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Review

Enrichment and separation techniques for large-scale proteomics analysis of the protein post-translational modifications☆

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ABSTRACT

Comprehensive analysis of the post-translational modifications (PTMs) on proteins at proteome level is crucial to elucidate the regulatory mechanisms of various biological processes. In the past decades, thanks to the development of specific PTM enrichment techniques and efficient multidimensional liquid chromatography (LC) separation strategy, the identification of protein PTMs have made tremendous progress. A huge number of modification sites for some major protein PTMs have been identified by proteomics analysis. In this review, we first introduced the recent progresses of PTM enrichment methods for the analysis of several major PTMs including phosphorylation, glycosylation, ubiquitination, acetylation, methylation, and oxidation/reduction status. We then briefly summarized the challenges for PTM enrichment. Finally, we introduced the fractionation and separation techniques for efficient separation of PTM peptides in large-scale PTM analysis.

Contents

1. Introduction

Protein PTMs play important role in regulating most of the critical biological processes and are close related to the development of many serious human diseases, such as cancer, neurodegenerative disease and diabetes [1]. Large-scale and in-depth analysis of the protein PTMs is crucial to elucidate the mechanisms of different physiological and pathological events. However, comprehensive characterization of protein PTMs still challenges the modern analytical platforms due to the inherent nature of low abundance and low stoichiometry of PTMs. More than 300 types of protein PTMs are known to occur physiologically within the living organism [2]. Each PTM has different characteristics and requires different

☆ Invited paper for the Honor Issue of Professor Peichang Lu's 90th birthday.

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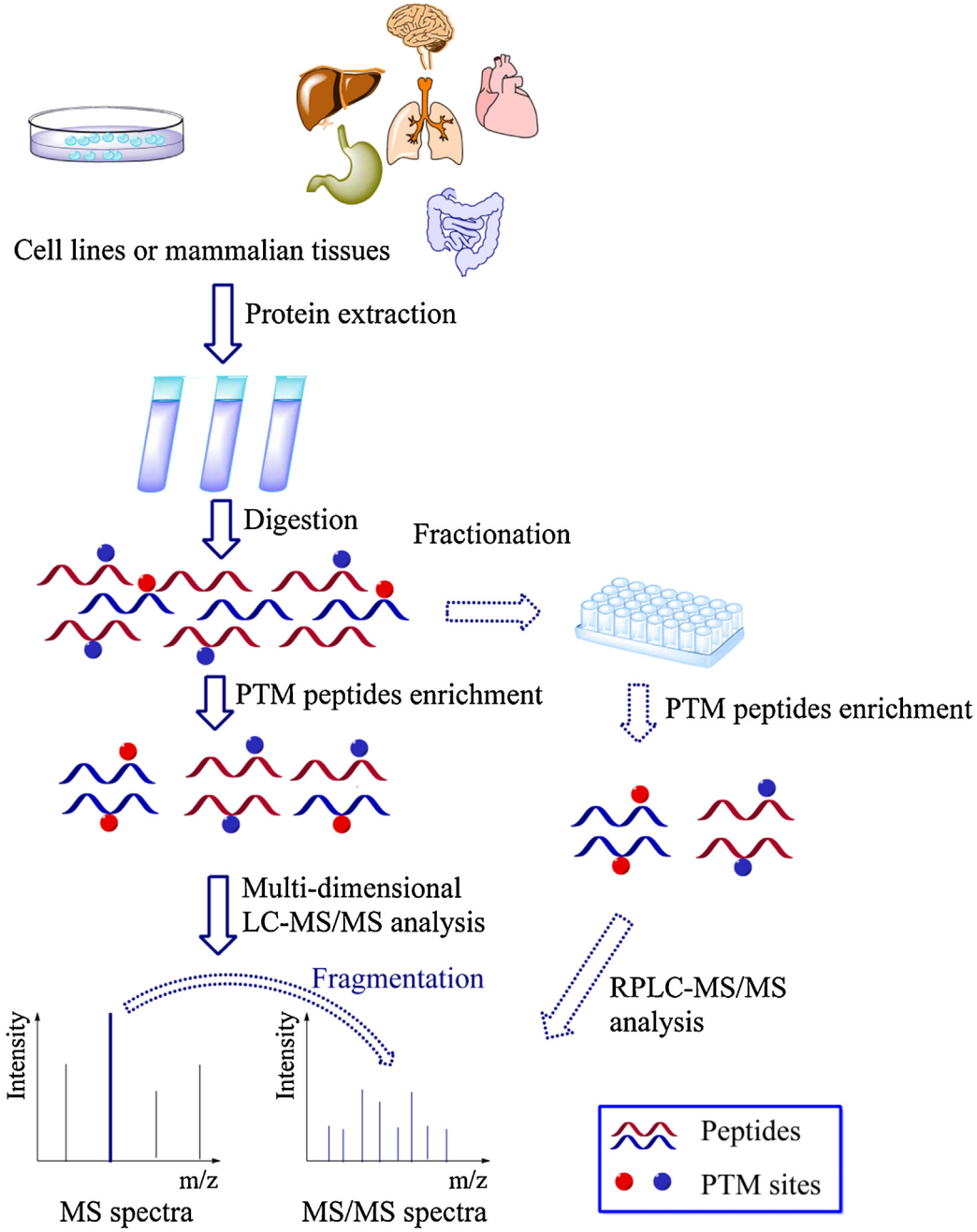

Fig. 1. The general workflow for large-scale PTM analysis by bottom-up proteomics.

approaches to analyze. The high diversity of PTMs makes the systematically analysis of PTMs extremely challenge.

The bottom-up proteomics (i.e. shotgun proteomics), which is based on mass spectrometry (MS) analysis of proteolytic peptides, has become the most powerful technique for in-depth characterization of protein PTMs [3,4]. The general shotgun proteomics workflow for analysis of protein PTMs mainly includes the steps of protein digestion, specific PTM peptide enrichment, pre-fractionation and LC separation, and MS detection (Fig. 1). Among these steps, the detection of PTM peptides by MS is vital for the identification of PTMs. A variety of fragmentation techniques including collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), electron-transfer dissociation (ETD) have emerged to generate information rich spectra for identification and localization of PTMs [5]. Although the mass spectrometers have obtained a rapid advance in past decades, it is still impossible to directly identify most of the PTMs from complex protein digests in a standard LC–MS/MS analysis [6]. Because the low stoichiometry of the PTMs and low abundance of the proteins carrying PTMs, the PTM peptides coexists with huge amount of unmodified peptides which seriously suppress the detection of PTM peptides [7]. To sensitively identify PTM peptides, these peptides must be specifically enriched. It should be mentioned the enriched PTM peptides are still very complex. To achieve large-scale analysis, the enriched peptides must be subjected to efficient separation prior to MS analysis.

Generally speaking, there are two types of approaches, i.e. chemical approaches and biochemical approaches, to enrich PTM peptides (Fig. 2, Table 1). The first type, chemical approaches, exploits the different chemical properties of PTM and non-PTM peptides. The chromatography-based purification methods (Fig. 2A) and the chemical derivatization methods (Fig. 2B) are

