylation is the most widespread post translational protein modification (PTM) in (eukaryotic) cells, and it is the main chemical protein modification in cellular signalling, metabolism, protein transport or cell division and apoptosis, when proteins interact with each other [5]. Recent proteomic research revealed that the onset of many severe diseases, especially many cancer types, is influenced by the activity of tyrosine kinases in certain regulation/signal transduction pathways [6]. Recently developed anticancer drugs like imatinib, desatinib or bosutinib act as direct inhibitors of the respective protein tyrosine kinases in cancer specific signalling networks [7-9]. New proteins in protein interaction pathways could successfully be inferred by LC-MS/MS identification coupled with phosphopeptide enrichment methods [10-13]. These achievements show that

phosphoprotein identification and phosphorylation site determination is of high clinical and research interest. O-phosphorylation is the most common type of protein phosphorylation. It occurs mainly on the hydroxyl-containing amino acids serine, threonine and tyrosine in a distribution of approximately 1800:200:1 [14], and far less frequently abundance as N-, S- and acyl-phosphorylation on histidine, lysine, cysteine and aspartic or glutamic acid residues [15]. The covalent attachment of the phosphate group to the respective amino acid residues is catalyzed by a class of enzymes called kinases which use the energy of adenosine triphosphate (ATP) or guanine triphosphate (GTP) hydrolysis for the transfer of the phosphate group onto the substrate protein [16, 17]. This leads to a conformational change and thus alteration of the activity of the respective substrate proteins or attracts phosphospecific binding domains from other proteins constituting a protein interaction [18].

Although some proteins remain phosphorylated, the larger amount, especially those involved in signalling pathways show a highly dynamic and regulated interaction between kinases and phosphatases, which rapidly dephosphorylate the proteins after a phosphorylation event, resembling an on/off mechanism. Even though phosphoproteins account for approximately 30% of the eukaryotic proteome, the ratio of the phosphorylated to unphosphorylated form of the protein is rather small, so that the phosphoproteins will be present in substoichiometric concentrations [19]. A couple of years ago researchers believed that the sensitivity and dynamic range of mass spectrometry analysers would increase in a few years so much that it would be possible to identify even low abundance proteins of complex samples like body fluids of cell lysates in a single run (“shotgun” proteomics) without the need of any further enrichment or separation technique. But like many dreams, this dream did not come true. Even with the availability of more powerful mass analysers (with improved selectivity, sensitivity, resolution power paired with a high dynamic range) like the linear ion trap (LIT), the Orbitrap or Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) that were designed for proteomics applications, there is still the need for an efficient and selective separation of peptides before mass spectrometry analysis, able to identify even low abundant protein species from a complex biological sample. A typical (phospho) proteomic experiment workflow is shown in Figure 1. Depending on the requirements one might start with a more or less specific prefractionation for the proteins of interest. This comprises methods like targeted protein pull downs (affinity purification of protein complexes), 1D or 2D gel electrophoresis or other common protein fractionation techniques.

After separation or fractionation on the protein level, proteins are typically digested with site specific enzymes to obtain smaller peptide fragments, which are accessible to a wider range of separation techniques and mass analysers, show higher ionisation efficiency and give a more predictable fragmentation pattern compared to whole proteins when using MS/MS fragmentation techniques. After protein digestion, peptides are injected onto a micro or nano

high performance liquid chromatography (nanoHPLC) system which is in most cases coupled online to the mass analyser. A digest of a phosphorylated protein will result in an excess of nonphosphorylated peptides. In a biological sample like cell extracts or body fluids, which consists of several thousand different proteins and phosphorylated proteins present in low abundances, the ratio between nonphosphorylated to phosphorylated peptides will be much higher. So even when using high performance liquid chromatography separation techniques, coelution of peptides will be unavoidable. It has been shown that phosphopeptides are more difficult to detect in complex mixtures than their unphosphorylated analogs. This is either attributed to lower ionization efficiency of phosphopeptides compared to unphosphorylated peptides [20], or to their sub stoichiometric occurrence [21]; the reports on this are contradictory. In any case, an enrichment step for phosphopeptides prior to reversed phase separation will help to overcome this limitation. Many different phosphopeptide enrichment strategies were therefore developed and are in permanent improvement. Nowadays a combination of phosphoselective prefractionation and enrichment is most widely used to obtain an increase in selectivity and to achieve better orthogonality for the following reversed phase separation. Figure 2 gives an overview about present enrichment methods. In following chapters methods are discussed in detail.

Here it is important to mention that some of these techniques, primarily developed for phosphoproteomic purposes, may also be applied to other classes of phosphorylated biomolecules. Due to the fact that there is an increased interest of academic researchers, but also from the pharmaceutical industry for the selective enrichment and separation of phospholipids, DNA (plasmids), or phosphorylated metabolites, a short comment about recent applications and developments will be included in this review.

2 Starting an experiment

When starting a proteomic experiment, a careful and elaborate sample preparation is necessary. Therefore, all sample preparation steps should be carried out at low temperature, and when working with cells or body fluids, protease inhibitors have to be added in an early stage to avoid protein artefacts which may lead to false positive identification. Phosphatase inhibitors have also to be added to avoid cleavage of the phosphate group upon cell lysis. The molecules of interest should exist in their native state, which means that all possible degradation during the preparation steps should be minimized. When analysing biological samples like tissue, cells or body fluids, one needs to remove interfering sample constituents such as DNA or RNA and cell debris. Therefore crude pretreatment steps such as centrifugation, precipitation, protein extraction, or protein pull downs, or some chromatographic steps are necessary. The resulting protein lysates are in most cases treated with site specific proteolytic enzymes like trypsin or Lys-c to obtain smaller fragments, for which LC-MS analysis can be realized more easily than when working with whole proteins. Peptides are then separated by liquid chromatography -mass spectrometry. The obtained peptide- and fragment mass data allows the identification of proteins from protein or genome databases.

3 Immunoaffinity chromatography

Affinity chromatography or immunoprecipitation are widely used enrichment strategies in biochemistry. Antibodies are known to have a high affinity and selectivity for their target epitopes. Commonly used for trapping whole proteins, they are even used for enrichment of smaller molecules like peptides or amino acids, although the generation of good working antibodies for smaller molecules is more elaborate.

In a traditional experiment to analyse protein phosphorylation the setup was focused on one phosphoprotein of interest. For detection and purification specific antibodies directed against the phospho-epitopes had to be generated. The production and validation of proper antibodies is a time consuming and expensive process taking up to one year to generate and validate an effective antibody [22].

Antibodies used for phosphopeptide or phosphoprotein enrichment on a proteomic scale should therefore be directed against the phosphosite solely, so they should have the same or similar affinity and selectivity for all the phosphopeptides or proteins in a biological sample to obtain a balanced enrichment of all phosphorylated members. However the proximity of phosphate groups in peptides is affected by surrounding amino acids so it is very difficult to generate adequate antibodies. Although there are antibodies against most common phosphosites like phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) available, their use is limited by the specificity of the antibodies [14, 23]. Highly specific pY antibodies exist and are predominantely used. Because phosphorylation on Serine, threonine and tyrosine occurs in a ratio of 1800:200:1, so the occurrence of phosphotyrosine compared to the other common phosphorylation sites is rather low. Tyrosine kinases play an important role in human cancer, taking part in oncogenic signalling for cellular proliferation and survival. Therefore an unregulated tyrosine kinase activity can lead to malignancy and tumor formation [24]. In a global phosphoproteomic approach the number of identifications of phosphotyrosine containing peptides would be rather small because of its low abundance. Repetition! Pandey et al.[23] used immunoprecipitation of tyrosine phosphorylated molecules of EGF stimulated HeLa cells in an LC-MS based proteomic approach. They used a mix of different anti phosphotyrosine antibodies for enrichment followed by a 1D electrophoresis and in gel digestion before LC-MS analysis. This approach led to the identification of nine signalling molecules, seven of it had previously been implicated in EGFR signalling. For example Vav-2, STAM and Odin could be identified from epidermal growth factor stimulated HeLa cells.

A more comprehensive proteomic study of tyrosine phosphorylation from Jurkat cells was done by Rush et al.[24]. Phosphotyrosine containing peptides from a cell digest of

pervanadate treated Jurkat cells were immunoprecipitated with P-Tyr-100, a phosphotyrosine specific antibody noncovalently coupled to protein G agarose. Pervanadate was used as a specific inhibitor of tyrosine phosphatases. The enriched peptides were later on analysed by conventional reversed phase chromatography – tandem massspectrometry. Using this strategy 688 phosphotyrosine containing peptides and 628 phosphotyrosine sites could be identified. The same strategy was used by Rukova et al. for a large scale analysis of tyrosine kinase activity in non-small cell lung cancer (NSCLC) cells lines [25]. Known tyrosine kinases such as EGFR and c-Met as well as new oncogenic tyrosine kinases not known to play a role in lung cancer were identified. In summary over 50 different tyrosine kinases and over 2500 downstream substrates were identified by this approach After elution an additional enrichment of phosphorylated species on the peptide level with less phosphosite specific enrichment or separation methods like immobilized metal affinity chromatography may be performed [26, 27].

4 Immobilized Metal Affinity Chromatography

Cysteines and Histidines are known to form stable complexes with zinc and copper ions in an aqueous solution [28]. This effect was used for selective enrichment of proteins via certain transition metals trapped in a chelating matrix. The concept, termed immobilized metal affinity chromatography, is a sort of pseudo affinity chromatography. The materials show affinity to a motif like a certain amino acid or functional group and not to a specific epitope like in biospecific affinity chromatography. The IMAC technique is widely used for the enrichment of recombinant histidine-tagged proteins for biotechnological purposes. A string of six His residues is genetically attached to the C- or N-terminus of proteins of interest and the high affinity of histidine to covalently fixed Ni^{2+} is used for enrichment of the tagged protein. The concept was expanded for enrichment of phosphorylated proteins by Andersson and Porath in the year 1986 [29]. In their experiments they used Fe^{3+} bound via iminodiacetic acid (IDA) to a sepharose matrix. Phosphorylated amino acids like phosphoserine, phosphothreonine or phosphotyrosine were retained by the chromatographic material whereas nonphosphorylated amino acids were not, or in some cases, like aspartic acid or glutamic acid, were only weakly bound. A separation of ovalbumin phosphoisoforms, carrying different numbers of phosphates, succeeded using this technology [49]. An advantage of the method was that all steps could be carried out in water or buffer and no protein denaturing components were needed.

The affinities of phosphorylated amino acids, peptides, and proteins to Fe^{3+} ions was characterised by Muzynska et al.[30]. The binding and release of analysed compounds were observed to be strongly pH dependent. The release of phosphorylated compounds occurred at higher pH than their nonphosphorylated counterparts. An improved application of the technique was reported by Scanff et al. [31] who did a phosphopeptide enrichment of a tryptic digest of the phosphoprotein casein with Fe^{3+}-IDA-Superose-beads.

Neville et al. [22, 32] observed an increase in selectivity towards phosphorylated peptides when using the tetradentate ligand nitrilotriacetic acid (NTA) instead of the tridentate ligand IDA for fixing the iron ion in the chromatographic matrix when analysing the phosphorylation sites of the cystic fibrose transmembrane conductance regulator (CFTCR). The phosphopeptides were identified using MALDI-MS^2. Meanwhile IMAC is the most frequently used method for enrichment of phosphopeptides also due to the fact that commercial kits are available from different suppliers. However, the high level of unspecific binding of acidic peptides via their carboxylic residues remains a challenging problem. A method to circumvent unspecific binding of acidic peptides was introduced by Ficarro et al. [33]. In his approach a tryptic digest of a yeast whole cell lysate was analysed and carboxylic residues

on peptides were chemically derivatised by O-methyl esterification with methanolic HCl before Fe^{3+}-IMAC enrichment. The esterification led to an elimination of unspecifically bound peptides without loss in sensitivity. Using this method the authors could identify 200 phosphopeptides from *S. cerevisiae*.

This technology increased the selectivity of IMAC phosphopeptide enrichment considerably and was used in numerous publications [26, 27, 33-38].

However, authors reported that the chemical derivatisation reaction does not have a 100% turnover, so some carboxylic residues remain unmethylated. This led to an increase in sample complexity originating from peptides with different degrees of O-methylation [39]. Other authors reported sample loss when performing methyl esterification before IMAC enrichment [36, 37]. Additionally this chemical modification is time consuming especially when one has to deal with many samples.

Another way to increase selectivity of the IMAC method towards phosphorylated peptides is to adjust the pH of the loading buffer. Acidifying the sample with strong acids like TFA, formic acid or acetic acid before enrichment protonates the carboxylic residues on peptides and will therefore reduce non specific binding. The pKa value of phosphate residues from peptides is significantly lower than that of carboxylic ones, roughly 1-2 units [40]. So the pH of the loading buffer has to be adjusted to a value where the carboxylic residues are protonated and the phosphate residues remain ionized to keep their affinity to the IMAC resin. Kokubu used 50% acetonitrile 0.1% TFA as loading buffer for Fe^{3+}- IMAC enrichment when analysing a tryptic digest of a mouse brain protein extract [41]. With TFA, a selective protonation of carboxylic residues on peptides was reported. Additionally the high acetonitrile content alleviated hydrophobic interaction between peptides and the IMAC resin. Using this loading buffer a reduction of nonspecific binding of acidic peptides was obtained. Just 96 nonphosphopeptides and 1654 phosphopeptides were identified using Mascot interpretation. After manual validation 166 phosphosites on 135 different proteins were identified using this approach. Although iron is used as central ion in most IMAC methods, other metal ions have been evaluated for selective phosphate affinity. Posewitz tested different metal ions like Ga, Sn, Ge, Fe, and others for their applicability in IMAC phosphopeptide enrichment [42]. With Ga^{3+} a better selectivity compared to conventional Fe^{3+}–IMAC was reported when analysing a tryptic digest of phosphoproteins. An interesting approach was reported from the group of Zou [43, 44]. They used a phosphate polymer to coordinatively bind Ti^{4+} or Zr^{4+} ions. The resulting IMAC resin was used for phosphopeptide enrichment and compared to Fe^{3+}-IMAC, TiO_2 and ZrO_2 enrichment methods. For the preparation of the IMAC resin they used a special chemistry to attach the phosphate groups to the matrix via a linker. The binding of the metal ions via phosphonate groups provides a beneficial structural orientation for the selective binding of phosphorylated biomolecules. Additionally phosphates form a stable

MO6 octahedron structure with Ti and Zr ions. So the metal ions stably bound to the phosphonate groups form an interface on the surface of the resin which is very attractive for phosphate ions to form a stable double layer. A mix of a standard phosphoprotein digest and BSA digest as nonphosphoprotein in a ratio of 1 to 500 was anaylsed. Further the method was applied to the phosphoproteome analysis of mouse liver. The method was reported to outperform conventional metal oxide enrichment (see following chapter) and Fe^{3+}-IMAC in terms of efficacy and selectivity, even when using optimized conditions for the respective methods and a bias of Ti^{4+}-IMAC towards monophosphorylated peptides and of Zr^{4+}-IMAC towards multiply phosphorylated peptides was reported. The results of the new methods were ascribed on one hand to the highly specific interaction between the Ti and Zr ions and the phosphate groups on peptides and on the other hand to the novel resin design which is composed of phosphonate- immobilized titanium or zirconium ions with a flexible spacer arm linked to polymer beads. Another way to increase the specifity and efficiacy of the IMAC technology is to combine it with other enrichment or fractionation methods. In most cases ione exchange chromatography (IEX) or hydrophilic interaction liquid chromatography (HILIC) chromatography is used for prefractionation before a specific enrichment for phosphopeptides with IMAC [39, 45-47] or metal oxides is performed. A detailed discussion is given in the chapter combination of methods.

5 Metal Oxide Affinity Chromatography

From the beginning of the 1990s TiO_2 and ZrO_2 was increasingly used as a new chromatographic material for high performance liquid chromatography in normal phase mode. The advantages of this material included large adsorption capacities, chemical stability when used under extreme pH ranges, mechanical stability and unique amphoteric ion exchange properties [48-53]. Phosphates are known to bind to metal oxide materials [3] and in 1990 Matsuda et al. [51] reported the selective adsorption of organic phosphates to ceramic TiO_2 material. Henceforward several studies concerning enrichment of phosphate containing biomolecules with metal oxide materials emerged [49, 51], but not a lot was known about the surface chemistry and binding properties of these materials. Connor et al. [3] investigated the phosphate adsorption onto TiO_2 from aqueous solutions with infrared spectroscopy. They proposed a pH dependent bidentate binding of monosubstituted phosphate groups to TiO_2 sol gel films. In another work, the surface chemistry of metal oxide materials was characterized by their acidity and basicity which are from Lewis and Bronsted base type [4]. Alumina, TiO_2 and ZrO_2 possess strong Lewis acid properties with increasing Lewis acidity from aluminia to ZrO_2. In addition, the high coordination numbers of Ti-O and Zr-O are responsible for strong complexation properties of these oxides. Both the Lewis acid and the strong complexation properties may be a possible explanation for the affinity of some metal oxides for phosphate molecules.

Tani et al.[54] characterized the chromatographic properties in terms of ion exchange and ligand exchange behaviour of different calcinated TiO_2. TiO_2 was calcinated in an oven from 200 to 800° C and a strong temperature dependence of the ion - and ligand exchange behaviour could be observed. At 700° C TiO_2 is completely converted from anatase to rultile form and no ion-exchange or ligand-exchange behaviour could be detected any more. The authors ascribed the absence of these properties to the loss of the surface hydroxyl groups and coodinatively bonded water by calcination at high temperatures. In another experiment Tani et al.[55] compared the chromatographic behaviour of TiO_2 and ZrO_2 with hydroxyl and other substituent aliphatic carboxylic acids. The highest retention times were achieved with alpha hydroxyl carboxylic acid because they can form a stable five-membered ring between metal ion and acid. Alpha hydroxy acids were later used as additives to avoid unspecific binding of carboxylic acid containing peptides to TiO_2 when used for selective phosphopeptide enrichment [56]. The stable ring formation can be a possible explanation why alpha hydroxyl acids are able to displace acidic peptides from TiO_2 or ZrO_2. A work with a focus to screen differently treated metal oxide materials for their phosphate affinity was done by Leitner et al. [57]. They compared different surface treated tin dioxide microspheres,

an alternative metal oxide used for phosphopeptide enrichment, for phosphopeptide affinity and selectivity purposes. The results gave evidence that parameters like calcination temperature, acid or base surface treatment can differently affect the surface chemistry and morphology of metal oxide materials, which will affect the affinity and selectivity of the materials to phosphates, respectively.

The first proteomic application of TiO_2 for phosphopeptide enrichment was done by Pinkse et al.[58] In his work a novel automated method for the enrichment of phosphopeptides from complex mixtures with TiO_2 was developed. A two dimensional chromatographic setup with titanium dioxide-based solid-phase material (Titansphere) as the first dimension and reversed-phase material as the second dimension was employed. Phosphorylated peptides were separated from nonphosphorylated peptides in the first dimension by trapping them under acidic conditions on the TiO_2 precolumn (0,1M acetic acid). Nonphosphopeptides were not retained in the first dimension but trapped in the second dimension precolumn before they were analysed by nanoflow LC-ESI-MS/MS. The phosphopeptides were eluted from the column under alkaline condition (ammonium bicarbonate pH 9.0), concentrated on the second dimension and analysed on nanoflow LC-ESI-MS/MS. 125 fmol of a phosphopeptide in a 1:1 mixture of the phosphorylated to unphosphorylated form could be successfully identified with a recovery rate of above 90 %. Additionally from a digest of the cGMP dependent protein kinase a novel autophosphorylation target could be identified. A drawback of this strategy was the high level of unspecific binding of acidic non-phosphorylated peptides to TiO_2 under these conditions which were reported to be in a comparable level to the IMAC method. This may be attributed to the relatively high pH in the binding step. The authors recommended O-methyl esterification [33] to reduce unspecific binding. Larsen[59] used 2,5-dihydroxy benzoic acid (DHB), a matrix component used in MALDI mass spectrometry, to increase selectivity without a reduction in phosphopeptide recovery. In this procedure, peptides from a tryptic digest of the phosphoproteins ovalbumin and α-and β- casein were used for phosphopeptide enrichment with TiO_2 columns. Peptides were loaded onto TiO_2 columns with different concentrations of DHB (0-200 mg/ml in 80% acetonitrile, 0.1% TFA) in the loading buffer. The column was washed with 20 mg/ml DHB in 50% acetonitrile and subsequently with an 80% acetonitrile 0.1% TFA solution. Bound peptides were eluted with NH4OH pH 10.5. With increasing concentrations of DHB in the loading buffer the number of non-phosphorylated peptides bound to the column decreased dramatically with no unspecifically bound peptides observed at 200 mg/ml DHB. When using very complex samples the author recommended the use of high concentrations of DHB, up to 400 mg/ml, to effectively exclude unspecific binding of non-phosphorylated peptides. A possible reason why DHB is able to avoid unspecific binding of acidic non-phosphorylated peptides without reducing phosphopeptide binding was explained by different binding properties of these

molecules to TiO_2. DHB binds to TiO_2 via a chelating bidentate geometry whereas a phosphate group binds in a bridging bidentate way, so the two molecules target different binding sites on the TiO_2 surface and DHB is directly competing with carboxylic residues from acidic peptides. One big disadvantage of this protocol is that one has to get rid of the DHB before LC-MS analysis, because the high amount of DHB would interfere with the reversed phase separation and the ionisation. Meanwhile several attempts to increase selectivity without decrease in sensitivity were done by screening different loading buffer additives to suppress unspecific binding [56, 60]. Mazanek [60] and co workers claimed that the high concentration of DHB used in the enrichment protocol from Larsen et al. [59] could be problematic with the following LC-MS analysis. Although washing with high organic content is performed, the remaining DHB causes a high background in MS. They suggested using a mix of DHB and octanesulfonic acid (OSA), an ion pairing agent used for improved peptide separation in reversed phase chromatography, to reduce unspecific binding. The additives were used in lower concentration and should therefore be less problematic with the following analysis. Additionally OSA as ion pairing reagent should improve the quality of the reversed phase separation. The quality of the protocol was shown with a mix of phosphorylated and non-phosphorylated peptides and the applicability of the protocol to more complex samples like a tryptic HeLa digest was demonstrated. More recently, this method was further optimized in terms of selectivity using slightly increased concentrations of DHB and OSA, and with the addition of heptafluorobutyric acid (HFBA) [61]. The optimized protocol allowed improved recovery of phosphorylated peptides and a reduction of unspecific binding from a trypsinized bovine serum albumin matrix, when compared to a previous protocol [60]. The new method was applicable to both titania and zirconia enrichment. The applicability to complex samples was demonstrated by the identification of in vivo phosphorylation sites of the affinity enriched anaphase promoting complex (APC/C) and the mitotic complex, condensin-I. Sugiyama et al screened different hydroxyl carboxylic acids as alternative displacement additives in TiO_2 and ZrO_2 enrichment. The best results in terms of selectivity were achieved when using lactic acid in a concentration of 300 mg/ml in 80% acetonitrile 0.1% TFA for TiO_2 and 100 mg/ml β-hydroxpropanoic acid in 80% ACN 0.1% TFA for ZrO_2 enrichment . Again the applicability to complex samples was successfully demonstrated by analysing a tryptic HeLa sample [56]. Lactic acid modified TiO_2 gave the highest selectivity as well the largest number of identified phosphopeptides. In total, 1100 phosphopeptides could be identified by four replicated experiments using TiO_2 and ZrO_2 enrichment. An alternative to optimizing the loading buffer composition for TiO_2 enrichment would be to test out other metal oxide materials for phosphopeptied enrichment purposes. At the same time when TiO_2 was tested as affinity chromatography material ZrO_2 was also evaluated due to

structural, chemical and physical similarities but phosphopeptide enrichment with ZrO_2 did not offer noteworthy alternatives or improvements to TiO_2 enrichment. $Al(OH)_3$ was reported as a cheap and robust alternative to TiO_2 or IMAC enrichment. In a work of Wolschin et al.[62], phospho*protein* enrichment was performed with $Al(OH)_3$ and two commercial available Phosphoprotein enrichment kits (BD, QIAGEN) were used as benchmark. Additionally phospho*peptide* enrichment was done with a tryptic digest of the phosphoprotein α-casein. To increase selectivity 0.2 M sodium glutamate and 0.2 M potassium aspartate was used in the loading buffer, which again will not allow coupling online to LC-MS. Although the authors succeeded to outperform the commercial kits, not a lot of further applications were published using this protocol since then. Porous tin dioxide microspheres, a new material for phosphopeptide enrichment, were tested in our group as novel phosphoaffinity chromatographic material [19]. The spheres were manufactured by the nanocasting process [63] which allows the fine tuning of material properties like particle size, morphology and pore size of different metal oxide materials. Using a tryptic digest of 10 proteins with ovalbumin as the only phosphorylated protein, phosphopeptide enrichment with SnO_2 and a simple loading buffer composition without additives performed on the same level to TiO_2 with the use of 300 mg/ml lactic acid as additive [56] which was used as benchmark protocol for all experiments in this approach. Later, a tryptic digest of a HeLa cell lysate was analysed on a FT-ICR mass spectrometer. With both protocols similar numbers of peptides were identified, although the SnO_2 protocol provided lower phospho-specifity. The overall specifity of SnO_2 with around 70% phosphopeptides in the eluent is however comparable to IMAC enrichment.

Recently, several authors reported the preparation of magnetic iron oxide (Fe_3O_4) / metal oxide - core/ shell particles for the enrichment of phosphopeptides [64-69]. The focus of this work was to facilitate the enrichment procedure by the fact that coated beads could be easily captured when applying a magnet to the sample vials during enrichment process, so that incubation and washing steps can be performed with a minimum of sample loss. Members from the Zhang group used known and novel metal oxide shell material for phosphopeptide enrichment [65, 66, 68, 69]. For example Dawei and colleagues coated the magnetic core particles with a tin oxide layer [69] and got comparable results to TiO_2 coated beads from a previous work without the use of high concentrated additives in the loading buffer. Also novel oxide materials like Nb_2O_5 [70] or Ta_2O_5 [68] were analysed for phosphopepetide enrichment application. Both materials in the near vicinity to TiO_2 and ZrO_2 in the periodic table of elements gave comparable performance to the benchmark material TiO_2 using common protocols, but were also able to reveal a useful degree of divergence in peptides selectivity between the compared materials.

Summing up, no other enrichment material can provide such a plethora of benefits for phosphopeptide enrichment than metal oxides. Different types metal oxide materials have proven their quality for selective phosphopeptide enrichment, with every material displaying its own (characteristic) bias, giving the possibility of enriching a wide range of differently phosphorylated peptides or other phosphorylated species. The materials have a high phosphate affinity, exhibit chemical and physical stability and can be manufactured in different morphologies and sizes, like porous microspheres [19, 63] or magnetic nanobeads [64, 67]. Additionally, MeOAC has shown to be more tolerant against buffer additives like salts, detergents and denaturing components than IMAC enrichment. These unique properties make metal oxides the ideal materials when an automated online enrichment/ LC-MS analysis is performed which would allow faster and more reproducible proteomic data acquisition [1, 71].

6 Ion exchange chromatography

In early proteomic approaches proteins were separated by their size and pI by 2D-PAGE before they were in gel digested and analysed by reversed phase liquid chromatography and mass spectrometry. The gel-electrophoresis step was reported to be tedious and time consuming, and portions of proteomes such as proteins with high molecular weight, extreme pI and low abundance proteins as well as hydrophobic proteins were rarely found in a 2D-PAGE study [72, 73]. Non gel based chromatographic systems for proteomic investigation were developed [72, 74] and it was found that ion exchange chromatography as an orthogonal resolving system to reversed phase separation performed sufficiently well especially when working with complex protein/ peptide samples. Multidimensional protein identification technology (MudPIT) used strong cation exchange chromatography in the first chromatographic dimension to separate peptides depending to their solution charge state, and reversed phase chromatography in the second dimension where analytes are separated by their hydrophobicity, which provided an unbiased chromatographic tool for proteome analysis [72]. The MudPIT technology was introduced to obtain a higher resolving power of the chromatographic system which was necessary for the analysis of complex protein samples. Beausoleil et al. used MudPIT in a phosphoproteomic approach [10] when analysing a tryptic digest of a HeLa cell lysate. For loading of peptides to the SCX, peptides were acidified to a pH of 2.7 and bound peptides were eluted with increasing salt and pH. At the pH 2.7 Lys, Arg, His and the amino-terminus of peptides are charged. Tryptic proteolysis produces peptides with an Arg or Lys on the C-terminus. Consequently, tryptic peptides should carry a solution charge of 2+ and because phosphate groups retain their negative charge at this pH, the net charge state of phosphopeptides should be 1+. Thus the phosphopeptides tend to elute earlier from the SCX column. The phosphopeptide containing fractions were analysed by LC-MS^2 and additional data dependent MS^3 for improved phosphopeptide identification was performed. This chromatographic setup combined with elaborate mass spectrometric identification allowed the determination of 2002 phosphorylation sites on 967 phosphoproteins. But the combination of SCX with RP chromatography also revealed some difficulties, especially when both columns were coupled online. For example, the interaction of peptides with the SCX resin is proposed to be (mainly) of electrostatic and (partially) of hydrophobic character, arising from the hydrophobic character of the sufonyl polymer backbone, so that structurally similar peptides with the same net charge may be separated. For the increase of peptide recovery from the SCX column, the use of organic modifier is recommended [75], which can on other hand interfere with the following RP separation. As a consequence both dimensions cannot be optimized

independently [76]. On the other hand, when using strong anion exchange chromatography (SAX) instead of SCX, phosphopeptides are typically more retained than non phosphorylated ones. Uridine monophosphate, uridine diphosphate, uridine triphosphate, and their unphosphorylated uridine were baseline separated on an anion-exchanging solid-phase extraction (SPE) column by a four step elution with a gradient of salt concentration and pH values in an approach from Zhang et al. [77]. Zhang et al. also demonstrated the applicability of anion exchange chromatography (AEC) for selective phosphopeptide enrichment when analysing a tryptic digest of the standard phosphoprotein β-casein. SAX was used for the enrichment and separation of phosphopetides from a digest of human liver cancer tissue from Han and co-workers [78]. In contrast to SCX chromatography, the authors claimed that not only phosphopeptide enrichment, but also fractionation according to the number of phosphate groups was possible. This was demonstrated when analysing tryptic a digest of the standard phosphoproteins α- and β-casein. For separation of bound phosphopeptides a salt gradient of NH_4Cl was used and the pH of the solvent was set to a value of 4. At this pH phosphopeptides which are more acidic than nonphosphorylated ones and should therefore remain ionized and hence are able to interact with the SAX resin via their phosphate groups. Consequently, multiply phosphorylated peptides should show a higher affinity to the SAX resin than singly phosphorylated ones. The performance of the technology was compared to a Fe^{3+}-IMAC enrichment with POROS 20 MC beads with a commonly used protocol. For comparison of the two methods only one fraction (5-28 minutes retention time) of retained peptides was collected from the SCX column due to the assumption that nonphosphopeptides would not be retained by the SCX column and will therefore elute with the flow through. A higher number of unique phosphopeptides, 47 to 24 with IMAC, could be identified using the SAX method and an overlap of 12 unique phosphopeptides was identified with both methods. Although both methods suffer from unspecific binding, the nonphosphopeptides identified with SAX were mainly acidic peptides whereas IMAC enriched peptides were reported to be more heterogeneous (histidine binding to IMAC). Additionally the ability for fractionating phosphopeptides was investigated. Two minutes fractions from the liver digest were taken and analysed by MALDI-MS. This led to the identification of 274 phosphorylation sites from 305 unique phosphopeptides corresponding to 168 proteins at FDR of 0.96%. A disadvantage arising from this method is that the solvents used in SAX chromatography are not optimal for online LC-MSMS coupling because the weak acidic to neutral pH and the aqueous buffer lowers the ionisation efficiency when LC-ESI-MS is used.

