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Review article

Advances in enrichment methods for mass spectrometry-based proteomics analysis of post-translational modifications

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ABSTRACT

Post-translational modifications (PTMs) occur during or after protein biosynthesis and increase the functional diversity of proteome. They comprise phosphorylation, acetylation, methylation, glycosylation, ubiquitination, sumoylation (among many other modifications), and influence all aspects of cell biology. Mass-spectrometry (MS)-based proteomics is the most powerful approach for PTM analysis. Despite this, it is challenging due to low abundance and labile nature of many PTMs. Hence, enrichment of modified peptides is required for MS analysis. This review provides an overview of most common PTMs and a discussion of current enrichment methods for MS-based proteomics analysis. The traditional affinity strategies, including immunoenrichment, chromatography and protein pull-down, are outlined together with their strengths and shortcomings. Moreover, a special attention is paid to chemical enrichment strategies, such as capture by chemoselective probes, metabolic and chemoenzymatic labelling, which are discussed with an emphasis on their recent progress. Finally, the challenges and future trends in the field are discussed.

1. Introduction

Post-translational modification (PTM) is a biochemical process by which amino acids in a protein are covalently modified after protein biosynthesis [1]. PTMs represent the result of the activity of specific enzymes (protein kinases, transferases, ligases, etc.), the consequence of *in vivo* production of reactive oxygen and nitrogen species, or the effect of spontaneous modifications (such as deamidation, cyclization of N-terminal). PTMs can involve the addition to one or more amino acids of a chemical group (such as phosphoryl, acetyl, methyl groups), of complex molecules (such as sugars, lipids), of proteins and peptides (ubiquitin and small ubiquitin-like modifiers), as well as proteolytic cleavage. They lead to the presence of different forms of a single gene product in a cell. Accordingly, the term "proteoforms" [2] is nowadays accepted and used to indicate all the different molecular forms in which the protein product of a single gene can be found, including forms due to PTMs. These modifications can impact the structure, activity, location and interactions of proteins, and therefore increase the functional diversity of a given proteome. Practically, PTMs represent a strategy of prokaryotic and eukaryotic cells to respond quickly to environmental changes, thanks to the rapid modification of key protein activities. Indeed, PTMs control almost all cellular events, including gene expression, signal transduction, metabolism, cell division, differentiation, immune-response, and apoptosis [3], and their dysregulation can lead to the development of diseases. Currently, a total of 1,950 disease-associated PTM events in 749 different proteins for 275 diseases have been manually curated and collected into the PTMD database [4].

Given the impact of PTMs in biological functions, the interest in their investigation in different types of cells (mammalian, plant, bacteria, and yeast cells) and functional states has progressively increased over the years [5,6]. However, the analysis of many PTMs has always posed difficulties due primarily to the substoichiometric nature of modified proteins within a proteome. The analysis of PTMs has thus evolved, from the first classical analytical and biochemical methods based for example on radioisotopes or antibodies, to global proteomic approaches initially based on two-dimensional electrophoresis coupled to PTM-specific fluorescent dyes [7] and then, in the most modern approaches, on bioelectrochemistry [8], enhanced Raman spectroscopy [9], and especially mass spectrometry (MS) [10]. MS has become the method of choice for qualitative and quantitative proteomic analyses of PTMs, thanks to improvements in the accuracy and sensitivity of instrumentation

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Table 1
Post-translational modifications and their target amino acids, mass shift, main functions, affinity and chemical enrichment strategies for MS-based proteomic analysis.

Protein PTMs	Target amino acid residues	ΔMass (Da)	Main biological functions	Affinity enrichmentstrategies	Chemical enrichment strategies	Refs
Phosphorylation	Ser, Thr, Tyr, His, Arg, Lys, Asp, Cys, Glu	+79.9663 (HPO_3)	protein activity, stability, cellular location, signalling pathways	IMAC, MOAC, SIMAC, SIMAC-HILIC, SCX, SAX, immunoenrichment, superbinder SH2 domains, ERLIC, hydroxyapatite AC, HILIC-IMAC	DBHA probe, HA-yne probe, isoDTB tag, photo-pTyr-scaffold probe, sulfonyl-triazole probe	[51,53,56-59, 65-68]
Acetylation	Lys, Ser, Thr, N terminus	+42.0106 (CH_3CO)	protein folding, epigenetics, gene transcription, DNA damage repair, cell division, signal transduction, autophagy and metabolism	immunoenrichment, SCX, ZIC-HILIC, COFRADIC, antibody-IEF, antibody-SCX	alkyne-containing thioester probe, metabolic labelling by ethyl fluoroacetate	[54,72,73,76,77, 88,89]
Methylation	Lys, Arg, His, Ala, Asn	+14.0157 (CH_3) +28.0314 (C_2H_6) +42.0471 (C_3H_9)	activity of specific proteins, epigenetics, cell cycle progression	immunoenrichment, IEF, SCX, HILIC, antibody-SCX, antibody-HpH RP, 3xMBT methyl-binding domains, antibody-propionylation	azide- and alkyne-analogues of SAM, chemoenzymatic labelling by ProSeAM	[21,80-86, 90]
Glycosylation	Asn, Arg (N-linked) Ser, Thr, Tyr (O-linked)	>800 +162.0528 (Hexose) +203.0793 (HexNAc) +291.095 (Sialic acids) +365.148 (HexHexNAc)	protein function, structure, stability, cellular adhesion, enzyme activity, protein trafficking	lectins, modified glycosidases, immunoenrichment, IMAC, MOAC, HILIC	metabolic labelling by: Ac_4ManNAz, Ac_4GalNAz, Ac_4FucAl, Ac_4ManNAl, GalNAz, 1,3-Pr_2GalNAz, GalNAzMe; chemoenzymatic labelling by UDP-GalNAz, Glyco-TQ	[91-97, 99-102]
Ubiquitination	Lys, N terminus, Cys, Ser, Thr,	+114.043 (Gly-Gly) >1,000	protein degradation, trafficking, regulation of enzymes, translation, DNA repair	tagged-Ub, immunoenrichment, COFRADIC	n/a	[106–108]
Sumoylation	Lys	600.2503 (SUMO-1) 599.2663 (SUMO-2/3)	protein interactions, subcellular localization, enzymatic activities	tagged-SUMO, immunoenrichment, SUMO-interacting motifs	n/a	[109–111]

and to advancements in the computational and statistical methods to analyse MS data. Although some complete and updated reviews of PTM analysis via MS which discuss the technical and experimental aspects, from ionization and fragmentation strategies to bioinformatics, have already been published [11–14], the different enrichment methods applied prior to MS for global proteomics analysis of PTMs deserve in our opinion further study and updating. Indeed, even though the enrichment methods for PTMs have already been reviewed, these appear to be either not recent [15], or referring to one [16–18] or different particular PTMs [19], or only on affinity [20] or chemical [21] enrichment strategies. The novelty of this review lies in offering an overview and an update of the enrichment methods for the MS-based, proteome-wide analysis of the most common and at the same time biologically relevant PTMs (phosphorylation, acetylation, methylation, glycosylation, ubiquitination and sumoylation). In particular, together with the more traditional enrichment approaches involving affinity-based capture, novel chemistry-based methods are reviewed. Finally, we provide some insights in existing challenges and future directions in this field of research.

2. Main types of PTMs

To date, over 500 unique PTMs have been distinguished and annotated [22], among these phosphorylation, acetylation and methylation, glycosylation, ubiquitination and sumoylation represent the most prevalent and well-studied (Table 1).

Phosphorylation, the reversible attachment of a phosphoryl group, is the most common and best-studied PTM. About 30% of the human proteome is phosphorylated, and each phosphoprotein may exist as multiple phosphorylated forms. There are two main kinds of phosphorylation: O-phosphorylation, which is more common and occurs on serine, threonine, and tyrosine residues; and N-phosphorylation, which take places on histidine, arginine, and lysine [14]. Other less common phosphorylation events involve aspartic acid, cysteine, and glutamic acid residues [23]. From bacteria to humans, phosphorylation regulates protein activity, stability, cellular location, substrate affinity, and complex formation. Therefore, this PTM plays a key role in essential events, such as signalling, cell division, growth, metabolism, development and aging [24]. In particular, phosphorylation at aspartic acids represents an abundant and critical PTM in prokaryotes which is crucial in their signalling pathways. Furthermore, phosphorylation at tyrosines, a rare PTM in eukaryotic cells (∼1% of the human phosphoproteome), is important in kinase regulation and implicated in many diseases. Given the functional relevance of phosphorylation and its involvement in a multitude of cellular functions, great efforts have been devoted to the development of methods for the analysis of the phosphoproteome. Such methods try to deal with the challenges related to substoichiometric character of this modification, the existence of multiple residues where proteins can be phosphorylated, and the lower MS detection sensitivity of phosphorylated species [13].

