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Post-translational modifications (PTMs) are covalent additions of functional groups to proteins and are known to play essential roles in biological processes. Covalently attached PTMs are usually present at substoichiometric levels, implying that a PTM proteome is often present in only a small fraction of the entire proteome. The low abundance of PTMs creates a tremendous analytical challenge for PTM proteomics. New analytical strategies, especially enrichment approaches, are required to allow the comprehensive determination of PTMs. Solid-phase capture of PTMs through chemical reactions provides the most specific approach for fishing the PTM proteome, and based on these chemical reactions, a variety of novel functional nanomaterials have been developed. This review mainly focuses on the currently available chemical approaches for investigating PTMs, as well as the functional solid phases used for PTM proteome separations.

1. Introduction

Innovations in proteomics technologies that enable biological process profiling at the protein level have led to the unveiling of the human proteome, as two separate groups recently generated near-complete drafts of the human proteome.[1,2] However, because post-translational modifications (PTMs) modulate the activity of most eukaryote proteins and control many biological processes, explaining their various biological functions or regulation mechanisms by simply examining the protein sequence is not possible. Protein PTMs contribute to a much broader diversity of a protein than its sequence alone, and studying protein PTMs will provide a foundation for better understanding the mechanisms of cell regulation.[3]

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Mass spectrometry (MS)-based proteomics is the most vital and versatile technique available for profiling the proteome. MS-based proteomics has identified approximately 10,000 different proteins in a single mammalian cell within one experiment.[4] However, MS-based, in-depth analysis of the PTM proteome still presents formidable challenges.[5] For example, considering the Swiss-Prot database (Released 2015_06), although it predicted that more than 16,000 N-glycosylation sites exist in the human proteome, approximately 2500 sites were confirmed experimentally, only accounting for 15% of the total predicted sites. The difficulties associated with PTM proteome analysis are as follows. First, considering PTMs, the proteome may be two to three orders of magnitude more complex than the encoding genomes would predict. Second, some PTMs are highly dynamic and often involve reversible processes. Third, most PTMs often exist at substoichiometric levels, and if the PTM events occur more than once on a given protein, the total level of certain

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PTMs will be even less than predicted. Fourth, the MS responses of PTM peptides are always severely suppressed by non-PTM peptides. Therefore, effective identification of the PTM proteome is orders of magnitude more difficult in complex mixtures when compared to traditional proteome profiling.

As modified peptides are normally present at substoichiometric levels compared to non-modified peptides, they are generally not detected by MS without specific enrichment. Advances in enrichment techniques (e.g., chromatographic, affinity purification and chemical methods) and in MS instrumentation have greatly promoted the development of PTM proteome analyses. Enrichment of the PTM proteome at either the protein, peptide or both levels leads to a reduction in sample complexity and thereafter an increase in proteome coverage.[6] Classical biochemical methods which exploit the specific interaction of antibody and antigen are widely used. Antibodies toward several PTMs, such as phosphorylation, glycosylation, methylation, acetylation, sumoylation and ubiquitination, are now commercially available. Some PTM proteome can also be enriched through chemical approaches which typically show higher selectivity towards the PTM proteome. MS characterization of the PTM proteome has benefited greatly from chemical methods that enable their specific enrichment. Typically, in a chemical enrichment approach, PTM-containing peptides are captured on a solid phases as a result of a specific reaction between the modification group and the functional group on the solid phase, while non-modified peptides do not bind are washed away. Thus, proteins carrying a certain PTM can be specifically isolated. Additionally, the PTM proteome can be enriched using a tag that is first introduced at modification site, using in vivo metabolic labelling with an analogue of the PTM or in vitro labelling by chemical or enzymatic means, followed by a selective chemical reaction between the tag and the solid phase to enable the capture of the PTM proteome. To obtain successful separation, the reaction between the PTM groups (or tags) and the functional groups on the solid phases is of primary importance. The reaction should be specific and produce a high yield with minimal or no side reactions. Furthermore, the reaction should proceed under mild conditions, which is important for protecting the peptides against degradation. The type of solid phases used to immobilize the functional groups is another important factor required for successful enrichment. The solid phase should be easily modified with functional groups and easily separated from the solution phase. Additionally, having a high surface area for grafting the functional groups and a low nonspecific adsorption towards non-PTM peptides would be beneficial. Nanomaterials are newly developed nanostructures that are constructed from many different chemicals and compounds (e.g., metal, carbon composites and dendrimers). Once formed, these materials are extremely small, ranging from one to several hundred nanometers, which provides the nanoproduct with unique properties. Due to their nano-scale size, their electrical, optical and chemical properties are very different than those at larger scales. Thus, nanomaterials possess a variety of applications in proteomics.[7,8] Advances in chemical approaches have accordingly inspired the design and synthesis of new functionalized nanomaterials for the solid-phase capture of PTM proteome. In this review, the current available chemical approaches for investigating PTMs are

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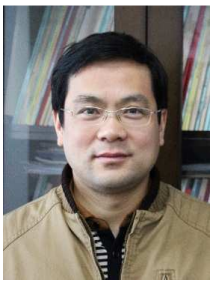

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discussed, and the recent developments and potential impacts regarding new functional nanomaterials applied to PTM proteome separations are highlighted. More specifically, the chemical approaches herein primarily include the formation of ionic and covalent bonds between a PTM and a solid phase, as well as the introduction of a tag on the PTM using chemical reactions to promote enrichment. Therefore, some important modifications like ubiquitination, and acetylation, which have significant biological roles but do not have corresponding chemical enrichment methods are not included in this review. To make the review more concise and holistic, PTMs are first assigned into two categories; one is by the modification type, the other is by the modification site. Under the first category, we further assigned phosphorylation, glycosylation, and other types of modification as the three subcategories, based on the fact that different chemical reactions are used to target different modification groups. Specifically, enrichment methods targeting phosphorylation and glycosylation are relatively common and plentiful, while methods for all other types are relatively limited. Thus, phosphorylation and glycosylation were discussed separately in the two subcategories, and all other types of modification were summarized in the third. Similarly, under the second category, we assigned cysteine modification and termini modification as the two subcategories. This categorization is based on the fact that modification can occur on a certain amino acid site, like cysteine (C) and (N/C-) termini of peptides. At a certain amino acid site, various types of modification may occur, yet they can be enriched with the same chemical method. Therefore, we assigned enrichment methods by modification site in this category.

2. Enrichment of protein PTM types

2.1 Phosphorylation

Reversible protein phosphorylation is a widespread and important PTM that dynamically modulates numerous signalling pathways and is controlled by the complementary action of protein kinases and phosphatases.[9] Approximately 30% of all cellular proteins are phosphorylated,[10] serving important roles in inter- and intracellular signalling events, protein synthesis, gene expression, cell survival and apoptosis.[11] Until recently, four types of phosphorylation have been acknowledged: 1) O-phosphorylation (serine, threonine and tyrosine), 2) N-phosphorylation (arginine, histidine and lysine), 3) S-phosphorylation (cysteine) and 4) acyl-phosphorylation (aspartic acid and glutamic acid).[12,13] Owing to the chemical stability in both acid and neutral conditions, O-phosphorylation is most popular among researchers; however, the other types of phosphorylation also attract significant attention. O-phosphorylation accounts for nearly 98% of all types of phosphorylation and is important in protein phosphorylation modifications. Recent examinations of the Swiss-Prot database determined that the relative abundances of pS, pT and pY are 76.4%, 16.8% and 6.8%, respectively.[10] Classical studies of the O-phosphoproteome involve radiolabeling combined with capillary electrophoresis, amino acid analysis or Edman radio sequencing. However, this workflow is not quite suitable for detailed analyses of phosphorylation state in biological processes and signaling networks at the molecular level. Currently, MS is the most powerful tool available for studying the phosphoproteome.[12] Although phosphorylated proteins/peptides can be directly analyzed by MS, the low abundance of phosphopeptides and low ionization efficiency of phosphopeptides require the development of strategies for phosphoproteome enrichment. In addition to traditional antibody-based methods that have been used to enrich the phosphoproteome, the chemical-based strategies discussed below play important roles in phosphoproteome extraction.

2.1.1 Chemical derivatization. Enrichment of phosphotyrosine (pY)-containing proteins can be achieved using antibodies.[14] However, antibodies for phosphoserine (pS) and phosphothreonine (pT) suffer limited specificity, and therefore techniques for pS/pT-containing protein enrichment are required. To achieve this goal, β-elimination combined

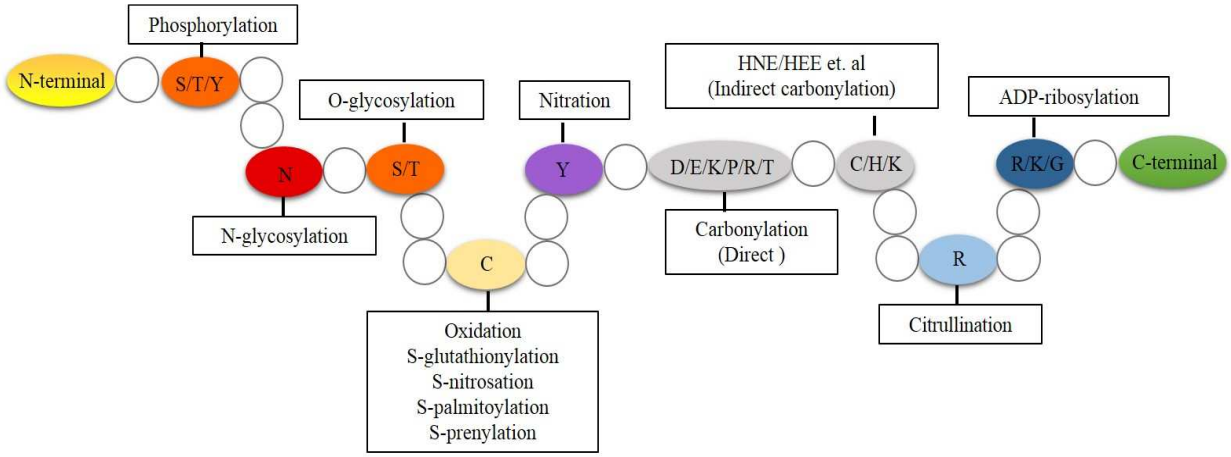

Figure 1. Overview of the post-translational modifications (PTMs) discussed in this review.

with Michael addition has been used to enrich pS/pT-containing peptides. The standard workflow includes elimination of phosphate from pS and pT together with the β-H, resulting in the formation of α, β-unsaturated carbonyl compounds. Then, a nucleophilic compound containing an enrichment tag is conjugated to the unsaturated carbonyl compound, allowing the phosphopeptides to directly or indirectly link to the functional materials.[15-17] Finally, mass tag-containing phosphopeptides are eluted from the material and then analyzed by MS. The nucleophile, which contains a chemical cleavage site between the reaction group and the core of the material, can also be applied to the surface to simplify the process.[18] This strategy is designed specifically for pS/pT-containing peptide enrichment because pY peptides do not undergo β-elimination. The shortcomings of this workflow are inevitable because other modified residues, such as O-glycosylated residues, can also be β-eliminated. Furthermore, unmodified serine and threonine residues will be β-eliminated during the process.[19]

Another chemical derivatization method used for the enrichment of phosphopeptides is based on phosphoramidate chemistry (PAC).[20] This method relies on a carbodiimide-catalyzed condensation reaction to attach cystamine to phosphate groups. Using a thiol group as an enrichment tag, phosphopeptides can be extracted from peptide mixtures using glass beads modified with iodoacetyl groups. Due to the multiple processes involved, optimization of this strategy with amino-derivatized dendrimers[21] or maleimide-derivatized porous glass[22] has simplified the process. Using PAC methods, the phosphate group remains attached to the peptide after isolation; therefore, PAC is suitable for enrichment and analysis of all pS-, pT- and pY-containing peptides.[19] However, as PAC methods require a series of chemical reaction steps, side reactions leading to sample complexity and loss are inevitable. Due to these shortcomings, relatively few phosphoproteome studies are being conducted using chemical derivatization strategies. However, overlap between the current enrichment methods for phosphoproteomics suggests that the PAC method is still a considerable choice.[23]

2.1.2 IMAC. Immobilized metal ion affinity chromatography (IMAC) has been one of the most extensively used enrichment techniques for phosphoproteome investigations. IMAC, introduced in 1975 and referred to as metal chelate affinity chromatography,[24] was originally designed as a method to fractionate and purify proteins in solutions based on the affinity of transition metal ions, such as Zn(II), Cu(II), Ni(II) and Co(II), for cysteine and histidine. Application of IMAC in phosphoproteome research provides a sensitive and selective purification procedure for studying the phosphoproteome. This method is based on the coordination between an immobilized metal ion and electron donor groups, producing metal chelates. With negatively charged phosphate groups, the electron donor is an oxygen atom. More accurately, other electron donor groups in proteins beside phosphate groups exist. In addition to hydroxyl groups on unmodified serine/threonine/tyrosine residues, carboxyl groups (e.g., α-carboxyl and carboxyl groups on aspartic/glutamic acid residues) and sialic acid (containing carboxyl and hydroxyl groups) possess the same electron donor atom as phosphate groups. Sulfhydryl groups on cysteine residues (sulfur as an electron donor) and imidazole groups (nitrogen as an electron donor) on histidine residues can also act as electron donor groups. Cysteine sulfhydryl groups become alkylated during standard sample preparation processes in phosphoproteome research; thus, interference by sulfhydryl groups can be eliminated using the IMAC process. As a hard Lewis base, phosphate groups prefer hard Lewis metal ions,[25] such as Al(III), Ca(II), Fe(III), Yb(III), Zr(IV) and Ti(IV). Interference caused by carboxyl and hydroxyl groups can be countered by regulating the pH of the coupling buffer used in the IMAC process. Carboxyl and hydroxyl groups are more easily protonated than phosphate groups because their pKa values are in the following order: hydroxyl group > carboxyl group > phosphate group. At a low pH, hydroxyl and carboxyl groups are protonated, which weakens the coordination between the oxygen atom and the metal ion. However, the phosphate groups remain unaffected. This difference can be exaggerated in high concentration acetonitrile solutions, which facilitate the highly selective enrichment of phosphoproteins/peptides.[26] Anchored phosphoproteins/peptides can be released from IMAC materials by acid, base or competitive chelating agents that contain phosphate ions. During elution with acid, a decrease in pH will result in phosphate group protonation, disrupting the coordination bond between the metal ion and the phosphate group. Elution with base or other compounds containing phosphate groups firmly attaches the metal ion to the material, removing the phosphoproteins/peptides from the material.