An anion-cation (ACE) -mixed bed system for shotgun proteome analysis was introduced by the Yates group [76]. They used self assembled columns filled with C-18 (upstream) and

SCX material or a mixture of SCX and WAX material. Increased recovery of peptides from the SCX resin and a better orthogonality of the 2D system were reported when using the anion-exchange-SCX mixed bed material. The “Donnan” effect [reference] was used to explain the beneficial behaviour of this novel chromatographic setup for proteome analysis. The opposite charges of the mixed bed material would lead to a separation of the salt ions used for elution. Salt cations were repelled from the anion exchange resin whereas the salt anions were attracted, resulting in a increased reflux of salt cations in the SCX proximity [76] improving elution efficacy. Beyond partitioning by charge state, HILIC provides an additional orthogonal separation tool to reversed phase chromatography for proteomic applications. In contrast to SCX, which is the standard first dimension in proteomic applications, in HILIC analytes are separated according to their polarity. HILIC is a special form of normal phase chromatography run using mobile phases that are 10-40% aqueous solutions. In HILIC, the interaction occurs via hydrogen-bonding of the analytes to a neutral hydrophilic resin within a small aqueous film at the interface between the stationary phase and the highly organic eluent. So the more polar the eluent becomes the more elution strength it has. Typical starting conditions for HILIC separations are up to 90% organic solvent. HILIC as part of a multidimensional peptide separation strategy was used for the phosphoproteomic analysis of a tryptic HeLa cell digest [79]. Phosphopeptides with the highly polar phosphate group should therefore be strongly retained on the HILIC stationary phase. IMAC phosphopeptide enrichment was added before or after HILIC chromatography to increase selectivity of the setup. Nearly 100% selectivity for phosphopeptides was achieved when IMAC enrichment was performed after HILIC chromatography, and over 1000 phosphorylation sites on 914 peptides were identified, demonstrating HILIC chromatography as a powerful prefractionation tool before selective phosphopeptide enrichment is done. Additionally phosphopeptides elute at a buffer composition of 70-50% ACN containing 0,1% TFA which is asserted to be the optimal loading buffer composition for IMAC phosphopeptide loading [41].

Somewhat similar to HILIC, electrostatic repulsion - hydrophilic interaction liquid chromatography (ERLIC) uses electrostatic repulsion as an additional chromatographic property to adjust selectivity in HILIC chromatography, as introduced by Alpert et al. [80]. Superimposing the properties of ion exchange and HILIC chromatography, selectivity can be adjusted by changing the organic content or the pH of the solvent and additionally by using a salt gradient. Using a weak anion exchange column and a solvent with a pH of 2(because carboxylic residues of acids peptides are not ionized at this pH) tryptic peptides are repelled from the stationary phase into the mobile phase via their N-termini and basic amino acids at their C-terminus. On the other hand the phosphate groups on phosphopeptides remain ionized and are retained by the WAX resin. A high organic content in the mobile phase will therefore trap these peptides on the stationary phase increasing the retention. With

decreasing organic content in the mobile phase nonphosphorylated peptides will elute whereas phosphopeptides remain trapped. Applying a salt gradient will elute remaining phosphopeptides. With this setup phosphopeptide separation and fractionation in a single step is possible [46]. In an elaborate comparative study, ERLIC chromatography was compared to SCX and SCX-IMAC for phosphopeptide enrichment purposes by Gan et al. In their approach human epithelial carcinoma cell line A431 cells were analysed leading to a total identification of 2058 unique phosphopeptides of which 1801 were identified by only one of the methods. Of these phosphopetides, ERLIC accounted for 38%, SCX-IMAC for 57% and SCX alone for 5%. The overlap between ERLIC and SCX-IMAC identified phosphopeptides was 12% showing that for a comprehensive phosphoproteome analysis a combination of different enrichment strategies is sometimes necessary. Currently most LC-based methods described in this chapter are used for crude phosphopeptide prefractionation before more selective enrichment with IMAC or MOAC is done (see also chapter 2.10). In this way superior selectivity up to 99% is archieved.

7 Chemical modification based methods

In addition to chromatography based methods, tagging phosphate species with certain compounds using a specific chemical derivatisation reaction is another strategy for phosphopeptide enrichment. Site specific modification of phosphoseryl and phosphothreonyl residues using a combination of β-elimination and Michael addition is a way to introduce phosphosite specific tagging. Oda and co-workers used this technology to introduce a biotin tag for biotin/ avidin enrichment [81]. Under strongly alkaline conditions the phosphate moiety from phosphoserine or phosphothreonine undergoes β-elimination to form a dehydroalanyl or β-methyldehydroalanyl residue, respectively. These α,β-unsaturated residues are Michael acceptors, which can react with nucleophiles like ethandithiole, which is further coupled to biotin. The benefits for this method are that one can selectively enrich via different types of tags available, and the tags can be isotope labelled for quantification purposes [81] or carry functional groups to increase ionization efficiacy or to facilitate phosphorylation site determination [82]. However, there were some drawbacks reported from the authors. The high concentration of hydroxyl ions, necessary for efficient β-elimination, can cause some proteine / peptide degradation, also the reactivity of free cysteine residues can lead to unwanted derivatisation. In addition, O-glycosylated serines could be converted to dehydroalanine as well. These drawbacks can be avoided by additional protecting reactions [81] or method modifications [83] but this may head to a more complicated realisation and potential sample losses, respectively. Besides only phosphoserine and phosphothreonine containing peptides can be enriched using β-elimination/ Michael addition because tyrosine is not able to form an α,β-unsaturated bond and additionally β-elimination on free serines were observed as well [84]. Aebersold and co-workers used a carbodiimide catalyzed reaction for a reversible capturing of phosphate groups on phosphopeptides [85]. A six step protocol was developed. First, the free amino groups had to be protected by t-Boc chemistry to avoid intra – and intermolecular condensation in the following steps. The main reaction used carbodiimide to catalyze the condensation reaction between the peptides and excess amine to derivatize phosphate and carboxylate groups. The phosphoamidate bonds had to be cleaved off again by a brief acid hydrolysis and the carbodiimide catalyzed condensation reaction had to be repeated with cysteamine as condensating agent. The newly introduced thiol groups were covalently bound to immobilized iodoacetyl groups. Phosphopeptides were released from the resin by acidic hydrolysis revealing the native phosphate form. The applicability of this method for complex samples was proven by analysing the phosphoproteome of a yeast cell lysate. Although no low abundance phosphoproteins were

identified using the method, this was referred to an inadequate post enrichment separation [85]. Because the six step strategy seems to be time consuming and prone to sample losses, this method was further refined to a more simplified realization and the implementation of quantitation by introduction of isotope labelling [86].

The resulting three step strategy includes the protecting of free carboxylate groups by methylation with methanolic HCl which also offers the possibility for the introduction of deuterium labelled methyl groups, the coupling of the methylated peptides to a dendrimer via their phosphate groups, catalyzed by imidazole and carbodiimide in a one pot reaction, and the release of the peptides by acidic hydrolysis with 10% TFA. Using a tandem purification protocol with anti phosphotyrosine immunoprecipitation followed by phosphoserine and threonine enrichment by implementing the above stated protocol, 80 serine and threonine phosphorylation sites on 97 tyrosine phosphoproteins from Jurkat T cells could be identified. A recent application of a similar protocol was reported by Bodenmiller et al. when performing an elaborate phosphosite assignment of *D. melanogaster* KC167 cells [84].

8 Ca^{2+}-Precipitation

In the year 1994 Reynolds et al. used an excess of Ca^{2+} (20 mol/ mol protein) in 50% ethanol for precipitating phosphopeptides from a tryptic casein hydrolysate. At lower pH, only peptides containing multiple phosphoserines were enriched. At a pH of 8 all phosphopeptides except two monophosphorylated could be found in the precipitate [87]. Calcium phosphate – calcium-phosphopetide–coprecipitation in combination with two subsequent IMAC enrichment steps were used when analysing the phosphoproteome of rice embryonic cells. Peptides were mixed with disodium phosphate (Na_2HPO_4) and ammonia solution (NH_3 x H_2O) followed by the addition of calcium chloride ($CaCl_2$) at a pH of 10. In total 242 phosphopeptides, representing 125 phosphoproteins could be identified [88]. Although no tyrosine phosphorylated peptide was detected in this study, this was attributed to the low abundance of these in rice, rather than lacking selectivity for it. A similar method was recently used for the phosphoproteomic analysis of human brain by Xia et al. allowing the identification of 551 phosphopeptides with 466 unique phosphorylation sites [89]. Again, no phosphotyrosine containing peptides could be found.

9 Combination of methods

On the one hand, a combination of methods can be employed to combine different enrichment methods in parallel to obtain a more comprehensive view of the phosphoproteome, on the other hand it is possible to combine them in a serial workflow to maximise selectivity. For example, several groups reported that using TiO2 enrichment preferably singly phosphorylated peptides are isolated, whereas IMAC enrichment shows a bias towards multiply, predominantly doubly phosphorylated peptides [1, 88, 90]. This may be explained by the different binding properties and /or some microstructural preferences, but also by the different peptide to resin ratios of both materials [91]. In most recent phosphoproteome studies TiO2 and IMAC enrichment are carried out in parallel [11, 91, 92] utilizing the different preferences of the methods. A comprehensive comparison of TiO2, PAC and IMAC enrichment was carried out by Bodenmiller et al. In their experiments they obtained good reproducibility within the same method when analysing repeated isolates but the overlaps of peptide identification, generated between different methods were rather low (around 30% between all methods) [91]. In a recent work from our group the phosphoproteome of HeLa cells was screened by combining different metal oxide protocols (nanocast SnO_2 and TiO_2, commercial TiO_2) in a parallel setup [93]. In sum 1595 unique phosphopeptides were identified. The overlap of identifications between the methods was less than one third, almost 140 phosphopeptides were identified by SnO_2 solely, but more surprisingly both titania materials isolated exclusively for more than 20% of the total identifications. These results are indicating that for a complementary coverage of the phosphoproteome, a combined approach may be necessary.

LC based methods are used preliminary in the first dimension and fractions are collected according to resolving power of the respective method used. One has to pay attention that the elution conditions used for the first dimension do not interfere with the following enrichment step. Primarily high salt concentration or low pH elution conditions, which may be used to elute peptides from prefractionation columns, can cause some problems with following enrichment methods. Therefore a desalting step or other sample buffer adjustment may be required to allow further phosphopeptide enrichment. Trinidad et al. analysed the postsynaptic density (PSD), a part of the mammalian central nervous system using SCX or SCX-IMAC phosphopeptide enrichment. 88 SCX fractions were collected and subjected either direct to LC-MS analysis or to further IMAC enrichment (for fractions 24 to 88). In the early fractions the highest amount of phosphopeptides were found, but phosphopeptides were identified throughout the whole fractions. Thus 311 phosphopetides were identified with SCX alone and with the addition of IMAC chromatography a 3 fold increase of identification

could be achieved summing up to a total of 998 unique phosphopeptide identifications on 287 phosphoproteins out of 1263 proteins which were identified in total with this approach. Using SILAC peptide labeling in combination with SCX-IMAC phosphopeptide enrichment and high accuracy mass spectrometry with MS^3 peptide sequencing, double auxotroph yeast strains were analyzed for pheromone response by the Jensen group. More than 700 phosphopeptides could be identified of whom 18% were regulated by pheromone response. Another comprehensive phosphoproteomic approach using SILAC-quantification, but using SCX-TiO2 enrichment and high resolution mass spectrometry with multistage activation peptide sequencing [94] for accurate phosphopeptide identification was done by Olsen et al. [13]. Analysing EGF treated HeLa cells, more than 99% confidence of phosphopeptide identification could be achieved by combining high precursor mass accuracy information and two stage fragmentation of peptides losing a phosphate group and 6600 phosphorylation sites on a total of 2244 proteins could be identified, 14% of identified phosphorylation sites were modulated more than two fold upon EGF stimulation.

SCX fractionation and a combination of IMAC and TiO_2 enrichment with high mass accuracy peptide identification and SILAC quantification was used for the analysis of the phosphorylation dependent cell cycle regulation of HeLa cells [11]. Cells were arrested in the G1 or M-phase of the cell cycle and –grown in media supplemented with differently isotope labeled amino acids to be able to distinguish between the different cell cycle phases. After SCX chromatography, fractions were split into halves and equally enriched with either TiO2 or IMAC. Differently enriched fractions were pooled again and analysed by mass spectrometry. The combined setup allowed the identification of over 14.000 sites of phosphorylation on 3682 proteins with over 1000 proteins being upregulated phosphorylation during mitosis. The authors reported nearly one order of magnitude increased number of sites identified from nocodazole arrested HeLa cells compared to previous work [10].

Studies have shown that IMAC shows a bias towards multiply phosphorylated peptides whereas preferentially singly phosphorylated peptides elute from TiO_2 [1, 33]. Utilizing this behavior, Thingholm developed a strategy for sequential separation of monophosphorylated peptides and multiplyphosphorylated peptides. The strategy named sequential elution from IMAC (SIMAC) uses IMAC in the first dimension and TiO_2 for secondary enrichment of IMAC flow through and mild condition elutes. Peptides were therefore eluted from IMAC under acidic conditions (1% TFA, pH 1.0) when preferably monophosphorylated peptides should elute followed by TiO_2 enrichment to remove remaining nonphosphorylated peptides which may coelute from IMAC under these conditions, respectively. Subsequently basic elution with ammonium hydroxide solution (pH 11.3) is applied to IMAC to recover remaining multiply phsophorylated peptides. Combining the advantages of IMAC and TiO_2 it was possible to identify 306 monophosphorylated peptides, 186 multiply phosphorylated peptides and 716

unique phosphosites of a tryptic digest of human mesenchymal stem cells. These numbers were in contrast to 232 monophosphorylated and just 54 multiphosphorylated peptides and 350 unique sites when using an optimized TiO_2 protocol alone.

10 Application to other biological phosphocompounds

As already mentioned in the Introduction, other phosphorylated biocompounds such as phosopholipids (PL), DNA or RNA, and phosphorylated metabolites play an important role in biological and pharmaceutical research as well.

Phospholipids are the main constituent of the cellular membrane bilayer but also play an important role in cellular signalling [95]. Lipidomics provides analytical strategies for the analysis of lipid metabolism and lipid mediated signalling processes [96].

Mass spectrometry is currently the method of choice for straightforward lipidomic analysis [references], however, elaborate separation or specific enrichment is also in this case essential for efficient MS identification.

Analytical tools such as gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC) with MALDI-TOF-MS can be used [97] but HPLC-ESI-MS is currently the most preferred combination for lipidomic analysis [95-97].

Therefore, in general, total lipids are extracted from biological samples like cells or tissue according to the Bligh-Dyer or the Folch method [98, 99] and extracts are separated by normal phase or reversed phase chromatography using diol, C-18 or amino- phase columns before mass spectrometric identification [97]. A more phosphospecific method was first introduced by Ikeguchi and co workers. In their approach they used TiO_2 for selective enrichment of PLs from egg yolk prior to LC analysis. Recovery rates up to 70 % for diverse PLs could be achieved and the method was also effective for removement of fatty acids and neutral lipids [49]. A further refinement of the method was done by Calvano et al. [100]. TiO_2 was packet into frits allowing a fast and uncomplicated SPE enrichment of PLs from dairy products. Similar to the protocol of Larsen for phosphopeptide enrichment they used DHB to reduce unspecific binding from nonphosphorylated lipids. Only PLs could be identified in the elution fraction, proving the high selectivity for PLs of the method. In a recent work from our group, ZrO_2 packed into SPE cartridges were used to selectively isolate phosphatidylcholines (PCs) from natural samples like milk, or human or mouse plasma [101]. Recovery rates up to 100% were achieved using an optimized extraction, incubation and elution protocol. Using the protocol optimized for ZrO_2, TiO_2 and SnO_2 were also tested for PC enrichment, whereas ZrO_2 performed superior compared to other materials tested

The negatively charged phosphate diester backbone of DNA can also serve as ligand for purification with phosphoaffine enrichment materials.

Due to the advancements of gene therapy and genetic vaccines during the last decade, the demand for highly purified plasmid DNA is still increasing [102]. pDNAs are mainly produced by recombinant *E.coli* fermentation. So, for research and clinical applications pDNA has to

be purified and separated from genomic DNA, RNA, remaining proteins and endotoxins. Secondly, only the super coiled conformation of pDNA (sc pDNA) is biologically active and hence has to be separated from other isoforms [103]. Therefore chromatographic methods such as size exclusion (SEC), hydrophobic interaction (HIC), hydroxyapatite (HA), reversed phase (RP), or thiophilic adsorption and affinity chromatography (AC) mostly in combination with ion exchange chromatography (IEX) as the second dimension is used [104]. To date, no phosphatespecific method for pDNA purification was reported, although Sousa et al. reported the complete separation of sc pDNA and open circular pDNA by arginine affinity chromatography. The specific recognition was described to be the result of multiple interactions between arginine and pDNA, including electrostatic interaction with the pDNA phosphate backbone and also some degree of biorecognition of nucleotide bases by the arginine ligand [105].

The highly active antiretroviral therapy (HAART) is currently the common treatment for human immunodeficiency virus (HIV)-infected patients. HAART is a combination of different antiretroviral drugs and one of its main constituents are nucleoside reverse transcriptase inhibitors (NRTIs). The active form of NRTIs is not the drug itself but its triphosphorylated metabolites in the cytosol. Analysis of NRTIs and their phosphorylated metabolites is needed to prove individual effectiveness of therapy. In a recent review by Jiaping Lai [106] analysis methods of HAART drugs constituents and their phosphorylated metabolites are discussed. The chromatographic methods range from SPE, GC to RP-LC with either spectroscopic or mass spectrometric detection. For triphosphorylated NRTIs (tp NRTIs), nano or micro RP-LC combined with triple quad MS in multiple reaction monitoring mode (MRM) was recommended due to the high selectivity and sensitivity of the method, but is still suffering from some serious problems. Ion pairing reagents (IPRs) are necessary to reach sufficient separation of the highly polar tp NRTIs, but IPRs lead to high signal background and reduced ionization efficiency during the ESI-MS analysis. Even though no work on phosphate specific enrichment of phosphorylated metabolites was published to date, according to the authors, more sensitive and selective methods for the trace analysis of tp NRTIs in biological samples need to be developed [106].

Summing up, the most important property of phosphorylated biomolecules is their low concentration in a complex biological matrix. Therefore, specific enrichment methods for phosphorylated biomolecules, that were initially developed for phosphoproteome analysis may help to overcome limitations which currently hamper effective LC-MS analysis.

11 Mass spectrometry based protein analysis

Before the introduction of mass spectrometry for (phospho-) proteome analysis, phosphosite specific antibodies or the incorporation of (^{32}P) orthophosphate into proteins with later detection on electrophoresis gels were the traditional methods for the detection of phosphoproteins. Phosphorylation site determination was accomplished using Edman degradation of ^{32}P- labelled peptides. These methods were robust and well established, but showed limitations when analysing complex samples and were labour extensive and prone to sample losses, so a lot of sample material was needed [107-110]. With the introduction of mass spectrometry for proteome analysis, the possibilities increased dramatically. Mass spectrometers provided accurate, fast, sensitive and selective instruments for peptide or protein mass determination. Depending on the type of mass spectrometer, data-dependent fragmentation [111] of measured peptides(tandem mass spectrometry) delivered additional peptide sequence information and/or allowed the identification of post-translational modification sites. During data dependent acquisition, the most abundant peptides from a survey scan are selected for further fragmentation. Depending on the analytical requirements, different types of mass analysators are available ranging from cheap and robust low resolution analysers like quadrupole, (linear) ion trap, or time of flight (TOF) mass spectrometers with higher resolving power, to highly accurate and sensitive, high resolution mass spectrometers like Orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) mass analysers. The application of the different analysers also depends on the budget of the laboratories. Nowadays most instruments are combinations of different analysers (“hybrid” instruments). Here, the first part is used for precursor selection or accumulation and highly sensitive, high resolution detection is accomplished in the second part of the instrument. Recent mass spectrometers used in proteomic studies are listed in Table 1 with their pros and cons and types of application.

12 Technologies for peptide sequencing by mass spectrometry

With the introduction of soft ionization techniques like electrospray ionization (ESI) or matrix assisted laser desorption / ionization (MALDI) analytes can be ionized in the gas phase, mostly in the positive mode by protonation, without inducing fragmentation. This allowed the introduction of the intact peptide or protein into the analysator.

After an enzymatic protein digest by site specific proteases, peptides are either analysed direcly without additional separation, e.g. by MALDI-TOF-MS, or using liquid chromatographic separation coupled to ESI-MS, and determined peptide masses are then compared to predicted peptide masses from genomic or proteomic databases. This technology called peptide mass fingerprinting is a useful tool when proteomic samples are not too complex or only mass spectrometers without fragmentation capabilities are available. Nevertheless this identification tool has its limitations when more complex protein samples like body fluids or cell lysates have to be analysed. There is a much higher risk of false positive identifications in comparison with methods that rely on MS/MS information as well [112]. Protein identification and peptide sequencing using tandem mass spectrometry revealed additional sequence specific data which allowed a higher identification confidence. Using this technique, precursors (peptides) are fragmented by colliding peptides with an inert gas (collision induced fragmentation, CID), or by capture of low energy electrons (electron capture dissociation, ECD) or by reduction with anions in the gas phase (electron transfer dissociation, ETD). These techniques predominantly induce fragmentation of the peptide backbone and sequence informative fragments designated as a, b, c and their complementary counterparts, x, y, z ions are generated [figure 3].

Each fragmentation technique produces a distinct series of ions (y and b for CID, c and z for ECD and ETD respectively). By determining the mass differences of ions of one ion series, a particular amino acid can be assigned to the next larger fragment and sequence determination can be realized.

For protein identification by database search, search programs need a predictable fragmentation pattern for generating possible ions out of DNA or protein databases [113-116]. Therefore an understanding of the mechanisms underlying this fragmentation pattern is necessary. The mobile proton model gives a good explanation of peptide fragmentation behaviour in CID experiments [117]. Briefly, the protons for ionisation need to move along the peptide backbone and the cleavage is initiated at the site where the proton is located at the time point of collision. The energy necessary for fragmentation depends on the sequence of the peptide and on the charge state. In the case of CID fragmentation, basic residues like lysine or arginine can attract or sequester protons required for fragmentation, and additional

energy is needed to move (“mobilize”) the protons from the residue to the backbone. If a second proton is available less activation energy is necessary because the most basic site is already occupied. Side-chains from acidic residues like aspartic acid, glumatic acid or protonated histidine can favour a cleavage in their proximity by providing additional protons.

Collision induced fragmentation

With Collision induced dissociation (CID), also called collision activated dissociation (CAD), peptide ions in the mass spectrometer are accelerated by applying additional kinetic energy and fragmented by collision with an inert gas such as nitrogen or helium.

Thereby imparted translational energy is converted into vibrational energy which is rapidly dispersed over the whole molecule where the weakest bonds are cleaved.

With CID, mainly sequence informative b and/or y fragment ions are generated, which contain the N- and C-terminus, respectively [figure 4]. In addition, neutral loss of water, ammonia, carbon dioxide and - in the case of phosphopeptides - phosphoric acid can occur [94, 118]. The phosphate group on serine or threonine is the energetically most labile bond on phosphorylated peptides. Frequently, a neutral loss of phosphoric acid (H_3PO_4 (98 Da)) is induced upon CID fragmentation and dehydroalanine or dehydroaminobutyric acid is generated due to an internal β-elimination reaction in the gas phase. A neutral loss of a phosphate group on phosphotyrosine can also occur (HPO_3 (80 Da)) but is less common [119]. Using the neutral loss as indicator to selectively target for phosphopeptides, a precursor ion scan or a neutral loss scan (on triple quadrupole mass spectrometers) can be used to specifically detect phosphorylated peptides [94, 120-123].

When a phosphopeptide is selected for fragmentation and a neutral loss is detected, the ion originating from the neutral loss can be selected for further fragmentation by MS^3 [10]. MS^3 provides a more sequence specific fragmentation spectra of the product ion of the neutral loss. In addition, phosphorylation site identification is enabled, for example by the detection of dehydroalanine instead of pSer. As a disadvantage, the authors [10] stated that this technology only worked well when relatively abundant phosphopeptides were selected, whereas low abundant phosphopeptides did not provide evaluable MS^3 spectra. This was attributed to insufficient trapping of ions over several MS stages. An alternative to MS^3, called pseudo MS^3 or multistage activation (MSA), to circumvent several trapping stages was introduced by Schroeder et al. Here, peptides and possible (multiple) neutral loss products are activated for fragmentation during one MS stage. Hence a composite spectrum containing fragments from precursor (MS^2) and the possible neutral loss products (MS^3) are generated and the analysis time per peptide is reduced. A comprehensive comparison of MS^2-only, MS^2/MS^3 and MSA workflows was carried out by Ulintz et al. When analysing the phosphoproteome of yeast and fruit fly, the MSA method showed the highest number of

identifiable peaks and the obtained spectra had the highest information content compared to the other methods used in the study [124].

Electron capture dissociation

In 1998 Zubarev introduced electron capture dissociation as a more gentle fragmentation method compared to CID. ECD rarely causes loss of labile groups such as post-translational modifications. In the static magnetic field of an FT-ICR mass spectrometer peptides are trapped and irradiated with a beam of thermal electrons. The absorption of the electron is a non-ergodic process and the intra-molecular energy dispersion by atomic vibration and rearrangements is slower than the bond cleavage mechanism [125]. Via ECD mainly c and z fragments are generated [figure 4], which results from the cleavage of the N-Cα amine backbone bond [126]. As a consequence of the electron capture and the ensuing charge state reduction, peptide ions have to be multiply protonated for fragmentation. In general, ECD generates a more randomized fragmentation pattern than CID and is less affected by acidic or basic side-chains. Only N-Cα cleavage N-terminal to proline residues is not observed due to the tertiary nature of the amide nitrogen. Disulfide bonds are reduced due to their high affinity towards radicals [125].

Electron transfer dissociation

Due to the fact that ECD is limited to FT-ICR mass spectrometers a further development of ECD, which can be applied to more accessible instruments like linear ion traps or ion trap hybrid mass spectrometers was developed by Syka and co- workers [126]. In a static radio-frequency field, like it is present in quadrupoles or ion traps, thermal electrons cannot be trapped [126]. Using anions with low electron affinities like anthracene as one electron donor, electron transfer to protonated peptides occurs and initiates the same non-ergodic fragmentation process like ECD. The anion beam is generated by a chemical ionization source and guided via octopole lenses to the ion trap where fragmentation occurs. With ETD, thermal electron triggered fragmentation is accessible to cheaper mass spectrometers like 3D-ion traps (IT) or linear ion traps (LTQ). The disadvantage of these analysers is the lower mass accuracy compared to FT-ICR mass spectrometers. With the introduction/commercialization of the LTQ-Orbitrap mass analyser, a cheaper and more robust mass spectrometer with similar mass accuracy and superior sensitivity compared to FT-ICR is available, which can use either CID or ETD fragmentation.