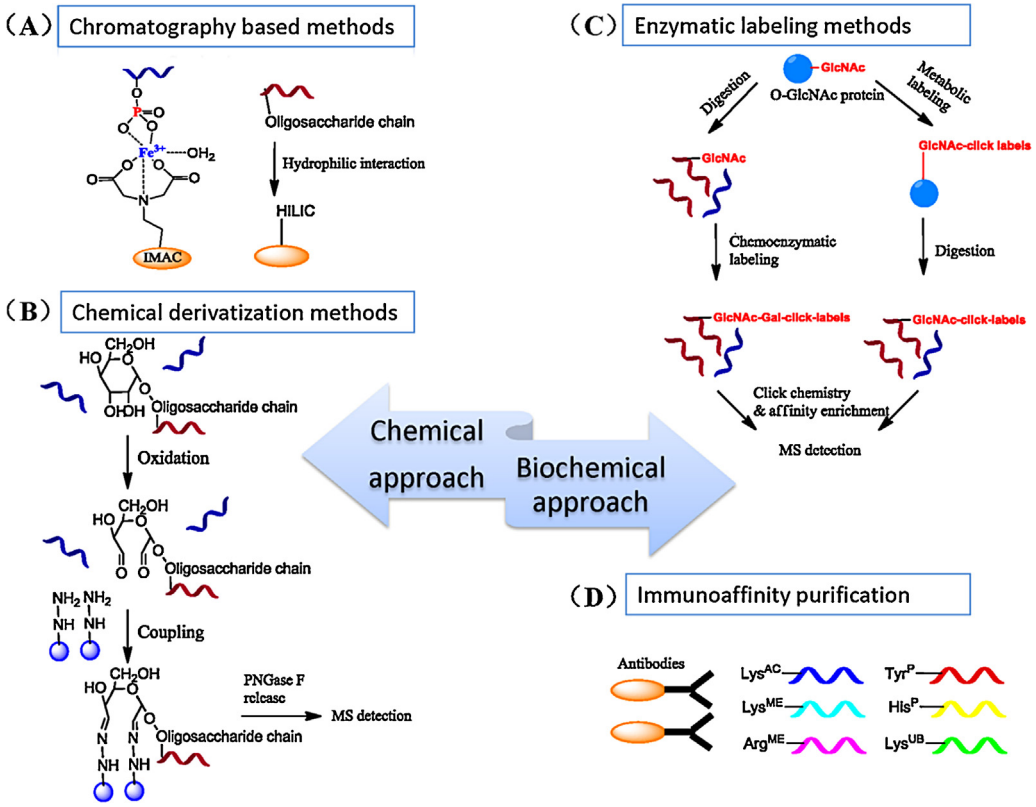

Fig. 2. Illustration of major strategies for enrichment of PTM peptides. Type I: chemical approaches, (A) chromatography-based purification methods for isolation of PTM peptides like phosphopeptides (IMAC), glycopeptides (HILIC), (B) chemical derivatization methods for isolation of PTM peptides like N-linked glycopeptides (Hydrazide chemistry method). Type II: biochemical approaches, (C) enzymatic approaches for isolation of PTM peptides like O-GlcNAc peptides, (D) immuaffinity purification for isolation of PTM peptides like acetylated, methylated, ubiquitinated, and tyrosine and histidine phosphorylated peptides.

belong to this type. For example, glycopeptides can be enriched by hydrophilic interaction chromatography (HILIC) because they are more hydrophilic than non-glycopeptides due to the highly hydrophilicity of the glycan chains; the glycopeptides can also be enriched by a chemical derivatization method (hydrazide chemistry method) because the glycopeptides could be oxidized and covalently coupled to hydrazide beads while generally the non-glycopeptides cannot. The second type is the biochemical methods which exploit the specific interaction between biomolecules. The enzymatic labeling approach (Fig. 2C) which are based on the specific interaction of enzyme and substrate and the immunoaffinity chromatography methods (Fig. 2D) which are based on the specific interaction of antibody and antigen belong to this type. The enzymatic methods have been applied to enrich O-GlcNAc and S-glutathionylation peptides while immunoaffinity purification methods are applied to enrich a variety of PTM peptides (Table 1).

Because the PTM peptides enriched from the protein digest sample overwhelming the peak capacity of one-dimensional LC separation, efficient fractionation prior to RPLC–MS/MS is indispensable for large-scale analysis [8]. The proteome-wide PTM analysis by elegantly coupling of the specific enrichment method and efficient fractionation strategies has extremely expanded our vision on protein PTMs. For example, the cutting edge phosphoproteomics approach is capable of mapping more than 50,000 distinct phosphorylated peptides in a single human cancer cell line [9].

Table 1
Overview of the enrichment methods for some PTMs.

Enrichment methods		PTMs	Applicable for large-scale analysis
Chemical approach – chromatography	IMAC or MOAC	pSer/pThr/pTyr	Yes
	HILIC	N-linked glycopeptides	Yes, relative poor specificity
	Boric acid	N-linked glycopeptides	No, poor specificity
Chemical approach – chemical derivatization	Hydrazide chemistry	N-linked glycopeptides	Yes, high specificity
	Biotin switch technique	Redox modification	Compromised by incomplete reaction and side reactions
Biochemical approach – enzymatic labeling		O-GlcNAc S-glutathionylation	Long preparation cycle and multiple steps
Biochemical approach – immunoaffinity purification		^{Ac}Lys	Yes
		^{Me}Lys, ^{Me}Arg,	Yes, relative poor specificity
		^{Ub}Lys	Yes
		pTyr	Yes
		pHis	No, poor specificity

This ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. Recently, there is a review nicely summarizing the protein-level PTM enrichment and separation methods [10], and another review giving a brief introduction of the status of PTM analysis by MS [11]. In this review, we will mainly summary recent progresses in the enrichment and fractionation strategies for large-scale protein PTM analysis.

2. Protein PTMs and their enrichment strategies

2.1. Protein phosphorylation

About 30% of cellular proteins can be phosphorylated during the cell cycle [12], and the abnormal protein phosphorylation events are always accompanied by diseases [13]. Phosphoproteomics analysis can identify thousands of phosphorylation sites from cell lysates which provides valuable information to elucidate the biological regulation mechanisms. In recent years, phosphoproteomics has made great progress and can be mined much deeper than before largely due to the emerging of excellent enrichment phosphopeptide methods [14]. A variety of phosphopeptide enrichment methods were developed to enrich phosphopeptides. The chemical derivation methods including using Michael addition/beta-elimination reaction [15] and phosphoramidate chemistry [16] were reported to enrich phosphopeptides 10 years ago. However, these methods are rarely used in the current phosphoproteomics analysis due to their poor yield and tedious procedure. Instead, the two chromatography based approaches, i.e. the metal oxide affinity chromatography (MOAC) and immobilized metal ion affinity chromatography (IMAC), are the most popular approaches because of the straight forward procedures and good performance.

MOAC was introduced for enrichment of phosphorylated substances in 1990 [17] and is currently widely applied to enrich phosphopeptides from the protein digests prior to MS analysis. Two kinds of metal oxides, ZrO_2 and TiO_2 [18,19], are often used in large-scale phosphoproteome studies [20–22] due to their relative high enrichment efficiency and easy availability. Recently, many composite materials with either ZrO_2 or TiO_2 such as the TiO_2/grapheme composites [23,24], Fe_3O_4@TiO_2–ZrO_2 microspheres [25], SiO_2/TiO_2 composite monolithic capillary column [26], TiO_2-modified porous nylon membranes [27], magnetic TiO_2-coated carbon-encapsulated iron nanoparticles [28], and magnetic yolk–shell Fe_3O_4@mTiO_2@mSiO_2 nanocomposite [29] were developed to enrich the phosphopeptides. These new MOAC materials generally have high surface area and so have high enrichment capacity. It was reported that Fe_3O_4@TiO_2–ZrO_2 microspheres enabled the enrichment of phosphopeptides from as low as 5×10^{-10} M tryptic digests of β-casein (500 μL) [25]. However, these new materials were seldom used in large-scale phosphoproteome analysis probably because they were not available to biological labs.

In addition to the development of new enrichment materials, the sample loading conditions also have significant effect on phosphopeptide enrichment performance in MOAC [30–32]. The enrichment selectivity of TiO_2 was found to increase from 12% to 58% when 0.7 M trifluoroacetic acid (TFA) was added in the loading buffer [33]. The non-specific adsorption of peptides through hydrophobic interaction could be prevented by addition of high concentration of organic solvent such as acetonitrile [34]. As a result, the addition of TFA and acetonitrile in loading buffer has been the standard protocol for the phosphopeptide enrichment in MOAC [9,35]. Because the non-specific adsorbed peptides are rich in acidic residues (aspartic acid (D) or glutamic acid (E)), a variety of organic acids or organic acid salts were added as competing agents to improve the enrichment specificity. For example, addition of 2,5-dihydroxybenzoic acid (DHB) and phthalic acid in the loading buffer was reported to improve the enrichment specificity [34]. However, increased loading pressure on the LC systems, short column lifetime and contamination of the MS extraction cone were observed when these compounds were used [30]. Alternatively there are many other organic acids including citric acid [30], ammonium glutamate [36], 1-octanesulfonic acid (OSA) [37], glycolic acid, lactic acid and β-hydroxypropanoic acid [38] could be used without the occuring of above problems. All of these compounds contain carboxyl and hydroxyl group which will compete with the carboxyl groups on D and E containing peptides to be adsorbed on MOAC beads. Therefore they are served as “non-phosphopeptide excluders” [30]. It should be mentioned that the concentration of these competing compounds must be carefully optimized. Low concentration of these compounds may serve as non-phosphopeptide excluders, while high concentration may lead to the loss of monophosphorylated peptides. For example, a recently study showed that addition of high concentration citric acid in the loading buffer of TiO_2 based phosphopeptide enrichment method had been verified as a good approach to enrich multi-phosphorylated peptides [39]. This is because most of the mono-phosphorylated peptides are not bound under this condition. By diluting the flow through from above enrichment, the mono-phosphorylated peptides could be enriched by TiO_2 again. Thus this facile two-step enrichment method enabled the identification of both mono-phosphorylated and multi-phosphorylated peptides which improved the overall phosphoproteome analysis coverage [39]. Recently, Fukuda et al. reported that addition of polyhydric alcohols, such as glycerol in the loading and washing buffer of MOAC also improved phosphopeptide selectivity [40].

