Development of phosphopeptide enrichment techniques for phosphoproteome analysis†

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Protein phosphorylation is one of the most biologically relevant and ubiquitous post-translational modifications. The analysis of protein phosphorylation is very challenging due to its highly dynamic nature and low stoichiometry. In this article, recent techniques developed for phosphoproteome analysis are reviewed with an emphasis on the new developments in this field in China. To improve the performance of phosphoproteome analysis, many novel methods, either by application of new separation mechanisms or by adoption of new separation materials, were developed to specifically enrich phosphopeptides from complex protein digests. A series of new materials, including nanostructure materials, magnetic materials, and monolithic materials, were applied to prepare immobilized affinity chromatography or metal oxide affinity chromatography to improve the performance of phosphopeptide enrichment. Besides, new software tools were also developed to validate phosphopeptide identification and predict kinase specific phosphorylation sites.

1. Introduction

Protein phosphorylation is one of the most biologically relevant and ubiquitous post-translational modifications (PTMs), and it is a reversible event affecting both the folding and function of proteins, regulating essential functions such as cell division, signal transduction and enzymatic activity. It is estimated that one third of all proteins is phosphorylated *in vivo* in mammalian cells at any time point.[1,2] There are four different types of phosphoamino acid residues: *O*-phosphates (*O*-phosphomonoesters), *N*-phosphates (phosphoamidates), acylphosphates (phosphate anhydrides) and *S*-phosphates (*S*-phosphothioesters). Among them, *O*-phosphates are by far the most abundant, mostly attached to serine, threonine, and tyrosine residues.[3,4] The occurrence of phosphorylation on Ser and Thr residues is more frequent than that on Tyr residues, with the ratio of pSer/pThr/pTyr in the order of 1800 : 200 : 1.[2,5] The protein *N*-phosphorylation on His/Lys residues, *S*-phosphorylation on Cys and acyl-phosphorylation on Glu/Asp residues also occur in different organisms, although they are less abundant.[6]

The analysis of protein phosphorylation has been recognized as one of the

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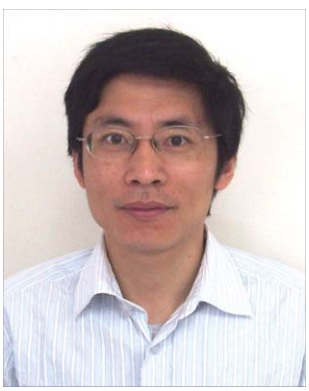

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most challenging tasks due to its highly dynamic nature and low stoichiometry. The development of new analytical methods for phosphoproteome analysis has drawn great attention all over the world. Protein phosphorylation has been conventionally analyzed by using radioactive labeling followed by 2D gel electrophoresis,[7] phosphopeptide mapping by thin-layer chromatography[8,9] or electrophoresis,[9,10] sequencing by Edman degradation,[11,12] or site-directed mutagenesis.[13–18] Recently, mass spectrometry (MS) has emerged as a key technology for mapping protein phosphorylation sites, owing to its high sensitivity and speed of analysis.[19–21] Phosphopeptides are fragmented in MS to pinpoint the specific Ser, Thr, or Tyr modified by a protein kinase. MS-based proteomics provides a framework for large-scale identification and characterization of phosphorylation sites.

Comprehensive analysis of the phosphoproteome is one of the most exciting and challenging tasks in current proteomics research. Owing to its highly dynamic nature and low stoichiometry, analysis of protein phosphorylations requires an enrichment method to decrease complexity of the samples and increase the detection sensitivity for low-abundant phosphoproteins.[20] At the protein level, tyrosine-phosphorylated proteins have been successfully enriched by immunoprecipitation,[22–26] but the application of this approach to serine- and threonine-phosphorylated proteins is limited. Enrichment strategies on the level of phosphopeptides have the advantage of identifying both the protein and the phosphorylated residues. Enrichment of phosphopeptides can be achieved by two types of methods. The first type is chromatography-based methods which include immobilized metal ion affinity chromatography (IMAC) (with Fe^{3+}, Ga^{3+}, $Ni^{2+}$and Zr^{4+} metal ions),[27–36] metal oxide affinity chromatography (MOAC) (with TiO_2, ZrO_2, Al_2O_3 and Nb_2O_5),[37–43] ion-exchange chromatography[44–49] and hydrophilic-interaction chromatography (HILIC).[50] The second type is chemical reaction-based methods. Phosphopeptides could be covalently conjugated to a polymer support and then released[51] or covalently attached with an affinity tag followed by affinity purification.[52,53] After enrichment, phosphopeptides are characterized by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS or submitted to liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis for the identification of phosphopeptides and phosphorylation sites. Recently, it has been demonstrated that different methods are complementary and can be combined to provide an aggregated data set which is larger than that obtained by single method alone.[54] By combining different methods, thousands of identified phosphoproteins and their phosphorylation sites from a single biological sample have been reported.[55,56]

The technologies for phosphoproteome analysis are developing rapidly. A series of novel methods, either by application of new separation mechanisms or by adoption of new separation materials, were developed for phosphopeptide enrichments. A new type of IMAC, *i.e.* immobilized Zr^{4+} or Ti^{4+} with phosphonate chelating groups, was developed, which has been demonstrated to be of extremely high specificity. A series of new materials including nanostructure materials, magnetic materials, and monolithic materials were applied to prepare IMAC or MOAC to improve the performance of phosphopeptide enrichment. Besides, new software tools were also developed to validate phosphopeptide identification and predict kinase-specific phosphorylation sites. In this review, we aim at offering an overview on the recent development of techniques for phosphoproteome analysis with an emphasis on new developments in this field in China.