13 Quantitation Methods

Measuring the proteomic response of cells to certain stimuli like drug treatment, changes in the environment, or disease, changes can be determined by differences in the amount of affected proteins. Mass spectrometry offers a wide range of applications for differential quantitative proteomics. In contrast to non mass spectrometry based quantification tools like staining of gels or western blots, mass spectrometry analysis delivers both, identification and quantitative information. For the determination of cellular phosphorylation dynamics phosphospecific enrichment is in most cases required before quantitation can be realized [127]. So the phosphorylation dynamics can be inferred from the respective number of phosphorylated peptides. Taking into account that the mass spectrometric response of peptides can depend on their size, charge or the hydrophobicity, it is necessary to compare each individual peptide during analysis. In general this can be accomplished by introducing a stable isotope label for the respective peptides. Isotopes used for this task are ^{2}H, ^{13}C, ^{18}O, or ^{15}N and can be introduced as internal standard during cell culture (SILAC, labelled amino acids), using enzymes (incorporation of ^{18}O during digestion),chemical tagging (iTRAQ, ICAT) or by spiking samples with known amounts of synthetic peptides as external standard (absolute quantification, AQUA). Isotopically labelled peptides are chemically identical to their native counterparts and exhibit the same chromatographic (with the exception of deuterium, which might lead to small shifts in retention time) and mass spectrometric properties. Thereby “light” and “heavy” phosphopeptide species can be distinguished in the spectra by a distinct mass difference and quantitation can be accomplished by comparing signal intensities [128].

In general it is recommended to pool sample and reference in the earliest stages of sample preparation. Thus, from the time point of combination, both samples are treated identically and errors in quantitation resulting from variations during sample preparation can be compensated for. Metabolic labeling allows the combination of samples in early stages right after cell harvesting. For SILAC, cells are grown in amino acid depleted media in which labelled essential amino acids are substituted. In most cases ^{13}C and ^{15}N labelled arginine and lysine are used, generating singly labelled peptides after trypic protein digestion ensuring a defined mass difference between peptides. SILAC quantification provides probably the most accurate quantitation method and applications are numerous [13, 129-134]. The disadvantages of SILAC are that the method is expensive and limited to cell culture.

Enzymatic and chemical tagging allows the integration of stable isotopes on the protein or peptide level. ^{18}O can be incorporated into peptides during protein digestion with proteolytic

enzymes [135]. For example trypsin and Glu-C introduce two oxygens per peptide which provides a mass shift of 4 Da, which is also sufficient for discrimination of isotopomers with higher charge states.

Reactive side chains offer a possibility to attach isotopic mass tags to peptides or proteins via a chemical reaction. Isotope coded affinity tags (ICAT) use iodoacetamide functional groups to selectively bind to the sulfhydryl group of cysteines [136]. The tag consists of the reactive group, a linker which can be isotopically labelled, and a biotin group for affinity purification of labelled peptides. Taking into consideration that cysteine is a rare amino acid, this method is not suitable when analysing proteins with low numbers of cysteines or for analysing post-translational modifications like phosphorylation [128]. The N-terminus of peptides and the epsilon amino group of lysines can be used to introduce an isobaric tag by specific N-hydroxysuccinimide (NHS) chemistry, a method called isotope tags for relative and absolute quantification (iTRAQ). During analysis, iTRAQ tagged peptides behave identical until CID fragmentation is initiated. During fragmentation, the tag loses its balancer group and peptide abundances can be determined by the ratio of the generated reporter ions [137]. Methyl esterification of carboxyl residues with deuterated alcohols can also be used to introduce isotopic tags. This modification is additionally attractive for phosphopetide enrichment, because it reduces unspecific binding to IMAC resins. As well as direct tagging of phosphorylated amino acids by using β-elimination/Michael addition reaction with deuterated nucleophiles is possible [138, 139]. However, a variety of additional chemical reaction for selectively tagging certain amino acids exist which can be used to introduce stable isotope labels [140-147].

Absolute protein quantification is generally used when only one or a few proteins are in focus, e.g. for analysis and validation of biomarkers in a large number of clinical samples [148]. In its simplest case, synthetic peptides in known amounts are added to a protein digest and quantities are determined by comparing signal intensities (absolute quantification by adding isotope-labelled peptides, AQUA) [149, 150].

More recently label free quantitation proved a straightforward technique for screening experiments, because no extra sample preparation or specific growth media are required. Label free quantitation uses global standards instead of peptide specific standards and quantitative information can be acquired by either counting the frequency of acquired MS^2 spectra [151] or by comparing the signal intensities of each peptide [150, 152], respectively .

More recently, liquid chromatography coupled with inductive coupled plasma mass spectrometry (LC-ICP-MS) was introduced for specific phosphopeptide quantification [153-156]. With ICP-MS, heteroatoms like metals and semimetals but also biochemically important non-metals like sulphur, phosphorous, and iodine on biomolecules can be selectively detected. ICP-MS delivers no sequence information and thus no discrimination between

different phosphopeptides is possible. Therefore one has to ensure that phosphopeptides are sufficiently separated into discrete peaks by chromatography. Secondly, decreasing ionization efficacy due to increasing organic solvent has to be considered. With the help of ICP-MS, relative and absolute specific phosphopeptide quantification is possible. The phosphorylation degree of proteins can be determined using the sulphur signal from cysteine or methionine as a probe to quantify both, the phosphorylated and the nonphosphorylated form of the protein [155].

This chapter describes only a brief and generalized comment on recent quantification methods. For further information the reader is referred to recent reviews [127, 128].

Conclusion

Phosphospecific enrichment combined with liquid chromatography – mass spectrometry analysis has enabled a more comprehensive proteome-wide view of cellular phosphorylation networks. But the analysis of the phosphoproteome is still a challenging task due to high phosphorylation dynamics and abundance differences between analytes and matrix components, respectively. Therefore, the utilization of new materials as well as improvements of protocols and strategies are offering solutions which may help to overcome these limitations. The combination of ion exchange chromatography with specific phosphopeptide enrichment enabled a large increase in phosphopeptide selectivity. On the other hand, the parallel implementation of different enrichment methods allowed a more complementary coverage of the phosphoproteome. Improvements in MS-instrumentation offer increased resolution and dynamic range, high mass accuracy and sensitivity with fast scan speeds, enabling the identification of thousands of analytes in a single run. The additional implementation of new fragmentation tools such as neutral loss-triggeredMS3, MSA, ECD and ETD allow a more specific phosphopeptide identification and a more detailed sequencing of phosphopeptides for accurate phosphorylation site identification.

A major protocol improvement for metal oxide-based phosphopeptide enrichment was achieved by the addition of specific buffer additives which allowed higher selectivity and better recovery rates of phosphopeptides. For further optimization, a fine tuning of material properties which may influence phosphopeptide binding and selectivity may be necessary. First attempts were already made in our group by screening different enrichment materials for material properties like surface area, surface treatment, or manufacturing process in terms of phosphopeptide affinity and selectivity, but there is still more information needed to allow a dedicated rational material design.

As a future perspective, a robust and automated phosphoproteome analysis for clinical application is intended. Therefore both physically and chemically robust enrichment materials are necessary which may be packed into chromatographic columns for online analysis. By the nanocast process metal oxides can be manufactured in different diameters and pore sizes. Especially porous tin dioxide, which can be used without the use of highly concentrated additives, may be a suitable material for this purpose.

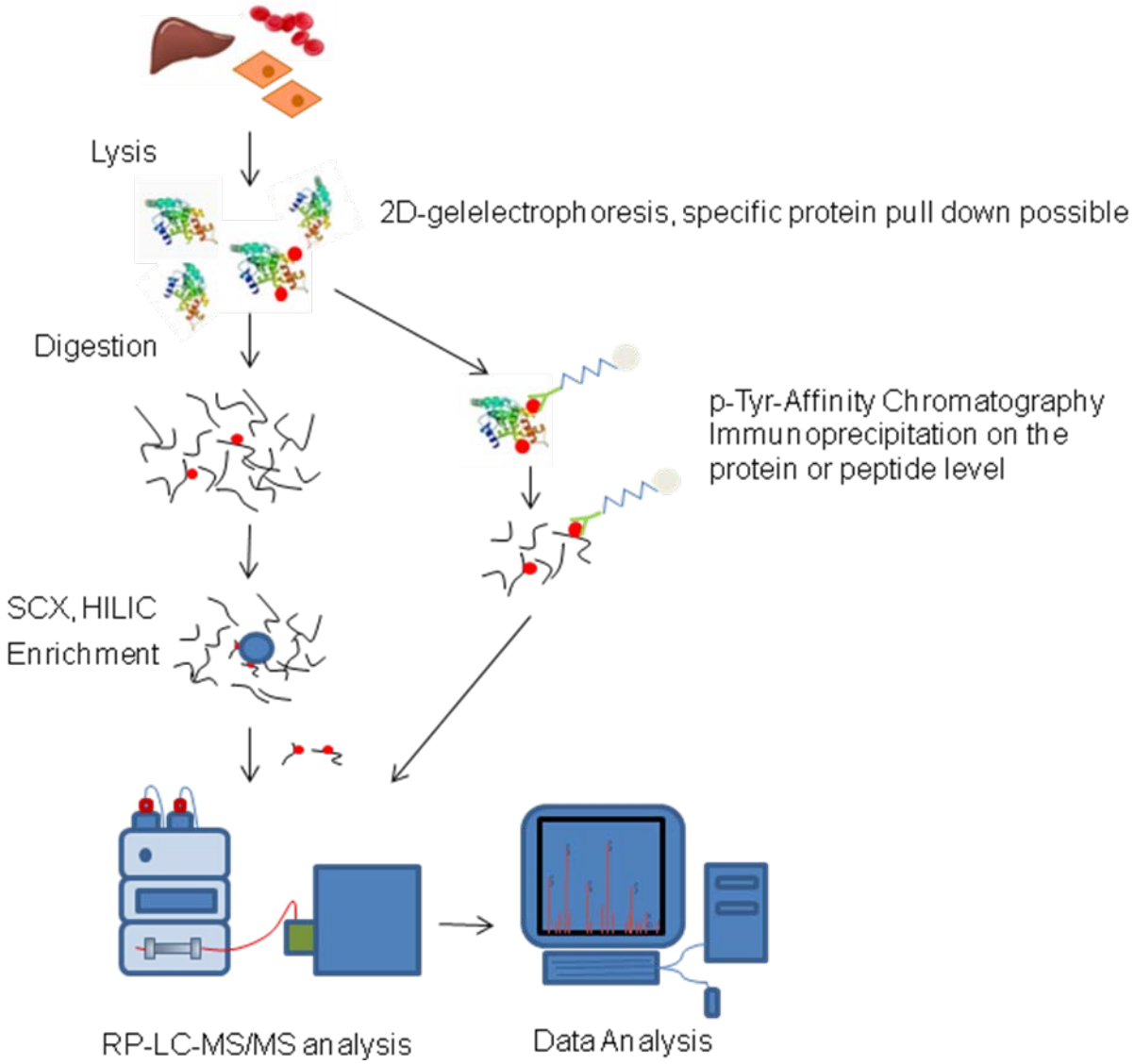

Figure 1. A general workflow of an MS based phosphoproteomic experiment is depicted. Phosphorylated species can be fractionated (SCX, HILIC) or specifically enriched (IMAC, MeOAC), furthermore p-Tyr containing peptides/proteins may be enriched by specific antibodies.

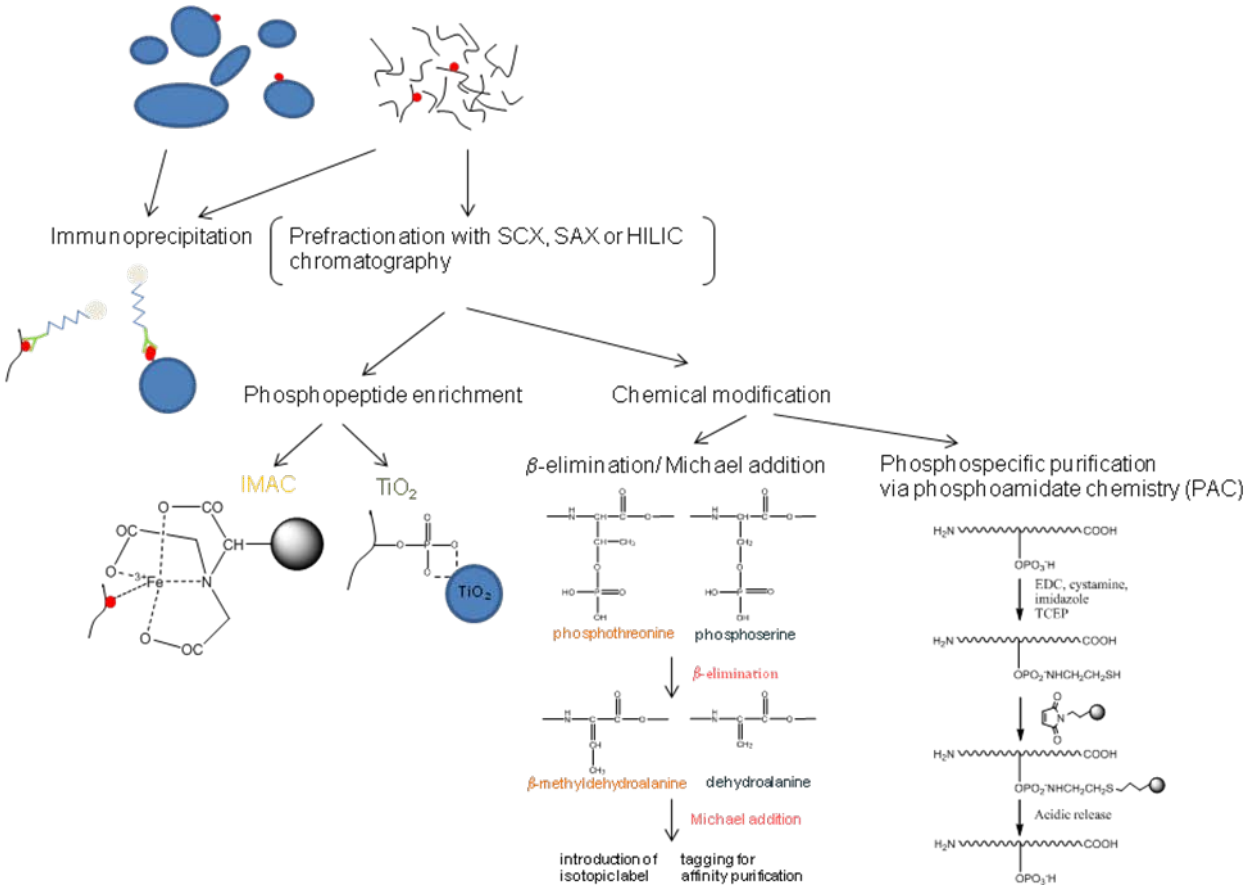

Figure 2. Overview about most common enrichment strategies for specific phosphopeptide enrichment. A combination of methods increases the amount of possible unique phosphosite identifications. By the use of prefractionation before specific enrichment a marked increase in selectivity can be achieved.

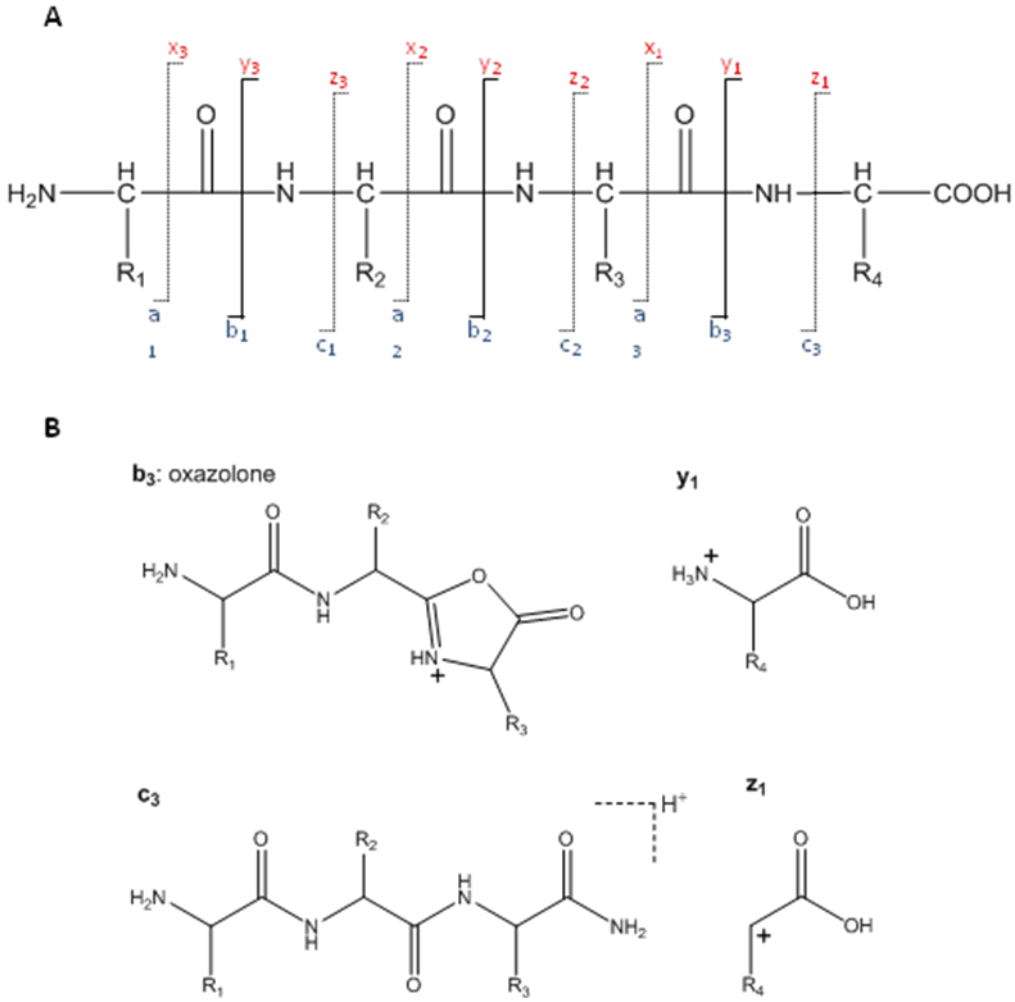

Figure 3. (A) Nomenclature of peptide fragmentation patterns with (B) predicted ion structures commonly induced by CID-type fragmentation (b and y ions) or ECD/ETD fragmentation (c and z ions) [157].

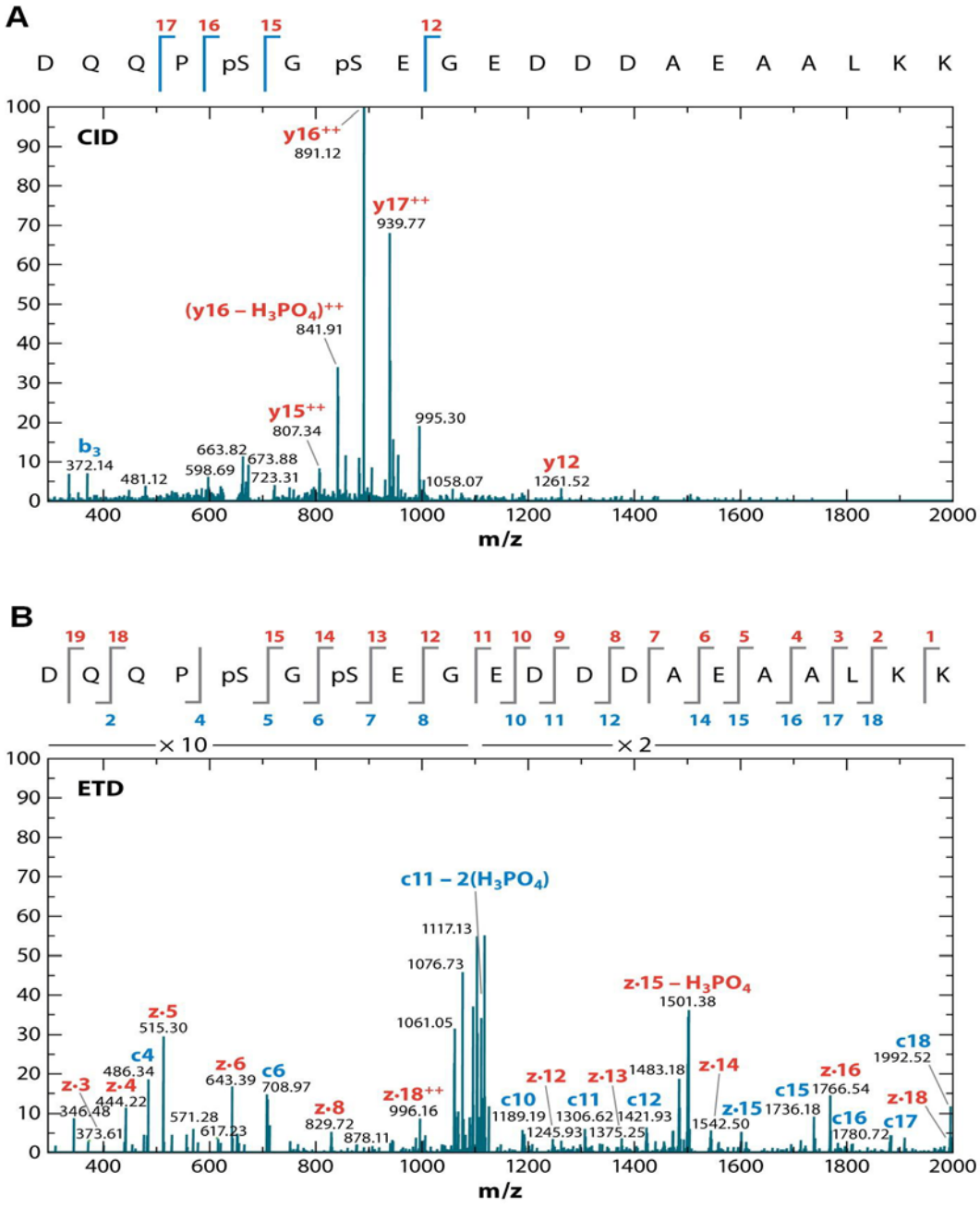

Figure 4. Only poor sequence information can be obtained from CID spectra of many phosphopeptides, because CID preferably induces dissociation of the most labile bonds. Predominant signals are caused by neutral loss of phosphoric acid (H_3PO_4), from the cleavage of the labile O-phosphate bond and by site specific fragmentation of the amide bond c-terminal to proline. Mainly y and b ions are generated. (B) Cleavage energy is dispersed more randomly on peptide backbone during ETD fragmentation, leading to a more sequence informative fragment spectra. Notably, only z and c ions are generated. Commonly no neutral losses of H3PO4 are induced by ETD. Neutral losses observeable in this spectrum are caused by a higher activation energy during fragmentation to increase signal intensity. Reprinted from [158] with permission of Annual Reviews of Pharmacology and Toxicology.

Type of analyzer	Pros	Cons	Supplier	Applications
Triple Quadrupole instruments (QqQ)	moderately expensive instrument, robust; Ion filtering, MS^2, CID fragmentation high dynamic range, very high sensitivity when used in MRM (SRM) mode – good for quantitation	Low scan speeds, Low sensitivity in scan mode, low resolution (unit resolution), only CID fragmentation	AB Sciex, Thermo Scientific, Agilent, Waters, Varian	Good for quantitation (SRM/ MRM) , but also peptide sequencing possible
(Linear) Ion Trap (L)IT Mass spectrometers	moderately expensive, robust; Ion selection, MS^n, CID & ETD fragmentation possible, Fast scan speeds, high sensitivity	relatively low (typically ~unit) resolution, higher scan speeds,sensitivity and enhanced resolution mode with latest generation instruments	Thermo Scientific, Bruker, Agilent, Varian	Peptide (protein) sequencing, quantification
Time of flight (TOF) Mass spectrometers	cheap High resolution, high sensitivity, wide mass range	Low dynamic range, no fragmentation, generally not applicable for quantitation purposes	Shimadzu, Bruker, Waters, Agilent	Peptide mass fingerprinting. Mainly provided with MALDI-ionization
TOF-TOF	High resolution and high sensitivity, high energy (20keV) CID, MS^2	Low dynamic range, minor application for quantitation	Bruker, AB Sciex, Shimadzu	Peptide sequencing, accurate peptide and fragment ion mass determination. Mainly provided with MALDI-ionization
Q-TOF	High resolution, high sensitivity (TOF), Ion filtering (Q) MS^2 (CID) Quantification		AB Sciex, Bruker, Waters, Agilent	Accurate mass determination (TOF). Peptide sequencing, quantification. Combines qualities of quadruplole and TOF MS
LIT-TOF	High resolution (TOF), CID fragmentation, high scan speeds, MS^n (LIT)		Shimadzu	Protein and peptide sequencing, quantification (LIT), Accurate mass determination (TOF)
LTQ (LIT)-Orbitrap	Very high resolution and sensitivity with Orbitrap High dynamic range Fast scan speeds,	expensive	Thermo Scientific	Peptide and protein sequencing in LIT, quantification combines fast scan speeds (MS^n) of LIT

	multiple fragmentation MS^n, CID & ETD fragmentation with LTQ			with high resolution and sensitivity of Qrbitrap
FT-ICR	Delivers highest resolution of all mass spectrometers, ECD fragmentation	Slow scan speeds, Low sensitivity, Very expensive, costly in operation (needs liquid He for cooling)		Accurate mass determination. Peptide and protein sequencing with ECD. Exclusively provided as hybrid instrument (Qq-FTMS, QqQ-FTMS, LTQ-FT-ICR)
LTQ-FT-ICR-MS	High resolution (FT-ICR) High scan speeds (LTQ) MS^n CID, and ECD fragmentation possible	Expensive, sensible, costly	Thermo Scientific	Peptide and protein sequencing, quantification. Combines fast scan speeds (MS^n) of LIT with high resolution of FT-ICR
Qq(Q)-FTMS	High resolution (FT-ICR-MS) Ion filtering, SRM High dynamic range CID, ETD, ECD fragmentation possible	Expensive, sensible, costly	Bruker, Varian	Peptide and protein sequencing, quantification. Combines the power of QqQ and FT-ICR

Table 1. Overview about recent MS technologies used in proteomic experiments. Their pros and cons, types of application and suppliers are listed. Abreviations: FT-ICR-MS Fourier transform-ion cyclotron resonance-MS; SRM selected reaction monitoring; MRM multiple reaction monitoring (same technology, differently named by supplier); CID collision induced fragmentation; ECD electron capture dissociation; ETD electron transfer dissociation.

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3 A Chemically Cleavable Linker Concept

Aim

During the second project the development and evaluation of a new method for affinity purification of target proteins was realized. Therefore a new linker chemistry was designed, which allows the release of bait – target complexes via specific chemical linker cleavage.
In the field of chemical proteomics, targeted protein pull-downs are generally used to selectively enrich for specific bait interactors, enabling their identification by liquid chromatography – (tandem) mass spectrometry. Commonly, baits are coupled to beads via different linker chemistries, allowing the isolation of target proteins from matrix components. Elution is generally performed by the use of denaturing buffers. However, this may also elute proteins which are unspecifically bound to the bead matrix, increasing the risk of false positive identifications. By elution-independent affinity chromatography, intact target–bait complexes can be eluted, commonly by specific linker cleavage. In our group a cleavable core structure, based on an indoleacetic acid-malondialdehyde derivative, was designed, which can be selectively cleaved at the indole nitrogen by incubation with pyrrolidine. This derivative was coupled to hydrazide functionalized agarose or acrylamide beads via the reactive allylaldehyde group of the derivative. As bait we used bosutinib, a drug recently used in the therapy of chronic myeloid leukemia, which was available in its amino functionalized form. Bosutinib was coupled to the linker via an amidation reaction with the help of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as coupling reagent. A protein pull down was performed on a K562 cell lysate and isolated interaction partners were identified by immunoblot and LC-MS/MS analysis.

Synthesis and results of this work can be found in the manuscript “A New Chemically Cleavable Linker of an Immobilized Bait for Target Protein Pull-down Concept” to be submitted (see Appendix Manuscript no. 1).

4 Appendix

Publication 1

ADVANCED FUNCTIONAL MATERIALS

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Tin Dioxide Microspheres as a Promising Material for Phosphopeptide Enrichment Prior to Liquid Chromatography-(Tandem) Mass Spectrometry Analysis**

By *Martin Sturm, Alexander Leitner,* Jan-Henrik Smått, Mika Lindén,* and *Wolfgang Lindner**

Functional materials are frequently used to identify and characterize proteins and their modifications in complex biological samples throughout the analytical workflow. Protein phosphorylation is a highly important post-translational modification that is being examined with the help of diverse materials that exhibit affinity towards the phosphate group of phosphoamino acids. Titanium dioxide and zirconium dioxide particles are increasingly used to enrich phosphopeptides from proteolytic digests of protein mixtures, although specificity and recovery still leave room for improvement. Here, we present tin dioxide (SnO_2, stannia) microspheres as a new type of metal oxide material for phosphopeptide enrichment. The microspheres are produced by a nanocasting process, starting from silica particles as a template, which allows the tuning of material properties such as particle diameter and porosity of the microspheres in a straightforward manner. For the first time, we are able to show that tin dioxide can be used to enrich phosphopeptides from mixtures such as enzymatic digests of proteins, followed by analysis by liquid chromatography-mass spectrometry (LC-MS). For optimization of the enrichment protocol, we use synthetic phosphorylated and nonphosphorylated peptides, and test different solvent compositions for loading and washing steps to enhance the selectivity of the material without compromising phosphopeptide recovery. Furthermore, the selectivity and phosphopeptide binding properties of tin dioxide are compared to the established metal oxide materials, titanium dioxide and zirconium dioxide, using mixtures of model proteins. Even without the use of additional additives such as α-hydroxy acids, which have been used to enhance the specificity of TiO_2-based enrichment, we show that a comparably good performance can be achieved for the SnO_2 spheres.

