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Label-free designed nanomaterials enrichment and separation techniques for phosphoproteomics based on mass spectrometry

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The surface chemical characteristics of nanomaterials have a substantial impact on the affinity probe used to enrich proteins and peptides for MALDI-MS analysis of a real human sample. Detecting phosphoproteins involved in signalling is always difficult, even with recent developments in mass spectrometry, because protein phosphorylation is often temporary from complicated mixtures. This review summarizes current research on the successful enrichment of various intriguing glycoproteins and glycol peptides using surface affinity materials with distinctive qualities such as low cost, excellent structural stability, diversity, and multifunction. As a consequence, this review will provide a quick overview of the scholars from various backgrounds who are working in this intriguing interdisciplinary field. Label-free cancer biomarkers and other diseases will benefit from future challenges.

KEYWORDS

label-free, nanomaterials, MALDI-MS, proteins, peptides

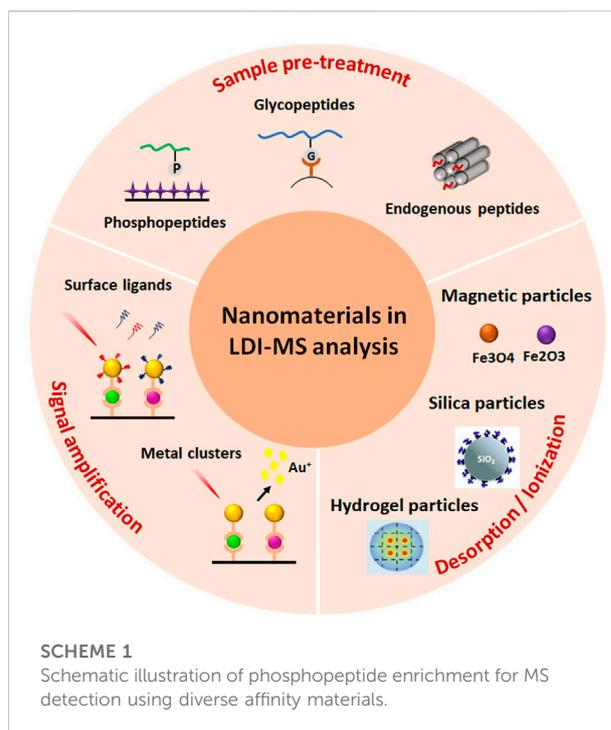
Introduction

Phosphorylation is the main post-translational protein adjustment, influencing signal transduction, quality articulation, cell cycle, cytoskeletal guideline, and apoptosis, among other cell flagging pathways (Wang et al., 2014; Müller, 2018; Ramazi and Zahiri, 2021). Phosphoproteomics has been a prominent study issue since it is critical for interpreting biological changes and discovering new techniques to identify and treat sickness (Arrington et al., 2017; Farooq et al., 2022). Enrichment of objective biomolecules is usually required in today's biological examinations because direct detection of target biomolecules is often difficult due to numerous typical constraints (e.g., low abundance of objective biomolecules, the presence of contaminants) (Angel et al., 2012; Rejeeth et al., 2018). Affinity materials with unique benefits such as reasonable price range, amazing physical consistency, diversity, and functionality have been widely used for enriching objective biomolecules from complicated biological samples based on the peculiar affinity

communication systems between the affinity materials and the objective biomolecules (Limo et al., 2018; Zhou and Pang, 2018). In response of the fast development in material science, particularly the development of nanotechnologies, a variety of excellent affinity materials have been developed to enrich different biological molecules (Ray et al., 2010; Tiambeng et al., 2020). Interestingly, in the field of phosphoproteomics, affinity materials for increasing phosphorylated proteins/peptides are getting a lot of attention (Wang et al., 2015) (Riley and Coon, 2016).