Many species of IMAC materials have been prepared by changing the metal ion, chelating ligands and the solid support. Metal ions, such as Fe(III),[27-31] Ga(III),[32] Al(III)[33] and Ce(IV),[34] have an enhanced affinity for phosphate groups and can be included in IMAC strategies (Table 1). Typically, Fe(III) can be immobilized on iminodiacetate (IDA)-agarose gels to enrich phosphoproteins.[27] Bound proteins can be eluted by increasing the pH or by including phosphate ions in the eluant. However, acidic and poly-His-containing peptides tend to be co-purified when using IDA-agarose gels. To improve selectivity, nitrilotriacetic acid (NTA) was used as the chelating ligand to optimize agarose gel efficiency. The specificity of NTA is attributed to the following characteristics: 1) NTA-agarose gels have a higher affinity for metal ions with coordination numbers of four and six compared to agarose gels; because Fe(III) has a coordination number of six, NTA-agarose gels should be much more stable than IDA- agarose gels; 2) Fe(III) loaded onto IDA-agarose gels may be lost during sample loading due to unstable affinity and 3) the free coordination site in IDA-agarose gels may lead to greater nonspecific interactions than with NTA-agarose gels.[28] During the past few decades, many types of Fe(III)-IMAC nanoparticles have been synthesized, such as poly(glycidyl methacrylate/divinylbenzene)-IDA-Fe^{III} (GMD-IDA-Fe^{III})[29] and Fe(III)-immobilized magnetic silica microspheres.[30,31] Ga(III) is

another metal ion that has been previously used to enrich the phosphoproteome. A Ga(III)-IMAC microtip, inspired by microtips containing reversed-phase perfusion beads, has also been developed. The results obtained demonstrated that microtip Ga(III)-IMAC is a highly efficient and versatile technique for enriching phosphorylated peptides from

Table 1. Different IMAC materials used for phophoproteome enrichment.

Metal Ion	Solid Support	Chelating Ligand	Selectivity	Sensitivity	Recovery	Ref.
Fe(III)	Agarose gel	Iminodiacetate	--	--	--	27
	Agarose gel	Nitrilotriacetic	--	--	--	28
	Poly(Glycidyl Methacrylate/Divinylbenzene)	Iminodiacetate	1:2 (GST-pERK2:GST-ERK2, tryptic digest)	0.2 μg/μL (β-casein, tryptic digest)	92% (β-casein, tryptic digest)	29
	Silica coated magnetic microspheres	Silane coupling agent (derived from reacting 3-glycidoxypropyltrimethoxysilane (GLYMO) with iminodiacetic acid)	--	20 nM (β-casein, tryptic digest)	--	30
			1:20 (α-casein:BSA, tryptic digest)	0.05 nM (standard peptide)	--	31
Al(III)	Agarose gel	Iminodiacetate	--	--	--	33
Ga(III)	Poros MC resin from PerSeptive Biosystems		1:2 (standard peptide: tryptic digest of β-galactosidase)	--	>80% (Standard peptide)	32
Ce(IV)	Silica coated magnetic microsphere	Silane coupling agent (derived from reacting 3-glycidoxypropyltrimethoxysilane (GLYMO) with iminodiacetic acid)	1:50 (β-casein:BSA, tryptic digestion)	20 nM (β-casein, ovalbumin, mixed with cytochrome C, myoglobin and BSA, tryptic digestion)	--	34
Ti(IV)	Polymer bead prepared by copolymerization of an ethylene glycol methacrylate phosphate (EGMP) and bis-acrylamide (BAA)	Phosphate group	1:500 (tryptic digestion of β-casein/ovalbumin/Standard peptide)	0.5 nM, 5 fmol (α-casein, tryptic digestion)	--	35
	Poly(GMA-co-TMPTMA-NH_2) microspheres	Phosphate group	1:1000 (α-casein:BSA, tryptic digestion)	20 nM, 50 fmol (peptide mix of α/β-casein and BSA, tryptic digestion)	--	36
	MCNC@PMAA@PEGMP	Phosphate group	1:500 (β-casein:BSA, tryptic digestion)	0.5 nM (β-casein, tryptic digestion)	87% (Standard peptide)	37
Zr(IV)	Poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads	Phosphate group	1:100 (α/β-casein:BSA, tryptic digestion)	20 nM (standard peptide, tryptic digestion of α/β-casein)	--	39
	α-Zirconium phosphate nanoplatelet		1:500 (α-casein/standard peptide:BSA, tryptic digestion)	2 fmol (α-casein, tryptic digest)	--	40
	Silica coated magnetic microsphere	Arsenate	1:100 (α-casein:BSA, tryptic digestion)	10 fmol (β-casein, tryptic digestion)	--	41

proteolytic digests prior to mass analysis.[32] Although other metals, such as Al(III) and Ce(IV), can also be used for phosphopeptide enrichment, few reports of their use in phosphoproteome component extraction from actual samples exist. This fact may be attributed to the inferior selectivity and preparation methods for these materials compared to the Ti(IV) and Zr(IV) IMAC methods discussed below.

Metal (IV) phosphate/phosphonate chemistry has emerged over the past three decades and is currently the most widespread IMAC method. The coordination of phosphate/phosphonate groups to metal (IV), especially Ti(IV) and Zr(IV), has now been extensively illustrated in the literature. Various types of Ti(IV)- and Zr(IV)-functionalized nanoparticles have been developed. For example, a porous phosphate polymer monolith was prepared by co-polymerization of a monomer containing phosphate groups with a crosslinker in the presence of porogenic solvents. Titanium ions were then immobilized by incubating the polymer beads with a $Ti(SO_4)_2$ solution after the polymer monolith was ground onto beads. Results have shown that Ti(IV)-IMAC beads can effectively isolate phosphopeptides when the ratio of phosphoprotein to non-phosphoprotein (α-casein:BSA) is 1:500.[35] To obtain increased enrichment performance, the preparation of Ti(IV)-IMAC beads were further optimized as follows: 1) monodisperse polystyrene seed microspheres were synthesized by dispersion polymerization; 2) monodisperse poly(GMA-co-TMPTMA) microspheres were prepared using polystyrene microspheres as seeds; 3) hydrophilic porous monodisperse poly(GMA-co-TMPTMA-NH_2) microspheres were prepared by reacting ethane-1,2-diamine with epoxide groups on poly(GMA-co-TMPTMA) microspheres; 4) poly(GMAco-TMPTMA-NH_2) microspheres were modified with phosphonate groups and 5) Ti(IV) was immobilized on the monodisperse microspheres via phosphonate group chelation. The Ti(IV)-IMAC adsorbent prepared by this method performed well in phosphopeptide enrichment.[36] To further facilitate the separation of phosphopeptides, magnetic composite microspheres have attracted significant interest due to their unique magnetic responsiveness. Combining magnetic nanomaterials with metal(IV) phosphate chemistry resulted in a simple and efficient separation of phosphopeptides from peptide mixtures using magnetic separation. We designed and presented a novel synthetic route for the preparation of high quality, Ti(IV)-IMAC, involving a two-step distillation–precipitation polymerization (DPP) method. This new material has a remarkable selectivity for phosphopeptides at a low molar ratio of phosphopeptides/nonphosphopeptides (1:500), as well as excellent recovery (87%) and high magnetic susceptibility.[37]

As a quadrivalent metal ion, Zr(IV) has attracted tremendous attention in phosphoproteome research. Using phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR), the coordination of Zr(IV) with phosphate groups is similar to that of Ti(IV) with phosphate groups.[38] Inspired by the fact that self-assembled monolayers and multilayer thin films of phosphate-containing organic molecules can be

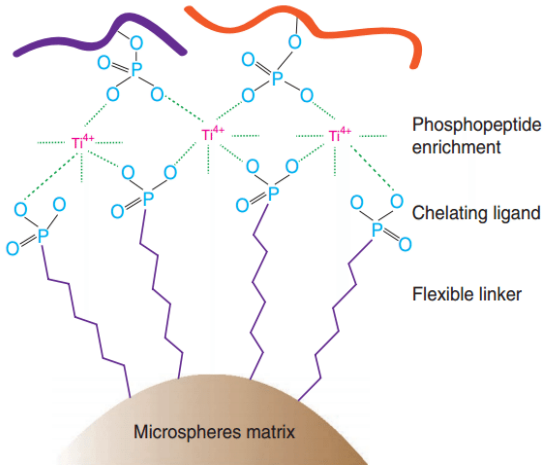

Figure 2. Proposed mechanism for phosphopeptide enrichment using Ti^{4+}-IMAC. [Reprinted by permission from Macmillan Publishers Ltd: [Nature Protocols] (H. Zhou, M. Ye, J. Dong, E. Corradini, A. Cristobal, A. J. Heck, H. Zou and S. Mohammed, *Nat. Protoc.,* 2013, **8**, 461-480), copyright (2013)]

prepared based on the strong interaction between phosphate groups on target molecules with Zr(IV) phosphonate on the surface of a solid matrix, a porous silicon wafer with its surface chemically modified with Zr(IV) phosphonate was prepared to specifically capture phosphopeptides followed by direct matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. The Zr(IV)-IMAC adsorbent was prepared by modifying poly(glycidylmethacrylate-co-ethylene dimethacrylate) beads with $POCl_3$, followed by immobilization of Zr(IV) using a $ZrOCl_2$ solution. The IMAC material was applied to mouse liver phosphoproteomics. In three replicate analyses, a total of 1,681 unique peptides were identified with an average of 109 non-phosphopeptides and 539 phosphopeptides in each run. Among the 1,681 unique peptides, 87.2% were phosphopeptides, and only 12.8% were non-phosphopeptides.[39] Similarly, Zr(IV)-immobilized nanoparticles, such as α-zirconium phosphate nanoplatelets (α-ZrPN)[40] or zirconium arsenate-modified magnetic nanoparticles ($ZrAs–Fe_3O_4@SiO_2$)[41], were synthesized and successfully applied to phosphoproteome analyses.

2.1.3 MOAC. Metal oxide affinity chromatography (MOAC) is another powerful and promising approach that permits selective phosphoproteome enrichment. The interaction of metal oxides with phosphate group-containing compounds has been extensively studied in catalysis. Infrared (IR) spectroscopy was applied to study phosphate protonation on goethite (α-FeO(OH)) surfaces, revealing three different types of complexes: protonated and nonprotonated bridging bidentate and non-protonated monodentate between orthophosphate ions and surface Fe(III) of α-FeO(OH) particles in aqueous suspensions. Changes in speciation of the phosphate surface complexes proved to be a function of pH and phosphate surface coverage.[42] Phosphate ion adsorption from aqueous solutions onto thin films of colloidal TiO_2 has also been studied.

Analyses have indicated that phosphate binds strongly as a bidentate surface species. Kinetic data has also supported a coverage-dependent model of the adsorption rate, discovered on the surface of goethite.[43] Further studies have suggested a bridging bidentate model identical to that of Ti(IV)-IMAC.[44]

Among the various types of metal oxides (Table 2), TiO_2 was first used as a chromatographic resin for the selective enrichment of phosphopeptides prior to MS analysis. The mechanism of selective phosphorylated peptide enrichment by TiO_2 was investigated in this work, determining that the matrix

Table 2. Different MOAC materials used for phophoproteome enrichment.

Metal oxide	Structure Characteristics	Selectivity	Sensitivity	Recovery	Ref.
TiO_2	TiO_2 beads were obtained from a disassembled cartridge	1:50 (phosphoprotein(α-casein, β-casein, ovalbumin):nonphosprotein(BSA, β-lactoglobulin, carbonic anhydrase), tryptic digestion)	50 fmol (peptide mixture from 12 standard protein, tryptic digestion	--	45,46
	TiO_2 nanocrystal clusters	--	--	50% (β-casein)	55
	Core/shell structure, magnetic core, mesoporous TiO_2 shell	1:1000 (β-casein:BSA, tryptic digestion)	0.1 nM (β-casein, tryptic digestion)	93% (standard peptide)	56
ZrO_2	Microtips filled with ZrO_2	--	1 pmol (α-casein, tryptic or Glu-C digestion)	--	47
	Mesoporous ZrO_2	7:93 (mass ratio, α-casein:5 nonphosphoproteins, tryptic digestion)	800 nM (α-casein, mixed with 5 nonphosphoproteins, tryptic digestion)	--	57, 58
HfO_2	Microtips filled with HfO_2	--	0.32 mM (β-casein, tryptic digestion)	--	48
	Mesoporous HfO_2	7:93 (mass ratio, α-casein:5 nonphosphoproteins, tryptic digestion)	800 nM (α-casein, mixed with 5 nonphosphoproteins, tryptic digestion)	--	58
Al_2O_3	Core/shell structure, magnetic core, Al_2O_3 shell	1:50 (phosphoprotein(α/β-casein):nonphosphoprotein(BSA, cytochrome C), tryptic digestion)	0.5 nM (α-casein, tryptic digestion)	--	49
Ga_2O_3	Core/shell structure, magnetic core, Ga_2O_3 shell	1:50 (β-casein:BSA, tryptic digestion)	0.2 nM, 40 fmol (β-casein, tryptic digestion)	--	50
ZnO	Core/shell structure, magnetic core, ZnO shell	--	0.25 nM, 2.5 fmol (β-casein, tryptic digestion)	--	51
Ta_2O_3	Core/shell structure, magnetic core, Ta_2O_3 shell	1:100 (phosphoprotein(β-casein, ovalbumin):nonphosphoprotein(BSA), tryptic digestion)	0.02 pmol (β-casein and ovalbumin, mixed with 2 pmol BSA, tryptic digestion)	--	52
La_2O_3	SiO_2 –La_2O_3 Composite	1:1:100 (α-casein:β-casein:BSA, tryptic digestion)	50 fmol (β-casein, tryptic digestion)	--	53
Fe_2O_3	Mesoporous γ-Fe_2O_3 nanocrystal clusters	1:100 (β-casein:BSA, tryptic digestion)	0.5 nM, 50 fmol (β-casein, tryptic digestion)	89.4% (standard peptide)	59
$NiZnFe_2O_4$	Magnetic nanoparticles	--	0.3 pmol (standard peptide)	--	64

DHB or other substituted aromatic carboxylic acids can enhance phosphorylated peptide binding selectivity due to the effective competition between DHB and non-phosphorylated peptides for binding sites on TiO_2.[45] This new procedure was developed as an important protocol for phosphoproteome sample preparation.[46] Subsequently, ZrO_2,[47] HfO_2,[48] Al_2O_3,[49] Ga_2O_3,[50] ZnO,[51] Ta_2O_3[52] and La_2O_3[53] were also evaluated for their ability to enrich phosphopeptides based on the same principle. A simple comparison between these metal oxides was also reported, and a more selective isolation of singly phosphorylated peptides was observed with ZrO_2 when compared to TiO_2, whereas TiO_2 preferentially enriched multiply phosphorylated peptides.[47] As a remarkable enrichment strategy for phosphoproteomics, MOAC, especially TiO_2-MOAC, has been widely used for large-scale phosphoproteomics.[54]

