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Review

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Development of the Affinity Materials for Phosphorylated Proteins/Peptides Enrichment in Phosphoproteomics Analysis

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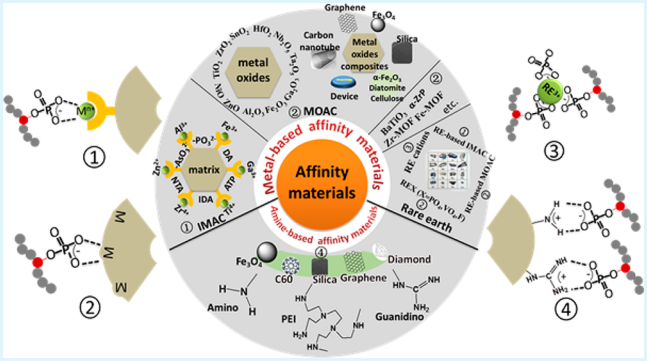
ABSTRACT: Reversible protein phosphorylation is a key event in numerous biological processes. Mass spectrometry (MS) is the most powerful analysis tool in modern phosphoproteomics. However, the direct MS analysis of phosphorylated proteins/peptides is still a big challenge because of the low abundance and insufficient ionization of phosphorylated proteins/peptides as well as the suppression effects of nontargets. Enrichment of phosphorylated proteins/peptides by affinity materials from complex biosamples is the most widely used strategy to enhance the MS detection. The demand of efficiently enriching phosphorylated proteins/peptides has spawned diverse affinity materials based on different enrichment principles (e.g., electronic attraction, chelating). In this review, we summarize the recent development of various affinity materials for phosphorylated proteins/peptides enrichment. We will highlight the design and fabrication of these affinity materials, discuss the enrichment mechanisms involved in different affinity materials, and suggest the future challenges and research directions in this field.



KEYWORDS: *affinity materials, development, phosphoprotein, phosphopeptide, enrichment*

1. INTRODUCTION

In modern biological analysis, the enrichment of target biomolecules is often required because the direct detection of target biomolecules is usually challenging because of some common problems (e.g., low abundance of target biomolecules, existence of contaminants). Currently, affinity materials with unique merits such as low cost, good structural stability, diversity, and multifunction have been widely used for enriching target biomolecules from complex biosamples based on the specific affinity interactions between the affinity materials and the target biomolecules.[1,2] Thanks to the rapid development in material science, especially the emergence of nanotechnologies, varied efficient affinity materials have been developed for the enrichment of different biomolecules.[3−6] Remarkably, affinity materials for enrichment of phosphorylated proteins/peptides have attracted tremendous attention in phosphoproteomics research.

Phosphorylated proteins/peptides originated from the dynamic and reversible protein phosphorylation involve in many biological processes, such as the cellular signaling and communication,[7] transcriptional and translational regulation,[8] and cell division and apoptosis.[9] Phosphoproteomics[10] has been a hot research topic because it is vital for unraveling the biological processes and finding new strategies for the diagnoses and treatment of diseases. Today, mass spectrometry (MS) with high sensitivity and fast data handling has become the most powerful and essential tool for the phosphoproteomics research.[11] However, the direct MS-based phosphoproteomics analysis still faces great challenges due to the well-known obstacles including the low-abundance of phosphorylated proteins/peptides resulting from high heterogeneity and low stoichiometry of protein phosphorylation, and the signal suppression effect of high abundance component (e.g., salts, lipid, and nonphosphorylated molecules) as well as the low ionization efficiency of phosphorylated proteins/peptides in MS analysis.[12] Therefore, an enrichment treatment prior to MS has become an imperative and crucial step to effectively decrease the complexity of proteome samples and concentrate the phosphorylated proteins/peptides so as to enhance the MS analysis results.

On the basis of the existence of phosphate groups, the phosphorylated proteins/peptides can be enriched by special strategies such as immunoaffinity and chemical modification.[13] Unfortunately, these strategies suffer from unavoidable problems such as high-cost antibodies, limited general applicability, and degradation of biosamples. Affinity material-based enrichment strategies, by contrast, possess unique superiorities such as low-cost, universality, and negligible effect

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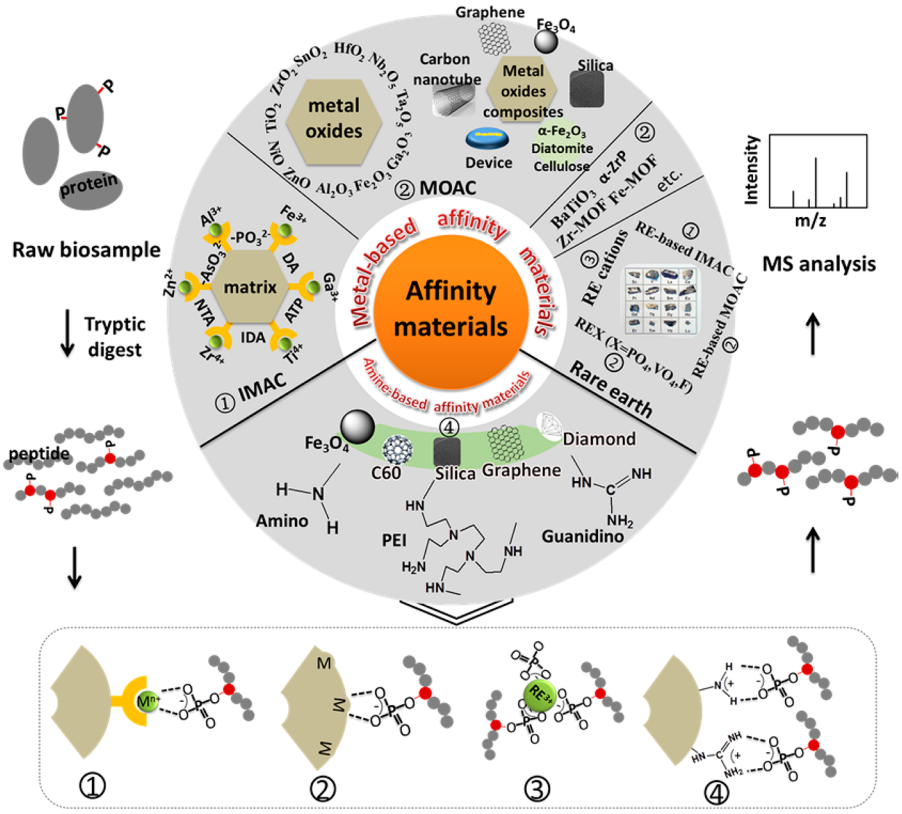

Figure 1. Schematic illustration of phosphopeptide enrichment for MS detection using diverse affinity materials. The numbers "①②③④" represent the main enrichment mechanisms for different affinity materials. The pie chart summarizes the affinity materials.

to the structural integrity of phosphorylated proteins/peptides. However, it should be pointed out that the affinity material-based strategies still face some problems such as the nonspecific binding of nonphosphorylated proteins/peptides which can seriously hamper the selective enrichment of phosphorylated proteins/peptides. Despite taking some auxiliary measures (e.g., chemical treatment of biosample,[14] pH regulation of loading buffers,[15] and addition of additives[16]), developing high-performance affinity materials is still the most significant and widely used way to improve the enrichment performance of affinity material-based strategies.

In this review, an overview on recent development of affinity materials for phosphorylated proteins/peptides enrichment is presented. The emphasis will be placed on the design and fabrication of these affinity materials as well as the introduction and discussion of relevant enrichment mechanisms. The challenges and future directions in this field will also be suggested. Details of some related issues such as the MS technology, enrichment conditions, and enrichment protocols are not introduced here because there have been a number of reviews on these topics already.[13,17,18]

2. AFFINITY MATERIALS FOR THE ENRICHMENT OF PHOSPHORYLATED PROTEINS/PEPTIDES

On the basis of the affinity site species, the reviewed affinity materials here are divided into two classes: metal-based and amine-based affinity materials (Figure 1). Meanwhile, the enrichment mechanisms involved in these affinity materials will be introduced and discussed. Specially, in the section of metal-based affinity materials, the newly emerging rare earth (RE)-based affinity materials will be presented as a separate subcategory. Moreover, affinity materials that showed enrichment preference for multiphosphopeptide will also be highlighted.

2.1. Metal-Based Affinity Materials. It is well-known that the metal cations are typical Lewis acids and that the phosphate groups with low acid−base ionization equilibrium constant (pK_a = 1−2) show typical Lewis base properties in a wide pH range. Thus, according to the Lewis acid−base theory, metal cations can interact with the phosphate groups in phosphorylated proteins/peptides through electrostatic interaction or/and chelation. Accordingly, a diverse range of metal-based affinity materials have been developed for the enrichment of phosphorylated proteins/peptide.

