REVIEW

Analytical strategies for phosphoproteomics

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Protein phosphorylation is a key regulator of cellular signaling pathways. It is involved in most cellular events in which the complex interplay between protein kinases and protein phosphatases strictly controls biological processes such as proliferation, differentiation, and apoptosis. Defective or altered signaling pathways often result in abnormalities leading to various diseases, emphasizing the importance of understanding protein phosphorylation. Phosphorylation is a transient modification, and phosphoproteins are often very low abundant. Consequently, phosphoproteome analysis requires highly sensitive and specific strategies. Today, most phosphoproteomic studies are conducted by mass spectrometric strategies in combination with phospho-specific enrichment methods. This review presents an overview of different analytical strategies for the characterization of phosphoproteins. Emphasis will be on the affinity methods utilized specifically for phosphoprotein and phosphopeptide enrichment prior to MS analysis, and on recent applications of these methods in cell biological applications.

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1 Introduction

Protein phosphorylation is one of the most widespread regulatory mechanisms in nature. Phosphorylation is a key regulator of intracellular biological processes and at present, it is the most studied and best understood PTM [1, 2]. Protein phosphorylation is known to be involved in the regulation of diverse processes including metabolism, transcriptional, and translational regulation, degradation of proteins, homeostasis, cellular signaling and communication, proliferation, differentiation, and cell survival [3, 4].

Phosphorylation events frequently initiate and propagate signal transduction pathways. It is a transient, reversible PTM that typically leads to changes in the conformation, activity, and interactions of a protein within a very short time-frame. Since the modification is reversible, changes in protein activity can be tightly controlled by phosphorylation/dephosphorylation in response to cellular or environmental stimuli. Phosphorylation often occurs at multiple residues within a protein and in most cases by different protein kinases. Multiple sites of phosphorylation allow a protein to adapt several different functions, depending on which phosphorylation site becomes occupied. For example, phosphorylation at one particular amino acid may lead to conformational changes that in turn allow for the phosphorylation of different amino acid residues within the same protein, or it can prevent the phosphorylation of nearby amino acids through sterical hindrance.

Phosphorylation is regulated by a complex interplay between specific protein kinases and protein phosphatases, which keep a strict temporal and spatial control of the phosphorylation and dephosphorylation events at specific sites in target proteins. In eukaryotes, phosphorylation takes place at serine, threonine, or tyrosine residues. Phosphorylation also occurs on histidine residues, however,

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Abbreviations: SIMAC, sequential elution from IMAC; **TiO_2,** titanium dioxide

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phosphohistidine is highly labile and therefore rarely identified in phosphoproteomic studies [5]. Genomic sequencing has revealed that 2–3% of all eukaryotic genes are likely to code for protein kinases [6] and more than 100 human protein phosphatases have been predicted by genome annotation [7], emphasizing the ubiquitous role of protein phosphorylation. At present, no studies have been able to elucidate how many proteins are in fact regulated by phosphorylation, but it has been estimated that more than 50 percent of all proteins are phosphorylated during their lifetime [2], and that more than 100 000 phosphorylation sites may exist in the human proteome [8]. Despite its ubiquitous role, phosphoproteins are generally present at relatively low abundance within the cell and phosphorylated forms of individual proteins also tend to be present at much lower ratios than their native counterparts. While some proteins are constitutively phosphorylated, most are only transiently phosphorylated following key cellular cues.

Due to the central regulatory role phosphorylation plays in maintaining the dynamic of a living cell, it is of key interest to develop analytical methods and strategies for the characterization of phosphorylated peptides. However, low relative abundance of many phosphoproteins, low phosphorylation stoichiometry and the dynamic regulation of phosphoproteins put high demands on the analytical methods required for the studies of phosphoproteins. Sensitive, comprehensive and highly specific analytical strategies are needed in order to characterize a meaningful number of modified proteins, including those present at very low abundance.

In this review, an overview of sample preparation and different strategies for the characterization of phosphoproteins will be given together with recent applications of the methods.

2 Important considerations for initial sample preparation

The first step, when studying phosphoproteins, is the isolation of proteins from a given sample originating from an entire cell lysate or a sub-cellular compartment. Upon lysis of a cell, many proteases and protein phosphatases will become active and if precautions are not made, proteins will be degraded and phosphate groups on phosphoproteins will be lost. By performing all sample preparations at 4°C, the activities of these enzymes will be reduced, however, it is advantageous to also add protease and phosphatase inhibitors to all buffers used in the initial steps of the sample solubilization and enzymatic digests [2, 9]. Protease inhibitor cocktails are generally used, as they will give the best overall protection against protease activity. It is also important to choose the correct mix of phosphatase inhibitors as each will have specific enzymatic reactions that they are capable of inhibiting [10]. For example, some phosphatase inhibitor cocktails may only inhibit the removal of phosphate from serine/threonine residues, but allow removal of phosphate from tyrosine. Thus, different types of phosphatase inhibitors produce distinct phosphoprotein populations, and a single phosphatase inhibitor cocktail may not be enough to control all phosphatase activities and combinations might need to be used. One also needs to inhibit the activities of protein kinases in order to identify phosphorylation sites of true biological relevance. This is most commonly achieved by adding EDTA, EGTA, or specific kinase inhibitors to the buffers. It is especially important to inhibit both protein kinases and protein phosphatases when studying dynamic protein phosphorylation. Furthermore, it is also critically important that the buffers and reagents used in the initial sample preparation will not interfere with the downstream analytical methods thus decreasing the sensitivity of a subsequent phospho-specific enrichment method.

3 Detection of phosphoproteins

There are several strategies for detecting phosphoproteins. Highly complex protein samples can be separated according to their p*I* and their molecular weight using 2-DE [11–15]. The separated proteins can subsequently be visualized using standard protein stains (*e.g.*, Coomasssie blue and silver staining). By this method the post-translationally modified proteins, such as phosphoproteins, can appear as multiple p*I* variants according to their modification states, since the addition or subtraction of a chemical group can subtly, but detectably, alter the p*I* of a protein. This is especially evident for multiply phosphorylated proteins. Phosphoproteins separated by gel electrophoresis can also be visualized by the use of a phosphospecific stain such as Pro-Q Diamond [16–19]. Alternatively, phosphoproteins can be radioactively labeled using ^{32}P or ^{33}P [16, 17] and detected by autoradiography, which is the most sensitive method available, but may not be compatible with downstream analytical procedures such as MS. Radioactive labeling is possible both *in vivo* as well as *in vitro*. In *in vivo* studies, cells are incubated with ^{32}P, however, the presence of endogenous ATP pools within cells can interfere with the incorporation of the label and result in inefficient radioactive labeling [20]. Furthermore, ^{32}P is toxic for many cells and will over time cause damage to the cell. In *in vitro* studies, proteins are incubated with specific kinases in the presence of [γ-^{32}P]-ATP, and under appropriate conditions ^{32}P is incorporated at the phosphorylated amino acid residue. A major drawback of this method, however, is that kinases may phosphorylate target proteins promiscuously. The occurrence of promiscuous phosphorylation events is due to the unnaturally high concentration of kinase compared to the substrate concentration [2], and kinases that would not phosphorylate a particular protein *in vivo* might still induce phosphorylation *in vitro* [21].

Phosphoproteins can be visualized by Western blotting using antibodies against general phosphoserine, phosphothreonine, or phosphotyrosine residues [22]. Detection with such antibodies provides no information of specific phos-

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phorylation sites. The protein bands of interest must be excised from the gels and the proteins analyzed by MS for further validation, which can be difficult due to co-migrating proteins.

Phosphoproteins can also be visualized by Western blotting using antibodies against specific phosphorylation sites in known proteins [23–25]. However, only information on a subset of phosphoproteins can be obtained at a time when using these antibodies.

In a recent study by Sevecka and MacBeath, antibodies were combined with microarrays for the detection of the abundance and phosphorylation state of selected proteins in order to study ErbB signaling [26, 27].