Recently, a large number of mesoporous metal oxides have been produced with excellent surface properties due to rapid progress in mesoporous materials synthesis. Mesoporous materials are nanostructured materials with pore sizes typically between 2–50 nm and with high surface areas. Together with the many active surface sites, these materials can possess an even higher loading capacity for binding phosphate groups than micro- and nanoparticles. These attributes make them ideal for phosphopeptide enrichment applications. Furthermore, adding superparamagnetic iron oxide nanocrystals to these clusters allows their efficient removal from analyte solutions after selective adsorption using an external magnetic field. Mesoporous TiO_2 has been exploited as a typical adsorbent for phosphopeptides, having a high binding capacity due to its surface area. It has the advantage of both the specific affinity offered by mesoporous TiO_2 and the size exclusion mechanism enabled by the mesoporous structure. The outer surface of each cluster can be made highly hydrophilic, such that nonspecific binding of many hydrophobic proteins/peptides can be avoided.[55] Our group further improved the synthetic process to obtain magnetic mesoporous Fe_3O_4@mTiO_2 microspheres with a well-defined core/shell structure, pure and highly crystalline TiO_2 layer, high specific surface area (167.1 m^2/g), large pore volume (0.45 cm^3/g) and high magnetic susceptibility. Due to the 100% purity and high crystallinity of the TiO_2 composition with no impurities remaining within the shells, Fe_3O_4@mTiO_2 possessed remarkable selectivity for phosphopeptides, even at a very low molar ratio of phosphopeptides/non-phosphopeptides (1:1000). Additionally, due to the exceptional porosity and large surface areas of this material, Fe_3O_4@mTiO_2 has a high enrichment capacity (as high as 225 mg/g, >10-fold that of Fe_3O_4@TiO_2 microspheres without mesoporous structure).[56] Similarly, other types of mesoporous metal oxides have also been synthesized for phosphopeptide enrichment. Mesoporous ZrO_2 and HfO_2 were synthesized using block co-polymer, template-directed sol-gel reactions.[57] Both preparations of mesoporous ZrO_2 and HfO_2 were demonstrated to be superior to TiO_2 for phosphopeptide enrichment from a complex mixture with high specificity (>99%). However, this method was demonstrated only with standard model protein mixtures.[58] To further simplify the enrichment process, we developed a novel strategy for fabricating magnetic mesoporous γ-Fe_2O_3 nanocrystal clusters for selective phosphopeptide enrichment. To obtain magnetic mesoporous γ-Fe_2O_3 nanocrystal clusters, mesoporous Fe_3O_4 nanocrystal clusters were synthesized using a solvothermal reaction and then subjected to calcination in air to form the final product. Distinct from previous studies, these magnetic iron oxide materials were used only as the magnetic cores to accelerate separation, while Fe_3O_4 can enrich phosphopeptides without the magnetic response. This new type of nanoparticle is a two-in-one functional material that possesses both the magnetic response and the ability for phosphopeptide enrichment.[59]

Compared with typical IMAC, MOAC is less sensitive to interfering compounds, such as salts and detergents.[60,61] Another difference between IMAC and MOAC is that MOAC favors binding with monophosphorylated peptides. TiO_2 is able to bind multiply and monophosphorylated peptides; however, multiply phosphorylated peptides are difficult to elute from TiO_2 due to their high binding affinity. In addition, multiply phosphorylated peptides are suppressed in the MS ionization process in the presence of monophosphorylated peptides and non-modified peptides, rendering them less detectable. Thus, monophosphorylated peptides are predominantly identified in large-scale phosphoproteomics experiments using TiO_2-MOAC. This difference is attributed to the development of the sequential elution from IMAC (SIMAC) technique. Acidic conditions primarily elute monophosphorylated peptides from IMAC materials, whereas subsequent basic elution recovers the multiply phosphorylated peptides that are normally not readily detected. Compared with TiO_2-MOAC, SIMAC is able to better enrich multiply phosphorylated peptides.[62] This method has now been used in large-scale phosphoproteomics studies.[63] As recently reported, metal ions at exposed octahedral sites of nano-ferrites, including Fe_3O_4, $NiFe_2O_4$, $ZnFe_2O_4$ and $NiZnFe_2O_4$, have distinctly selective coordination abilities with mono- and multiply phosphopeptides. When these nano-ferrites were applied to profiling the zebrafish egg phosphoproteome, $NiZnFe_2O_4$ was highly selective for multiply phosphopeptides, while Fe_3O_4, $NiFe_2O_4$ and $ZnFe_2O_4$ had a relatively stronger affinity for monophosphopeptides. According to these results, the author speculated that the selectivity was dependent on cation interactions at the octahedral and tetrahedral sites. For example, $NiZnFe_2O_4$ is highly selective for multiply phosphopeptides due to the increased surface area and decreased magnetic field of exposed surface octahedral sublattices.[64]

2.2 Glycosylation

As one of the most common, complex and important PTMs, protein glycosylation is of great biological significance. Glycosylation can affect the three-dimensional structure of proteins and serves key roles in various biological processes, such as cell division, tumor immunology, inflammation and so on.[65] Aberrant glycosylation is closely related to the initiation

and progression of tumors, and to date, more than half of all cancer biomarkers are glycosylated proteins or peptides.[66] Therefore, specific profiling of glycoproteins is highly inclined toward the discovery of disease biomarkers and clinical diagnosis. Although glycosylation occurs on more than 50% of the proteins in mammalian cells,[67] glycopeptides usually exist at a relatively low abundance (2% to 5%) compared with non-glycosylated peptides.[68] The microheterogeneity of glycans further reduces the relative amount of glycosylation, and the low ionization efficiency of glycopeptides reduces the detection sensitivity of MS analysis. Therefore, highly selectively enrichment of glycopeptides from complex biological samples is a critical prerequisite for in-depth glycoproteome research. The most common studied types of protein glycosylation are N- and O-glycosylations. In this review, we will focus primarily on these two glycosylation types.

N-glycosylation occurs by carbohydrate attachment to the carboxamide nitrogen atoms of asparagine in the consensus sequence Asn-X-Ser/Thr, where X is any amino acid residue with the three types: high mannose, complex and hybrid. These glycans are generally large, typically 10–20 monosaccharide residues, with a single common core.[69] O-glycans are mainly linked to a serine or threonine. There is no consensus sequence for O-glycans, and the glycan moiety is generally less complex than in N-glycans, consisting exception of proline. N-glycosylation can be further divided into of 3-10 monosaccharide residues. O-GlcNAcylation, distinct from "classical" O-glycosylation, is monosaccharide modification of serine and threonine hydroxyl groups and is not further elongated to form complex sugar structures.[65] Enrichment and separation of N-glycoproteins/glycopeptides can be realized by chemical attachment between N-glycans and specific functional groups on a solid phase. The chemical reactions involved in N-glycoproteins/glycopeptides enrichment include boronic acid chemistry, hydrazide chemistry, reductive amination chemistry, oxime click chemistry, TiO_2 coordination chemistry and alkyne click chemistry (Figure 3). Separation of O-GlcNAc modified proteins/peptides can be accomplished using hydrazide chemistry, tagging-assisted enrichment and β-elimination/Michael addition (BEMA)-based enrichment. In the following section, enrichment methods will be discussed according to the involved chemical reactions involved.

2.2.1 Boronic acid chemistry. Boronic acid can form five or six-membered cyclic esters with cis-diol compounds under alkaline conditions; the cyclic esters dissociate under acidic conditions. Therefore, the capture and release of glycoproteins/glycopeptides on a solid support can be performed using boronic acid chemistry and by simply adjusting the pH of the solution.[70] To enrich glycoproteins/glycopeptide, several types of functional materials modified with boronic acid groups, including agarose resins, magnetic nanoparticles, dendrimers and mesoporous silica, carbon nanotubes have been reported. Each type of functional material provides specific enrichment features, as described below.

Magnetic nanoparticles possess a large magnetic response that can be easily fished out of solution using an external magnet. Therefore, this type of material is an ideal matrix for the immobilization of boronic acid groups and the enrichment

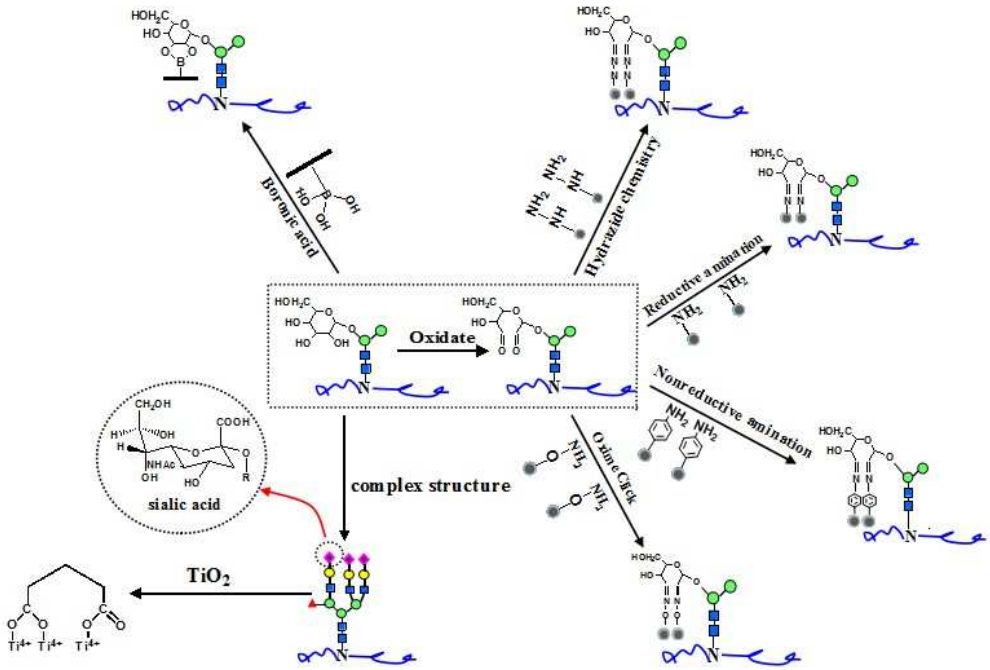

Figure 3. Enrichment strategies for N-glycoprotein/glycopeptides.

of glycoproteins/glycopeptides. Our group successfully synthesized a boronic acid-functionalized core–satellite-structured composite material, composed of a silica-coated ferrite "core" and numerous "satellites" of boronic acid-functionalized gold nanoparticles. Because numerous gold nanoparticles increase the surface area, and long organic chains reduce the steric hindrance and suppress nonspecific binding, the enrichment and release of glycosylated samples is quite specific and effective. These new composite nanoparticles were used to enrich glycosylated proteins from human colorectal cancer tissues and identify N-glycosylation sites. In all, 194 unique glycosylation sites mapped to 155 different glycoproteins were identified, of which 165 sites (85.1%) were novel.[71] Covering the surface of materials completely with boronic acid groups is difficult due to synthetic limitations, and the selectivity of traditional materials are not satisfied due to nonspecific adsorption. We also developed a new synergistic method using two different nanomaterials: boronic acid-functionalized Fe_3O_4 nanoparticles for enriching glycopeptides and poly(methyl methacrylate) nanobeads for strongly adsorbing non-glycopeptides. The synergistic enrichment provided by these two materials resulted in extremely high sensitivity and selectivity for the capture of glycopeptides from solution. A washing step is not necessary because the non-glycopeptides were separated by the poly(methyl methacrylate) nanobeads. Thus, the enrichment process was simplified, and the recovery efficiency of glycopeptides reached 90%.[72] Similarly, it has been reported that boronic acid can be immobilized on other magnetic particles like magnetic carbon nanotubes,[73] and zwitterionic polymer-coated core-shell magnetic nanoparticles.[74]

To enhance the binding affinity of boronic acid-functionalized materials for glycoproteins at low concentration, dendrimeric boronic acid-functionalized magnetic nanoparticles were synthesized and exhibited significantly enhanced binding strength for glycoproteins. Due to synergistic dendrimer-assisted multivalent binding, these materials exhibited dissociation constants of 10^{-5} to 10^{-6} M for glycoproteins, which was 3 to 4 orders of magnitude higher than the affinities associated with single boronic acid binding (Figure 4). The avidity of the boronate materials was tolerant of interference by abundant competing sugars. Moreover, these nanoparticles exhibited two additional advantages: high binding capacity (0.41±0.06 μmol/g, HRP, 9 glycosylation sites) and rapid binding/desorption kinetics (binding speed: 1 min; desorption speed: 5 min; common boronate affinity MNPs: 1 h) due to the abundant amount of boronic acid on the dendrimers.[75]

The above-mentioned boronic chemistry-based enrichment methods were mainly performed under alkaline conditions, which differ from the neutral physiological conditions of glycoproteins and may cause some unpredictable degradation. To capture the original glycoproteins using functionalized material at physiological pH values, Fe_3O_4/PAA-AOPB composite microspheres were designed and constructed, having a high magnetic response magnetic superparticle (MSP) core and a crosslinked poly(acrylic acid) (PAA) shell that

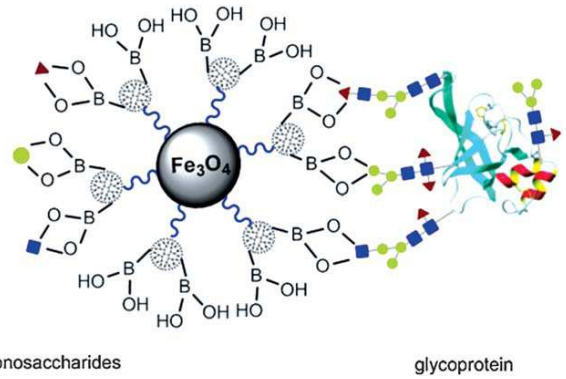

Figure 4. Principle of enhanced binding strength of boronic acid-functionalized magnetic nanoparticles for glycoproteins through synergistic dendrimer-assisted binding. (Reproduced from Ref. 75).

anchors abundant benzoboroxole functional groups on the surface. The choice of benzoboroxoles is significant for the capture of glycoproteins in neutral physiological environments because the pKa of benzoboroxoles is approximately 7.0, and when the enrichment pH is >7.0, benzoboroxole reacts with cis-diols to form five-membered cyclic esters. After optimizing the procedure, the prepared Fe_3O_4/PAA-AOPB microspheres displayed high selectivity for glycoproteins in complex biological samples (HRP/BSA of 1:80).[76]

Mesoporous nanoparticles are another important type of substrate material. With prominent features that include a high surface area, large pore volume and narrow distribution of regular pore size, mesoporous naonoparticles have great advantages in adsorption processes, leading to promising applications in glycoproteins/glycopeptides enrichment. Our group developed novel boronic acid-functionalized mesoporous silica for the enrichment of glycopeptides. To synthesize boronic acid-functionalized FDU-12 (FDU-12-GA), a two-step post-graft method was adopted, as illustrated in Figure 5 (left). The specific enrichment of glycopeptides is also shown in Figure 5 (right). Compared to the direct analyses of glycopeptides without enrichment, this method enabled two orders of magnitude improvement in the detection limit of glycopeptides. The high surface area and mesoporous confinement effect accelerated the reaction, and the loading time was shortened to only 15 min.[77] Subsequently, another boronic acid grafted mesoporous nanoparticle with MCM-41 as the matrix was synthesized for use in glycopeptidome research. With the combination of the size exclusion effect of MCM-41 and the selectivity of boronic acid chemistry, this material exhibited excellent selectivity (glycopeptide/non-glycopeptide analyses at molar ratios of 1:100), extreme sensitivity (fmol level detection limitation), good binding capacity (40 mg/g) and a high post-enrichment recovery of glycopeptides (up to 88.10%).[78]

As discussed above, boronic acid methods have several prominent advantages. First, many types of novel boronic acid materials are available because boronic acid can be simply and