2. Phosphopeptide enrichment methods with new mechanisms

Mainly two types of separation mechanisms were applied to enrich phosphopeptides by chromatography-based methods. The first type is based on the strong specific interaction between metal ions and phosphate groups on the molecule of phosphopeptides. Both IMAC and MOAC belong to this type. For IMAC, adsorbents with chelating ligands such as iminodiacetic acid (IDA, a tridentate metal-chelator) and nitrilotriacetic acid (NTA, a quadradentate metal-chelator) are typically used for the immobilization of Fe^{3+} or Ga^{3+} *via* chelating to the carboxylic groups and amino groups on IDA or NTA ligands. It has been observed that those conventional IMAC methods lack enough specificity for enrichment of phosphopeptides.[57] Acidic peptides and peptides containing histidine are also enriched which suppress the detection of phosphopeptides. The poor specificity may be partly because the chelating ligands not only bind phosphate groups but also the carboxylic and amino groups on the peptides. In order to reduce the non-specific binding to the IMAC resin, methyl-esterification of the carboxyl group prior to IMAC enrichment was reported by Ficarro *et al*.[57] Nevertheless, incompleteness and side reactions of the derivatization often complicate MS analysis and subsequent data interpretation, leading to decreased sensitivity. Recently, optimized IMAC protocols to improve its performance have been reported. Imanishi *et al.*[58] have optimized phosphopeptide elution conditions for Fe^{3+}-IMAC and found that the combination of phosphoric acid and acetonitrile generated an excellent IMAC eluent for subsequent MALDI-MS and electrospray ionization mass spectrometry (ESI-MS). Instead of using IMAC with Fe^{3+}, higher specificity was observed for MOAC using some metal oxides such as TiO_2 and ZrO_2.[38,59–61] Considerable efforts have been made to improve the MOAC protocol by introducing hydroxy acids such as 2,5-dihydroxybenzoic acid (DHB),[37,38] β-hydroxypropanoic acid,[60] phthalic acid,[54] ammonium glutamate[62] and glutamic acid[63] in the loading and washing buffer. However, big phosphopeptides may not be enriched effectively by metal oxides due to the presence of steric hindrance, and inorganic adsorbents are not suitably biocompatible in principle. Therefore, it is of interest to develop immobilized Ti^{4+} or Zr^{4+} affinity chromatography adsorbents by using new chelating ligands on polymer materials.

The second type of separation mechanism is based on the electrostatic interaction between phosphopeptides and a chromatographic stationary phase. Since phosphopeptides have extra negatively charged groups, they have a weaker interaction than non-phosphopeptides with strong cation-exchange chromatography (SCX). Therefore, they will be eluted in early fractions in SCX and can be separated from non-phosphopeptides which are more strongly retained on the SCX

column.[44,45,64] As many as 2002 phosphorylation sites in the nuclear fraction of HeLa cell lysate were located after analysis of the 40 phosphopeptide fractions resulting from the SCX runs of the tryptic digests from ten regions of the gel.[45] However, the application of SCX for phosphopeptide enrichment has its disadvantages. This approach is not very specific for phosphopeptides as strong acidic peptides are also eluted in the first few fractions. To further improve the specificity, IMAC was used to purify phosphopeptides from the early eluted SCX fractions.[64,65] Some phosphopeptides, especially those with multiple phosphate groups, have net negative charge and may not bind to the SCX column. An alternative way is the use of strong anion-exchange chromatography (SAX). Phosphopeptides are strongly retained on the SAX column because of their negatively charged groups.

2.1 IMAC with phosphonate chelating groups

In metal(IV) phosphonate chemistry such as Zr^{4+} or Ti^{4+}, it is well known that the layer structure of the metal phosphonate can be obtained by MO_6 octahedra,[66] with each Zr or Ti sharing six oxygen atoms with different monohydrogen phosphate groups. In turn, each monohydrogen phosphate group behaves as a tridentate anionic ligand and shares three oxygen atoms with three different metal atoms.[67] Therefore, each metal ion coordinates to more than one phosphate molecule and the phosphates bind to more than one metal ion. The extremely strong binding of the zirconium ions or titanium ions stacks the original monolayer and provides a very stable, well-defined interface of metal phosphonate sites, where the metal ions located on the surface are very active and readily react with other phosphate groups and bind them to the surface.[68] It is reasonable to think that if Zr^{4+} or Ti^{4+} is immobilized onto a material with its surface-containing phosphate groups as ligands, the resulting IMAC adsorbents should also be able to enrich phosphopeptides. In addition, the unique coordination specificity of Zr or Ti ions immobilized on the phosphate materials may also greatly improve the selectivity of phosphopeptide binding by preventing acidic peptide binding.