2.1.1. Metal Cation-Immobilized Affinity Materials for IMAC. Since Andersson et al. used Fe^{3+}-immobilized iminodiacetate-agarose gel to isolate phosphoproteins in 1986,[19] immobilized metal affinity chromatography (IMAC) has long been a widely used enrichment technique in phosphoproteomics. For IMAC, metal cations with unoccupied coordination orbitals are the prerequisite, and the main enrichment mechanism is the chelation between the immobilized metal cations and the phosphoryl oxygens in phosphorylated proteins/peptides (Figure 1①) besides electronic attraction. For fabricating IMAC affinity materials, the matrix supports carrying chelating ligands are usually first prepared and then the metal cations are immobilized onto the chelating ligands.

To date, a series of metal cations including Cu^{2+}, Zn^{2+}, Fe^{3+}, Ga^{3+}, Al^{3+}, Ti^{4+}, and Zr^{4+} have been used for IMAC.[20−24] Fe^{3+} and Ga^{3+} are the most widely used metal cations in traditional IMAC materials. But then, they have the weakness of nonspecific binding for nontarget biomolecules carrying acidic

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moieties. Nowadays, high-valence metal cation (e.g., Ti^{4+} and Zr^{4+}) immobilized IMAC materials are increasingly used. For example, the Zr^{4+}-IMAC and Ti^{4+}-IMAC with phosphate group as chelating ligands showed higher selectivity for phosphopeptides compared with traditional Fe^{3+}-IMAC beads because of the unique coordination specificity of metal(IV)-phosphate chemistry.[25,26]

Traditionally, iminodicitic acid (IDA) and nitrilotriacetic acid (NTA) are typically used as chelating ligands for immobilizing metal cations (e.g., Fe^{3+} and Ga^{3+}). However, each metal cation can coordinate with only one NTA or IDA ligand, which may result in loss of the immobilized metal cations during sample loading and washing. Thus, novel chelating ligands with higher chelating ability are desired. Accordingly, arsenate ($-AsO_3^{2-}$), phosphate ($-PO_3^{2-}$), adenosine triphosphate (ATP) and dopamine were successfully employed as novel highly efficient chelating ligands to immobilize metal cations, especially Ti^{4+} and Zr^{4+}.

(1). IMAC Affinity Materials with Arsenate Chelating Ligands. Arsenate-based IMAC materials, as new enrichment tools for phosphoproteomics, can be prepared by modifying arsenate groups (As) on matrix materials (e.g., silica[27] and magnetic silica nanoparticles[28]) (Figure 2a). For instance, ZrAs-Fe_3O_4@SiO_2 exhibited a higher enrichment selectivity and a better capture capability toward multiphosphopeptides than commercial ZrO_2.[28] Significantly, results of enrichment test using ZrAs-SNP indicated that the human serum phosphopeptides were differently expressed between healthy and adenocarcinoma individuals, implying that the ZrAs-SNP may have high application potential in biomarker discovery and clinical research.[27]

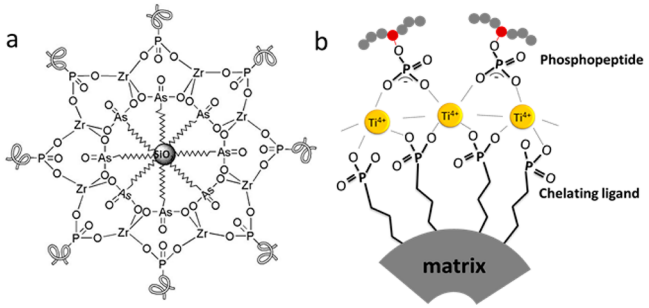

Figure 2. Scheme of phosphopeptide capture by (a) Zr^{4+}-arsenate IMAC affinity materials (Reproduced with permission from ref 27. Copyright 2011 Springer-Verlag) and (b) Ti^{4+}-phosphate IMAC affinity materials.

(2). IMAC Affinity Materials with Phosphate Chelating Ligands. Based on metal(IV) phosphate chemistry, the metal(IV) phosphate solids possess a unique layer structure and metal(IV) cations coordinate with phosphate groups by a MO_6 octahedral coordination model in which each immobilized metal(IV) cation strongly coordinates with more than one phosphate groups.[29] Thus, the phosphate-based IMAC materials can possess a very stable, well-defined metal-phosphate interface with low-coordinated surface active metal(IV) sites for capturing other phosphate groups (Figure 2b).[26,30,31]

In some early works, phosphate ligands for metal(IV) cations immobilization were derived on surfaces of matrix supports (e.g., porous silicon wafer[32] and Fe_3O_4@SiO_2 nanoparticles[33]) by the chemical reaction between $-NH_2$ and POCl. The obtained metal(IV)-phosphate IMAC affinity materials can enrich phosphopeptides from complex biosamples (e.g, Chang liver cells), but the single-layer phosphate ligands on these IMAC materials can only immobilize a limited amount of metal(IV) cations, which is undesirable for high enrichment capability. Hence, polymeric materials carrying abundant phosphate ligands were designed.[24,34,35] Briefly, polymeric matrixes were first obtained through polymerization of monomers carrying active groups (e.g., epoxy and amino), and phosphate ligands were then derived on the active groups via chemical reactions. The resulting materials with abundant phosphate ligands can be used as ideal matrix supports to immobilize lots of metal cations for enrichment applications. For example, Zou et al. fabricated the polymeric Fe_3O_4@SiO_2@PEG-Ti^{4+} affinity microspheres (Figure 3).[35] Thanks to the magnetic cores, the resistance of PEG chains to nonspecific adsorption,[36] and the high-abundance Ti^{4+}, the Fe_3O_4@SiO_2@PEG-Ti^{4+} exhibited outstanding enrichment performance such as rapid isolation, remarkable specificity (molar ratio of BSA/α-casein up to 2000:1), high recovery (over 70%), very low detection limit (0.5 fmol β-casein), and a much higher binding capacity (i.e., enriching 2447 unique phosphopeptides from

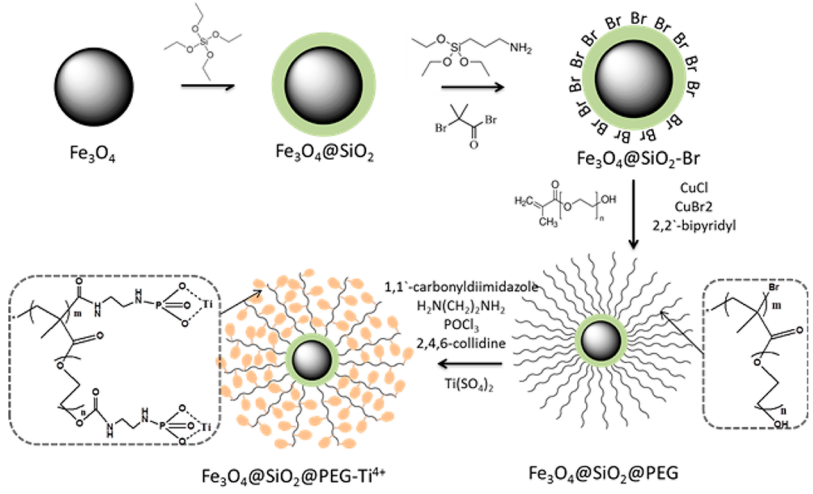

Figure 3. Scheme for the fabrication of Fe_3O_4@SiO_2@PEG-Ti^{4+} IMAC affinity microspheres for phosphopeptide enrichment.

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tryptic digests of *Arabidopsis*) than Fe_3O_4@SiO_2−Ti^{4+} (1186) and commercial TiO_2 microspheres (961).

To simplify the preparation process, some phosphate-based matrix supports have been made from precursors containing phosphate group or phosphate analogs, such as 3-(trihydroxysilyl)propyl methylphosphonate,[37] diethoxyphosphorylethyl-triethoxysilane,[38] and the ethylene glycol methacrylate phosphate (EGMP) monomer.[39] The work of Deng et al. indicated that the ZrP-functionalized mesoporous silica enriched 153 phosphopeptides with 218 phosphorylation sites from the lysate of rat brain.[37] Zou et al. synthesized the p(EGMP) matrix with abundant phosphate ligands through the polymerization of EGMP.[39] The numbers of phosphopeptides enriched by Ti^{4+}-p(EGMP) and Zr^{4+}-p(EGMP) from tryptic digests of mouse liver lysate were 216 and 185, respectively, which were much higher than those enriched by TiO_2 (102), ZrO_2 (95) and Fe^{3+}-IMAC (47). Further, Wang et al. rationally synthesized the MCNC@PMAA@p(EGMP)-Ti^{4+} composite via a two-step distillation−precipitation polymerization method (Figure 4).[40] Benefiting from the abundant Ti^{4+} cations (4.1 wt

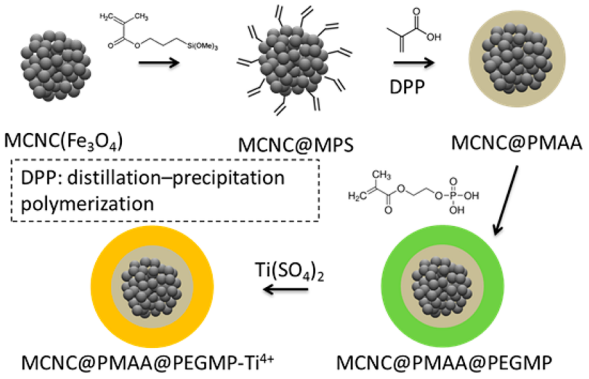

Figure 4. Scheme for the preparation of MCNC@PMAA@PEGMP-Ti^{4+} affinity microspheres for phosphopeptide enrichment.

