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INVITED REVIEW

Advances in phosphopeptide enrichment techniques for phosphoproteomics

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Abstract Phosphoproteomics is increasingly used to address a wide range of biological questions. However, despite some success, techniques for phosphoproteomics are not without challenges. Phosphoproteins are present in cells in low abundance relative to their unphosphorylated counterparts; therefore phosphorylated proteins (or phosphopeptides after protein digestion) are rarely detected in standard shotgun proteomics experiments. Thus, extraction of phosphorylated polypeptides from complex mixtures is a critical step in the success of phosphoproteomics experiments. Intense research over the last decade has resulted in the development of powerful techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. Here, we review how the development of IMAC, MOAC, chemical derivatization and antibody affinity purification and chromatography is contributing to the evolution of phosphoproteomics techniques. Although further developments are needed for the technology to reach maturity, current state-of-the-art techniques can already be used as powerful tools for biological research.

Keywords Mass spectrometry · Proteomics · Systems biology · Cell signaling

Abbreviations

2D	Two-dimensional
2,5-DHB	2,5-Dihydroxybenzoic acid
AA	Acetic acid
ACN	Acetonitrile
DDA	Data dependent acquisition
EGF	Epidermal growth factor
ESI	Electrospray ionization
FA	Formic acid
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IMAC	Immobilized metal affinity chromatography
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption ionization
MOAC	Metal oxide affinity chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
pS	Phosphorylated serine
pT	Phosphorylated threonine
pY	Phosphorylated tyrosine
RP	Reverse phase
SAX	Strong anion exchange
SCX	Strong cation exchange
SIMAC	Sequential elution from IMAC
TFA	Trifluoroacetic acid
TiO_2	Titanium dioxide

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Introduction

Phosphorylation is a reversible post-translational modification that serves as a key mechanism for the regulation of protein function (Cohen 2001; Manning et al. 2002; Ubersax and Ferrell 2007). Phosphorylation, which in eukaryotes usually occurs upon serine, threonine or tyrosine residues (Ubersax and Ferrell 2007), has the potential to induce conformational changes in protein structure thereby enabling the activation or deactivation of enzymes

(Olsen et al. 2006). In addition, phosphorylation can also affect cellular function by mediating recognition of proteins by interaction partners and by affecting their subcellular localization. The dynamics of protein phosphorylation is tightly regulated by kinases, which phosphorylate proteins, and phosphatases, which dephosphorylate proteins (Cohen 2001; Cutillas and Timms 2010). Proteins can be phosphorylated on multiple sites and each event may lead to a distinct biological output. Extrapolating from genomic data, there are potentially around 700,000 phosphorylation sites in a typical eukaryotic cell (Ubersax and Ferrell 2007). Empirical observations from phosphoproteomics experiments have so far identified around 100,000 phosphorylation sites in cells across all species, 27,000 of which are from human cells (Gao et al. 2010).

Protein phosphorylation plays a fundamental role in signal transduction and thus is a key regulator of essential cellular processes including metabolism, growth, cell cycle progression, migration and apoptosis (Cohen 2001; Manning et al. 2002; Ubersax and Ferrell 2007). Disruption of signalling pathways is associated with the pathology of many diseases including cancer, diabetes, autoimmune diseases and neurodegenerative conditions (Cohen 2001). Hence, understanding the activity of signal transduction pathways can lead to the identification of novel therapeutic targets and biomarkers. Such biomarkers may be used to stratify patients according to their defects in signalling, therefore enabling the implementation of targeted treatments based on the activity status and circuitry of affected cells (Cutillas and Jorgensen 2011).

Disruption to signalling pathways can arise through a number of mechanisms including mutations or deregulated expression of kinases or phosphatases, which result in increased or decreased enzymatic activity. In addition, there are many other mechanisms, such as substrate availability and epigenetic modifications that can also regulate pathway activity through protein phosphorylation. Thus, when designing methods to quantify signalling, a direct measurement of protein phosphorylation most faithfully represents signalling activity as protein phosphorylation is the end product of all of the potential molecular events that can lead to changes in kinase and phosphatase activities (Cutillas and Jorgensen 2011).

Protein phosphorylation can be quantified using immunochemical techniques. However, global mass spectrometry (MS) analysis of protein phosphorylation is currently the preferred phosphoproteomics approach. Indeed, this technique is emerging as a powerful method for the investigation of signalling pathway activity (Cutillas and Jorgensen 2011; Olsen et al. 2006). Current state-of-the-art MS-based techniques permit cross-comparison of thousands of phosphoproteins and phosphorylation sites in an unbiased manner, i.e. without preconceptions of the pathways or activities that may be affected. The method can also be extremely sensitive permitting the detection of phosphopeptides present in low femtomole levels (Pinkse et al. 2004; Stensballe et al. 2001) and, with more recent advances, even at low attomole levels (Dong et al. 2010).

Although MS is a very sensitive technique its dynamic range of detection is limited, especially when using discovery MS techniques such as data dependent acquisition (DDA), tandem mass spectrometry (MS/MS). Untargeted large-scale proteomics experiments do not normally identify or quantify a significant number of phosphorylated proteins. This is because the number of peptides from a complex mixture that are selected for MS/MS in DDA experiments is limited by the analyzer duty cycle. Due to this "under-sampling" effect, only the most abundant peptides are fragmented and many low-abundance peptides are not identified. This phenomenon is a particular problem for phosphoproteomics as phosphoproteins are usually present in very low stochiometry compared to their non-phosphorylated counterparts and are therefore less likely to be identified. In addition, commonly used fragmentation methods for MS/MS frequently result in the neutral loss of phosphate and/or phosphoric acid from phosphopeptides, thus reducing fragmentation efficiency and sequence information (Molina et al. 2007). Another challenge in phosphoproteomics is that phosphorylated peptides have been found to ionize with lower efficiency than non-phosphorylated peptides, using both electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) (Miliotis et al. 2001).

Methods to enrich phosphorylated proteins are thus required prior to analysis to successfully perform MS-based phosphoproteomics experiments. A plethora of techniques for the enrichment of phosphoproteins prior to MS analysis have emerged in the last few years and these have been instrumental in the development and success of phosphoproteomics. These techniques can be divided into two groups: those that perform the enrichment at the protein level (Fig. 1a) and those that enrich phosphopeptides after digestion of whole cell lysates by proteases (Fig. 1b).

One approach for phosphoproteomics involves enriching full-length phosphorylated proteins by affinity purification for phosphorylated sites (e.g. anti-phosphotyrosine antibody immunoprecipitation or by other chromatographic methods). The amounts of protein present in phosphoprotein-enriched eluents can then be quantified by MS (Blagoev et al. 2004; Cutillas et al. 2005). In this technique, phosphorylation sites are not necessarily identified or quantified directly. Instead, it is assumed that the relative amounts of proteins after enrichment are a reflection of their extent of phosphorylation.