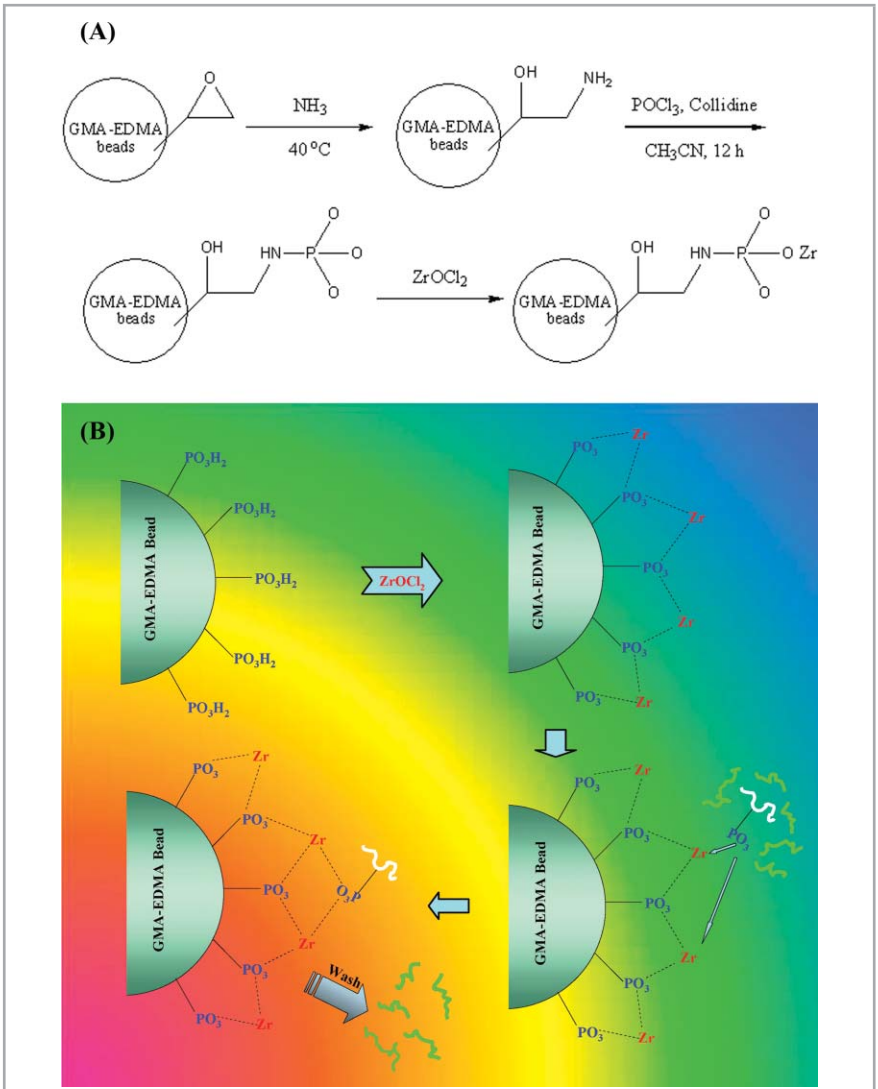

Fig. 1 (A) Scheme for the preparation of Zr^{4+}-IMAC beads; (B) scheme for the enrichment of phosphopeptides by using Zr^{4+}-IMAC beads. [Part (A) reproduced with kind permission from ref. 31. Copyright 2007, American Society for Biochemistry and Molecular Biology.]

Based on the above mechanism, Zr^{4+}-IMAC was successfully developed in our lab recently.[31,69] Fig. 1(A) gives the scheme for the preparation of polymer bead-based Zr^{4+}-IMAC adsorbents. Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (GMA-EDMA) polymer beads were selected to prepare such adsorbents because they have neutral hydrophilic surface and epoxide groups. Epoxide groups on the polymer surface were first transferred to amino groups by incubation in ammonium hydroxide solution. Phosphonate groups were subsequently introduced by reaction of amino groups with $POCl_3$. Finally, Zr^{4+} was immobilized by incubation of the modified beads in $ZrOCl_2$ solution. Compared with inorganic supports such as silica beads, GMA-EDMA polymer beads have the advantage of good chemical stability in a wider pH range. The scheme for the isolation of phosphopeptides by Zr^{4+}-IMAC is shown in Fig. 1(B), which is very similar to other IMAC methods. Peptide samples are typically loaded at low pH and the captured phosphopeptides are eluted at high pH. The high specificity of the Zr^{4+}-IMAC adsorbent was demonstrated by effectively enriching phosphopeptides from the digest mixture of phosphoprotein (α- or β-casein) and bovine serum albumin (BSA) at a molar ratio of 1 : 100. The Zr^{4+}-IMAC adsorbent was also successfully applied to the analysis of mouse liver phosphoproteome which

resulted in the identification of 153 phosphopeptides (163 phosphorylation sites) from 133 proteins in mouse liver lysate. Significantly more phosphopeptides were identified than that of the conventional Fe^{3+}-IMAC procedure, which indicated the excellent performance of the Zr^{4+}-IMAC approach.

Recently, Ti^{4+}-IMAC was also successfully developed in a similar way in our lab.[70] It was demonstrated that the resulting Ti^{4+}-IMAC can specifically isolate phosphopeptides from the digest mixture of standard phosphoproteins and non-phosphoproteins even at a molar ratio of 1 : 500 (phosphoproteins/non-phosphoproteins). Its performance was comprehensively compared with four other phosphopeptide isolation methods including Fe^{3+}-IMAC, Zr^{4+}-IMAC, TiO_2 and ZrO_2. It was found that Ti^{4+}-IMAC had the best selectivity and Fe^{3+}-IMAC was the poorest for enrichment of the phosphopeptides. It was found that the immobilized metal ions (Ti^{4+}-IMAC and Zr^{4+}-IMAC) have a better performance than their corresponding metal oxides (TiO_2 and ZrO_2). This is because the presence of the spacer arm in IMAC reduced the steric hindrance and facilitated the binding of the phosphopeptides, and polymer beads improved the compatibility with biological samples. In conventional IMAC, metal ions like Fe^{3+} were immobilized by use of chelating groups such as IDA or NTA. However, in Zr^{4+}- or Ti^{4+}-IMAC, phosphonate groups are acting as the chelating group which may be one reason that Zr^{4+}- or Ti^{4+}-IMAC has a higher specificity. Besides, the specific interaction of immobilized Zr^{4+} or Ti^{4+} with phosphate groups on phosphopeptides also accounts for the high specificity.

2.2 Strong ion-exchange chromatography (SAX)

Phosphopeptides are more acidic than non-phosphopeptides due to the extra negative charges on the phosphate groups. Therefore, phosphopeptides will bind more strongly than non-phosphopeptides on SAX. To the best of our knowledge, only a few publications have been reported for the application of SAX in phosphoproteomics analysis. Nühse *et al.* used SAX chromatography with salt gradient elution as a pre-fractionation approach before IMAC for the analysis of phosphopeptides from *Arabidopsis*,[49] and found that the 2D separation decreases the complexity of samples by the IMAC method and yields a far greater coverage of phosphorylated peptides. A 'Yin-Yang' multidimensional liquid chromatography coupled with a mass spectrometry system[46] was developed by Zeng *et al.* to profile protein phosphorylation. Protein digests were first loaded on an SCX column, and the flow-through peptides from SCX were collected and further loaded onto a SAX column. It was found that SCX cannot bind with most of the phosphorylated peptides if their precursor p*I* values are below 4.5. Loss of many phosphopeptides is unavoidable if only SCX is used to enrich phosphopeptides. However, phosphopeptides in the SCX flow-through could be recovered by SAX and many more phosphopeptides were identified. A recent study by Nühse *et al.* indicated that SAX and SCX pre-fractionation preferentially enriched different subsets of phosphopeptides.[48] These studies demonstrated the potential for the application of SAX in the enrichment of phosphopeptides.