1. Introduction

Proteomics, as a scientific discipline, aims to characterize the protein complement – the ''proteome'' – of a cell, a body fluid or another complex biological sample. It is estimated that the human proteome consists of approximately 30 000 genes, each gene coding for a specific protein. But the number of different proteins present in an organism at any given time could rise over one million, and this is mainly the result of splice variants and posttranslational modifications (PTMs). These PTMs comprise chemical modifications ranging from the formation of disulfide bonds via oxidation of the thiol groups from cysteines during protein folding, to phosphorylation, glycosylation and other covalent modifications when the protein is already folded.[1] Protein phosphorylation is known to be a key event in intracellular signaling and other important cellular processes such as metabolism, transcription and apoptosis. Thus, the identification and localization of phosphorylation sites in proteins is very important to get insights in the regulation of cellular signaling cascades and other biological networks. It is estimated that phosphoproteins account for more than 30% of the proteome,[2] but on the level of a particular protein, phosphorylation is typically transient and only present in substoichiometric amounts. Mass spectrometry (MS) is the technique of choice for proteome analysis due to its ability to not only identify a large number of proteins, mostly from their corresponding peptides, but also to identify, characterize and locate post-translational modifications.[1]

In a typical ''bottom-up'' analysis, single proteins or more complex samples like whole cell lysates or plasma are enzymatically digested into peptides, because they are more amenable to mass spectrometric analysis. Peptide mixtures are then most commonly separated by one- or two-dimensional high performance liquid chromatography (HPLC) prior to MS analysis. However, after the digestion step, phosphopeptides are usually present in an overwhelming excess of unphosphorylated peptides, which usually have higher ionization

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efficiencies and are preferentially selected for peptide sequencing by tandem mass spectrometry (MS/MS) in so-called ''data-dependent'' experiments. For this reason it is necessary to develop efficient and selective phosphopeptide enrichment methods to reduce the complexity of the peptide mixture to ease later analysis and allow the identification of phosphorylation sites. Different affinity-based preseparation strategies have been used with varying degrees of success.[3] These include immunoprecipitation, immobilized metal affinity chromatography (IMAC), strong cation exchange chromatography, metal oxide affinity chromatography and chemical modifications like β-elimination. Some of these methods have recently allowed the identification of several hundred and up to thousands of phosphoproteins in large-scale phosphoproteomics studies.[4]

While IMAC continues to be widely used in phosphoproteomics, studies on different metal oxide materials such as titanium dioxide (TiO_2, titania),[5] zirconium dioxide (ZrO_2, zirconia)[6] and aluminum oxides,[7] which are said to enrich phosphopeptides due to their Lewis acid properties have shown promising results, and frequently outperform the IMAC materials. The number of studies that have used metal oxide-based phosphopeptide enrichment has increased rapidly as a result of these initial reports. Beside the use of commercially available particles, several groups have also reported the preparation of different types of metal oxide materials and morphologies (nanoparticles, magnetic beads, surfaces and membranes) and their application to phosphopeptide enrichment. TiO_2,[8] ZrO_2,[8d,9] Al_2O_3,[10] and, most recently, Ga_2O_3[11] have been demonstrated to bind phosphopeptides with varying degrees of specificity, although in most cases, the materials were only evaluated with samples of limited complexity (single proteins or mixtures of two to three proteins).

Unfortunately, at present little is known about the exact binding characteristics of the phosphate groups to these metal oxide materials. It is known that under acidic conditions, titania shows amphoteric ion-exchange properties depending on the pH-value of the solvent[12] and the adsorption of soluble phosphate to titania presumably occurs in a bidentate manner.[13] Different material properties may arise from variations of the manufacturing process of the oxides, such as the use of different calcination temperatures. This may in turn affect the binding properties, as exemplified by the different performance of commercially available TiO_2 materials compared in a recent study by Yates and co-workers.[14] Calcination of commercial TiO_2 spheres – thereby changing its crystalline properties – was recently found to increase the specificity of phosphopeptide enrichment on titania as reported by Ishihama and co-workers.[15] But even with these newly introduced metal oxide materials sufficient selectivity for the analysis of complex samples may not always be achievable and was found to be highly dependent on the solvents used for loading of the sample and during washing steps. Larsen et al.[5c] and Mazanek et al.[16] added dihydroxybenzoic acid (DHB) or DHB in combination with octanesulfonic acid to the loading buffer and achieved a remarkable gain in chemoselectivity, but these additives may complicate direct LC-MS analysis because residual DHB interferes with peptide detection. Less interference can be expected with the use of different aliphatic hydroxy acids as first reported by Sugiyama et al.[17]

Phosphate groups have been shown to have high affinity towards a number of metal oxides,[13] so it is desirable to test new porous metal oxide materials that may show even better chemoselectivity and phosphate binding properties at less stringent binding and release conditions. For this purpose, we evaluated porous tin oxide (SnO_2, stannia) as a novel oxide material for phosphopeptide enrichment. SnO_2, for example, has a different, slightly lower surface isoelectric point than the presently used oxides and may also differ in other properties that may influence phosphate affinity. In our experiments we used SnO_2 microspheres that are manufactured in a so called ''nanocasting'' (replication) process[18] allowing the convenient preparation of dedicated materials and tailoring of material properties like shape, particle diameter and pore size. Previously, the nanocasting method has successfully been used to prepare a series of mesoporous metal oxide powders,[19] and it is easier to control the morphology/porosity of the material with this technique than with the coacervation (polymer-induced colloid aggregation) process commonly used for the preparation of TiO_2[20] and ZrO_2.[21] Starting from porous silica particles as a ''negative'' template, the particles are first soaked in a metal salt solution, and metal oxides are formed *in situ* by evaporation of the solvent followed by the thermal decomposition of the metal salt and a calcination step. In the final step, the silica portion is leached from the particles by treatment with HF or NaOH solution. Nanocast stannia material obtained by the above-mentioned procedure was evaluated for its suitability for phosphopeptide enrichment, using a set of model peptides and protein digests. Our results demonstrated that SnO_2 is a promising phosphopeptide affinity material that can complement existing sorbents in the study of the phosphoproteome.

2. Results and Discussion

2.1. Structural and Chemical Properties of the Nanocast SnO_2 Spheres

A scanning electron microscopy (SEM) image of SnO_2 spheres replicated from 10 μm silica spheres is shown in Figure 1a. The spheres have a uniform shape and a narrow size distribution similar to the starting silica spheres (results not shown). For comparison, a SEM image of commercial TiO_2 spheres (Sachtopore NP), which is used as reference, is shown in Figure 1b. For the latter, we observed a slightly broader size distribution compared to the SnO_2 spheres. Furthermore, we have used the nitrogen physisorption technique to evaluate the intra-particular porosity of the spheres and the adsorption isotherms of the SnO_2 and TiO_2 microspheres are plotted in

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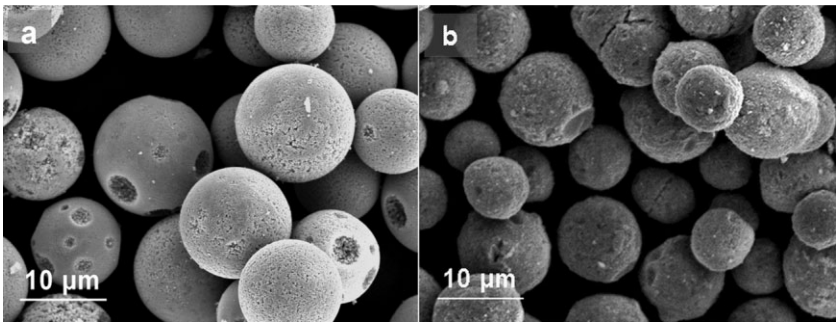

Figure 1. SEM images of a) tin dioxide microspheres prepared from 10 µm silica as starting material and b) Sachtopore TiO_2 spheres for comparison.

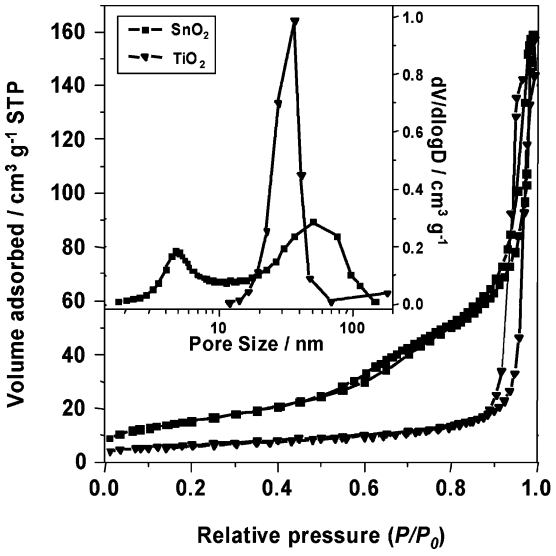

Figure 2. Nitrogen physisorption isotherms of the SnO_2 replica spheres (squares) and the Sachtopore TiO_2 spheres (triangles). Inset: The corresponding BJH pore size distribution plots derived from the desorption branch.

Figure 2. From the inset in Figure 2 it is evident that the SnO_2 replica spheres have a bimodal pore structure, while the Sachtopore TiO_2 spheres have a sharp monomodal mesopore size distribution. The data on the textural properties of the spheres obtained from the nitrogen physisorption measurements is summarized in Table 1. The SnO_2 spheres have a specific surface area of $55.9\,m^2\,g^{-1}$ and a pore volume of $0.19\,cm^3\,g^{-1}$, which is lower than for the starting silica template ($374\,m^2\,g^{-1}$ and $1.21\,cm^3\,g^{-1}$, respectively). For comparison, these values are $23.5\,m^2\,g^{-1}$ and $0.19\,cm^3\,g^{-1}$ for the commercial TiO_2 spheres. While the SiO_2 and the TiO_2 spheres have monomodal pore size distributions (centered around 10.7 nm and 32.1 nm, respectively), the SnO_2 replica spheres have a bimodal distribution of pores centered around 50.8 nm and 5.0 nm, and which comprise of about 73% and 23% of the total mesopore volume, respectively. The larger pore region consists of inter-aggregate pores and the smaller pore region is an inverse pore structure of the silica mesopores. A more complete structural study of the SnO_2 spheres has been published earlier.[18b] Electrokinetic titrations were conducted to shed some light on the differences in the surface chemistry of the various metal oxides used in this study and the results are presented in Figure 3. The isoelectric point (IEP), defined as the point at which the electrokinetic potential (ζ-potential) equals zero, is a measure of the acidity of the metal oxide. The IEP values (listed in Table 1) determined for the three oxides are 3.2 for SiO_2, 4.1 for SnO_2, and 5.0 for TiO_2, confirming the higher acidity of tin dioxide compared to titanium dioxide as reported previously.[22]

2.2. Optimizing a Phosphopeptide Enrichment Procedure for SnO_2 with Standard Peptides

The binding properties of tin dioxide as an affinity material were unknown. Thus, as a first step it was necessary to set up an enrichment protocol using a set of synthetic peptides with a focus on the optimization of loading solvent conditions (binding pH, organic solvent content, type of acid used) in order to obtain a method for the chemoselective binding of phosphopeptides in the presence of nonphosphorylated peptides. Throughout the study, enrichment experiments were performed in ''batch-mode'' by mixing the sample solutions with the metal oxide spheres in microcentrifuge tubes (Fig. 4). Unbound fractions were combined with the supernatants from washing steps to constitute the ''binding/washing'' fraction. Peptides bound to the material were eluted by a combination of high pH (10.5, adjusted with NH_4OH) and addition of inorganic phosphate ($(NH_4)_2HPO_4$) as a competitor for phosphate binding sites. These elution conditions even allowed the recovery of multiply phosphorylated peptides in high yield. Liquid chromatographic separation in combination with electrospray ionization mass spectrometry was then used as

Table 1. Textural properties of the spheres obtained from the nitrogen physisorption measurements together with the isoelectric points obtained from electrokinetic titrations.

Sample	BET surface area [$m^2\,g^{-1}$]	Pore volume [$cm\,g^{-1}$]	Pore diameter [nm]	IEP [pH]
SiO_2	374.0	1.205	10.7	3.2
SnO_2	55.9	0.188	5.0; 50.8	4.1
TiO_2	23.5	0.193	32.1	5.0

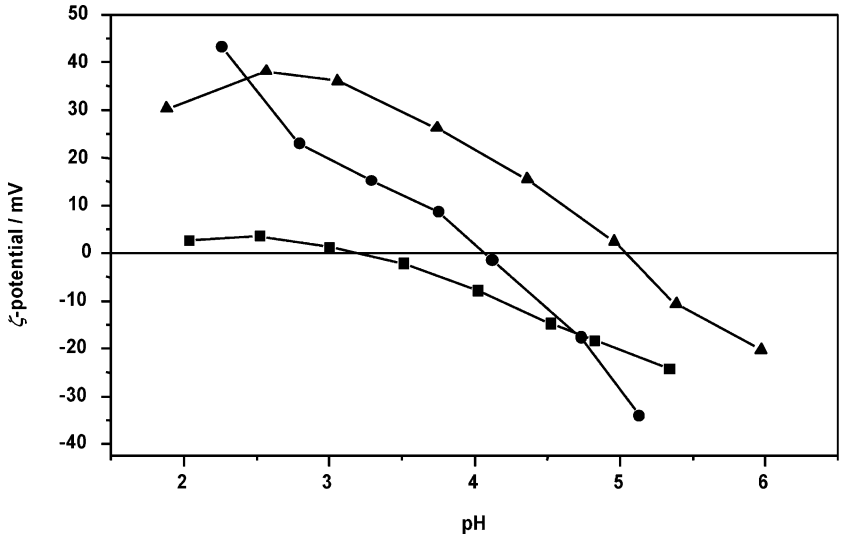

Figure 3. Electrokinetic titration curves for the starting SiO_2 spheres (squares), the SnO_2 replica spheres (circles), and for the Sachtopore TiO_2 spheres (triangles). The metal oxide spheres were titrated from their native pH to a pH value around 2.

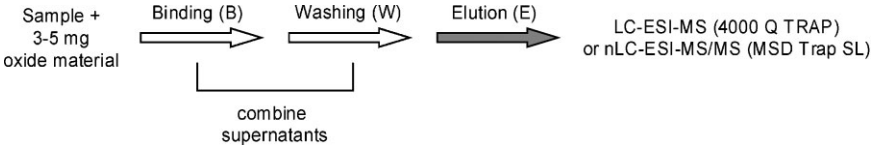

Figure 4. Workflow of the phosphopeptide enrichment protocol. Model peptides or protein digests were applied to the oxide materials in loading buffer, mixed for 30 min, washed twice with the same solvent and supernatants of the binding and washing steps were pooled. Bound phosphopeptides were eluted twice with elution buffer, and supernatant fractions were again pooled. Final analysis of the collected fractions was performed by LC-MS or -MS/MS as described in the Experimental Section.

an analysis platform to allow the sensitive and specific detection of the peptides.

2.2.1. Influence of pH and Content of Organic Solvent in the Loading Buffer on Selectivity and Recovery

As a first step towards optimizing a protocol for phosphopeptide enrichment with SnO_2 we chose a ''mild'' loading buffer composition to better allow the monitoring of possible differences in binding properties of peptides to SnO_2 and other metal oxides. For this reason we used formic acid in different concentrations.

Selected synthetic peptides (Table 2) were dissolved in various solvents differing in their pH and content of organic solvent (acetonitrile, ACN) and loaded onto the tin oxide material. The results show a strong pH-dependency of the binding properties of phosphopeptides to tin oxide, as already an increase from 0.1% to 0.5% formic acid noticeably reduced the binding of phosphopeptides to the metal oxide material. The influence of content of organic solvent was found to be less significant, but 50% acetonitrile showed the best compromise in selectivity and phosphopeptide recovery. Therefore we chose a solvent containing 50% ACN and 0.1% formic acid (FA) for further experiments.

To prove the competitiveness of the tin oxide material compared to already established metal oxide materials used for phosphopeptide enrichment and for the selection for a representative control during optimization, we evaluated titanium oxide and zirconium oxide in terms of their binding properties for selected phospho- and nonphosphopeptides (data not shown). Titanium oxide achieved better results than zirconia and was used as a control to compare selectivity and recovery rates for further experiments.

Synthetic peptides (Table 2) were applied to tin oxide and titanium oxide with the optimized solvent system and the enrichment procedure was performed as described in the Experimental section. The nonphosphorylated peptides chosen in this study deliberately represented ''sticky'' peptides with one or more acidic residues that should exhibit particularly strong nonspecific binding. Results showed a reduced tendency of certain very acidic nonphosphorylated peptides to bind to tin oxide compared to titanium oxide (the recovery rates of ''acidic'' unphosphorylated peptides in the elution fraction were lower by up to 50%) whereas the phosphopeptide recoveries were slightly reduced (around 10% lower compared to titania) in the case of tin oxide. These results allow the assumption that titania possesses a higher affinity to Lewis bases (i.e., in this case the phosphopeptides) than tin oxide, which might be an advantage because of higher recovery rates achievable for phosphopeptides, but also a disadvantage in the case of lower selectivities in the presence of acidic peptides.

Table 2. Amino acid sequences (in one-letter code) of the synthetic phospho- and nonphosphopeptides used in this study. Phosphorylated serine (pS), threonine (pT) and tyrosine (pY) residues are highlighted in bold.

Abbreviation	Sequence	M_r
PP1	N**pS**VEQGRRL	1137.5
PP2	SVENLPEAGI**pT**HEQR	1758.8
PP3	LIEDNE**pY**TAR	1302.5
PP4	QLGEPEK**pS**QDSSPVL**pS**ELK	2230.0
PP5	QLGEPEK**pS**QD**pS**SPVL**pS**ELK	2310.0
PP6	KFL**pS**LASNPELLNLPS**pS**VIK	2329.2
NPP1	RPPGFS (*internal standard*)	659.6
NPP2	WAGGDASGE	848.8
NPP3	RNIAEIIKDI	1184.4
NPP4	PTHIKWGD	953.1
NPP5	EGVNDNEEGFFSAR	1569.7

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Nonetheless, we observed that a complete removal of nonphosphorylated peptides in the elution fraction was not attainable with this simple experimental setup for both stannia and titania. Thus, the selectivity achieved in this first optimization step may not be adequate for more complex samples such as protein digests or cell extracts. The problem could be that the high abundance of nonphosphorylated peptides in such samples will prevent binding of phosphorylated peptides if the selectivity of the enrichment material is not good enough. Several groups have reported the effectiveness of hydroxy acids to reduce unspecific binding of acidic peptides to titanium oxide. In our study, we tested some α-hydroxy acids (lactic acid, tartaric acid, malic acid in a concentration of 300 mg ml^{-1}; according to Ref. [17a]) and 2,5-dihydroxybenzoic acid (300 mg ml^{-1}; Ref. [5c]) for both tin oxide and titanium oxide. Both protocols were previously optimized for a different TiO_2 material and reevaluation for the present titania spheres was necessary. The best selectivity and phosphopeptide recovery were obtained by titania with 0.1% trifluoroacetic acid (TFA) and 300 mg ml^{-1} of lactic acid (LA), in line with data reported by Sugiyama et al.,[17a] while for the SnO_2 material the use of hydroxy acids was not found to further improve specificity.

2.2.2. *Influence of pH and Type of Acid in the Solvent for Binding Properties of Enrichment Materials*

Despite favorable binding of phosphopeptides to SnO_2 even at relatively high pH, the lack of specificity led us to investigate the influence of the acidity of the loading solvent in more detail. It was expected that working at a lower pH would improve the discrimination between phosphopeptides and unphosphorylated, acidic peptides when the pH is lower than the pK_a of the carboxyl groups (~4). Thus, in accordance with results previously observed for other oxides, carboxylic acids should be present in protonated form to a large degree and not compete with phosphopeptides for binding.

In the next experiment we therefore compared the selectivity achieved by the use of different acids in varying concentrations for the tin oxide material. Figure 5 shows the recovery rates of unphosphorylated (NPP2-NPP5) and phosphorylated peptides (PP1-PP4, see Table 2) in the elution fraction after enrichment using tin oxide and different compositions of the loading solution. Solvents used were 50% ACN with 0.05% TFA, 0.1% TFA or 0.1% FA. It is evident that there is no sufficient selectivity achievable for a satisfactory phosphopeptide enrichment with formic acid, particularly for complex samples where the excess of acidic peptides is much higher than in this case: up to 80% of the unphosphorylated controls were retained under these conditions. The lowest recovery rates for nonphosphopeptides in the elution fraction (i.e., less than 3% of nonspecific binding) were obtained with 0.1% TFA. Considering the fact that in complex samples the excess of acidic peptides will be much higher than in this experiment, we decided to use 0.1% TFA in the loading solution instead of 0.05% TFA for further experiments, although higher phosphopeptide recovery rates would be

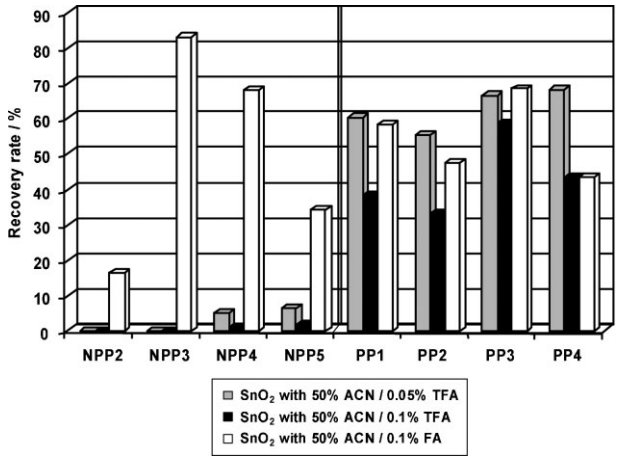

Figure 5. Recovery rates (relative to an unprocessed sample) of nonphosphorylated (NPP) and phosphorylated (PP) peptides after the phosphopeptide enrichment procedure with SnO_2 and different loading buffer compositions. ACN = acetonitrile, FA = formic acid, TFA = trifluoroacetic acid.

feasible with 0.05% TFA. In the following, we compared our protocol for SnO_2 to an optimized protocol for titania, as reported previously by Sugiyama et al.[17a] The percentage of peptides recovered in the elution fraction of peptides (relative to an unprocessed sample) was determined for three different experimental set-ups: SnO_2 and 0.1% TFA as additive in the loading solvent; TiO_2 with 0.1% TFA, or 0.1% TFA and 300 mg ml^{-1} LA as solvent. The results (shown in Fig. 6) reveal that the recovery rates of nonphosphopeptides are comparably low for SnO_2 and TiO_2 with lactic acid – indicating a sufficient reduction in nonspecific binding – while in the case of TiO_2 with TFA alone the selectivity was not satisfactory (e.g., 65% nonspecific binding for NPP5). The comparable performance of SnO_2 using TFA alone with an optimized TiO_2 method demonstrates that the optimized protocol for SnO_2 is able to

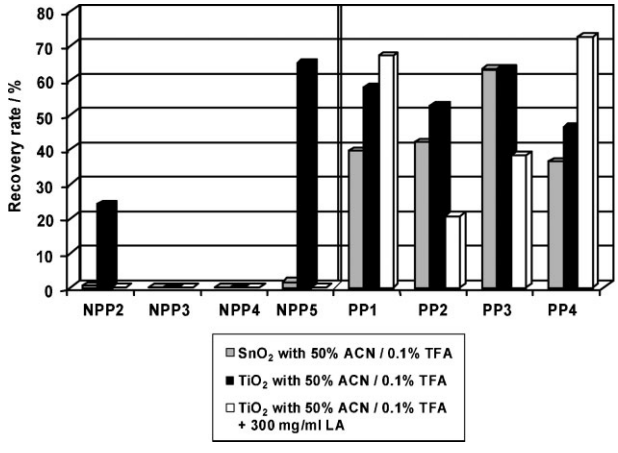

Figure 6. Recovery rates (relative to an unprocessed sample) of selected peptides after phosphopeptide enrichment using optimized procedures for SnO_2 and TiO_2. ACN = acetonitrile, TFA = trifluoroacetic acid, LA = lactic acid.

Table 3. Peptides and proteins identified from a tryptic digest of α-casein and fetuin with phosphopeptide enrichment using either SnO_2 or TiO_2, analysis by nanoLC-MS/MS and subsequent database search using Mascot. Shown are the numbers of identifications from six repeat analyses.

Protein	Peptide	Identified by SnO_2 enrichment[a]	Identified by TiO_2 enrichment[b]
α_{S1}-casein	YKVPQLEIVPN**pS**AEER	6 of 6 runs	6 of 6 runs
	VPQLEIVPN**pS**AEER	5/6	5/6
	HIQKEDVPSER	2/6	2/6
α_{S2}-casein	TVDME**pS**TEVFTKK	4/6	4/6
	NMAINP**pS**KENLCSTFCK	1/6	1/6
Fetuin	HTFSGVASVE**pS**SSGEAFLIVGK	2/6	2/6

[a] Using 50% ACN/0.1% TFA as loading buffer. [b] Using 50% ACN/0.1% TFA + 300 mg ml^{-1} lactic acid as loading buffer.

compete with other established enrichment materials and protocols, at least for model peptides. As a next step it was necessary to prove whether our material and protocol was applicable to more complex samples such as protein digests.

2.3. Tryptic Protein Digests

α-casein and fetuin represent highly phosphorylated proteins and are therefore often used as model proteins for phosphopeptide enrichment studies. We analyzed a tryptic digest of a mixture of α-casein (containing both α_{S1}- and α_{S2}-forms) and fetuin after a phosphopeptide enrichment step using the two optimized protocols for SnO_2 (50% ACN and 0.1% TFA as loading buffer) and TiO_2 (50% ACN/0.1% TFA + 300 mg ml^{-1} LA), respectively. The elution fractions recovered from the materials were analyzed by nanoflow LC-MS/MS as described in detail in the Experimental section.

For evaluation of the results, we decided to rely on the qualitative interpretation of database search results using the Mascot search engine, as this reflects a typical ''discovery-mode'' type of phosphopeptide profiling. Quantitative strategies in complex samples need to rely on stable isotope labeling or related approaches,[23] as (relative) quantitation based on extracted ion chromatograms exclusively cannot be expected to yield reliable results when comparing samples differing enormously in their complexity such as a whole protein digest and a phosphopeptide-enriched sample in the present case.

As Table 3 shows, the data obtained from the two materials were highly comparable. One phosphorylation site from α_{S1}-casein was identified in all analyses (three parallel enrichment procedures, two nanoLC-MS/MS runs per sample), in most cases with two peptides, one of which contained a missed cleavage site. A phosphopeptide from α_{S2}-casein was identified in four out of six runs, while a second phosphopeptide from this casein variant and a phosphopeptide from fetuin were only infrequently detected. This may be due to several reasons such as low phosphorylation stoichiometry, poor spectral quality or lower recovery from the enrichment procedure. For both the stannia and the titania material, only one peptide (from α_{S1}-casein) was found to bind nonspecifically under the conditions employed, and this peptide was identified in less than half of the analyses. While a tryptic digest of these phosphoproteins is expected to produce more than the phosphopeptides identified here, the reason for the inability of identifying further phosphopeptides may be the generally unfavorable fragmentation properties, the reliance on identification by MS/MS, not MS alone (thus requiring good spectral quality), and the low ionization efficiency of these peptides.

After these encouraging results, a tryptic digest of a ten protein mix was analyzed by nano-LC-MS/MS after phosphopeptide enrichment by both SnO_2 and TiO_2. Ovalbumin represented the only phosphorylated protein in the sample (containing two annotated phosphorylation sites) and ideally should be the only protein identifiable after the enrichment step. The results for this sample are shown in Table 4. With the

Table 4. Peptides and proteins identified from a tryptic digest of the ten protein mix with phosphopeptide enrichment using either SnO_2 or TiO_2, analysis by nanoLC-MS/MS and subsequent database search using Mascot. Shown are the numbers of identifications from four repeat analyses.

Protein[a]	Peptide	Identified by SnO_2 enrichment[b]	Identified by TiO_2 enrichment[c]
Ovalbumin, chicken	EVVG**pS**AEAGVDAASVSEER	4 of 4 runs	2 of 4 runs
	ISQAVHAAHAEINEAGR	0/4	2/4
α1-acid glycoprotein 1, human	EQLGEFYEALDCLR	1/4	0/4
Carbonic anhydrase, bovine	AVVQDPALKPLALVYGEATSR	0/4	3/4
β-lactoglobulin, bovine	TPEVDDEALEK	4/4	0/4
	TPEVDDEALEKFDK	4/4	0/4
	VYVEELKPTGDLEILLQK	2/4	0/4
Lactotransferrin, bovine	LCALCAGDDQGLDK	1/4	0/4
Lysozyme C, chicken	FESNFNTQATNR	4/4	0/4
	NTDGSTDYGILQINSR	4/4	0/4

[a] No peptides were identified from the following proteins: bovine κ-casein, α-lactalbumin, equine myoglobin and human transferrin. [b] Using 50% ACN/0.1% TFA as loading buffer. [c] Using 50% ACN/0.1% TFA + 300 mg ml^{-1} lactic acid as loading buffer.