More recently, technical advances have allowed the implementation of phosphoproteomic approaches in which

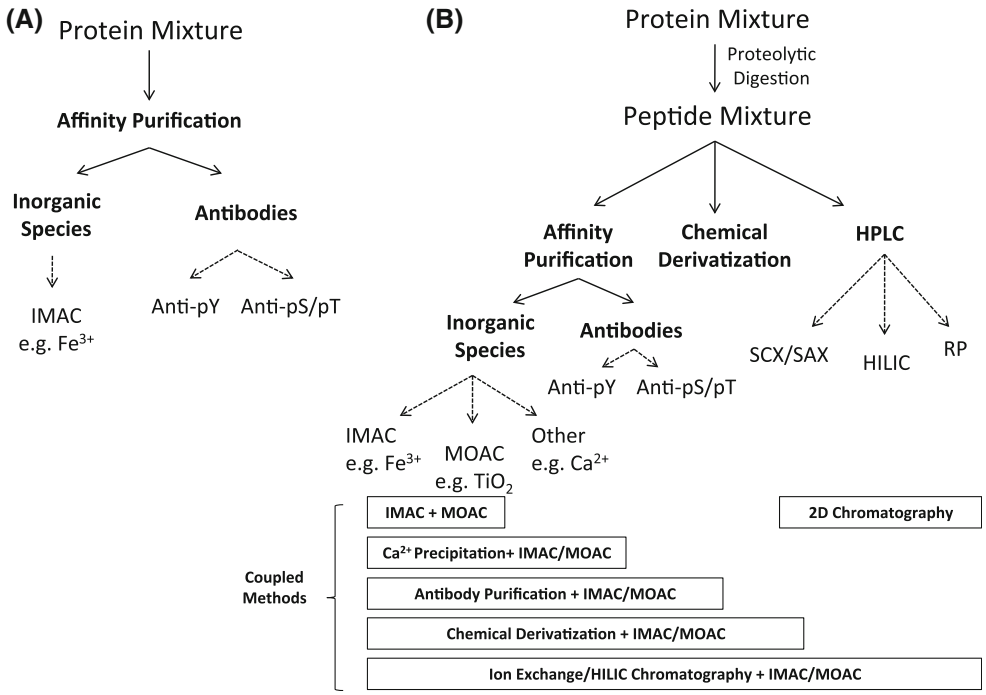

Fig. 1 Summary of enrichment strategies commonly used for phosphoproteins (**a**) and phosphopeptides (**b**)

the enrichment is performed at the peptide level. In these methods, purified proteins or crude protein mixes are subjected to proteolytic digestion, enrichment of the peptide mixture for phosphopeptides and subsequent analysis by MS and MS/MS. The resulting data permit the identification of peptides and any existing phosphorylated residues along with their quantification. Since by definition each phosphorylation site is the result of a kinase reaction, phosphorylation data can be used as a measure of signalling activity (Cutillas and Timms 2010). Thus, this technique permits the unprecedented opportunity to carry out the qualitative and quantitative evaluation of hundreds, or even thousands, of phosphorylation events in a single analysis. In addition, no prior knowledge of a phosphorylation site is necessary and novel sites can be concomitantly identified and quantified. However, the technique is not without challenges. Careful sample preparation and phosphopeptide enrichment, at the protein or peptide level, are essential for the success of these global MS-based phosphoproteomics methods.

This review aims to give an overview of current phosphopeptide and phosphoprotein enrichment techniques used in the field prior to MS analysis. It should be noted that mass spectrometric strategies to enhance phosphopeptide identification have also contributed significantly to advances in this field, but a detailed description of such techniques is beyond the scope of this review. For accounts on mass spectrometry approaches to enhance the identification of phosphorylation, we refer the reader to recent reviews that cover the topic in depth (Palumbo et al. 2011; Thingholm et al. 2009).

Techniques for phosphopeptide enrichment

Phosphorylation is a dynamic post-translational modification (Cohen 2001) and specimens must be handled appropriately to prevent protein degradation and loss of phosphate groups due to protease and phosphatase activity upon cell lysis (Thingholm et al. 2008b). Phosphatases are highly efficient and robust enzymes (Barford et al. 1998), therefore measures must be taken to restrict their activity. Cells or tissues should be lysed in the presence of appropriate phosphatase inhibitors to minimize phosphate hydrolysis (Reinders and Sickmann 2005; Thingholm et al. 2008b). It is also advisable that proteins in the sample should be rapidly denatured, for example by the use of chaotropes (Ahmed 2009). Phosphopeptide enrichment procedures can be lengthy and care should be taken to minimise steps that may enhance hydrolysis of phosphate groups, such as exposure to heat and high pH.

Proteolytic digestion of specimen proteins is a key step in phosphopeptide enrichment protocols and many different approaches have been reported (reviewed in reference (Cañas et al. 2007)). An important consideration when carrying out proteolytic digestion is that many detergents and reducing agents used in protein harvesting can inhibit protease activity. One approach to proteolytic digestion is

to first immobilize proteins in a solid support, for example using polyacrylamide gel electrophoresis (PAGE). The proteins embedded in gels are washed clean of interfering substances before digestion and then peptides are eluted after proteolysis. An example of an alternative method, which also makes use of a solid support for protein washing prior to digestion, is filter-assisted sample preparation (FASP) (Wisniewski et al. 2009). A different, more commonly used, approach is to carry out proteolytic digestion in solution (Cañas et al. 2007). In such methods, samples are typically diluted after protein solubilisation with chaotropes, such as urea or thiourea, to reduce the impact of interfering substances on digestion.

Selection of an appropriate protease for sample digestion is also important in phosphopeptide analysis. Trypsin, which cleaves proteins at the carboxyl terminus of arginine and lysine residues, is the most commonly used protease for protein digestion (Cañas et al. 2007). However, it should be taken into consideration that several features of phosphorylated peptides, including the frequent occurrence of basic residues adjacent to the phosphorylation site, possible interactions between the phosphate group and arginine or lysine residues on side chains and the presence of proline adjacent to the cleavage site, can impair the efficiency of trypsin digestion (Siepen et al. 2007). This phenomenon should be taken into account during data analysis by giving appropriate consideration to peptides with missed cleavage sites during identification and, if appropriate, quantification. Other proteases that are also widely used include: chymotrypsin, which cleaves proteins at tyrosine, tryptophan or phenylalanine; Lys-C, which cleaves proteins at the carboxyl terminus of lysine; and Asp-N, which cleaves proteins at the N-terminal side of aspartic acid. A combination of proteases results in more complete protein digestion (Wa et al. 2006). Also, when used in parallel experiments, the use of multiple proteases produces phosphopeptides of complementary sequences, resulting in greater coverage of the phosphoproteome. It is essential to select the most appropriate protease for the type of enrichment to be used. For example, hydrophilic interaction chromatography (HILIC) is poorly suited to trypsin digestion because internal arginine and lysine residues, which do not result from the use of this protease, are advantageous for separation (McNulty and Annan 2008).

This review focuses on the discussion of phosphopeptide enrichment techniques; however, it should be noted that some of the methods discussed are also applicable to phosphoprotein enrichment prior to proteolytic digestion. There are also many different formats in which to apply the phospho-enrichment methods discussed below. Methods for enriching phosphopeptides can be used off-line or on-line, where the enrichment method is coupled directly to the mass spectrometer. A wide range of affinity purification reagent solid phases is also available and includes beads, columns, tips and nanoparticles.

Affinity purification: inorganic species

Phosphorylated peptides or proteins can be purified from a mixture by exploiting the affinity of phosphate groups, which are negatively charged, for certain chemical species bound to solid affinity matrices. Such affinity reagents can be used as the solid phase of liquid chromatography methods. The most popular of these techniques are immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) (Fig. 2a).