4 Affinity enrichment of phosphoproteins and phosphopeptides

Phosphoproteins are mostly characterized using MS methods after proteolytic processing, however, phosphopeptides are notoriously difficult to analyze by MS, especially in the presence of nonmodified peptides. This is mainly due to lower ionization efficiency of phosphopeptides resulting in lower signal intensities in the presence of nonphosphorylated peptide ions. Thus, efficient enrichment of the phosphorylated species (proteins or peptides) prior to MS analysis will result in increased sensitivity and more efficient characterization. There are various phosphoproteomic enrichment strategies to choose from, depending on the sample type and study aims. Fig. 1 gives an overview of strategies currently available. Methods used mainly for prefractionation prior to phosphopeptide enrichment are not included.

4.1 Immunoaffinity chromatography

Traditional biochemical approaches have often focused on the analysis of a single phosphoprotein and entire signaling pathways are studied protein by protein. Single phosphoproteins can be purified from a cell lysate by immunoprecipitation using antibodies [23]. After immunoprecipitation,

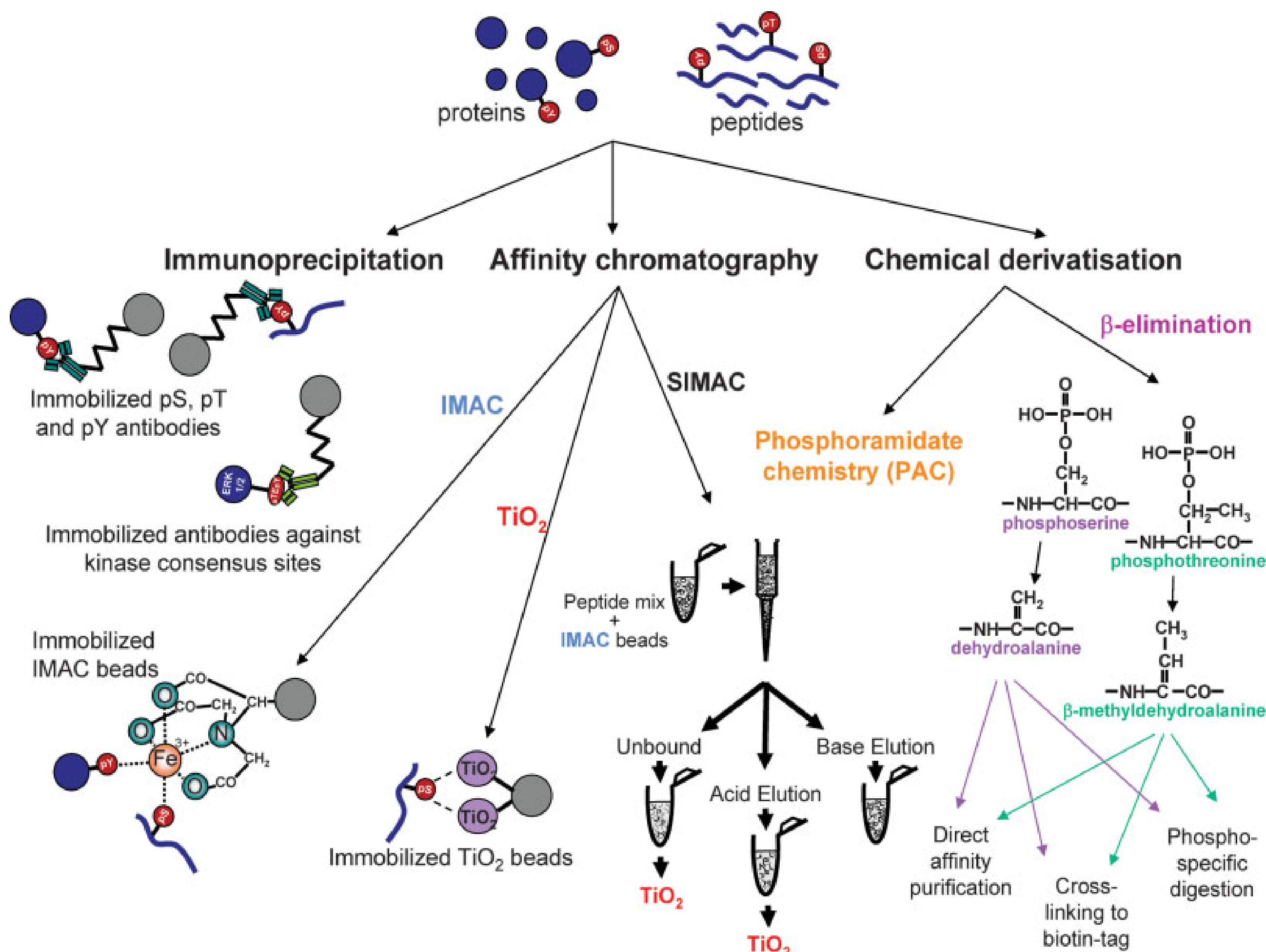

Figure 1. Strategies for phospho-specific enrichment. Most commonly used strategies for immunoprecipitation, affinity chromatography or chemical modification applied for enrichment of phosphoproteins and phosphopeptides are illustrated.

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the sample can be separated by SDS–PAGE and the correct protein band of interest is excised and further analyzed by MS. Antibodies against phosphorylated serine, threonine, and tyrosine residues can be used for the overall enrichment of phosphoproteins from a complex sample, though, this method is limited by the specificity of the antibodies [28, 29]. Tyrosine phosphorylation, in particular, is of very low abundance. In nature, the ratio of phosphoserine, phosphothreonine, and phosphotyrosine is reported to be 1800:200:1 [30], however, highly selective phosphotyrosine-specific antibodies exist and can be utilized for the enrichment of tyrosine phosphorylated proteins prior to analysis by MS [26]. Pandey *et al.* used this approach to characterize the epidermal growth factor (EGF) receptor signaling pathway and they identified a novel immunoreceptor tyrosine-based activation motif-containing protein (STAM2) and characterized its role in growth factor and cytokine receptor signaling pathways. Steen and co-workers applied anti-phosphotyrosine antibodies for immunoprecipitation of tyrosine phosphorylated proteins in EGF-treated or untreated HeLa cells. The proteins were subsequently separated using SDS–PAGE and analyzed using a tandem MS-based technique that relied on screening for the specific immonium ion for phosphotyrosine at m/z 216 (phosphotyrosine-specific immonium ion, PSI) [20]. They identified eight proteins known to be involved in EGF receptor signaling. In addition, they identified several known tyrosine phosphorylation sites as well as five novel sites [20]. Grønborg and co-workers tested a number of anti-phosphoserine and anti-phosphothreonine antibodies for immunoprecipitation and Western blotting detection and found several novel phosphorylation sites [28]. However, anti-phosphoserine and threonine antibodies are not yet routinely used in phosphoproteomics, mainly due to their limited specificity [31].

Enrichment of phosphoproteins from complex biological samples using antibodies is an efficient prefractionation method prior to further enrichment at the peptide level using immobilized metal ion affinity chromatography (IMAC) or titanium dioxide (TiO_2) chromatography [32]. Zheng *et al.* combined immunoprecipitation and IMAC in a study of interferon α (IFNα) signal transduction in Jurkat cells [33]. They isolated tyrosine phosphorylated proteins from whole cell lysates by immunoprecipitation using anti-phosphotyrosine antibodies. Following tryptic digestion of the isolated proteins, phosphopeptides were enriched using IMAC [33]. They characterized IFNα-induced changes in tyrosine phosphorylation and identified increased phosphorylation at several known, as well as novel, tyrosine phosphorylation sites on proteins known to be involved in IFNα signaling [33].

Phosphospecific antibodies can also be used for enrichment at the peptide level. Rush *et al.* applied anti-phosphotyrosine antibodies for the enrichment of peptides containing phosphotyrosine residues. They tested the efficiency on trypsinized protein extracts from several cell systems, including cancer cell lines, and identified 185 tyrosine phosphorylation sites in 3T3-Src cells [34]. Using a similar method Villén and co-workers identified 385 unique tyrosine phosphorylation sites from mouse liver [35]. While this method worked well in cell culture systems, large amounts of protein starting material are required for the immunoprecipitation steps. As an example, Villén *et al.* used 80 mg protein for their experiments [31, 35], an amount which may often not be possible to generate for many cell lines, tissues, and sub-cellular fractions.