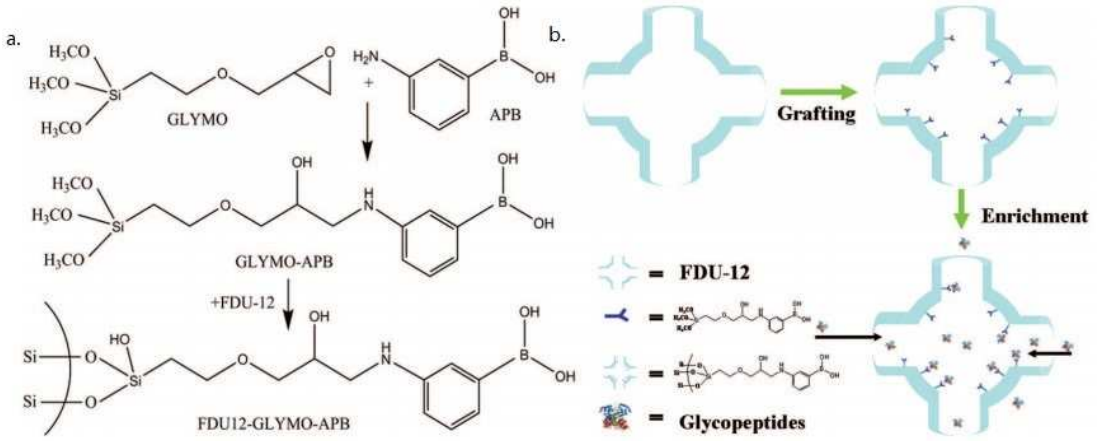

Figure 5. a. Post-synthetic steps of ordered boronic acid-functionalized mesoporous silica materials; b. specific enrichment of glycopeptides from peptide solutions using FDU-12-GA as a nanoreactor through the specific binding of di-boronic acid groups and glycopeptides. [Reprinted with permission from (Y. W. Xu, Z. X. Wu, L. J. Zhang, H. J. Lu, P. Y. Yang, P. A. Webley and D. Y. Zhao, Anal. Chem., 2009, 81, 503-508). Copyright (2009) American Chemical Society.]

conveniently modified on different types of matrices. Second, the conditions for capture/liberation of glycopeptides are mild and easy to alter. Third, without enzymatically removing the glycans, the released glycopeptides can directly afford sugar chains for MS analysis, which can provide specific information regarding the sugar chains at certain binding sites. However, boronic acid-functionalized materials often have associated non-specific binding due to low binding constants and relatively low selectivity. Immobilization of boronic acid on low-specific adsorption surfaces would help to solve this problem.

2.2.2 Hydrazide chemistry. Hydrazide chemistry-based methods are another important type of N-glycoproteome enrichment strategy. Due to high specificity and commercially available reagents, this method is now widely applied to profiling N-glycoproteomes of complex biological samples and is extensively combined with stable isotope labeling for the high-throughput identification and quantification of glycosylation sites and glycopeptides. The solid-phase extraction method to enrich glycoproteins based on hydrazide chemistry was first developed by Zhang et al. This methodology includes the following four steps: 1) periodate oxidation of carbohydrate cis-diol groups to aldehydes; 2) hydrazone formation between aldehydes and hydrazide groups; 3) tryptic digestion of immobilized proteins to remove non-glycosylated peptides and 4) liberation of glycosylated peptides from a support with PNGaseF.[79,80] Recently, to make hydrazide chemistry enrichment of glycoproteome easier and more efficient, different matrices, such as magnetic beads,[81] gold nanoparticles,[82] polymers [83] and dendrimers [84] were exploited to be functionalized with hydrazide groups for selective capture of glycopeptides from complex biological samples. Our group fabricated a new type of hydrazide-functionalized core–shell magnetic nanocomposites, Fe_3O_4@poly(methacrylic hydrazide) (Fe_3O_4@PMAH). Polymer-coated magnetic nanocomposites with a large number of carboxyl terminal groups were prepared by reflux precipitation polymerization, which is a very rapid, simple and effective method for introducing a large amount of functional groups onto the surface of the magnetic core. After coating with polymer, adipic acid dihydrazide (ADH) was reacted with the surface carboxy groups to generate hydrazide functionalized polymer magnetic nanocomposites. Abundant hydrazide groups can specifically enrich glycopeptides, and the magnetic core facilitates the separation of nanocomposites from solution. In addition, the hydrophilic polymer surface can minimize nonspecific adsorption of other peptides.[83] To explore the significant biological importance of trace glycosylated proteins or peptides, developing a more sensitive enrichment approach for in-depth glycoproteome research is urgent. We then designed and synthesized hydrazide-functionalized dendrimer materials for efficient and selective enrichment of N-linked glycopeptides from complex biological samples using filter-aided sample preparation (FASP). Polyamidoamine (PAMAM) dendrimer materials possess an ideal high molecular weight, enabling thorough separation from non-bound peptides. The molecular size of this material is comparable to many folded proteins and will promote interactions during incubation with target species. Another attractive feature of PAMAM as a matrix is that it possesses abundant reactive groups for surface conjugation due to higher generation dendrimers, which have more exposed functional groups on their surface. This hydrazide-functionalized, PAMAM-based enrichment strategy was successfully applied in comprehensive glycosylation site characterization using complex biological samples, and 158 unique glycopeptides from human serum were mapped, identifying 60 different glycoproteins.[84]

In recent years, different research groups have performed in-depth glycoproteome profiling by combining hydrazide chemistry-based enrichment with other complementary enrichment methods. Combined with a lectin enrichment

approach, 6,367 N-glycosylation sites on 2,352 proteins from four mouse tissues and from blood plasma were mapped using high-accuracy MS.[69] Combining click maltose–hydrophilic interaction chromatography (HILIC) with improved hydrazide chemistry helped to identify 14,480 N-glycopeptides corresponding to 2,210 N-glycoproteins and 4,783 N-glycosylation sites in the human liver.[85]

When an attached glycan is terminated with sialic acid (SA), mild periodate oxidation (usually performed at a temperature of 0-4°C, 1-2 mM sodium periodate and within 10 min) can be employed to selectively oxidize the polyhydroxy side chain of sialic acid. The three linearly adjacent hydroxyl groups at the C7, C8 and C9 carbons in sialic acids are highly susceptible to periodate oxidation. Periodate oxidation of sialoglycoproteins, followed by coupling to hydrazide beads, trypsin digestion and acid hydrolysis to cleave SA glycosidic bonds releasing desialylated glycopeptides, has been used for profiling glycoproteins in human cerebrospinal fluid.[86] As mentioned, hydrazide chemistry is commonly used for N-glycoprotein/glycopeptide enrichment because the glycans are usually detached using enzymes, such as PNGase F; however, no available enzyme for O-glycosylation exists. Recently, a similar method was developed for the enrichment of O-linked glycoproteins. The method includes three steps: 1) periodate oxidation of O-GlcNAc groups and conversion to its dialdehyde derivative; 2) oxidized O-GlcNAc protein capture using a hydrazide resin; 3) removal of non-glycosylated proteins, 4) enzymatic digestion and removal of non-glycopeptides and 5) release of O-linked peptides by glycosidic bond acid hydrolysis using hydroxylamine. With this enrichment strategy and MS analyses, four O-GlcNAc sites were identified in Drosophila proteins. However, the main shortcoming of this method is that to derivatize the less active O-GlcNAc moiety, harsher conditions must be used, leading to undesired side reactions (e.g., oxidation of N-terminal Ser/Thr residues). At the same time, the release of tagged O-GlcNAc peptides is not efficient and specific enough for large-scale O-GlcNAc site mapping. At present, hydrazide chemistry enrichment method for O-GlcNAc is not a mature technology and is used as an alternative to existing methods to yield complementary information.[87]

Due to the covalent capture of target molecules, the selectivity of hydrazide chemistry-assisted enrichment for N-glycoproteome can be as high as 90%.[88] Another distinct advantage is that the hydrazide chemistry method can be easily combined with other enrichment approaches, such as lectin, HILIC and so on, to conduct in-depth glycoproteomic profiling. However, massive sample loss during the harsh washing process to remove nonspecific binding represents the major limitation of this method. Furthermore, enrichment requires a relatively long time and a desalting step before MS analyses can be performed because the enrichment process utilizes a large amount of salt. In addition, when using hydrazide chemistry-based enrichment, information regarding glycans from the isolated glycoproteins is lost during hydrazide-based capture, as the reaction between the glycan and the hydrazide group is irreversible and enzymatic detachment of the glycans (e.g., PNGase F) from the glycopeptides to release the peptides for MS analyses results in glycan information loss.

2.2.3 Reductive amination chemistry. Reductive amination, including Schiff base formation and its subsequent reduction to a secondary amine with a reducing agent, has been widely used for glycan derivatization. This reaction involves the initial formation of an intermediate carbinol amine, which dehydrates to form an imine. With acid, the imine is rapidly protonated to form an iminium ion. Subsequent reduction of this iminium ion enables the formation of an alkylated amine product.[89] Although reductive amination has been extensively studied in glycomics, our group introduced reductive amination to enrich N-glycopeptides. The enrichment strategy is based on the reaction between aldehyde groups, which are produced by oxidation of the cis-diol groups on glycans and amino groups attached to magnetic nanoparticles. The amino groups from 3-aminopropyltriethoxysilane (APTES), assembled on the surface of nanoparticles through a one-step silanization reaction, conjugated with aldehydes from oxidized glycopeptides, therefore completing the extraction. Compared to hydrazide chemistry-based solid-phase extraction, this protocol eliminated the desalting step and shortened the extraction time to 4 h, improving the detection limit of glycosylated peptides by two orders of magnitude. A total of 111 N-glycosylation sites were identified in 108 glycosylated-peptides, using only 5 μL of human serum.[90] The requirement for a reductive reagent to generate the stable C-N bond between the N-glycopeptides and the amine functionalized magnetic nanoparticles suggests a major limitation of this reductive amination-based method. Different from alkylamines, aniline can react with the aldehyde group without the addition of a reductive reagent.[91] The generated aromatic Schiff base could be stable in a wide pH range without reducing the C=N bond to C–N bond. Therefore, very recently, we have developed a novel N-glycoproteome enrichment method based on the conjunction of aldehydes from oxidized glycopeptides to aniline groups on magnetic nanoparticles via nonreductive amination. Correspondingly, a new type of aniline-functionalized nanoparticle has been designed and synthesized.[92]

2.2.4 Oxime click chemistry. The oxime click reaction is a classical reaction between an aldehyde group and an aminooxy group, usually proceeding under mild conditions to produce stable oxime bonds because aminooxys are more nucleophilic than hydrazides due to the α-effect.[93] Oxime click reactions have been studied extensively for decades in the area of glycomics, e.g., labeling the reductive end of a glycan prior to glycoblotting.[94] Inspired from the original concept of the glycoblotting method, an enrichment method directed toward SA-containing glycopeptides was developed, which first selectively oxidized the SA residues of glycopeptides and then subsequently enriched them by chemical ligation with a polymer reagent.[95] Similarly, a simple method for efficiently labeling and enriching cell surface SA–containing glycans on living animal cells was reported. This method used mild

periodate oxidation to generate an aldehyde on sialic acids, followed by aniline-catalyzed oxime ligation with a biotin tag for the corresponding streptavidin enrichment. Due to the mild reaction condition, a majority of cell surface sialylated glycoproteins can be efficiently labeled while maintaining high cell viability.[96] In a recent study, to enrich glycoproteins with specific glycans (i.e., terminated with SA or galactose), oxime click reaction was employed to introduce an aminooxy-functionalized biotin onto glycoproteins. These biotinylated glycoproteins were then captured on streptavidin-coated beads, thus enabling the targeted glycoproteins to be obtained with high specificity.[97] Our group further improved this oxime click chemistry-based enrichment strategy by introducing a novel solid-phase extraction method. With the use of newly synthesized aminooxy-functionalized magnetic nanoparticles, oxidized glycan chains on glycopeptides readily reacted with the aminooxy groups through oxime click chemistry, resulting in the highly selective extraction of glycopeptides. This new method demonstrated excellent enrichment performance within 1 h, with improved enrichment sensitivity (fmol level), selectivity (extracting glycopeptides from mixtures of non-glycopeptides at a 1:100 molar ratio), and reproducibility (CVs of <20%).[98]

2.2.5 TiO_2 coordination chemistry. TiO_2 is often used to capture phosphopeptides. Negatively charged SA, which contains carboxylic acid and hydroxyl groups, may also interact with TiO_2 via multipoint binding. Therefore, after dephosphorylation with alkaline phosphatase, an interaction will occur specifically between TiO_2 and SA. Based on this principle, a simple, yet highly selective strategy for the enrichment of SA-containing glycopeptides was implemented. A total of 192 and 97 SA-containing glycosylation sites were identified from depleted human plasma and saliva, respectively.[99] Combined with HILIC enrichment, a protocol for the comprehensive analysis of SA-containing glycopeptides was established. To improve the binding efficiency and selectivity of SA-containing glycopeptides to TiO_2 resin, a low pH buffer containing a substituted acid, such as glycolic acid, was used. The efficiency of the method was illustrated by the identification of 1,632 unique sialylated glycopeptides from 817 sialylated glycoproteins.[100] Recently, profound profiling of sialylated N-glycoproteomics in human plasma was conducted by an optimized strategy that consisted of a combination of FASP, TiO_2 enrichment, multiple enzyme digestion and high pH reversed-phase peptide separation. A total of 982 glycosylation sites were identified in 413 proteins, providing to date, the largest SA glycosite database in human plasma.[101] Because both phosphopeptides and N-linked sialylated glycopeptides can be enriched using TiO_2, this method can be utilized for studying these two PTMs simultaneously by varying the enrichment conditions. [102]

2.2.6 Tagging-assisted enrichment. By adding a functional tag to a glycosylation conjugate, tagged glycopeptides can be readily captured onto chemically modified materials. This type of enrichment method is mainly used for O-GlcNAc analysis. For example, analogs of GlcNAc, such as N-azidoacetylglucosamine (GlcNAz) or other similar chemical handles, have been introduced into O-GlcNAc glycoproteins as a labeling tag by chemoenzymatic or metabolic methods. An enrichment tag (biotin or others) via copper-catalyzed azide-alkyne cycloaddition between the azide group of GlcNAz and an alkyne reagent were then introduced onto the labeling tag, and finally the enrichment tags were immobilized on a solid support to obtain labeled O-GlcNAc peptides. Chemoenzymatic labeling and metabolic labeling represent ideal choices for in vitro and in vivo analyses of O-GlcNAcylation, respectively. Chemoenzymatic labeling makes use of the merits of the traditional β-1-4-galactosyltransferase (GalT) labeling, and GalNAz can be added to the GlcNAc moiety on target proteins by GalT. Metabolic labeling offers an in vivo method for O-GlcNAc protein enrichment by feeding cells with an appropriate amount of peracetylated GlcNAz, and proteins that were initially modified with O-GlcNAc will be substituted with GlcNAz.

GlcNAz was first used in metabolic labeling in 2003, and the GlcNAz-tagged proteins were then chemoselectively conjugated with a biotinylated phosphine reagent (enrichment tag) via Staudinger ligation. After streptavidin-conjugated bead enrichment, tagged proteins are then digested and subjected to MS analyses.[103] Using a similar strategy, 199 putative O-GlcNAc modified proteins from HeLa cells were identified, among which 23 were confirmed using reciprocal immunoprecipitation.[104] Although the above-mentioned biotin tag and streptavidin procedure is standard for O-GlcNAc enrichment, the release of the tagged peptides is difficult due to the strong interaction between biotin and streptavidin.