Immobilized metal affinity chromatography (IMAC)

Enrichment of phosphopeptides by IMAC exploits the affinity of phosphate groups for transition metal ions, which include Cu^{2+} (copper), Ni^{2+} (nickel), Zn^{2+} (zinc), Fe^{3+} (iron), Co^{2+} (cobalt), Al^{3+} (aluminium) and Ga^{2+} (gallium) (Gaberc-Porekar and Menart 2001). The interaction is based on the coordinate bonds formed between the electron donor groups of the oxygen species in the negatively charged phosphate group and the metal ions (Gaberc-Porekar and Menart 2001). Transition metals also have affinity for nitrogen and sulphur containing functional groups; therefore, many amino acids, although principally histidine, also bind metal ions. Hence, IMAC was initially developed as a method for separating histidine-rich peptides and proteins from complex mixtures (Porath et al. 1975). The binding efficiency of metal ions for each reaction species varies greatly and thus selection of the appropriate ion and reaction conditions can enhance the specificity of IMAC for the desired purification process. For example, Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} show greater affinity for nitrogen containing functional groups whereas Fe^{3+}, Ga^{2+}, Al^{3+} and Ca^{2+} show greater affinity for oxygen-rich functional groups, such as phosphate groups (Gaberc-Porekar and Menart 2001). Fe^{3+} has a high affinity for phosphate groups and a relatively low affinity for carboxyl and phenolic groups (Muszynska et al. 1992), and thus is the most frequently used metal for phosphopeptide enrichment (Li and Dass 1999). Ga^{3+} has also been used successfully for phosphopeptide enrichment (Aryal et al. 2008; Posewitz and Tempst 1999) and other metals, including Zn^{2+} and Zr^{4+} (zirconium), have also been reported to be effective although their use has not been extensive (Feng et al. 2007; Kinoshita et al. 2005). IMAC has been used for the enrichment of both full length phosphoproteins and phosphopeptides derived from the proteolytic cleavage of proteins. Neville et al. (1997) were one of the first groups to report the use of Fe^{3+}-IMAC to enrich samples for phosphopeptides, which they applied to

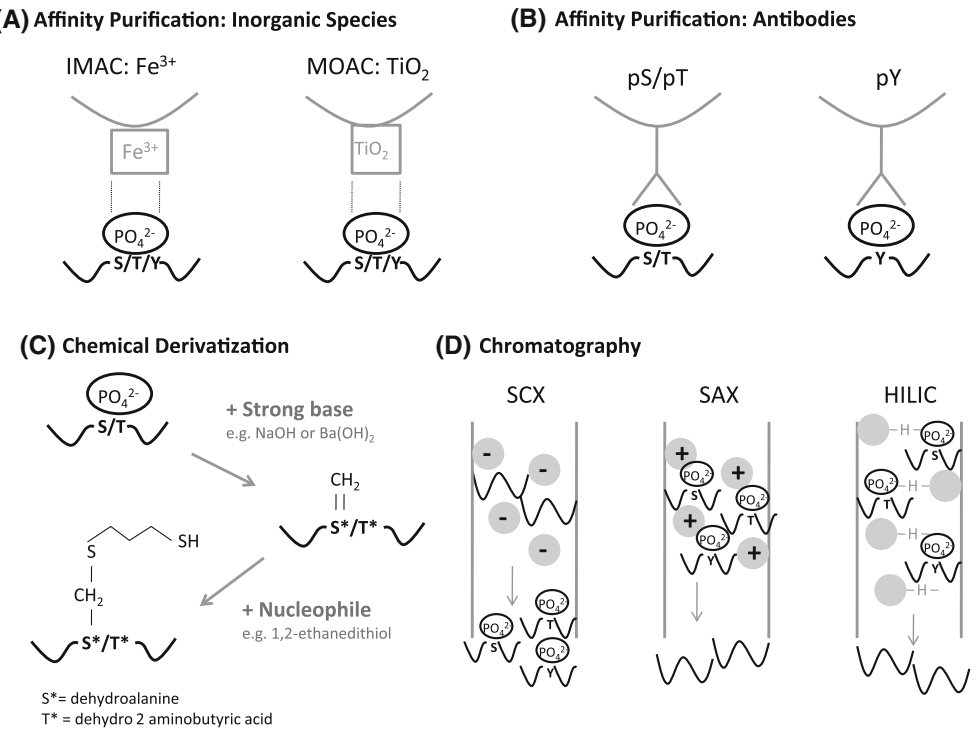

Fig. 2 Principles of commonly used phosphopeptide enrichment methods

identify sites of phosphorylation on purified cystic fibrosis transmembrane conductance receptor. Other early papers further demonstrated the utility of IMAC in phosphoproteomics (Anguenot et al. 1999; Ficarro et al. 2002; Hart et al. 2002; Li and Dass 1999; Posewitz and Tempst 1999; Stensballe et al. 2001).

One of the major limitations in the use of IMAC is that its specificity is limited by the concomitant binding of carboxyl groups to IMAC material, thus resulting in co-purification of acidic peptides alongside phosphopeptides. In this respect, IMAC material behaves like an anion exchange solid phase as changes in pH and ionic strength of solvents can be used to control the specificity of binding and elution. Theoretical calculations showed that an increase in the concentration of the organic solvent acetonitrile (ACN) reduced the ionization of acidic residues, such as aspartate and glutamate, whilst the ionization of phosphate groups was unaffected, thus enhancing the specificity of phosphopeptide binding to IMAC material (Ye et al. 2010). Indeed, a higher concentration of ACN was also demonstrated to increase the strength of binding between phosphopeptides from the tryptic digest of α and β casein and Fe^{3+} IMAC material (Ye et al. 2010). It was also found that disruption of the secondary structure of phosphopeptides with the fluorinated solvent 1,1,1,3,3,3-hexafluoroisopropanol led to a greater efficiency of phosphopeptide enrichment (Barnouin et al. 2005). These findings indicated that steric hindrance and secondary structure conformations are factors affecting the binding of phosphates to IMAC material.

Methods to increase the specificity of phosphopeptide binding to IMAC material relative to acidic peptides have been reported. Tsai et al. (2008) showed that modulation of pH can result in an increase in the specificity of phosphopeptide enrichment and, more specifically, that the pH range optimal for phosphopeptide binding to Fe^{3+} IMAC material is between 2.5 and 3.5. However, the pH value used must be carefully chosen to optimize specificity as well as sensitivity; lowering the pH was found to minimize the binding of non-phosphorylated peptides at the cost of decreasing the number of phosphopeptides binding due to incomplete deprotonation of the phosphate group (Tsai et al. 2008). Thus, acid choice and concentration, as well as pH, are important factors in determining the efficiency of IMAC. Formic acid (FA), known to enhance IMAC specificity through competition with non-phosphorylated peptides for binding, was also found to compete with phosphopeptides (Tsai et al. 2008). It was also demonstrated that loading under acidic conditions using 0.1 % trifluoroacetic acid (TFA) resulted in a loss of monophosphorylated but not multiply phosphorylated peptides in Fe^{3+} IMAC flow-through (Thingholm et al. 2008a). Therefore, the use of acids to improve IMAC specificity may result in the loss of some phosphopeptides, particularly those that are monophosphorylated.