Mass spectrometry (MS) with great specificity and faster data processing has been the most dynamic and crucial tool for phosphoproteomics research in recent years (Iwamoto and Shimada, 2018; Macklin et al., 2020). Regardless, due to significant issues such as the low abundance of phosphorylated proteins/peptides resulting from high heterogeneity and low stoichiometry of protein phosphorylation, the significant impact of high-overflow parts (e.g., nonphosphorylated atoms, lipids, and salts), and the low ionisation productivity of phosphorylated proteins/peptides in MS examination, direct MS-based phosphoproteomics investigation is not recommended (Goel et al., 2018; Bekker-Jensen et al., 2020). As a result, performing preliminary analysis before MS has become a crucial step in efficiently reducing the complexity of proteome preliminaries and concentrating phosphorylated proteins/peptides to improve MS investigation outcomes (Angel et al., 2012). Enrichment analysis prior to MS has become a vital step in successfully lowering the complexity of proteome trials and concentrating phosphorylated proteins/peptides in order to improve MS analysis outcomes up to this point (Nakayasu et al., 2021). The employment of a linker molecule to render metal ions impotent on a porosity bead, polymer bead, or nanomaterial is one of the most extensively used technologies, and great effort has gone into constructing the IMAC material (Colón and Furukawa, 2020). Linkers like iminodiacetic acid and nitrilotriacetic acid are commonly used to chelate metal ions. The attached metal ions were easily washed away during the sample loading and washing procedure due to the comparatively anaemic interaction, dramatically lowering the enrichment efficiency (Rodzik et al., 2020) (Fang et al., 2017).

Unfortunately, intrinsic difficulties such as high-cost antibodies, limited universal applicability, and biological sample decay hamper these approaches (Morofuji et al., 2016; Zhou and Rossi, 2017). Affinity material-based enrichment approaches, on the other hand, have a number of advantages, including universality, low cost, and minimal impact on the structural integrity of phosphorylated proteins/peptides (Wang et al., 2015; Rejeeth et al., 2018; Zhang et al., 2019; Rejeeth et al., 2022). However, affinity material-based approaches still face nonspecific binding of nonphosphorylated proteins/peptides, which can significantly hamper the selective enrichment of phosphorylated proteins/peptides (Delom and Chevet, 2006;

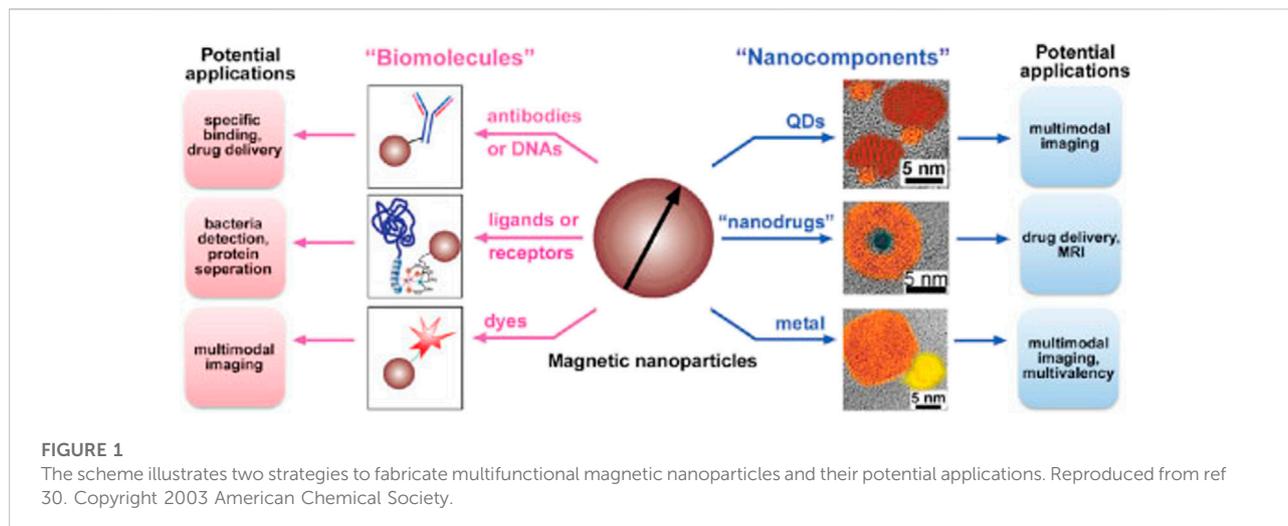


Rainer and Bonn, 2015). Developing high-performance affinity materials remains the most important and broadly used way to improve the enrichment performance of affinity material-based strategies, despite the addition of some auxiliary measures (e.g., chemical treatment of the biological sample, pH directive of loading buffers, and additive addition) (Gabriel et al., 2019; Keeble Anthony et al., 2019).