Among the common types of PTMs there are also acetylation and methylation, which involve the transfer to a protein of an acetyl group from acetyl-CoA or of a methyl group from S-adenosyl methionine (SAM), respectively. Acetylation occurs at the N-terminal of 90% of all human proteins and at lysine, as well as with a less extent at serine or threonine [25], whilst methylation modifies mainly lysine and arginine, and -to a lower extent- also

histidine, alanine and asparagine, and cysteine [26]. Traditionally, acetylation and methylation have been closely associated with histones and represent the chemical basis for epigenetics. The analysis of histone PTMs faces different challenges compared to global PTM analysis. Indeed, modifications on histones are not substoichiometric, but exist in many different combinations that make their analysis challenging [27]. Moreover, an increasing number of non-histone proteins are emerging as target of acetylation and methylation. In particular, lysine acetylation of non-histone proteins is involved in gene transcription, DNA damage repair, cell division, signal transduction, protein folding, autophagy and metabolism [28], while lysine/arginine methylation of non-histone proteins regulates cell cycle progression, and the activity of specific proteins involved in translational and post-translational regulation, mitochondrial activity and muscle contractility [29]. Notably, the comprehensive analysis of the methyl-proteome faces several challenges, which include for example the lack of significant physicochemical changes that can be exploited for modified peptide enrichment, and the existence of different degrees of methylations.

Glycosylation involves the covalent attachment of sugar moieties, represents one of the most complex PTM and it influences almost half of all proteins in nature [30]. There are two main types of glycosylation: N-linked that occurs on asparagine or less commonly on arginine; and O-linked which take places on serine and threonine and, less often, on tyrosine. N-linked glycoproteins exist in three different subtypes having all a common core glycan structure made up of two N-acetyl glucosamine (GlcNac) and three mannoses, and named high-mannose (glycan chains linked to the core structure via mannose), hybrid (glycan chains linked to the core via GlcNAc and mannose) and complex glycans (glycan chains linked to the core via GlcNAc). O-linked glycoproteins include, among the common types, O-GlcNAcylated and O-N-acetylgalactosamine (O-GalNAc) glycosylated proteins. O-GlcNAcylation, comprises the attachment of a single GlcNAc to serine and threonine, is highly dynamic, often transient, characterized by cross talk with phosphorylation, and implicated in transcription, epigenetics and cell signalling dynamics [31]. Whereas O-GalNAc glycosylation, also named mucin-type O-glycosylation, involves the addition of complex structures which can include galactose, O-GlcNAc, fucose and sialic acid. O-GalNAc glycoproteins, usually secreted and ubiquitous on cell surfaces and in body fluids, are implicated in a variety of functions, including adhesion, cell–cell communication, host–pathogen interactions, and metastatic process. Other less frequent glycosylations include C-glycosylation on tryptophan, S-glycosylation on cysteine, P-glycosylation on serine and threonine, and GPI anchors on protein anchored to the membrane. Glycosylation influences the function, structure, stability, oligomerization and aggregation of proteins, and it is implicated in host cell-surface interactions, enzyme activity and protein trafficking. The MS analysis of this PTM is difficult due to the high complexity and heterogeneity of glycosylated proteins.

Ubiquitination determines a covalent addition of ubiquitin (Ub, an ~8.6 kDa protein ubiquitously expressed) to the epsilon-amino group of lysine, and to a much lower extent to cysteine, serine, threonine, and the protein N-terminus. This PTM works as a regulatory signal that depends on the type of linkage of the Ub chains. Indeed, the target protein can be modified with a single or multiple Ub, forming homo- or heterotypic (mixed or branched) chains wherein Ub are interconnected via lysine (K) or methionine (M) [32]. The most common types of poly-ubiquitination reactions are K48, K29 (which tag proteins for proteasomal degradation), K63, K11, K6 and M1, which are involved, together with mono-ubiquitination, in lysosomal degradation, trafficking, regulation of enzymes, translation, and DNA repair [33]. Another layer of complexity is added by the generation of hybrid chains containing Ub and Ub-like proteins, such as small ubiquitin-like modifier (SUMO), interferon-stimulated gene 15 (ISG15), and neuronal precursor cell expressed developmentally downregulated protein 8 (NEDD8). In particular, sumoylation implicates a covalent addition of SUMO (a highly conserved ~12 kDa proteins) to lysine [34]. There are four different human SUMO isoforms, all with similar functional properties. Unlike ubiquitination, sumoylation does not tag proteins for degradation, but alters their interactions with other proteins and with DNA, and dysregulates their subcellular localization and enzymatic activities [35]. Dysfunction of ubiquitination and sumoylation are implicated in cancers, neurodegenerative and infectious diseases.

3. Mass spectrometry-based analysis of protein PTMs

Exploring PTMs by MS is a rapidly evolving area of analytical chemistry, with methods for the identification and quantification being updated and advanced on a regular basis [13]. The development of sensitive high-resolution mass spectrometers and of highly sophisticated search algorithms and bioinformatic tools [36,37] has also contributed to the advancement of PTM-site identification and quantification. MS represents the "gold standard" for the proteomic analysis of PTMs and it is commonly performed by using bottom-up approaches, which involve the digestion of proteins into peptides (reviewed in [38,39]). Briefly, a bottom-up workflow for PTM analysis implies protein extraction from biological samples, digestion of protein mixtures using proteases, enrichment and/or fractionation of peptides, and separation of peptides via liquid chromatography (LC) (Fig. 1). After ionization, peptides are injected in the mass spectrometer, where their mass/charge (m/z) ratio is accurately measured. The peptides are then subjected to MS/MS fragmentation, usually obtained by collision-induced dissociation (CID) or high-energy collision dissociation (HCD) fragmentation (reviewed in [40]), for peptide sequencing and assignment of PTMs to specific sites. Modified peptides display a predictable shift in their mass (see ΔMass in Table 1) and in the mass of the fragment ions when the PTM is non-labile and therefore retained after fragmentation (Fig. 2). Conversely, less stable PTMs, such as phosphorylation, give rise to neutral losses (*i.e.* loss of parts of the modified side chain) during fragmentation, which aid in the PTM identification [41]. In addition, after fragmentation, some modified peptides can produce specific diagnostic ions (*i.e.* immonium ions of the modified residue), in the low mass region of the MS/MS spectrum, which are useful markers for the assignment of PTMs. For example, specific immonium ions are released by modified peptides containing phospho-lysine [42], phospho-tyrosine [43], N-acetyl lysine [44], and N-methyl amino acids [45]. Notably, to improve the detection of labile PTMs the electron capture dissociation (ECD) and electron transfer dissociation (ETD) soft fragmentation techniques can be employed (reviewed in [40]).

Beyond identification, various MS-based setups allow PTMs quantification by label-free and label-based approaches (reviewed in [46]). In particular, the label-based methods mainly include stable isotope labeling with amino acids in cell culture (SILAC), isobaric tags for relative and absolute quantitation (iTRAQ), tandem mass tag (TMT) and dimethyl labelling. Notably, PTM stoichiometry, i.e. the fraction of modified peptides as a percentage of the total protein amount, can be assessed in parallel with global protein expression profiling and provide useful insight into the extent and functional role of PTMs [47]. Whatever the type of quantification, fragmentation and -more in general- of the workflow chosen, exploring PTMs by MS-based proteomics is experimentally challenging due to the low abundance, the labile nature and the heterogeneity of most PTMs. Therefore, enrichment of modified peptides is required to be able to identify and quantify them by MS from complex samples, such as cell lysates and tissue extracts [12].