A strategy to increase specificity of enrichment is to derivatise carboxylic acids on peptides by esterification

prior to IMAC, thus reducing the acidity of peptides. For example, Ficarro et al. (2002) treated tryptic peptide mixtures with methanolic hydrochloric acid to convert carboxyl groups to methyl esters, thus eliminating their interaction with Fe^{3+} IMAC material and improving selectivity for phosphopeptides. Another elegant strategy to increase specificity of enrichment by IMAC is to specifically elute phosphopeptides from the IMAC material, whilst leaving acidic peptides behind. For example, Thompson et al. (2003) used beta-elimination, a reaction that disrupts the bond between the peptide and the phosphate group, to specifically release phosphopeptides from IMAC material. Beta-elimination at phosphoserine and phosphothreonine groups results in the formation of dehydroalanine and dehydro 2 aminobutyric acid, respectively, thus permitting the precise identification of phosphorylated residues by MS/MS. However, as phosphotyrosine residues are not susceptible to beta-elimination, this method is only suitable for the enrichment of phosphorylated serine and threonine residues (Thompson et al. 2003).

IMAC material has also been shown to bind multiply phosphorylated peptides with higher affinity than that for singly phosphorylated peptides, thus producing a bias in phosphopeptide enrichment (Thingholm et al. 2008a; Ye et al. 2010). Phosphopeptides can be eluted from Fe^{3+} IMAC material under acidic conditions, for example using phosphoric acid or TFA, or under basic conditions, for example using ammonium hydroxide (Thingholm et al. 2008a; Ye et al. 2010). Monophosphorylated peptides were shown to elute from Fe^{3+} IMAC material with increasing efficiency as TFA concentration was increased, although multiply phosphorylated peptides were fully retained (Thingholm et al. 2008a). Basic conditions are more effective for the elution of multiply phosphorylated peptides (Thingholm et al. 2008a). Ye et al. (2010) have also suggested that time may be an important factor in elution efficiency, as their data found that acidic and multiply phosphorylated peptides eluted more rapidly than non-acidic monophosphorylated peptides. This paper, which examined the optimization of Fe^{3+} IMAC in depth, also noted that an excess of IMAC material did not appear to affect the selectivity of the method and that performance varied widely between materials from different suppliers (Ye et al. 2010).

Phosphopeptides can be eluted from Ga^{3+} IMAC material using a 0.075 % solution of ammonim hydroxide (pH 10), which is directly compatible with MALDI analysis (Posewitz and Tempst 1999). Furthermore, "direct analysis" of phosphopeptide-loaded Ni^{2+} IMAC resin applied to the target and mixed with 2,5-dihydroxybenzoic (2,5-DHB) matrix has also been reported (Hart et al. 2002). This analysis was shown to be possible through ligand-displacement of phosphopeptides from IMAC material by 2,5-DHB (Hart et al. 2002). Hart et al. (2002) also demonstrated that phosphopeptides can be eluted from Fe3+ IMAC material using 2,5-DHB matrix solution, although they reported a strong bias for monophosphorylated peptides. In further work, Stensballe and Jensen (2004) showed that the addition of phosphoric acid to 2,5-DHB matrix, when used as an elution solution, improved phosphopeptide analysis by increasing elution and enhancing phosphopeptide ion signals.

Whilst IMAC is widely used to enrich for phosphopeptides after proteolytic digestion of proteins, this technique has also been used to enrich for full length phosphoproteins prior to MS analysis. In one study, lymphoma cells were stimulated with a tyrosine phosphatase inhibitor, before and after treatment of cells with a PI3K inhibitor, and IMAC was then used to enrich for full length phosphoproteins (Cutillas et al. 2005). The relative amounts of all purified proteins were quantified by LC–MS and, in addition to finding potential new phosphoproteins downstream of PI3K, the study demonstrated the application of label-free MS to profile phosphorylation (Cutillas et al. 2005). In another study, the so-called "double IMAC" method was reported as a highly efficient enrichment technique and applied to identify phosphopeptides downstream of the EGF receptor (Cantin et al. 2008). This technique involved enriching phosphoproteins from total cell lysates using IMAC, before digesting these phosphoproteins and further enriching for phosphopeptides with a second round of IMAC (Cantin et al. 2008). Multiple rounds of IMAC purification at the peptide level have also been demonstrated to improve the efficiency of phosphopeptide enrichment, particularly in the recovery of monophosphorylated peptides (Ye et al. 2010).

Metal oxide affinity chromatography (MOAC)

An alternative form of affinity chromatography for phosphopeptide isolation is based upon the affinity of phosphate groups for metal oxides, sometimes referred to as MOAC. The interaction between phosphopeptides and metal oxides is based on the affinity of oxygen in the phosphate group for metal atoms. Titanium dioxide (TiO_2) is the chemical species most frequently used for MOAC, although zirconium dioxide (ZrO_2) can also be used. Both have been shown to have similar efficiency for the enrichment of singly phosphorylated peptides, however, TiO_2 shows better efficiency for the purification of multiply phosphorylated peptides (Aryal and Ross 2009).

Pinkse et al. (2004); Sano and Nakamura (2004) were the first groups to show that TiO_2 chromatography was suitable for the selective isolation of phosphopeptides from protein digests. Peptides from a purified phosphoprotein were separated using a column packed with porous titania

microspheres and phosphopeptide-rich fractions were analyzed by reverse-phase liquid-chromatography MS/MS (RP LC–MS/MS) (Pinkse et al. 2004). The authors reported 90 % enrichment for phosphorylated peptides (Pinkse et al. 2004). TiO_2 affinity purification has since also been demonstrated to be suitable for enriching phosphopeptides from more complex mixtures, including total cell lysates (Aryal and Ross 2009; Choudhary et al. 2009; Olsen et al. 2006; Wu et al. 2010).

As with IMAC, acidic residues also bind TiO_2 with high affinity, thereby resulting in the isolation of acidic peptides alongside phosphopeptides. The addition of organic acids to binding solvents minimizes the binding of carboxyl groups to TiO_2, thus resulting in greater specificity for phosphopeptides (Aryal and Ross 2009; Gates et al. 2010). Indeed, increasing concentrations of acetic acid (AA), FA and TFA, from 0.1 to 5 %, were all demonstrated to increase the specificity of isolation of phosphorylated peptides from a tryptic digest of α and β casein using TiO_2 (Aryal and Ross 2009). The same article confirmed that TFA, at an optimum concentration of 1 %, was the most efficient organic acid of those tried at reducing the binding of non-phosphorylated peptides, followed by FA then AA (Aryal and Ross 2009).

In addition to the use of acidic conditions during loading and washing steps, the use of non-phosphopeptide excluders in solution has been shown to improve the specificity of TiO_2 affinity purification. Non-phosphopeptide excluders are typically organic acids that bind metal oxides more weakly than phosphate groups, but more strongly than carboxyl groups (Aryal and Ross 2009), and include 2,5-DHB and glycolic acid. Larsen et al. (2005) were the first group to report on the use of 2,5-DHB to significantly enhance the specificity of phosphopeptide enrichment from casein tryptic digests using TiO_2 microcolumns. The same group also reported on the use of glycolic acid as a non-phosphopeptide excluder to significantly improve the selectivity of TiO_2 affinity purification (Thingholm and Larsen 2009). However, results reported from the use of these compounds are variable. Aryal and Ross (2009) found no increase in the recovery of phosphopeptides from a tryptic digest of α and β casein using 2,5-DHB or glycolic acid in loading solution and reported that the latter increased the recovery of non-phosphorylated peptides. Mazanek et al. (2007) also reported that 2,5-DHB, when used at high concentrations, caused high background in MS spectra and ionization suppression. However, when DHB was used at a low concentration alongside the sodium salt of 1-octane-sulfonic acid, a second ion-pairing agent, the efficiency of purification of phosphorylated peptides from a bovine serum albumin digest using TiO_2 was significantly improved (Mazanek et al. 2007). Chemical derivatization has also been applied to help overcome specificity limitations and it has been shown that esterification of carboxyl groups reduced the retention of highly acidic peptides by TiO_2 (Pinkse et al. 2004).