%), the pure Ti^{4+}-phosphate interface and the high magnetic susceptibility, this composite showed excellent selectivity (molar ratio of BSA/β-casein up to 500:1), high recovery (87%) and a detection limit as low as 50 fmol β-casein.

Natural polymers such as chitosan (CS) and cellulose possess unique merits (e.g., low-cost, easy availability, abundant functional groups and facile chemical modification), which makes them ideal IMAC matrix materials. Ren's group developed the Ti^{4+}-phosphate functionalized cellulose and chitosan by esterifying cellulose and chitosan using H_3PO_4.[41,42] Results showed that 30 phosphoproteins were enriched by the Ti^{4+}-phosphate functionalized cellulose from salt-stressed rice leaf protein lysates.[41] Our group lately designed the MPCS-Ti^{4+} magnetic microspheres containing *N*-methylene phosphonic chitosan by a facile one-pot solvothermal strategy (Figure 5).[43] Profiting from the good dispersibility, abundant Ti^{4+} sites and magnetic responsiveness, the MPCS-Ti^{4+} exhibited high affinity selectivity for phosphopeptides and rapid separation as well as good reusability. Similarly, Zou et al. designed the novel Fe_3O_4@SiO_2@ $(HA/CS)_{10}$-Ti^{4+} nanoparticles via a phosphate-derivation strategy, which showed outstanding selectivity (molar ratio of BSA/β-casein up to 2000:1), excellent sensitivity (0.5 fmol β-casein) and good application performance for real biosamples (i.e., human serum and nonfat milk).[44]

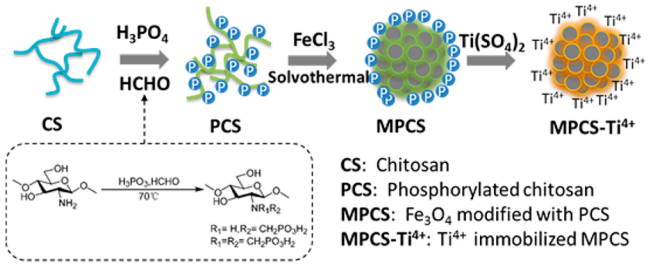

Figure 5. Scheme for the synthesis of MPCS-Ti^{4+} microspheres for phosphopeptide enrichment.

(3). IMAC Affinity Materials with Other Novel Chelating Ligands. Based on the Phos-tag technology, Tohru Koike et al. developed Zn^{2+}-Phos-tag magnetic beads for simple and comprehensive separation of phosphomonoester-type biomolecules.[45] Notably, Zhang et al. recently adopted the adenosine triphosphate (ATP) molecules as novel chelating ligands which were grafted onto amine-functionalized magnetic nanoparticles using glutaraldehyde as linker (Figure 6a).[46] The prepared Ti^{4+}-

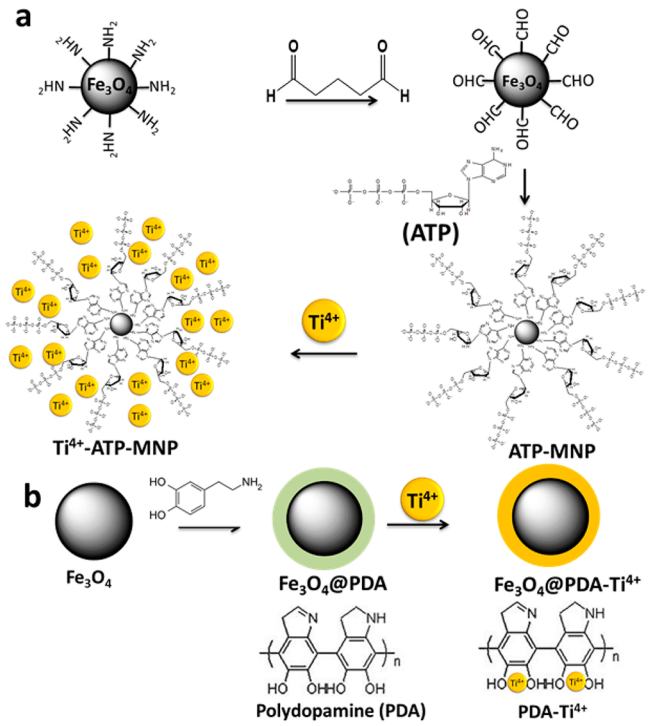

Figure 6. Scheme for the synthesis of Ti^{4+} immobilized Fe_3O_4 nanoparticles (a) modified with ATP and (b) coated with polydopamine (PDA) for phosphopeptide enrichment.

ATP-MNPs showed superhigh sensitivity (3 amol β-casein), much higher specificity (molar ratio of BSA/β-casein up to 5000:1) than conventional Ti^{4+}-IMAC and TiO_2 beads, high recovery (84.76 ± 2.9%), and better applicability than commercial TiO_2 beads for rat liver mitochondria samples. The terrific enrichment performance of the Ti^{4+}-ATP-MNPs was attributed to the abundant phosphate group in ATP which can help the ATP ligand chelate abundant metal cations, and the outstanding hydrophilicity of ATP which can avoid the nonspecific binding and help phosphopeptides retain on the affinity microspheres by the strong hydrophilic interaction (e.g.,

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hydrogen bonding) between the groups of ATP and phosphopeptides.[46]

Another exceptional novel chelating ligand is dopamine, which can be easily polymerized onto a variety of substrates including Fe_3O_4 particles,[47] graphene,[48] eppendorf tube,[49] and porous silica[50] for metal cation (e.g., Ti^{4+}) immobilization based on the strong chelation between the catechol hydroxyl groups and metal cations. Because of the abundant Ti^{4+} sites, strong chelation of catechol hydroxyl-groups for Ti^{4+}, and unique hydrophilicity of polydopamine, these resulting dopamine-based IMAC materials showed high application potential in phosphoproteomics. For example, the Fe_3O_4@PD-Ti^{4+} microspheres (Figure 6b) exhibited excellent specificity (molar ratio of BSA/β-casein up to 500:1) and high sensitivity (2 fmol β-casein).[47]

2.1.2. Metal Oxide (MO)-Based Materials for MOAC. Metal oxide affinity chromatography (MOAC) is another widely used enrichment technology in phosphoproteomics (Figure 1②). Similar to IMAC, the principle affinity sites in MOAC are also metal cations. But these metal cations have robust chemical bonds to their neighbor oxygen anions and thus can overcome the problem of metal cation loss in IMAC. As most of MOs in MOAC are amphoteric (e.g., TiO_2 with pK_{a1} = 4.4 and pK_{a2} = 7.7),[51] these MOs can act as solid Lewis acids with positively charged surfaces in acid solutions but solid Lewis bases with negatively charged surfaces in basic solutions. Consequently, phosphorylated proteins/peptides can be captured at low pH (e.g., pH 2.7) and then be eluted at high pH (e.g., pH 10.5) based on the reversible Lewis acid−base interactions (e.g., ion-exchange, electrostatic interaction) between the surface metal cations and the phosphate groups.[18] Up to now, a series of MOs have been used for MOAC, including NiO,[52] ZnO,[53] Al_2O_3,[54] Fe_2O_3,[55] Ga_2O_3,[56] TiO_2,[57] ZrO_2,[58] SnO_2,[59] HfO_2,[60] Nb_2O_5,[61] and Ta_2O_5.[62]

2.1.2.1. Pure MOs. Most of the MOs have initially been used in their most common forms (e.g., bead),[59] which unfortunately have limited surface areas and are hard to meet the increasing demand. Compared with their solid counterparts, the mesoporous MOs not only have larger surface areas and more active affinity sites, which mean higher capturing capacity but also possess specific advantages such as the size-exclusion effect resulting from the mesoporous structure. Thus, mesoporous MOs affinity materials were fabricated by alternative methods such as hydrothermal reaction, supercritical drying, and self-assembly. For example, the TiO_2 nanocrystal clusters (TiO_2 NCC) fabricated by a self-assembly method not only showed better enrichment efficiency for phosphopeptides than solid TiO_2 particles owing to its high surface area[63] but also realized the selective enrichment of intact phosphoproteins profiting from the size-exclusion effect.[64] MO (e.g., TiO_2[65] and ZrO_2[66]) aerogels with large specific surface areas also showed much better enrichment performance than their solid counterparts. Moreover, mesoporous γ-Fe_2O_3 nanocrystal clusters can capture 11 phosphopeptides from tryptic digests of drinking milk.[67]