4.2 Immobilized metal ion affinity chromatography

IMAC is a widely used affinity based technique for enrichment of phosphopeptides prior to MS analysis. Metal ions (Fe^{3+}, Al^{3+}, Ga^{3+}, or Co^{2+}) are chelated to nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) coated beads, forming a stationary phase to which negatively charged phosphopeptides in a mobile phase can bind. The technique was initially used for the affinity-purification of proteins based on the interactions of histidine and cysteine residues with the IMAC resin [36–38]. However, the binding of phosphoproteins and phosphoamino acids to metal ions, first demonstrated by Andersson and Porath in 1986 [39], gave the technique new potential. IMAC was further extended by Neville *et al.* to the enrichment of phosphopeptides obtained from proteolytically digested proteins [40], and the IMAC technique has since been used extensively for enrichment of phosphopeptides prior to MS analysis [41–47].

One of the problems associated with the IMAC technique is the often high level of nonspecific binding when used for phosphopeptide enrichment of highly complex peptide samples. Nonphosphorylated peptides containing multiple acidic amino acid residues co-purify with the phosphopeptides, thereby reducing the selectivity of the method. This is a serious problem, due to the more efficient ionization of nonphosphorylated peptides in subsequent MS analyses resulting in the suppression of signals from phosphopeptides. Ficarro and co-workers circumvented this problem by derivatizing the carboxylic groups on acidic amino acid residues in peptides by *O*-methyl esterification [48] and thereby improving phosphopeptide enrichment considerably [48–50]. Further experiments have shown, however, that *O*-methyl esterification does not derivatize 100% of the available carboxylic acid groups, and that it can increase the complexity of the analysis due to signals originating from peptides with different degrees of *O*-methylation [51]. Furthermore, the buffer used for *O*-methyl esterification can result in partial deamidation forming glutamic acid from glutamine as well as aspartic acid from asparagine [52]. These amino acid residues are subsequently *O*-methylated, adding further complexity to the analysis. In addition, *O*-methyl esterification requires extensive lyophilization of the sample, which causes adsorptive losses of phosphopeptides to surfaces [53, 54].

Another approach to increase the efficiency of IMAC is to adjust the pH. Acidification of a sample prior to IMAC anal-

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ysis protonates the carboxyl groups on the highly acidic amino acid residues and reduces nonspecific binding [47]. In most studies, 0.1–0.25 M acetic acid (pH ~2.7) has been used as a loading buffer for IMAC, however, Saha *et al*. showed that the pKa value of phosphoric acid decreased to 1.1 upon methylation [55]. The pKa values of phosphopeptides would therefore be expected to be significantly lower than that of phosphoric acid due to the organic environment provided by the surrounding amino acid residues. By decrease in the pH of the IMAC loading conditions further (below pH 1.9), more acidic peptides will become neutralized while phosphopeptides will retain their negative charge and their binding affinity toward the IMAC resin. Kokubu and co-workers showed that 0.1% TFA, 50% ACN as IMAC loading buffer reduced the level of nonspecific binding and improved the specificity of the method toward phosphopeptides [56]. Yet another approach to improve the performance of IMAC is to reduce the complexity of the peptide samples by prefractionation using methods such as IEF [57–60], Ion Exchange Chromatography [41, 43, 46, 61] or hydrophilic interaction chromatography (HILIC) [62] prior to IMAC purification.

4.3 Titanium dioxide chromatography

Recently, TiO_2 chromatography was introduced as an alternative to IMAC. For some years, TiO_2 was used in bioelectrochemical studies of protein functions using the adsorption of proteins to TiO_2 films [63]. More interestingly for phosphoproteomic studies, TiO_2 was shown to have affinity for phosphate ions from aqueous solutions [64–71]. In 2004, Pinkse and co-workers presented an online 2-D LC strategy for phosphopeptide analysis with spherical particles of titanium dioxide (Titansphere) as the first dimension and RP material as the second dimension. The peptide sample was loaded onto TiO_2 columns in 0.25 M acetic acid (pH 2.9), which promoted the binding of phosphopeptides. Unbound, nonphosphorylated peptides were trapped on the RP column, and subsequently eluted and analyzed using nanoLC-ESI-MS/MS. The phosphopeptides were eluted from the TiO_2 column using an alkaline buffer (pH 9.0), concentrated on the RP precolumn and analyzed using nanoLC-ESI-MS/MS [72]. The strategy was tested on a 153 kDa homo-dimeric cGMP-dependent protein kinase, and eight phosphorylation sites were identified, two of which were novel. However, a number of nonphosphorylated peptides were also observed and the authors recommended *O*-methyl esterification of the acidic residues prior to analysis [72].

Recently, our group introduced a new protocol for phosphopeptide enrichment using TiO_2. In this offline strategy, peptide loading is performed at highly acidic loading conditions using various substituted organic acids including 2,5-dihydroxybenzoic acid (DHB), phthalic acid or glycolic acid to efficiently eliminate binding of acidic peptides to the TiO_2 resin [73–76]. In addition, by increasing pH of the elution buffer from 9.0 to 11.3 (using ammonia solution), phosphopeptides are more efficiently eluted from the TiO_2 resin, resulting in increased sensitivity [75]. TiO_2 chromatography provides a high selectivity toward phosphopeptides in the optimized loading buffer and the offline setup is simple, fast, and does not require expensive equipment. Furthermore, TiO_2 is extremely tolerant toward most buffers and salts used in biochemistry and cell biology laboratories [73]. This has resulted in a robust method for enrichment of phosphopeptides, which has already become a highly popular method for in large-scale phosphoproteomic studies [10, 77–90].

4.4 Sequential elution from IMAC

Despite the highly sensitive phosphopeptide enrichment methods that have been developed in recent years, the analysis of multiply phosphorylated peptides by MS is still a major challenge in large-scale phosphoproteomics. A general assumption has been that TiO_2 chromatography has a preference for mono-phosphorylated peptides, and the level of multiply phosphorylated peptides identified by TiO_2 chromatography is consequently rather low. We believe that TiO_2 does, in fact, bind multiply phosphorylated peptides as well as mono-phosphorylated peptides, but that they are difficult to elute due to their extremely high binding affinity [74]. On the other hand, IMAC, has been argued to be less efficient for large-scale phosphoproteomic studies, and to have a preference for multiply phosphorylated peptides [48, 73, 74].

Multiply phosphorylated peptides ionize poorly and are suppressed in the presence of both mono- or nonphosphorylated peptides. Due to the low intensities of multiply phosphorylated peptide ions, they are rarely selected for fragmentation during the analysis of complex samples, which is why mono-phosphorylated peptides are predominantly identified in large-scale experiments, particularly when TiO_2 is used for phosphopeptide enrichment. In 2007, our group presented SIMAC (Sequential elution from IMAC), a new method, which combines the strengths of IMAC with the strengths of TiO_2 [74]. SIMAC is a simple and straight forward strategy, in which mono- and multiply phosphorylated peptides are efficiently enriched from highly complex samples into separate pools, which can be analyzed using different MS/MS settings targeting either mono-phosphorylated or multiply phosphorylated peptide ions. In SIMAC, binding to IMAC beads enriches phosphopeptides from tryptic digests, and unbound peptides, including unbound phosphopeptides, are collected for further enrichment using TiO_2 chromatography. Mono-phosphorylated peptides are eluted from the IMAC beads using acidic conditions (pH 1.0), and the multiply phosphorylated peptides are subsequently eluted from the same beads using basic conditions (pH 11.3) [74]. Due to the limited selectivity of the IMAC material for highly complex samples, the acidic elution fraction is subsequently enriched using TiO_2 chromatography prior to MS/MS analysis. The basic elution fraction contains a relatively low level of nonphosphorylated peptides and thus no further enrichment is necessary. The fractions

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containing mono-phosphorylated peptides (unbound and acidic fractions) are analyzed using standard $pdMS^3$ (phosphorylation-directed fragmentation) [61], whereas the fraction containing multiply phosphorylated peptides (basic fraction) is analyzed using optimized $pdMS^3$ [74]. Figure 2 illustrates the different selectivity of the IMAC, TiO_2, and SIMAC methods for phosphopeptide enrichment from 500 fmol semi-complex tryptic digestion.