Phosphopeptides are typically eluted from TiO_2 using basic solutions. NH_4OH solutions are most commonly used; however, $NH_4H_2PO_4$ was found to have a similar efficiency of elution to NH_4OH, whereas the use of NH_4HCO_3 resulted in a lower number of phosphopeptides purified from a tryptic digest of α and β casein (Aryal and Ross 2009). Acidic elution solutions, such as phosphoric acid, have also been used, although they were found to be less efficient, even in the presence of non-phosphopeptide excluders (Aryal and Ross 2009).

TiO_2 affinity purification has been widely used for phosphopeptide enrichment in proteomic studies. Greater depth of analysis using this enrichment method has been obtained by performing multiple rounds of enrichment from a single sample (Choudhary et al. 2009). The method has also been adapted by Eriksson et al. (2010) to permit on-target phosphopeptide enrichment for "direct analysis" by MALDI.

TiO_2 affinity purification enriches monophosphorylated species to a greater extent than multiply phosphorylated species (Aryal and Ross 2009; Larsen et al. 2005). This is because the latter bind to TiO_2 with very high affinity making them more difficult to elute (Aryal and Ross 2009; Thingholm et al. 2008a). Elution buffers of high pH and ionic strength are often used to overcome this limitation, but care should be taken as high pH may increase the hydrolysis of phosphates. In addition, it should be noted that a comparative study found that the performance of a number of commercially available MOAC resins varied widely, although all were considered suitable for the enrichment of phosphopeptides from a tryptic digest of α and β casein (Gates et al. 2010). Work by Li et al. (2009a) has also demonstrated that the ratio of peptides to TiO_2 material is important for phosphopeptide enrichment efficiency and for the selectivity profile. The authors reported that recovery of multiply phosphorylated peptides was increased when insufficient TiO_2 beads were used (Li et al. 2009a), although such an approach could adversely affect quantitative approaches if the affinity reagent becomes saturated.

Comparison between IMAC and MOAC

IMAC, using Fe^{3+} and Ga^{2+}, has been shown to be less selective than TiO_2 MOAC in the enrichment of phosphopeptides from a tryptic digest of α and β casein, but more multiply phosphorylated peptides are identified using the former method (Aryal and Ross 2009). Larsen et al. (2005) also noted that TiO_2 phosphopeptide enrichment is

more selective and sensitive than that obtained with Fe^{3+} IMAC.

TiO_2 chromatography is less sensitive to the presence of interfering compounds, such as salts and detergents, than IMAC. Indeed, the sensitivity and selectivity of IMAC can be greatly affected by buffers and detergents (Tsai et al. 2008; Ye et al. 2010) and it has also been shown that nucleic acids can compete with phosphopeptides for binding to IMAC material (Li et al. 2009b). However, several protease and phosphatase inhibitors are also known to reduce the efficiency of phosphopeptide enrichment using TiO_2 when included in the loading solution (Aryal and Ross 2009; Rogers et al. 2010). Thus, peptide purification from crude extracts is advisable prior to affinity chromatography-based enrichment methods. Peptide purification can be carried out using various methods, including reversed phase extraction and graphite chromatography, although the latter is reported to retain phosphopeptides with greater efficiency (Larsen et al. 2004).

As of yet, there is no single phosphopeptide enrichment method that is capable of providing full coverage of the whole phosphoproteome. This was demonstrated by a study comparing various methods of phosphopeptide enrichment, including MOAC and IMAC, which demonstrated that different methods produced distinct datasets, which overlapped by around just 30 % (Bodenmiller et al. 2007). One strategy to overcome this method bias is to combine multiple enrichment techniques to improve phosphoproteome coverage (this will subsequently be discussed in more detail).

Alternative affinity purification methods

Other affinity purification methods for phosphopeptide enrichment have also been reported. Phos-tag (1,3-bis [bis(pyridin-2-ylmethyl)-amino]propan-2-olato dizinc(II)) is an agarose-coupled dinuclear zinc complex, which was specifically designed to have an extremely high affinity for phosphate groups at neutral pH (Kinoshita et al. 2005). Phos-tag was reported to perform more efficiently than TiO_2 in the affinity purification of phosphopeptides from HeLa cell lysate and was used to evaluate the temporal dynamics of TNF-α signalling in HeLa cells (Nabetani et al. 2009). 802 phosphopeptides were identified and quantified from four 50 μg HeLa lysate protein samples, thus demonstrating the suitability of this method for the analysis of limited amounts of protein material (Nabetani et al. 2009).

Other phosphopeptide enrichment methods that are dependent on the chemical behaviour of phosphate groups have also been reported. In calcium precipitation, phosphopeptides in solution form an insoluble calcium phosphate deposit upon the addition of calcium salts and thus can be isolated from the peptide mixture (Xia et al. 2008). The use of calcium precipitation as a stand-alone phosphopeptide enrichment method was reported in the identification of over 500 phosphopeptides from postmortem brain samples (Xia et al. 2008). Comparative studies also demonstrated that this method was as efficient as IMAC and had less bias for multiply phosphorylated peptides (Xia et al. 2008). However, this method is most frequently used in combination with other phosphopeptide enrichment methods (discussed below). More recently, the use of crystalline calcium phosphate (hydroxyapatite), which strongly binds phosphorylated proteins, has been reported in the affinity purification of phosphopeptides (Mamone et al. 2010). Affinity purification of tryptic digests of α and β casein using this method demonstrated that phosphopeptides bound strongly to this material and could be displaced by an increasing gradient of phosphate buffer (Mamone et al. 2010). Enrichment was shown to be highly specific and phosphopeptides were sequentially eluted in a stepwise manner dependent on the number of phosphorylations present on the peptide (Mamone et al. 2010).

Affinity purification: antibodies

Antibody-based affinity purification involves selective enrichment of peptides or proteins that contain phosphorylated residues in regions that are recognized by specific antibodies (Fig. 2b). As such, these methods are limited by the specificity of the antibodies available to the researcher. There are many antibodies commercially available that claim to be specific for phosphorylated serine (pS) and threonine (pT) residues. The suitability of a number of these antibodies for affinity purification of phosphoproteins was evaluated by Gronborg et al. (2002). Of those considered, only a few were found to perform well for immunoprecipitation and these were used to purify phosphoproteins from HeLa cells treated with calyculin A prior to 1D gel electrophoresis, in-gel protein digestion of resolved bands and MALDI-TOF or LC–MS/MS analysis (Gronborg et al. 2002). The authors reported the identification of several known phosphoproteins and a novel PKA substrate, designated Frigg (Gronborg et al. 2002). However, their study identified only seven phosphoproteins and therefore cannot be considered a general approach to study the phosphoproteome.