Recently, octahedral SnO_2 nanoparticles exposing high-index {221} crystal facets were proved to have better enrichment performance than SnO_2 nanospheres and commercial TiO_2, which could be attributed to the abundant unsaturated coordination Sn atoms with high chemical activity on the high-index facets.[68] This work provided a new thought to enhance the enrichment efficiency of crystalline MOs by tailoring their exposed crystal facets. Notably, Zhong et al. demonstrated that Fe_3O_4, $NiFe_2O_4$ and $ZnFe_2O_4$ can capture both mono- and multiphosphopeptides but have relatively stronger affinity toward monophosphopeptides, whereas the $NiZnFe_2O_4$ showed a strong affinity preference for multiphosphopeptides.[69] This interesting phenomenon was speculated to result from the increased area and decreased magnetic field of exposed surface octahedral sublattices of $NiZnFe_2O_4$.[69]

Some MOs have been demonstrated to show complementary enrichment preference for phosphopeptides. For example, TiO_2 preferred to enrich multiphosphopeptides whereas ZrO_2 preferentially enriched monophosphopeptides.[58] Therefore, integrating different MOs may exhibit superior enrichment efficiency over single MO. Accordingly, binary TiO_2−ZrO_2 mixed oxides were fabricated and showed better enrichment capability than singly TiO_2 and ZrO_2, which may be because there were larger amount of surface acidic sites on the TiO_2−ZrO_2 mixed oxides.[70] Another interesting binary affinity probe is SnO_2−$ZnSn(OH)_6$, which can realize global phosphopeptide detection.[71] SnO_2 is propitious to the detection of monophosphopeptides because of the incomplete elution of the strongly adsorbed multiphosphopeptides induced by the high Lewis acidity of SnO_2. On the contrary, in view of the low Lewis acidity of ZnO, $ZnSn(OH)_6$ may have much lower Lewis acidity, resulting in its strong binding force to the multiphosphopeptides but weak binding force to the monophosphopeptides. Consequently, comprehensive phosphopeptide analysis can be achieved by using SnO_2 and $ZnSn(OH)_6$ together.

2.1.2.2. MO-Based Affinity Composites. Despite their contributions to phosphoproteomics, pure MOs still face problems such as laborious separation processes (e.g., repeated centrifugation), which can cause loss of affinity materials and biosamples; aggregation of MOs particles, especially the nanosized ones in the enrichment solutions, which will lead to decreased available active affinity sites; and single function (i.e., affinity), which makes it difficult to satisfy the increasing application demands. Conjugating MOs with other functional materials/devices has become a widely used strategy to overcome these problems, which can endow the resulting affinity composites with other unique functional properties available besides the affinity ability of MOs, thereby greatly promoting the phosphoproteomics research.

(1). Magnetic MO Affinity Composites. Because of the superparamagnetic property of magnetic components (e.g., Fe_3O_4, γ-Fe_2O_3), magnetic MO affinity composites can not only be rapidly isolated with an external magnetic field but also be easily redispersed after removing the external magnetic field.[72] This excellent magnetic responsiveness will greatly facilitate the separation procedure and reduce the possible loss of affinity materials and biosamples, and thus greatly enhance the enrichment efficiency.

Magnetic MO affinity composites are usually core−shell structured. MOs can be coated onto the magnetic cores directly[73] or via assistant linkers (e.g., SiO_2,[53] carbon,[74] and PMAA[75]). For instance, the Fe_3O_4@ZnO magnetic composites fabricated by the aid of SiO_2 can not only efficiently enrich and rapidly isolate phosphorylated peptides/proteins from biosamples (e.g., saliva) but act as microwave absorbers to assist tryptic digestion of trapped proteins under microwave heating.[53] Moreover, to improve enrichment performance by means of MOs shells with favorable surface properties such as high surface area and porous structure, several kinds of magnetic composites with porous TiO_2 shells were synthesized

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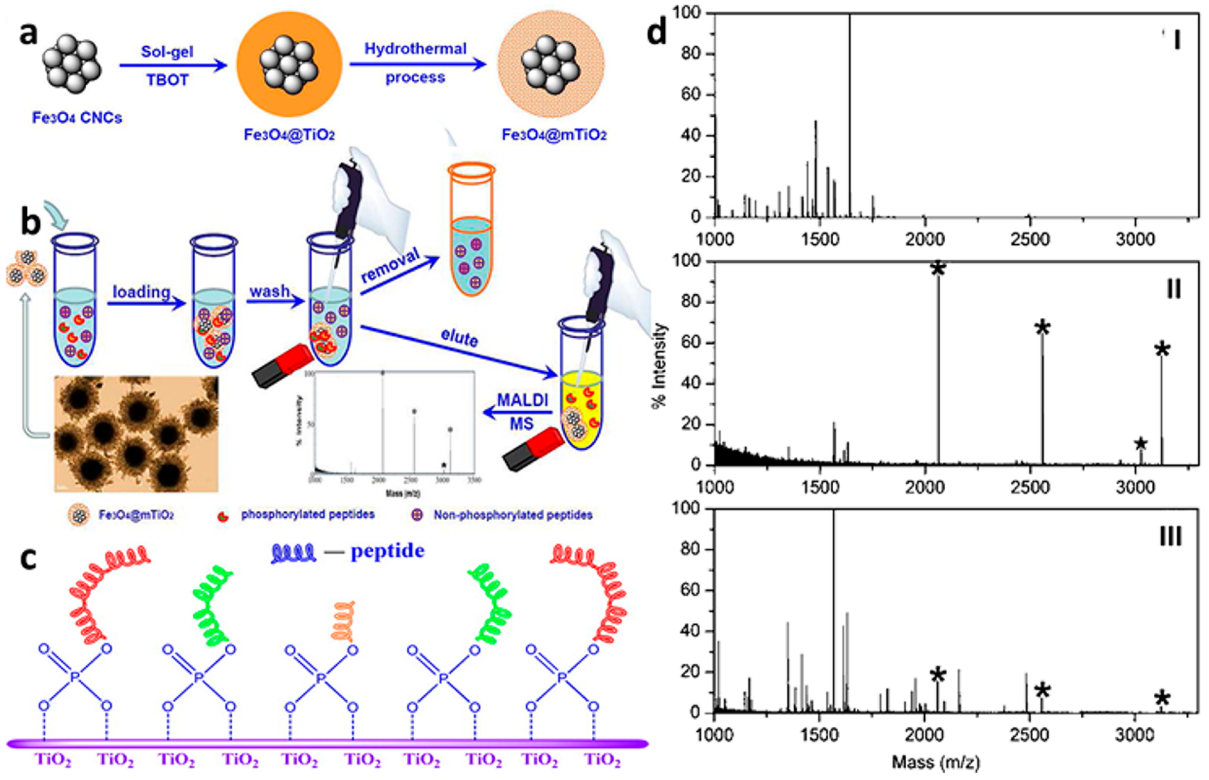

Figure 7. (a) Scheme for the synthesis of Fe_3O_4@mTiO_2 microspheres; (b) schematic representation of phosphopeptide enrichment process using Fe_3O_4@mTiO_2 microspheres; (c) schematic illustration of the binding of phosphopeptides on TiO_2. (d) MALDI MS spectra of the digest mixture of β-casein and BSA at a molar ratio of 1:1000: (I) direct analysis, analysis after enrichment using (II) Fe_3O_4@mTiO_2, and (III) Fe_3O_4@TiO_2. '*' indicates phosphopeptides. Reproduced with permission from ref 76. Copyright 2012 American Chemical Society.

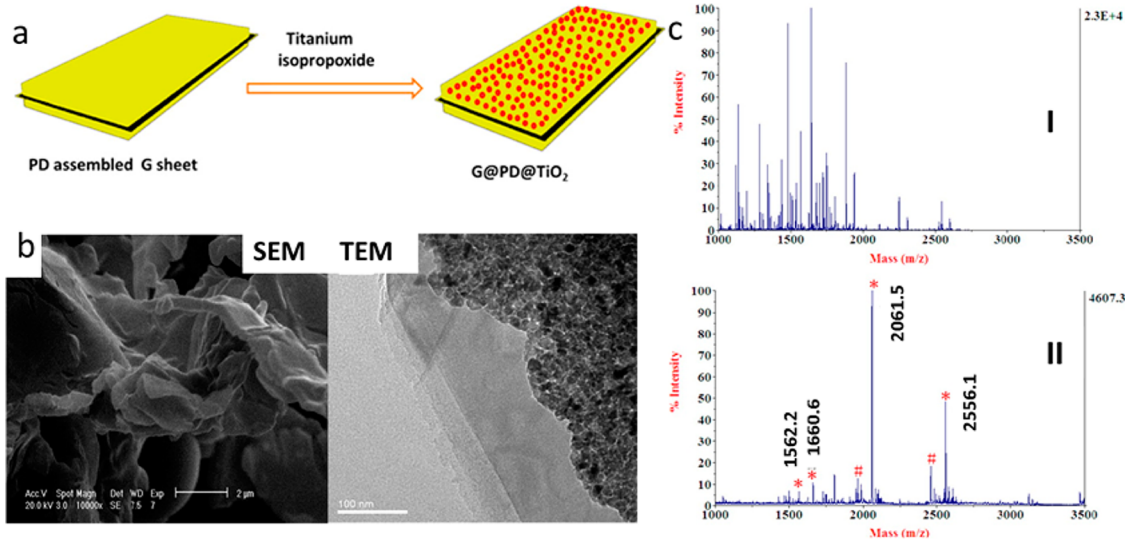

Figure 8. (a) Scheme for the synthesis of G@PD@TiO_2 composite. (b) SEM and TEM images of the G@PD@TiO_2 composite. (c) MALDI MS spectra of the tryptic digest mixture of β-casein and BSA at a molar ratio of 1:1000: (I) direct analysis, (II) analysis after enrichment by the G@PD@TiO_2 composite. '*' and '#' indicate the phosphopeptides and their dephosphorylated counterparts, respectively. Reproduced with permission from ref 87. Copyright 2014 American Chemical Society.