The present state of affinity materials for the enrichment of phosphorylated proteins and peptides is discussed in this review. The focus will be on the design and creation of affinity materials, as well as the presentation and discussion of relevant enrichment methods. The difficulties and future directions of this discipline will also be highlighted. Because several assessments on specific topics, such as MS technology, enrichment conditions, and enrichment protocols, have already been completed, the details of some related issues, such as MS technology, enrichment conditions, and enrichment protocols, are not presented here.

Affinity-bound nanoparticles: Manufacturing and properties

Biomolecule-conjugated nanoparticles (NPs) have been shown to have promising uses in bioanalysis. The reviewed affinity materials are grouped into three types depending on the species of the affinity site: magnetic-based affinity materials, silica-based affinity materials and hydrogel-based affinity materials (Scheme 1). In the meantime, the enrichment



processes utilised by these affinity materials will be described. In general, the three particles involve the use of various reducing agents, formation management, size preparation, and the discovery of some interesting previous literature reports for medication and gene delivery against various cancers and other ailments.

Magnetic nanoparticles

Magnetic nanoparticles are made up of an inorganic core containing paramagnetic/superparamagnetic structures like magnetite (Fe_3O_4) or maghemite (Fe_2O_3), as well as a shielding layer, which is often made up of polymers and gives colloidal stability and solubility (Gao et al., 2009; Wu et al., 2015). Magnetic nanoparticles (MNPs) have been studied for a variety of uses. In order to provide imaging, treatments, and targeting modalities, MNPs must be encased in biocompatible materials, primarily polymeric ligands containing chemical functional groups to conjugate biomolecules and ligands (See Figure 1).

Due to their unique properties and modalities in fields such as imaging, drug administration, magnetic separation, and purification, magnetic particles, particularly superparamagnetic MNPs, have increased in prominence in biomedical research in recent decades (Ali et al., 2021). Superparamagnetic iron oxide nanoparticles (SPIONs) have been used in tumour imaging, stem cell detection, metastatic breast cancer imaging, Alzheimer's disease detection, lymph node imaging, and many more imaging applications as an intrinsic T2 magnetic resonance imaging (MRI) contrast agent (Cheng et al., 2014; Fatima et al., 2021). Readers can be enticed to look into extensive evaluations in this sector. SPIONs have been used in a variety of applications, including chemotherapeutics, gene transfer, and hyperthermia (Musielak et al., 2019; Suci et al., 2020).

Magnetic separation has significant potential in biological research because it allows for targeted capture that is selective, sensitive, and regulated (Yildiz, 2016). Magnetic separation, which employs an external magnetic field to trap target molecules with MNPs *via* affinity interaction, may be more time, labour, and yield efficient than complex and time-consuming chromatographic separations and purifications (Zborowski and Chalmers, 2011; Musielak et al., 2019). To create a design a superparamagnetic-magnetically responsive only in the presence of an external magnetic field-adsorbent or catcher with high magnetization must be used with proper features such as stability, biocompatibility, and having binding units affinity ligands to capture the target of interest with high specificity and selectivity, and a superparamagnetic-magnetically responsive only in the presence of an external magnetic field-adsorbent or catcher with high magnetization must be used with proper features such as stability (Suci et al., 2020).

The immobilisation of affinity ligands on the surface of particles toward the target proteins is the basis for magnetic particle-based separation of proteins such as antibodies, biomarkers, and enzymes, and a comprehensive list of applications, including homemade and commercial magnetic particles, has been reviewed elsewhere (Mani et al., 2011; Yildiz, 2016). On the other hand, the goal of this article is to discuss recent research in this field. Immobilized metal affinity chromatography (IMAC) has long been a favoured enrichment method in phosphoproteomics (Ndassa et al., 2006). Apart from electronic attraction, IMAC is prepared using metal cations with unoccupied organisation orbitals, and chelation between the immobilised metal cations and the phosphoryl oxygen in phosphorylated proteins/peptides is the primary enrichment mechanism (Balmant et al., 2016). The matrix supports carrying chelating ligands are normally synthesised first, and then the metal cations are immobilised onto the chelating ligands to form IMAC affinity materials. Cu^{2+} , Zn^{2+} , Fe^{3+} , Ga^{3+} , Al^{3+} ,