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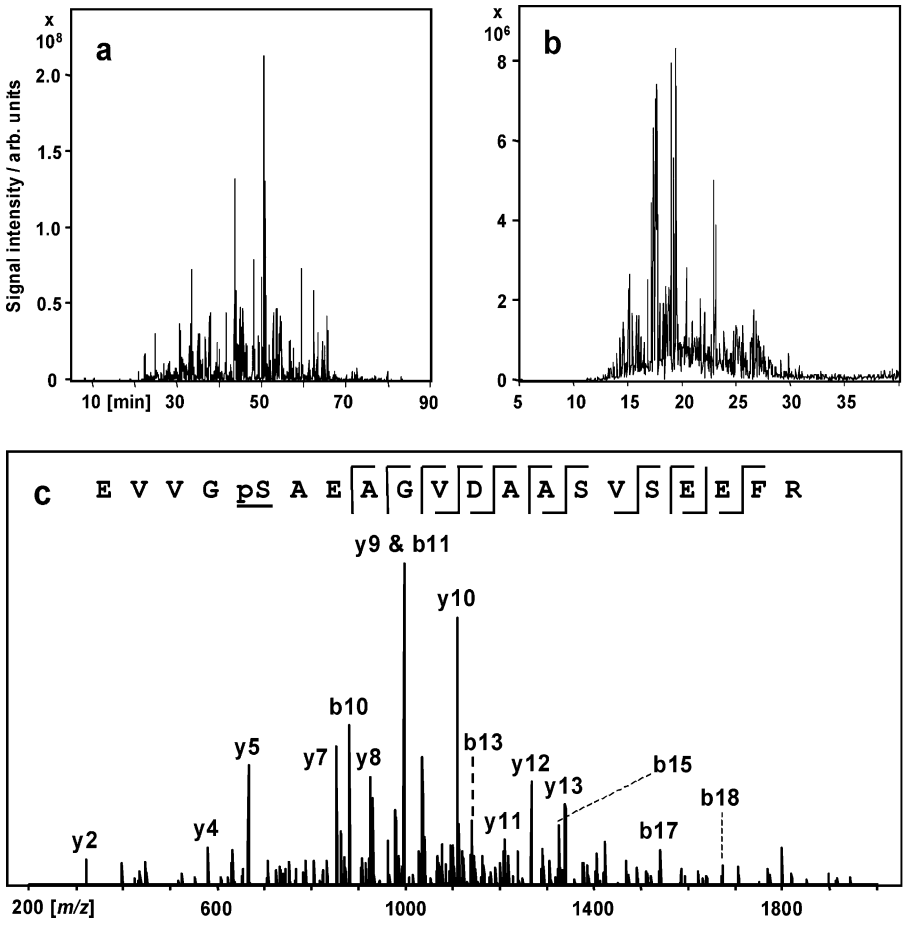

Figure 7. a) and b) Total ion chromatogram of a nanoLC-MS/MS run of the tryptic digest of the ten protein mix (a) before and (b) after an enrichment step using SnO_2 microspheres and 50% acetonitrile/0.1% trifluoroacetic acid as loading buffer. c) shows the annotated MS/MS spectrum of the phosphopeptide identified from ovalbumin (EVVG**pS**AEAGVDAASVSEEFR). Peptide identification was carried out by Mascot as described in the Experimental section.

optimized SnO_2 protocol it was possible to identify ovalbumin via one of its phosphopeptides in every single LC-MS/MS analysis of duplicate runs from two separately processed samples. As an example, Figure 7a and b depicts the MS^2 total ion chromatograms of a nanoLC-MS/MS analysis of the ten protein mix before and after phosphopeptide enrichment with stannia. It is apparent that a significant reduction of matrix complexity was achieved, as the intensity of the most abundant peak is reduced by a factor of ~25 (note that an extended gradient for the unprocessed sample was used to allow a more accurate comparison of the results). The annotated MS/MS spectrum of the phosphopeptide EVVG**pS**AEAGVDAASVSEEFR after the respective enrichment step is shown in Figure 7c.

In the case of stannia, four other proteins (β-lactoglobulin and lysozyme C in all four analyses, lactotransferrin and α_1-acid glycoprotein 1 in one analysis) were also identified with up to two nonphosphopeptides. For enrichment with titania the selectivity appeared to be better, because only in three cases one unphosphorylated protein (carbonic anhydrase 2) was identified. However, the identification of ovalbumin via its phosphopeptides succeeded in only two of four attempts, indicating that the use of lactic acid as a displacer in the loading solvent for the titania material also causes significant losses of phosphopeptides during loading and washing. To demonstrate the degree of enrichment, we analyzed the ten protein digest also without an enrichment step. In this experiment all ten proteins could be identified, and in the case of ovalbumin, no phosphopeptides but eight unphosphorylated peptides containing up to five acidic amino acid residues per peptide were identified, whereas in the enriched sample only the phosphopeptide could be identified. This is a further indicator for the specificity of the phosphopeptide enrichment step using SnO_2 microspheres.

By using the simple argumentation that the total surface area of the employed spheres in the enrichment experiments is enormous compared to what is needed to completely adsorb the phosphopeptides (about 1 million times larger), we conclude that the surface area of the spheres is not the decisive factor for the good performance observed for the SnO_2 material. Furthermore, since we also have smaller pores in the SnO_2 replica spheres compared to the commercial TiO_2, it is more likely that this is limiting rather than promoting the accessibility of the SnO_2 surface to some degree, because larger peptides entering the 5 nm pores is not favored. Therefore, we can presume that the variations in the phosphopeptide enrichment behavior observed between the studied materials are mainly consequences of the surface chemistry rather than the morphology.

3. Conclusions

We have shown that porous tin oxide microspheres represent a valuable new material for phosphopeptide enrichment purposes. Using an optimized protocol, comparable selectivity to titania was achievable, with the advantage that no solvent additives in high concentrations are necessary to gain sufficient selectivity. This minimizes the risk of problems with nanoscale HPLC columns and/or nanoelectrospray in the downstream analysis and should lead to a more robust analytical platform for phosphoproteomic studies. The

microspheres can be manufactured using readily available starting materials, and their properties (thermal, physical, and chemical stability) make them an interesting alternative to presently used material. Further studies are currently ongoing to test the performance of the SnO_2 replicas on more complex biological samples. Moreover, the flexibility of their preparation will allow the in-depth study of factors influencing the phosphate group affinity that will help in the design of improved metal oxide affinity materials.

4. Experimental

Metal Oxide Materials: Porous tin oxide microspheres were prepared according to the protocol of Smått et al. [18b], starting from Daisogel silica particles (Daiso Co. Ltd., Osaka, Japan) with a particle size of 10 μm and 120 Å pore size. Briefly, the silica spheres were impregnated with an excess amount of an aqueous $SnCl_2$ solution (1.8 g mL^{-1}, Riedel-de Haën, Seelze, Germany). After an impregnation time of 1 h, centrifugation was used to remove the excess precursor solution. The samples were then directly heated at 150 °C (3 h) to remove the solvent and further heated up to 250 °C (3 h) and 550 °C (5 h) in order to decompose the precursor salt to SnO_2. In a final step, the silica portion was removed by leaching 2 times in aqueous NaOH (2 M) at 90 °C, and the obtained spheres were dried in vacuo. Prior to use, the SnO_2 material was treated with aqueous HCl (0.1 M) for 24 h under reflux for activation, followed by drying in vacuo. Titania (Sachtopore NP titanium dioxide, particle size 10 μm/pore size 300 Å) and zirconia (ZirChrom-PHASE zirconium dioxide, particle size 10 μm/pore size 300 Å) were obtained from ZirChrom Separations, Inc. (Anoka, MN, USA) and used as received.

Characterization of the SnO_2 and Reference Spheres: The sphere morphology was studied using SEM (Jeol JSM-6335F, Jeol Ltd., Japan). The surface area, mesopore dimensions and the mesopore volumes were determined by nitrogen physisorption measurements at 77 K (ASAP 2010, Micromeritics Co., Norcross, GA). The total mesopore volume was taken at 0.98 P/P0 and the BJH model (desorption branch) was used for pore size calculations. Electrokinetic titrations to obtain the IEP were performed on a Malvern ZetaSizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK) coupled to a MPT-2 titrator unit. The zeta potential was measured as a function of pH by titrating with aqueous HCl (0.1 M) at 25 °C. The data was monitored and analyzed using the Malvern Dispersion Technology software v. 4.20.

Peptides and Proteins: Synthetic unphosphorylated peptides were obtained from Bachem (Weil am Rhein, Germany), while phosphopeptides were prepared by standard Fmoc solid phase chemistry and kindly provided by K. Mechtler from the Institute of Molecular Pathology (IMP, Vienna, Austria). All peptides used in the study are listed in Table 1. Model proteins used in our experiments were obtained from Sigma–Aldrich (Deisenhofen, Germany).

Tryptic digest of standard phosphoproteins: A total of one milligram of the respective proteins was dissolved in aqueous urea solution (8 M). Disulfide bonds were reduced using dithiothreitol (10 mM, 55 °C, 60 min) followed by alkylation of free thiol groups with iodoacetamide (50 mM, room temperature, 90 min) according to standard procedures. Samples were diluted 1 to 10 with aqueous NH_4HCO_3 (50 mm), and trypsin (proteomics grade, Sigma) was added at an enzyme-to-substrate ratio of approximately 1:25. Samples were digested overnight at 37 °C and purified by solid-phase extraction (C_{18} material) prior to evaporation under a stream of nitrogen.

Enrichment of Phosphopeptides: In brief, the workflow is shown in Figure 4. Different phosphorylated and nonphosphorylated peptides (see Table 1) were used in a quantity of 300 ng (amounting to approx. 200 fmol) per peptide per sample for an enrichment experiment. In the case of tryptic digests, 20 μg of starting material for the digest of casein and fetuin and 50 μg for the ten protein digest were applied.

Approximately 4 mg of oxide material (SnO_2, TiO_2, or ZrO_2) was weighed in 1.5 ml microcentrifuge tubes and equilibrated for 10 min with 100 μl of the respective solvent (as noted in the Results and Discussion section) prior to loading of the samples. After equilibration the suspension was centrifuged and the supernatant was discarded. Peptide samples diluted in loading solvent (100 μL) were applied to the oxide material and incubated for 30 min, and after centrifugation and removal of the supernatant the materials were washed twice for 10 minutes with loading buffer (100 μL). When hydroxy acids were used as additives the hydroxy acid was omitted in the second washing step to avoid carry-over into the elution fraction. The supernatants of the binding and two washing steps were pooled and stored for later MS analysis. For elution of bound phosphopeptides, the material was incubated twice with elution buffer (100 μL of 50 mM of $(NH_4)_2HPO_4$ adjusted to pH 10.5 with NH_4OH) for 10 min and supernatants were again pooled for analysis. All steps (equilibration, binding, washing, eluting) were carried out on an IKA MS3 digital minishaker (IKA, Staufen, Germany) to keep the metal oxides in suspension during the procedure.

Pooled samples were dried in a nitrogen stream and redissolved in water/acetonitrile/formic acid (95:5:0.5, v/v/v) prior to LC-MS analysis as described below. All enrichment experiments were performed at least in duplicate, and all collected fractions were analyzed twice by LC-MS.

LC-MS System: For the analysis and quantification of the standard peptides after the enrichment procedure we used an Agilent 1200 HPLC system coupled with an Applied Biosystems 4000 Q TRAP (triple quadrupole linear ion trap hybrid) mass spectrometer equipped with a Turbo V electrospray ion source (Applied Biosystems, Foster City, CA, USA). For gradient LC separation a Thermo BetaBasic-18 reversed phase column (150 × 2.1 mm; particle size 3 μm; Thermo Scientific, Runcorn, UK) was used with a flow rate of 200 μL min^{-1}. The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A gradient elution, starting with 5% to 50% B in 30 min, followed by 50% to 80% B in 5 min, 80% B for 5 min and reequilibration of the column with 5% of solvent B for 10 minutes was employed for reversed phase separation of the peptides. The mass spectrometer was operated in positive enhanced MS scan (EMS) mode where the third quadrupole is operated as a linear ion trap. The *m/z*-range was set from 300 to 2000 with a scan rate of 4000 per second. Data acquisition and analysis was performed with Analyst software, version 1.4.2 (Applied Biosystems).

For peptide quantification, an internal standard (300 ng of bradykinin fragment 1–6, Table 1) was added to the sample after the phosphopeptide enrichment procedure to correct for signal variabilities. The recovery rates were calculated as a quotient of the corrected peptide signal of the peptide in the elution fraction and the corrected peptide signal of the corresponding peptide in an untreated sample (100%, no enrichment).

Nanoflow LC-MS/MS System: Protein digests were analyzed using an Agilent Nanoflow Proteomics Solution in data-dependent MS/MS mode essentially as described previously [24]. An Agilent Zorbax 300 SB-C18 column (50 mm × 75 μm i.d.) was used for all samples with the exception of the unprocessed 10 protein mix, which was analyzed on a 150 mm column to avoid undersampling due to the higher sample complexity. Peak lists generated by the instrument software (LC/MSD Trap software 4.2, Bruker Daltonik, Bremen, Germany) were exported in Mascot-MGF format and searched against the UniProtKB/SwissProt database (release 47.5, dated 19/07/05, 186882 entries) using an in-house Mascot server running version 2.0.5 (Matrix Science Ltd., London, UK). Search parameters were set as follows: Taxonomy = chordata (vertebrates and relatives), enzyme = trypsin, fixed modifications = carbamidomethylation on Cys, variable modifications = phosphorylation on serine or threonine, peptide tolerance = ±1.5 Da, MS/MS tolerance = ±0.8 Da, peptide charge = 2+/3+, instrument = ESI trap. Peptides were considered identified if they were

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assigned to the main proteins in the sample (excluding possible low-level contaminants) and the ions score reported by Mascot was at least 20.

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Optimizing the performance of tin dioxide microspheres for phosphopeptide enrichment

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ABSTRACT

Phosphopeptide enrichment based on metal oxide affinity chromatography is one of the most powerful tools for studying protein phosphorylation on a large scale. To complement existing metal oxide sorbents, we have recently introduced tin dioxide as a promising alternative. The preparation of SnO_2 microspheres by the nanocasting technique, using silica of different morphology as a template, offers a strategy to prepare materials that vary in their particle size and their porosity. Here, we demonstrate how such stannia materials can be successfully generated and their properties fine-tuned in order to obtain an optimized phosphopeptide enrichment material. We combined data from liquid chromatography–mass spectrometry experiments and physicochemical characterization, including nitrogen physisorption and energy-dispersive X-ray spectroscopy (EDX), to explain the influence of the various experimental parameters.

1. Introduction

The central role of protein phosphorylation in biological signaling processes has been the driving force for the development and advancement of methods for the characterization of this post-translational modification in biological samples. Recent years have shown a notable increase in the number of analytical tools available to study phosphorylation events on a large scale [1–5]. These methods for "phosphoproteomics" have complemented previously available tools such as phosphospecific antibodies and immobilized metal affinity chromatography (IMAC) and include methods based on metal oxide affinity chromatography (MOAC, see below), ion exchange chromatography [6–9] as well as methods involving chemical modification reactions [10,11].

MOAC has arguably been the most widely adopted strategy in recent years and has yielded very promising results. While the affinity of inorganic phosphates to oxides such as titanium dioxides had been demonstrated earlier [12], TiO_2 was introduced for the purpose of phosphopeptide enrichment only in the beginning of this decade [13–15]. This was followed by reports that other oxides are suitable for the same purpose as well, including zirconium [16], aluminum [17], gallium [18], cerium [19], tin [20] and niobium [21] oxides. Titania is currently the most widely used oxide material and has been applied in a number of large scale studies, allowing the identification of up to thousands of phosphorylation sites in complex biological samples. For example, Mann and co-workers used two-dimensional phosphopeptide enrichment based on strong cation exchange and titanium dioxide fractionation to study signaling dynamics in HeLa cells upon epidermal growth factor stimulation, identifying approximately 6600 phosphorylation sites in the study [22]. The use of two complementary fragmentation techniques, collision-induced dissociation and electron transfer dissociation, in combination with TiO_2-based MOAC allowed the identification of nearly 1500 phosphorylation sites in human embryonic kidney 293T cells [23], as shown by Molina et al. and Heck and co-workers developed a titania-based on-line enrichment strategy and profiled 2152 phosphopeptides from *Drosophila melanogaster* cell lysate [24]. Using both TiO_2 and ZrO_2 material, and IMAC as complementary methods for enrichment, Sugiyama et al. characterized 2172 phosphorylation sites in *Arabidopsis* [25].

Despite the successful implementation of MOAC, it is evident that there is little detailed knowledge available on the intricacies of the binding mechanism of phosphopeptides to metal oxides.

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Fundamental studies carried out on small model compounds may not adequately reflect the binding behavior of polyfunctional biomolecules. It is well known that unphosphorylated peptides carrying acidic residues also bind to metal oxides to some degree, and this binding may be reduced by the addition of different additives/displacers in the solution used during the binding step (see below). While nonspecific binding of acidic peptides has been attributed to a bidendate binding of their carboxylic groups on the surface of the metal oxide, this model does not offer a full explanation for the observed interactions. Presently, a more systematic study of the influence of different material properties of MOAC sorbents is limited by the availability of the respective materials, although there are different parameters that are expected to contribute to the enrichment process. For example, pore size distribution and total surface area of the particles will influence the capacity of the material. Also, different MOAC materials may not always be chemically homogeneous as is evident from differences in performance of titania materials from different manufacturers [26] or with different crystallinity [27].

Recently, we have evaluated porous tin dioxide (SnO_2, stannia) as an alternative material for phosphopeptide enrichment by MOAC [20]. We could not only demonstrate that stannia gives comparable or even superior results than titania when used under the same conditions, but the material is also produced by a strategy that allows considerable flexibility in material design. The so-called "nanocasting" process [28,29] uses porous silica spheres as a template for the generation of metal oxide replicas in the pores of the silica material. Thus, it offers a straightforward preparation of metal oxide spheres of different particle size and type of porosity. Here, we take advantage of the flexibility of the nanocasting process to prepare different stannia materials and study the factors affecting the performance of this novel phosphopeptide affinity material.

2. Experimental

2.1. Preparation of porous tin dioxide microspheres by nanocasting

SnO_2 materials were prepared according to the nanocasting method described previously [30] from different silica templates. These included SiO_2 spheres with different particle sizes (5, 10, 20 μm) and porosities (120, 200, 300, 1000 Å nominal pore size). Typically, the nanocasting process involves impregnation of the porous silica spheres with $SnCl_2$ solution, followed by decomposition of the salt to SnO_2 by heat treatments in air, and finally removal of the silica portion by leaching in either HF or NaOH solutions. An overview of the different materials prepared and evaluated in this study is given in Table 1.

For hydrochloric acid activation, larger amounts (>500 mg) of tin dioxide materials were suspended in 0.1 M aqueous HCl and refluxed overnight (approximately 16 h) with overhead stirring. The spheres were collected by filtration, thoroughly washed with water and dried in vacuo for up to two days at 60 °C. Alternatively, smaller batches of material (~100 mg) were weighed in 1.5 mL microcentrifuge tubes (Eppendorf Safe-Lock), 1.5 mL 0.1 M aqueous HCl was added and the tightly sealed tubes were placed in an Eppendorf Thermomixer set to 95 °C. After overnight shaking at 1400 rpm, samples were thoroughly washed and dried as above.

2.2. Commercially available porous metal oxides

For comparison purposes, the following materials were used: GL Sciences Titansphere TiO_2 (10 μm particle size, 80 Å nominal pore size; MZ Analysentechnik, Mainz, Germany), Sachtopore NP TiO_2 (10 μm/300 Å; ZirChrom, Anoka, MN, USA) and ZirChrom-PHASE ZrO_2 (10 μm/300 Å; ZirChrom).

2.3. Physicochemical characterization

The size and the morphology of the tin dioxide spheres were studied using scanning electron microscopy (SEM) with a Jeol JSM-6335F (Jeol Ltd., Tokyo, Japan) and prior to the analysis the samples were sputtered with platinum. The surface areas, mesopore dimensions and mesopore volumes were determined by nitrogen physisorption measurements at 77 K (ASAP 2010, Micromeritics Co., Norcross, GA, USA). The total mesopore volume was acquired at 0.98 P/P_0, while the BJH model based on the desorption branch was used in the pore size calculations. For the elemental analysis, both a Link Inca 300 (Oxford Instruments, Abingdon, UK) energy-dispersive X-ray spectroscopy (EDX) instrument and a Quantum 2000 (Physical Electronics, Chanhassen, MN, USA) X-ray photoelectron spectroscopy (XPS) instrument were used. The XPS instrument was equipped with a monochromatic AlKα X-ray source and operated at 25 W with a spot diameter of 100 μm.

2.4. Phosphopeptide enrichment by metal oxide affinity chromatography

The enrichment procedure was performed essentially as described previously [20]. Peptide mixtures (1 μg per peptide, see Table 2) were dissolved in 100 μL of 50% CH_3CN, 0.1% TFA in water and added to microcentrifuge tubes containing 4.5 ± 0.2 mg metal oxide beads that had been prewashed with 200 μL of the same solution. After incubation for 30 min on the thermomixer (1400 rpm, room temperature), the beads were settled by centrifugation and the supernatant was recovered. The beads were then washed twice

Table 1
Overview of the materials used in this study.

Sample code	Starting material	Number of impregnation steps	Leaching agent	Average pore size of SnO_2 replica (Å)	Surface area of SnO_2 replica ($m^2\ g^{-1}$)
SnO_2 120-5-1-NaOH	Daisogel 5 μm, 120 Å average pore size (Daiso Co. Ltd., Osaka, Japan)	1	NaOH	50; 520	55.9
SnO_2 120-10-1-HF	Daiso 10 μm, 120 Å average pore size	1	HF	54; 540	53.4
SnO_2 120-10-1-HF *HCl-treated*	Daiso 10 μm, 120 Å average pore size	1	HF, HCl-treated	52; 620	46.4
SnO_2 120-10-1-NaOH	Daiso 10 μm, 120 Å average pore size	1	NaOH	49; 530	55.9
SnO_2 120-10-1-NaOH *HCl-treated*	Daiso 10 μm, 120 Å average pore size	1	NaOH, HCl-treated	48; 580	48.7
SnO_2 120-10-2-NaOH	Daiso 10 μm, 120 Å average pore size	2	NaOH	51; 490	52.8
SnO_2 120-10-3-NaOH	Daiso 10 μm, 120 Å average pore size	3	NaOH	52; 540	50.4
SnO_2 120-20-1-NaOH	Daiso 20 μm, 120 Å average pore size	1	NaOH	52; 400	62.1
SnO_2 200-5-2-NaOH	Daiso 5 μm, 200 Å average pore size	2	NaOH	100; 500	44.7
SnO_2 300-10-2-NaOH	Kromasil 10 μm, 300 Å average pore size (Eka Chemicals, Bohus, Sweden)	2	NaOH	500 (monomodal)	40.3
SnO_2 1000-10-2-NaOH	Daiso 10 μm, 1000 Å average pore size	2	NaOH	530 (monomodal)	18.3

Table 2
Overview of the synthetic peptides used in this study, and their typical retention times in HPLC. Phosphorylated amino acid residues are highlighted in bold.

Sequence	M_r	t_R (min)
Mix 1		
WAGGDASGE	848.3	16.4
RNIAEIIKDI	1183.7	27.0
PTHIKWGD	952.5	19.3
EGVNDNEEGFFSAR	1569.7	23.8
N**pS**VEQGRRL	1137.5	16.2
SVENLPEAGl**pT**HEQR	1758.8	20.3
LIEDNE**pY**TAR	1302.5	17.3
QLGEPEK**pS**QDSSPVL**pS**ELK	2230.0	24.1
Mix 2		
WWGSGPSGSGGSGGGK (P0)	1419.6	21.7
WWGSGPSGSGG**pS**GGGK (P1)	1499.6	22.4
WWGSGPSG**pS**GG**pS**GGGK (P2)	1579.5	22.9
WWGSGP**pS**G**pS**GG**pS**GGGK (P3)	1659.5	22.8
WWG**pS**GP**pS**G**pS**GG**pS**GGGK (P4)	1739.5	21.8
Internal standard		
DRVYIHPF	1045.5	23.6

with 100 μL of the same solvent and the supernatants combined with the first solution. Elution was performed by incubating the beads two times for 15 min with 100 μL of a solution containing 50 mM $(NH_4)_2HPO_4$, adjusted to pH 10.5 by addition of ammonia solution (25% in water). Again, the supernatants from the two elution steps were combined. All experiments were performed in triplicate.

All collected fractions (binding/washing and elution) were then evaporated to dryness on a SpeedVac vacuum concentrator (ThermoFisher Scientific, Waltham, MA, USA) and stored at −20 °C prior to LC–MS analysis.

2.5. Liquid chromatography–mass spectrometry

Samples were reconstituted in 200 μL of an aqueous solution containing 5 μg mL^{-1} angiotensin II (Bachem) as internal standard. 60 μL each of this solution was used for three separate LC–MS analyses.

Peptides were separated on an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a Thermo BetaBasic-18 column (150 mm × 2.1 mm) at a flow rate of 200 μL min^{-1} at 25 °C. Gradient elution was performed using solvent A = 0.1% formic acid in water and solvent B = 0.1% formic acid in acetonitrile and the following gradient table: 0–5 min, 5%B; 5–35 min, 5–50%B; 35–40 min, 50–80%B, followed by reequilibration at 5%B.

The eluent from HPLC was introduced into the TurboV electrospray ion source of an Applied Biosystems 4000 Q TRAP triple quadrupole linear ion trap instrument (Applied Biosystems, Foster City, CA, USA). Detection was performed in enhanced MS (linear ion trap) scan mode using the following settings: Scan range, m/z 400–1700 (Mix 1) or 500–900 (Mix 2); scan speed, 4000 amu s^{-1}; positive ionization, voltage 4000 V; declustering potential, 50 V.

2.6. Data analysis

For quantitative analysis, extracted ion chromatograms were obtained for the one or two most abundant charge states of every peptide using a ±0.5 m/z window. Peak heights were used for further evaluation, as they were found to provide more reliable results than peak areas in preliminary experiments (data not shown). Material characteristics were compared based on two parameters defined as follows: Specificity = peak height in the elution fraction divided by combined peak heights in the binding/washing and elution fractions (ideally 100% for phosphopeptides and 0% for unphosphorylated controls), and Recovery = peak height in elution fraction divided by peak height of an unprocessed control. Statistical significances between materials were determined by one-way analysis of variance (ANOVA) performed using Microsoft Excel.

3. Results and discussion

3.1. Design of the study

While the general suitability of porous stannia microspheres for phosphopeptide enrichment has been described previously [20], the manufacturing process of this material allows to study in detail the influence of different material properties on performance. For this purpose, a set of SnO_2 spheres were prepared that differed in the particle and pore size of the starting material (silica), the number of impregnation steps, the process used for leaching out the silica (using hydrofluoric acid versus sodium hydroxide) and the use of an additional acid treatment step of the final material. For example, in the SEM images in Fig. 1(a) and (b), silica spheres with mean sizes of 5 μm and 10 μm have been used as templates for the preparation of differently sized SnO_2 spheres. In the insets clear differences in the interior structure of the replica spheres depending on the starting silica materials can be observed. In Fig. 1(a), one can see primary particle aggregates which give rise to the interaggregate porosity observed at ~500 Å typical for all samples. Generally the smallest mode of pores is not detectable by SEM. However, in the case of sample SnO_2 1000-10-2-NaOH, which was replicated

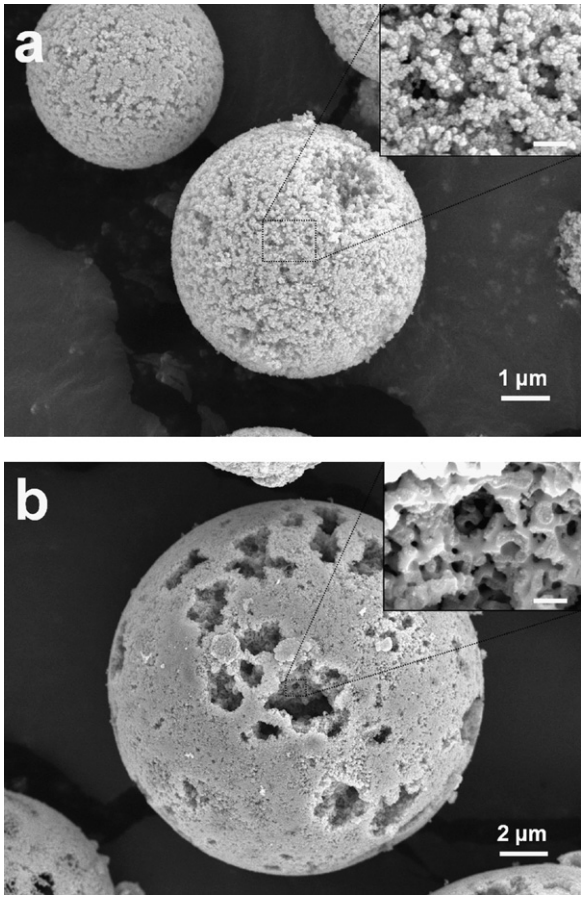

Fig. 1. Examples of SEM images of nanocast tin dioxide materials prepared from different silica materials: (a) Sample SnO_2 200-5-2-NaOH prepared from 5 μm sized silica spheres (pore size: 200 Å) and (b) sample SnO_2 1000-10-2-NaOH prepared from 10 μm spheres (pore size: 1000 Å). Insets show details of the textural properties of the interior of the replica spheres. Scale bar for the insets equals 200 nm.

from silica spheres with a pore size of 1000 Å, it can be observed (inset of Fig. 1(b)).

Regardless of the starting material used, all replicas were prepared successfully; details about the materials used in the present study can be found in Table 1. With the exception of materials prepared from silica with higher porosities, average pore sizes and surface areas are very similar.

For practical applications in phosphoproteomics, two properties play a major role for the end user of the affinity material, namely specificity and recovery. The discrimination between phosphopeptides and unphosphorylated, but frequently highly acidic peptides carrying several aspartic or glutamic acid residues remains a challenge for different phosphopeptide enrichment strategies. The problem of nonspecific binding has been well documented for IMAC-based protocols and has been addressed by methyl esterification of the carboxyl groups [31]. While the effects have not been as dramatic in the case of metal oxide affinity chromatography, some type of ion exchange or acid-base interaction mechanism appears to play a role also in this case, resulting in the binding of unphosphorylated peptides. To overcome this, a number of solvent additives have been proposed in the literature to reduce nonspecific interactions, these include dihydroxybenzoic acid [15], phthalic acid [11], aliphatic hydroxy acids such as lactic acid [32], glutamic acid [33] or its ammonium salt [34]. Previously, we have shown that tin dioxide appears to exhibit intrinsically less nonspecific binding than titanium dioxide [20] so that these additives are not required in the enrichment protocol for mixtures of moderate complexity.