Several methods have been developed for easily releasing captured O-GlcNAc peptides. An ultra violet (UV)-cleavable linker was introduced to improve the release efficiency of tagged peptides from streptavidin-conjugated beads. This novel biotinylation reagent contained a UV-cleavable reagent that reacts with the azido group on GlcNAz. Tagged peptides were enriched by affinity chromatography, released from a solid support by photochemical cleavage and finally analyzed by electron transfer dissociation (ETD) MS.[105] Using a combination of GlcNAz labeling, click chemistry, UV-cleavage and ETD analysis, 458 O-GlcNAc sites in 195 proteins from mouse cerebrocortical brain tissue were identified.[106] Making use of the reversible reaction between TiO_2 and phosphate groups, another procedure for releasing O-GlcNAc peptides was introduced. O-GlcNAc peptides were enzymatically labeled with GlcNAz. The azide was then reacted with a phospho-alkyne group using click chemistry and then enriched by TiO_2 chromatography (Figure 6). This enrichment method was applied to a nuclear preparation that was generated from HeLa cells. A total of 42 unique O-GlcNAcylated peptides were identified, including seven novel O-GlcNAc sites.[107] Because the reaction between a hydrazide and a carbonyl is chemoselective and reversible, a combination of hydrazide chemistry and GalNAz followed by click chemistry was introduced for O-GlcNAc enrichment. The azide group on the GalNAz residue reacts with 3-ethynylbenzaldehyde via a click reaction to form an aromatic aldehyde group, which could be enriched by reversible hydrazine formation with a hydrazide

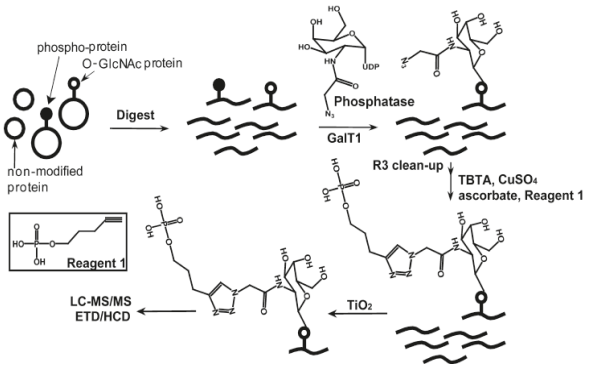

Figure 6. Enrichment of O-GlcNAcylated peptides by GalNAz enzymatic labeling and cycloaddition of a phosphoalkyne, followed by TiO_2 chromatography. [Reprinted with permission from (B. L. Parker, P. Gupta, S. J. Cordwell, M. R. Larsen and G. Palmisano, J. Proteome Res., 2011, 10, 1449-1458). Copyright (2011) American Chemical Society.]

resin. All of the materials and chemicals required for this method are commercially available, and no special reagents are required, facilitating the introduction of this method in any laboratory.[108] To easily separate biomolecules from complex samples, disulfide- and terminal alkyne-functionalized magnetic silica particles (DA-MSPs) were synthesized and used for azido-labeled glycopeptide enrichment. Azido glycopeptides could be captured on the DA-MSPs via click chemistry and eluted following disulfide cleavage after washing. This enrichment process is expected to be a promising technique for the future analyses of O-GlcNAcylation.[109]

2.2.7 β-elimination and Michael addition (BEMA)-based enrichment. Because O-GlcNAc can be removed from proteins/peptides by β-elimination under basic conditions with serine and threonine residues being converted into their dehydrated equivalents, a subsequent Michael addition with a nucleophile, such as dithiothreitol (DTT) (referred to as "BEMAD") or a biotinthiol probe to label the β-eliminated O-GlcNAc site, produces a peptide that can then be enriched with a thiol-capture resin or with streptavidin-conjugated beads.[110] Recently, an adapted method involving BEMA with a biotin-cystamine tag, followed by processing with streptavidin-conjugated beads was developed. The specificity of the reaction was increased using differential isotopic labeling with either "light" biotin-cystamine or deuterated "heavy" biotin-cystamine. The enriched peptides were analyzed by liquid chromatography (LC)-MS and relatively quantified. Using this approach, five novel and one known O-GlcNAc site on the murine 20S proteasome core complex, and two novel O-GlcNAc sites on murine Hsp90 were identified.[111] The most attractive features of BEMA-based enrichment is that, unlike the O-GlcNAc moiety, the final resulting sulfide derivatives are stable during fragmentation and are thus suitable for detection and site mapping by CID-MS/MS. Quantitative O-GlcNAc site information can be readily achieved using isotopic labels.

Because phosphorylated or other modified peptides can also be subjected to BEMAD under harsh basic conditions, easily eliminated O-glycosidic linkages should be used under milder conditions, and the samples should be treated by enzymatic dephosphorylation, passivation of cysteine residues or other treatments.

2.3 Other PTMs

In addition to the two most prevalent types of PTMs, phosphorylation and glycosylation, other PTMs can be enriched using covalent chemical reactions. Different from the above two most prevalent PTMs, chemical reactions will not be further divided into subtypes in the following sections because the chemical reactions involved in these PTMs are not as comprehensive as those for phosphorylation and glycosylation.

2.3.1 Nitrotyrosine. Protein tyrosine nitration (PTN) involves nitration of tyrosine residues to produce 3-nitro-tyrosine. PTN is associated with significant changes in protein functions, such as hindering tyrosine phosphorylation, and is implicated in a variety of biological processes, including immunogenicity and signal transduction in cells.[112] The low abundance of nitrated tyrosine residues (only five TN residues per 10,000 tyrosines) has hampered the determination of the modified sites.[113] Therefore, selective enrichment becomes a prerequisite for the detection of PTN. Due to the poor chemical reactivity of the nitro group on the tyrosine side chain, most of the chemical enrichment strategies for PTN are performed after conversion to the corresponding aminotyrosines because the amino group offers a more convenient chemical handle for tagging, enabling the enrichment of nitropeptides from the matrix of unmodified species. Generally speaking, this enrichment includes the following four steps: 1) blocking the ε-amino group of lysine and N-terminal amines; 2) reducing 3-nitrotyrosine to 3-aminotyrosine; 3) capturing the 3-aminotyrosine-containing peptides on a solid-phase support through tagging 3-aminotyrosine with an enrichment tag or without tagging and 4) washing away the nonspecifically bound peptides and releasing the captured 3-aminotyrosine-containing peptides. Strategies for enriching the PTN products are shown in Figure 7.

The first chemical enrichment strategy involved reducing 3-nitrotyrosine to 3-aminotyrosine with sodium dithionite at the protein level, then selectively coupling 3-aminotyrosine to a cleavable biotin affinity tag and capturing it with a streptavidin affinity column. Although this approach was sound in principle, it suffered from nonspecific derivatization reactions to aminotyrosine in the presence of other primary amines due to the abundant free amino groups in proteins. Additionally, the biotin derivatization was performed at the protein level, which may further limit tagging efficiency. This approach has only been applied for analyzing single proteins.[114] Subsequently, a more selective method than biotin tagging for avidin enrichment to capture aminotyrosine peptides utilizing solid-phase chemistry ("chemoprecipitation") was reported. Instead

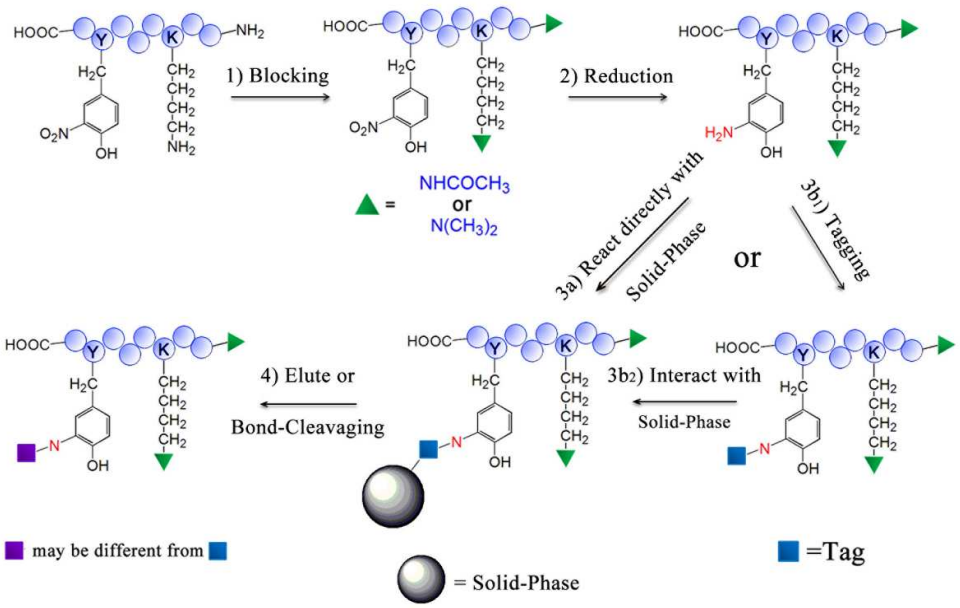

Figure 7. General enrichment strategies for PTN.

of tagging the aminotyrosine peptides with a biotin affinity tag, N-succinimidyl S-acetylthioacetate (SATA) was employed as the tag, followed by deprotection of S-acetyl on SATA to form free sulfhydryl groups. Enrichment was obtained by capturing sulfhydryl-containing peptides with thiopropyl Sepharose beads. In this work, to improve selectivity, another chemical reaction was included before biotin tagging that involved blocking all free amines by acetylation. The blocking reaction prevented the protein free amino groups to be tagged by biotin, thus improving the selectivity of this method.[115] Alternative chemical reactions for tagging and enrichment have been suggested. Aminotyrosine can be reacted with a large excess of pyridine-2-carboxaldehyde to form a Schiff base, followed by reductive amination upon treatment with $Na(CN)BH_3$, resulting in a bispyridinylated peptide. The resulting bispyridinylated tyrosine can form a stable complex with Ni^{2+}-NTA magnetic agarose beads through strong and specific metal chelating interactions, leading to the enrichment of PTN peptides. The utility of this approach was demonstrated by nitrated peptide detection in complex samples, such as tryptic peptide mixtures of BSA and HeLa cell lysates.[116] Subsequently, an enrichment method was developed by the same group that involved several chemical modification steps, including acetylation of ε-NH_2 groups on lysine residues and the N-terminus, reduction of NO_2 groups on the nitrated tyrosine residues to NH_2 groups, fluorinated carbon attachment of NH_2 groups on tyrosine residues and enrichment of tagged peptides. Using this method, 28 nitrated peptides corresponding to the same number of proteins from the Huh7 human hepatoma cell line were identified.[117]

In general, the above-mentioned enrichment strategies involved multiple-step chemical conversions to obtain the corresponding tagged peptides. The efficiency of each step can severely impact the overall enrichment efficiency, and multiple sample preparation steps would lead to sample loss. To overcome these shortcomings, a simpler, yet highly specific method for identifying nitration sites in peptides/proteins by significantly decreasing the number of chemical modifications was reported. Free amino groups were blocked, and then nitropeptides were reduced to the corresponding aminopeptides. Subsequently, aminopeptides were selectively chemoprecipitated on glass beads using a novel designed solid-phase active ester reagent (SPAER), whereas other peptides carrying no free amino groups were separated from the immobilized species by thoroughly washing the beads. The tagged peptides could be recovered via acid-catalyzed hydrolysis at room temperature for MS analyses. This method was demonstrated to be useful in the analysis of the nitroproteome in human serum, as 261 nitroproteins were identified out of 1815 identified proteins.[118] This SPAER approach was recently described as a protocol for the analysis of nitropeptides from complex proteomes.[119]

2.3.2 Carbonylation. Protein carbonylation is considered to be a universal indicator of oxidative stress. Carbonylation groups can be directly formed in proteins by the oxidation of specific amino acids (Pro, Arg, Lys, Thr, Glu and Asp) and can be also indirectly formed by modification of lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (HEE), 2-propenal and malondialdehyde to Cys, His or Lys residues. Proteomics has revealed the relevance of protein carbonylation in several pathological conditions, such as those involved in the inflammatory response, metabolism and tissue remodeling.[120,121] In this review, we limit our discussion to representative works using chemical enrichment methods to enrich certain types of carbonylated proteins.

The existence of carbonyl groups provides a readily available functional group for the enrichment of carbonylated peptides. Reaction with a carbonyl-reactive reagent via formation of a Schiff base, hydrazone or oxime provides unique opportunities to enrich the carbonylated peptides, and discuss of these reactions can also be found in the glycoprotein enrichment parts. To enrich the carbonylated peptides, a biotin hydrazide (BHZ) was reacted with carbonyl groups to form the corresponding Schiff base, which was then reduced to a stable hydrazone, thereby tagging the carbonylated peptides with biotin. Then, avidin affinity chromatography was used employed to purify the biotin-tagged carbonylated peptides. This work allowed the isolation of oxidized proteins from the remainder of the proteome and facilitated their identification, making the identification of the site of oxidation site possible.[122] The same group then reported the first proteomic-based identification and characterization of oxidized proteins in human plasma using this method., with oxidation products arising from direct oxidation of amino acid side chains in proteins, formation of advanced glycation endproducts (AGEs) adducts, and formation of adducts with lipid peroxidation products were simultaneously identified. It is noteworthy that, hydrazine is highly reactive towards all types of reactive carbonyls formed in the various carbonyl modifications. Therefore, the differentiation of different types of carbonylation relied on MS analyses, which requires mass spectrometric MS analysis with high accuracy.[123]

Similarly, the hydroxylamine that can react with carbonyl groups to generate the corresponding aldoxime/ketoxime derivatives was also used as an alternative method for the enrichment of carbonylated peptides. The advantages of a hydroxylamine probe are: 1) first, the bond formed between the hydroxylamine and the carbonyl group is more stable; and second2), the conjugation condition required for the reaction between the hydroxylamine and the carbonyl group is milder. To perform biotin tagging of the carbonylated peptides, N'-amino-oxymethylcarbonyl-hydrazino D-biotin was reacted with aldehyde/keto groups to obtain chemically stable oxime derivatives through formation of oximes instead of hydrazones. The biotin tagged peptide was then amenable to enrichment using avidin affinity capture. Using the hydroxylamine probe, seven mitochondrial proteins that showed an age-dependent increase in protein carbonyls were profiled.[124] The biotin tag can also be detected by avidin affinity staining, and the detected proteins are subsequently subjected to in-gel proteolysis and MS analysis to identify the modification sites.[125] Methodological details of this strategy can be found in a book chapter, in which additional model studies are presented.[126]

Modified biotin-avidin enrichment methods that are combined with other tagging techniques (e.g., click chemistry, also known as the copper-catalyzed Huisgen 1,3 dipolar cycloaddition reaction) can be used to probe the reactivity of HNE with proteins in vivo. In these cases, azido or alkynyl analogues of HNE were synthesized to induce heme oxygenase and apoptosis in colon cancer cells. Cells exposed to the HNE analogues were lysed and treated with conjugated HNE analogue-modified proteins with corresponding alkynyl- or azido- biotin through click chemistry to form a stable triazole for subsequent affinity purification. Proteomic analysis revealed that several proteins involved in stress signaling were selectively adducted by azido- and alkynyl-HNE. The use of azido and alkynyl derivatives in conjunction with click chemistry appears to be a valuable approach for identifying protein targets of HNE. Compared with biotin-avidin-based methods, click chemistry was found to be superior for the recovery of biotinylated proteins from streptavidin-coated beads.[127] Furthermore, combined with isotopically tagged photocleavable azido-biotin reagent, a new quantitative chemoproteomic platform to selectively capture and quantify the cellular targets were reported very recently.[128] In addition to the biotin-avidin enrichment methods, other chemical enrichment methods exist. For enriching HNE peptides, a reversible hydrazone reaction was employed using hydrazide-functionalized resins that allow the efficient capture-release of carbonylated peptides by covalent chromatography. Using a reversible hydrazone reaction, enriched HNE peptides can be eluted at elevated temperatures (60°C) and low pH values (10% TFA or formic acid).[129]

Although the developed approaches provided methods for analyzing carbonylation modifications, several pitfalls must be considered to further improve these methods. First, tagging efficiencies have an important impact on enrichment efficiency and vary with the nature of reaction buffer, reaction time period and reaction temperature. However, studies that focus on tagging efficiency are rare. Second, the stabilities of these derivatives must be considered, especially under different washing and elution conditions. Third, considering that hydrazines/hydroxylamines react with all carbonyl-containing compounds, which will complicate data interpretation, characterization of different protein carbonyls therefore requires mass analyzers with the highest possible resolution and mass accuracies.