Antibody-based enrichment techniques have demonstrated more success for the enrichment of peptides and proteins phosphorylated on tyrosine residues. Tyrosine phosphorylation is typically found at very low levels compared to serine and threonine phosphorylation. For example, the distribution of phosphorylation sites in EGF-stimulated HeLa cells was estimated to be 86.4 % on serine, 11.8 % on threonine and 1.8 % on tyrosine residues

(Olsen et al. 2006). Methods that are not residue specific typically result in the enrichment of low numbers of tyrosine phosphorylated peptides relative to other phosphopeptides. Thus, antibody purification methods specific for phosphorylated tyrosine (pY) have shown increased enrichment of this species relative to other methods. In addition, there are a number of antibodies commercially available that are specific for pY and highly efficient for immunoprecipitation (Zhang and Neubert 2006), whereas those for pS and pT have been found to be less specific. Anti pY antibodies may be more easily produced than anti pS/pT because the aromatic ring in pY is more electron rich than the aliphatic side chains of pT and pS. Thus, pY has more opportunities to form bonds with the antibody binding pocket, resulting in stronger and therefore more specific binding.

Antibody affinity purification has been applied to targeted phosphoproteomics. In one study phosphorylation patterns of the receptor EphB5 were investigated in HEK293 cells (Kalo and Pasquale 1999). The GST tagged receptor was overexpressed and, after purification, digested using a combination of endoproteinases. pY-containing peptides were enriched by immunoprecipitation using pY-specific antibodies before analysis using MALDI-TOF. In another study, novel pY sites on gelsolin, an actin-binding protein, were identified (De Corte et al. 1999). pY containing peptides were purified from a tryptic digest of phosphorylated gelsolin using immunoprecipitation followed by MALDI-MS analysis.

In an approach similar to that described for purification of pS and pT containing proteins above, proteins containing pY were purified from HeLa cells by immunoprecipitation using a mixture of pY-specific antibodies, before or after stimulation with EGF (Pandey et al. 2000). The purified proteins were separated by 1D electrophoresis, resolved by silver staining, excised and analyzed by MALDI and LC–MS/MS. Two novel tyrosine-phosphorylated second messengers in the EGF pathway were identified, as well as seven previously known ones (Pandey et al. 2000). More recently, Blagoev et al. (2004) used anti-pY antibody enrichment, followed by in-gel trypsin digestion and LC–MS/MS analysis, to identify 202 proteins from HeLa cell lysate, 81 of which were found to be dynamically regulated in response to EGF stimulation. A similar approach, which also demonstrated the application of label-free quantification in phosphoproteomics, identified more than 200 proteins purified by anti-tyrosine antibody enrichment from lymphoma cells, several of which were found to be sensitive to inhibition of the PI3K signalling pathway (Cutillas et al. 2005). Antibody affinity phospho-enrichment has also successfully been applied to the global analysis of pY in human cancer cell lines and tumours (Rikova et al. 2007; Rush et al. 2005). Proteins were extracted from three cancer cell lines; Jurkat, Karpas 299 and SU-DHL-1, which were either treated with a tyrosine phosphatase inhibitor or were expressing a constitutively active tyrosine kinase. The proteins were digested and fractionated, prior to enrichment by pY immunoprecipitation and LC–MS/MS analysis. A pY enrichment of around 90 % was obtained and 688 pY sites were identified across 628 phosphopeptides, of which 70 % was estimated to be novel (Rush et al. 2005). This is the first study in which the enrichment was performed at the peptide level using anti pY antibodies.

Chemical derivatization

Phosphopeptide enrichment techniques using chemical derivatization involve the selective modification of the phosphate group on phosphoproteins or phosphopeptides prior to their enrichment (Fig. 2c). The rationale of this approach is to increase sensitivity and sequence coverage of phosphopeptide analysis by removal of the phosphate group before MS analysis. This avoids phosphate and/or phosphoric acid neutral loss during MS/MS, a phenomenon which decreases fragmentation efficiency of the rest of the peptide thereby reducing sequence information. In addition, by reducing the negative charges of phosphopeptides, ionization efficiency in positive ion mode is thought to increase as a result of phosphate removal. The most common approach to phosphopeptide enrichment using chemical derivatization involves β-elimination of phosphate and Michael addition with a chemical group. In this method, the phosphate group is eliminated by a strong base and then a chemical tag, which is capable of forming a more stable chemical bond, is introduced at the unsaturated residue.

Substituting phosphates with a derivatized moiety containing a tag for affinity purification has been exploited for the isolation of phosphoproteins and phosphopeptides. Such a concept was used for the selective analysis of pS and pT (Adamczyk et al. 2001). In this study, phosphorylated residues in a mixture of three proteins were converted to S-(2-mercaptoethyl)cysteinyl or β-methyl-S-(2-mercaptoethanol)-cysteinyl residues by Ba(OH)2-catalyzed β-elimination and the addition of 1,2-ethanedithiol (Adamczyk et al. 2001). The proteins were then reversibly biotinylated followed by proteolytic digestion, affinity purification using avidin and LC–MS/MS analysis. The authors reported the identification of all known phosphorylation sites of the proteins in a standard phosphoprotein mixture. It was also noted that the derivatization reaction was slower and rate limiting if the amino acid proline was located next to the phosphorylated residue (Adamczyk et al. 2001).

Limitations of chemical derivatization strategies include potential sample loss during multiple reaction steps and unavoidable side reactions. However, one advantage of chemical derivatization strategies is that sample labelling for quantification can be incorporated into the phosphopeptide enrichment workflow. For example, β-elimination was followed by Michael addition of ethanethiol or ethane-d_5-thiol to create distinct isotopic labels for two samples that were subsequently quantitatively compared (Weckwerth et al. 2000). In a similar approach, Goshe et al. (2001) followed β-elimination with phosphoprotein isotope coded affinity tag (PhIAT) labelling, a biotinylated tag that permitted high-specificity affinity purification of modified peptides and isotopic labelling for relative quantification of samples. It was also proposed that in the use of methanolic HCl for the methyl esterification of carboxylate groups to enhance IMAC selectivity, deuterated and unlabelled methanol could be used during enrichment thus allowing quantitative comparison of two samples (Ficarro et al. 2002).

Other chromatographic methods

Other forms of chromatography have also been proposed as direct methods for phosphopeptide enrichment. Strong cation exchange (SCX) chromatography separates molecules based on their charge (Fig. 2d). The stationary phase displays a negatively charged functional group and thus retains cations, which are sequentially eluted by displacement by an increasing gradient of salt (such as NaCl or ammonium based salts) or pH. Strong anion exchange (SAX) is based on the same principles but polarities are reversed; i.e. the stationary phase is positively charged and retains anions (Fig. 2d).