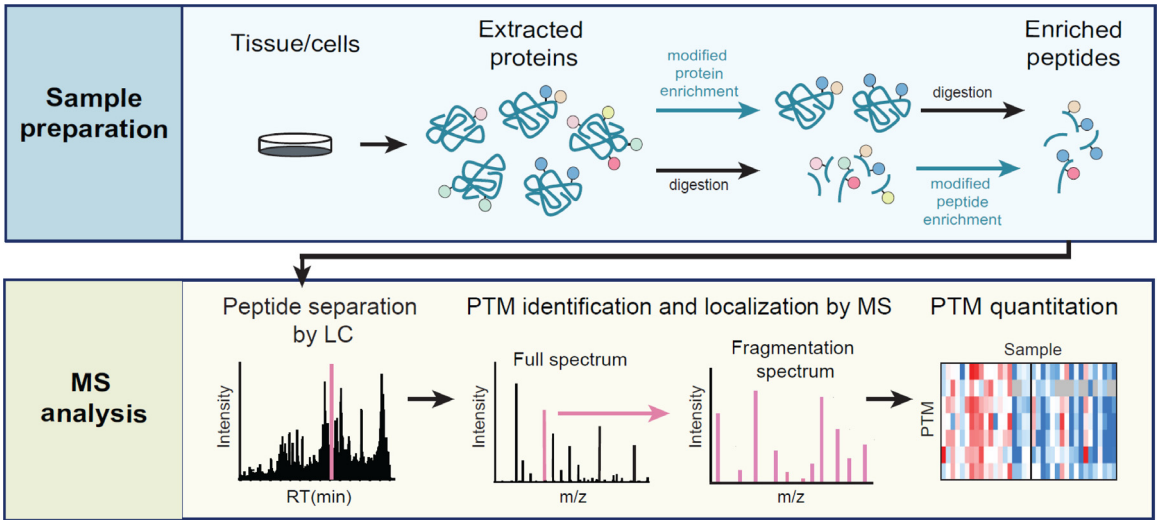

Fig. 1. Mass spectrometry-based workflow for the identification of PTMs. After protein extraction, proteins are digested into peptides. PTMs can be enriched at the protein- (before digestion) or, more commonly, at the peptide- (after digestion) level. Enriched peptides are then separated by liquid chromatography (LC) and analyzed in the mass spectrometer, allowing not only the identification of PTMs, but also their quantitation.

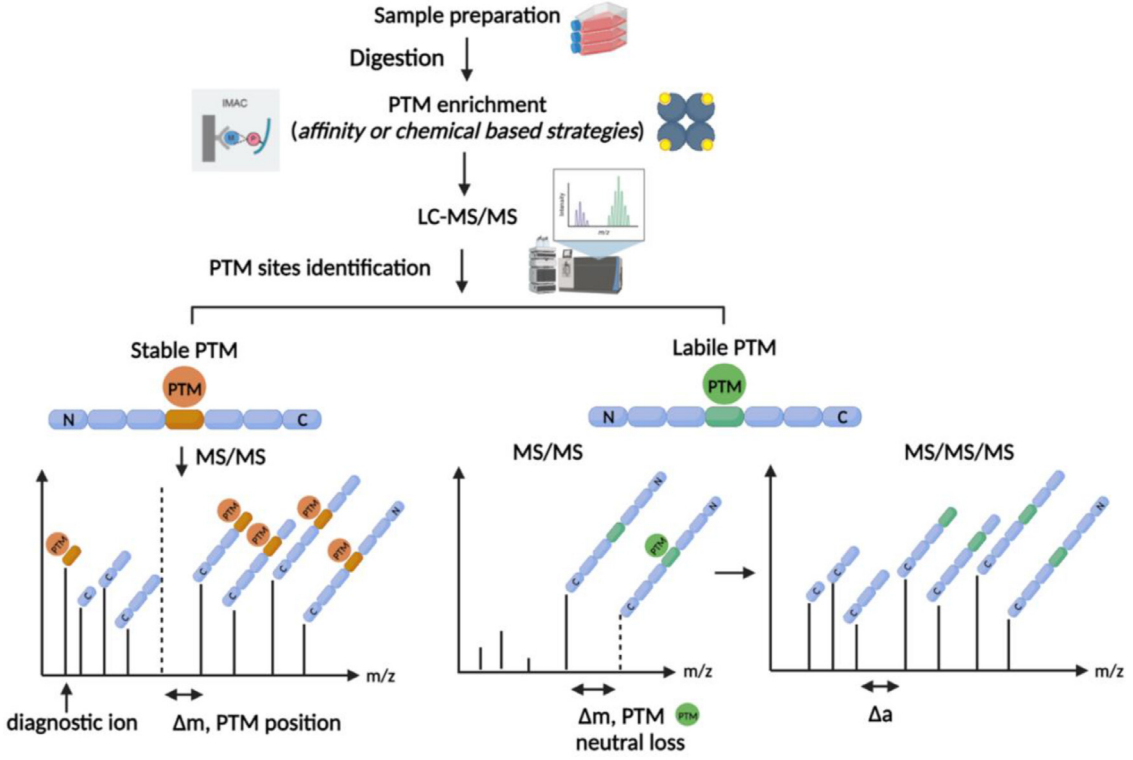

Fig. 2. MS/MS fragmentation of PTMs. Stable PTMs are retained by the precursor peptide after fragmentation giving, together with a delta mass in the MS spectrum, a predictable mass shift in parts of the fragment ions. Labile PTMs give rise to an intense neutral loss peak after fragmentation located at a lower mass than the precursor peptide due to lose of a PTM-related a neutral specie. Usually the neutral loss peak is selected for further fragmentation (MS/MS/MS) and peptide sequencing. This image was created with BioRender (https://biorender.com/).

3.1. Affinity and chemical enrichment strategies

The enrichment of modified peptides/proteins can be achieved by affinity and/or chemical strategies. Affinity strategies involve purification based on antibody/protein domain recognition or chromatographic separation based on specific physico-chemical properties of the PTM under consideration (Table 1). These PTM-specific enrichment strategies separate modified proteins/peptides from their unmodified counterparts, reduce the complexity of the sample and increase the efficiency and reliability of the MS analysis. However affinity enrichment strategies suffer some times of non-specific binding and of lack of appropriate antibodies.

In light of these problems, chemical proteomics methods were applied to alleviate them. Accordingly, over the past decade, a variety of chemical enrichment strategies has significantly enhanced the proteomic investigation of PTMs (Table 1). These strategies can involve capture by chemoselective probes, metabolic labelling by unnatural precursors and chemoenzymatic labelling [48,49] (Fig. 3). By using these approaches the enrichment of modified peptides/proteins prior to MS is usually carried out by means of streptavidin beads (or columns) that capture the PTM previously

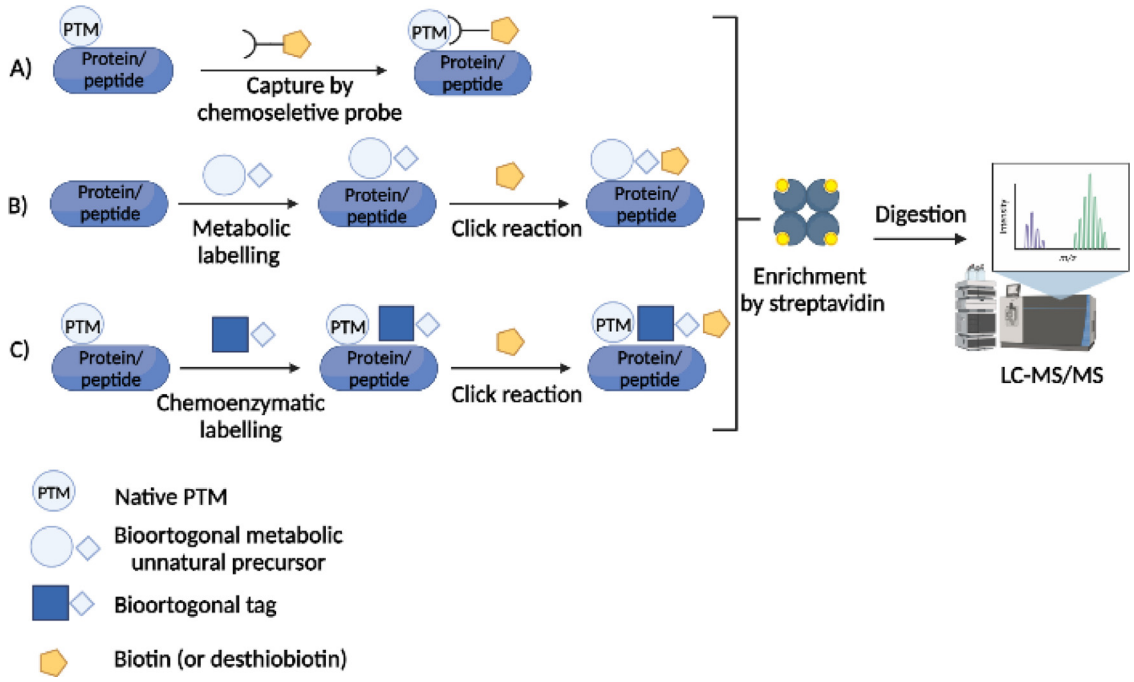

Fig. 3. Illustration of major chemical strategies for the enrichment of PTMs based on capture by chemoselective probes, metabolic labelling by unnatural precursors and chemoenzymatic labelling. This image was created with BioRender (https://biorender.com/).

labelled with a biotin tag. Notably, this biotin tag can be present in the probe structure or can be added through a "click" chemistry reaction, such as a copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC), i.e. a reaction that is selective, modular and bioorthogonal [50].