The SIMAC strategy was tested on 120 µg whole-cell extract from human mesenchymal stem cells (hMSCs) and compared to the phosphopeptide enrichment provided by TiO_2 chromatography alone. The SIMAC method resulted in the identification of 716 phosphorylation sites as opposed to 350 by TiO_2 chromatography alone, mainly due to a significant increase in the number of multiply phosphorylated peptides identified [74].

The SIMAC approach is the first to make it feasible to study multiply phosphorylated peptides in large-scale setups from low microgram amounts of starting material, and the method is expected to be even more powerful when combined with prefractionation methods such as ion exchange chromatography or HILIC.

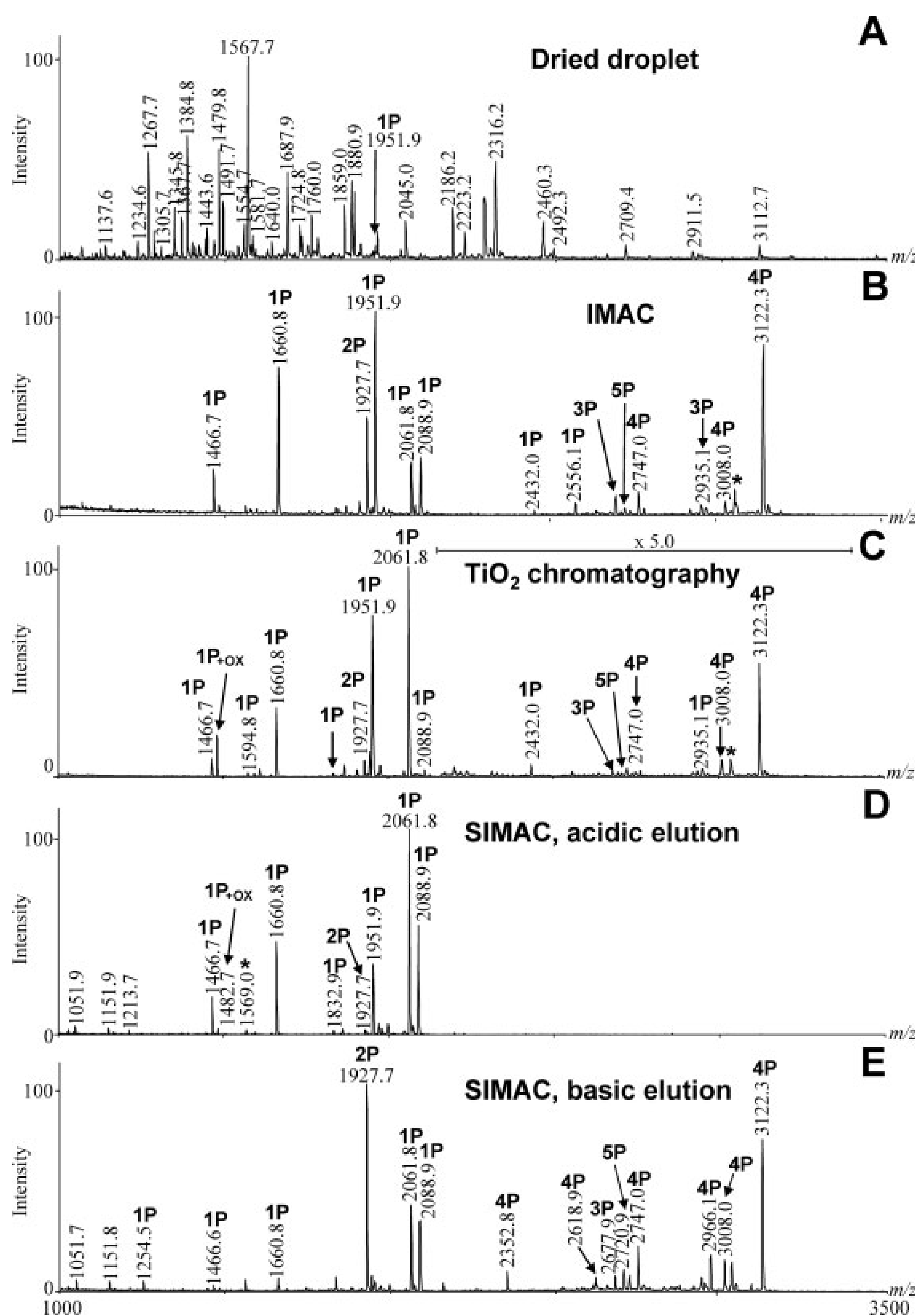

Figure 2. Phosphopeptide enrichment by IMAC, TiO_2 chromatography, and SIMAC from 500 fmol peptide mixture originating from tryptic digestion of 12 standard proteins; transferrin (human), serum albumin (bovine), beta-lactoglobulin (bovine), carbonic anhydrase (bovine), beta-casein (bovine), alpha-casein (bovine), ovalbumin (chicken), ribonuclease B (bovine pancreas), alcohol dehydrogenase (Baker yeast), myoglobin (whale skeletal muscle), lysozyme (chicken), alpha amylase (bacillus species). The underlined proteins are phosphorylated. (A) MALDI MS peptide mass map of an aliquot of the peptide mixture (500 fmol) analyzed using the dried droplet sample preparation method. (B–E) MALDI MS peptide mass maps of phosphorylated peptides originating from 500 fmol peptide mixture from tryptic digestion of the 12 proteins, after enrichment using IMAC (0.1% TFA, 50% ACN as loading buffer), TiO_2 chromatography (0.1%TFA, 80%ACN, 1M glycolic acid as loading buffer), SIMAC (eluted using 1% TFA), and SIMAC (eluted using pH 11.3), respectively. Phosphopeptides are marked by “#P” referring to the number of phosphate groups on the particular peptide. Asterisk indicates the loss of phosphoric acid.

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Recently, a new and interesting method employing poly-arginine-coated beads was introduced for purification of multiple phosphorylated peptides prior to MS analysis [91]. This method, however, has yet to be tested for its efficiency in larger phosphoproteomics studies and in those where only low amounts of starting material (micrograms) are available.

4.5 Calcium phosphate precipitation

In 1994, Reynolds and co-workers presented a strategy for phosphopeptide enrichment. using calcium ions and 50% ethanol to precipitate peptides containing phosphorylations on multiple serines from a tryptic digest of casein [92]. Recently, Zhang and colleagues presented a method for calcium phosphate precipitation of phosphopeptides from tryptic digests using disodium phosphate (Na_2HPO_4), ammonia solution ($NH_3 \cdot H_2O$), and calcium chloride ($CaCl_2$) [93]. When combined with phosphopeptide enrichment methods (*e.g.*, IMAC), the method offers an efficient enrichment strategy for phosphoproteomic studies. In total, 227 unique phosphorylation sites were identified (213 sites on serine residues and 14 on threonine residues) from a rice embryo preparation. No tyrosine phosphorylated peptides were identified in this study, likely due to the low abundance of these in rice, rather than due to poor selectivity of the method [93].

4.6 Ion exchange chromatography

Anion exchange chromatography has previously been applied as prefractionation of phosphorylated peptides prior to phosphopeptide enrichment [46, 94]. In anion exchange chromatography, phosphopeptides are typically more retained than nonphosphorylated peptides. However, it is our experience that acidic peptides bind strongly to anion exchange material and that phosphopeptides can be difficult to recover from this resin (Larsen *et al.*, unpublished results). In a study by Nühse *et al.*, more than 200 phosphopeptides were identified in plasma membrane (PM) fractions from *Arabidopsis thaliana* using strong anion exchange (SAX) combined with IMAC enrichment [46]. A recent study by Han and coworkers identified 274 phosphorylation sites in human liver tissue using SAX without further phosphopeptide enrichment [95].