by alternative methods such as hydrothermal[76,77] and solvothermal[78] treatment. A prominent example is the magnetic Fe_3O_4@mTiO_2 microspheres which were rationally prepared by Wang et al. via a sol−gel method followed by a hydrothermal process (Figure 7a).[76] The tailor-made mesoporous TiO_2 shell possess unique textural properties such as high specific surface area (167.1 m^2/g), large pore volume (0.45 cm^3/g), and tunable pore size (8.6−16.4 nm). In virtue of the magnetic responsiveness of the Fe_3O_4 cores and the unique textural properties of the TiO_2 shells, the Fe_3O_4@mTiO_2 microspheres possessed outstanding phosphopeptide enrichment features including rapid separation (less than 5 min), much better selectivity (molar ratio of BSA/β-casein up to 1000:1) (Figure 7d) and higher enrichment capacity (225 mg/

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g) than Fe_3O_4@TiO_2 microspheres (20 mg/g), remarkable sensitivity (10 fmol β-casein), and high recovery (93%).

(2). Carbon Nanotube-Based and Graphene-Based MO Affinity Composites. In view of their unique properties (e.g., good structure stability, high specific surface area, and easy modification), carbon nanotube[79] and graphene[80] have been successfully employed as strong matrixes for supporting MOs nanoparticles, which can avoid the problems of aggregation and loss of affinity MOs, and thus improve the enrichment efficiency. MOs (e.g., TiO_2 and ZrO_2) can be loaded on the surfaces of carbon nanotube and graphene by in situ growth processes using proper precursors such as titanium isopropoxide (TTIP).[81−83] For instance, Tang et al. prepared the titania-decorated graphene (G-TiO_2) using TTIP by a sol−gel method.[83] Enrichment test results indicated that from the sample of Hela cells, 62.5 μg of G-TiO_2 captured 967 phosphopeptides, whereas 2000 μg of commercial TiO_2 beads captured only 640 phosphopeptides. Notably, the G-TiO_2 can also serve as a platform for the direct MS analysis of phosphopeptides. To facilitate the enrichment process, we can further load the magnetic particles on carbon nanotube and graphene.[84−86] The magG/(Ti−Sn)O_4 composite, for instance, can selectively capture and rapidly isolate phosphopeptides from the peptides mixture of β-casein and BSA with a weight ratio of 1:1500 and enrich 170 phosphopeptides from mouse brain lysates.[85]

The method of in situ growing MOs on the graphene sheets often resulted in graphene-based composites with randomly distributed MOs, which may cause undesirable challenges such as limited affinity sites and poor dispersibility. Differently, the G@PD@TiO_2 composite was recently synthesized by coating polydopamine(PD) on graphene followed by the deposition of a fine layer of TiO_2 nanoparticles (Figure 8).[87] The polydopamine layer not only serves as the middle coupling linker but also improves the dispersibility of the composite. Because of its high surface area, excellent dispersibility, and large amount of TiO_2, the G@PD@TiO_2 exhibited intriguing enrichment features, including very low detection limit (5 fmol β-casein), excellent selectivity (molar ratio of BSA/β-casein up to 1000:1) (Figure 8c), and high application ability for real-life biosamples (e.g., enriching 334 phosphopeptides from mouse brain tissue).

(3). Silica-Based Metal Oxide Affinity Composites. Since being synthesized via the simple "Stöber method" in 1968,[88] silica (SiO_2) has become an incredibly powerful material. Its remarkable properties such as controlled morphologies, tunable structures, excellent hydrophilicity and outstanding modifiability make silica an ideal material for fabricating MOs-based composite. For instance, fibrous TiO_2/SiO_2 composites obtained by an electrospinning method exhibited better enrichment performance than commercially available TiO_2 particles because of the large surface area and appropriate Lewis acidity of the TiO_2/SiO_2 composite.[89]

Particularly, considering the unique properties of mesoporous silica (e.g., easy modifiability, high surface area, stable skeleton, and size exclusion effect) and the affinity ability of MOs, MO modified mesoporous silica composites with abundant affinity sites were fabricated via facile methods (e.g., sol−gel).[90,91] These composites can efficiently and selectively capture phosphopeptides from complex biosaomples (e.g., human serum).[90,91] Compared with mesoporous silica, macroporous silica with open large pores is very beneficial for both the MOs loading and the later binding of phosphorylated proteins/peptides to the affinity composites. Importantly, the open macroporous structure can ensure the efficient mass transport and avoid the so-called "shadow effect" in some mesoporous materials with long channels. For example, the Al-MOSF and Ti-MOSF were fabricated by modifying Al_2O_3 and TiO_2 onto the macroporous ordered silica foam (MOSF).[92,93] Intriguingly, the Al-MOSF can be used as a multifunctional nanoreactor where the digest of proteins and the capture of phosphopeptides can be done subsequently, thereby greatly simplifying the whole protocol (Figure 9aII).[92] Moreover,

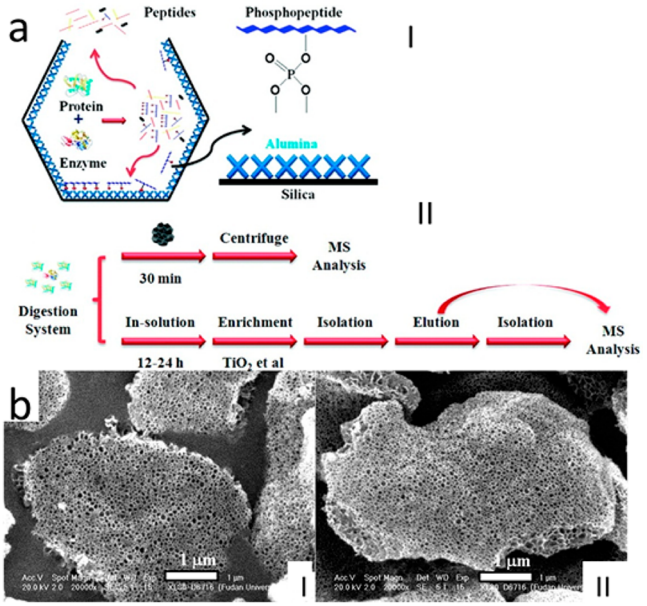

Figure 9. (a) (I) Schematic illustration of protein digestion and phosphopeptide enrichment in the Al-MOSF nanoreactor, (II) schematic comparison between the overall phosphopeptide enrichment strategy of using Al-MOSF nanoreactor (upper) and traditional workflow (below). (b) SEM images of (I) MOSF and (II) Al-MOSF. Reproduced with permission from ref 92. Copyright 2009 American Chemical Society.

different from TiO_2 nanoparticles which can enrich the mono- and multiphosphopeptides simultaneously, the Ti-MOSF showed a preferential enrichment toward multiphosphopeptides.[93] This phenomenon was attributed to the coordination difference of Ti atoms between the Ti-MOSF and TiO_2 particles. Additionally, TiO_2-loaded hierarchically ordered macro/mesoporous silica composites prepared via a template method were demonstrated to have excellent enrichment features such as low detection limit (8 fmol β-casein), great specificity (molar ratio of BSA and β-casein up to 1000:1), and rapid enrichment speed (within 1 min).[94]

(4). MO-Based Affinity Devices. In phosphoproteomics analysis, well-designed enrichment devices are important for simplifying enrichment protocols. Traditionally, the MO particles are packed in a chromatography column or a pipet tip, and then the enrichment manipulation can be achieved with the help of other equipment (e.g., pump and pipet).[57,95] However, the packed MOs were randomly cumulated together, which may seriously impede the mass transport. Consequently, researchers have tried loading MOs on certain fine materials/devices. Bruening et al. reported TiO_2 nanoparticles-decorated porous nylon membrane for rapid phosphopeptide enrichment.[96] Moreover, magnetite-doped polydimethylsiloxane[97]

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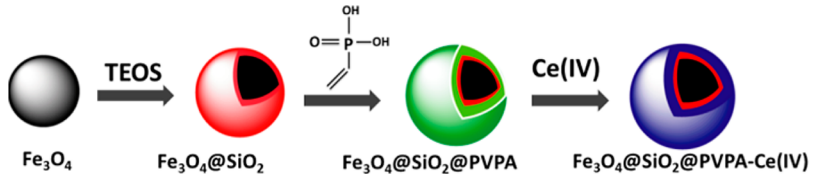