Formation of AGE product adducts is another type of carbonylation, and these adducts can then be oxidized to carbonyls. Protein glycation may occur at lysine or arginine amino groups or at the N-terminal amino acid residues of proteins. In principle, methods based on traditional techniques for glycoprotein enrichment could be used for selectively enriching non-enzymatically glycated proteins. These strategies are usually based on affinity chromatography using lectins, hydrazide and boronic acids supports. Among the above-mentioned methods, boronic acid chromatography has proven to be a highly efficient method for enrichment of glycated protein enrichments. The first effective high-throughput methods for identifying glycated proteins were reported using phenylboronate affinity chromatography. After a two-step enrichment scheme to selectively isolate glycated proteins and glycated tryptic peptides, the enriched peptides obtained from human serum were subsequently analyzed. In particular, using the ETD fragmentation mode for analyzing the enriched glycated peptides permitted the identification of a significantly higher number of glycated peptides (87.6% of all identified peptides).[130] Recently, this strategy was extended

further to perform comprehensive proteomic analyses of glycated proteins in control and diabetic human plasma samples and in erythrocytes. Using immunodepletion, boronic acids enrichment and fractionation strategies, 7,749 unique glycated peptides, corresponding to 3,742 unique glycated proteins were identified, which represented the largest scale glycated proteome dataset at that time.[131] In addition, through the incorporation of methacrylamido phenylboronic acid in acrylamide gels, glycated proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[132]

2.3.3 Citrullination. Protein citrullination results from the enzymatic deimination of peptidylarginine, converting a positively charged guanidino group into a neutral ureido group. Protein citrullination has been reported to play an important role in several physiological processes, including cell differentiation and apoptosis.[133] Although the role of protein citrullination is being increasingly recognized, chemical-based techniques available for enriching citrullinated proteins are rare. To accomplish enrichment, "citrulline reactive resin beads" were designed and synthesized with 4-hydroxyphenylglyoxal as the reactive group attached to the resin via a base-labile linker. Based on the specific reaction of glyoxal derivatives with the ureido group on citrullinated peptides under acidic conditions, citrullinated peptides can be captured on glyoxal-modified beads. Unbound noncitrullinated peptides are removed by extensive washing. Finally, citrullinated peptides carrying a modified ureido group can be released from the beads at high pH values to cleave the base-labile bond and analyzed by MS. In a preliminary study, this enrichment procedure was validated by synthetic citrulline-containing peptide enrichment from peptide mixtures and applied to enrich citrullinated peptides from a digest of deiminated myelin basic protein.[134] To further improve the enrichment sensitivity for in-depth proteomic analyses of biological samples, this group reported an alternative enrichment strategy based on the same chemical reaction, generated from the in-solution biotinylation of citrulline residues followed by selective enrichment of modified peptides using streptavidin beads. Due to this in-solution reaction, the enrichment of biotinylated citrullinated peptides demonstrated 20-fold higher sensitivity when compared to the enrichment strategy in which beads were functionalized with 4-hydroxyphenylglyoxal via a cleavable linker, thus enabling additional citrullinated peptides to be detected.[135] To improve the synthesis of "citrulline reactive beads", synthesis of phenylglyoxal compounds with an azido group at the meta position that could be reacted with alkyne-modified rhodamine via Cu-catalyzed azide–alkyne cycloaddition was reported; the derivatization of phenylglyoxal onto the solid phase via the para position reported previously is not amenable to the solution phase. After optimization of the conditions, enrichment sensitivity was improved, as demonstrated by the selective analysis of low fmol level citrullinated histone H3 from a peptide mixture. However, due to the lack of a cleavable linker, bound citrullinated proteins cannot be analyzed by MS.[136] Recently, the first large-scale citrullinome was reported in rheumatoid arthritis using a biotin-tag and avidin enrichment method. From the analyses of total and depleted synovial fluid after enrichment, the number of citrullinated peptides was estimated to be approximately 3,600 and 2,100, respectively, according to the signature ions generated by the biotin tag. However, identification of these biotinylated peptides by MS/MS was very difficult due to biotin moiety fragmentation, as only 13 citrullinated peptides derived from a total of 17 proteins could be identified.[137]

Notably, because the above-mentioned enrichment procedures are all based on the specific reaction of a glyoxal moiety with an ureido group, other ureido containing peptides, e.g., protein carbamylation generated during sample preparation in the presence of urea, could also be simultaneously captured. These types of PTMs should be verified when searching MS/MS data with respective variable modifications. Additionally, as citrullination blocks the tryptic cleavage site at arginine residues, the average peptide length may be significantly increased when using traditional trypsin, or even LysC digestion. Therefore, the combined use of different proteolytic enzymes would benefit the MS identification of citrullinated peptides.

2.3.4 Ribosylation. Poly(ADP-ribose) (PAR) is a reversible PTM found in higher eukaryotes. PAR is composed of linear and/or branched repeats of ADP-ribose, up to a length of 200 units. Although the role for this modification in the identification and repair of DNA nicks and double-stranded breaks has been considered for a long time, this modification has received increasing attention due to a number of recent studies reporting that the role of poly(ADP-ribose) goes beyond DNA repair in the cell. Presently, PARylation sites have been determined for only a few proteins, due to challenges relating to the modification itself, which may be labile, large, and highly charged, but also primarily due to the lack of enrichment methods on a global scale. In a recent report, boronic chemistry was used to isolate ADP-ribosylated peptides for large-scale proteomic studies of protein ribosylation. The enrichment is attributed to the formation of ester bonds between boron and a 1,2-cis-diol moiety within ADP-ribose. The captured ADP-ribosylated peptides were then eluted by NH_2OH treatment because the ester bond between the first ADP-ribose unit of PAR and the side chain carboxyl group of an aspartic acid and glutamic acid residue is susceptible to NH_2OH attack (Figure 8). This method strategically used NH_2OH to detach the modification group while retaining a fixed mass tag of 15.0109 Da due to the generation of a hydroxamic acid derivative at the modification site. Therefore, the use of NH_2OH could further facilitate confirmation of the site of modification. Compared with the method of detaching the entire ADP-ribosylated peptide from the solid phase under acid conditions by the breaking of the ester bonds between boron and the 1,2-cis-diol moiety, the introduction of a characteristic mass signature can be used as a fixed modification for a database search. In contrast, the entire ADP-ribosylated peptide presents difficulties for traditional spectrum interpretations and database searches because PARylation is a heterogeneous modification. Compared with the conversion of an ADP-

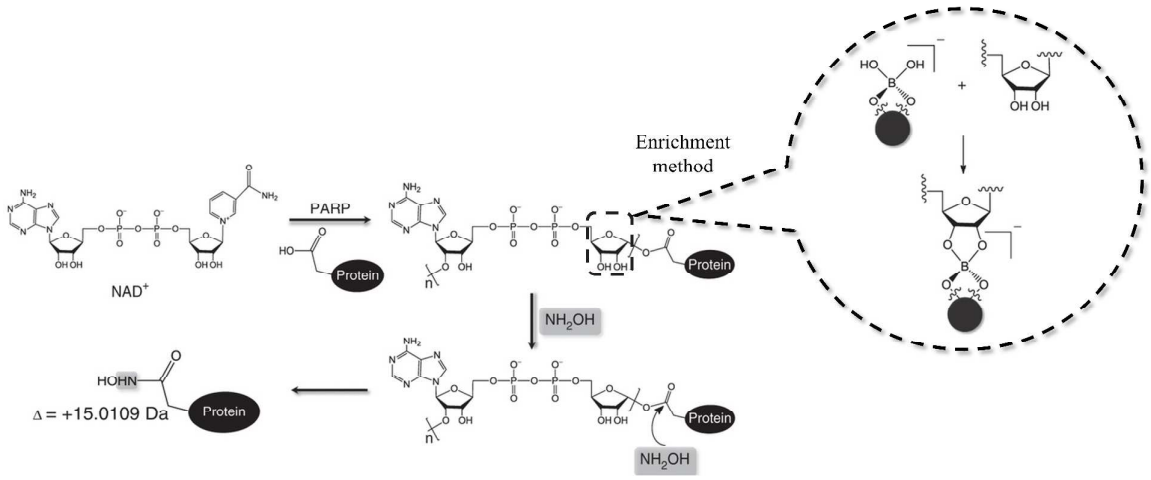

Figure 8. General scheme for the isolation and site determination of protein Asp- and Glu-ADP-ribosylation. {Reprinted by permission from Macmillan Publishers Ltd: [NATURE METHODS] (Y. Zhang, J. Wang, M. Ding and Y. Yu, 2013, Nat Methods, 10, 981-984), copyright (2013)}

ribosylated aspartic acid or glutamic acid back to its unmodified form, which results in the loss of site information, the introduced mass tag can be readily distinguished by MS. Additionally, fragmentation of NH_2OH-derivatized peptides yielded typical b- and y-ion series, allowing easy localization of the ADP-ribosylation sites. In total, 1,048 unique, unambiguously localized Asp- and Glu-ADP-ribosylation sites on 340 proteins were identified from H_2O_2-stimulated, PARG-knockdown human HCT116 cells, which provided the largest dataset of Glu-ADP-ribosylation in human cells at that time. Notably, this approach cannot identify nonacidic ADP-ribosylated residues because only acid residues are susceptible to NH_2OH attack, and thus, modifications at lysine and arginine sites would not be recognized. Additionally, this approach cannot be used to characterize hydroxylamine-insensitive ADP-ribosylated amino acid residues, which have been shown to account for up to 33% of the total ADP-ribosylated sites.[138]

To enable a global ribosylation proteome analyses, a novel strategy that allows for the identification of mono(ADP-ribosyl)ation and poly(ADP-ribosyl)ation sites via the enzymatic product of a phosphodiesterase-treated sample has been reported. This strategy involves an enrichment protocol based on the digestion of ADPr by snake venom phosphodiesterase (SVP), a pyrophosphatase that cleaves ADPr subunits down to phospho(ribose) and 5'-AMP, and then the enrichment of the phosphor -group containing peptides. The digestion by SVP produces a single phospho(ribose) group at the site of ribosylation that can be enriched by IMAC or MOAC, as was used for phosphopeptides enrichment, and modification site identification by MS can be obtained because of a fixed mass increase of 212.01 Da. Therefore, this enrichment technique allows researchers to characterize global ADP-ribosylation not only on acidic sites but also on nonacidic sites. This strategy also can be used to study the changes in the ADP-ribosylated proteome alongside the co-enriched phosphoproteome.[139]

3. Enrichment of protein PTM sites

3.1 Cysteine modifications

Cysteine is one of the least abundant amino acids found in proteins in prokaryotes and eukaryotes (1–2%).[140] However, cysteine plays a significant role in protein scaffolds as sites of nucleophilic and redox catalysis, metal binding and allosteric regulation.[141] For most cysteines in proteins, thiol pKa values lie in the range of 8 to 9, which is close to physiological pH. As a result, the ionization state of cysteine is highly sensitive to minute changes in the surrounding environment, causing cysteine to be the most reactive amino acid in proteins.[142] Due to the reactivity of the thiol group, a multitude of electrophilic and oxidative PTMs can be obtained. PTMs on cysteines can be spontaneous or enzyme-catalyzed. Spontaneous cysteine PTMs, such as reactive oxygen/nitrogen species (ROS/RNS) and lipid-derived electrophiles (LDEs), are usually driven by endogenous oxidants and reactive electrophiles. Enzyme-catalyzed modifications of cysteines include many acylations, e.g., prenylation and palmitoylation. Some modifications, such as disulfide formation, can be both spontaneous and enzyme-catalyzed. Chemical-proteomic approaches to enrich, identify and quantify cysteine-containing peptides have been widely used in proteomics research.[143] In the meantime, techniques for study cysteine PTMs have also been developed over several decades, ranging from radioisotope labeling to chemical probe design combined with MS analyses.[144] However, methods for evaluating all types of cysteine PTMs have not yet been established; therefore, our focus will be on methods directed toward frequently occurring cysteine PTMs, such as cysteine oxidation (S-sulfenylation), S-glutathionylation, S-nitrosylation, S-palmitoylation and S-prenylation (including S-farnesylation and S-geranylgeranylation) (Figure 9).