Strong cation exchange (SCX) was originally used for prefractionation of proteins and peptides, where positively charged groups (cations) interact with negatively charged functional groups on the SCX particles. The pH and ionic strength of the buffers used for loading and elution of peptides can be modified to promote the binding and elution of different molecules. Multi-dimensional protein identification technology (MudPIT) combines SCX with online RP chromatography [96]. In 2004, Beausoleil and co-workers introduced the use of SCX for phosphopeptide enrichment [61]. Tryptic peptides were acidified in 5 mM KH_2PO_4, 30% ACN, pH 2.7. At this pH many tryptic peptides will have a charge state of +2 due to the charge at the *C*-terminal arginine or lysine as well as the charge on their *N*-terminus. Tryptic mono-phosphorylated peptides carrying the negative charge from a phosphate group would therefore have a net charge of +1 at pH 2.7. Phosphopeptides were therefore expected to display weakened binding to SCX material, and therefore elute before nonphosphorylated peptides with multiple positive charges. Multiply phosphorylated peptides would have net charges at ≤ 0, and would therefore not be retained by the SCX material. However, in this particular study, the authors did not examine the unbound peptides in the SCX flow-through. Bound tryptic peptides were separated by SCX chromatography using a gradient of KCl and finally an elution step using pH 7.0. The early eluting peptides were desalted and analyzed by RP LC-MS/MS. Their results confirmed a high level of phosphopeptides in the early fractions, which had been separated from nonphosphorylated peptides found in later fractions. Beausoleil *et al.* identified 2 000 phosphorylation sites from 8 mg nuclear extract of HeLa cell lysate using this approach [61].

For large-scale phosphoproteomic studies, SCX is most efficient as a prefractionation technique prior to phosphopeptide enrichment using either IMAC or TiO_2 chromatography. Trinidad and co-workers compared the enrichment efficiency of SCX as a prefractionation method prior to IMAC to both IMAC and SCX alone [51]. They found that the combination of SCX and IMAC led to at least a three-fold increase in the number of phosphopeptides identified relative to either approach alone. In addition, they found that only 35% of the tryptic peptides possessed a +2 charge, with most peptides carrying higher charge states. This resulted in a shift in the elution of phosphopeptides, which were not concentrated in the early SCX fractions, but distributed among all fractions. The trypsin digestion was performed in 1 M guanidine hydrochloride, and the authors suggested that the guanidinium ions could have acted as a competitive inhibitor of trypsin, thereby reducing its activity. This would lead to a high level of missed cleavages, which could explain the higher charge state on the observed peptides [51]. However, recent work by Villén *et al.* in which they fractionated tryptic digests from murine liver using SCX and enriched all 15 fractions for phosphopeptides using IMAC, revealed that phosphopeptides were spread throughout all SCX fractions [35]. The phosphopeptides were eluted according to their charge states, which seemed to be more dependent on the content of histidine, arginine, or lysine residues in their sequences and the charge on their *N*-terminus than the negative charges from the phosphate groups [35]. Indeed, by combining SCX with TiO_2 enrichment we have found phosphopeptides eluting at 500 mM KCL (Thingholm *et al.*, unpublished results).

Overall, SCX greatly improves phosphopeptide recovery when used as prefractionation prior to subsequent phosphopeptide enrichment. Using a combination of SCX and IMAC, Gruhler *et al.* performed a large-scale phosphoproteomic study on the yeast pheromone signaling pathway and

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identified and quantified more than 700 phosphopeptides of which 139 were differentially regulated at least two-fold in response to mating pheromone [43]. Olsen and co-workers identified 6 600 phosphorylation sites in 2 244 proteins in HeLa cells upon stimulation using EGF by combining SCX and TiO_2 chromatography [86]. Once again, it should be noted that in both cases large amounts of starting material were used and it remains to be determined whether the combination of prefractionation and enrichment will be ideal for very low microgram levels of starting protein.

4.7 Hydrophilic interaction chromatography

Hydrophilic interaction chromatography (HILIC) is a separation technique that is used for the separation of polar biomolecules [97]. The principle of this method is that peptides in an organic solution (mobile phase) bind to a neutral, hydrophilic stationary phase by hydrogen bonding. These hydrogen bonds can subsequently be disrupted by decreasing the organic environment in the mobile phase and peptides will elute according to their polarities (hydrophilicities). McNulty and Annan introduced HILIC as a peptide prefractionation technique prior to phosphopeptide enrichment by IMAC [62]. Using a TSKgel Amide-80 HILIC column and a shallow inverse organic gradient of ACN, water and TFA, they fractionated 1 mg of tryptic peptides from HeLa cells, which had been starved and treated with the phosphatase inhibitor, calyculin A. They tested the efficiency of HILIC as a phosphopeptide enrichment technique and compared it to SCX and IMAC as well as to the combination of HILIC with IMAC for more efficient phosphopeptide recovery [62]. They found that peptides were uniformly distributed throughout the HILIC gradient, containing mainly short, hydrophobic peptides in the early fractions and larger, hydrophilic, and highly acidic peptides in the later fractions. In contrast, SCX fractionation resulted in an uneven distribution of peptides. They found that the combination of HILIC and IMAC improved phosphopeptide recovery significantly. When IMAC was performed prior to HILIC, a high level of nonspecific binding due to nonphosphorylated peptides was observed, whereas HILIC separation prior to IMAC enrichment improved the phosphopeptide selectivity to more than 99% [62]. Thus, HILIC seems highly efficient for prefractionation of highly complex samples prior to specific phosphopeptide enrichment methods such as IMAC, TiO_2 chromatography, or SIMAC.

5 Chemical derivatization strategies

Chemical derivatization is yet another strategy for phosphopeptide enrichment. A variety of chemical derivatizations have been used for the tagging of phosphate groups on both proteins and peptides. Under alkaline conditions, β-elimination of phosphate groups present on serine and threonine residues produces dehydroalanine and β-methyldehydroalanine (dehydroaminobutyric acid), respectively. By subsequently adding a chemical compound containing a free sulfhydryl group to dehydroalanine or β-methyldehydroalanine acid by Michael addition, a di-thiol is created in the peptide, which can serve as a cross-linker to a biotin-tag [98] or for direct affinity purification [99]. Knight and co-workers used the combination of β-elimination, Michael addition reaction and proteolysis to aid the assignment of phosphorylation sites using tandem MS analysis [100]. In this study, phosphoserine and phosphothreonine were converted to aminoethylcysteine and β-methylaminoethylcysteine, respectively, both targets for lysine specific proteases (*e.g.*, trypsin, and lysyl endopeptidase (Lys-C)). In this way peptide fragments cleaved at the specific site of serine or threonine phosphorylation were generated. However, during aminoethylcysteine modification, diastereomeric aminoethylcysteine peptides (R, S) are generated in an approximately 1:1 mixture. Only peptides containing the R stereochemistry are substrates for lysine-specific proteases, resulting in cleavage only at 50% of the modified phosphorylation sites.

The general drawback of β-elimination is the contamination from peptides with other modifications that also undergo β-elimination, such as *O*-glycosylation. Furthermore, the strategy cannot be applied for tyrosine phosphorylated peptides, and tagging of nonphosphorylated serine residues has also been observed [31, 99].

In 2001, Zhou and co-workers introduced a multistep chemical modification strategy (phosphoramidate chemistry, PAC), which can be applied to all phosphorylated residues [101]. Firstly, the carboxyl groups on the peptides are *O*-methyl-esterified to prevent them from interacting in subsequent steps and causing nonspecific purification. Phosphopeptides are derivatized by a sulphydryl group and subsequently linked to iodoacetyl groups immobilized on a synthetic polymer solid support [102] or glass beads [103] through phosphoamidate chemistry. The phosphate groups are reconstituted by acid hydrolysis of the phosphoramidate bonds (*e.g.*, using TFA [101]), facilitating the identification of the phosphorylation sites by MS. However, the workload of the strategy is extensive due to the many chemical reactions and sample may be lost at each step, especially during the O-methylesterification steps. Bodenmiller and co-workers used PAC for the isolation of phosphopeptides from *Drosophila melanogaster* Kc167 cells in a new integrated chemical, MS, and bioinformatic approach to improve the confidence in the identification and assignment of phosphorylation sites [103].