The second important issue is the recovery of phosphopeptides from different affinity materials. Previous studies have shown that an experimental bias may exist towards the enrichment of polyphosphorylated peptides. Obviously, their interaction with the sorbent is much stronger and can lead to two scenarios: multiply phosphorylated peptides may be over represented when washing conditions are relatively harsh (or the binding to the material inherently weak) so that a considerable number of singly phosphorylated peptides are removed along with nonspecific binders. On the other hand, when mild elution conditions are used for desorption, recovery of multiply phosphorylated peptides may be compromised. Different elution conditions from metal oxides have been used in the literature, and we have obtained the most satisfying results with a combination of high pH and the addition of inorganic phosphate to maximize the yield of phosphopeptides. However, intrinsic material properties may still limit the achievable recoveries for a given material.

In order to systematically evaluate tin dioxide as an MOAC sorbent, we chose a set of synthetic peptides listed in Table 2. Most of the work described herein was carried out using Mix 1 containing four singly or doubly phosphorylated model peptides along with four unphosphorylated controls. Additional experiments were performed on a set of peptides with identical amino acid sequence that differed only in the number of phosphorylation sites, ranging from zero to four (Mix 2). We have previously demonstrated that the general enrichment procedure works well also in more complex samples [20], so the composition of the two sample mixtures is ideal for a focused study on material-related effects.

LC–MS analysis was optimized to achieve highly reproducible results in order to allow minor differences between the materials to be observed. Thus, we favored the use of conventional HPLC column diameters (2 mm I.D.) because of the increased robustness in terms of signal stability. Additionally, the peptide amounts used for the enrichment experiments, in the range of several hundred pmols, reduce the effects of any external factors such as adsorption on surfaces that may be considerable for analyte amounts several orders of magnitude lower [35,36]. Initial experiments using standard mixtures revealed that the chosen set-up including detection in the linear ion trap mode of the Q TRAP instrument allowed us to obtain relative standard deviations of peak heights in the range of ~1% which was also the case throughout the further study. To ensure reliability of the data, three experimental replicates (individual enrichment procedures) were performed for all materials, and bound and unbound fractions were each analyzed in triplicate by LC–MS.

Typical retention times for all peptides are given in Table 2. Interestingly, the retention times of Mix 2 do not correspond to the widespread assumption that retention time in reversed-phase chromatography decreases with an increase in phosphorylation: actually, the doubly phosphorylated form of the peptide is most strongly retained, while the unphosphorylated and the quadruply phosphorylated form showed the least retention for the chromatographic system chosen. The retention behavior is most likely influenced by different factors such as the mobile phase system used, the activity of residual silanol groups on the stationary phase and the presence of interactions within the peptide chain (intramolecular ion pairing) that affect the interaction of hydrophobic regions with the stationary phase.

3.2. Material properties influencing phosphopeptide enrichment specificity

We have previously observed that when tin dioxide replicas were prepared using different leaching agents (sodium hydroxide versus hydrofluoric acid), their specificity differed somewhat, and NaOH-leached SnO_2 appeared to have higher specificity towards phosphopeptides. Moreover, we observed that when the spheres were refluxed overnight in diluted hydrochloric acid, an additional increase in performance was obtained, although the reason for this was not clear. In the present, systematic study, we were able to confirm these results. For this purpose, tin dioxide replicas were prepared from a single starting material, 10 μm silica particles with a nominal pore size of 120 Å, and part of the spheres was treated with either NaOH or HF to remove the silica portion. Additionally, part of the NaOH- and HF-leached spheres was treated with 0.1 M HCl, as described in the Section 2. All four materials were evaluated using the previously established protocol, and selected results are shown in Fig. 2. From these data, it is evident that the amount of nonspecific binding of unphosphorylated peptides noticeably differs between the differently prepared spheres, and it was found to be lowest for the NaOH- and HCl-treated spheres.

For example, EGVNDNEEGFFSAR (Glu^1-fibrinopeptide B), exhibited the strongest binding among the unphosphorylated peptides, which is due to the highly acidic character of the peptide (four acidic residues, pI = 4.00). For SnO_2 particles that were not treated by HCl, 37% and 14% of the total amount were bound to the affinity material under the conditions chosen for the HF- and the NaOH-leached spheres, respectively. This binding was reduced to values of 15% and 9% for the HCl-treated samples, showing a dramatic improvement in specificity. At the same time, the amount of singly phosphorylated peptides that bound to SnO_2 was also noticeably reduced, which we attribute to the fact that phosphopeptides are also partially bound via nonspecific interactions.

Thus, leaching by sodium hydroxide and further "activation" using HCl afforded a material with substantially reduced nonspecific binding, although at the cost of phosphopeptide recovery in the elution fraction. This is particularly relevant for the weakest binding phosphopeptide, NpSVEQGRRL. We propose that for this peptide, strong intermolecular ion pairing occurs between the phosphorylated serine residue located near the N-terminus and the two strongly basic arginine residues near the C-terminus. This could be confirmed by covalent modification of the arginines with malondialdehyde according to a procedure previously developed by us [37,38]. The reduced basicity of the modified Arg residues resulted

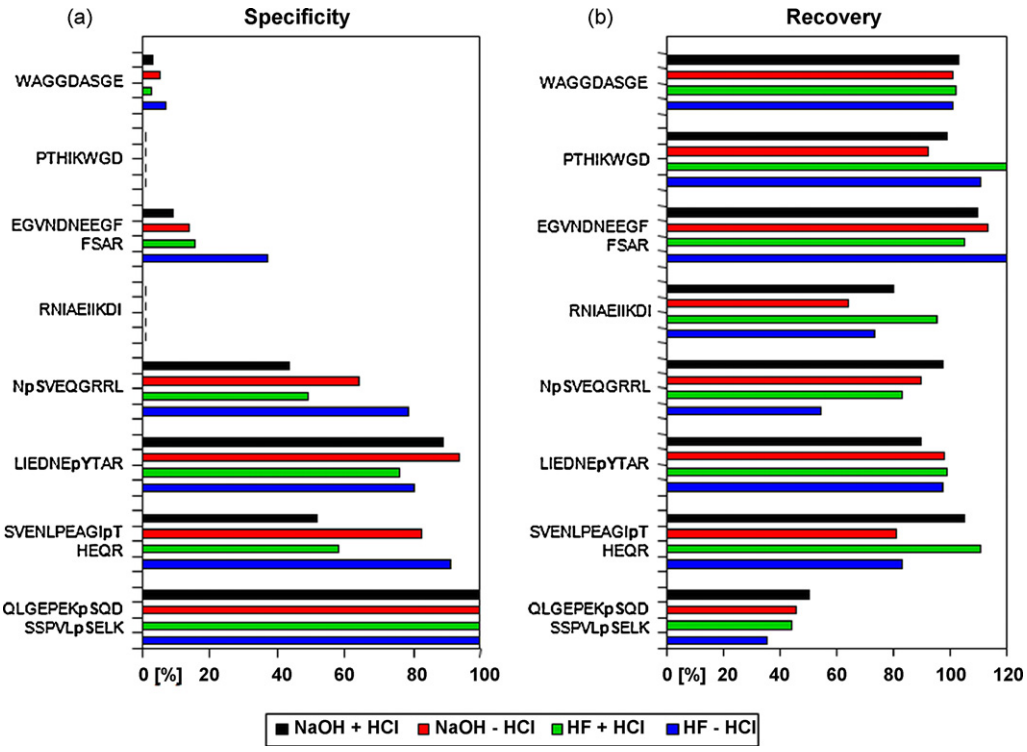

Fig. 2. Specificity (a) and recovery (b) values for phosphopeptide Mix 1 using differently treated tin dioxide materials for enrichment. Leaching was performed either with NaOH or HF, and the materials were optionally refluxed in diluted HCl. Values for individual peptides are given for (from top to bottom) NaOH + HCl (black), NaOH – HCl (red), HF + HCl (green), and HF – HCl (blue). For details, see text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

in improved binding of the phosphopeptide to the metal oxide: The amount of unbound peptide decreased from ~57% to ~31%, reflecting the weaker intramolecular ion pair formation. While the presence of two arginine residues in a peptide is not typical for complex samples where trypsin is most commonly used as the protease, this observation demonstrates that intramolecular ionic interactions can interfere with the binding process in particular cases.

The effect of preparation conditions on the material properties was further studied by EDX and XPS to estimate the remaining amount of silicon after the leaching steps, and nitrogen physisorption to reveal any effects on the porosity of the materials. EDX data from materials that had been exposed to NaOH for different periods of time (12–96 h) showed that after approximately 24 h, a constant level of remaining Si is obtained at ~0.75 at%, while shorter contact with the strong base resulted in more residual silica. XPS results show the same tendency. (Note that the NaOH leaching solution was changed every 24 h in order to not saturate the solution.) The constant level suggests that the remainder is not easily accessible for dissolution and may therefore also play a minor role in generating unspecific interactions. Interestingly, HCl treatment increased the apparent amount of Si as determined by EDX to ~1%, although the difference to the untreated spheres was not statistically significant. We propose that this is the result of a removal of SnO_2 aggregates that are only weakly attached to the rigid core of the particle. This assumption is again corroborated by XPS values and by nitrogen physisorption data that show a relative increase in larger pores upon HCl treatment (Fig. 3). Like for the Si content, the changes in porosity are not expected to be the predominant cause for changes in specificity, as pore sizes and surface areas do not influence the performance to a large degree, as will be shown below. Instead,

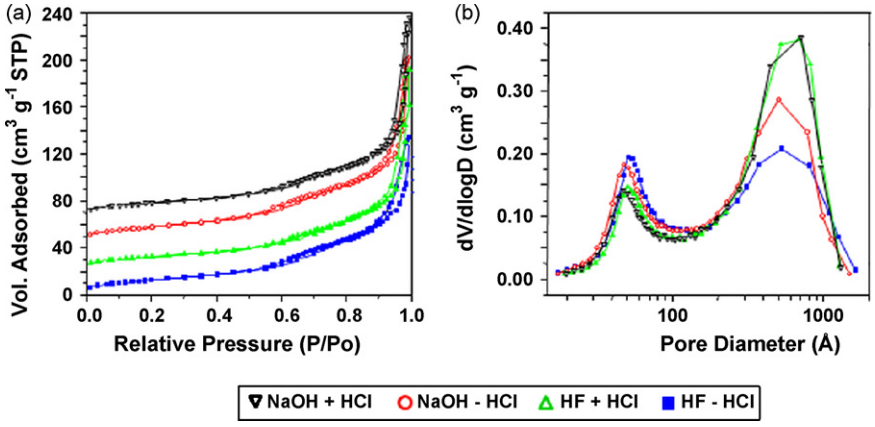

Fig. 3. Influence of additional HCl treatment on the physicochemical properties of 10 μm tin dioxide particles: (a) nitrogen physisorption isotherms (offsets: NaOH + HCl (black): 60 $cm^3\ g^{-1}$, NaOH – HCl (red): 40 $cm^3\ g^{-1}$, HF + HCl (green): 20 $cm^3\ g^{-1}$, HF – HCl (blue): no offset); (b) the corresponding BJH pore size distributions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

it seems more likely that the acid treatment results in chemical changes to the tin dioxide surface that affect the affinity properties of the material.

Other factors that could possibly influence the specificity of the material were also investigated. Subjecting the materials to repeated impregnation steps affords particles with increased robustness by continuously increasing the wall thickness of the SnO_2 replicas. This does not lead to a dramatic reduction in pore volume or surface areas of the material, as shown in Table 1, and should therefore only have a minor influence on specificity. Accordingly, no significant differences were found for materials that had been impregnated once, twice, or three times. The multiply impregnated spheres could be better suited for high-pressure applications.

In contrast, notable differences were observed in some cases when SnO_2 particles were prepared from silica templates with different pore sizes. Silica spheres with nominal porosities of 120, 200, 300 and 1000 Å were used for nanocasting. Table 1 shows that the SnO_2 replica prepared from the 200 Å shows a bimodal pore size distribution similar to the spheres obtained from 120 Å templates, although with larger mesopores in the range of 100 Å (compare also the data shown in Fig. 3). In contrast, the bimodal distribution changes to a monomodal one when silica of larger pore size is used, as the two modes of pores merge into one.

When the affinity towards phosphopeptides was compared, small to medium pore size silica (120–300 Å) afforded material with similar properties, despite the change in pore size. The replica of the 1000 Å silica spheres gave the worst performance, as less than 40% of the two phosphopeptides NpSVEQGRRL and SVENLPEAGIpTHEQR were recovered in the elution fraction. The poor binding efficiency is related to the dramatic reduction in available surface (less than half than for the 200 and 300 Å replicas), resulting in reduced binding capacity. Thus, while it is generally feasible to generate nanocast porous spheres from such a wide pore material, it comes at the cost of loading capacity.

Similarly, preparation of stannia materials of different particle size is easily achieved by varying the particle size of the silica. In this study, spheres with average diameters of 5, 10 and 20 μm were compared. This aspect is of particular relevance for set-ups where the particles are packed in capillaries or columns, as the use of larger particles results in lower back-pressures. We found that 5 and 10 μm particles showed almost identical affinities, while the specificity of 20 μm replica was lower, resulting in increased binding of both phosphopeptides and unphosphorylated controls. Whether this is a result of the somewhat higher surface area compared to the smaller particles (see Table 1), cannot be said with certainty.

In summary, the major influencing factors that were apparent are the "chemistry-related" preparation conditions, which not only appear to influence the porosity of the material to some degree, but also the surface of the material. Unfortunately, more detailed information on surface properties is not easily obtainable from methods available to us.

3.3. Comparison of nanocast tin dioxide material with titania and zirconia sorbents: influence of the number of phosphorylation sites

The stannia material with the best performance as determined above, i.e. spheres leached with NaOH and further treated with HCl, was compared to commercially available oxides in terms of the binding behavior towards mono- and polyphosphorylated peptides. 10 μm particles were chosen for all oxides, and the same enrichment protocol was used for all materials (without using additives), as nonspecific binding of unphosphorylated peptides was irrelevant in this study. This was corroborated by the fact that ZrO_2 was the only material that showed any noticeable binding of the unphosphorylated form of the peptide (~2%). This is in line with our previous observations that ZrO_2 shows higher nonspecific binding.

In contrast, significant differences were apparent for the differently phosphorylated forms, as shown in Fig. 4. SnO_2 and ZrO_2 showed much higher recoveries of the singly phosphorylated peptide than the two titania materials – above 75% for stannia and zirconia compared to only 13 and 33% for Sachtopore and Titansphere, respectively – and similar results were obtained for the doubly phosphorylated form. In contrast, the best recovery values for the triply and quadruply phosphorylated peptide were obtained using the Titansphere TiO_2 and the ZrO_2 material. Quantitative binding on all materials (100% specificity) was obtained for the peptides carrying two to four phosphate groups so that any losses can directly be attributed to incomplete elution from the materials. (No reductions in signal intensities were observed when peptide solutions were incubated without affinity material under binding and elution conditions, respectively, so that a significant influence of peptide degradation or adsorptive loss during the enrichment procedure can be ruled out.)

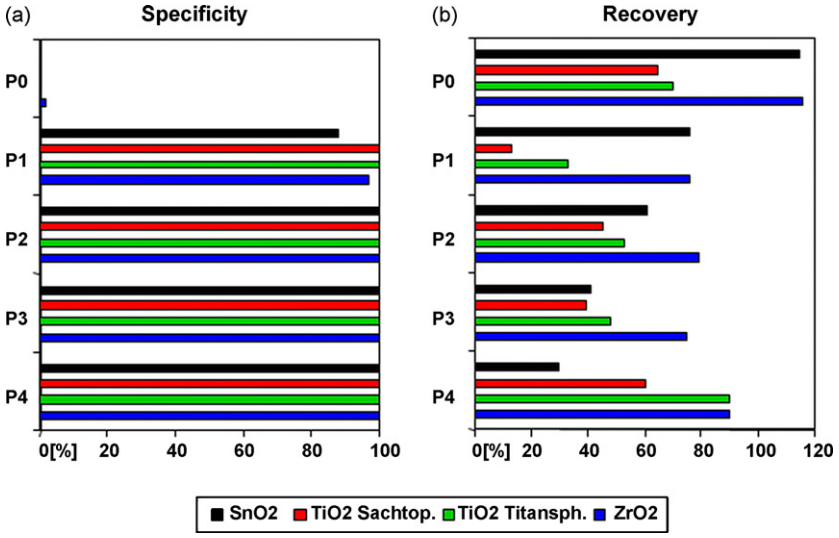

Fig. 4. Specificity (a) and recovery (b) values for phosphopeptide Mix 2 using different metal oxides for enrichment. Values for individual peptides (for sequences, see Table 2) are given for (from top to bottom) SnO_2 (black), Sachtopore TiO_2 (red), Titansphere TiO_2 (green) and ZrO_2 (blue). For details, see text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Clearly, additional experiments on rather complex samples are necessary to compare in more detail the affinities of the stannia material to other metal oxides, and are presently being carried out using samples of biological interest. However, judging from this set of data, it appears that SnO_2 allows for a high recovery of singly and doubly phosphorylated forms of the same peptide, while still giving acceptable values for higher phosphorylated forms (>30%). Recoveries of multiply phosphorylated peptides may be improved by a further optimization of elution conditions, for example using reagents proposed recently by Kyono et al. [39].

4. Conclusion

While different porous metal oxides are increasingly used for phosphopeptide enrichment purposes, little is known about the factors influencing their performance. Commonly, structural variations of the affinity material are limited by commercial availability or the flexibility of the manufacturing process. Using the nanocasting technique, we have shown that a wide range of tin dioxide materials may be prepared, and the process should be equally adaptable to other oxides such as TiO_2 or ZrO_2. Differently porous particles were found to give comparable performance (with the exception of replicas from 1000 Å material), which may be relevant to particular experimental set-ups such as packed beds for on-line enrichment columns. Interestingly, acid or base treatment during the preparation of the spheres was found to have the most significant influence. While this was connected at least to some extent to a change in the pore size distribution of the material, the decisive changes seem to take place on the surface of the material. Further investigations in this direction are therefore necessary. Finally, experiments with peptides differing in the number of phosphorylation sites revealed that phosphopeptide enrichment using the stannia material allows for the adequate representation of both singly and multiply phosphorylated peptides, which is important for the analysis of biological samples.

Preliminary data from an ongoing study on the analysis of whole cell lysates have already demonstrated that tin dioxide affinity chromatography is also applicable to this type of sample, as several hundred phosphopeptides were tentatively identified from HeLa lysate. Results from this study will be reported in due course.

Acknowledgements

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Publication 3

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Probing the Phosphoproteome of HeLa Cells Using Nanocast Metal Oxide Microspheres for Phosphopeptide Enrichment

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Metal oxide affinity chromatography (MOAC) has become a prominent method to enrich phosphopeptides prior to their analysis by liquid chromatography−mass spectrometry. To overcome limitations in material design, we have previously reported the use of nanocasting as a means to generate metal oxide spheres with tailored properties. Here, we report on the application of two oxides, tin dioxide (stannia) and titanium dioxide (titania), for the analysis of the HeLa phosphoproteome. In combination with nanoflow LC−MS/MS analysis on a linear ion trap-Fourier transform ion cyclotron resonance instrument, we identified 619 phosphopeptides using the new stannia material, and 896 phosphopeptides using titania prepared in house. We also compared the newly developed materials to commercial titania material using an established enrichment protocol. Both titania materials yielded a comparable total number of phosphopeptides, but the overlap of the two data sets was less than one-third. Although fewer peptides were identified using stannia, the complementarity of SnO_2-based MOAC could be shown as more than 140 phosphopeptides were exclusively identified by this material.

The biological significance of protein phosphorylation has stimulated the development and refinement of countless analytical tools for profiling the "phosphoproteome".[1−5] Difficulties in studying protein phosphorylation on a large scale are well documented and have been addressed by improvements on all levels of the proteomic workflow. For mass spectrometry-based proteomics, this begins with the optimization of extraction procedures for biological material (e.g., the addition of phosphatase inhibitors[6]) to ensure that the phosphorylation state is accurately represented in the sample that is analyzed and ends with the use of highly sensitive, fast scanning mass spectrometers that employ improved fragmentation techniques to maximize the sequence information from phosphopeptides.[7,8] In between, new enrichment and fractionation strategies have been developed in recent years to complement the established techniques of immobilized metal affinity chromatography (IMAC) and immunoaffinity precipitation. Among those, metal oxide affinity chromatography (MOAC) takes advantage of the particular affinity of metal oxides such as titanium dioxide to phosphate groups.[9] Various materials have been proposed although the majority of studies were carried out using TiO_2 as affinity material.[10−12] In addition, zirconium dioxide[12−14] and, more recently, niobium pentoxide[15] have been used in large-scale studies. Frequently, it has been reported that metal oxides vary in their affinity to different groups of phosphopeptides, e.g., mono- and multiply phosphorylated forms. Therefore, several groups have reported the use of two metal oxides and/or different enrichment techniques (MOAC and IMAC in parallel) to allow for a more comprehensive representation of the phosphoproteome.[16,17]

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One obstacle in further improving MOAC as an enrichment technique is the limitation in materials that are commercially available for a systematic comparison. Titania and zirconia have been used as stationary phases in HPLC,[18,19] and materials with small particle sizes are offered from several suppliers. For other metal oxides, researchers need to rely on particles that have not been specially optimized for chromatographic purposes. To overcome these limitations, several groups have reported the synthesis of metal oxide nanoparticles[20,21] although controlling the morphology of these particles is far from straightforward. On the other hand, the concept of nanocasting[22,23] offers more freedom to prepare materials with desired particle and pore sizes. This process relies on the formation of a "replica" in the pores of a porous template particle such as a silica sphere by filling the pores with a metal salt solution. Following this "impregnation" step, metal oxides are formed in situ by evaporation of the solvent and calcination in air.

We have recently shown that tin dioxide microspheres synthesized this way can also serve as supports for MOAC[24] and that nanocasting allows the generation of a "library" of SnO_2 materials to be used for optimizing the material properties.[25] Here, we expand the nanocasting concept to the generation of titanium dioxide microspheres suitable for MOAC. Both SnO_2 and TiO_2 materials were used to isolate phosphopeptides obtained from a digest of soluble proteins from a HeLa cell lysate. This cell line is a commonly used model system for phosphoproteomic studies, and different groups have reported the enrichment of HeLa phosphopeptides.[12,26−28] In our case, we were able to identify 600 to 900 phosphopeptides from less than 100 μg of HeLa proteins per run, depending on the material. We compare the performance of the nanocast material to a commercial titania sorbent (Sachtopore NP, Sachtleben Chemie, Germany). Although nonspecific binding was found to be higher with our in-house materials, we could demonstrate that the nanocast spheres perform well for complex biological samples and possess complementary affinity.

EXPERIMENTAL SECTION

Synthesis and Characterization of SnO_2 and TiO_2 Microspheres. Nanocast tin dioxide microspheres were prepared as described previously.[24] Ten micrometer silica spheres (120 Å, Daiso, Osaka, Japan) were used as templates and were impregnated with an aqueous solution of 1.8 g mL^{-1} $SnCl_2$. After removing excess solution by centrifugation, the wet samples were heat treated at 150 °C (3 h), 250 °C (3 h), and 550 °C (5 h) with heating ramps of 1 K min^{-1} between the different steps. For the preparation of titanium dioxide spheres, a modified method previously described by Lee et al.[29] was employed. In this case, 5 μm silica spheres (120 Å, Shiseido Co. Ltd., Tokyo, Japan) were impregnated with an excess solution of 50% (v/v) titanium isopropoxide (≥97.0%, Aldrich) in ethanol (99.5%). After stirring the suspension for 20 h, the excess precursor solution was discarded from the sample using centrifugation and finally ethanol was evaporated in a vacuum oven. Subsequently, a mixture of ethanol/water (1:1 volume ratio) was added to the dried sample, upon which the sol−gel reaction of the titanium precursor was initiated. In order to ensure mechanically stable replica spheres, the impregnation/sol−gel steps were repeated once. Finally, the composite spheres were calcined at 550 °C to obtain crystalline anatase. The silica template was removed from both materials by two sequential treatments in 2 M NaOH at 90 °C. The obtained metal oxide particles were then refluxed in 0.1 M aqueous HCl, as this was previously found to increase specificity of the enrichment process.[25]

The materials were characterized using scanning electron microscopy, SEM (Jeol JSM-6335F, Jeol Ltd., Tokyo, Japan), nitrogen physisorption (ASAP 2010, Micromeritics Co., Norcross, GA), energy-dispersive X-ray spectroscopy (Link Inca, Oxford Instruments, Abingdon, UK), X-ray diffraction (XRD, X'pert X-ray diffractometer, Philips PANalytical, Almelo, The Netherlands), and electro-kinetic titrations (Malvern ZetaSizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK).

Preparation of HeLa Protein Sample. HeLa cell lysate was prepared as described in Poser et al.[30] Dried HeLa proteins (1 mg) were resuspended in 8 M urea/500 mM NH_4HCO_3, reduced using dithiothreitol (DTT, 50 μg per mg protein, 30 min, 56 °C), and alkylated by iodoacetamide (250 μg per mg protein, 30 min, room temperature). Alkylation was stopped by adding an excess of 250 μg of DTT. Enzymatic digestion was first carried out by diluting the samples to 6 M urea, adding lysyl endopeptidase (mass spectrometry grade, Wako Chemicals, Neuss, Germany) at an enzyme-to-substrate ratio of 1:50, and incubating at 30 °C for 2 h. The sample was then diluted to 0.8 M urea and digested using trypsin (proteomics grade, Sigma-Aldrich, Vienna, Austria) by adding two 16.5 μg aliquots initially and after 2 h, respectively (final enzyme:substrate ratio 1:33). Tryptic digestion was carried out overnight at 37 °C.

The protein digest was acidified using trifluoroacetic acid (TFA) and purified by solid-phase extraction using C_{18} cartridges (Phenomenex, Torrance, CA). The SPE eluate was evaporated to dryness and stored at −20 °C until further use.

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Phosphopeptide Enrichment. Phosphopeptides were enriched by MOAC according to two different protocols separately optimized for SnO_2 and TiO_2. In addition to the nanocast metal oxides synthesized in-house, Sachtopore NP TiO_2 (10 μm, 300 Å, Sachtleben Chemie, Duisburg, Germany) was used as reference material. All steps were carried out at room temperature in a thermomixer, and the MOAC material was settled by centrifugation after each step.

Tin Dioxide Enrichment. Aliquots (200 μg) of digest were redissolved in 100 μL of loading buffer (LB, 50% acetonitrile/0.1% TFA). Five milligrams of stannia material was preincubated in LB for 10 min, and afterward the supernatant was discharged. HeLa sample was added and incubated for 30 min at room temperature. After enrichment, the supernatant was discarded and beads were washed twice with 100 μL of LB for 10 min. Finally 2 × 100 μL of a 50 mM $(NH_4)_2HPO_4$ solution (pH 10.5, adjusted with ammonium hydroxide) was used for the elution of bound peptides.

Titanium Dioxide Enrichment. Phosphopeptide enrichment using titanium dioxide was performed as above with the following exceptions: 50% acetonitrile/0.1% TFA containing 300 mg mL^{-1} lactic acid was used as buffer for pretreatment, incubation, and the first washing step. For the second washing step, 50% acetonitrile/0.1% TFA was used. All eluates were acidified to a pH of 4 to 5 with TFA and stored at −20 °C prior to analysis.

NanoLC−MS Analysis. LC−MS/MS analyses were carried out on an Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) connected to a LTQ FT Ultra mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a Proxeon nanospray source (Proxeon, Odense, Denmark). MOAC eluate (90 μL, corresponding to ~90 μg of starting material) were loaded onto a trap column (Dionex PepMap C18, 5 mm × 300 μm ID, 5 μm particles, 100 Å pore size) at a flow rate of 25 μL min^{-1} using 0.1% TFA as mobile phase. After 40 min, the trap column was switched in line with the analytical column (Dionex PepMap C18, 250 mm × 75 μm ID, 3 μm, 100 Å). Peptides were eluted using a flow rate of 275 nL min^{-1}, and a ternary gradient described in detail in Mitulovic et al.[31] was used with the following mobile phases: A (water/acetonitrile/formic acid, 95/5/0.1, v/v/v), B (water/acetonitrile/formic acid, 70/30/0.08, v/v/v), and C (water/acetonitrile/trifluoroethanol/formic acid, 10/80/10/0.08, v/v/v/v). The total run time was 240 min.

The LTQ FT was operated in data-dependent mode using a full scan in the ICR cell (m/z range 400−1800, nominal resolution of 100 000 at m/z 400, ICR target value 500 000) followed by MS/MS scans of the five most abundant ions in the linear ion trap. MS/MS spectra (normalized collision energy, 35%; activation value q, 0.25; activation time, 30 ms; isolation width, ±3 Da) were acquired in the multistage activation mode, where subsequent activation was performed on fragment ions resulting from the neutral loss of −98, −49, or −32.6 m/z. Precursor ions selected for fragmentation (charge state 2 and higher) were put on a dynamic exclusion list for 180 s. Monoisotopic precursor selection was enabled.