Figure 10. Scheme for the fabrication of Fe_3O_4@SiO_2@PVPA-Ce(IV) polymeric affinity microspheres for phosphopeptide enrichment.

and microfluidic devices supporting TiO_2 nanotube array[98] or TiO_2–ZrO_2[99] have also been designed for efficient on-chip enrichment of phosphopeptides through a rapid and straightforward protocol. The microfluidic device supporting TiO_2 nanotube array, for example, realized the efficient enrichment of serum phosphopeptide, which proved the differential expression of serum phosphopeptides between ovarian cancer patients and healthy women. He et al. fabricated a device by coating TiO_2 onto ZnO nanorod arrays grown on the inner wall of a capillary microchannel.[100] This device can rapidly and specifically capture phosphopeptides through an automatic continuous-flow protocol, which is expected to achieve cost-effective and high-throughput phosphopeptide enrichment from large volumes of complex biosamples. Additionally, omitting any extra synthesis processes, Corthals et al. directly applied the commercial indium tin oxide-coated glass slides as a straightforward platform for phosphopeptide purification, providing a simple, fast, and sensitive enrichment method.[101]

(5). Other MO-Based Affinity Composites. Besides above-mentioned composites, several other functional materials including hematite (α-Fe_2O_3) nanotubes,[102] diatomite,[103] and cellulose,[104] as supports, have also been explored. The α-Fe_2O_3 nanotubes had well-defined morphology, high surface-to-volume ratio, and accessibility to inner and outer surfaces. After loading the tiny SnO_2 nanoparticles, the resulting α-Fe_2O_3@SnO_2 successfully enriched 139 phosphopeptides from the tryptic digestion of rat brain.[102] With merits such as low-cost, porous structure, and good chemical stability, diatomite and cellulose can be used as ideal supports. Yang et al. prepared the TiO_2-modified diatomite. Compared with pure TiO_2, it could be more easily separated and had a larger maximum dynamic adsorption capacity.[103] Zhang et al. designed robust magnetic cellulose microspheres loaded with tiny TiO_2 nanocrystallines, which can selectively capture phosphopeptides from a mixture of β-casein and BSA with a molar ratio of 1:1000.[104]

2.1.3. Rare Earth (RE)-Based Affinity Materials. Rare earth (RE) is a vital class of metal elements (i.e., 15 lanthanide elements, scandium and yttrium) and RE-based materials have been extensively used. The stable RE ions are usually trivalent/tetravalent cations which, as typical hard acids, can preferentially interact with hard bases (e.g., phosphate group). In fact, RE-based materials have been applied in biomedical areas related with phosphorylated molecules for several decades. For instance, lanthanum ions have been used for the dephosphorylation of nucleotides since 1950s.[105] At present, lanthanum carbonate is administered to patients with hyperphosphonatemia as a phosphate-binding agent for the control of serum phosphates.[106] Therefore, the RE-based materials theoretically bear high potential for the enrichment of phosphorylated proteins/peptides.

2.1.3.1. RE Cations. Considering the overwhelming preference of free RE cations for phosphate, free RE cations have been successfully used as precipitating agent to isolate phosphorylated proteins/peptides from complex biosamples (e.g., egg white, milk, and nonmalignant liver tissue) (see Figure 1③).[107−109] For instance, La^{3+} together with phosphate anions can precipitate 27 phosphoproteins with as many as 33 phosphorylation sites from lysed pig urothelial cells.[107] With unique merits such as the weak nonspecific binding, high recovery of the target proteins/peptides, and the simple one-step isolation process without any sophisticated equipment, the RE cation-based precipitation method provides an alternative enrichment strategy for the phosphoproteomics research.

2.1.3.2. RE-Based IMAC Nanocomposites. Similar to other cations (e.g., Ti^{4+}), RE cations can also be readily immobilized onto the IMAC matrixes for enriching phosphorylated biomolecules. Li et al. reported the Ce^{4+}-chelated magnetic affinity microspheres using IDA as the chelating group, which showed better enrichment capability than the Fe^{3+}-chelated magnetic microspheres.[110] Recently, our group facilely coated poly(vinylphosphonic acid) (PVPA) with abundant phosphonate groups onto the Fe_3O_4@SiO_2 to immobilize Ce(IV) for phosphopeptide enrichment (Figure 10).[111] Benefiting from the abundant Ce(IV), the excellent dispensability and the magnetic responsiveness, the Fe_3O_4@SiO_2@PVPA-Ce(IV) can selectively and rapidly enrich phosphopeptides from biosamples. Moreover, the catalytic ability of Ce(IV) for phosphate ester bond cleavage[112] can also assist the MS identification of phosphopeptides based on the principle similar to that of CeO_2 as discussed later. Interestingly, RE cations (i.e., La^{3+}, Ho^{3+}, and Er^{3+}) immobilized PVPA affinity resins were demonstrated to rank as Er-PVPA > Ho-PVPA > La-PVPA in terms of enrichment specificity and MS peak intensity, which was attributed to the different charge transfer effects for the three RE cations according to the calculation of quantum mechanics.[113]

2.1.3.3. RE-Based MOAC Composites. At present, La_2O_3 and CeO_2 have been explored as enrichment materials. F. Jabeen et al. prepared the silica-La_2O_3 composite by a four-step chemical derivatization method.[114] This composite not only showed high enrichment ability for phosphopeptides, but also can be used as an efficient carrier material for the material-enhanced laser desorption/ionization (MELDI) MS analysis.

It is particularly worth noting that besides affinity ability, CeO_2 can dramatically catalyze the dephosphorylation of phosphopeptides (i.e., the phosphate ester bond cleavage).[115,116] Thus, in MS analysis, phosphopeptides treated with CeO_2 will produce MS peaks with characteristic mass loss of $n \times 80$ Da ($n \geq 1$) which can serve as marker peaks for direct identification of phosphopeptides. Significantly, our latest pioneering work revealed that exposed crystal facets can greatly influence the performance of CeO_2 nanocrystals for adsorption and dephosphorylation of phosphorylated molecules (Figure 11).[117] Briefly, all three facets (i.e., {100}, {111}, and {110}) can dramatically catalyze the dephosphorylation of the adsorbed phosphorylated molecules while the {111} and

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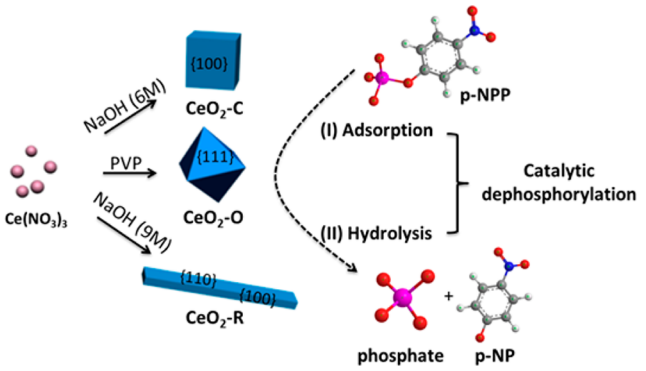

Figure 11. Scheme for the synthesis of CeO_2 nanocrystals with different morphologies and their catalytic dephosphorylation for the model phosphorylated molecule. (p-NPP and p-NP represent p-nitrophenyl phosphate and p-nitrophenol, respectively).

{110} facets possess much higher adsorption capacities and kinetic catalytic activities than {100} facets, which should result from the different surface structures of the three facets. This work is believed to present valuable references for the design and fabrication of highly efficient affinity materials.

2.1.3.4. Other RE-Based Affinity Materials. On the basis of the remarkable physicochemical properties (e.g., chemical stability, catalysis, and coordination) of rare-earth phosphates ($REPO_4$), our group have pioneeringly developed $REPO_4$ as novel affinity probes for enriching phosphopeptides. For instance, the $REPO_4$ (RE = La, Nd, and Eu) nanorods loaded on the MALDI target plate can selectively capture and then in situ MS identify the phosphopeptides, which reduced loss of sample, facilitated the enrichment process and accelerated the MS detection.[118] In another work, the mesoporous $REPO_4$ (RE = Y, Gd, and Yb) hollow microspheres covered with nanothorns (Figure 12a, b) can not only specially capture phosphopeptides (Figure 12c) based on their large specific surface area and the unique coordination affinity of RE cations for phosphate groups but also can assist the identification of phosphopeptides owing to the RE^{3+}-mediated dephosphorylation reaction (Figure 12d).[119] Further, to facilitate the isolation process, we prepared magnetic core–shell structured Fe_3O_4@$REPO_4$ microspheres by combining a homogeneous precipitation method and an ion-exchange process (Figure 12e).[120,121] These microspheres were demonstrated to have high application potential in phosphoproteomics. For example, the Fe_3O_4@$hYPO_4$ microspheres successfully enriched 2678 unique phosphopeptides from the tryptic digest of proteins extracted from mouse brain.[121] Additionally, to realize the sequence identification of different kinds of peptides, we prepared a multifunctional affinity probe composed of graphene, $LaPO_4$ nanorods, and Fe_3O_4 nanoparticles.[122] On the basis of the hydrophobic interaction between graphene and low-abundance peptides, the affinity ability of La^{3+} for phosphate groups, and the magnetic responsiveness of Fe_3O_4, this multifunctional composite realized the simultaneous extraction, fast magnetic separation, and sequential MS detection of two types of peptides (i.e., the low-abundance peptides and phosphopeptides) under optimized enrichment and elution conditions.