Beausoleil et al. (2004) were, to the best of our knowledge, the first group that implemented ion exchange chromatography as a direct enrichment method for phosphoproteomics. SCX HPLC was used under acidic conditions (pH <2.7), whereby only lysine, arginine and histidine residues and the amino terminus of a peptide are ionized. Thus, at this pH phosphorylated tryptic peptides have an overall charge of 1+ and interact poorly with the SCX column. Accordingly, it was shown that early fractions in the SCX separation were highly enriched for phosphopeptides compared to fractions eluted with a higher salt gradient later in the run (Beausoleil et al. 2004). It has also been demonstrated that phosphopeptides can additionally be found in the unretained SCX fraction (Dai et al. 2006; Nie et al. 2010). SCX phosphopeptide enrichment was applied to identify 2,002 distinct phosphorylation sites from isolated HeLa cell nuclei, all of which were found on serine or threonine residues (Beausoleil et al. 2004). However, later studies showed that highly hydrophobic phosphopeptides could be retained by the SCX column (Dai et al. 2006; Lim and Kassel 2006), and that highly acidic non-phosphorylated peptides often co-eluted with phosphopeptides (Lim and Kassel 2006). It was also noted that in cases when trypsin digestion is incomplete there may be internal arginine or lysine residues that would result in such phosphopeptides having a charge greater than 1+, and thus they would not elute in phosphopeptide-rich fractions (Lim and Kassel 2006).

Due to the highly acidic nature of phosphopeptides, SAX HPLC was later proposed as a more suitable method for phosphopeptide enrichment. Under weakly acidic conditions phosphate groups are fully ionized and phosphopeptides are more strongly retained by SAX columns than their non-phosphorylated counterparts, thus providing the basis for this enrichment method. Han et al. (2008) demonstrated that, indeed, phosphopeptides obtained from the tryptic digestion of α and β casein were strongly retained by SAX columns and could not be identified in the flow-through fraction. They also showed that mono- and multiply phosphorylated peptides from a casein tryptic digest could be eluted separately, as two distinct "retained" fractions, and that very few non-phosphorylated fractions were retained by the SAX column (Han et al. 2008). Analysis of a human liver protein digest demonstrated that, although SAX provides more specific enrichment than Fe^{3+} IMAC, around the same number of phosphopeptides were identified by each method (Han et al. 2008). However, the authors noted that SAX showed less bias for multiply phosphorylated peptides than IMAC, thus the two methods were proposed to be complementary (Han et al. 2008). Nie et al. (2010) later showed that a high number of phosphopeptides could be found in the unretained fraction of SAX chromatography when used to analyze complex mixtures, such as a whole cell lysate.

Hydrophilic interaction chromatography (HILIC) has also been proposed as a method for phosphopeptide enrichment (Fig. 2d). HILIC is a form of chromatography based on the interaction between molecules and the neutral, hydrophilic stationary phase due to hydrogen bonding. The strength of interaction with the stationary phase increases with increasing polarity and molecules are eluted by a polar hydrophilic mobile phase. Since phosphorylated peptides have greater hydrophilic character than non-phosphorylated counterparts, these are retained more strongly by the column, thus providing the basis for separation. However, the enrichment efficiency of HILIC is relatively low and phosphopeptides co-elute in non-phosphorylated peptide rich fractions (McNulty and Annan 2008).

Collectively, the accumulated data indicate that HILIC, SCX and SAX, by themselves, are not suitable for phosphopeptide enrichment due to their low specificity (Gruhler et al. 2005; Lim and Kassel 2006; McNulty and Annan

2008; Nie et al. 2010). These techniques are nevertheless powerful and widely used as first separation steps prior to more specific enrichment using IMAC or TiO_2 in coupled strategies. These coupled methods will be subsequently discussed in detail.

Coupled methods

Multi-dimensional chromatography

Several chromatographic methods have been applied for phosphopeptide enrichment; however, various studies, some of which are mentioned above, have demonstrated that these methods alone do not provide sufficient coverage of the phosphoproteome. Nevertheless, these methods may be combined to provide two dimensional (2D) or even multidimensional chromatographic separation for phosphopeptide enrichment. The rationale behind 2D chromatography is to use two orthogonal chromatographic methods (i.e. two methods that produce non-overlapping separation) to comprehensively fractionate a peptide mixture. Although such strategies do not specifically enrich samples for phosphopeptides, the comprehensive separation of complex peptide mixtures results in an increased identification of these low abundance and modified peptides. There are multiple methods of chromatography used for this purpose. In addition to SCX, SAX and HILIC, described previously, reverse phase (RP) chromatography has also been used for phosphopeptide enrichment. RP chromatography is based upon the interaction between analytes and the non-polar stationary phase, such as C18. Molecules are then separated by polarity as they are eluted by the polar organic mobile phase, for example ACN.

Both SCX, SAX and HILIC are orthogonal to RP LC–MS, although HILIC and RP at high pH have been shown to produce a more even fractionation of peptides than SCX (Gilar et al. 2005). Thus, it has been proposed that HILIC and RP at high pH may be more useful separation techniques for the purpose of reducing sample complexity prior to LC–MS analysis (Gilar et al. 2005; McNulty and Annan 2008).

Link et al. (1999) were among the first to demonstrate the power of multidimensional separations in proteomics. Later, other groups have shown modifications of this analytical concept for phosphopeptide enrichment. A recent example is the study by Dai et al. (2006) that proposed the use of SCX and SAX as orthogonal chromatographic methods for the comprehensive profiling of protein expression and phosphorylation in the so-called "Yin-Yang Multi-Dimensional Liquid-Chromatography" method. In this method, a peptide mixture was fractionated by SCX and the flow-through from the first separation step was fractionated by SAX, thus providing extensive separation of both acidic and basic peptides prior to LC–MS/MS analysis of all fractions. Although this approach is not strictly a method for phosphopeptide enrichment, over 800 unique phosphopeptides were identified from mouse liver protein digest (Dai et al. 2006). The overlap of peptides identified by both SCX and SAX fractions was just 23.5 %, thus demonstrating the orthogonality of these two methods (Dai et al. 2006). In a similar approach, Motoyama et al. (2007) combined anion and cation exchange (ACE) chromatography and RP LC–MS/MS in a 2D chromatography approach to separate a complex peptide mixture from a HeLa nuclear extract. ACE chromatography, in which anion and cation resins are combined thereby creating a phase containing opposite charges within a single column, was shown to improve recovery of phosphopeptides, particularly neutral and basic phosphopeptides, compared to SCX and was also found to provide better orthogonality to RP (Motoyama et al. 2007). Dai et al. (2008) later combined SAX chromatography and RP LC–MS/MS in a fully automated, online method for the enrichment of phosphopeptides. Using this method, 1,497 distinct phosphorylation sites were identified from HeLa cell protein digests, providing comprehensive coverage of the phosphoproteome with no apparent bias for singly or multiply phosphorylated species (Dai et al. 2008). The advantage of these approaches over more traditional phosphopeptide enrichment techniques is that there is no loss of unmodified peptides, which may also provide informative data.

Coupling of traditional enrichment methods

Several phosphopeptide enrichment techniques have been coupled together to increase phosphopeptide enrichment efficiency. As discussed previously, MOAC and IMAC isolate distinct subsets of phosphopeptides. Similarly, a study comparing TiO_2 affinity purification, SAX and SCX followed by TiO_2 affinity purification of enriched fractions identified around just 10 % of phosphopeptides from HeLa cell digests by all three methods (Nie et al. 2010). Thus, as a strategy to increase the coverage of the phosphoproteome Thingholm et al. (2008a) developed a method termed sequential elution from IMAC (SIMAC). In this technique, monophosphorylated and acidic peptides were eluted from Fe^{3+} IMAC material under acidic conditions prior to a second round of enrichment for monophosphorylated peptides using TiO_2 chromatography, whilst multiply phosphorylated peptides were eluted from IMAC material under basic conditions (Thingholm et al. 2008a). The mono and multiply phosphorylated peptide fractions were analyzed separately by LC–ESI–MS/MS. This method was found to identify twice the number of phosphopeptides compared to TiO_2 chromatography alone (Thingholm et al. 2008a). When SIMAC was coupled to SCX, several

thousand phosphopeptides were identified in the NIH-3T3 cell line (Alcolea and Cutillas 2010).