Here the affinity and chemical enrichment strategies are discussed in the context of the most common and best-studied PTMs.

3.1.1. Phosphorylated proteins/peptides

Phosphopeptide enrichment by affinity approaches relies on the specific interaction of phosphorylated amino acids with different binding reagents, which can be generally categorized into affinity-based chromatography, ion-exchange chromatography, and antibody / protein domain-based enrichment of phospho-tyrosines (pTyr) (Fig. 4).

The immobilized metal ion affinity chromatography (IMAC) technique exploits the affinity of phosphate groups for positively charged metal ions such as Fe^{3+}, Ga^{3+}, Zr^{4+}, Sn^{4+}, and Ti^{4}. The metal ions are non-covalently attached to a chromatographic resin, and interact though metal chelation and electronic attraction with negatively charged phospho-peptides. A limitation of IMAC resins is that they can also bind non-phosphorylated peptides with multiple negatively charged residues. Another limitation consists in the relatively low recovery of mono-phosphorylated peptides compared with multiply-phosphorylated ones, due to their weaker interaction with metal cations. To solve this issue, enrichment workflow involving a double round of IMAC enrichment were developed [51]. Another kind of affinity chromatography (AC) is metal oxide affinity chromatography (MOAC), which exploits the affinity of the phosphate group to metal oxides. The extraction is based on Lewis acid–base interaction, and the phosphate group assumes a bidentate binding mode to the surface of metal oxide. Phosphopeptides are therefore enriched by MOAC based on the affinity of the negatively charged phosphate group to metal oxides, most commonly TiO_2. Compared with IMAC, MOAC is more tolerant towards low pH and the presence of detergents. Although the most common metal oxide (MO) used for phospho-enrichment is TiO_2, approaches involving many other reagents have been developed. Because different MOs display different affinities and specificities for phosphopeptides, enrichments involving multiple MOs can be combined to maximize phosphopeptide recovery. For instance, sequential elution from IMAC (SIMAC) couples Fe^{3+}-IMAC and TiO_2 enrichment to capture multi-phosphorylated and mono-phosphorylated peptides, respectively [52]. A recent application reported by Engholm-Keller et al. [53], combined SIMAC with hydrophilic interaction liquid chromatography (HILIC) which is based on a hydrophilic stationary phase and a hydrophobic organic mobile phase, and permits the longer retention and subsequent enrichment of hydrophilic compounds, such as phosphopeptides, than hydrophobic ones. The combination of SIMAC with HILIC in the TiO_2-SIMAC-HILIC (TiSH) approach, improves the phosphoproteome coverage when dealing with limited sample amounts.

The ion exchange chromatography separates proteins based on their net charge, by taking advantage of positively or negatively charged ion exchangers. In strong cation exchange (SCX) chromatography, the ion exchangers present in the column possess negatively charged groups, which attract positively charged cations. In acidic pH, typical tryptic peptides are positively charged, while phosphate groups retain a negative charge, resulting in a weaker interaction with the anionic SCX material, which can be used to isolate phosphopeptides. This principle was first applied as a standalone technique, and then incorporated into more complex workflows [54]. Opposite from cation exchanges, strong anionic exchangers (SAX) have positively charged groups that attracts negatively charged anions, including phosphate groups. Once again, SAX can be employed individually, or in combination with other enrichment approaches. For instance, a multidimensional LC setup coupling SCX, SAX, and reversed-phase (RP) separation has been reported [55].

While the methods described above are efficient for isolating peptides containing phospho-serines and phospho-threonines, they typically do not perform well for pTyr, due to the lower abundance of Tyr and maybe lower degree of their phosphorylations in general. Anti-pTyr antibodies can be employed to enrich Tyr-phosphorylations at the protein or peptide level [51]. Because immunoprecipitation (IP) of whole proteins generates a high number

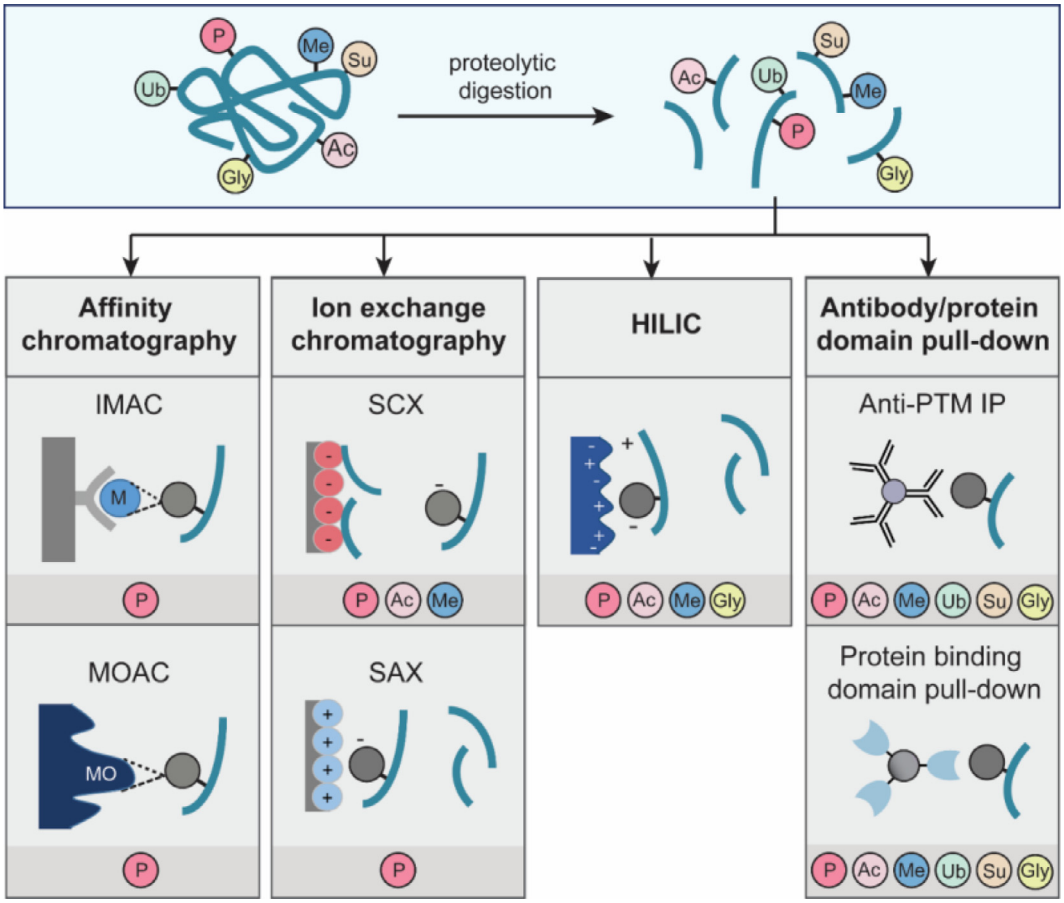

Fig. 4. Summary of the most common affinity enrichment strategies for PTMs analysis involving affinity chromatography (IMAC and MOAC), ion exchange chromatography (SCX and SAX), hydrophilic interaction liquid chromatography (HILIC), antibody immunoprecipitation (IP) and protein binding domain pull-down.

of non-modified peptides present in much higher amounts compared with pTyr-peptides, enrichment of peptides is currently the method of choice. As an alternative to immunoenrichment, protein domains recognizing pTyr, such as a pull-down purification based on Src homology 2 (SH2)-domain-derived super-binder protein that shows enhanced affinities for pTyr-containing peptides, has been proposed [56,57]. This approach provided a deeper coverage of pTyr compared with antibody-based methods, allowing the identification of >10000 pTyr sites.