Recently, we systematically investigated the performance of SAX in enriching phosphopeptides.[71] Tryptic digests of proteins were directly loaded onto a SAX column and then separated under gradient elution. It was found that the phosphopeptides were strongly retained on the SAX column and the majority of non-phosphopeptides were presented in the flow-through fraction. It was also observed that the more phosphate groups the phosphopeptides have, the longer it takes for them to be eluted, which indicated that SAX has the ability to fractionate phosphopeptides based on their different interactions with the SAX beads. The performance of SAX for phosphoproteome analysis was compared with Fe^{3+}-IMAC by analysis of digest of human liver lysate. Totals of 47 and 24 unique phosphopeptides at the false discovery rate (FDR) of less than 1% were identified with SAX and Fe^{3+}-IMAC, respectively, by one-step elution of the adsorbed phosphopeptides on those materials. Fewer phosphopeptides were identified by Fe^{3+}-IMAC, probably because the weak interactions between singly-phosphorylated peptides with the Fe^{3+}-IMAC beads resulted in loss of some of these peptides. Compared with conventional methods for the isolation of phosphopeptide by Fe^{3+}-IMAC, a significant advantage of SAX is that not only can it specifically enrich phosphopeptides but it can also fractionate phosphopeptides to reduce the complexity of the samples for large-scale phosphoproteome analysis. SAX was further applied to enrich and fractionate phosphopeptides in the tryptic digest of proteins extracted from human liver tissue adjacent to tumorous region for phosphoproteome profiling. This resulted in the highly confident identification of 274 phosphorylation sites from 305 unique phosphopeptides corresponding to 168 proteins at the FDR of 0.96%.

3. Phosphopeptide enrichment with new adsorbent materials

The performance of phosphopeptide enrichment also strongly depends on the physico-chemical properties of materials used to prepare the affinity absorbents. Because of their unique properties, nanomaterials were utilized for phosphopeptide enrichment. For example, recent work by Chang *et al.* reported that 100 nm polyarginine-coated diamond nanoparticles[72] were used for the selective extraction and enrichment of multiphosphorylated peptides based on multiple arginine–phosphate interactions. New materials were also designed to facilitate the procedure of phosphopeptide enrichment. For example, magnetic materials were prepared to enrich phosphopeptides to conveniently and efficiently separate absorbents from solution,[41,42,73,74] Blacken *et al.* prepared zirconium oxide-coated plates[75] and Dunn *et al.* prepared gold plates with an Fe^{3+}-NTA complex[76] to facilitate the MALDI-TOF analysis of phosphopeptides. Several research groups in China have explored many new materials including nanostructure materials, magnetic materials, *etc.*, for phosphopeptide enrichment. Some of these materials made of metal oxides such as ZrO_2 and TiO_2 could be applied directly for phosphopeptide enrichment. For other materials, the immobilization of metal ions or coating with a layer of metal oxides is required.

3.1 Nanostructure materials

The phosphopeptides on IMAC beads could be detected directly by MALDI-TOF MS which avoided possible sample

loss during elution. However, because of the unavoidable 'shadow effect' of the porous beads at the micron scale, most of the phosphopeptides bound in the pores would probably be inaccessible during the direct laser desorption. Therefore, for MALDI-MS analysis, it is preferable to prepare IMAC with all the chelation occurring on the surface of the nanoparticles with a large external surface area. Based on this consideration, Tang and Yang's group prepared Fe^{3+}-immobilized zeolite nanoparticles[77] to isolate phosphopeptides with direct analysis by MALDI-TOF MS/MS. It was demonstrated that these Fe^{3+}-nanozeolites with a large external surface area and high dispersibility could facilitate the MALDI identification of phosphopeptides. Mesoporous molecular sieve MCM-41 has been widely studied in the chemistry of materials because of its structural simplicity and ease of preparation. For its large pore volumes and very high surface area, MCM-41 shows the adsorptive capacity of more than an order of magnitude higher than that of conventional adsorbents.[78–80] We prepared a type of IMAC on nanoparticles by chemical modification of MCM-41.[81] The highly ordered MCM-41 with a particle size of *ca.* 600 nm and pore size of *ca.* 3 nm was synthesized, and Fe^{3+} was then immobilized on its surface through the modification of silicate hydroxyl (silanol) groups with IDA. The specificity and performance of the Fe^{3+}-immobilized MCM-41 was demonstrated by selectively enriching phosphopeptides from the tryptic digest of phosphoprotein standards as well as mouse liver lysate.

The nanostructure materials which could be used directly for the enrichment of phosphopeptides are metal oxides such as ZrO_2, TiO_2 and Fe_2O_3. We have developed a phosphopeptide enrichment method using ZrO_2 nanoparticles with an average size of 20–30 nm.[39] The study showed that ZrO_2 nanoparticles enabled the specific enrichment of phosphopeptides from a complex peptide mixture. ZrO_2 nanoparticles were also applied to selectively isolate phosphopeptides from the tryptic digestion of mouse liver lysate for phosphoproteome analysis by nano-LC–MS^2 and MS^3, where 248 phosphorylation sites and 140 phosphorylated peptides were identified. Because of their tiny size, nanoparticles are difficult to be packed into chromatographic columns due to the high back-pressure and lack of the appropriate frits. To solve this problem, Chen and co-workers reported the preparation of TiO_2 nanocomposites which could be packed in cartridges to achieve phosphopeptide enrichment.[82] In their study, TiO_2 nanoparticles were silanized and photopolymerized in the presence of a diacrylate cross-linker. The cross-linking of the TiO_2 particles with organic groups to form the composites helps to prevent the loss of the particles during flushing of the solutions through the packed cartridges. It was found that the TiO_2 nanocomposites have twice as much phosphate binding capacity and 2–5 times the capture efficiency compared to 5 μm TiO_2 particles. Recently, Feng and co-workers have deposited a thin layer of TiO_2 nanoparticles onto the surface of a capillary column by a liquid phase deposition technique and applied this to selectively concentrate phosphopeptides from protein digest products.[83]

While Fe^{3+}-IMAC is the most common IMAC, its corresponding metal oxide, Fe_2O_3, is seldom reported for the enrichment of phosphopeptides. Recently, Zhao and Yang's group have synthesized mesoporous Fe_2O_3 microspheres[84] for the enrichment of phosphopeptides under mild conditions in a relatively short time. They presented a process of polymerization (urea and formaldehyde)-induced ferric hydroxide colloid aggregation to prepare mesoporous Fe_2O_3. The obtained materials showed microspherical morphology with a particle size of *ca.* 3 μm and large pore size of *ca.* 48 nm. The affinity of Fe_2O_3 particles for phosphopeptides was demonstrated by enriching phosphopeptides from tryptic digests of two standard phosphoproteins. The high surface area, large pore size and porosity enabled the mesoporous Fe_2O_3 microspheres to achieve fast and efficient enrichment of phosphopeptides.