Another approach that uses different enrichment methods in tandem involves selective precipitation followed by further enrichment using a chromatographic method. In one study demonstrating this concept, phosphopeptides were precipitated from solution by the addition of $CaCl_2$ prior to further enrichment using Fe^{3+} IMAC (Zhang et al. 2007). This study showed that an additional enrichment step prior to IMAC significantly increased the number of phosphopeptides identified resulting in an enrichment greater than 90 % (Zhang et al. 2007). Multiply phosphorylated peptides still predominated in the data, although this study also demonstrated that a second round of IMAC enrichment can significantly increase the number of monophosphorylated peptides detected (Zhang et al. 2007). In an alternative approach, pY-specific immunoprecipitation from leukaemia cell lysate was followed by trypsin digestion, methyl esterification of carboxylic groups and Fe^{3+} IMAC (Salomon et al. 2003). Around 30 novel tyrosine phosphorylation sites were identified and phosphorylation in response to inhibition of the oncogenic BCR-ABL protein was investigated (Salomon et al. 2003). In another study, β-casein peptides were pre-enriched using Fe^{3+} IMAC prior to chemical derivatization using modified β-elimination to enhance phosphoproteomic analysis (Ahn et al. 2004). Tagged peptides produced good quality MS/MS results and thus improved the quality of phosphopeptide identifications and enabled the identification of more multiply phosphorylated species (Ahn et al. 2004).

Chromatographic separation of peptides followed by affinity purification of phosphopeptides is also emerging as a powerful strategy for global phosphoproteomic analysis. As previously mentioned, current mass spectrometers have limited duty cycles and thus are still restricted in their capacity to analyse complex samples. Therefore, the incorporation of separation steps into the workflow improves depth of analysis. One approach is to carry out separation at the protein level, for example using 1D or 2D electrophoresis, prior to protein digestion and phosphopeptide enrichment. An alternative is to perform the separation at the peptide level after digestion of whole cell lysates, thus reducing sample complexity of individual fractions prior to phosphopeptide enrichment, although at the expense of increasing analysis time.

In one example of a coupled-method approach to phosphopeptide enrichment, peptide mixtures from yeast cells were separated by SCX prior to Fe^{3+} IMAC of early fractions (Gruhler et al. 2005). The depth of phosphoproteome coverage was noted to be incomplete, but the dataset was the largest reported at the time for yeast and sufficient to fully characterise the pheromone response pathway (Gruhler et al. 2005). In another study, McNulty and Annan (2008) used HILIC in combination with Fe^{3+} IMAC to enrich phosphopeptides with efficiencies of 99 % from HeLa total cell lysate. It was also noted that more monophosphorylated phosphopeptides were found than those usually encountered with IMAC. One major advantage of HILIC over other chromatographic techniques is that elution is carried out with a volatile, salt-free buffer system which is directly compatible with affinity purification techniques such as IMAC.

TiO_2 affinity purification has also been used in coupled approaches. Choudhary et al. (2009) performed multiple rounds of TiO_2 enrichment on a single sample or SCX followed by TiO_2 enrichment of all fractions to identify over 14,700 phosphorylation sites from murine myeloid cells. Nie et al. (2010) proposed that SAX and TiO_2 should be highly complementary for phosphopeptide enrichment as the former shows bias for acidic, multiply phosphorylated peptides and the latter preferentially enriches for more basic, singly phosphorylated peptides. Using a strategy that coupled both methods, 3,462 unique phosphopeptides were identified from HeLa cells and it was demonstrated that this approach performed better than SCX coupled to TiO_2, showed good reproducibility and provided more comprehensive coverage of the phosphoproteome (Nie et al. 2010). Solution-based isoelectric focusing has also been used as a peptide separation step prior to TiO_2 enrichment (Rogers et al. 2010). The chromatography coupled approach has also been used in the reverse order i.e. enriching phosphopeptides using TiO_2 prior to SCX chromatography and LC–MS/MS analysis of fractions (Wu et al. 2010). This approach was used to identify and quantify several thousand phosphopeptides obtained from a HeLa cell lysate digest (Wu et al. 2010).

Concluding remarks

As summarised in this article, a plethora of methods and approaches to selectively enrich samples for phosphorylated polypeptides are now available to the researcher and have been, and are being, applied extensively to investigate a wide array of biological processes. A detailed discussion of such applications is beyond the scope of this paper but they have been reviewed elsewhere (Macek et al. 2009; Harsha and Pandey 2010). In-depth understanding of the particular strengths and limitations of the available techniques will aid in the selection of a workflow or analytical strategy that will best fit the biological question to be addressed.

It should be noted that none of the methods described above for phosphopeptide enrichment were reported to be optimized for quantitative analysis. However, particular care should be taken when the aim of the investigation is to

provide relative quantification of phosphorylation across experimental conditions. In such quantitative phosphoproteomics methods, phosphopeptide extraction has to be robust, reproducible and the amounts of phosphopeptides extracted must correlate with their abundance in samples. Therefore, enrichment techniques used for quantitative phosphoproteomics require greater consideration than those applied to identify sites of phosphorylation on proteins. An important feature of quantitative analysis is that the relationship between analyte concentration in the sample and signal intensity must be linear. This typically requires the construction of calibration curves for each of the analytes quantified. However, this is not possible in global phosphoproteomic experiments where the aim is to quantify thousands of phosphorylation sites per experiment. One approach to assess the performance of phosphopeptide enrichment strategies in quantitative experiments involves mixing samples at different proportions prior to analysis. This method enables the assessment of the linearity of detection for each phosphopeptide analysed in large-scale phosphoproteomic experiments (Casado and Cutillas 2011). Application of this technique to assess the performance of the methods described above will result in a better understanding of their performance for quantitative work.

It should also be noted that improvements to methods for phosphopeptide enrichment are not the only option to enhance the characterization of the phosphoproteome. Mass spectrometric strategies to enhance phosphopeptide identification have also been reported. Although a full description of these methods is outside the scope of this review, classical approaches include precursor ion scanning (Steen et al. 2001), MS^3 (Schroeder et al. 2004) and the related multistage activation (Boersema et al. 2009). More recently, it was also shown that collision induced dissociation (CID) in quadrupoles and linear ion traps produce complementary information and that their combination enhances phosphopeptide identification by reducing the number of false negatives (Alcolea et al. 2009). This paper also showed that spectral matching may be an alternative to classical methods to identify phosphopeptides from MS/MS data, which are based on searching protein databases. The use of electron transfer dissociation (ETD) has also shown promise as an alternative fragmentation method to CID in phosphoproteomics (Chi et al. 2007; Molina et al. 2007).

In conclusion, MS-based phosphoproteomics is emerging as a powerful technology for biological research. The implementation of developments in enrichment techniques, MS methods and bioinformatics tools (not reviewed here) are contributing to the success of this technique and will continue to advance research in different fields of biological investigation.

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