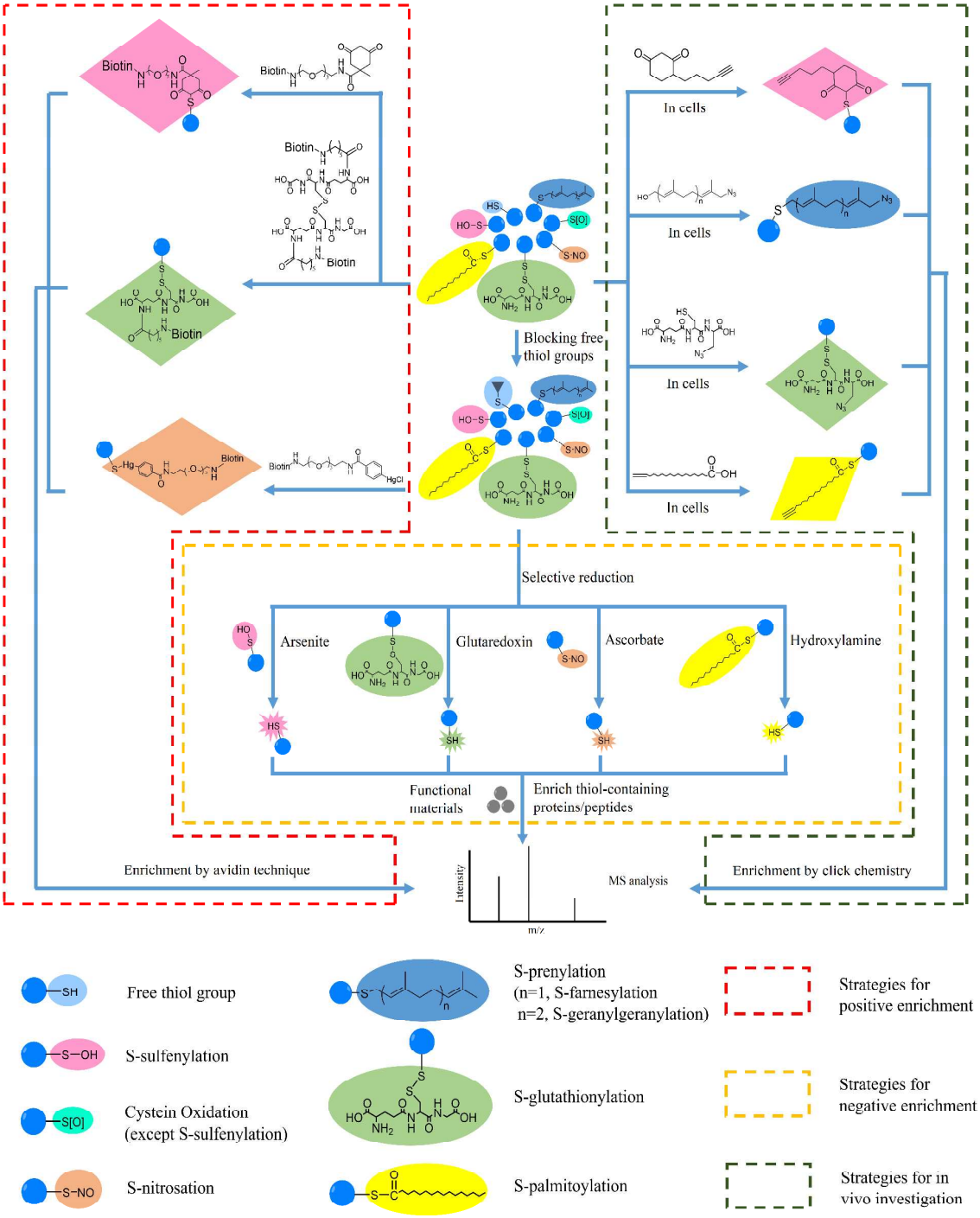

Figure 9. Chemical methods for enriching different cysteine PTMs.

3.1.1 Cysteine oxidation. Cysteine oxidation includes the formation of sulfenic/sulfinic/sulfonic acids and intra- and inter-chain disulfides.[145] Several methods have been developed to monitor global changes in cysteine oxidation, but do not reveal the chemical nature of certain modifications. An acid-cleavable isotope-coded affinity tag (ICAT) reagent was used in the identification and quantification of oxidant-sensitive protein thiol groups. This approach is based on the fact that only free cysteine thiol groups are capable of being labeled with the iodoacetamide-based ICAT reagent and that MS can be used to quantitate the relative labeling of free thiols.[146] Alternative approaches have introduced reducing

reagents into the workflow. Such protocols usually include alkylation of free thiols, reduction of modified thiols with DTT or tris(2-carboxyethyl)phosphine (TCEP) and labeling of new thiols with a tagged alkylating agent, such as biotinylated iodoacetamide (BIAM)[147] and N-(6-(biotinamido)hexyl)-3'- (2'-pyridyldithio)propionamide (biotin-HPDP),[148] which can promote the enrichment of labeled proteins/peptides.

In addition to enrichment and quantification of global changes related to cysteine oxidation, strategies to investigate certain types of cysteine oxidations, e.g., S-sulfenylation, have also been established. S-sulfenylation (SOH) is one type of cysteine oxoform, generated by the reaction of a cysteine thiol group with biological oxidants, such as hydrogen peroxide, peroxynitrite, hypochlorous acid and hydroxyl radicals. Hydrolysis of S-nitrosothiols also results in sulfenic acids.[145] Further oxidation of sulfenic acids results in sulfinic and sulfonic acids. As sulfenic acids are transient and highly reversible, methods to selectively identify sulfenic acids within a complex mixture are required to better understand the targets, abundance and biological functions of these modifications.[149] Based on the fact that sulfenic acids can be reduced to thiol groups by arsenites, an arsenite-based strategy was established to selectively enrich sulfenylated proteins. Free thiol groups were first blocked by pretreatment with maleimide. Then, sulfenated cysteine was reduced to normal cysteine using sodium arsenite in the presence of biotin–maleimide. Labeled proteins were purified using avidin technology.[150] In addition to this arsenite-based strategy, the dimedone-based approach is another method that can be used to study protein S-sulfenylation, which is more widely used in the proteome-wide analyses of S-sulfenylation. This strategy relies on the nucleophilic addition of an enolate intermediate, which is generated from the 1,3-dicarbonyl moiety, onto sulfenic acid to form a stable covalent adduct.[151] This method was first used for in fluorescence detection of sulfenated proteins.[152] Subsequently, conjugation of dimedone or its derivatives with biotin allowed avidin enrichment of target proteins.[153] However, biotin-conjugated dimedone analogs cannot be used to study protein sulfenylation in vivo. To achieve this goal, azide-[154] or alkyne-[155] tagged probes, which were expected to be cell permeable and minimally disruptions the local redox environment, were designed using the Staudinger ligation or click chemistry to investigate native protein S-sulfenylation. Dimedone-based strategies have also been used in the quantification of sulfenated proteins, either with isotope-coded dimedone derivatives[156] or with a metal-containing dimedone reagents.[157] In addition to dimedone and its derivatives, other dicarbonyl compounds (e.g., 1,3-cyclopentanedione[158] and β-ketoester[159]) have been used as chemical probes to investigate protein sulfenylation. More recently, new types of chemical probes, highly strained bicyclo[6.1.0]nonyne (BCN) derivatives, were developed to trap sulfenic acids. These strained cycloalkynes react efficiently with sulfenic acids in proteins and also provide bioorthogonal reactivity with sulfenic acid by not reacting with the thiol group, disulfide, sulfinic acid or S-nitroso oxoforms of cysteine.[160]

3.1.2 S-glutathionylation. S-glutathionylation is another important PTM that occurs on protein cysteine residues. This event is a redox-dependent post-translational modification with growing relevance in signal transduction.[161] Glutathione has three forms in an organism, reduced (GSH), oxidized (GSSG) and modified (S-glutathionylation). The formation of S-glutathionylated proteins is dependent on the GSH/GSSG ratio and involves a thiol/disulfide exchange between a protein thiol and GSSG, also resulting from the reaction of S-nitrosated cysteines or sulfenic acids with glutathione.[162] Early methods for studying protein S-glutathionylation used radiolabeled GSH.[163] However, this method requires specialized training for use of radioactivity and the need to inhibit global protein synthesis. Furthermore, other forms of S-thiolation, such as S-cysteinylation, will result in false positives, and enrichment processes for isolating modified proteins remains lacking.[144] To overcome the limitations associated with the use of radiolabeled glutathione, biotinylated glutathione analogs have been developed. For example, N,N-biotinyl glutathione disulfide (Biotin-GSSG)[164,165] and biotin-labeled glutathione ester (Bio-GEE)[166] were designed to detect, purify and visualize proteins that underwent S-glutathiolation. To study protein S-glutathionylation in vivo, a mutant of glutathione synthetase was used to catalyze azido-Ala in place of Gly with high catalytic efficiency and selectivity. Azido-tagged glutathione allowed the modified proteins to be selectively and sensitively detected by Western blotting or fluorescence after click reaction with biotin-alkyne or rhodamine-alkyne.[167] Glutaredoxin (GRX)-mediated reduction is another method that can be applied in proteome-wide studies of S-glutathionylation. In the GRX-mediated reduction method, proteome samples are first treated with cysteine-reactive reagents, such as N-ethylmaleimide (NEM), to cap reduced cysteines. Then, GRX is used to selectively reduce glutathionylated cysteines to produce new thiol groups. The new thiol groups are subsequently labeled with a thiol-reactive reagent that contains an enrichment tag, enabling selective enrichment of glutathionylated proteins and peptides.[168]

3.1.3 S-nitrosation. Protein S-nitrosation is defined as the conversion of cysteine thiols to S-nitrosothiols (SNO).[169] SNO synthesis in cells can result from three pathways: 1) reaction of cysteine thiols with N_2O_3 resulting from autooxidation of nitric oxide (NO); 2) recombination of NO with thiyl radicals and 3) transition metal-catalyzed addition of NO to a thiol.[170] Direct enrichment and identification of S-nitrosation is challenged by the low abundance and poor stability of endogenous SNO. Early methods for studying S-nitrosation were based on the detection of liberated NO after UV-visible, chemiluminescence detection and photolysis, which could homolytically cleave S-N bonds. These methods can quantify total nitrosothiol content in a sample, but do not allow for direct visualization or identification of individual S-nitrosated proteins.[171] For proteome-wide analyses of protein S-nitrosation, the biotin-switch technique (BST) is the most widely utilized method. This method includes three steps: 1) blocking of free thiols by incubation with methanethiosulfonate (MMTS); 2) decomposing nitrosothiol bonds with ascorbate to generate

new thiol groups and 3) reacting the newly formed thiol groups with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP). After purification using streptavidin–agarose, target proteins are digested with trypsin prior to MS analyses.[172] Optimization of this method introduced a proteolytic digestion step before the avidin capture step. This added step provides for the selective isolation of peptides that previously contained SNO Cysteine residues, rather than intact SNO proteins.[173]

In addition to biotin-tagged reagents, His-tagged reagents have also been designed and used to conduct enrichment of target S-nitrosated peptides.[174] To simplify the enrichment process, resin-assisted capture (SNO-RAC) was applied in the study of S-nitrosation. SNO-RAC uses a thiol-reactive resin instead of thiol-reactive biotin, thus combining the obligatory 'labeling' and 'pulldown' steps in the BST. On-resin trypsin digestion and peptide labeling can be performed because SNO-RAC results in a covalent disulfide linkage between the SNO site and the resin, allowing it to be easily combined with exiting strategies in proteomics studies.[175] For example, this method can be easily combined with multiplex isobaric labeling techniques, such as iTRAQ and TMT, for the quantification of S-nitrosated proteins. Recently, a sequential iodoTMT-switch procedure, which was also derived from BST, was established to conduct the identification and quantification of S-nitrosated proteins.[176,177] Another strategy for isolating and enriching S-nitrosated proteins/peptides is based on organomercury resins (MRC). Reactions of phenylmercury compounds with S-nitrosocysteine result in the formation of relatively stable thiol–mercury bonds. The MRC-based strategy consists of three steps: 1) blocking of reduced cysteine residues with methyl methanethiosulfonate (MMTS); 2) capturing and releasing of S-nitrosylated proteins or peptides and 3) LC-MS/MS analyses.[178] To increase the confidence in the identified targets and mitigate limitations, combining orthogonal methods, such as BST and MRC, to identify S-nitrosation have been proposed.[179]

3.1.4 S-palmitoylation. S-palmitoylation is the incorporation of palmitic acid onto cysteine residues of proteins.[180] This PTM is enzymatically mediated by protein S-acyltransferases (PATs), which contain a cysteine-rich domain and a conserved Asp-His-His-Cys (DHHC) signature motif. This process is a reversible lipid PTM that occurs on proteins in diverse cell types. The covalent attachment of the 16-carbon palmitic acid to specific cysteine side chains regulates protein trafficking to membrane compartments in numerous biological processes.[144] Similar to S-glutathionylation, early methods to study S-palmitoylation relied on metabolic labeling with radiolabeled lipid species.[181] To circumvent the limitations of using radiolabeled palmitic acid to monitor S-palmitoylation, two complementary chemical approaches have been established, greatly facilitating the investigation of S-palmitoylation. One of these two approaches is the acyl-biotin exchange (ABE) method. This method relies on the sensitivity of the fatty acylthioester linkage to cleavage by hydroxylamine to generate a free cysteine thiol and includes three steps: 1) capping of all free cysteine thiols with NEM; 2) selective cleavage of palmitoyl groups using hydroxylamine to reveal free thiols and 3) conjugation of biotin to these newly exposed thiols using biotin-BMCC or biotin-HPDH. Biotinylated proteins can be visualized by immunochemistry or enriched and then identified by MS analysss.[182] A modified version of the ABE method is S-acylation by resin-assisted capture (acyl-RAC). Free thiols generated after hydroxylamine treatment are captured on a thiol-reactive Sepharose resin.[183] However, as in the use of glutathione mutants in the study of S-glutathionylation, several azide- and alkyne-functionalized fatty acids have been utilized as tools to tag endogenously palmitoylated proteins.[184-186] When compared with azide tags, alkyne-functionalized lipid derivatives are more efficient for metabolic labeling.[187]

3.1.5 S-prenylation. S-prenylation is the attachment of a farnesyl (C15) or geranylgeranyl (C20) isoprenoid to one or more cysteine residues located near the C-terminus.[188] These modifications are enzymatically installed by farnesyl transferase (FTase) or geranylgeranyltransferase (GGTase-I/II). As a kind of PTMs modulating protein cellular localization, signaling and degradation, the recognition motif for this modification is a the sequence known as CAAX-box, where C is a cysteine, A stands for aliphatic amino acid, and X can be any amino acid and usually determines the nature of the anchored isoprenoid.[189] Similar to S-glutathionylation and S-palmitoylation study, identification of endogenously prenylated substrates begins with the application of radiolabeled isoprene derivatives, while these methods need to handle radiolabeled material and are incapable of identifying unknown prenylated proteins from a complex mixture. To avoid the use of radiolabels, fluorescent[190] and biotin-tagged isoprene derivatives were introduced.[191] These methods provide powerful tools to study prenylation, especially biotin-tagged switch, which allows the enrichment of targeted proteins from a complex sample for MS analysis. Metabolic labeling switches were also used for proteome-wide analysis of both S-geranylgeranylation[192] and S-farnesylation.[193] Compared with azido-isoprenes, alkyne-isoprenes can reduce the influence of background labeling,[194] drawing more attention in S- prenylation research.[195] These technologies provided a powerful method to monitor S-prenylation both in vitro and in living cells.

3.2 N-terminus or C-terminus modifications

N/C-terminal processing are crucial post-translational modifications. N-terminal modifications affect protein activation, conversion, and degradation, while C-terminal modifications affect the activity, functionality and stability of proteins. Therefore, the study of N/C-terminal modifications is gaining increased interest. However, direct identification of N/C-terminal peptides by mass spectrometric analysis without pre-separation is often difficult, because N/C-terminal peptides are typically overshadowed by enzyme-generated peptides. Enrichment approaches can be generally categorized into positive and negative enrichment. Positive enrichment is directly enriching the N/C-terminal peptides while negative enrichment is depleting the non-N/C-terminal peptides.