Apart from $REPO_4$, several other RE-based materials including magnetic Fe_3O_4@$La_xSi_yO_5$,[123] γ-Fe_2O_3@$REVO_4$ (RE = Sm, Dy, and Ho),[124] and γ-Fe_2O_3@$xNH_4F{\cdot}yLuF_3$[125] as well as bonelike GdF_3[126] were also explored for enrichment and detection of phosphopeptides. Moreover, Messner et al. explored the metal–organic framework (MOF) consisting of Er^{3+} and 1,4-phenylenediacetate for capturing phosphopeptides, which implied that the RE-based MOF materials may serve as favorable affinity tools in phosphoproteomics.[127]

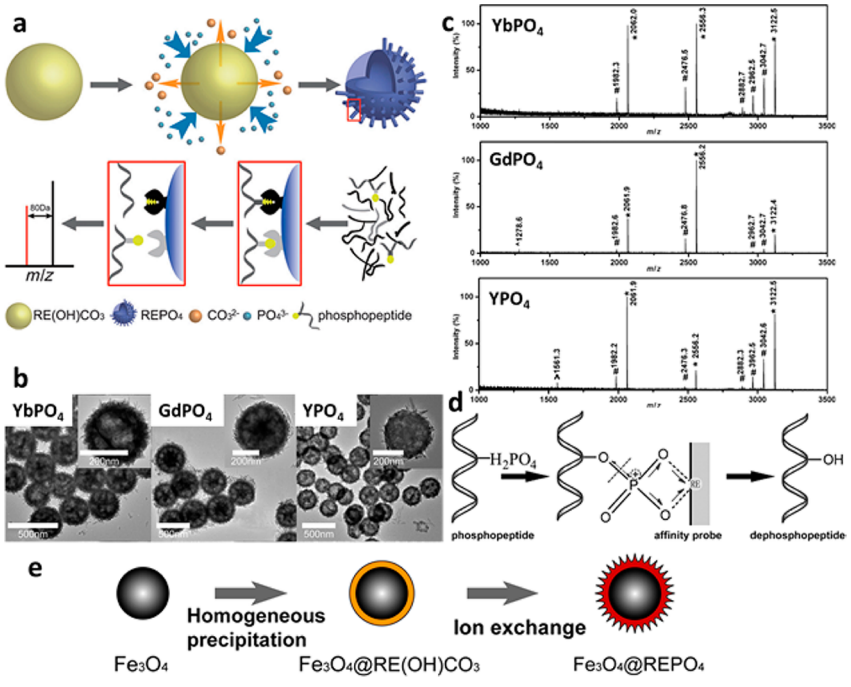

Figure 12. (a) Scheme for the synthesis of prickly $REPO_4$ hollow microspheres for phosphopeptide enrichment and dephosphorylation; (b) TEM images of the prickly $REPO_4$ hollow microspheres; (c) MALDI-TOF mass spectra of β-casein digest treated with the prickly $REPO_4$ affinity microspheres; (d) illustration of RE^{3+}-mediated dephosphorylation reaction on the $REPO_4$ affinity probe; (e) scheme for the synthesis of magnetic Fe_3O_4@$REPO_4$ (RE = Eu, Tb, and Er) microspheres. '*' and '#' indicate the phosphopeptides and their dephosphorylated counterparts, respectively.

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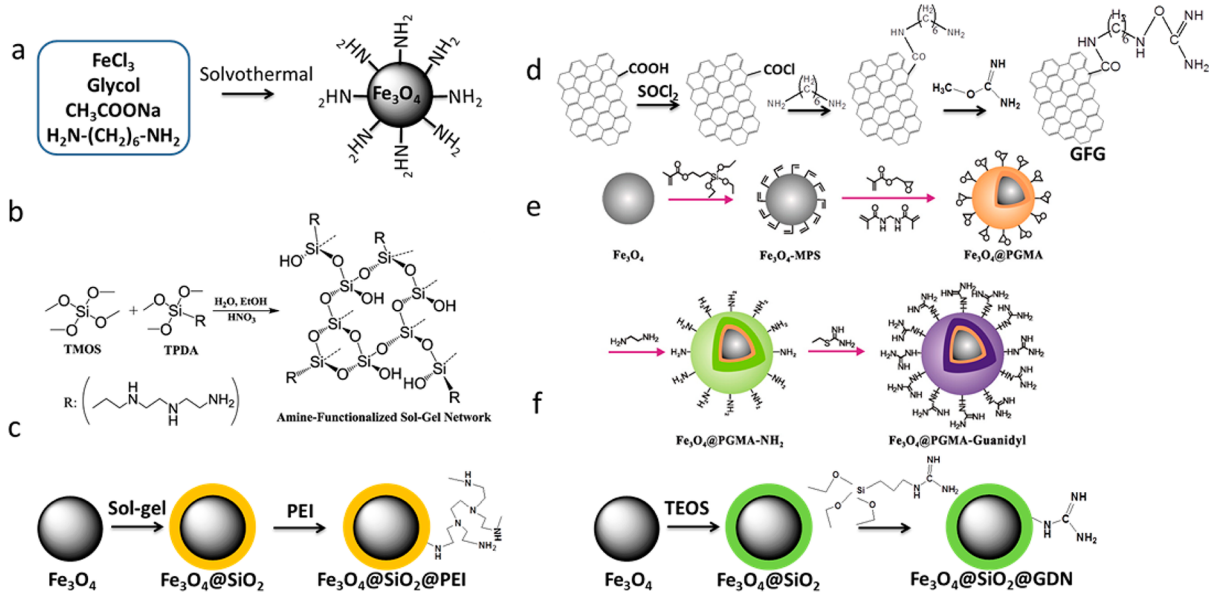

Figure 13. Scheme for the fabrication of several amine-based affinity materials: (a) Fe_3O_4@NH_2 nanoparticles, (b) amine-functionalized silica sol–gels (reproduced with permission from ref 139; copyright 2012 American Chemical Society), (c) Fe_3O_4@SiO_2@PEI, (d) Guanidino-functionalized graphene (GFG), (e) Fe_3O_4@PGMA-Guanidino nanoparticles (reproduced with permission from ref 144; copyright 2014 American Chemical Society), (f) Fe_3O_4@SiO_2@GDN (GDN = guanidinium) microspheres.

2.1.4. Other Metal-Based Affinity Materials. Ti-doped mesoporous silica (Ti-MPS)[128] and Ti-aluminophosphonate-5 molecular sieves[129] were adopted as novel affinity materials utilizing the properties of mesoporous texture (e.g., large surface area and high capacity) and the strong affinity of incorporated Ti sites in the framework for the phosphate groups. For example, the Ti-MPS with Si/Ti molar ratio of 8:1 enriched 396 phosphopeptides from the sample of human placenta mitochondria, whereas only 162 and 175 phosphopeptides were enriched by commercial P25 TiO_2 and TiO_2 beads, respectively.[128]

Due to the unique properties such as plenty of Lewis acid sites, high surface area, wide chemical tunability, and excellent mechanical stability, the porous MOF materials have aroused increasing research interest in separation area.[130] For phosphopeptide enrichment, Fe-MOF(iron(III) benzenetricarboxylate)[131] and Zr-MOF (zirconium(IV) 1,4-benzenedicarboxylic acid)[132] have been explored as novel affinity materials. For example, the Fe_3O_4@PDA@Zr-MOF with better sensitivity than Fe_3O_4@PDA@Zr^{4+} can specifically enrich phosphopeptides from the high complex peptides mixture with a mole ratio of β-casein to BSA as 1:500 and capture four endogenous phosphopeptides from the human serum of a hepatocellular carcinoma patient.[132]

In addition, on the basis of the affinity theory similar to that of MOAC, solid materials such as $BaTiO_3$,[133] α-zirconium phosphate,[134] and hydroxyapatite[135] have also served as novel affinity materials for phosphopeptide enrichment.

2.2. Amine-Based Affinity Materials. Interactions (e.g., electrostatic attraction and hydrogen bonding) between amine and phosphate groups are ubiquitous in nature and play an important role in various areas. For example, amine-based groups involved in host–guest chemistry can be used for the selective extraction and rapid detection of phosphate compounds.[136] Hence, the amine-based materials are expected to provide a means for enriching phosphorylated proteins/peptides (Figure 1④). Briefly, in acidic buffers (e.g., pH 2), the positively charged amine groups ($pK_a \geq 9$) prefer to interact with the negatively charged phosphate groups (pK_a = 1–2) over noncharged carboxyl groups, and thus the phosphorylated proteins/peptides can be selectively captured. The captured phosphorylated proteins/peptides can then be eluted in high pH (e.g., pH 10) or extreme low pH (e.g., pH 1) buffers in which the interaction between the amine and phosphate groups can be disrupted. Therefore, by finely controlling the pH of the buffers, the phosphorylated proteins/peptides can be selectively enriched.