Data Analysis. Xcalibur .raw files were searched against the human subset of the IPI database (www.ebi.ac.uk/IPI/, version 3.55 (dated 12 Feb 09, 75 554 entries)) using the SEQUEST search option of Proteome Discoverer (version 1.1, ThermoScientific). Search parameters were set as follows: enzyme name, trypsin (full); maximum missed cleavage sites, 2; precursor mass tolerance, 5 ppm; fragment mass tolerance, 0.5 Da; static modifications, carbamidomethyl-Cys; dynamic modifications, oxidation on Met and phosphorylation on Ser/Thr/Tyr. The maximum number of identical modifications per peptide was set to three, and the number of maximal modifications per peptide was set to four. The output was filtered to a false discovery rate of 1% based on the built-in decoy search functionality of the software.

The resulting peptide identifications from three technical replicates were combined, and a nonredundant list of peptide IDs was generated. The peptide counts reported here are based on the following conventions: An identical amino acid sequence differing only in Met oxidation (present/not present) is counted as one. Phosphopeptides differing only in the number of phosphorylation sites (0, 1, 2, 3, 4) are counted as different peptides. Peptides with identical numbers of phosphorylation sites, but different localization of phosphate group (as assigned by SEQUEST), were also counted as different peptides.

A list of all phosphopeptide identifications is given in the Supporting Information (Tables S1−S3). The complete data set will be deposited in the IMP Vienna SpectrumDB database accessible at http://www.imp.ac.at/research/protein-chemistry/labhomepage/spectrumdb/?expname=Nanocast%20MOAC. Selected peak areas were determined by manual integration in Xcalibur.

Analysis of Peptide Properties. Isoelectric points of both phospho- and nonphosphopeptides were calculated using the program pICalculator,[32] version 1.0. The pK settings "optimized" (for unphosphorylated amino acids) and "default" (for phosphoamino acids) were used. Amino acid distributions were calculated by PROMPT,[33] version 0.9.6, downloaded from http://www.geneinfo.eu/prompt/downloads.php. Proportional Venn diagrams were created by Venn Diagram Plotter, version 1.3.3103, available from http://ncrr.pnl.gov/software/.

Caution: Corrosive substances such as tin chloride, titanium isopropoxide, and concentrated acids and bases should be handled with care and in a well ventilated fume hood. No further special precautions apply.

RESULTS

Preparation of Nanocast Metal Oxide Materials for Phosphopeptide Enrichment. A more thorough structural investigation of the SnO_2 replica as well as the commercial Sachtopore TiO_2 spheres has been reported previously.[24] However, the main properties of these materials are listed in Table 1 for comparison with the new nanocast TiO_2 spheres. On the basis of our previous experience with tin dioxide spheres,[24,25,34] we prepared nanocast titanium dioxide material according to a similar procedure (see Experimental Section). Both MOAC materials were prepared from 120 Å pore size silica and treated with diluted hydrochloric acid prior to use. This "activation" procedure has been found beneficial for the specificity of the stannia material,[25] presumably due to changes in surface chemistry.

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Table 1. Textural Properties for the Different Particles Determined by Nitrogen Physisorption and Electro-Kinetic Titrations

	surface area[a] [$m^2\ g^{-1}$]	pore volume[b] [$cm^3\ g^{-1}$]	pore diameter[c] [nm]	crystallite size[d] [nm]	isoelectric point[e] [pH]
Nanocast SnO_2	55.9	0.188	5.0; 50.8	18.8	4.1
Nanocast TiO_2	246.1	0.844	16.9	7.0	4.7
Sachtopore TiO_2	23.5	0.193	32.1	35.2	5.0

[a] Determined using the BET model. [b] Determined from the adsorption branch at $P/P_0 = 0.98$. [c] Determined using the BJH model from the desorption branch. [d] Approximated from the XRD data using the Scherrer equation. [e] Values obtained using electro-kinetic titrations starting from the native pH of the metal oxides to a pH of about 2.

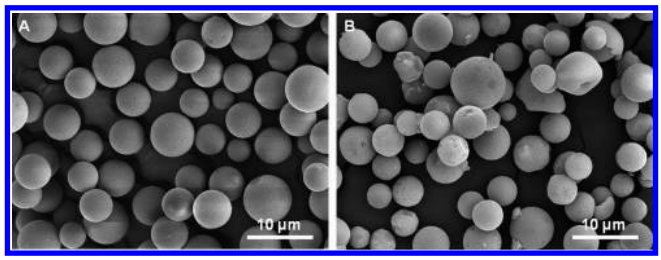

Figure 1. SEM images of (a) the SiO_2 template together with the novel TiO_2 replica (b) spheres used in the enrichment protocol.

SEM characterization of the nanocast titania microspheres in relation to the starting silica template is shown in Figure 1. This comparison indicates that we are able to fully replicate the sphere structure on a macroscopic length scale as was shown earlier for the stannia materials.[24,25,34] Even if we have shown that the morphology does not play a crucial role in phosphopeptide enrichment performance of stannia spheres,[25] it is important to be able to control the size and shape of the particles for further adaptations of these materials in column applications. Simply using silica spheres with different morphology as templates, we have the possibility to control the structure of the replica.

Nitrogen physisorption was carried out to reveal the textural properties on a nanometer length scale and the results are listed in Table 1 as well as shown in the Supporting Information (Figure S1). While the nanocast SnO_2 and the Sachtopore TiO_2 spheres show comparable properties with regard to the specific surface area and mesopore volume (25−50 $m^2\ g^{-1}$ and 0.19 $cm^3\ g^{-1}$, respectively), it is essential to point out that the nanocast TiO_2 displays an almost 10 times higher surface area (250 $m^2\ g^{-1}$) and more than 4 times higher pore volume (0.85 $cm^3\ g^{-1}$). In addition to this, the nanocast TiO_2 exhibits a sharp pore size distribution around 17 nm. The reason for the higher surface area compared to the stannia replica spheres is that titania is forming a more true replica of the silica mesopore structure on the nanometer length scale, which was also shown previously for nanocast zirconia spheres.[34]

As we observed previously for the SnO_2 materials, the surface chemistry of the MOAC materials plays a larger role for the enrichment efficiency of phosphopeptides than the structural properties do.[25] Using X-ray diffraction (XRD), we are able to determine the crystalline form and estimate the crystal size (using the Scherrer equation) of the different MOAC materials, and the results are shown in Figure S2 in the Supporting Information. For the nanocast SnO_2 spheres, we can only observe the cassiterite crystalline phase with approximately 19 nm crystallite domains. Furthermore, the XRD patterns obtained for the two different TiO_2 materials can both be assigned to the anatase form. However, there is a clear difference in the domain size of the crystallites as the Sachtopore material displays fairly large domains (~35 nm), while the nanocast TiO_2 spheres are nanocrystalline with a crystallite size of about 7 nm. Moreover, a larger portion of the nanocast titania particles seems to be amorphous, because a broad hump can be observed in the range of 10−20° 2θ.

Energy-dispersive X-ray spectroscopy (EDX) was used to determine the remaining amounts of silica in the two replica materials after the etching step. It was clear that the silica part could be more easily removed from the tin dioxide spheres compared to the titania spheres (~1% and ~3% remaining Si, respectively). A probable reason for this behavior is that the impregnated titania phase is in more intimate contact with the silica template (better wetting of the surface), and thus, there is a larger probability for the oxides to form a chemically stable mixed oxide at the interface.

Because of the differences in the amount of residual silica in the materials, we also studied the electro-kinetic behavior using zeta potential titrations in order to determine the isoelectric point (pI) of the different MOAC materials (listed in Table 1). The stannia material clearly displayed the lowest pI value at about 4.1, while for the Sachtopore TiO_2 spheres the pI was higher (~5.0). Interestingly, the pI of the nanocast TiO_2 was slightly lower compared to the commercial TiO_2 (~4.7), which one would expect for adding Si atoms in the TiO_2 structure. The small pI shift in addition to the smaller crystallite size could at least partially explain the differences in the enrichment behavior that we observe for both titania materials described below.

Analysis of the HeLa Phosphoproteome by Tin Dioxide and Titanium Dioxide MOAC. *Methodology [Figure 2].* The three metal oxide materials, nanocast SnO_2 and TiO_2 and commercial TiO_2, were used to enrich phosphopeptides from a HeLa cell lysate. To provide a fair comparison between the materials, separately optimized protocols were used for stannia and titania, respectively. Enrichment with tin dioxide was carried out using 50% acetonitrile and 0.1% TFA during binding and washing steps, while for titanium dioxide, binding and initial washing was carried out in the presence of 300 mg mL^{-1} lactic acid to reduce nonspecific binding. The latter protocol, following a procedure initially introduced by Sugiyama et al.,[12] provides in our hands the best compromise between recovery and nonspecific binding for titania, and no other protocols were evaluated as part of this study. Phosphopeptides bound to the metal oxides were eluted by an inorganic phosphate solution adjusted to pH 10.5, ensuring efficient elution resulting from the pH shift and the competition of the phosphate anions for binding sites on the oxides.

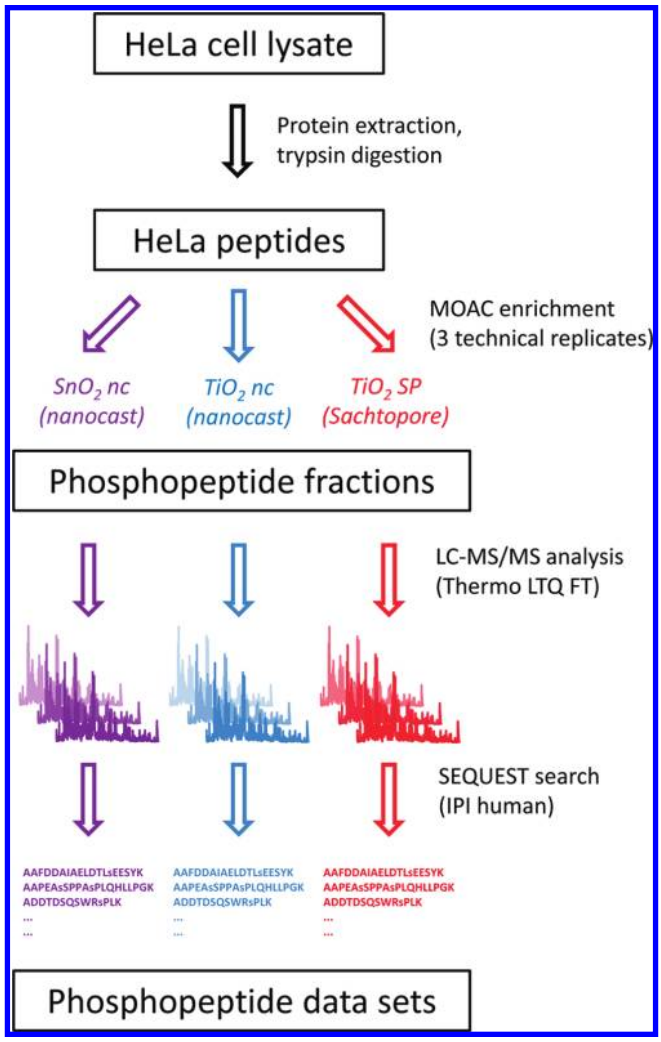

Figure 2. Analytical strategy for the enrichment of HeLa phosphopeptides by metal oxide affinity chromatography.

MOAC eluates were subsequently analyzed by reversed-phase HPLC−MS/MS on a linear ion trap-FTICR hybrid mass spectrometer. Because of the facile neutral loss of the phosphate group (in the form of H_3PO_4) from many phosphoserine and phosphothreonine peptides that can compromise spectral quality,[35] we employed multistage activation[36] for sequencing. This technique, which activates both the precursor ion and product ions resulting from neutral loss of H_3PO_4 without an intermittent isolation step, shows better performance compared to a MS^2/MS^3 strategy and facilitates data analysis, as also recently confirmed by Ulintz et al.[37] Following a database search using SEQUEST against the human subset of the IPI database and a decoy database approach, peptide identifications were filtered to a false discovery rate of less than 1% for every individual LC−MS/MS run.

Number of Phosphopeptides. Figure 3 gives an overview of the performance of the three MOAC materials. Combining the results of three technical replicates (separate MOAC enrichment followed by a single LC−MS/MS run), more than 600 phosphopeptides were confidently identified from each material in at least one LC−MS/MS analysis (Figure 3a). Average numbers per individual run ranged from ~330 to 570 for the different materials. Using the two titania materials, a larger number of phosphopeptides could be identified compared to stannia, and enrichment by the Sachtopore material yielded roughly 10% more identifications than the in-house made material (1010 vs 896). When grouping the phosphopeptides according to the number of phosphorylation sites (Figure 3b), we found that 71% of the peptides identified from the Sachtopore samples were singly phosphorylated, while the number was higher for the two nanocast materials (82% and 84%, respectively). The majority of the remaining peptides were diphosphorylated (>85% for all materials), although triply and (few) quadruply phosphorylated peptides were also identified by all materials.

Ishihama and co-workers previously reported specificities of >90% in the elution fractions of Titansphere TiO_2-MOAC using lactic acid as modifier.[14] We could corroborate these results for the Sachtopore material, where we achieved 94% specificity. In contrast, the amount of nonspecific binding was higher for the nanocast materials: Specificities of 67% and 60% were obtained for titania and stannia, respectively. These results are, however, comparable to reported values obtained for zirconia-based MOAC, and it is important to consider that no additional additive was used for the stannia material. This confirms our previous observation made using model peptides that stannia possesses a significantly higher intrinsic specificity for phosphopeptides than titania,[24] which has also been recently confirmed by Zhang and co-workers using magnetic tin dioxide-coated particles.[38]

Overlaps in the Data Sets. Every sample obtained from MOAC was analyzed using a single dimension chromatographic separation followed by tandem MS. In order to examine the reproducibility of the enrichment strategy, MOAC was carried out in triplicate. Figure 4a shows the overlap in phosphopeptide identifications of the three materials used for this study. Less than one-third of the phosphopeptides were identified in all three replicate analyses, with the overlap being the highest for the nanocast titania material. In at least two runs, 40−50% of the peptides were detected. In both cases, the overlap was lowest for the tin dioxide material. The (small) difference to the titania materials may be caused not only by the material properties but also by slight variations in instrument performance over time. In general, the values obtained reflect the enormous complexity of the phosphopeptide sample which would require a more comprehensive (multidimensional) separation strategy for in-depth characterization.

However, it is still of interest to confirm the overlap in phosphopeptide IDs between the different metal oxide materials. Figure 4b shows the overlap for all peptides identified in at least one run per material. Only 297 phosphopeptides (18.6% of the total pool) were identified with all three materials. Compared to the Sachtopore data set, the nanocast materials contributed 586 additional identifications, of which 363 were from nanocast titania and 144 from nanocast stannia (79 were found in both). Despite

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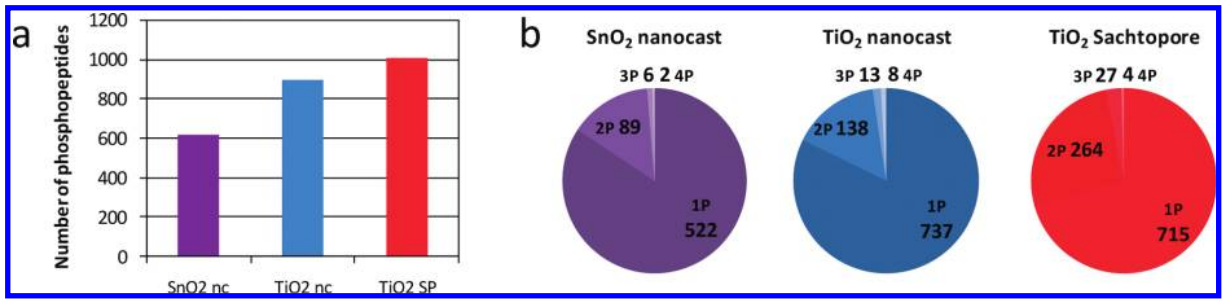

Figure 3. Comparison of phosphopeptides identified by the three metal oxide materials. (a) Total number of phosphopeptides identified (in at least one of three replicates) and (b) distribution according to the number of phosphorylation sites per peptide.

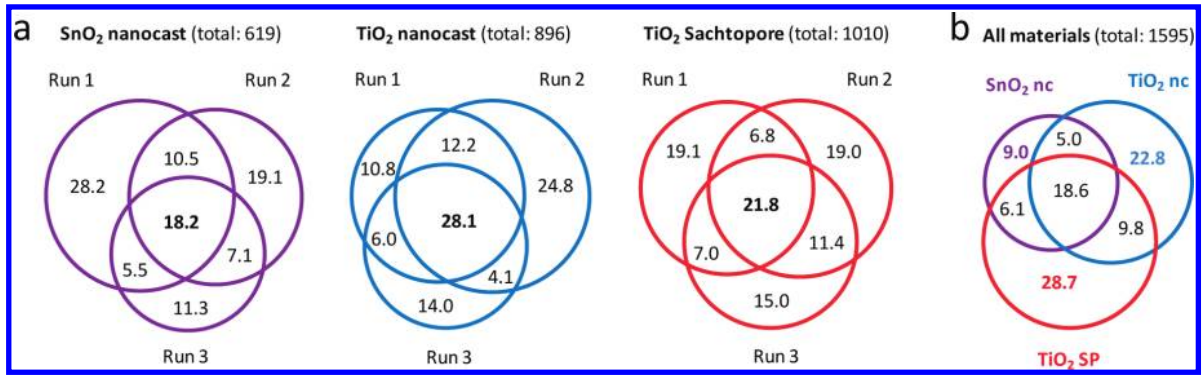

Figure 4. Overlaps (in %) in the phosphopeptide identifications from the three metal oxide materials. (a) Overlap of phosphopeptide identifications in different technical replicates. (b) Overlap of phosphopeptides (identified at least in one of three replicates) for the three metal oxide materials.

the low degree of overlap between the two materials, predicted isoelectric points of the phosphopeptides and distribution according to phosphorylated amino acids were very comparable (see Figure S3 in Supporting Information).

At first glance, this might suggest that there is no significant difference in the affinities of the different materials. However, a more detailed analysis of the data reveals additional qualitative and quantitative differences. For example, when only the subset of phosphopeptides is considered that is identified in all three technical replicates for a given material ($n = 392$), only a small fraction of the peptides (56) were identified three times with all three metal oxides. However, 74 phosphopeptides (37 for Sachtopore TiO_2, 35 for nanocast TiO_2, and 2 for nanocast SnO_2) were identified in all replicates of one material, but not a single time with any other material.

More importantly, a comparison of peak areas of selected identifications revealed that relative amounts of phosphopeptides differed significantly between the materials, sometimes even by a factor of 10. In contrast, reproducibility between technical replicates was very good on the basis of signal intensities. Selected examples are shown in Figure 5, which also provide evidence that SnO_2 shows clear quantitative differences in affinity for a subset of peptides. Note that quantitative differences in affinity do not always correspond to differences in identification. Frequently, a given peptide was still identified although it was present at levels of less than 10% compared to the better performing material. However, it is obvious that with decreased sample amounts the differences in identification would become more apparent.

Characterizing Nonspecific Binding. As mentioned above, the nanocast materials showed a larger amount of nonspecific binding when compared with the commercial material. Thus, we examined the lists of nonspecifically bound unphosphorylated peptides to find out whether any differences in the physicochemical properties of these peptides exist. Figure 6a compares the distribution of isoelectric points among the three sets of peptides. It is evident that a clear majority of peptides that nonspecifically bind to stannia are strongly acidic, with a pI < 5. It is very likely that this is a consequence of the enrichment protocol that does not use any additional modifier, in contrast to the titania protocol. In previous work on stannia-based MOAC, we could not find any additive that improved the specificity without significantly compromising recoveries. However, the amount of nonspecific binding is still at a level that should not cause problems for phosphopeptide identification, as more than half of the identified peptides were phosphorylated. Interestingly, despite the higher numbers of unphosphorylated peptides identified using the nanocast titania material, differences in the pI distributions between the two sets of peptides were small, with a slightly larger contribution of more acidic peptides for the nanocast material. Therefore, a higher degree of nonspecific binding may at least in part be explained by the much larger surface area of the replica material, resulting in more binding sites per milligram of material. As the same amount of material by weight was used, nonspecific binding may be reduced if smaller amounts proportional to the surface areas are used in this case.

As a second parameter that affects nonspecific binding, we looked for differences in amino acid distribution among the nonphosphopeptides (Figure 6b). As could be expected, the stannia data set contained elevated levels of aspartic and glutamic acid, although both titania materials also showed increased levels of these two residues relative to the average amino acid composi-

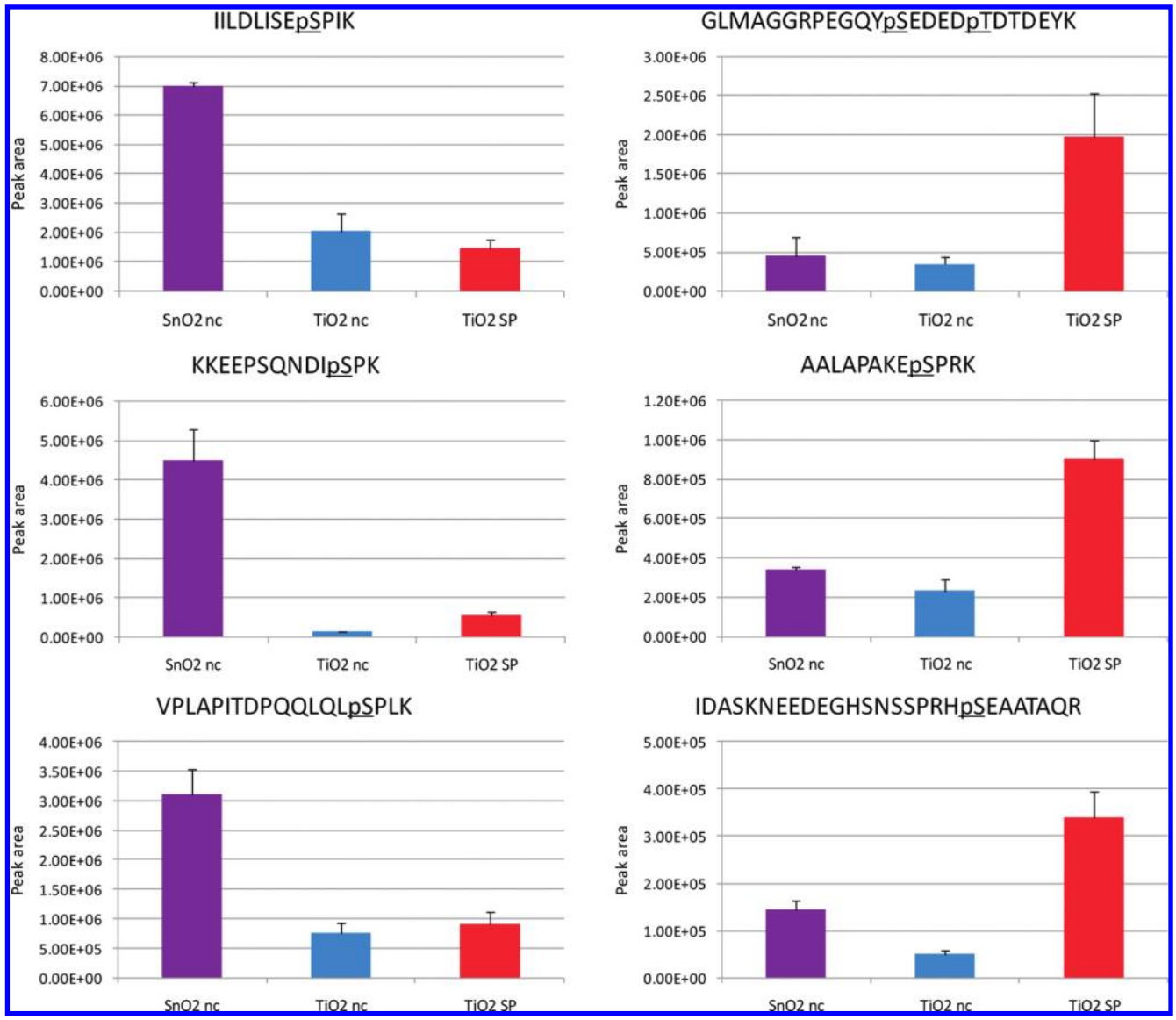

Figure 5. Examples for different affinity of phosphopeptides to tin dioxide and titanium dioxide. Peak areas given are averages of three technical replicates, error bars represent one standard deviation.

tion of the SwissProt database (version 57.6, 28/07/09) taken as a reference. Other notable differences to SwissProt for all three materials were also observed, with reduced levels of Phe, Ile, Leu, Met, and Tyr. While overall differences between the two titania materials were again rather small, enormous differences in histidine content are noticeable when comparing tin dioxide and titanium dioxide: The average His content in SwissProt is 2.3%, identical to the number in the SnO_2 data set. The corresponding numbers for Sachtopore and nanocast titania are 10.2 and 8.4%, amounting to a 4-fold increase in relative His content. In fact, in the Sachtopore data set, 73% of the unphosphorylated peptides contain at least one His residue and 48% contain two or more. Although there have been some reports about the histidine affinity of titanium dioxide,[39,40] it has not been discussed in the context of proteomic research. Tin dioxide, in contrast, does not appear to bind His peptides preferentially.

DISCUSSION

The results presented above provide convincing evidence that nanocast metal oxide materials are suitable for application to complex biological samples such as whole cell lysates. However, in the case of the TiO_2 material, nonspecific binding still is higher than for a well established commercial chromatographic material. Further studies to address this issue are ongoing in our laboratories, although even the presently achievable specificities provide samples that are highly enriched for phosphopeptides and commonly available instruments with high sequencing speeds are able to deal with the slightly elevated number of peptides present. In fact, the largest number of phosphopeptides identified in any single run (636) as part of this study was achieved using the nanocast titania. The aspect of nonspecific binding would be even less of a concern in combination with other chromatographic or electrophoretic separation methods in a two-dimensional setup.

Importantly, the use of tin dioxide has resulted in the identification of additional phosphopeptides not covered by the titania materials, and even the two titania materials differ in their

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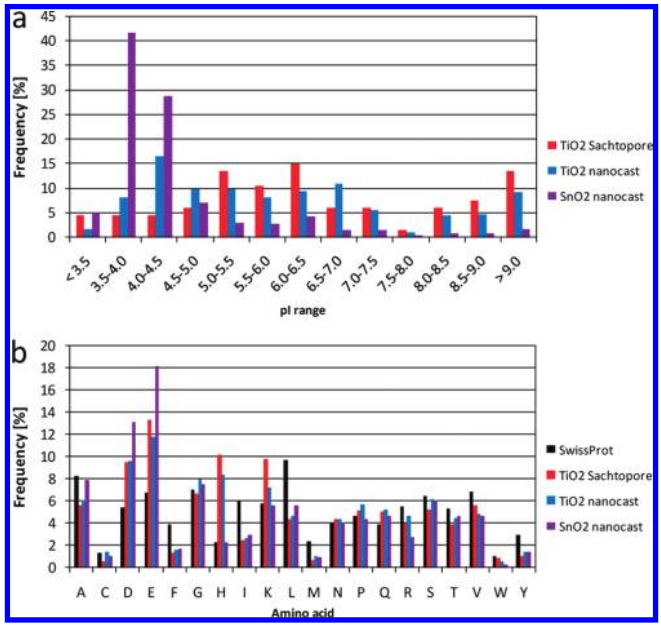

Figure 6. Physicochemical characterization of nonspecifically bound peptides. (a) pI distribution and (b) amino acid frequencies of unphosphorylated peptides bound to the three metal oxide materials.

specificities. This not only confirms previous data from studies in which different metal oxides have been used (Ti and Zr, Ti and Nb) but also increases the number of MOAC sorbents available to enhance the coverage of the phosphoproteome.

Further improvements in the design of nanocast metal oxides will focus on the optimization of surface chemistry which is still not fully explored and is potentially very dependent on the synthesis conditions. Improving the specificity of the materials may also be achieved by refinement of the enrichment protocol, in particular the binding and washing steps. Finally, the concept is expandable to other metal oxides of interest that have not yet been widely used, such as the recently described hafnium[41] or tantalum[42] oxides. A detailed comparison of such materials is only possible when different metal oxides are prepared in highly similar procedures, thereby reducing the influence of parameters such as particle and pore size.

In summary, we have used two metal oxide affinity materials prepared by the nanocasting technique to analyze the phosphoproteome of HeLa cells. Both materials significantly enriched for phosphopeptides and provided complementary coverage of the phosphoproteome. The final data set obtained from the nanocast materials alone (provided in the Supporting Information) contains 1137 phosphopeptides.

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SUPPORTING INFORMATION AVAILABLE

Detailed list of all identified phosphopeptides for the three materials (Table S1, tin dioxide; Table S2, nanocast titanium dioxide; Table S3, Sachtopore titanium dioxide). Additional figures as reported in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Manuscript 1

A Chemically Cleavable Linker for Immobilizing Bait Compounds in a Target Protein Pull-down Concept

Abstract

A novel linker chemistry based on a malondialdehyde - indole condensation reaction has been developed for the affinity-independent elution of targeted protein pull downs. Recently developed in our lab for tagging of tryptophan residues on proteins or peptides, the concept was extended for the design of a chemically cleavable linker system. In our setup a specific kinase inhibitor (bosutinib) was coupled to agarose and acrylamide beads respectively via the new linker system and a protein pull down experiment of possible interaction partners of a K562 whole cell lysate was performed. After purification, the linker can be cleaved from the beads by incubation with 50mM pyrrolidine. The system is especially compatible with targeted protein pull downs because during cleavage step no protein hydrolysis or any degradation of amino acid side-chains was induced. From the pull-down experiment, key targets of bosutinib such as the tyrosine kinase, Btk, were identified.