6 Comparison of enrichment methods

The different methods for phosphoprotein or phosphopeptide enrichment provide different results. Some methods selectively enrich for specific species, such as antibodies

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against phosphotyrosine in immunoprecipitation techniques, or the combination of β-elimination and Michael addition reactions for the study of serine and threonine phosphorylated peptides. Other enrichment methods, such as calcium phosphate precipitation, SCX, SAX, and HILIC, function better as preseparation techniques to reduce sample complexity prior to more specific phosphopeptide enrichment methods including IMAC, TiO_2 chromatography, SIMAC and PAC. The enrichment techniques themselves also have specific advantages and disadvantages, including different specificities. In our hands, TiO_2 chromatography enrich mono-phosphorylated peptides more efficiently than multiply phosphorylated peptides as the latter bind too strongly for sufficient elution, whereas IMAC has a preference for multiply phosphorylated peptides, but is limited by a low capacity and selectivity when used for highly complex samples. SIMAC seems to provide a significant improvement by allowing the identification of more multiply phosphorylated peptides than any other enrichment method currently available. However, this technique also needs to be combined with a prefractionation technique in order to identify more phosphopeptides from complex samples. For example, when SIMAC was compared to TiO_2 chromatography, some phosphopeptides (approximately 10%) were solely identified by TiO_2 chromatography alone [74]. Bodenmiller and co-workers made a comprehensive comparison study, in which they examined the reproducibility, specificity and efficiency of IMAC, PAC, and two protocols for TiO_2 chromatography (pTiO_2 (phthalic acid in the loading buffer) and dhbTiO_2 (DHB in the loading buffer) [79]. Each method was tested for the enrichment of phosphopeptides from 1.5 mg tryptic peptides from cytosolic fractions from *D. melanogaster* Kc167 cells. The authors found, when studying the LC-MS patterns, that the overlap between replicate injections of a specific enriched sample was nearly equal to the overlap of independently enriched samples using the same method. This was evident for any one of the tested methods. The internal patterns also shared high similarity. As a conclusion, all tested enrichment methods were highly reproducible and suitable for quantitative phosphoproteomics [79]. Bodenmiller *et al.* also found that none of the methods tested were able to identify the entire phosphoproteome, but that the different methods identified distinct and partially overlapping segments of the cytosolic Kc167 phosphoproteome [79]. This is not surprising as the different methods have different binding affinities for phosphate groups and neighbouring amino acid residues will also contribute to this binding affinity.

In general, it is desirable to keep a phosphoproteomic strategy simple with relatively few sample preparation steps in order to maximize the sensitivity. The more sample handling, the greater the risk of losing phosphopeptides through adherence to the surfaces of tubes and tips. However, this is less critical with a high amount of starting material. Furthermore, the efficiency of a given enrichment method also dependents on the amount of starting material, as well as on the expertise of the scientist performing the enrichment. This is why detailed quality protocols of enrichment methods are highly recommended [76, 104–108]. A summary of the main phosphoproteomic methods is supplied in Table 1.

7 Phosphopeptide sequencing by tandem mass spectrometry

In order to make site-specific phosphorylation studies, it is important to be able to assign the specific amino acid that becomes phosphorylated upon a biological event. Today most phosphorylation sites are identified by Tandem MS, however, sequencing of phosphopeptides by MS/MS is not a trivial task due to the low ionization efficiency as well as the loss of the labile phosphate group resulting in low number of peptide fragment ions for identification.

7.1 Collision-induced dissociation

Peptide ions fragmented by CID in the mass spectrometer, produce series of y- and b-ions [109, 110] (Fig. 3), from which the peptide sequence can be interpreted (Fig. 4). Phosphorylation on serine and threonine residues are often labile and conventional CID will typically result in the partial loss of phosphoric acid (H_3PO_4 (98 Da), neutral loss) due to the gas-phase β-elimination of the phosphoester bond, generating dehydroalanine and dehydroaminobutyric acid. Unfortunately, most collision energy is used for this fragmentation pathway resulting in inadequate fragmentation of the peptide backbone. Frequently, only limited sequence information is obtained, and the identification of phosphorylation sites is therefore compromised. Partial neutral losses are also observed for phosphotyrosine residues (80 Da (HPO_3)), however, the phosphate group on tyrosine residues is much more stable than on the two other phosphoamino acids described. A characteristic phosphotyrosine immonium ion at m/z 216 in the fragment ion spectrum can be used as an indicator of the presence of a phosphotyrosine in the peptide sequence. Steen *et al.* specifically targeted tyrosine-phosphorylated peptides by product ion scanning using the m/z 216 immonium product as a diagnostic ion [20, 111]. In another product scanning strategy, a triple quadrupole instrument can be set to automatically switch between negative ion mode, where it scans for the precursor at m/z 79 (PO_3) and positive ion mode for MS/MS analysis of the detected precursors to specifically identify serine and threonine phosphorylated peptides [112, 113].

Since the phosphate groups on serine and threonine residues are labile, the ion originating from the loss of phosphoric acid can be selected for further fragmentation by MS^3 (pdMS^3) [61]. Here, a phosphopeptide is selected for fragmentation and when a neutral loss is detected, this ion is automatically isolated for further fragmentation. This method provides

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Table 1. A summary of the main methods used in phosphoproteomic studies

	Principle	Origin of sample	Results	Ref.
Sample preparation				
The use of protease and phosphatase inhibitors	Protecting phosphoproteins from degradation and loss of phosphate groups during sample preparation	Plasma membranes from human mesenchymal stem cells	Identifying clear differences between the use of different phosphatase inhibitors	[10]
Detection methods				
Phosphospecific staining/labeling using Pro-Q Diamond, ^{32}P or ^{33}P	Phosphospecific staining using Pro-Q Diamond upon gel electrophoresis to visualize phosphorylated proteins by fluorescence Labeling of phosphoproteins with ^{32}P or ^{33}P *in vivo* or *in vitro*. Subsequent detection using autoradiography	Whole-cell extracts of *M. genitalium and M. pneumoniae*	Showed that phosphorylation in mycoplasmas is comparable to that of other bacterial species	[16]
Western blotting using phosphospecific antibodies	Detection of phosphoproteins upon gel electrophoresis by transferring the proteins to a membrane and probing them with specific antibodies against phosphorylated residues	ARPE-19 cell line	Found that cross talk between EGFR and c-Met may play a key role in regulating RPE cell migration, proliferation and wound healing	[23]
Affinity enrichment methods				
Immunoprecipitation using antibodies against specific phosphorylated peptide sequences	Isolation of phosphoproteins by binding to phospho-specific antibodies (*e.g.*, anti-phosphotyrosine antibodies)	HeLa cells, 293T cells and BaF3 cell	Identification of a novel immuno receptor tyrosine-based activation motif-containing molecule, STAM2, and characterization of its importance in growth factor and cytokine receptor signaling pathways	[29]
Immobilized Metal ion Affinity Chromatography (IMAC)	Purifying phosphoproteins and phosphopeptides from complex samples by their affinity toward positively charged metal ions (Fe^{3+}, Al^{3+}, Ga^{3+} or Co^{2+}) chelated to a solid support	His_6-tagged NBDI-R domain (amino acids 404–830 of human CFTR)	First report of phosphorylation of ^{753}Ser in full-length cystic fibrosis transmembrane conductance regulator (CFTR.	[40]
Titanium dioxide (TiO_2) chromatography	Highly selective enrichment of phosphopeptides from complex samples by their affinity toward TiO_2-coated beads packed in a micro-column	Human mesenchymal stem cell plasma membrane fractions	703 unique phosphorylation sites were identified in 376 phosphoproteins	[10]
Sequential elution from IMAC (SIMAC)	Method in which mono-and multi-phosphorylated peptides are efficiently enriched from highly complex samples and separated prior to subsequent MS/MS analysis	Whole-cell extracts of human mesenchymal stem cells	716 phosphorylation sites were identified from just 120 μg protein	[74]
Calcium Phosphate Precipitation	Preisolation of phosphopeptides by precipitation using disodium phosphate (Na_2HPO_4), ammonia solution ($NH_3 \cdot H_2O$) and calcium chloride ($CaCl_2$) prior to other enrichment strategies (*e.g.*, IMAC)	The seed of rice (*Oryza sativa* L. ssp. *indica*) cultivars, 9311	More than 90% of the enriched peptides proved to be phosphorylated	[93]