A typical amine-based affinity material is the 1,6-hexanediamine-functionalized magnetic nanoparticles (Fe_3O_4@NH_2) (Figure 13a).[137] The phosphopeptides can be selectively captured by the Fe_3O_4@NH_2 nanoparticles in the loading buffer containing 1% TFA and then be eluted by 5% $NH_3 \cdot H_2O$ solution. In another case, the C_{60}-fullerene bound aminopropylsilica allowed a straightforward and selective isolation of mono- and multiphosphorylated peptides according to their different isoelectric point (pI) values through a stepwise elution strategy.[138] However, these two affinity materials have the limited number of amine groups, resulting in relatively low enrichment efficiency. Differently, amine-functionalized silica sol–gels with abundant amine groups were prepared by cohydrolyzation of N′-[3-(trimethoxysilyl)-propyl]-diethylenetriamine and tetramethylorthosilicate (Figure 13b). After being packed into a pipet tip, the sol–gel material can capture 25 phosphopeptides from the tryptic digest of nonfat milk under the optimized conditions.[139] Fe_3O_4@SiO_2@PEI carrying abundant amine groups was also synthesized by modifying polyethylenimine (PEI) on the magnetic particles (Figure 13c).[140] The ammonium groups in PEI can strongly interact with phosphate groups in phosphopeptides by forming bidentate complexes, leading to high enrichment specificity for phosphopeptides. Interestingly, the Fe_3O_4@SiO_2@PEI mainly enriched multiphosphopeptides when the binding solvent contained protic components such as water, whereas both the mono- and multiphosphopeptides can be enriched

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simultaneously when only using acetonitrile as the solvent. This phenomenon might be because that the water not only has a stabilizing hydration effect on the bidentate complex of ammonium-phosphate but can reduce the ammonium-phosphate binding strength through solvation of phosphate groups, which thus favors the detection of multiphosphopeptides.

Carrying three amine groups, guanidino groups have been proved to strongly interact with phosphate groups by virtue of the electrostatic interaction and hydrogen bonds.[141] Accordingly, Wu et al. prepared the polyarginine(PA)-coated diamond nanoparticles as a novel affinity probe.[142] As the guanidino moieties in adjacent arginine side chains are able to generate stable "covalent-like" complexes with phosphate groups, the PA-coated diamonds can capture phosphopeptides from protein digests as low as 50 fmol α-casein. However, this affinity material was high-cost and the separation process was inconvenient. Recently, guanidino-modified materials including graphene (Figure 13d),[143] magnetic poly(glycidyl methacrylate) (PGMA) microspheres (Figure 13e)[144] and Fe_3O_4@SiO_2 nanospheres (Figure 13f)[145] were employed as novel multifunctional affinity materials. For instance, the Fe_3O_4@SiO_2@GDN microspheres achieved the efficient and selective enrichment of phosphoproteins from real biological samples such as nonfat milk, egg white and mouse liver.[145] Moreover, the GFG[143] showed better enrichment performance than amino-functionalized graphene and can serve as support for direct MALDI MS analysis.

Particularly, an extraordinary enrichment phenomenon is that the GFG and Fe_3O_4@PGMA-Guanidino can enrich mono- and multiphosphopeptides simultaneously in acetic acid (HAc) buffer (pH ~3.4), while only multiphosphopeptides can be extracted in trifluoroacetic acid (TFA) buffer (pH ~2) (Figure 14).[143,144] This is because at pH 3.4, phosphate groups are highly deprotonated with negative charge and thus show strong affinity toward positively charged guanidino groups, resulting in enrichment of both mono- and multiphosphopeptides. However, at pH 2, the affinity between phosphate and guanidino groups was weakened due to partially protonation of phosphate groups, and thus only multiphosphopeptides exhibited sufficient affinity to the guanidino groups.[143] Additionally, only multiphosphopeptides with consecutive phosphorylated serine residues can be extracted by GFG in TFA buffer, but those ones with discrete phosphorylated sites can only be adsorbed in HAc buffer.[143] The reasons are still unknown although possible mechanisms such as "salt bridge" interaction (i.e., phosphate(−)· guanidino(+)·phosphate(−))[143] were proposed.

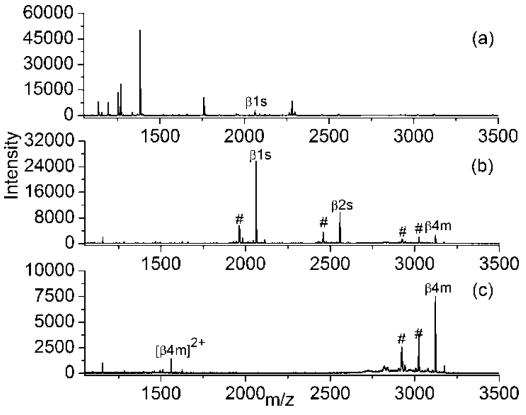

Figure 14. MALDI-TOF mass spectra of β-casein tryptic digest (1 pmol): (a) Direct analysis, (b, c) after enrichment by Fe_3O_4@PGMA-Guanidino in (b) 50% ACN, 0.1 M HAc and (c) 66% ACN, 0.02% TFA. (s, monophosphopeptide; m, multiphosphopeptide; #, peptide residue originating from loss of the phosphate group in phosphopeptide). Reproduced with permission from ref 144. Copyright 2014 American Chemical Society.

2.3. Affinity Materials with Enrichment Superiority for Multiphosphopeptides. Multiphosphorylation has been found to play a unique role in some biological processes. For instance, multiphosphorylation of p53 can enhance binding of p53 to CBP/p300, which is important in activation of CBP/p300-dependent p53 transcriptional pathways.[146] Thus, the efficient MS analysis of multiphosphopeptides will provide compelling insights into some biological processes. However, the MS detection of multiphosphopeptides is often challenging because of their low-abundance and poor ionization efficiency in the presence of nonphosphopeptides and monophosphopeptides. Therefore, it is very necessary to separate multiphosphopeptides from both nonphosphopeptides and monophosphopeptides so as to enhance the MS detection efficiency of multiphosphopeptides.

Up to now, a few affinity materials have been found with enrichment superiority for multiphosphopeptides under optimized enrichment conditions, including $NiZnFe_2O_4$,[69] $ZnSn(OH)_6$,[71] Ti-MOSF,[93] Fe_3O_4@SiO_2@PEI,[140] and guanidino-based affinity materials (i.e., PA-coated diamonds,[142] GFG,[143] and Fe_3O_4@PGMA-guanidino[144]). For these interesting enrichment superiorities, several speculative reasons have been proposed, including the increased area and decreased magnetic field of exposed surface octahedral sublattices for $NiZnFe_2O_4$, the proper Lewis acidity for $ZnSn(OH)_6$, the abundant surface 4-coordinated Ti^{4+} species for TiO_2-modified MOSF, the hydration and solvation effect of water for Fe_3O_4@SiO_2@PEI, and the unique interaction (e.g., "salt bridge") between guanidino groups and phosphate groups for guanidino-based affinity materials. Nevertheless, these speculative reasons still need validations, and further detailed studies are essential to reveal the real principles behind these superiorities.

3. CONCLUSIONS AND PERSPECTIVES

Over the past decades, a wide variety of affinity materials have been developed for enrichment of phosphorylated proteins/peptides based on different enrichment mechanisms. Despite the significant progress, the applications of most of these materials are still in their infancy. Most of these affinity materials were evaluated for their enrichment performance by similar or even rigid evaluation systems (e.g., digest mixtures of standard pure phosphorylated and nonphosphorylated proteins, human serum, and nonfat milk), thus more comprehensive and detailed work are required to test these affinity materials. It should be noted that tailored suitable enrichment conditions such as pH and solvent component are also essential for successful applications of these materials in phosphoproteomics. Moreover, as different affinity materials can exhibit complementary enrichment performance, combination of different affinity materials can be adopted when a comprehensive analysis of highly complex biosamples is expected. Furthermore, the interaction mechanisms and principles between affinity materials and target phosphorylated molecules are highly significant for the design of desired efficient affinity

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materials, however, most of which are still unclear and need more in-depth works.

Along with the advancement of phosphoproteomics analysis, an enormous amount of research efforts will be dedicated to the improvement and development of affinity materials to meet the phosphoproteomics analysis needs. More powerful multifunctional affinity materials are believed to emerge from the integration of the conventional preparation methods and burgeoning technologies (e.g., nanotechnology). To understand the fundamental microscopic enrichment mechanisms and principles could be another issue, and more and more related research will be carried out so as to provide theoretical guidance for the design and fabrication of powerful affinity materials.

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Notes

The authors declare no competing financial interest.

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