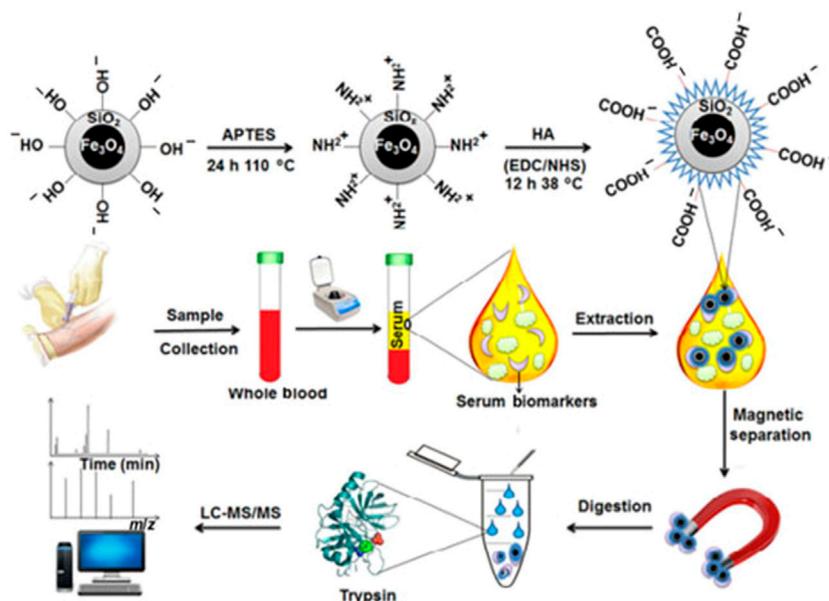


FIGURE 2

Schematic illustration for the preparation of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{HA}$ particles and their application in the extraction and detection of biomarkers from human serum samples by MS. Reproduced from ref 7. Copyright 2018 Springer.

Ti^{4+} , and Zr^{4+} are some of the metal cations that have been employed in IMAC thus far. In traditional IMAC materials, the most frequent metal cations are Fe^{3+} and Ga^{3+} (Ruprecht et al., 2015). Antibody-free approaches are promising and need to be developed for real-case applications in serum to address the limitations of antibody-based techniques in terms of robustness, expense, and throughput. In this work, we demonstrated a novel approach using hyaluronic acid (HA)-modified materials/devices for the extraction, detection, and profiling of serum biomarkers *via* ligand-protein interactions. Figure 2 shown using hyaluronic acid (HA)-modified materials/devices (e.g., $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{HA}$) (Rejeeth et al., 2018).

Due to the unusual coordination specificity of metal (IV)-phosphate chemistry, Zr^{4+} -IMAC and Ti^{4+} -IMAC with phosphate group as chelating ligands displayed greater selectivity for phosphopeptides than regular Fe^{3+} -IMAC beads (Ruprecht et al., 2015). Iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are two chelating ligands that are frequently employed to immobilise metal cations (e.g., Fe^{3+} and Ga^{3+}) (Blankespoor et al., 2005). Sample loading and washing could impair the immobilised metal cations since each metal cation can only bind to one NTA or IDA ligand. Figure 3 discusses, scientists are on the lookout for new chelating ligands with greater chelating ability. As a result, novel, extremely effective chelating ligands such as arsenate (AsO_3^{2-}), phosphate (PO_3^{2-}), adenosine triphosphate (ATP), and dopamine have proved successful in immobilising metal cations, particularly Ti^{4+} and Zr^{4+} (Yan et al., 2013).

As affinity probes, magnetic nanoparticles ($\text{Fe}_3\text{O}_4@\text{Al}_2\text{O}_3$) efficiently concentrate polyhistidine (His)-tagged proteins/peptides from complicated materials (Zhang et al., 2013). This method can be used to quickly determine whether or not a sample contains His6-tagged organisms. Cell lysates containing recombinant Shiga-like toxins labelled with His6 were used as samples to demonstrate that this technology may be used to examine exceedingly complex samples (Schmit et al., 2019). The entire analysis procedure takes around 10 min, including on-plate enrichment and enzymatic digestion, followed by MALDI-MS analysis (Rühl et al., 2017). For similar reasons, magnetic nanoparticles (MNP) with amine functionalization are roughly four times smaller than those previously utilised. Researchers compared the enrichment performance of the magnetic nanoparticle approach to a commercially available glycopeptide enrichment kit offered by EMD Millipore using tandem mass tags from Thermo Scientific (Bodnar and Perreault, 2013). The MNP approach is easy to apply and takes less than 10 min, and it enriches sialylated glycopeptides quantitatively and qualitatively more than a commercially available kit (Bodnar and Perreault, 2013). Another study used magnetic nanoparticles modified with polyethyleneimine (PEI) as a highly selective affinity probe. The particles collected phosphopeptides from a tryptic digest of a protein mixture that contained 0.07% (mole/mole) phosphoproteins, which is the highest selectivity ever observed. In matrix-assisted laser desorption/ionization mass spectrometry analysis, the detection limits of phosphopeptides generated from bovine casein and casein were 5 fmol (Chen et al.,