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Table 1. Continued

	Principle	Origin of sample	Results	Ref.
Strong cation exchange chromatography (SCX)	At pH 2.7 phosphopeptides will have a +1 charge and bind weakly to the cations in the SCX particles; they can therefore be eluted prior to nonphosphorylated peptides with higher charge states; SCX is most efficient as a pre-fractionation tool prior to subsequent phosphopeptide enrichment (*e.g.*, TiO_2 chromatography)	Cytosolic and nuclear fractions from HeLa cells	Identified 6600 phosphorylation sites in 2244 proteins upon stimulation with EGF by combining SCX and TiO_2 chromatography	[86]
Hydrophilic Interaction Chromatography (HILIC)	For prefractionation of peptides prior to phosphopeptide enrichment method (*e.g.*, IMAC)	HeLa cells	Showed that prefractionation using HILIC improved phosphopeptide selectivity of IMAC to more than 99%	[62]
Chemical derivatization				
β-elimination and Michael addition reaction	Converting phosphoserine and phosphothreonine into dehydroalanine and β-methyldehydroalanine, respectively by β-elimination; subsequently generating aminoethylcysteine from dehydroalanine by Michael addition (and generating β-methylaminoethylcysteine from β-methyldehydroalanine), which are isosteric with lysine and are used as targets for lysine specific digestion to aid the assignment of phosphorylation sites	Tubulin purified from bovine brain and *in vitro* phosphorylated by recombinant G protein–coupled receptor kinase 2 (GRK2).	Presented an approach for phosphopeptide mapping by making phosphorylated threonine and serine residues recognizable for a protease	[98]
β-elimination and Michael addition reaction	To cross-link phosphate groups to a biotin-tag or for direct affinity purification	Bovine synapsin I, rat Ca2+/calmodulin-dependent kinase II (CaM kinase II) and rabbit calmodulin	Identification of four *in vitro* phosphorylation sites in bovine synapsin I by Ca2+/calmodulin-dependent kinase II, two of which were novel	[98]
Phosphoramidate Chemistry (PAC)	To link phosphate groups to immobilized iodoacetyl groups for purification	Different phosphoprotein standards and a whole yeast cell lysate	Demonstrate that PAC is efficient for the isolation and identification of both serine-, threonine- and tyrosine-phosphorylated proteins from highly complex samples	[101]

Each method is listed with details of the principle, an example of an application, the origin of sample for the application as well as a reference. The references in the table refer to the application studies, which are not necessarily the original references to the particular methods.

more phosphopeptide sequence information, and aids the specific assignment of the phosphorylation sites on serine or threonine, respectively. Naturally, the efficiency of $pdMS^3$ is best for abundant ions. In addition, the filling time needed and fragmentation setup will reduce the overall number of fragmented peptides.

Multi-stage activation (MSA), also known as pseudo-MS^3, is a further development of MS^3 [114]. In MSA, the fragmentation of the precursor ion occurs simultaneously with the fragmentation of the ion originating from the neutral loss. Hereby the MS^2 and the MS^3 are combined in a hybrid spectrum, which will result in more information for a data

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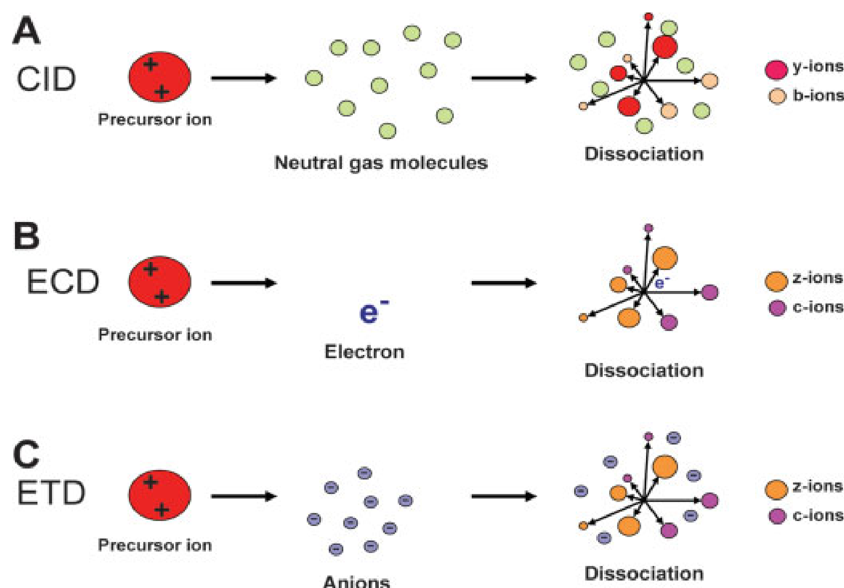

Figure 3. Principle of CID, ECD, and ETD. In CID fragmentation is induced by the collision of peptide ions with neutral gas molecules (A). In ECD the capture of a thermal electron causes peptide ions to fragment (B). Electron transfer dissociation (ETD) induces peptide fragmentation by the same nonergodic process as ECD, however, anions with sufficiently low electron affinities such as anthracene or azobenzene function as electron donors (C).

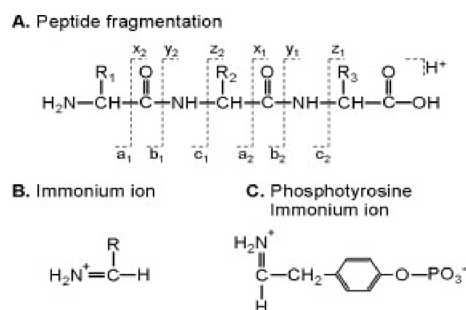

Figure 4. Common nomenclature of peptide fragment ions. The common nomenclature for different fragment ions generated in MS/MS was proposed by Roepstorff and Fohlman in 1984 [109], and slightly modified four years later by Biemann [110]. In CID, primarily y and b ions are produced, whereas z and c ions are generated by ECD and ETD.

base search and improved sequence information and thereby improved phosphorylation site assignment. MSA is implemented on quadrupole-IT [114] or linear IT-orbitrap instruments [86]. Figure 5 illustrates the different results obtained when using MS^2, MS^3, and MSA for the analysis of a diphosphorylated peptide with the sequence pSRpTPLLPR.

7.2 Electron capture dissociation

In 1998, Zubarev and co-workers presented a new strategy for the fragmentation of multiply charged protein and peptide ions [115]. The principle of this method is the generation of charge-reduced, radical peptide ions from the reaction of multiply charged peptide (or protein) ions with low-energy (thermal) electrons, a method termed electron capture dissociation (ECD). The capture of a thermal electron causes the peptide ion to fragment by a nonergodic process (*i.e.*, does not involve intramolecular vibrational energy redistribution, such as that observed in CID fragmentation). Fragmentation then occurs in the peptide between the backbone amide and the alpha carbon (Cα) generating c and z ions (Fig. 4). The fragmentation is largely independent on the peptide sequence except that it has a selectivity for disulfide bonds (due to the high radical affinity of the bond) and is inefficient at cleaving *N*-terminal to proline residues as they are cyclic around the amide-$^{\alpha}$C and require the breaking of two bonds [115]. A major advantage of ECD is that fragmentation occurs solely on the peptide backbone, and labile PTMs such as phosphate groups are therefore left intact on the resulting c and z fragment ions enabling identification of the specific phosphorylation sites [116, 117]. This also makes it a highly efficient fragmentation technique for the analysis of multiply phosphorylated peptides. The drawback is that ECD is only used in FT-ICR instruments due to the requirement of a static magnetic field for the thermal electrons.