3.2.1 N-terminus modifications. Positive enrichment of N-terminal peptides typically blocked the amino groups on the

protein side chain and then introduced an enrichment tag at the N-terminal peptides through chemical or enzymatic before digestion. Chemical labeling are frequently used. Chemical biotinylation approach termed "N-terminalomics by Chemical Labeling of the Alpha Amine of Proteins' (N-CLAPS)" was developed. In this approach, all amines in a protein were blocked using phenyl isothiocyanate (PITC), then trifluoroacetic acid (TFA) was added to selectively deblock the N terminus but not PITC-modified lysines. The newly generated amino groups at the N-termini were then reacted with an amine-specific labeling reagent EZ-Link Sulfo-NHS-SS-biotin. After protein digestion, the N-terminal peptides were recovered using avidin-based resins, and eluted with a reducing agent for following MS. In N-CLAP, simple and readily available reagents were used, thus this method was easily employed by other labs.[196] Similarly, thiol groups or phosphate group were introduced on N-terminal peptides followed by thiol-affinity resin or TiO_2 enrichment, respectively.[197,198] To achieve an easier separation, high molecular weight hyperbranched polyglycerol (HPG) functionalized with ester-linked aldehyde groups was used to capture the N-terminal peptides through binding primary-amine-containing peptides in a reductive alkylation reaction. Once bound, the high molecular weight of the polymer facilitates separation of the captured N-terminal peptides from a complex peptide mixture. Either a 30 kDa molecular weight cutoff membrane or precipitation in acetonitrile can be used. The ester linker between the N-terminal peptides and HPG was hydrolyzed to release the peptide into solution for MS analysis. The newly synthesized polymers were easy to separate through ultrafiltration, hadlower nonspecific peptide-binding properties, and possessed more reactive groups than commercially available resins.[199]

Alternatively, enrichment tags can be introduced on the N-terminal peptides through enzyme catalysis. Different from chemical introducing tags, free amino groups do not need to be blocked before labeling. Mahrus et al. used a modified subtiligase enzyme to conjugate a biotinylated peptide to the a-amino group of protein N-termini.[200] Following trypsination, biotinylated proteins were captured by immobilized avidin, then the N-terminal peptides for MS analysis were released from the biotinylated peptides by thetobacco etch virus (TEV) protease (biotinylated peptides contain TEV cleavage motifs). Similarly, Pan et al used alternative enzyme for introducing an enrichment tag on N-terminal peptides. A co-substrate with a stable isotope-coded Arg residue containing a biotin tag was synthesized and incubated with tryptic peptides and trypsin in ethanol solution. The stable isotope-coded affinity tag was specifically coupled onto the N-termini of peptides via the formation of new peptide bonds. Then the labeled peptides were specifically enriched by avidin affinity chromatography for MS analysis. A unique advantage of this method is that coupling a short sequence tag onto peptides can influence the fragmentation, thereby improving the coverage for proteome analysis. The enzymatically- introduced enrichment tag can avoid blocking primary amine groups on lysine side chains, but is limited by the ligation efficiency as well as the biased enzymatic labeling specificity.

Positive enrichment typically leaves a tag on the N-terminal peptides; the characteristically mass increase allows for convenient identification of N-terminal peptides. But the limitation of positive enrichment is that these enrichment approaches are not well suited to study naturally modified N-termini because the enrichment tag relies on free, unmodified a-amines of protein N-termini. In contrast, negative enrichment strategies to be discussed below can accommodate for both free and natively modified protein N-termini.

McDonald first reported a negative enrichment approach. At first, all amino groups were blocked by acetylation. Subsequently, proteolysis generated new peptides that were biotinylated. These biotinylated internal peptides were removed by recovery onto immobilized avidin or strepatividin. In contrast to the previously discussed negative enrichment, in this method, protein naturally acetylated N-termini were automatically included in the analyte set. Although this protocol was effective, it required multiple peptide-purification steps to separate the peptides from the excessive reagents.[201] The author further improved the method thereafter. Instead of capturing trypsin-generated peptides by biotinylation, a commercially available amine reactive immobilized reagent (NHS-activated sepharose) was used to react and retain internal peptides in one step. The sepharose beads with captured peptides were then removed by brief centrifugation, thereby leaving the N-terminal peptides for further analysis.[202]

Kleifeld et al. reported a beautiful terminal amine isotopic labeling of substrates (TAILS) approach using a new class of highly water-soluble polymers to selectively enrich N-terminal peptides by negative enrichment (Figure 10). Briefly, a series of aldehyde-functionalized hyperbranched polyglycerols (HPG-ALD) with molecular weight in the range of 100–600 kDa and narrow polydispersivity were synthesized. The HPG-ALD polymer readily reacted with all unblocked internal and C-terminal tryptic peptides through their free N-termini in sodium cyanoborohydride, and the captured peptides were removed along with the polymers through centrifugation. Authors showed that the enrichment capacity reached 2.5 mg peptide/mg polymer, which was more than ten-fold improvement in capacity over amine-reactive resins, allowing highly sensitive analysis of the N-terminomics. They identified 731 acetylated and 132 cyclized N-termini, and 288 matrix metalloproteinase (MMP)-2 cleavage sites in mouse fibroblast secretomes.[203,204]

3.2.2 C-terminus modifications. Compared to the analytical methods for N-terminus, there have been few strategies proposed for investigating the C-terminomics in the last couple of years. Development of enrichment methods is mainly limited by the completeness of the derivatization of carboxyl groups on both C-terminus and aspartic and glutamic acids, because these carboxyl groups exhibit similar reactivity. Additionally, the blocking efficiency of free carboxyl groups is frequently incomplete due to the low reactivity of side chain

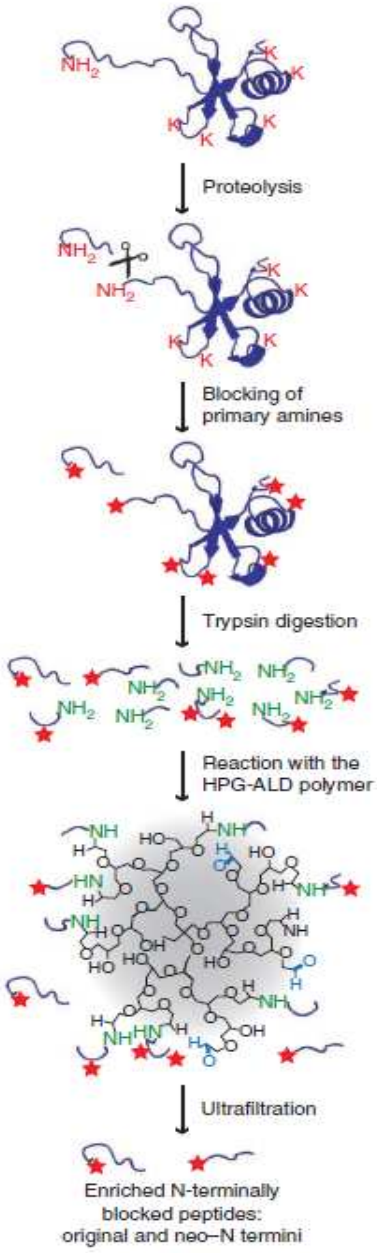

Figure 10. Schematic representation of the TAILS workflow. [Reprinted by permission from Macmillan Publishers Ltd: [Nature Protocols] (O1. Kleifeld, A. Doucet, A. Prudova, U. auf dem Keller, M. Gioia, J. N. Kizhakkedathu, C. M. Overall. *Nature Protocols*, 2011, **6**, 1578–1611.), copyright (2011)]

carboxyl groups.[205] Until rather recently, a range of C-terminomics technologies have been introduced. Similar to N-terminomics enrichment, both positive and negative enrichment are allowed in C-terminomics. Selective purification of C-terminomics is predominant through negative enrichment. The Overall's group, who developed a TAILS approach for enriching N-terminomics as discussed before, also reported a C-terminal amino-based isotope labeling of substrates (C-TAILS) as a negative selection strategy for C-terminomics enrichment. In this method, chemistry of the carboxyl group (i.e., carbodiimide chemistry) is used to blocking all carboxyl groups with ethanolamine at the protein level, and then the newly generated tryptic peptides were removed by covalent coupling to a high-molecular-weight (~56 kDa) linear polyallylamine polymer formed by EDC-mediated condensation of the free carboxyl groups to the primary amines of the polymer.[206,207] The advantage of this method is that both EDC-based carboxyl blocking and depleting proceed under mild conditions, and the polymer can be easily separated by ultrafiltration. Alternatively, oxazolone chemistry-based enrichment methods were developed because an oxazolone only targets at the C-terminus of a peptide chain, discriminating against all side-chain carboxyl groups.[208] Oxazolone-based chemistry is nowadays regarded as one of the few effective approaches to discriminate between the carboxyl groups at the C-terminus and the side chains. For example, highly selective derivatization of C-terminal COOH was achieved using 3-aminopropyltris-(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-propylamine) through oxazolone chemistry and amidation on protein level. GluC was then used to digest the protein and the generated new peptides were then treated with a COOH scavenger to deplete peptides containing free carboxyl groups.[209] However, the elaborate protocols for chemical manipulations, which are often unfriendly for following MS analysis, still need to be improved. On the other hand, incomplete derivatization of carboxyl groups might limit identification of C-terminal peptides.

Nika et al. recently proposed a solid phase enrichment strategy to isolate C-terminal peptides. Briefly, the protein was glycinamidated to block both the side chain and C-terminal carboxyl groups. The one-pot reaction mixture was subsequently digested by tryptic digestion. The digest was then adsorbed onto ZipTipC18 pipette tips for sequential solid-phase peptide amine acetylation to block the newly generated amino-groups and EDC-mediated peptide carboxylate condensation with ethylenediamine (EDA) to introduce a new amino-group on C-termini of these tryptic peptides. The products were transferred to NHS activated agarose for subsequent depletion of the amino group-functionalized N-terminal and internal peptides, leaving the carbamidated C-terminal peptide in the flow-through fraction for MS analysis. The advantage of this method is that reagents are exchanged directly on the support, eliminating sample transfer between the reaction steps. However, multiple chemical reactions and buffer change processes were involved which may lead to problems such as incomplete derivatization or sample loss.[210] Different from these above mentioned methods, Kuyama et al. described a method for negative enriching C-terminal peptide without derivatization of C-terminal carboxyl groups. This method employed endopeptidase Arg C digestion to generate arginine-terminated peptides, and then an m-aminophenylboronic acid–agarose was used to deplete these arginine-terminated peptides through the form phenylboronic acid diester between the boronic acid and the cis-

dihydroxyimidazoline. It was formed by adding 2,3-butanedione to the guanidino group of the arginine residue. The selectively recovered C-terminal peptide without derivatization can cause less artificial identifications.[211]

In contrast to all previous methods that are based on negative selection, positive enrichment methods are much less reported given that low chemical reactivity remains the major difficulty for labeling of carboxyl groups. Xu et al. reported a positive enrichment method termed "Profiling Protein C-Termini by Enzymatic Labeling (ProC-TEL)". ProC-TEL uses carboxypeptidase Y and other readily available reagents to selectively add abiotinylated derivative of lysine (biocytinamide) as the affinity tag to protein C-termini, which can capture C-terminal peptides from complex cell lysates. Compared with negative enrichment, less sample preparation steps were involved in this method. However, the shortcoming of this approach is that the efficiency of carboxypeptidase Y is highly dependent on the nature of the C-terminal amino acids, possibly introducing biases in the analysis of complex samples.[212] In principle, the oxazolone chemistry can also be used in the positive enrichment of C-terminal peptides, because oxazolone only targets at the C-terminal carboxyl groups and therefore can introduce an enrichment tag at the C-termini. Based on this principle, we are now developing a positive enrichment method for C-terminomics.

Conclusions and Perspective

Design and synthesis of new nanomaterials with functional groups for studying the PTM proteome has gained increasing attention in recent years. Advances in chemical-base enrichment methods using these nanomaterials have greatly facilitated the identification of thousands of PTM sites in cells, tissues and body fluids. Although many functional materials have been designed and synthesized, most of them focused on the two major forms of protein PTM, namely phosphorylation and glycosylation. Most other PTMs are difficult to address and remain poorly characterized due to the lack of such enrichment methods. Development of new functional materials that are PTM-specific would shed new light on the in-depth profiling of the PTM proteome. Difficulties associated with revealing novel modifications continue to drive the development of new approaches to enrich and detect these protein PTMs. For profiling the other poorly understood modifications, specific chemical reactions between the modification and the functional groups on nanomaterials must be explored, thereby promoting the development of tailor-made functional materials that can be used for the specific enrichment of these PTMs. Additionally, specific enrichment approaches developed for certain types of modifications may be generally applicable to other new types of PTMs with some modifications, thereby establishing new methods for the discovery of PTMs that do not have available chemical enrichment methods. For example, very recently Wu et al. described a new chemical proteomics approach for global analysis of lysine mono-methylation through combination of chemical derivatization and pan antibody enrichment.[213] Furthermore, to realize successful PTM proteome analyses, a key element is the specificity between the PTM groups and the functional groups, or the specificity between a tag and a functional group. Biotinylated tags offer a unique opportunity to specifically enrich PTM peptides/proteins from whole proteomes by tagging the PTM site with a specific biotinylated tag. Then, the PTM proteome can be subsequently captured through the biotin-avidin interaction to reduce sample complexity and hence increase identification rates by MS. However, non-specific binding of proteins/peptides to avidin beads via hydrophobic and/or electrostatic interactions can complicate analyses. Therefore, avidin immobilized on low non-specific adsorption nanoparticles would help enhance enrichment specificity. Additionally, the efficiency of detaching captured PTM peptides is an important factor that affects recovery. Development of cleavable-tags on functional nanoparticles would contribute to the high recovery enrichment of the PTM proteome. This technique is already being used in some modification type analyses.

While identification of an individual common PTM proteome (e.g., phosphorylation) in a complex sample is now fairly routine, simultaneous characterization of cross-talk PTMs is more difficult, as cross-talk between different types of PTMs adds additional difficulties to sample preparation.[214] Recently, sequential enrichment methods have demonstrated their utility in profiling multiple PTMs, which involve multiple enrichment strategies in a single experiment, allowing some types of PTMs to be characterized on a large-scale basis.[215] For example, by combining two methods, 466 proteins with 2,100 phosphorylation sites co-occurring with 2,189 ubiquitylation sites were identified in *Saccharomyces cerevisiae.* In the first enrichment approach, cobalt-NTA affinity purification was used to enrich for proteins modified with His-tagged ubiquitin, and after tryptic digestion, phosphopeptides in both the ubiquitin-enriched population and the flow-through were enriched.[216] Similarly, the phosphoproteome, ubiquitinome and acetylome were enriched by serial enrichments of different post-translational modifications (SEPTM) from the same biological sample, enabling quantitative analysis of > 20,000 phosphorylation, 15,000 ubiquitination and 3,000 acetylation sites per experiment.[217] Complementary use of multiple different enrichment approaches will likely be required to fully decipher the PTM cross-talk code, because a single enrichment technically cannot achieve the desired depth of proteome coverage.

Overall, our goal with this multi-discipline review is to stimulate nanomaterial scientist interest in designing and synthesizing new materials, chemist interest in exploring new chemical reactions specific for PTMs and establishing new analytical methods, biologist interest in using these novel methods to more deeply understand the human proteome and to afford methods for clinicians to discover additional PTM protein biomarkers. We also hope this review will motivate collaborations among nanomaterial scientists, chemists, biologists and clinicians.

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