Immobilized metal ion affinity chromatography (IMAC) for the enrichment of phosphopeptides is based on the specific chelating interaction of negatively charged phosphate group with the immobilized metal ions on the solid beads. Conventionally, metal ions are chelated to nitrilotriacetic (NTA) or iminodiacetic acid (IDA) of the IMAC matrix (Fig. 3). The initial metal ion used in IMAC is Fe^{3+} [41], after that, a lot of metal ions such as Al^{3+}, Ga^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+}, etc. have been used in IMAC. Unfortunately, this conventional IMAC suffers the problem of nonspecific binding of D and E containing peptides. To overcome this problem, methyl esterification of carboxyl groups on D and E residues prior to phosphopeptide enrichment was proposed [12]. This method is rarely used since its introduction because it suffers the disadvantages of incomplete esterification, occurring of some side reactions and substantial peptide loss [12]. Instead of chemical derivatization of peptides, optimization of the loading buffer is a simple approach to improve the enrichment specificity. The high enrichment specificity could be achieved by acidifying the loading buffer to pH 2–2.5 with an organic acid such as acetic acid [42] or TFA [43]. This is because the acid dissociation constant (pK_a) between acidic amino acids (pK_a (E) = 4.25, pK_a (D) = 3.65) and the phosphate group (pK_a = 2.1) are different. At above pH range, most acidic amino acids were protonated which cannot be bound to the positively charged metal ions, while most phosphate groups are still deprotonated, so they are able to bind to the positive metal ions.

The conventional IMAC method has been developed and optimized for a long time and has been used in many studies. However, the performance of this IMAC for phosphopeptide enrichment cannot compete with that of MOAC method [32,33,44,45]. Recently, a new type of IMAC, i.e. Ti^{4+} or Zr^{4+}-IMAC was developed in our lab [46–48]. It has much higher performance than either the conventional IMAC (Fe^{3+}-IMAC) or MOAC (TiO_2 or ZrO_2). Phosphoproteomics analysis using Ti^{4+}-IMAC leaded to identification of about 2 and 4 times of phosphopeptides that identified using TiO_2 and Fe^{3+}-IMAC [47]. Unlike in conventional IMAC where NTA or

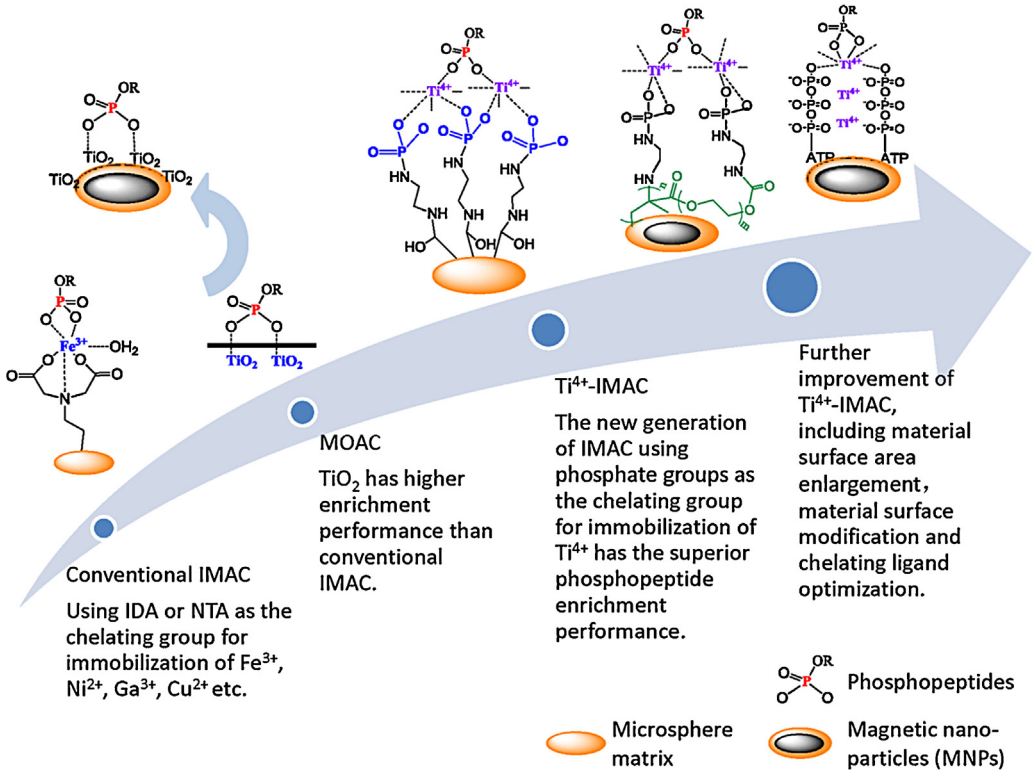

Fig. 3. Evolution of phosphopeptide enrichment methods.

IDA is used as chelating group, phosphate group is used as the chelating group in this new IMAC (Fig. 3). The immobilization of Ti^{4+} or Zr^{4+} ions onto the solid phase matrix is achieved by their strong and specific interaction with the phosphonate modified surface, and their specific enrichment of phosphopeptides is also achieved due to the specific interaction of the immobilized metal ions with the phosphate groups on the peptides [49,50]. The improved performance of Ti^{4+} or Zr^{4+}-IMAC for phosphopeptide enrichment is mainly because it integrates the advantages of conventional IMAC and MOAC. Firstly, as in the MOAC, it exploits the high specific interaction between metal (IV) and phosphate group. Secondly, as in conventional IMAC, a flexible "spacer arm" is introduced in Ti^{4+} or Zr^{4+}-IMAC which provides a beneficial spatial orientation for the phosphopeptide binding by reducing the steric hindrance [50,51]. Thus Ti^{4+} or Zr^{4+}-IMAC has higher specificity than conventional IMAC and is more accessible than MOAC to phosphopeptides. Due to its excellent performance, it is routinely used in our lab [52–59] and our collaborate labs [60–63]. A detailed protocol to synthesize the Ti^{4+}-IMAC microsphere and to use this IMAC for phosphoproteomics was published in Nature Protocols [50].

This new generation of IMAC has drawn more and more attention. Many new matrix materials have been developed. Besides the conventional micrometer scale matrix materials, nanomaterials have also been synthesized to prepare the Ti^{4+} or Zr^{4+}-IMAC [64–71]. Among them, Fe_3O_4@SiO_2@PEG-Ti^{4+}-IMAC nanoparticle is very attractive. The thick grafting layer of the PEG brushes has a high chelating capacity of titanium ions. Due to the combination of the superior hydrophilic surface and the high binding capacity of the grafted PEG brushes, these magnetic nanoparticles were demonstrated to have a high phosphopeptide recovery (over 70%) and low limit of detection (0.5 fmol). An exceptional great specificity to capture phosphopeptides from a tryptic digest of the mixture of a non-phosphorylated protein BSA and a phosphorylated protein α-casein with molar ratios of BSA/α-casein up to 2000:1 was achieved [62]. Recently, a new type of Ti^{4+}-IMAC using ATP as chelating groups was developed (Ti^{4+}-ATP-MNPs). The high recovery (84.76 ± 2.9%, n = 3) and low limit of detection (3 amol, phosphopeptides in β-casein digests detected by MALDI-TOF MS after on-target enrichment by Ti^{4+}-ATP-MNPs) were achieved by using this material. This may mainly due to the ATP molecule, which composes of three phosphate groups, offering strong and active phosphonate sites to bind metal ions. Besides, the hydrophilic purine base and pentose sugar groups of the ATP grafted on the material surface might also contribute to the low nonspecific adsorption of non-phosphopeptides [70].

It was reported that each method prefers to enrich different sets of phosphopeptides [72]. Combinational using of different enrichment methods is an effective approach to improve phosphoproteome analysis coverage [20]. A so-called SIMAC (Sequential Elution from IMAC) method by combining IMAC and TiO_2 has been developed to separately identify mono-phosphorylated peptides and multi-phosphorylated peptides [73]. Mono-phosphorylated peptides were eluted from Fe^{3+}-IMAC by ultra-acidic buffers (1% TFA/20% acetonitrile, pH = 1.0) due to its weak binding, and the eluate was further subjected to TiO_2 enrichment to remove the non-specific bound peptides. After the elution by acidic buffer, multi-phosphorylated peptides were still bound on the Fe^{3+}-IMAC and could be eluted by alkaline buffers. Mono-phosphorylated peptides and multi-phosphorylated peptides were separately analyzed by MS which resulted in improved coverage in phosphoproteome analysis [74,75]. Sequential enrichment of phosphopeptides from complex biological sample by repeatedly using the IMAC materials with the same or complementary characteristics greatly improved the phosphoproteome coverage, especially for the multi-phosphorylated peptides [76–78]. This may attribute to that the multi-phosphorylated peptides are separately enriched in the first weak IMAC enrichment fraction and therefore the ion suppression effect by mono-phosphorylated peptides during MS detection can be reduced [73].