7.3 Electron transfer dissociation

Electron transfer dissociation (ETD) was developed in an attempt to find an ECD-like dissociation method, which could be used on widely accessible instruments, such as quadrupole linear ITs [118, 119]. Syka and co-workers found that anions with sufficiently low electron affinities (*e.g.*, anthracene or azobenzene) could function as one-electron donors, and induce fragmentation by the same nonergodic process as ECD [119]. The implementation of ETD in lower-cost instruments makes it more readily used, however, the drawback of using instruments such as the quadrupole ITs as opposed to the FT-ICR, is reduced resolution and accuracy [119]. Chi and co-workers combined IMAC for phosphopeptide enrichment with ETD in a study of the yeast phosphoproteome, identifying 1252 phosphorylation sites [120],

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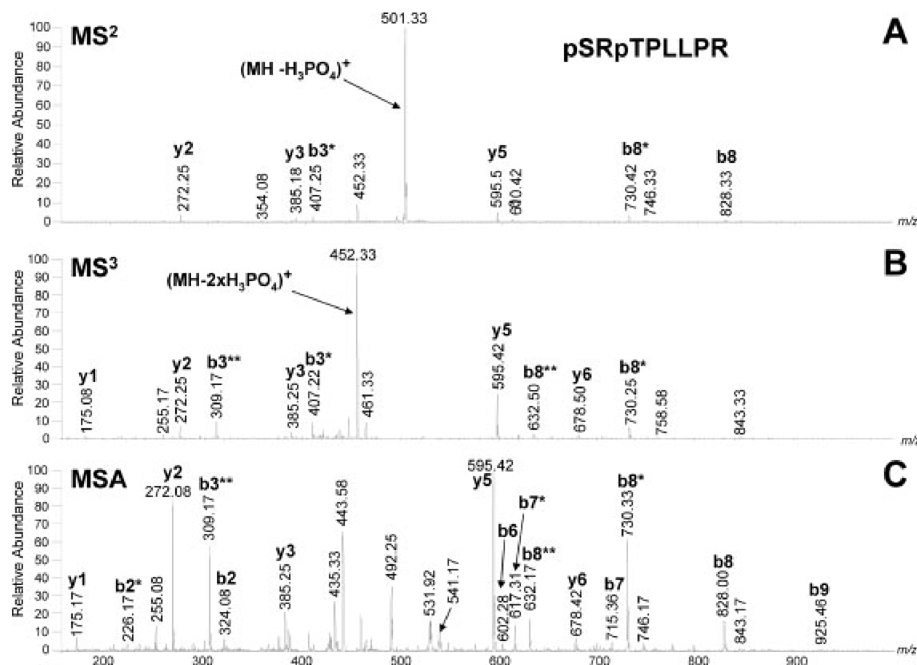

Figure 5. MS^2, MS^3, and MSA data obtained from a phosphopeptide with the amino acid sequence, pSRpTPLLPR. The doubly phosphorylated peptide originates from the serine/arginine repetitive matrix protein 2. As observed, poor sequence is obtained by MS^2 of this doubly phosphorylated peptide, due to the loss of phosphoric acid and site-specific fragmentation at the prolines (A). When the fragment ion originating from the loss of phosphoric acid is further fragmented (MS^3) more sequence information is obtained however still a pronounced ion originating from the loss of two phosphoric acids is observed (B). MSA results in this example in improved sequence information (C).

whereas Molina *et al.* combined TiO_2 chromatography with ETD in a large-scale study of human embryonic kidney 293T cells, and identified 1435 phosphorylation sites of which approximately 80% were novel [85]. Molina *et al.* also compared the use of ETD and CID for fragmentation, and identified 60% more phosphopeptides when using ETD, mainly due to an average of 40% more fragment ions. However, the authors found that a combination of ETD and CID by an automatic alternating mode gave the best results as little overlap was observed from the two fragmentation techniques [85].

Since ETD and ECD are optimal methods for fragmentation of multiply charged peptide ions (*e.g.*, charges of +3, +4), an alternative protease might be a better choice as trypsin mainly produces doubly charged ions, which are suitable for CID. Lys-C is a logical choice as it generates trypsin-like peptide ions, but only cleaves *C*-terminal to lysine, thus creating longer peptides. Molina and co-workers tested the efficiency of ETD on peptides originating from trypsin or Lys-C digests and surprisingly found no major improvement through the use of Lys-C. This was thought to be due to a high level of missed cleavages in the tryptic digest caused by the presence of phosphate groups or highly acidic residues [85]. Recently, our group has presented an alternative way to increase the charge state on peptide ions. By adding 0.1% *m*-nitrobenzyl alcohol (*m*-NBA) to the solvent for LC-MS, the predominant charge state of tryptic peptides and phosphopeptides increased from +2 to +3 or higher, improving their assignments by ETD MS/MS and database searching [121]. The strategy is currently being tested on more complex samples.

8 Quantitation strategies for phosphoproteomic studies

The development of highly sensitive and efficient strategies for large-scale phosphoproteomics, has taken the phosphoproteomic field into a new area. At a proteomic scale, protein changes in response to various stimuli, differentiation or disease progression are readily measured using specific quantitation techniques. These techniques can now be implemented to identify up- or down-regulation of phosphorylation at specific sites upon stimulation. This will provide insight into the specific regulation of different phosphorylation pathways and offer a better overview of the different target proteins affected by phosphorylation upon stimulation. However, one must bear in mind that such data need to be normalized to corresponding protein abundance in order to verify regulations as phosphorylation-directed, and not related to protein synthesis or degradation.

Many quantitation techniques introduce a stable isotope label or tag on proteins or peptides containing *e.g.*, ^{2}H, ^{13}C, ^{18}O, or ^{15}N. The abundance of the labeled species originating from *e.g.*, diseased tissue can then be compared to the abundance of the nonlabeled or differently labeled species originating from *e.g.*, healthy tissue.

Quantitation methods differ at the level in which the label is introduced into the sample. Some techniques introduce the label in viable cells in cell culture ($^{14}N/^{15}N$ labeling [122] and stable isotope labeling by amino acids in cell culture, SILAC [123, 124]). Others introduce the tag at the protein level (isotope-coded affinity tags, ICAT [125]) or peptide level (isobaric peptide tags for relative and absolute quantifi-

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cation, iTRAQ [126] or ^{18}O-labeling during tryptic digestion [127]), whereas yet others use a label-free strategy in which quantitative information is extracted from LC-MS (extracted ion current, XIC) or MS/MS data (protein abundance index, PAI) in sequential experiments [128–130]. Not all quantitation strategies are efficient for phosphoproteomic studies. Using the ICAT method, only cysteine-containing peptides will be labeled. During the development of the SIMAC strategy we found that only ~5% of the 492 phosphopeptides identified from 120 μg whole-cell extract from hMSCs did in fact contain cysteine residues (unpublished results). Overall, most proteomic quantitation strategies can be implemented on phosphoproteomic studies. The various quantitation strategies are outside the scope of this review. They are described in more details in other recent reviews *e.g.*, by Bantscheff and co-workers [131].

9 Conclusions and perspectives

Phosphoproteomics is a field that has made rapid progress in the last few years and promises to make even further progress in the near future. The importance of protein phosphorylation as a regulator of most intracellular biological processes in nature is driving the method development in phosphoproteomics. Sensitive techniques for phospho-specific enrichment of biological samples provide enabling tools for the era of phosphoproteomics. Today, most phosphoproteomic studies are conducted by MS strategies in combination with some of the phospho-specific enrichment methods described in this review. The constant development of MS instrumentation that offer increase in the resolution, better mass accuracy, large dynamic range and enormous sensitivity and speed has expanded the phosphoproteomics field tremendously. In addition, highly efficient phosphopeptide fragmentation strategies such as $pdMS^3$, MSA, ECD, and ETD enable better identification and sequencing of phosphopeptides for accurate assignment of phosphorylation sites. Combined with the recent development of new and highly specific and sensitive methods for phosphoprotein and phosphopeptide enrichment, MS has for the first time made it feasible to perform large-scale phosphoproteomic studies of highly complex samples [35, 43, 46, 51, 86]. However, we believe that further development of phosphoproteomic strategies is still needed in order to be able to characterize signaling pathways employing low abundant protein phosphorylation.

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