Introduction

For many drugs which are in pharmaceutical development or already in clinical use, their main targets and other possible interaction partners are still unknown. To facilitate further advances in drug effectiveness but also to get a better overview of possible side effects, all interacting proteins should ideally be known. In the field of chemical proteomics, proteins are selectively enriched by affinity chromatography for further analysis by 2D-gelelectrophoresis and/or liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). With the help of organic chemistry probes can be coupled to beads via certain linker chemistry to further purify targeted proteins. Thereby it is possible to purify targets from matrix components which may otherwise interfere with downstream analysis. However, in case of targeted protein pull downs, elution of enriched proteins in their native form can be difficult, since most conventional elution protocols are based on the use of denaturing components like detergents, low pH, or high salt concentrations for elution. Recently new affinity independent elution methods which do not affect the bioaffinity interaction were introduced [1-5]. Linkers can be cleaved enzymatically by cleaving a DNA-linker with nuclease [3] introduced by Santala et al., photochemically [5], or chemically by using an azobenzene linker which can be cleeaved by reduction with sodium dithionite [1, 4].

Here, we use a malondialdehyde-indole derivative as linker which can be chemically cleaved by incubation with pyrrolidine or hydrazine. This chemistry, using malondialdehyde bound to the indole nitrogen was previously used in our lab for the reversible and selective tagging of tryphophan residues on peptides or proteins [6, 7].

Experimental

Synthesis

The indole linker **3** ((E)-2-(1-(3-oxoprop-1-enyl)-1H-indol-3-yl)acetic acid, IAA-MDA) was designed to display two functional groups on opposite ends for coupling to the support and the drug ("bait"), respectively, and a chemically cleavable group in the middle of the linker moiety (scheme 1, a).

A condensation reaction of malondialdehyde (MDA) with 3-indolyl-acetic acid (IAA) **1** under strongly acidic condition [7], primarily described by Teuber et al.in 1964 [8],was used (scheme 1, a). As the first step, malondialdehyde (MDA) was applied in its commercial available form as tetramethoxypropane (TMP) **2**, which is converted to MDA in situ during reaction. The newly formed derivative contains a chemically cleavable group at the indole nitrogen which is linked to an activated α,β- unsaturated aldehyde group for coupling onto beads. Structure of the synthesis product was confirmed by mass spectrometry and NMR spectroscopy (see supplemental data). The reaction conditions had to be controlled accurately (not more than 2h reaction time, controlled temperature) to avoid polymerisation of the derivative, due to the high activity of the resulting acroleine type group. The reactive carbonyl group typically reacts with amine or hydrazide nitrogens to form Schiff base type products.

For affinity chromatography purpose, we used the Pierce Carbolink Coupling Gel, an agarose gel which contains hydrazide functionalities (coverage approximately 10 µmol/ml gel). Additional coupling experiments were carried out on hydrazide activated acrylamide beads for comparison of coupling and purification performance. Both materials performed in comparable manner. For simplicity only data gained by use of agarose beads is quoted. With the use of 10 mM aniline as catalyst [9] the reaction time could be reduced to two hours and the reaction was performed in slightly acidic environment (solvent buffered to pH 4.5) (scheme 1, b). During the reaction, the beads turned yellowish due to binding of the colored IAA-MDA derivate.

The coupling efficacy was around 50% as determined by liquid chromatography – mass spectrometry (LC-MS) in multiple reaction monitoring mode detection of the cleavage product 3-indolyl-acetic acid (IAA) after cleavage with 355mM pyrrolidine (complete cleavage). After

coupling, remaining hydrazide groups had to be saturated to avoid cross reactions in following steps. Therefore we used a 30x molar excess of N-acetoxysuccinimide in order to achieve complete saturation of remaining hydrazide groups (scheme 1, c). Completeness of saturation was demonstrated by trying to re-bind the derivative to already saturated beads. No coloring of beads, like it is normally the case upon binding of the derivative, could be detected upon twentyfold excess of N-acetylsuccinimide, and after treatment with pyrrolidine, no IAA could be detected by LC-MS analysis. A result of the saturation process was the competitive displacement of readily bound IAA from beads (up to 50%).

From this point, the beads were ready for the attachment of the desired affinity target. The free carboxylic residue of the linker was coupled to amino groups on affinity targets, using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as coupling reagent [10]. The coupling reaction with DMT-MM is a one step reaction, so carboxylic residues on the bait have to be protected first. Additional, only free amino groups in their non-ionized form are accessible for the reaction.

To prove the effectiveness of the reaction, we chose methyl 2-amino-3-(4-hydroxyphenyl)propanoate (tyrosine methyl ester) for coupling (scheme 1, d). As solvent, methanol was found to be the best candidate because it ensured good coupling efficiency without a collapse of the agarose beads. The binding efficiency was determined to be 50%, again evaluated by quantitative LC-MS (see supplemental data). As a reference for quantitation a 3-indolylacetic acid- tyrosine methyl ester conjugate **4** was used, which was synthesized in house. The coverage of baits could be varied by changing in the amount of IDA-MDA used for the initiation reaction. Consequently the protocol allows an easy control of the total loading of baits on the beads.

For specific linker cleavage 50mM pyrrolidine was used and a 0.1M Tris buffer system did not seem to interfere with the cleavage chemistry. The use of pyrrolidine was further tested to clarify whether or not the reagent causes protein precipitation on the beads or causes any protein degradation. Therefore beads were incubated overnight with a mixture of four proteins that were dissolved in 0,1M Tris buffer or 50mM pyrrolidine solution either (see supplemental data). The supernatant and possible remaining proteins from beads, once directly after incubation and once after three washing steps with 0.1M Tris buffer (beads were boiled in Lämmli buffer for elution) of both samples were analysed by 1D SDS-PAGE with silver staining. The two samples behaved similarly. In case of pyrrolidine, a small amount of protein could be detected after 3 washing steps, indicating a slight precipitating effect of pyrrolidine (see supplemental data). To prove the long term stability of the system, beads linked with the derivative alone, or linker coupled to bait were stored for 3 weeks in water containing 0.02% sodium azide at 4°C, respectively. After 3 weeks, no changes in the coupling rate could be detected for both samples.

Biological application

As a biological application we used the tyrosine kinase inhibitor bosutinib (SKI-606) [11] for enrichment of possible direct and indirect interactors from a K562 cell lysate. Bosutinib is an ATP-competitive third generation kinase inhibitor for the treatment of chronic myeloid leukemia (CML) [12], which is currently in phase III of clinical trials. Former investigations revealed SRC and ABL kinases as the main interactors of bosutinib, but also a lot of other affinity targets could be found [12-14]. The drug in its amino functionalized form (scheme 2) was kindly provided by the Center of Molecular Medicine, Austria (CeMM). The applicability of this functionalized form of bosutinib for targeted protein pulldowns was shown in a recent publication from Superti-Furga's group [13]. A coupling rate of 50% could be achieved with DMT-MM as coupling agent. Ready-made beads had an overall coverage of 0.7 µmol of bosutinib per ml beads suspension. The cleavage product (IAA-MDA-c-bosutinb) resulting from elution by pyrrolidine was found and confirmed by MS (see supplemental data). The efficacy of the c-bosutinb coupling reaction was calculated by the amount of remaining IAA after cleavage with pyrrolidine compared to an uncoupled control.

Affinity Purification

Protein pull down experiments were performed at the Center of Molecular Medicine on a K562 cell lysate according to a protocol from Rix et al. [15] with the exception that instead of eluting interactors by boiling with Lämmli buffer, elution was performed with 100mM pyrrolidine and incubation for 3h at 4°C. The higher amount of pyrrolidine (100mM instead of 50mM in previous experiments) was chosen to compensate for higher sample complexity and the lower incubation temperature (4 °C instead of RT in previous experiments). This step was at this point not optimized with regards to concentration needed to carry out a complete cleavage.

Immunoblot analysis

Immunoblot analysis was performed by 1D SDS-PAGE separation of the eluates (an elution by boiling beads with Lämmli buffer was used for comparison). A monoclonal anti BTK antibody was used for detection. The tyrosine kinase BTK is one of the main interactors of Bosutinib. The immunoblot is shown in figure 1, demonstrating the enrichment of BTK by affinity purification. Hence, the applicability of the purification protocol using indole linker beads could be successfully demonstrated (line C). In terms of efficacy a stronger signal could be achieved using the indole-beads compared to the reference (line D). This strong enrichment of BTK suggests that even lower concentrations of pyrrolidine might be used for cleavage if desired.

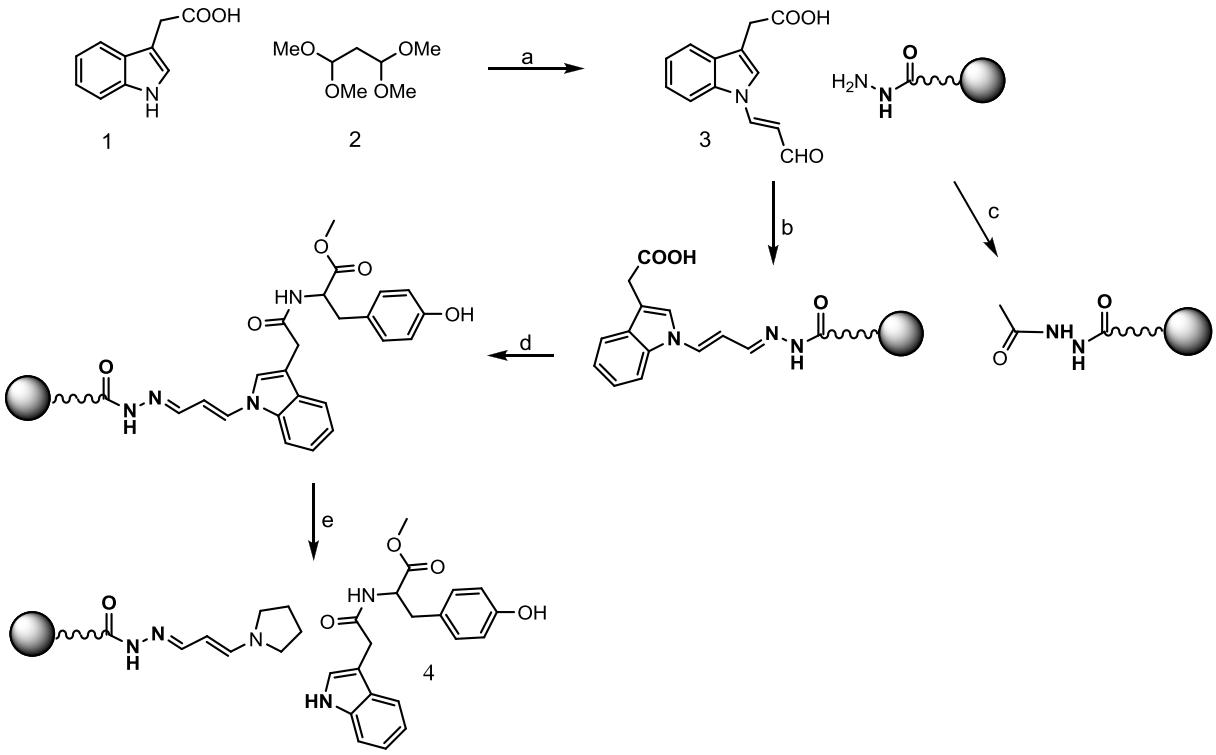
Liquid chromatography mass spectrometry

Along with the immunoblot analysis a LC-MS/MS protein identification of the pull-down was realized on a LTQ-Orbitrap XL mass spectrometer to identify specific interaction partners and to determine the degree of unspecific binding. Prior to LC-MS, 2D gel electrophoresis with tryptic in gel digestion of separated proteins was performed. For procedure information the reader is referred to the work of K. Bennett [15] and collegues.

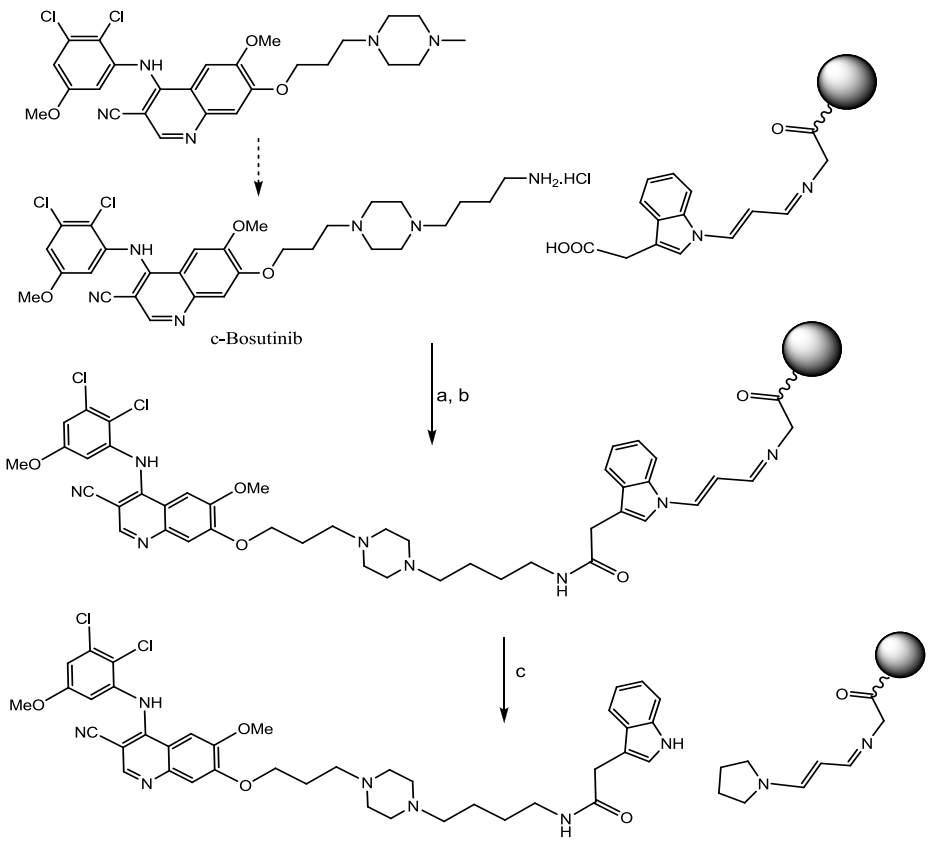
Target purification using the indole linker system allowed the identification of 32 targets of bosutinib (Table 1). Main interactors like SRC, ABL, BTK or TEC were successfully identified but also newly found targets like CAMK2G could be identified [16] (Table 1). However, also a high degree of unspecific bound proteins were found (see supplemental data). Agarose and acrylamid gels behaved similarly, whereby the latter showed a higher degree of unspecific binding.

Conclusion

A new linker chemistry, based on a reversible reaction of malondialdehyde with the indolyl group of 3-indolyl-acetic acid was demonstrated for an affinity chromatography concept in form of a pull-down protocol. The linker was coupled to hydrazide functionalized agarose beads via an α,β-unsaturated aldehyde group, and baits can be coupled to the linker via an amide groups with DMT-MM as coupling agent. A protein pull down was successfully performed using bosutinib as bait for purifying interaction partners of a K562 cell lysate. Efficient purification of the main target BTK was shown by immunoblot analysis. All main interactors could be identified by LC-MS/MS analysis. To further reduce unspecific binding of unwanted proteins the binding, washing and elution protocol may be further improved. The reduction of the pyrrolidine concentration used during cleavage seems particularly reasonable due to the high enrichment efficiacy achieved for the bosutinib target Btk when using 100mM pyrrolidine, compared to the purification using a noncleavable linker.



Scheme 1. Linker synthesis. (a) Synthesis of the cleavable linker building block. Reagents and conditions: Tenfold excess of tetramethoxypropane added dropwise to 3-indolyl-actetic acid in 80% TFA, 1h RT, 40% yield. (b) Coupling of linker to hydrazide functionalized agarose gel. Reagents and conditions: 50% acetonitrile/50% 100mM ammonium acetate pH 4.5, 10mM aniline as catalyst, 2h RT, 50% recovery rate. (c) Saturation of remaining hydrazide groups. Reagents and conditions: 30x molar excess of N-acetoxy succinimide in 50% acetonitrile/50% 50mM sodium phosphate pH 7.8 as buffer, 2h RT. (d-e) Coupling and release of affinity probe. Reagents and conditions: (d) MeOH, four fold excess of TyrOMe and 40x excess of DMT-MM for 1.4µmol linker groups per ml agarose beads, 3h RT; (e) 50mM pyrrolidine, 2h RT, 50% coupling efficiacy (determined with LC-MS/MS, see supplemental data).



Scheme 2. Coupling of c-bosutinib to linker. Reagents and Conditions: (a) Amino functionalized bosutinb was converted from HCl salt into free amino from by conversion with 3x excess of DIPEA, MeOH, 1h RT; (b) 10 times excess of DMT-MM to bosutinib was used, MeOH, 3h RT, slight agitation of beads, 50% coupling efficiacy. (c) pyrrolidine cleavage.

Table 1. List of direct interactors of bosutinib identified by affinity chromatography using the indole linker system (coupled to agarose- and acrylamide beads) and LC-MS/MS protein identification (LTQ-Orbitrap XL mass spectrometer). Complete list of identifications see supplemental data.

IPI Ac	Entrez Ac	Gene symbol	peptide counts - acrylamide beads	peptide counts - agarose beads
IPI00296337	5591	PRKDC, ISOFORM 1 OF DNA-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT.	189	116
IPI00031410	2475	FRAP1, FKBP12-RAPAMYCIN COMPLEX-ASSOCIATED PROTEIN.	65	45
IPI00029132	695	BTK, TYROSINE-PROTEIN KINASE BTK.	32	34
IPI00013212	1445	CSK, TYROSINE-PROTEIN KINASE CSK.	20	21
IPI00413961	5747	PTK2, PTK2 PROTEIN TYROSINE KINASE 2 ISOFORM B.	8	15
IPI00293613	29110	TBK1, SERINE/THREONINE-PROTEIN KINASE TBK1.	9	14
IPI00003783	5605	MAP2K2, DUAL SPECIFICITY MITOGEN-ACTIVATED PROTEIN KINASE KINASE 2.	9	11
IPI00003479	5594	MAPK1, MITOGEN-ACTIVATED PROTEIN KINASE 1.	10	8
IPI00219604	5604	MAP2K1, DUAL SPECIFICITY MITOGEN-ACTIVATED PROTEIN KINASE KINASE 1.	8	8
IPI00787531	2580	GAK, SIMILAR TO CYCLIN G-ASSOCIATED KINASE.	5	8
IPI00216378	818	CAMK2G, CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II GAMMA ISOFORM 1.	5	7
IPI00827625	817	CAMK2D, ISOFORM DELTA 11 OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE TYPE II DELTA CHAIN.	2	7
IPI00000878	7006	TEC, TYROSINE-PROTEIN KINASE TEC.	6	6
IPI00234463	1453	CSNK1D, ISOFORM 2 OF CASEIN KINASE I ISOFORM DELTA.	8	5
IPI00883914	5571	PRKAG1, AMP-ACTIVATED PROTEIN KINASE, NONCATALYTIC GAMMA-1 SUBUNIT ISOFORM 2.	7	5
IPI00029263	2241	FER, PROTO-ONCOGENE TYROSINE-PROTEIN KINASE FER.	3	5
IPI00377174	816	CAMK2B, CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE IIB ISOFORM 4.	2	5
IPI00029643	51776	NA, ISOFORM 2 OF MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE MLT.	5	5
IPI00872474	4067	LYN, LYN PROTEIN (FRAGMENT).	0	4
IPI00873480	4216	MAP3K4, UNCHARACTERIZED PROTEIN MAP3K4.	0	4
IPI00848174	NA	NA, SIMILAR TO CASEIN KINASE I ISOFORM DELTA.	14	4
IPI00411818	25989	ULK3, UNC-51-LIKE KINASE 3.	8	3
IPI00742900	5595	MAPK3, MITOGEN-ACTIVATED PROTEIN KINASE 3 ISOFORM 2.	4	3
IPI00513803	10746	MAP3K2, MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 2.	2	3
IPI00183400	1452	CSNK1A1, CASEIN KINASE I ISOFORM ALPHA.	3	2
IPI00410287	5562	PRKAA1, PROTEIN KINASE, AMP-ACTIVATED, ALPHA 1 CATALYTIC SUBUNIT ISOFORM 2.	2	2
IPI00006752	9448	MAP4K4, ISOFORM 1 OF MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE KINASE 4.	2	2
IPI00472717	3654	IRAK1, ISOFORM 2 OF INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE 1.	0	2
IPI00217437	146057	TTBK2, TAU-TUBULIN KINASE.	0	2
IPI00328867	6714	SRC, OTTHUMP00000030931.	0	2
bcrabl	NA	NA, Gene_Symbol=BCR-ABL fusion protein	0	2
IPI00412057	55589	BMP2K, ISOFORM 2 OF BMP-2-INDUCIBLE PROTEIN KINASE.	3	0
IPI00029196	5588	PRKCQ, ISOFORM 1 OF PROTEIN KINASE C THETA TYPE.	2	0
IPI00002857	1432	MAPK14, MITOGEN-ACTIVATED PROTEIN KINASE 14 ISOFORM 2.	2	0
IPI00384765	9088	PKMYT1, MEMBRANE-ASSOCIATED TYROSINE- AND THREONINE-SPECIFIC CDC2-INHIBITORY KINASE.	2	0

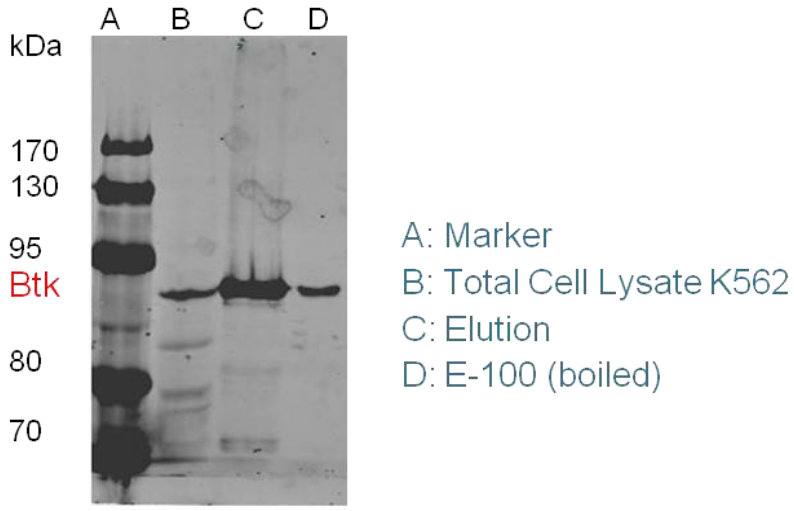


Figure 1. Immunoblot analysis of pull down experiment on K562 cell lysate. The bait was immobilized C-Bosutinib. Primary antibody (AB): monoclonal anti-BTK (Santa Cruz Biotechnology, Santa Cruz, CA); Secondary AB: Alexa Fluor 680 goat anti-mouse. Lines: (A) protein marker, (B) total cell lysate, (C) elution with 100mM pyrrolidine, (D) elution by boiling with Lämmli buffer.

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16. Rix, U., et al., A comprehensive target selectivity survey of the BCR-ABL kinase inhibitor INNO-406 by kinase profiling and chemical proteomics in chronic myeloid leukemia cells. Leukemia. 24(1): p. 44-50.

Curriculum Vitae

Persönliche Daten

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Nationalität	Österreich
Geburtsdaten	Klagenfurt, 31.10.1975
Familienstand	ledig

Ausbildung

10/06-06/10 Dissertation am Institut für Analytische Chemie und Lebensmittelchemie in der Arbeitgruppe von Prof. W. Lindner

Thema der Dissertation:

„ Preparation, implementation and evaluation of affinity chromatographic tools for targeted proteome identification by liquid chromatography – tandem mass spectrometry“

Die Dissertation wurde im Rahmen des APP II (Austrian Proteomics Platform II) durchgeführt.
Das Dissertationsthema umfaßte die Entwicklung und Evaluierung von porösen Metalloxidmaterialien zur selektiven Phosphopeptidanreicherung, sowie Phosphopeptidanreicherung unter Verwendung von Ionentauscher und Mixed Mode Phases im Ionenaustauscher- und HILIC-Modus. Evaluierung (Quantifizierung und Identifikation) mittels Flüssigkeitschromatographie und ESI-

Massenspektrometrie (Agilent 1200 HPLC System gekoppelt mit Applied Biosystems 4000 QTRAP, Agilent 1100 Series nano LC/MSD Trap);
Weiters die Entwicklung eines chemisch spaltbaren Linkers gekoppelt mit Wirkstoff an Agarose- oder Acrylamidbeads für Protein Pulldownexperimente.
Evaluierung der Kopplungs- und Abspaltraten mittels LC-MS² im MRM-Modus (Agilent 1100 HPLC system, Applied Biosystems 365).
Test des Konzepts mit Bosutinb-Wirkstoff an K562-Zellen am Center für Molekulare Medizin (CeMM).

11/05-08/06 Mitarbeit in der Arbeitsgruppe von Prof. Dr. G. Ammerer am Max F. Perutz Laboratories zur Weiterentwicklung eines genetischen Proteininteraktionassays für Modellorganismus S.Cerevisiae mit dem Ziel der Möglichkeit einer massenspektrometrischen Detektion.
Die praktische Arbeit beinhaltete die Konstruktion von Enzym/Substrat-getaggten Zielproteingenkonstrukten in E. coli, Durchführung des Assays und Proteinexpression in S. cerevisiae. Auswertung mittels Western Blot und Massenspektrometrie.

09/05 Abschluss des Chemiestudiums mit dem Titel Magister der Naturwissenschaften, mit Auszeichnung bestanden.

03/04-07/05 Diplomarbeit am Institut für spezifische Prophylaxe & Tropenmedizin am Zentrum für Physiologie und Pathophysiologie der Medizinischen Universität Wien unter der Betreuung von Ao. Univ. Prof. Dr. Ursula Wiedermann-Schmidt zum Thema: *„Mucosal vaccination against type I allergy: a new treatment approach using lactic acid bacteria as delivery systems for allergens“.*
Praktische Tätigkeit beinhaltete Herstellung von Zellkulturen von Mausmilzzellen, Evaluierung der Zellulären Cytokinantwort mittels ELISA, Immunoglobulin-Subklassen-ELISA, Western Blot, Proliferationskontrolle durch Thymidineinbau.

10/96-09/05 Studium der Chemie an der Universität Wien
Schwerpunkt: Biochemie, Analytik

09/90-10/96	Höhere Technische Bundeslehranstalt, Abteilung für Elektrotechnik
09/82-06/87	Realgymnasium Lerchenfeldstrasse 22, 9020 Klagenfurt

Berufspraxis

Seit Sept. 2009	Mitarbeiter der Firma Österreichisches Forschunginstitut - OFI am Projekt „COIN-QUANTUM“ zur massenspektrometrischen Detektion und Quantifizierung von Kunststoffadditiven.
11/05-03/09	Wissenschaftlicher Mitarbeiter an der Universität Wien und Lehrbeauftragter (Betreuung von Grund- und Fortgeschrittenenpraktika im Bereich Biochemie und Analytik).
03/04-07/05	Bezahlte Diplomarbeit an der Medizinischen Universität Wien.
05/03-09/03	Fa. Fluka Production GmbH, CH-9471 Buchs/Schweiz; Abteilung Qualitätskontrolle: Massenanalytische Gehaltsbestimmung mittels Titration (NaOH, HCl, Komplexometrie); UV-Spektroskopie (Gehaltsbestimmung, Qualitätsbeurteilung von Farbstoffen und org. Lösungsmittels); CHN-Analyse (Elementaranalyse) von organischen, anorganischen und biochemischen Substanzen. Während des Studiums Anstellungen im Gastgewerbe, Fixanstellung bei Firma T-Mobile im Bereich Verkauf, Promotiontätigkeit für Firma One, Siemens.

Vorträge, Posterpräsentationen, Workshops

01/09	Vortrag am Doktorandenseminar des AK Separation Science in Hohenroda zum Thema: *„Porous SnO_2 Microspheres a new Material for Phosphopetide Enrichment“*.
05/08	Vortrag am 4. ASAC JunganalytikerInnen Forum in Wien zum Thema*: „Porous SnO_2 Microspheres a new Material for Phosphopetide Enrichment“*.
02/08	Teilnahme am Glycoproteomics Workshop am Institut für Pharmazeutische Wissenschaften in Graz.
01/08	Posterpräsentation am 5. International Symposium of the Austrian Proteomics Platform in Seefeld, Tirol.
06/07	Vortrag am 3. ASAC Junganalytikerforum in Linz *„SnO_2 Microspheres als Material zur Phosphopeptidanreicherung“*
05/07	Teilnahme am Workshop *“Practical course about quantitative Proteomics”* am Institut für Molekulare Biotechnologie (IMBA) in Wien.

Publikationen

Sturm, M., A. Leitner, et al. (2008). "Tin dioxide microspheres as a promising material for phosphopeptide enrichment prior to liquid chromatography-(tandem) mass spectrometry analysis." Advanced Functional Materials **18**(16): 2381-2389.

Leitner, A., M. Sturm, et al. (2009). "Optimizing the performance of tin dioxide microspheres for phosphopeptide enrichment." Analytica Chimica Acta **638**(1): 51-57.

Leitner, A., M. Sturm, et al. "Probing the phosphoproteome of HeLa cells using nanocast metal oxide microspheres for phosphopeptide enrichment." Anal Chem **82**(7): 2726-33.

Sturm, M., A. Leitner, W. Lindner (2010). „A chemically cleavable linker for immobilizing bait compounds in a target protein pull down concept". Submitted to Bioconjugate Chemistry.

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Zusätzliche Qualifikationen

Sprachen	Englisch: fließend
EDV-Kenntnisse	Word, Exel, Powerpoint, Windows, Analyst, Chemstation.
Führerschein	Klasse B
Hobbies	Sport (Rennrad, Mountainbike, Badminton, Snowboard) Literatur, Theater, Film, Reisen, Kochen.