The method to efficiently elute the phosphopeptides from MOAC or IMAC is also very important [79]. The captured phosphopeptides can be eluted by using phosphate buffer [42,80]. However, the phosphate is not compatible with MS detection. Thus an additional desalting procedure is needed, which will generate substantial sample loss. The volatile salt, NH_4HCO_3, is compatible with the MS detection and it (200–250 mM, pH = 9) was used as the elution buffer in online phosphopeptide enrichment system [81]. However it is not strong enough to elute very acidic and multi-phosphorylated peptides [82] and it would also make LC system suffering from high back pressure [45]. The most popular elution strategy is using ammonium hydroxide (NH_4OH) solution [19,83] as it can be easily removed by vacuum drying prior to the LC–MS/MS analysis without additional desalting step. Interesting, Kyono et al. recently found that secondary amines, such as piperidine and pyrrolidine were of better efficiency than the NH_4OH and phosphate buffers for phosphopeptide elution. Based on this discovery, they established a successive elution approach which eluted the phosphopeptides from TiO_2 column by 5% NH_4OH, 5% piperidine and 5% pyrrolidine serially. Thus they got a 1.6-fold increase in the number of identified phosphopeptides comparing with conventional conditions [79].

MOAC and IMAC methods both are powerful tools for the in-depth profiling of phosphoproteome. However, a weakness is the poor binding of phosphopeptides containing multiple basic residues due to their low binding affinity to the enrichment materials and the competition of high abundant phosphopeptides containing multiple acidic residues. This leads to an underrepresentation of basophilic kinase substrates in current phosphoproteome studies [62]. Recently, a negative enrichment strategy was reported to enhance the identification of basophilic kinase substrates [84]. This method was based on an observation that high-pH strong anion exchange (SAX) chromatography can separate tryptic phosphopeptides according to the number of acidic amino acid residues that they have. Thus SAX could be used to deplete acidic phosphopeptides. When SAX was applied to deplete the acidic phosphopeptides from the phosphopeptides enriched by Ti^{4+}-IMAC, the coverage for detection of basophilic kinase substrates was significantly improved. Interestingly, basic phosphopeptides can also be enriched by sequential use of the strong cation exchange chromatography (SCX) at two different pH values [85]. This strategy was based on the change of phosphate groups of phosphopeptides with multiple basic residues at the two different pH values. At pH = 3, phosphopeptides with multiple basic residues were co-eluted with the unphosphorylated peptides with one basic residue (they contain the same net positive charge) in SCX. While at pH = 1, the phosphate groups on phosphopeptides were protonated and they had one more positive charge, therefore it could be separated from the formally co-eluted unphosphorylated peptides.

Serine and threonine are the major sites of O-phosphorylation in phosphoproteome, while tyrosine phosphorylation is less abundant despite its vital role in signaling transduction. Currently, the most successful enrichment method for tyrosine phosphoproteome analysis is immunoaffinity purification of tyrosine phosphopeptides using anti-phosphotyrosine antibodies. In addition to tyrosine phosphopeptide enrichment, immunoaffinity purification of tyrosine phosphoproteins, which showed complementary identifications with those at peptide level [86,87], could be also used for phosphoproteome analysis. Alternatively to antibody, SH2 domain, the phosphotyrosine peptide binding domains, could be used to enrich tyrosine phosphorylated peptides [88]. However, the performance cannot compete with the immunoaffinity approach up to now. Phosphoserine/phosphothreonine antibodies are commercially available, but the application of these antibodies to enrich phosphoserine/phosphothreonine peptides is not very successful due to their poor specificity [89,90].

In spite of the big breakthroughs in the enrichment of O-phosphorylation, N-phosphorylation is long considered as an experimental artifact or a product of nonenzymatic activity. Until recently, the significance of this modification was observed in a growing number of cellular processes [91]. Among different types of N-phosphorylation, reversible histidine phosphorylation is the most prevalent N-phosphorylation. It was estimated that it accounts for 6% of the total protein phosphorylation in eukaryotes [92]. However, they were typically undetected in conventional phosphoproteomics analysis because of the instability of the phosphate–nitrogen bond in acidic solutions [93]. The instability of these phosphorylations even hindered the development of antibodies for N-phosphorylation enrichment. Interestingly, many commercially available anti-phosphotyrosine antibodies also recognize phosphohistidine, but the specificities is relatively low [94]. Recently, Kee et al. developed a pan-specific antibody for direct detection of protein histidine phosphorylation, which used the stable phosphorylated histidine analog to produce the anti-phosphohistidine antibody [95]. This antibody was successfully used for identification of histidine phosphorylation in proteins from E. coli cell lysates. Though this method did not lead to large-scale histidine phosphorylation analysis, it really provided new ideas to resolve the problem of lacking effective tools for N-phosphorylation enrichment.

2.2. Protein glycosylation

Protein glycosylation plays structural, protective, and stabilizing roles in living cells and is involved in diverse biological processes [96,97]. The aberrant glycosylation is always accompanied with oncogenesis and tumor progression, and up to now, most of the clinically used cancer biomarkers are glycoproteins [98–100]. Comprehensive characterization of the glycoproteome is a challenge because of the vast dynamic range of protein concentration and the high diversity and microheterogeneity of glycan chains attached to proteins. The selective enrichment of the glycoproteins/glycopeptides is also the most efficient ways to reduce the sample complexity to achieve an in-depth glycoproteome analysis [101–103].

N-linked glycosylation is the most well studied form of glycosylation up to now. N-linked glycans are attached to the amide group of asparagine (N) residue with a consensus motif of N-X-S/T, where X represents any amino acid except proline. Three approaches, including two chromatography based approaches, i.e. the lectin affinity chromatography (LAC) [104–106] and HILIC [107], and one chemical labeling method using hydrazide chemistry (HC) [108–110], are the most widely used ones for the enrichment of N-linked glycoproteins/glycopeptides. In addition, glycoprotein and/or glycopeptide enrichment by using boronic acid [111,112], size-exclusion chromatography [113], and graphite powder adsorbents were also reported [114]. Recent studies had demonstrated that these methods were complementary in enrichment of glycoproteins with different glycan structures [115,116].

Among these enrichment methods, the obvious advantage of the hydrazide chemistry method is its high enrichment specificity. Generally over 90% of the enriched peptides are identified as glycopeptides by HC method, while only less than 50% specificity could be achieved by other methods [117]. The high enrichment specificity benefits from the formation of covalent bonds between the $NaIO_4$ oxidized glycans of glycoproteins/glycopeptides and the hydrazide groups on the beads. Because the glycoproteins/glycopeptides were covalently bound to the HC beads, the nonspecific adsorption of proteins or peptides on the beads could be well removed by the subsequent harsh washing procedures. While hydrazide chemistry method also has some disadvantage. One of them is its long sample processing time. Fortunately, Chen et al.

integrated all of sample preparation procedures into a hydrizide tips, both the digestion time and the glycan cleavage time were reduced. Thus the processing time was significantly decreased from 3 to 4 days to less than 8 h with excellent reproducibility [118]. Another disadvantage of hydrazide chemistry strategy is that the glycan structures are broken by the $NaIO_4$ oxidation, and thus it is impossible to elucidate the glycan structures.

Enrichment of glycopeptides by LAC is based on the specific affinity of lectin to glycans with certain structure. There are a number of well characterized lectins that could be used for glycopeptide enrichment. For example, concanavalin A (ConA) binds to mannose glycan; wheat germ agglutinin (WGA) preferentially recognizes N-acetylglucosamine as well as sialic acid; agglutinin RCA120 captures galactose modified at the 3–0 position (e.g. with sialic acid or another galactose) and terminal galactose; Jacalin lectin has affinity to galactosyl (β-1,3)N-acetyl-galactosamine. Multiple lectins can be used in tandem to improve the glycoproteome coverage, however, the enrichment specificity of lectins is usually very low [106]. To decrease the glycopeptides loss in sample preparation procedure, an N-glyco-FASP approach, which replaced the lectin column in conventional method with ultrafiltration units, was developed [119]. In this method, the glycopeptides were enriched by binding to lectins on the top of a filter, which greatly reduced the sample loss and improved the glycoproteome coverage. It was successfully used in the large-scale glycoproteome analysis and leaded to identification of 6367 N-glycosylation sites in 4 mouse tissues and plasma.

Enrichment of glycopeptides by HILIC is based on the fact that the glycopeptides are more hydrophilic than non-glycopeptides due to the attached carbohydrates. The HILIC method can enrich glycopeptides in a non-glycan-specific fashion, thus it is suitable to comprehensive glycoproteome analysis. Another advantage of HILIC method is that the attached glycan chains are kept intact and is well suited for the characterization of glycan structure [120]. However it also has the obvious disadvantage of poor specificity. The enrichment specificity could be improved by adding ion paring reagents like TFA to reduce the nonspecific adsorption [121] or by using novel matrix with strong hydrophilic oligosaccharides [122–124]. Even so, the specificity is still much poorer than the hydrazide chemistry method. To further improve the glycopeptide enrichment specificity, lectin and HILIC enrichment methods could be applied sequentially [125,126]. By using this strategy, the glycopeptide enrichment specificity can reach to 75.8% [126], which was already close to the hydrazide chemistry method.