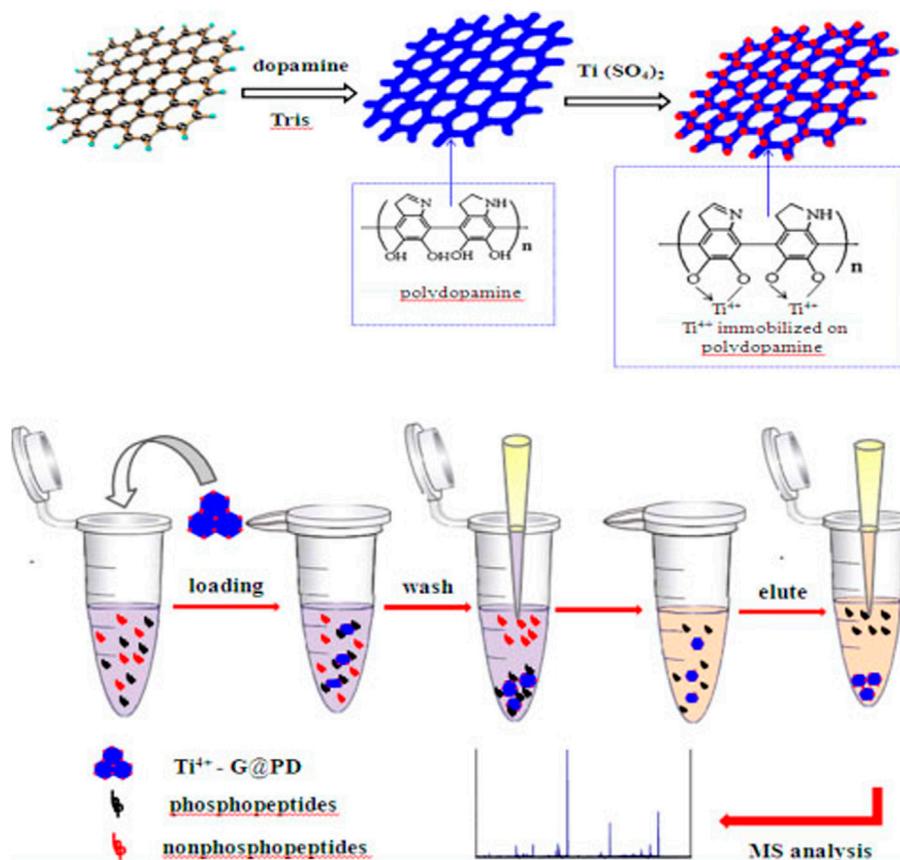


FIGURE 3

The Ti⁴⁺-G@PD with the above unique properties was anticipated to have excellent performance for the selective enrichment of phosphopeptides, which would be highly beneficial for mass spectrometric analysis. Reproduced from ref 45. Copyright 2003 American Chemical Society.

2011). This method was also utilised to boost the amount of phosphopeptides in a non-fat milk protein digest. Using antibodies against target peptides, MNPs could concentrate serum biomarker peptide fragments. MNPs were conjugated to polyclonal antibodies against the tumour necrosis factor (TNF) peptide and utilised to collect TNF from a TNF-spiked blood sample, followed by MS analysis (Dunning and Lame, 2018). These effective applications to single glycoproteins and complete proteome combinations collected from physiological fluids revealed magnetic practical flexibility that is unrivalled.