Since every enrichment method has strengths and limitations, and may be suitable for specific subclasses of phospho-peptides, combining complementary methods may provide a deeper coverage of the phosphoproteome. Besides the above-mentioned affinity approaches, other enrichment strategies can be employed for phosphopeptide enrichment (reviewed in [58]): electrostatic repulsion liquid interaction chromatography (ERLIC, a mixed-mode chromatography that combines the use of electrostatic repulsion and hydrophilic interaction); hydroxyapatite affinity chromatography, and HILIC. These approaches have been used as combined strategies, obtaining remarkable results. For instance, HILIC separation in combination with Fe^{3+}-IMAC enrichment allowed the detection of >16,000 phosphosites in a single experiment [59], while HILIC elution from an aliphatic hydroxy acid-modified TiO_2 column has been also exploited to selective elute first singly and then multiply phosphorylated peptides [60]. p-Tyr IP can also be coupled with other enrichment approaches, for instance IMAC [61].

In addition to the identification of phosphoproteins, quantitative phosphoproteomics is usually performed to decode the phosphorylation cascades in cell signaling pathways. To this end, isotope labeling, such as iTRAQ, TMT, or dimethyl labeling can be used for phosphopeptide quantification [62]. For example, by using dimethyl labeling it was determine the absolute phosphorylation stoichiometries in human cells [63]. This was obtained by a method involving the treatment of two aliquots of tryptic peptides with either mock or phosphatase, the isotopic labelling, a first IMAC step, the *in vitro* phosphorylation of the flow through by a kinase and purification of phosphopeptides by IMAC. Moreover, TMT labeling has been very recently applied to quantify alterations in protein phosphorylation pathways related to dysregulated kinases [64]. This was obtained by generating *in vitro* phosphopeptides using different kinases, these motif-centric peptides, together with tryptic peptides from study samples, were subjected to TMT labeling, mixed, and enriched by IMAC prior LC-MS/MS analysis.

In spite of phosphoproteomics has greatly benefited from affinity enrichment strategies, the introduction of chemical approaches has allow improvements for the analysis of some specific phosphorylations. Indeed, as regarding the analysis of pTyr signaling in human cells, Chu at el. [65] have recently developed a trifunctional chemical probe termed photo-pTyr-scaffold, which contains an engineered Src kinase SH2 domain for the selective capture of native pTyr signaling complexes, a photoreactive group for the covalent cross-linking of probe to proteins, and a biotin tag for the enrichment before MS. This enrichment strategy proved to be a powerful approach for the global profiling of pTyr signaling in clinically relevant samples and can represents an efficient method for biomarker discovery. Moreover, this probe could become useful for functionalizing different binding domains, particularly with low binding affinity, and for profiling other PTM-dependent protein complexes, such as ubiquitination and methylation. Alternatively, a clickable sulfonyl-triazole probe, based on electrophile sul-

fur for the selective detection of nucleophilic hyper-reactive pTyr sites, has been proposed by Hahm et al. [66]. In this case, after the labelling, a desthiobiotin azide tag was clicked by CuAAC to enable the enrichment of pTyr-containing peptides before MS. This sulfonyl-triazole probe is useful for the detection of hyper-reactive pTyr sites, which can be detected by comparative proteomics of labeled sample at low and high probe concentrations. Indeed, while the hyper-reactive pTyr sites, exhibit comparable labelling intensity at low and high concentrations of probe, less reactive ones show concentration-dependent increases in probe labelling. This probe proved to be a useful enrichment strategy also to analyse the pTyr regulation in live cancer cells. In addition, the sulfur-triazole exchange chemistry used for the synthesis of this probe can be applied for the development of other enrichment tools with tuned chemoselectivity to detect a larger number of pTyr sites.

The chemical enrichment strategies have also allow the analysis of unconventional phosphorylated amino acids. For example, the MS-based analysis of phospho-aspartate (pAsp) sites of bacteria, which are intrinsically labile and therefore incompatible with common proteomic methods, was achieved using a desthiobiotin-containing hydroxylamine (DBHA) probe by Chang et al. [67]. This probe contains an O-alkyl hydroxylamine group to capture pAsp sites and a desthiobiotin tag for the enrichment, which permits milder elution conditions than biotin. The workflow implies labelling of phosphoproteins with the DBHA probe, enrichment on streptavidin-agarose resin, trypsin digestion, collection of bulk tryptic peptides and their TMT labelling, elution of DBHA-modified pAsp-peptides and MS analysis. A possible drawback of this workflow is represented by possible non-selective labelling of pAsp, since DBHA probe may attach other electrophilic PTMs. However this limitation can be overcome by combining this enrichment strategy with label-based quantitative proteomics to ensure site-specific resolution. The DBHA probe has allows the first analysis of pAsp dynamics in native *E. coli* proteome, and could represents an important tool advancing bacterial proteomics. Very recently, an attempt has been undertaken by Allihn and coworkers [68] to facilitate the totally selective labelling of pAsp by using a hydroxylamine alkyne (HA-yne) probe. The lower steric hindrance of this probe, as compared to DBHA, enhances its access to sterically demanding protein pocket containing pAsp. In this case the capture of pAsp sites occurs directly during lysis of bacterial cells, which should be done under optimized conditions by using lauryldimethylamine oxide at pH 4. Following the HA-yne labelling, proteome is clicked by CuAAC to desthiobiotin azide, proteins digested and phosphopeptides enriched on streptavidin beads before MS analysis. This enrichment strategy proved efficacy in identification of hundreds of different pAsp sites in *B. subtilis* and *P. aeruginosa* proteomes. Moreover, the authors applied the isotopically labeled desthiobiotin azide (isoDTB) tags to detect pAsp modulation in *P. aeruginosa* treated with dynorphin A. These results shine a light on the possibility to detect and profile of novel and unknown pAsp sites, as well as to decipher signaling mechanism in bacteria.

In summary, as concerning phosphoproteomics, a single enrichment strategy that captures all phosphorylated peptides does not exist, and the application of different enrichment methods is therefore advisable. Moreover, the trend of phosphoproteomics involves also the development of novel sorbents, such as magnetic core-mesoporous shell variants and mesoporous SiO_2 supported nanocomposites [69,70], which however need further investigation before their large-scale application, due to the limited data on true biological samples.

3.1.2. Acetylated and methylated proteins/peptides

Acetylation and methylation have been extensively investigated at the level of histones, where they represent the most common and best-characterized modified sites. Specific histone extraction and enrichment approaches for the MS analysis of histone PTMs have been developed and already reviewed [27,71].

As concerning the global methylome/acetylome analyses, immunoaffinity purification of acetylated proteins or peptides using pan anti-acetyl-lysine antibodies is the most widely used enrichment method [72]. When applied at the peptide level, immunoprecipitation allowed the identification and quantification of 10000 acetylated lysine peptides from more than 3000 proteins [73].

Because no antibodies are available for N-terminal acetylation enrichment, alternative strategies must be used. For example, an alternative methodology that does not require antibodies, involved the protection by reductive dimethylation of primary amine groups of peptide N-termini and lysine, the *in vitro* lysine deacylase reaction to remove acetyl groups from lysine, the derivatization of unmodified lysines with biotin tag, and the enrichment by AC before nanoLC-MS/MS [74]. SCX separation can enrich N-terminally acetylated peptides based on the lack of a positive charge at their N-terminal amino acid groups [54]. A recent method coupled the TrypN digestion to generate protein N-terminal and internal peptides (with two positive charges at their N termini), with the SCX separation to isolate acetylated and unmodified protein N-terminal from internal peptides [75]. The SCX approach can also be combined with peptide dimethyl labelling, which increases the charge differences between N-terminally acetylated and internal peptides, and, as a consequence, the resolving power of the chromatographic separation [76]. The lack of the N-terminal positive charge in N-terminally acetylated proteins is also exploited by zwitterionic ZIC-HILIC, where the peptides are retained by electrostatic and hydrophilic interactions through a stationary-phase containing polar zwitterionic functional groups [72]. Combined fractional diagonal chromatography (COFRADIC) is another popular technique consisting of two successive identical chromatographic separations, with a modification step directed to different classes of modified peptides between the two separations, which alters the peptides chromatographic properties, facilitating their isolation. COFRADIC has been applied to specifically enrich N-terminal acetylated proteins through the derivatization of primary amines [77], usually in combination with downstream fractionation with SCX or RP chromatography.