The comprehensive N-linked glycoproteome analysis can be achieved by using complementary enrichment methods. Recently Li et al. combined two general glycopeptide enrichment approaches, hydrazide chemistry and zwitterionic HILIC, to in-depth characterization of N-glycoproteins in the secretome of two human hepatocellular carcinoma (HCC) cell lines with low (MHCC97L) and high (HCCLM3) metastatic potential. A total of 1212 unique N-glycosites from 611 N-glycoproteins were confidently identified and the N-glycosylation site overlap of two methods was only 28.4% [127]. A similar strategy which combined the click maltose-HILIC and the hydrazide chemistry method was used to comprehensively map the N-glycosylation sites of human liver tissue [128]. Altogether, 14,480 N-glycopeptides, corresponding to 2210 N-glycoproteins and 4783 N-glycosylation sites were identified.

Other than large-scale analysis of glycoproteome using large amount of sample, for analysis of precious little sample, reducing the sample loss is vital [129]. Thus it is of interest to develop the integrated and miniaturized devices. Recently, Qu et al. developed an online glycopeptide enrichment system, which composed a HILIC column, a SCX precolumn, and a PNGase F immobilized enzymatic reactor (IMER) together. This system allowed the glycopeptide enrichment, sample buffer exchange, and deglycosylation performed on-line. The detection limit of deglycosylated glycopeptide for digests of avidin was as low as 5 fmol and the sample pretreatment time was shortened to ~1 h. With such a system, a total of 196 N-linked glycosylation sites corresponding to 120 unique glycoproteins were identified from 6 μg rat brain proteins [130]. Similarly, a monolithic capillary column based glycoproteomic reactor was developed which exhibited higher detection sensitivity in mapping of N-glycosylation sites from minute amounts of sample. As many as 486 unique N-glycosylation sites were reliably mapped in three replicate analyses of a protein sample extracted from only ~10^4 HeLa cells (~1 μg) [122].

Except for the N-linked glycosylation, O-linked glycosylation is also an important PTM, which is localized on hydroxyl groups of Ser/Thr residues. There are many types of O-linked glycosylation [131], while mainly two of them are gaining intense attention. One is O-GlcNAcylation which attaches a single sugar (β-N-acetylglucosamine) to Ser/Thr residues [132] and another one is O-GalNAcylation which initially adds a N-acetylgalactosamine on the Ser/Thr residues and then this core unit could be elongated with simple or more complex carbohydrate structures [133,134]. Generally the O-glycosyl moieties involved are shorter and less complex than N-linked glycans. Characterization of O-linked glycosylation is especially difficult compared to N-linked glycosylation due to the lack of a reliable amino acid consensus sequence and the lack of a general enzyme that can cleave all O-linked glycans [135,136].

The first successful approach for the enrichment of O-GlcNAcylated peptides was achieved by enzymatic coupling of an azide containing GalNAc to O-GlcNAc using an engineered galactosyl transferase [137]. Then the derivated O-GlcNAc is enriched by the alkynyl biotin or photocleavage tag containing alkynyl beads [138,139]. Using this enzymatic enrichment strategy, hundreds of O-GlcNAc sites could be identified by MS analysis [140]. The disadvantage of this approach is its low enzymatic galactosyl transference efficiency. Thus the coverage of O-GlcNAc glycoproteome obtained by this method is limited. An alternative approach is to introduce the alkynyl or azide analogs of N-acetylglucosamine to the O-GlcNAcylated substrates by metabolic labeling approach, and then the affinity tags are covalently linked to the metabolic labels via click chemistry [141,142]. Hahne et al. used an alkyne-modified resin for immobilization of metabolic azide-labeled O-GlcNAcylated proteins. After on-resin trypsin digestion of the captured proteins, about 1500 O-GlcNAc proteins were identified by LC–MS/MS from a single cell line. This demonstrated that the metabolic labeling efficiency is not a problem anymore. However, it should be mentioned that the glycosites were not identified as the glycopeptides cannot be released from the resins by on-resin trypsin digestion. The glycopeptides could be released and labeled by β-elimination and Michael addition. However, only 185 O-GlcNAc sites on 80 O-GlcNAc proteins were identified [143]. It seems that this releasing strategy is not so successful.

Except for the enzymatic or metabolic labeling methods, Vosseller et al. developed a Lectin Weak Affinity Chromatography (LWAC) strategy to enrich O-GlcNAc peptides with relatively low specificity [144,145]. Trinidad et al. used this LWAC strategy identified over 1750 sites of O-GlcNAcylation from murine synaptosomes. Although the WGA lectin they used is not completely specific to O-GlcNAc peptides, they still obtained the largest O-GlcNAc glycoproteome dataset to date [146]. This result shines a light on the development of O-GlcNAc peptide specific lectins. Notably, it has been demonstrated that a so called Nictaba lectin which is extracted from tobacco (Nicotiana tabacum) has specific interaction with O-GlcNAc histone from calf thymus [147]. Thus it may be a useful tool to enrich GlcNAcylated peptides or proteins from complex samples, while further investigation is still needed.

As to the O-GalNAcylation identification, both lectin affinity chromatography and HILIC can be used to enrich O-GalNAcylated peptides. While unlike the O-GlcNAcylation, the glycan structures of O-GalNAcylation are of high heterogeneity. Thus the direct identification of intact O-GalNAcylated peptides is still challenge. To facilitate the identification of peptide backbones for glycopeptides, the glycans are typically removed from peptides. This is not a problem for N-linked glycopeptides since PNGase F can cleavage all glycans from the peptides. However, the enzyme to cleave all O-linked glycans from peptides is not available. To overcome this problem, Darula et al. utilized exoglycosidase digestion for partially deglycosylation of O-Glycopeptides to reduce the heterogeneities in glycan structures [148]. This facilitated the MS detection of glycopeptides and its attached sites. Therefore this strategy was used to analyze the O-linked glycoproteome of human serum which leaded to identification of 124 O-GalNAc glycosylation sites in 51 glycoproteins [149]. Other than in vitro partial deglycosylation of O-Glycopeptides, an alternative approach called "Simple cell" strategy, which applied zinc-finger nuclease (ZFN) gene targeting to interrupt the O-GalNAcylation glycan elongation pathway in human cells, was presented to generate glycoproteins with the short glycan homogenous O-GalNAcylation [150]. This strategy allowed straightforward isolation and sequencing O-GalNAcylated peptides from total cell lysate digest using lectin affinity chromatography. Recently, Steentoft et al. implemented this approach to analyze 12 human cell lines from different organs, and presented the first map of the human O-glycoproteome with almost 3000 O-GalNAcylation sites in over 600 O-glycoproteins. Based on these data, they established a bioinformatic tool NetOGlyc 4.0 for prediction of O-GalNAcylation [151].

2.3. Protein ubiquitination

Ubiquitination is a well-known regulator of protein stability, activity, cellular localization and degradation, and is involved in various biological processes, including cell meiosis, autophagy, DNA repair, immune response, and apoptosis [152]. Ubiquitin is a highly conserved small protein contains 76 amino acids. It is covalently coupled to target proteins by E3 ligases through the formation of isopeptide bonds between ubiquitin C-terminal carboxyl groups and target protein lysine ε-amino groups [153]. After proteolysis, C-terminal residues of the ubiquitins remain covalently attached to lysine residues on the target peptides, which could be recognized by antibodies. For example, trypsin digestion leaves a C-terminal glycine-glycine (Gly-Gly) dipeptide of ubiquitin at the ubiquitination site, and the well-developed Gly-Gly specific antibodies could be used to enrich the tryptic peptides with this dipeptide [154,155]. In addition, the appearance of signature mass shift of +114.1 Da is beneficial for the site localization of ubiquitination. Interestingly, Wagner et al. found that most (~95%) of the Gly-Gly modified peptides were identified with a charge state of +3 or higher. Thus, Gly-Gly modified peptides are present in a low mass/charge (m/z) range (m/z 300–1150). Therefore, by adjusting the MS parameter in data dependent acquisition mode, they minimized the mass spectrometric time to sequence unmodified peptides presented in the antibody enriched samples due to nonspecific adsorption. In this way, they improved the coverage to identify Gly-Gly modified peptides [155]. Up to now, the immunoaffinity purification using such antibodies was the most effective enrichment approach for large-scale analysis of ubiquitination. And tens of thousands of ubiquitination sites could be identified routinely by this method [156–159].