Another type of nanostructure material, porous anodic alumina (PAA) membrane,[85] was also used to selectively enrich phosphopeptides from a peptide mixture, as demonstrated by Xia, Guo and co-workers. The PAA film consists of a hexagonal close-packed array of *ca.* 200 nm-diameter channels. The pore size of the PAA membrane is uniform and the interval of the pores is almost equal. It was demonstrated that the PAA membrane could enrich phosphopeptides with high efficiency and selectivity. For example, phosphopeptides in the tryptic digest product of α-casein at a concentration as low as 4×10^{-9} M can be satisfactorily enriched and detected by MALDI-TOF MS.

3.2 Magnetic materials

Because of their high surface area, nanoparticles have strong potential for sample pre-treatment. However, the isolation of nanoparticles from the sample solution is not efficient. It may take a long time to centrifuge these particles down in sample solutions because of their tiny size. Magnetic materials, which can be isolated readily from the sample solutions by employing a magnetic field, have drawn attention for the enrichment of phosphopeptides. Chen *et al.* prepared nanocomposite magnetic particles coated with Al_2O_3,[41,86] TiO_2,[41,87] ZrO_2[88] as the affinity probes to selectively concentrate phosphopeptides. Only a short time is required to enrich the phosphopeptides into sufficient amounts for MALDI-MS analysis when these magnetic materials are used. Recently, Zhang and Deng's group presented a new approach for the synthesis of Fe_3O_4@TiO_2 microspheres with a well-defined core–shell structure.[73] The scheme is shown in Fig. 2. First, magnetite microspheres are synthesized *via* a solvothermal reaction. Then, the magnetite microspheres are coated with a thin layer of carbon by the polymerization and carbonization of glucose through a hydrothermal reaction, resulting in Fe_3O_4@C microspheres. Finally, tetrabutyltitanate is pre-hydrolyzed and absorbed onto the microspheres, and eventually converted into titania by calcination under nitrogen. It was found that the outer shell of the obtained Fe_3O_4@TiO_2 microspheres consisted of a large amount of titania nanoparticles, which provided a high specific area and were beneficial for the specific trapping of phosphopeptides. Besides Fe_3O_4@TiO_2 microspheres, Fe_3O_4@Al_2O_3,[42] Fe_3O_4@Ga_2O_3,[89] Fe_3O_4@ZrO_2[56] core–shell microspheres have also been synthesized in a similar way. These microspheres were successfully utilized to enrich phosphopeptides for phosphoproteome analysis.

In addition to magnetic materials coated with metal oxide, magnetic materials with immobilized metal ions were

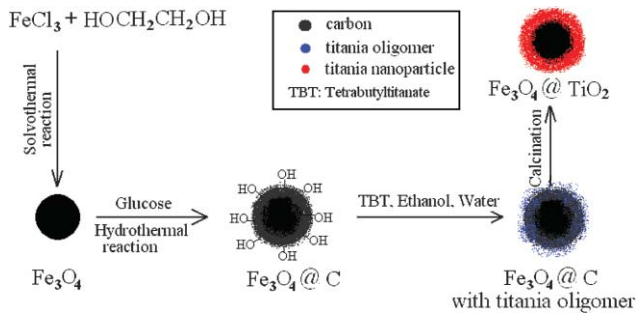

Fig. 2 Synthetic route to Fe_3O_4@TiO_2 core–shell microspheres. (Reproduced with kind permission from ref. 73.)

also prepared to enrich phosphopeptides. Zhang, Deng and co-workers reported the synthesis of Fe^{3+}-immobilized magnetic silica microspheres for the enrichment of phosphopeptides.[90] In their study, silica generated from the hydrolysis and condensation of tetraethyl orthosilicate (TEOS) was coated onto magnetite microspheres, and the chelating groups, *i.e.* IDA, were coupled onto the silica surface by chemical modifications. The resulting magnetic silica microspheres had core–shell structure, and the magnetic core was *ca.* 300 nm in diameter and the silica shell was *ca.* 70 nm in thickness. After immobilization of Fe^{3+}, the microspheres were successfully used for the enrichment of phosphopeptides followed by direct MALDI-TOF MS analysis. Recently, Ce^{4+} was immobilized onto magnetic silica microspheres and their performance for the enrichment of phosphopeptides was reported[91] for the first time. It was found that Ce^{4+}-IMAC exhibited more selective isolation ability than Fe^{3+}-IMAC for concentrating phosphopeptides from complex mixtures. Qian and co-workers have synthesized Fe^{3+}-immobilized magnetic silica microspheres with an average diameter of 15 nm.[92] After evaluation of the phosphopeptide enrichment performance using tryptic digests of α-casein as the sample, these nanoparticles were further applied to analyze the phosphoproteome of the plasma membrane of mouse liver which resulted in the identification of 217 unique phosphorylation sites corresponding to 158 phosphoproteins. Recently, we synthesized the zirconium phosphonate-modified magnetic Fe_3O_4@SiO_2 core–shell nanoparticles,[93] and successfully applied them to selectively capture phosphopeptides from a complex tryptic digest of a mixture of α-casein and BSA at a molar ratio of 1 : 100 for MALDI-TOF MS analysis. The zirconium phosphonate-modified magnetic nanoparticles were further applied to mouse liver lysate combining with nano-LC–MS^2 and MS^3 analysis, and a total of 194 unique phosphopeptides were identified at the FDR of <1%. It is interesting that when the eluents were collected by rinsing the nanoparticles with 6% TFA, most of the identified phosphopeptides in this step were singly-phosphorylated peptides. When the NH_4OH solution was subsequently used, almost all the identified phosphopeptides in this step were multiply-phosphorylated peptides just like those reported by Larsen and co-workers recently.[94] Magnetic silica microspheres with immobilized Zr^{4+} for phosphoproteome analysis were also synthesized by Qian's group in a similar way,[95] and were combined with SCX for the phosphoproteome analysis of Chang liver cell lysate.