As described above for phosphorylation, the combination of different enrichment/fractionation strategies often provides improved efficiency. Examples are the combination of immunoenrichment with OFFGEL isoelectric focusing (IEF) separation or the combination of SCX with IP enrichment or COFRADIC, which allows overcoming the poor specificity of SCX (e.g. SCX also enriches phosphopeptides) [72].

Moreover, as for phosphorylation, recently significant efforts have been expended to the development of nanomaterials for the enrichment of acetylated peptides [78]. For example, an efficient enrichment method for protein N-terminal peptides based on dimethylation of N-terminal and lysine amino groups, followed by digestion, sulfydryl tagging of internal peptides and depletion by gold-nanoparticles, was developed, improving the identification of acetylated N-termini in proteome sample [79].

The last years have witnessed various advances in the strategies for methyl-peptide separation and enrichment, particularly concerning arginine (R) methylation [26]. Several antibodies recognizing mono and symmetric R di-methylations have been developed and commercialized. While immunoenrichment was initially applied to intact proteins, the current consensus strategy involves immunoenrichment at peptide level [80]. Recently, this approach has been applied by Lim et al. [81] also to patient-derived colorectal cancer tissues, where 759 methylated peptides in 272 proteins were identified. In parallel to the optimization of pan-methyl-antibodies for peptide-IP, fractionation strategies such IEF, SCX or HILIC chromatography have been successfully applied to MS-based

methylation analyses. In particular, HILIC separation alone has led to the identification of 249 R methyl-sites on 131 proteins, while SCX separation at low pH, combined with methyl-peptide IP, allowed the detection of > 1000 R sites [26]. One strategy that can be applied also for methylated peptides fractionation is off-line high-pH (HpH) RP peptide chromatography, which was recently used by the Nielsen group [82], in combination with monomethylation immunoenrichment, allowing the annotation of more than 8000 monomethyl R sites on 3300 proteins. This remarkable result should however be taken with caution in light of the fact that methylation is isobaric to several amino acid substitutions, as well as to chemical methyl-esterification of aspartic and glutamic acid, which can occur during sample preparation protocols involving the use of methanol or ethanol. The confidence of methylation identification can be increased by using validation methods such as heavy methyl SILAC (hmSILAC) or isomethionine methyl SILAC (iMethyl-SILAC) [26], two metabolic labelling strategies that enable the discrimination of true methylations from artefactual ones. Specific computational tools have been also developed to deal with the data obtained using these strategies [83,84]. The investigation of the lysine methylome has proved to be more challenging, with approximately 160 lysine methylation sites identified by using specific antibodies [80].

To overcome the difficulty in developing pan-methyl-lysine-specific antibodies displaying the broad specificity and the selectivity required for proteome-wide analyses, methyl-binding domains have been proposed as an alternative method to enrich methyl-lysines. The three malignant brain tumour repeats (3xMBT) of the methyl-lysine binding L3MBTL1 protein were employed to enrich proteins carrying mono- and dimethyl- lysines [85], but can only be applied to whole proteins. Immunoaffinity enrichment of peptides was also used in combination with propionylation of mono-methylated lysines, allowing the identification of methylated lysines in 398 proteins [86].

During the last years, some chemical enrichment methods have been developed to enhance the detection of acetylated and methylated proteins/peptides overcoming some limitations of immunoaffinity approaches. Indeed, antibodies cannot distinguish between the enzymatic acetylation of proteins by acetyltransferases and the direct chemical modification by the reactive acetyl-CoA thioester [87]. Therefore, chemical enrichment methods specific for non-enzymatic or enzymatic acetylated proteins/peptides have been developed (Fig. 5).

Recently, to investigate the non-enzymatic acetylome, Kulkarni et al. [88] proposed an alkyne-containing thioester probe that mimics acetyl-CoA but cannot be used by acetyltransferases. This method involved the protein labelling with the alkyne-containing thioester probe, a click chemistry reaction to add a biotin-azide tag, the enrichment of modified proteins by streptavidin beads and the proteomic analysis by LC-MS/MS. The authors found that proteins containing acetylated lysines were heavily enriched in their probe dataset (~80%) as compared to the overall proteome (~18%), demonstrating that the alkyne-containing thioester probe is useful to investigate the biological role of non-enzymatic acetylation. Another recent strategy developed for acetylation analysis, is based on protein labelling using the pro-metabolite ethyl fluoroacetate [89] which is converted to fluoroacetyl−CoA and used by acetyltransferases to label proteins also in live cells. After the protein labelling, a fluorine-thiol displacement reaction is exploit to insert a biotin tag on fluorinated labeled proteins, which are then enriched using streptavidin magnetic beads. As proof-of-concept, the authors validated the acetylation sites on known protein substrates, such as histones and α-tubulin, by LC-MS/MS. Since the fluorine tag present less steric hindrance than the alkyne/azide tags commonly used for CuAAC chemistry, it has potential for identifying targets of acetylation, and possibly many other PTMs mediated by transferases with restricted active sites.

In addition, also the characterization of methylated proteins has taken advantage of chemical methods, particularly a series of clickable azide- and alkyne-analogues of SAM have been developed in the last decade [21]. In recent times, Sohtome et al. [90] used a propargylic Se-adenosyl-L-selenomethionine (ProSeAM) to improve the quantitative methylome analysis and detect poorly characterized protein methylation. This method exploits the chemoenzymatic labelling of proteins with ProSeAM by methyltransferase (MTase) that transfers the propargyl group to the proteins, a CuAAC click reaction to attach a biotin tag, the enrichment of modified proteins by streptavidin, digestion and finally the identification of methylated proteins by MS. This enrichment strategy can be useful to detect the substrates of specific MTases by label-free quantitative MS, for example by comparing cell lysates in the absence and presence of exogenous recombinant MTase. Moreover, the ProSeAM labelling can be also combined with SILAC to compare the proteome of cells with or without disease-associated MTase knockouts and thus to discover functionally unknown methylation events. Despite ProSeAM labelling is limited to cell lysates due to its low membrane permeability, and lead to identification of methylated proteins that need to be validated, certainly its use represents a step forward to identify biological relevant methylation events.

3.1.3. Glycosylated proteins/peptides

Glycosylation enrichment strategies have been adapted to the high heterogeneity of glycosylation, which occurs both at the level of the occupancy of different glycosylation sites and of the number of different structures that can be present at each site (reviewed in [91]).

Many affinity enrichment strategies exploit the different sugar recognition specificities of carbohydrate binding proteins, such as lectins. The most widely used lectins in glycoproteomic experiments are concanavalin A, sambucus nigra, wheat germ agglutinin and ricinus communis agglutinin. Multiple lectins with specificities for particular glycoforms can be incorporated in affinity columns to achieve a comprehensive enrichment of glycoprotein classes. For instance, a multi-lectin approach combined with immunodepletion was recently employed by Totten et al. [92] to investigate glycoproteomic changes in human serum, revealing differential glycoform levels between prostate cancer and benign prostatic hyperplasia samples. In addition to naturally occurring lectins, modified glycosidases acting as lectins have been employed, some of which display nanomolar affinities for specific glycoprotein classes [91].

Antibodies have also been employed for the enrichment of glycosylated proteins [93]; however, their application is limited by the highly dynamic nature of glycosylation. Approaches developed for phosphopeptide enrichment have been exploited for glycopeptide enrichment [91]: the IMAC and MOAC approaches can be used to enrich negatively charged (i.e., sialylated) glycans, while HILIC takes advantage of the higher hydrophilicity of glycopeptides compared with non-glycopeptides.