2.4. Protein acetylation

Acetylation, a PTM regulating diverse protein functions including apoptosis, cellular metabolism, protein stability, and neurodegenerative disorders, mainly occurs at lysine ε-amino or N-terminal amino groups of target proteins [160–163]. And it is demonstrated that acetylation has crosstalk with phosphorylation, methylation, ubiquitination, SUMOylation, and many other important PTMs to form dynamic regulatory programs [164]. To date, the main tool for lysine acetylation enrichment is acetyl-lysine antibody. Because the acetylation modification is still attaching on the target peptides during peptide fragmentation by CID, it could be easily identified by MS/MS. It is worth mentioning that lysine acetylation could make trypsin mis-cleaved at the modified lysine residues owing to the charge neutralization of lysine. Therefore, lysine acetylated peptides are detected as missed cleavage products. To date, by using antibody enrichment method tens of thousands of lysine acetylation sites were identified, which enabled the discovery of the sequence motifs of lysine acetylation [165,166]. While compared to the lysine acetylation, N-terminal acetylation has not been investigated globally. This is mainly due to the high diversity of N-terminal acetylation, and the lack of a specific enrichment method. Furthermore, the mature proteins are usually N-terminally processed, and the initial methionine residue could be removed, therefore, consideration of non-tryptic peptides would be essential to finding N-terminal acetylation peptides in database search based protein identification approach [161].

2.5. Protein methylation

Protein methylation is another frequently occurred PTM. The methyltransferase catalyzes the addition of methyl groups to carbon, nitrogen, sulfur, and oxygen atoms of several amino acid residues [167], of which the arginine and lysine methylations are the most widely studied [168]. Arginine methylation is always engaged in regulating RNA processing, gene transcription, DNA damage repair, protein translocation, and signal transduction [169]. Lysine methylation is known as histone function regulator and is involved in epigenetic regulation of gene transcription [170]. Methylation always has varied modification extents, which result in mono-, di-, and trimethylations, therefore it should take all the possibilities into consideration during the proteomic database search. Although trimethylated lysine (+42.04 Da) is similar in mass to acetyllysine, their difference can be distinguished by high-resolution mass spectrometers [171].

For the enrichment of methylated peptides, some anti-methyllysine and anti-methylarginine antibodies had been well developed [169]. And to increase the confidence of methylation identification, Ong et al. presented a modified SILAC method, in which the $^{13}CD_4$-methionine was metabolically converted to $^{13}CD_4$-S-adenosyl methionine, and the latter one was then served as the only methyl donor. The heavy methylated peptide and its light counterpart peptide had a signature mass difference and could be easily detected by MS. By using antibodies targeted to methylated residues, they successfully identified 59 methylation sites by LC–MS/MS [172]. With recently advances in MS technique and the enrichment protocols, the number of identified methylation sites had increased dramatically. Over 1000 arginine methylation sites in human cell line and mouse tissues, and about 160 lysine methylation sites in human cell line were identified [169]. Uhlmann et al. found that most tryptic peptides containing methylated arginine were highly basic and hydrophilic [173]. Therefore these peptides could be effectively enriched from total cell extract digests by chromatography or electrophoresis methods such as SCX, HILIC, and isoelectric focusing (IEF). Coupled with heavy methyl-SILAC and

MS analysis, this method enabled the identification of 249 arginine methylation sites on 131 proteins of T cells [173].

2.6. Protein redox modification

Proteins can be modified through redox reactions. Therefore proteomic studies focusing on these modifications are termed as the redox proteomics [174]. Among all amino acid residues, cysteine is the most susceptible residue as it contains an active thiol. The thiol of cysteine can be oxidized to a variety of forms, including disulfide bond between two cysteine residues from either different proteins or within the same protein, sulphenic acid (S—OH), sulphinic acid (SO_2H), sulfonic acid (SO_3H), S-glutathionylation, and S-nitrosylation [175,176].

As in analysis of many other PTMs, the low abundance of cysteine modifications makes the detection of them challenging. In addition, some of the redox modifications are quite labile and therefore they cannot be directly analyzed with conventional sample preparation and MS analysis methods. The main workflow to monitor changes in the redox state of cysteine is called the "biotin switch" strategy, which is performed as following: firstly, the free thiols need to be completely blocked. Then the modified cysteine is reduced by a suitable reductant (glutaredoxin for S-glutathionylation [177–179]; DTT or TECP for disulfide [180,181]; ascorbate for S-nitrosylation reduced by [182–184]; arsenite for sulphinic acids [185]). After that the reduced peptides were labeled with biotin derivatized thiol-specific alkylating agents such as biotin-N-ethylmaleimide (NEM) or biotin-iodoacetamide (IAM). The labeled peptides are enriched by biotin affinity chromatography and identified by MS analysis [175]. However, several concerns have been raised including the incomplete reduction and alkylation, the low specificity of the reductants for specific forms of oxidized cysteine (for example, the selectivity of ascorbate as an S-nitrosylation reductant has recently been doubted as it may also reduce some disulfides which generates false positive results [186] and the selectivity/efficiency of arsenite as sulphinic acids reductant also needs further investigation), and possible artificial oxidations in cysteine residues of proteins owing to ambient exposure in the sample preparation procedures. Moreover, these methods cannot tell different disulfide types, and do not know which two cysteine residues are linked.

Due to the above pitfalls, many new strategies were developed to improve or replace the conventional biotin switch strategy. For the analysis of S-glutathionylation, the enzymatic labeling method is an attractive strategy. Chiang et al. incorporated the gene of E. coli glutathionylspermidine synthetase (GspS) in human 293T cell lines, which allowed expression of the enzyme GspS. The GspS subsequently converted the endogenous GSH to biotinylated glutathionylspermine (Gspm-biotin) in vivo. Gspm-biotin conjugates to reactive cysteine residues of proteins, which could be effectively enriched by streptavidin resin. Biotin-spermine was enzymatically removed by Gsp amidase, and thereby the intact glutathione was leaved on the labeled peptides which could be analyzed by LC–MS/MS [187]. However, the Gspm-biotin is bulky due to the presence of biotin-spermine which may influence its conjugation efficiency. Samarasinghe et al. overcame this drawback by metabolic tagging of glutathione with a small functional group, i.e. azide or alkyne by bio-engineering introduced glutathione synthetase (GS) [188]. The subsequently introducing of fluorophore or biotin through bio-orthogonal click reaction in vitro enabled detecting and enriching target proteins or peptides of glutathionylation [189].

For the protein disulfide analysis, cysteine connection can be obtained by the use of partial reduction followed by differential alkylation at protein level based on the fact that different disulfide bonds have different reduction rates. This strategy was successfully used to assess the susceptibility of intrachain and interchain disulfide bonds of IgG1 molecules [190]. The most accurate way to determine disulfide bond arrangement is to directly analyze non-reduced peptides by MS with minimum sample preparation approaches. However, a variety of MS/MS fragment ions containing free cysteine residue, cysteine thioaldehyde (−2 Da), cysteine persulfide (+32 Da), and dehydroalanine (−34 Da) generated by the breakage of S—S and C—S bond, and the fragment ions linked by intact disulfide bonds can also be observed during the MS/MS fragmentation [191]. Thus it is labor intensive and time consuming to interpret all the MS/MS spectra for all possible cysteinyl peptide combinations due to spectra complexity. Therefore, more efforts should be made for data interpretation as the fragment ions are derived from multiple peptides, which can be either from interprotein or intraprotein [192–195].

As to the analysis of protein S-nitrosylation, sinapinic acid is utilized to specifically reduce the S-nitrosylation, which could eliminate the potential artifacts generated in the ascorbate reduction strategy [196]. An attractive strategy is to apply chemoselective reactions to directly label S-nitrothiol. And fortunately, the triarylphosphine reagents were demonstrated to be excellent as the probes for S-nitrosylation analysis [197–200]. For the detection of the sulphenic acid modifications, the 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and its analogous probes could be used because they can directly react with the sulphenic acid [201–203]. This method also permits relative quantitation of sulfenic acid modifications between different cellular states [204] and enables estimation of absolute sulfenylation site occupancy [205].