3.3 New target materials for MALDI analysis

The IMAC nanoparticles with captured phosphopeptides could be placed on a MALDI target plate for direct analysis. However, some of these nanoparticles may detach from the target plate during MALDI analysis and deposit in the chamber, which is detrimental to the MS instrument. To solve this problem, TiO_2 nanoparticles were sintered on a stainless steel plate for phosphopeptide enrichment and MALDI analysis by Liu and co-workers.[96] An array of sintered TiO_2 nanoparticle spots on a stainless steel plate provided a porous substrate with a very large specific surface and durable functions. These spots were used to selectively capture phosphorylated peptides from peptide mixtures, which could then be analyzed directly by MALDI MS after washing away the non-phosphorylated peptides. In this strategy, the steps of phosphopeptide capture, purification, and subsequent mass spectrometry analysis are all successfully accomplished on a single target plate, which greatly reduces sample loss and simplifies analytical procedures.

Instead of placing nanoparticles on a target plate, silicon chips with an affinity surface could be attached onto the plate for MALDI analysis. We have prepared porous silicon wafers with an immobilized Fe^{3+} affinity surface to analyze phosphopeptides by MALDI-TOF MS.[97] IDA-1,2-epoxy-9-decene was synthesized and covalently linked to the surface of a porous silicon wafer through a photochemical reaction, and then Fe^{3+} was immobilized *via* chelating with IDA. After sample loading and washing, the silicon chip with captured phosphopeptides was attached to a MALDI plate for analysis. Selective enrichment of phosphopeptides was observed. To further improve specificity, we also prepared porous silicon wafers with an immobilized Zr^{4+} affinity surface[69] (see Fig. 3). The porous silicon wafer was first modified with phosphonate groups, and then Zr^{4+} was immobilized *via* chelation. The excellent selectivity of this approach was demonstrated by analyzing phosphopeptides in the digest mixture of β-casein and BSA at a molar ratio of 1 : 100. High detection sensitivity has been achieved for the analysis of the phosphopeptides from the tryptic digest of 2 fmol α-casein on the silicon wafer surface.

4. Phosphopeptide enrichment with monolithic capillary column

MALDI MS can be applied to characterize and detect phosphopeptides presented in the digest of only one or a few proteins. However, a phosphopeptide mixture enriched from the digest of complex protein samples such as total cell lysate is extremely complex. Thus separation of phosphopeptides prior to MS analysis is required. In practical application, nanoflow RPLC–MS/MS, where a 75 μm i.d. separation column is typically used, is often applied to analyzing these samples. Considering the small size of the sample used, a nanoliter IMAC column is preferable for the enrichment of

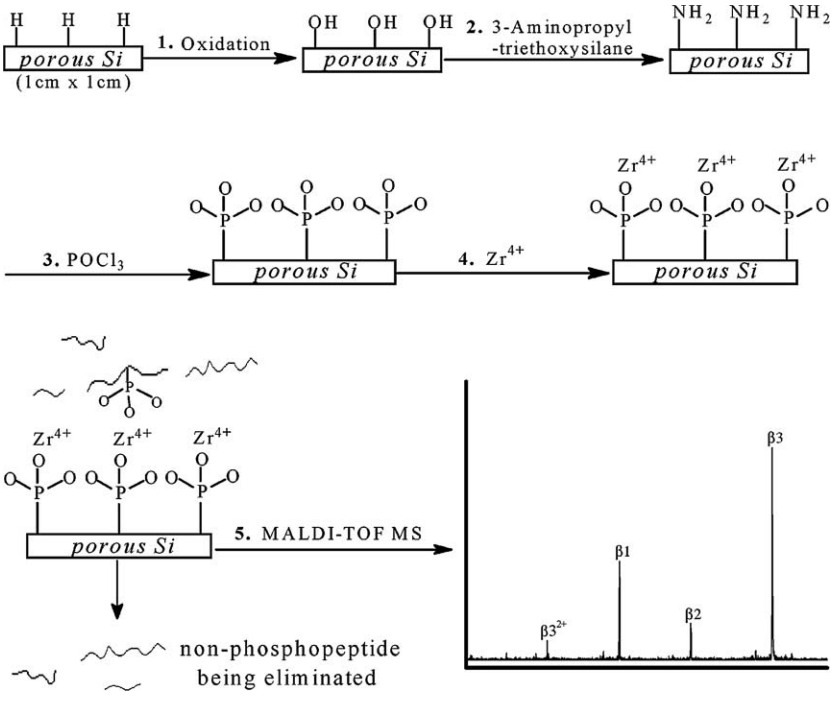

Fig. 3 Scheme for preparation of affinity silicon wafer with immobilized Zr^{4+} to trap phosphopeptides for MALDI-TOF MS analysis. (Reproduced with kind permission from ref. 69. Copyright 2006, American Chemical Society.)

phosphopeptides when only a limited sample is available. However, packing a nanoliter IMAC column with beads is labor-intensive and often difficult. Monolithic materials were developed as an alternative to bead-based columns for chromatographic separation. The key advantages of monolithic columns include the ease of preparation and modification, adjustable porosity, fast mass transport, and low column back-pressure under high flow rate.[98–101] Monoliths are also good support materials for IMAC.The macroporous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith was prepared in a HPLC column, and Cu^{2+} was immobilized *via* IDA for the purification of lysozyme from egg white.[102] For the enrichment of phosphopeptides, two types of monolithic capillary IMAC column were reported recently. These columns could be coupled directly onto nanoflow RPLC–MS/MS for phosphoproteome analysis.

4.1 Silica monolithic capillary column

Silica-based monoliths have small-sized skeletons with a large specific surface area, and a bimodal pore size distribution with millimeter-sized through-pores and nanometer-sized mesopores, which provides silica-based monoliths with favorable properties of a low-pressure drop across the column, fast mass transfer kinetics, and high binding capacity.[103–107] We have prepared an IMAC capillary column by chemical modification of a silica monolith with IDA followed with the immobilization of Fe^{3+} ions inside the capillary.[29] It was demonstrated that the Fe^{3+}-IMAC silica monolithic IMAC capillary column can specifically capture the phosphopeptides from a tryptic digest of α-casein for analysis by MALDI-TOF MS. The prepared silica monolithic IMAC capillary column had the dimensions of 75 μm × 100 mm and a volume of less than 0.45 μL, which makes the silica monolithic IMAC capillary column readily couple with nanoflow RPLC–MS/MS. After loading with tryptic digest, the IMAC column was washed thoroughly to remove non-specifically adsorbed peptides, and then manually connected to the 75 μm separation column. The retained phosphopeptides on the IMAC column were eluted directly onto a C18 separation column by phosphoric buffer at high pH, and analyzed by nanoflow RPLC–MS/MS under gradient elution. The above approach was validated by using tryptic digests of standard phosphoproteins and mouse liver lysate. As the sample loss was reduced by directly eluting the phosphopeptides onto the C18 separation column, this approach was proved to be very suitable for the phosphoproteome analysis of minute samples.