However, affinity enrichment strategies suffer from low specificity for particular carbohydrates and bind glycan structures with relatively low affinity. Thus, glycoproteomics has greatly taken advantage of chemical enrichment strategies, in particular of metabolic labelling with azido and alkynyl monosaccharide precursors, such as variants of GlcNAc, GalNAc, N-acetylmannosamine (ManNAc), and fucose [91]. Azides are usually used as bioorthogonal handle because they are small, unreactive, stable, and easy to add to synthetic sugars. Recently, peracetylated N-azidoacetylmannosamine (Ac_4ManNAz) and peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz) analogues have been proposed by the Bertozzi group [94] for the detection of glycoproteins bearing intact glycans. The workflow involves the metabolic

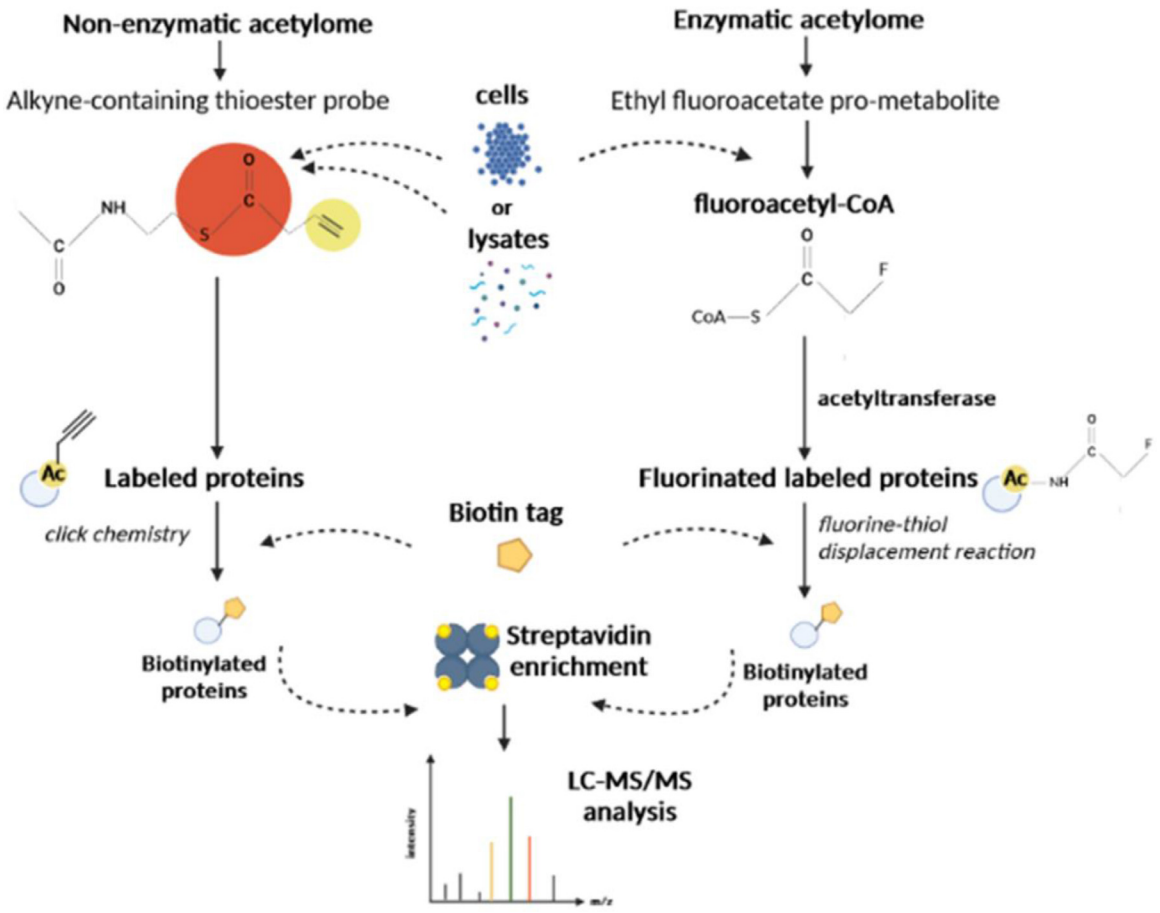

Fig. 5. Schematic illustration of two recent chemical enrichment methods for the analysis of acetylated proteins/peptides, based on alkyne-containing thioester probe [88] and ethyl fluoroacetate [89] useful for the detection of non-enzymatic or of enzymatic acetylome, respectively. This image was created with BioRender (https://biorender.com/).

labelling of glycans with azido-monosaccharide, a click chemistry reaction to add an isotope-targeted glycoproteomics (IsoTaG) tag containing a biotin and giving a recognizable ion for glycopeptide identification, an enrichment step and the MS analysis. Particularly, this method is useful for the glycoproteome characterization of human cell lines, and for the identification of N- and O-glycan structures over intact glycopeptides [94]. The same group applied the metabolic labelling based on peracetylated azido and alkynyl sugars (Ac_4ManNAz, Ac_4FucAl, Ac_4ManNAl) combined with IsoTaG, to detect the glycoproteome of prostate cancer cells, identifying several N- and O-glycans modified with sialylated glycan structures [95]. The same year, an attempt to apply the metabolic labelling to tissue has been done using Ac_4ManNAz in *ex-vivo* cultures of prostate cancer tissue [96]. Moreover, the dynamics of O-GlcNacylated proteins during T-cell activation was achieved by Woo et al. [97] using the Ac_4GalNAz combined with label-free MS quantification. However, if on the one hand the peracetylated monosaccharide analogues have good cell membrane permeability that makes them particularly suitable for the metabolic labelling, on the other hand it has been discovered that they induce an artificial S-glycosylation on cysteine [98]. This fact has raised the concern that some false positives might have been detected using these sugar analogues. To avoid artificial S-glycosylation new approaches have been proposed. For example, unacetylated GalNAz and UDP-GalNAz have been used by Qin et al. [99] to explore the O-GlcNAcylation in live cells and lysates by metabolic and chemoenzymatic labelling. The authors also exploited an isotope-tagged cleavable linker (isoTCL) consisting of a light or heavy alkyne-biotin tag, which assures enrichment of glycopeptides and subsequent relative quantification by MS. The isoTCL, similarly to the above described IsoTaG, generates specific MS peaks that enhance the glycopeptide identification. The chemoenzymatic labelling was employed by incubating lysates with a galactosyltransferase and UDP-GalNAz, which resulted in GalNAz-modified glycopeptides for following click chemistry, enrichment and proteomics. Overall, 1008 O-GlcNAcylation sites were identified in HeLa cells, of these only 24% were detected with both approaches while 61% were specifically identified by metabolic labelling which resulted to be more sensitive. Although naked monosaccharide analogues, such as GalNAz, can avoid S-glycosylation, they need to be administered using high concentrations in the millimolar range. Therefore the Chen group [100] developed the partially protected 1,3-di-O-propionylated Gal-NAz (1,3-Pr_2GalNAz) possessing a superb metabolic labeling efficiency in the μM range, which avoid S-glycosylation and assured a high-throughput enrichment of O-GlcNAcylation.

As concerning O-GalNAc glycoproteins, a particular drawback of most analogues is the low specificity due to their intracellular epimerization which lead to the labelling of others glycans. Recently, to specifically detect the O-GalNAc glycosylation of cell surface proteins, the N-(S)-azidopropionylgalactosamine (GalNAzMe) has been used by Debets et al. [101]. This azido sugar, thanks to branched acylamide side chains, is not subjected to epimerization and therefore is specific for O-GalNAc glycosylation. HepG2 cells must be first transfected with an engineered pyrophosphorylase (to assure the biosynthesis of UDP-GalNAzMe re-

quired for O-GalNAc glycosylation) and then fed with GalNAzMe. Azide-modified secreted proteins are de–N-glycosylated, subjected to CuACC to add a biotin-alkyne group, enriched, digested and analysed by MS. This chemical enrichment strategy assures labeling specificity, and therefore guarantees the identification of specific O-GalNAc glycosylated peptides.

Tremendous efforts have been devoted also to the development of selective enrichment methods for N-glycopeptides prior to MS analysis. Recently Li et al. [102] used a chemoenzymatic approach named Glyco-TQ to quantify the different glycan types. Glyco-TQ exploits three endoglycosidases that cleave the intact high-mannose, hybrid and complex glycans, with or without fucosylated core structure, from the glycopeptides. Peptides, obtained from the three enzymatic reactions, are treated with β-N-acetylhexosaminidase to remove O-GlcNAc, subjected to dimethyl labelling (with light, intermediate and heavy isotopes), combined and then incubated with a galactosyltransferase and GalcNAz to label the retained GlcNAc. Then an alkyne biotin tag is added by CuAAC for the subsequent enrichment of N-glycopeptides with streptavidin agarose. This method has higher specificity than those based on boronic acid and ZIC-HILIC, allows the identification of N-glycopeptides and also reveals the N-glycan type microheterogeneity.