2.7. The challenges of PTM enrichment

To date, most of the developed methods are only able to enrich one specific type of PTM. Because of the crosstalk between different types of PTMs, simultaneous analysis of multiple PTMs for the same sample is of particular interesting. Recently, Mertins et al. developed a promising approach termed serial enrichment of different PTMs (SEPTM) to enrich multiple types of PTM peptides from the same complex sample. This technology enabled identification and quantification of more than 20,000 phosphorylation, 15,000 ubiquitination and 3000 acetylation sites from the same bortezomib-treated human leukemia cell samples. Based on the above dataset, a systematic view of signal transduction processes across time and perturbational conditions was obtained [206]. The systematic study of PTM crosstalk is a major challenge because effective methods to determine whether two PTMs co-occur on the same protein are lacking. Swaney et al. developed a strategy to study the crosstalk between phosphorylation and ubiquitination [207]. They combined the SCX fractionation and Gly-Gly antibody enrichment methods, which successfully identified a total of 1008 peptides containing both ubiquitination and phosphorylation sites. They found that distinct phosphorylation sites often co-occur with ubiquitination. Other than the enrichment of phosphopeptides, TiO_2 has been demonstrated to have the ability to enrich sialic acid containing glycopeptides [208]. Based on this discovery, Larsen et al. developed a TiO_2 enrichment protocol to simultaneously enrich the phosphopeptides and sialylated glycopeptides. The peptides with these two PTMs were separated by HILIC prior to MS detection and a total of 7682 unique phosphopeptides and 3246 unique formerly N-sialylated glycopeptides were identified. This study provided the global view of cross-talk between N-linked sialylation and phosphorylation [209]. O-GlcNAcylation and phosphorylation could occur on the same Ser/Thr residues, and recently studies demonstrated that O-GlcNAcylation has extensive cross talk with phosphorylation [210,211]. Trinidad et al. established an approach, which combined the WGA lectin chromatography

and TiO_2 enrichment method, enabled the serial enrichment of O-GlcNAcylated and phosphorylated peptides [146]. Finally they identified 1750 O-GlcNAcylation and 16,500 phosphorylation sites, which is the first systematic analyses regarding crosstalk between O-GlcNAcylation and phosphorylation. They found 135 of all O-GlcNAcylation sites that were also found their phosphorylated counterpart and 66 peptides that were simultaneously modified with both phosphorylation and O-GlcNAcylation. These large-scale PTM crosstalk analyses substantially improved our knowledge about the collaborative or competitive regulation of cell signaling transduction by the co-occurrence PTMs on the same protein. However the datasets obtained are far from enough to interpret full PTMs regulation pattern in vivo, therefore new strategies should be continued to develop.

3. Fractionation and separation approaches for the analysis of PTMs

Enrichment of PTM peptides from the protein digest could significantly reduce the interference from non-modified peptides during MS analysis. However, the enriched PTM peptides are still very complex due to the extremely complexity of the proteome sample. Thus efficient fractionation and separation of them prior to MS analysis is critical to obtain high proteome PTM coverage. The well-established RPLC is the most efficient method for peptide separation, which has the highest separation capability and is compatible with ESI-MS detection. The detection sensitivity could be improved by reducing the column inner diameter and the flow rate, while the peak capacity could be improved by using small chromatographic particles and/or increasing the column length [212]. Despite recent progresses in RPLC, the complexity of biological samples still far exceeds the separation capabilities of this method, especially for the large-scale proteome PTM analysis of a whole cell line or mammalian tissue. Therefore separation by multi-dimensional chromatography is essential to in-depth analysis of PTMs, while the RPLC was always set as the last dimensional separation approach due to its good compatibility to MS [8,10]. In additional to chromatography, electrophoretic approaches are also powerful tool for fractionation of proteome sample. For example, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to fractionate proteins in whole cell extract for phosphoproteome analysis [213–215]. However, electrophoretic approaches were seldom used to fractionate PTM peptides for large-scale PTM analysis.

The coupling of SCX with RPLC–MS represents the classic 2D-LC system for protein PTM analysis. Most of the tryptic peptides have a positive charge at low pH and therefore they bind to the negatively charged SCX material. The separation of peptides by SCX is mainly based on charge, while RPLC separation is predominantly based on hydrophobicity. Therefore these two techniques have high orthogonality for peptide separation [216] and have been successfully applied to large-scale proteome PTM analysis [80,217]. For example, Villén et al. identified 5635 nonredundant phosphorylation sites on 2328 proteins from mouse liver by using this 2D separation approach [80]. An additional RP column was placed prior to the SCX column to increase the sample loading amount for SCX column [218,219]. Moreover it allowed on-line sample desalting, which reduced the sample loss. Acetonitrile was commonly added to the mobile phases of the SCX separation to reduce the hydrophobic interaction of peptides with the support material. As a consequence, the influence of the hydrophobicity of the peptide on the retention can be minimized, and thus the separation is mainly based on the net charge of the analyte. This will increase the orthogonality of SCX-RPLC 2D separation. After the SCX separation the acetonitrile in the collected fractions could be removed by vacuum centrifugation [220]. Instead of using only SCX resins, a mixed-bed format of WAX and SCX was reported to have improved orthogonality in separation, which led to the identification of much more phosphopeptides [221,222]. SCX fractionation method also has the advantage of identifying PTMs in a less biased manner than specific enrichment methods and may facilitate global simultaneous analysis of multiple relevant biological PTMs. In combination with Lys-N protease digestion, SCX fractionation enabled identification of multiple PTMs, including acetylated N-terminal peptides, singly phosphorylated peptides containing a single basic (lysine) residue, peptides containing a single basic (lysine) residue, and peptides containing more than one basic residue, simultaneously from complex mixtures [223,224]. This is because after Lys-N digestion, most of the peptides would contain a single basic lysine residue at the N termini of the peptides and therefore carried two positive charges in solution, while the acetylated protein N-terminal peptides would not contain any basic group and therefore be neutral. Thus the acetylated protein N-terminal peptides would be eluted from the SCX column first and be separated from other types of peptides. Similarly, singly phosphorylated peptides containing a single basic (lysine) residue would have one charge in solution and should thus be separated from the unphosphorylated single lysine containing peptides by SCX [224].

Although the combination of SCX with RPLC is successful for fractionation of tryptic peptides containing PTM, its performance for analysis of phosphorylation is hampered in-part by the poor binding of phosphopeptides with SCX adsorbents and thus significant sample loss could happen. Strong anion exchange (SAX) chromatography is a useful tool for separation of acidic peptides, such as phosphopeptides. For example, SAX has the ability to fractionate phosphopeptides under gradient elution. And when it was applied to enrich and fractionate phosphopeptides from human liver tissue, the largest human liver phosphoproteome dataset at that time was generated [225]. SAX was combined with SCX to further improve the coverage of phosphoproteome analysis [226,227]. After the complex peptide sample was loaded onto SCX, the flow through was collected and the peptides bound on SCX were eluted with 11 pH steps from pH 3.0 to pH 10.0. The collected flow through was loaded on a SAX column for further fractionation. Each fraction of SCX and SAX was analyzed by RPLC–MS/MS. The results showed that more basic peptides were detected in the SCX fractions, while more acidic and phosphorylated peptides were detected in the SAX fractions [226]. Hennrich et al. presented a similar strategy by combinational use of weak anion exchange chromatography (WAX) and SCX. In their strategy, each phosphopeptide fraction from the SCX was further fractionated by WAX. After all of the fractions were analyzed by RPLC–MS, 40% more phosphopeptides were identified by this new strategy compared to the SCX-RPLC separation in the same total gradient time [227].

HILIC is another alternative separation technique coupled with RPLC for multidimensional separation. The exact separation mechanism of HILIC is still not fully understood but the hydrophilic partitioning model is often used to explain the analyte retention. A water-enriched liquid layer around the polar stationary phase is generated when a low aqueous mobile phase (5–20% water in ACN) are employed. The retention is achieved by partitioning of analytes from the mobile phase into this aqueous layer on the surface of the stationary phase. Elution is then achieved by increasing the water content of the mobile phase. In HILIC, the retention of peptides increases with increasing polarity or hydrophilicity of peptides, which is opposite to the trends observed in RP. The high orthogonality to RP makes HILIC a suitable fractionation method in multi-dimensional separations for analysis of complex PTM samples [228–231].

ERLIC is a special form of HILIC, which uses a weak anion exchange (WAX) resin as the HILIC stationary phase [232]. In ERLIC, two mechanisms contribute to the peptide retention. Initially in the