4.2 Phosphate polymer monolithic column

The preparation of a silica-based IMAC column requires several chemical derivatization steps, which is labor-intensive and time-consuming. The silica-based monolith is not very stable at high pH, while the elution of phosphopeptides from IMAC usually uses alkaline solution. To circumvent these problems, we developed a novel approach to prepare an IMAC polymer monolithic capillary column for highly specific capture of phosphopeptides.[108] As shown in Fig. 4, a phosphate monolithic capillary column was prepared by direct copolymerization of the functional monomer-containing phosphate group and the cross-linker in a ternary porogenic solvent. Because the phosphate groups on the monomer do not take part in the polymerization, the resulting polymer contains a large number of phosphate groups on the surface. Zr^{4+} was then immobilized *via* chelating with the phosphate groups. The high specificity of this column was demonstrated by enriching phosphopeptides in the digest mixture of β-casein and BSA at a molar ratio of 1 : 200. In addition to the simplified procedure, the binding capacity of these columns can be easily controlled by adjusting the amount of functional monomer in the polymerizing mixture.

5. Software tools for phosphorylation analysis

With the availability of mass spectrometry methods to analyze complex biological samples on a large scale, computational tools to analyze and statistically evaluate data generated from LC–MS experiments are needed, thus catalyzing a new research direction in the field of bioinformatics.[21,109–111] A large number of software tools are now available that support various functionalities such as the pre-processing of MS data, evaluation and

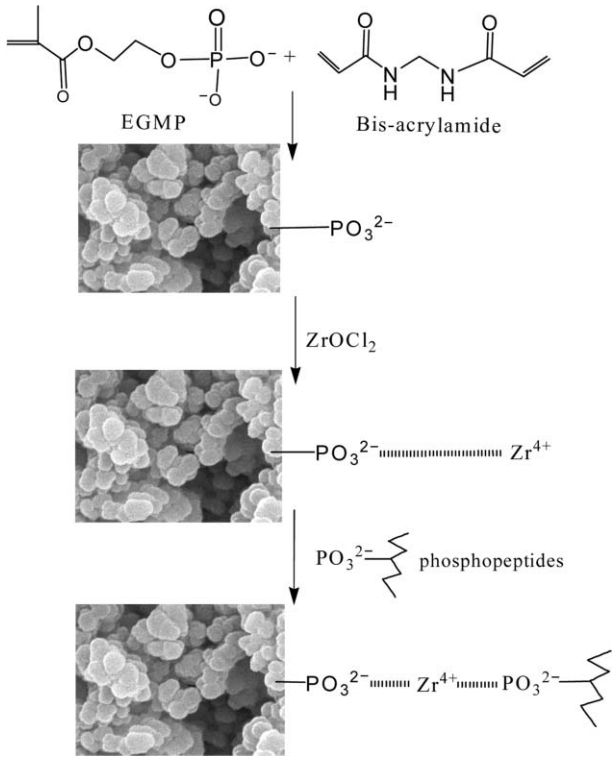

Fig. 4 Schematics for the preparation of phosphate polymeric monolithic column and its specific interaction with phosphopeptides. (Reproduced with kind permission from ref. 108. Copyright 2006, Wiley InterScience.)

assignment of MS/MS spectra to peptide sequences, comparison and quantification of multiple LC–MS experiments, as well as the integration of data generated by mass spectrometry with other available biological data resources. The analysis of the phosphoproteome is one of the most exciting and challenging tasks in current proteomics research. The huge amount of data generated will require the development of new and more robust and specific software tools.[2,56,112–125] Broadly speaking, there are two types of software tools developed for phosphoproteome analysis. The first type is developed to validate phosphopeptide identifications derived from database searching, and the second type is developed to predict the phosphorylation sites based on the primary protein sequence.

5.1 Validation of phosphopeptide identification

Identification of the peptide depends strongly on the quality of the MS^2 spectra. sHowever, the MS^2 spectra of phosphopeptides often lack enough fragment peaks because of neutral loss of phosphate groups. This reduces the ability of database searching algorithms to unambiguously identify phosphopeptides. To obtain more fragmentation information, the neutral loss ion in the MS^2 stage can be further fragmented to acquire the MS^3 spectrum. Both MS^2 and MS^3 spectra can be submitted to database searching for phosphopeptide identifications. Because of the poor spectrum quality, manual validation of these identifications is required to improve the identification confidence, which is time-consuming and not objective. Therefore, the development of new data processing methods or software tools to validate the phosphopeptide identifications automatically is needed. Methods incorporating a very high accuracy mass spectrometer with the MS^2 target-decoy search strategy have been reported to obtain high-confidence phosphopeptide identification and precise site location without manual validation.[2,121] However, the application of this approach is limited as mass spectrometers with very high mass accuracy are only available in a few labs. As a phosphopeptide independently identified by both MS^2 and its MS^3 spectra can be regarded with very high confidence,[126] another approach to automatically validate phosphopeptide identification is to combine MS^2 and MS^3 database search results. Nesvizhskii and co-workers presented a statistical model[113] for adjusting the peptide identification probabilities based on the combined information obtained by coupling peptide assignments of consecutive MS^2 and MS^3 spectra. We have also presented an approach, termed the MS^2/MS^3 target-decoy database search approach[127] or MS^2/MS^3 approach in short, for the automatic validation of phosphopeptide identification. The scheme is given in Fig. 5. Briefly, there are four modules in the software: (1) extraction of MS^2/MS^3 pairs with validated charge state; (2) performing MS^2 and MS^3 target-decoy database searches, separately; (3) reassignment of the peptide scores in SEQUEST output to generate a list of peptide identifications for pairs of MS^2/MS^3 spectra; (4) filtering the candidate phosphopeptides with new defined parameters (Rank'm, DelCn'm and Xcorr's) to achieve phosphopeptide identification with a specific FDR. Only phosphopeptides identified from both MS^2 and MS^3 were accepted for further filtering, which greatly improved the reliability in phosphopeptide identification. Because of the use of the target-decoy database, the FDR of the identified phosphopeptides could be easily determined and precisely reflect the actual FDR. Compared to the manual validation approach, the strength of this approach is that the confidence of the phosphopeptide identifications is high and the results are objective. However, this approach can only identify phosphopeptides with both MS^2 and MS^3 spectra. This will lead to a decrease of the number of phosphopeptide identifications as the phosphopeptides with only MS^2 spectra are not identified.