As summarized in recent review articles, a variety of functionalized nanomaterials have been investigated, including magnetic and mesoporous materials, metal frame compounds, graphene, and dendrimers for the enrichment of glycopeptides/glycoproteins [70,78,103]. For example, the enrichment of N-glycopeptides could be also obtained by covalent coupling approaches based on solid phase extraction using nanoparticles. Recently Cay Y. et al. [104] developed a method able to overcome the typical limitations of covalent coupling approaches, such as long coupling time and harsh coupling conditions. After the oxidation of glycan moieties the N-glycopeptides are capture by the beta-amino thiols groups on the surface of magnetic nanoparticles through thiazolidine formation. This solid-phase extraction proved to be ultrafast and highly efficient, reaching sensitivity in the low fmol levels, with high selectivity (enriching N-glycopeptides from 1:100 mixture of glycopeptides and non-glycopeptides, respectively) and reproducibility (CVs $<$ 26%).

Ultimately, with regard to glycoproteomics, it should be considered that there is currently not a single enrichment method that fully captures the diversity of the glycoproteome, however, as for other PTMs, combinations of different methods can be applied taking into consideration the strengths and limitations of each approach.

3.1.4. Ubiquitinated and sumoylated proteins/peptides

To date, the main tools for the enrichment of ubiquitinated and sumoylated proteins/peptides rely on affinity strategies. Trypsin cleaves after the first arginine residue of Ub, leaving a two glycine (GG) remnant attached to the ubiquitinated peptide, which can be exploited in MS-based workflows for the enrichment of this PTM. Different studies relied on the expression and enrichment of various tagged forms of Ub, such as His-, hemagglutinin-, or biotinylated-Ub [105]. These approaches however suffer from high background and uneven protein ubiquitination due to the presence of the tag.

The development of antibodies recognizing the di-GG remnant has greatly accelerated ubiquitinome research, allowing the remarkable identification of $\sim$19000 ubiquitination sites in $\sim$5000 proteins [106]. However, this enrichment strategy also has limitations, as each antibody has sequence context biases, and the K-GG epitope is also generated following trypsin digestion of ISG15- and NEDD8-modified proteins, which could represent a confounding factor. To overcome these limitations, an antibody recognizing the unique Ub remnant left on protein targets after proteolytic digestion with the LysC protease has been recently developed by Akimov et al. [107], allowing the detection of 64000 sites across the conditions tested, which included proteasome inhibition.

The COFRADIC approach has also been applied to Ub analysis and overcomes the need for tagged versions of Ub as well as the problems related with the use of sequence-biased antibodies against Ub remnants. In this case, protein primary amino groups are blocked by chemical acetylation, and Ub chains are proteolytically removed by a deubiquitinase and substituted by a handle useful for isolation. Through this approach $>$7500 ubiquitination sites were identified [108].

The analysis of sumoylation is challenging due to its substoichiometric nature and the activity of SUMO-specific proteases in cell extracts. In addition, trypsin digestion of sumoylated peptides generates a large mass remnant ($>$2 or 3 kDa, depending on the SUMO isoform), which limits the MS-based analysis of sumoylated peptides. This problem has been overcome by using alternative proteases [109] or by creating different SUMO point mutants that can be cleaved by trypsin to generate shorter peptide signatures that are more easily analysable by MS [110]. Affinity tags, such as histidine-, biotin-, Myc- or hemagglutinin -tags, are often conjugated to SUMO to facilitate purification [111]. Non-mutant methods to detect endogenous sumoylation involve immunoenrichment of sumoylated peptides using anti-SUMO antibodies or SUMO affinity traps based on the poly-SUMO-binding function of RNF4, which contains four SUMO-interacting motifs [111].

3.1.5. Multiple PTMs

Since multiple PTMs can positively or negatively affect each other, combinations of orthogonal enrichment and/or fractionation methods for individual PTMs have been developed to investigate PTM cross-talks. In the serial enrichments of different PTM (SEPTM) approach, protein expression, phosphorylation, ubiquitination and acetylation were analysed from the same sample, by using the flow-through from the first enrichment step as the input of the from the following one. By combining IMAC, immunoenrichment with anti K-GG antibodies and anti-lysine-acetylation, the quantitation of more than 20000 phosphorylation, 15000 ubiquitination and 3000 acetylation sites was obtained, generating a comprehensive view of cellular signal transduction pathways in cultured cell lines [112]. The crosstalk between phosphorylation and O-GlcNAcylation was also investigated in murine synaptosomes by combining lectin and TiO_2 affinity chromatography, through the identification of over 1750 O-GlcNAcylation and 16500 phosphorylation sites [113]. More recently, Song et al. [114] analysed the phosphorylation and O-GlcNAcylation cross-talk in hepatoblastoma cells by exploiting IMAC and immunoenrichment by O-GlcNAc antibody. They found that 52% of the O-GlcNAcylated proteins were also phosphorylated, and that some of the proteins modified by both PTMs could interact with each other and thus might play a synergistic role in signal transduction. In the context of clinical samples, a combination of IMAC and immunoenrichment of pTyr, acetylations and methylations was recently applied by Gu et al. [115] to serum samples derived from patient with different types of cancer, quantifying different PTM types. Among them, lysine acetylation and arginine mono-methylation were the most represented, with 796 and 808 unique sites identified, respectively, and showed distinct patterns in the sera from leukemia, breast cancer, and lung cancer patients.

4. Conclusions and perspectives

In summary, optimization and advances of affinity and chemical enrichment methods for modified proteins and peptides, as

well as continuous improvements in mass spectrometry, have enabled the comprehensive analysis of PTMs, leading to larger and deeper discovery of protein modifications in different proteomes. Notwithstanding the progress that has been made, there are still some challenges that need to be addressed. For example, the continuously expanding variety of protein PTMs requires the design of novel methods to enrich newly-identified PTMs in order to characterize their cellular abundance, discover the cellular pathways in which they are involved and, thus develop new hypotheses on their functional roles in the model systems under investigation.

It would be also important and urgent to expand, in a creative manner, the enrichment workflows by coupling multiple/orthogonal strategies to dissect PTM cross'talks, especially in the context of dynamic systems and/or perturbed states, to better understand how individual PTMs occurring on the same proteins/domains synergize or inhibit each other.

Another aspect that shall be developed in the future is the possibility to test the combination of affinity-capture strategies with chemical-labeling ones, aiming to profit from their specific strengths and overcome the respective limitations, pushing forwards the limits in the annotation of tricky-to-detect PTMs, for which proteome-wide analyses are still lagging behind. For example, the interplay between phosphorylation and O-GlcNAcylation has been recently investigated by the Heck group [116] through the combination of phosphopeptide enrichment by IMAC with subsequent chemoenzymatic labelling of O-GlcNAcylation.

Last but not least, clinical proteomics is gaining momentum and the global protein expression profiling of clinical patient samples is becoming an essential part of personalized medicine, especially thanks to the capability offered by proteomics to detect the dynamic states of organisms, such as the transition from health to disease, disease progression, or response to therapeutic treatment. However, the starting amount of material from patient-derived samples is often limiting, which represents a daunting task for modification proteomics experiments. Future efforts shall be devoted towards the optimization of biochemical work-flow and analytical methods in order to achieve a progressive scaling down of the amount required to obtain a reliable PTM characterization. In this contest, some achievements have been already accomplished for phosphoproteomics, for example by optimizing an approach for the high-throughput analysis of phosphoproteome dynamics at subcellular level [117], however much still remains to be done for the other PTMs.

Despite these open issues, the impressive number of novel strategies developed in the last 5 years for the enrichment of post-translationally modified proteins and peptides is very encouraging and suggests that several of the current limitations will be overcome in the near future, thanks to the synergic effort of the scientists bringing different and complementary expertise in this branch of proteomics research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jessica Brandi: Writing – original draft, Writing – review & editing, Visualization. **Roberta Noberini:** Writing – original draft, Writing – review & editing, Visualization. **Tiziana Bonaldi:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Daniela Cecconi:** Supervision, Conceptualization, Writing – original draft, Writing – review & editing.

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