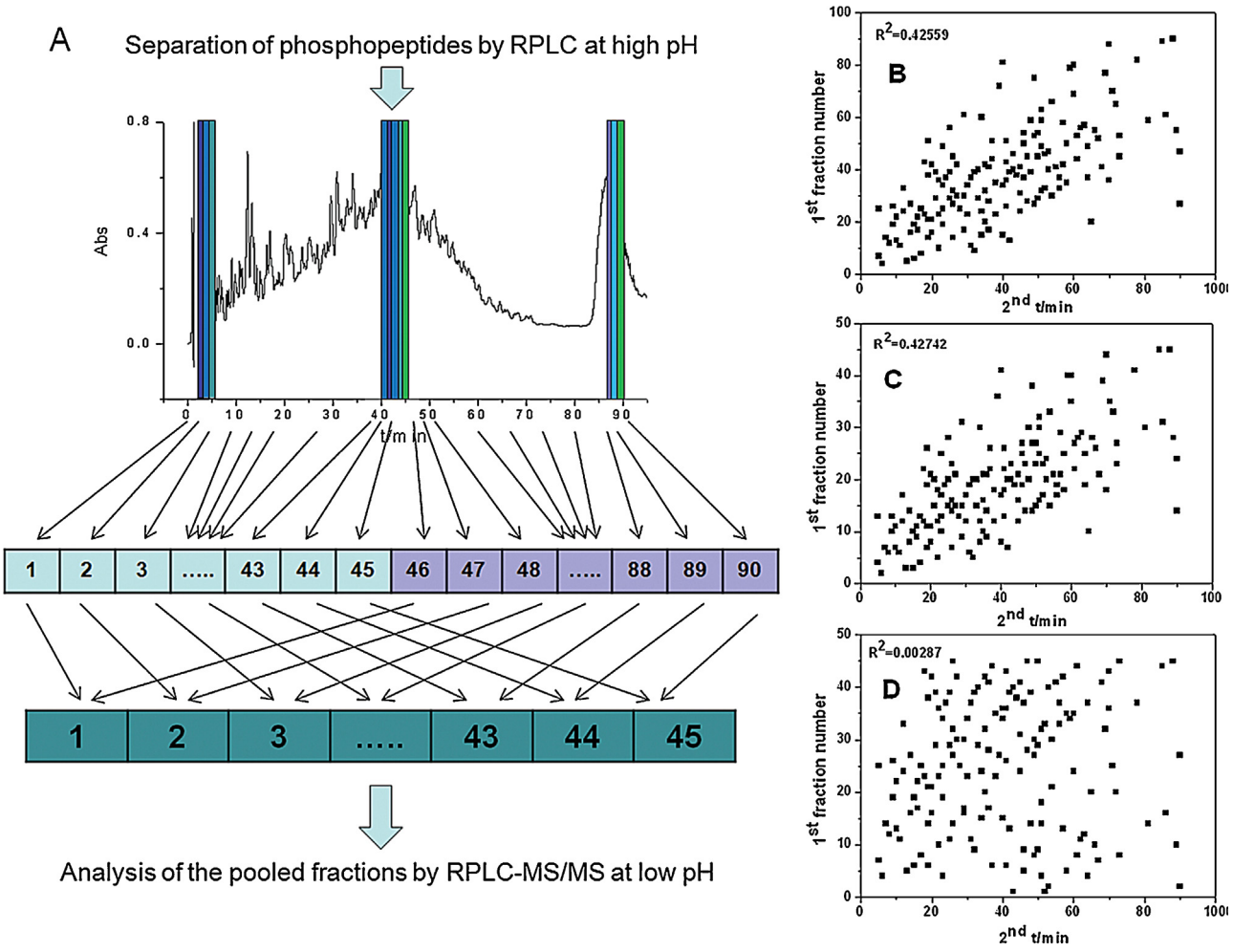

Fig. 4. The noncontiguous pooling strategy for RP–RPLC 2D separation. (A) The scheme for fractionation of peptides in the new strategy; (B) 2D retention plots for a hypothetical 2D separation of peptides, with 90 fractions; (C) reducing fraction number by pooling adjacent fractions; and (D) reducing fraction number by pooling equal interval fractions.

Reproduced from Ref. [48].

high organic solvent mobile phase, hydrophilic interaction mechanism is dominated, just as that in the HILIC. However, when aqueous content of the mobile phase is increased, the WAX resin will repel the basic peptides due to the electrostatic force, while it will retain the acidic peptides until a decreasing pH gradient is introduced. These combinational separation mechanisms make the ERLIC having better resolving power than SCX and HILIC to separate peptides [233]. Meanwhile ERLIC showed high orthogonality and even comparable peak capacity to RPLC. Reversing the RPLC and ERLIC separation order in 2D separation could also get satisfactory separation of peptides. It was successfully applied for the analysis of asparagine deamidation on peptides. This is because the peptide and its deamidation-related isoform was co-eluted and collected in the same fraction of the first RPLC separation. However, they could be separated and identified in the second dimensional separation with ERLIC. Thus the extent of asparagine deamidation on peptides could be simultaneously assessed [234]. ERLIC is also able to specifically enrich phosphopeptides, which is based on hydrophilic interaction and electrostatic repulsion. Therefore, enrichment and fractionation of phosphopeptides could be achieved simultaneously. Briefly, at low pH (pH <3), carboxyl groups on peptides are protonated, thus peptides with N-termini positive charge are generally electrostatically repulsed by the WAX resin. However, phosphopeptides are electrostatically attracted to WAX due to its negative phosphate group. The retention of phosphopeptides could be further enhanced by the usage of high concentration of organic solvent which promotes hydrophilic interaction of the phosphate group with the column. A salt gradient was then used to elute phosphopeptides from the column [235]. However, for phosphopeptide identification, the use of existing ERLIC as the first dimensional separation did not surpass the performance of SCX [236]. Nevertheless these two strategies are very complementary. As ERLIC is proved to be suited for the separation of multi-phosphorylated peptides, while SCX prefers the fractionation of mono-phosphorylated peptides, therefore loading the flow through of ERLIC to an additional SCX column would largely improve the coverage of phosphoproteome analysis [237,238].

Two-dimensional separation system with a RPLC separation at high pH in the first dimension and a RPLC separation at low pH (RP–RP) in the second dimension is a promising platform for large-scale proteome analysis [239]. As the organic solvents are used in both dimensional of RP, the compatibility of the mobile phase must be taken into consideration for online configuration [78]. Recently, SAX was added in the middle of RP–RP separation system to incorporate an online three-dimensional RP–SAX–RP configuration to resolve this problem. This online three-dimensional separation system significantly improved the peak capacity and ionization efficiency of peptides, which allowed the identification of proteins present at approximately 50 copies per cell from the entire yeast proteome [240]. This system was further applied to large-scale

analysis of murine embryonic stem cells proteome which achieved genome-scale proteome coverage with the mapping of 11,352 gene products [241]. It was also used in the phosphoproteome analysis and high performance both in terms of separation peak capacity and the number of unique phosphopeptides identified per microgram of cell lysate consumed was observed [242].

Even though the hydrophobicity of peptides changed significantly after changing of pH value for mobile phases, correlation of peptide retention times between the two dimensional separation was still observed, indicating that the conventional high pH RP-low pH RP 2D separation was not fully orthogonal. To improve the orthogonality of this 2D separation, a noncontiguous pooling strategy was proposed (Fig. 4A) [49]. After the peptides were prefractionated by RPLC at high pH, the collected fractions were then combined by pooling the one from the early eluted fractions and another one from the late eluted fractions. In this way, both the hydrophilic peptides and hydrophobic peptides are presented in the same fraction. When the combined fractions were submitted to RPLC–MS/MS at low pH, the peptides were more symmetrical distributed across the separation window [49] and the orthogonality of RP–RP had improved significantly (Fig. 4B–D). Because of the high peak capacity of the RPLC, more fractions could be pooled to further increase the orthogonality of the 2D separation [243]. This strategy allowed exploitation of the full separation window in the second dimensional RP separation. When this new RP–RP strategy was applied to phosphoproteome analysis, over 30% more phosphopeptides could be identified compared with the conventional RP–RP approach [49]. This new strategy was further applied to large-scale analysis of human liver phosphoproteome, which leaded to the generation of a large human organ phosphoproteome dataset with the identification of 9719 phosphorylation sites from 2998 phosphoproteins [244]. This new strategy had also been put into use for the proteome profiling of human cell line [243], identification of peptide–protein interactions [245], large-scale analysis of ubiquitination [158]. This new strategy was demonstrated to be best fitted for large-scale analysis of PTM peptides as reported in recent Nature Methods paper where more than 20,000 phosphorylation sites, 5000 ubiquitination and 3000 acetylation sites per experiment were obtained [206].

It should be noted that fractionation of peptides at high pH by RPLC has the risk to lose hydrophilic peptides as the hydrophobicity of peptides at this pH is low. Recently, we developed an enzyme assisted RP–RPLC approach which allowed the operation of both the RPLCs at low pH [52]. In this approach, two proteases with different specificities, e.g. Glu-C and trypsin, were used to digest proteins. The proteome sample was first digested by Glu-C which generated relative large peptides. The resulted peptides were first fractionated by RPLC with mobile phase containing 0.1% TFA representing the optimal condition for RPLC of peptides. The collected fractions were further digested by trypsin, which resulted in significant change of peptides' hydrophobicity. It was found the distribution of peptides in the second dimension RPLC at low pH was even and the proposed 2D separation was highly orthogonal [52]. This enzyme assisted RP–RPLC approach was successfully applied to large-scale analysis of human liver phosphoproteome. To further increase the analysis coverage, two types of instruments, i.e. TripleTOF 5600 and LTQ Orbitrap Velos, were used. A total of 22,446 phosphorylation sites, corresponding to 6526 nonredundant phosphoproteins were finally identified from human liver tissue [52].

4. Conclusion

In summary, tremendous progresses in PTM peptide enrichment and separation were achieved in the last few years which facilitated the proteome-wide analysis of protein PTMs. The workflow for large analysis of some major protein PTMs including phosphorylation, glycosylation, acetylation is well established which enabled the identification of huge number of modification sites. However, there is still lack of effective enrichment approach to analyze many not well characterized PTMs. Because of the crosstalk between different types of PTMs, simultaneous analysis of multiple PTMs for the same sample is of particular interesting. However, most of the developed methods are only able to enrich one specific type of PTM and there is still lack of full-spectrum PTM identification approach that could simultaneously identify multiple types of PTMs without bias in complex proteome samples.

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