5.2 Prediction of phosphorylation sites

Large-scale phosphoproteome analysis could identify thousands of phosphorylation sites; however, it could not provide information on which kinases are responsible for these phosphorylations. Kinase-specific phosphorylation sites on the substrates could be determined by *in vivo* or *in vitro* phosphorylation experiments; however, these experiments

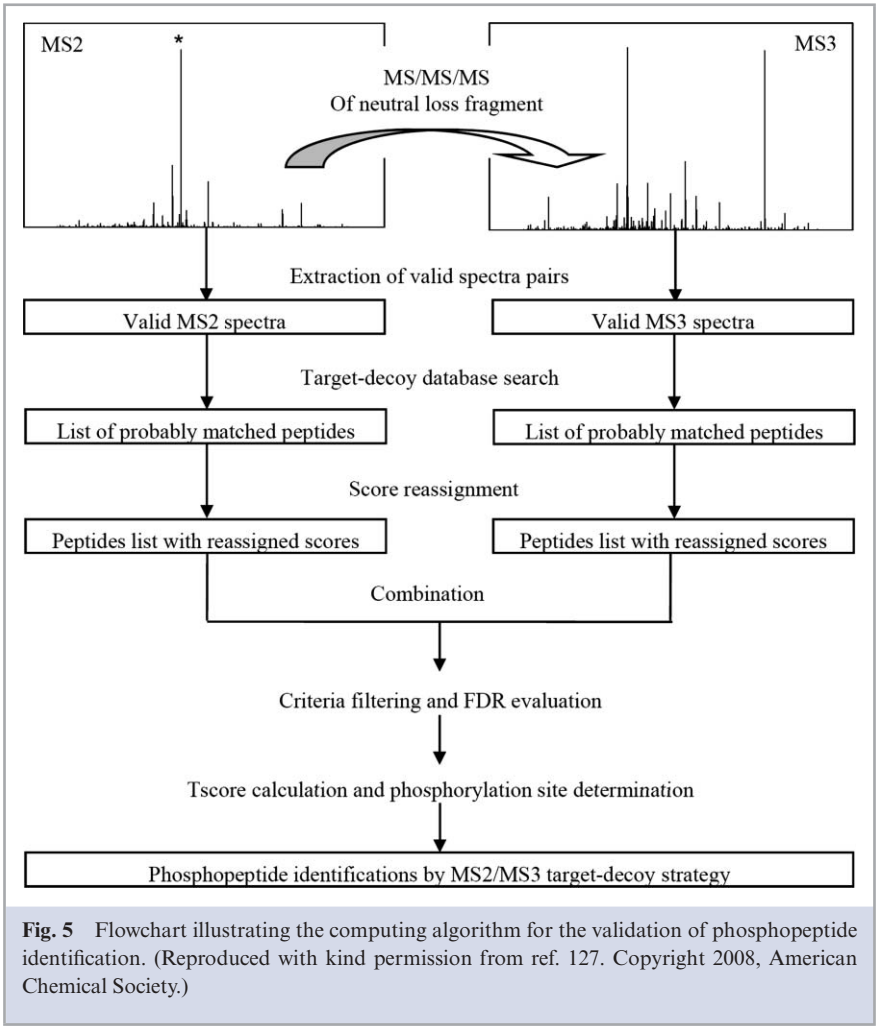

Fig. 5 Flowchart illustrating the computing algorithm for the validation of phosphopeptide identification. (Reproduced with kind permission from ref. 127. Copyright 2008, American Chemical Society.)

are labor-intensive and expensive. Because of its convenience and fast speed, *in silico* prediction of kinase-specific phosphorylation sites based on primary protein sequence is very desirable. Several excellent predictors, including GPS,[128] PPSP,[129] NetPhosK,[125,130] ScanSite,[118] KinasePhos,[122,123] and PredPhospho[131] have been implemented using various algorithms. Among them, GPS,[128] for group-based phosphorylation site prediction and scoring, was developed by Yao and co-workers. It was demonstrated that GPS offers greater precision and computing power over existing prediction systems. A comprehensive kinase-specific prediction server GPS (http://bioinformatics.lcd-ustc.org/gps_web/predict.php) can predict kinase-specific phosphorylation sites from protein primary sequences for 71 different protein kinase groups.[128,132] The same group also presented a program named PPSP[129] to predict protein kinase-specific phosphorylation sites with Bayesian decision theory (BDT). These are powerful tools for the experimentalists who are focusing on phosphorylation substrates with their protein kinase-specific site identification.

6. Conclusion and perspective

Phosphoproteome analysis depends heavily on the MS analysis of phosphopeptides. Because of the low stoichiometry of protein phosphorylation, phosphopeptides always co-exist with huge amounts of non-phosphopeptides which seriously depress the detection of the phosphopeptides. To specifically isolate phosphopeptides is one of the key issues in phosphoproteome analysis. A series of novel technologies have been developed recently to improve the performance of phosphopeptide enrichment. Selectivity and efficiency were improved by either employing new separation mechanisms or applying new adsorbent materials. Software tools have also been developed to validate phosphopeptide identifications and to predict phosphorylation sites, respectively. Despite the significant progress over the past decade, continuous improvements are still required to further overcome the complexity and dynamic range problem for phosphoproteome analysis. The development of highly efficient and automated platforms, including enrichment, elution and analysis, is needed for phosphoproteome analysis. New strategies for quantitative phosphoproteomics should be developed in order to analyze the evolution of the phosphoproteome across different cellular states. With the advancement of analytical techniques and detection methods, it can be expected that more insights for biological and medical research based on phosphoproteome analysis will be obtained in the future.

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