Silica particles

Two synthesis methods for silica nanoparticles include the Stober process and reverse micro emulsion (Kozłowski et al., 2016). A water in oil (w/o) micro emulsion is formed up of a homogeneous mixture of water, oil, and surfactant molecules (Kozłowski et al., 2016). The Stober method

involves hydrolysis and condensation of siloxane precursors (such as tetraethylorthosilicate TEOS) with ethanol and ammonia to produce silica particles (Kim and Kim, 2002). This method has been used to incorporate a variety of organic colour chemicals. They are classified as spherical, mesoporous, or macrospores in biomedical applications (Borzęcka et al., 2022). Spherical silica NPs, on the other hand, carry cargo through encapsulation or conjugation. Mesopores (pore sizes ranging from 2 to 30 nm) and macrospores are the two types of pores found in mesoporous silica NPs (50–100 nm pore size) (Narayan et al., 2018). Physical and chemical adsorption are frequently utilised to disperse active payloads. The release profile of payloads delivered by porous silica NPs is controlled by chemical linkers or silica matrix degradation, whereas the “gatekeeper” strategy of modifying the inner surface of the pores to control the binding affinity with drugs controls the release profile of payloads delivered by porous silica NPs (Jafari et al., 2019).

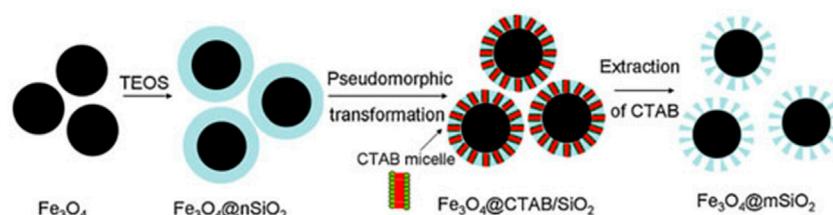


FIGURE 4

Schematic diagram for the preparation of monodisperse $\text{Fe}_3\text{O}_4@m\text{SiO}_2$ microspheres. Reproduced from ref 63. Copyright 2012 Elsevier.

MO modified mesoporous silica composites with various affinity sites were made using simple techniques that took into account the unique qualities of mesoporous silica (e.g., size exclusion effect, high surface area, easy modifiability, and stable skeleton) as well as MO affinity ability (e.g., solgel) (Mamaeva et al., 2013). These compounds can extract phosphopeptides from complicated biosamples quickly and selectively (e.g., human serum). Using mesoporous silica particles, titanium phosphonate was employed to selectively extract phosphopeptides from complicated peptide and protein combinations (Kim et al., 2010). Using MALDI-TOFMS detection, the limit of detection for phosphopeptides from -casein and standard phosphopeptide spikes in human serum was as low as 1.25 fmol (Karayel et al., 2020). The modified mesoporous silica particles were then utilised to enrich phosphopeptides from the serum of patients with hepatocellular carcinoma and healthy persons, which were subsequently evaluated using MALDI-TOFMS. MALDI-TOF MS/MS results for blood phosphopeptide profiling were also confirmed utilising a combination of isobaric tagging for relative and absolute quantitative labelling (Rauniyar and Yates, 2014). The substantial disparities in serum phosphopeptide profiling between cancer patients and healthy people emphasise the technique's utility in cancer detection and biomarker development. This approach could be applied to a wide range of biological samples and disorders. Microspheres with a $\text{Fe}_3\text{O}_4@m\text{SiO}_2$ core and a perpendicularly oriented mesoporous SiO_2 shell ($\text{Fe}_3\text{O}_4@m\text{SiO}_2@m\text{SiO}_2$) were used to develop a novel peptide enrichment approach (Chen et al., 2010). The magnetic receptivity of the $\text{Fe}_3\text{O}_4@m\text{SiO}_2@m\text{SiO}_2$ microspheres is advantageous, as it speeds up and simplifies the enrichment process. The microspheres' highly organised nanoscale pores (2 nm) and high surface areas were shown to have a good size-exclusion effect for peptide adsorption. A $\text{Fe}_3\text{O}_4@m\text{SiO}_2@m\text{SiO}_2$ microsphere was also investigated. The microspheres were used to concentrate endogenous peptides in rat brain extract on a large scale. After enrichment, the sample was examined with an Automated nano-LC-ESI-MS/MS, revealing a total of 60 distinct peptides (Bian et al., 2020). Figure 4 shown the

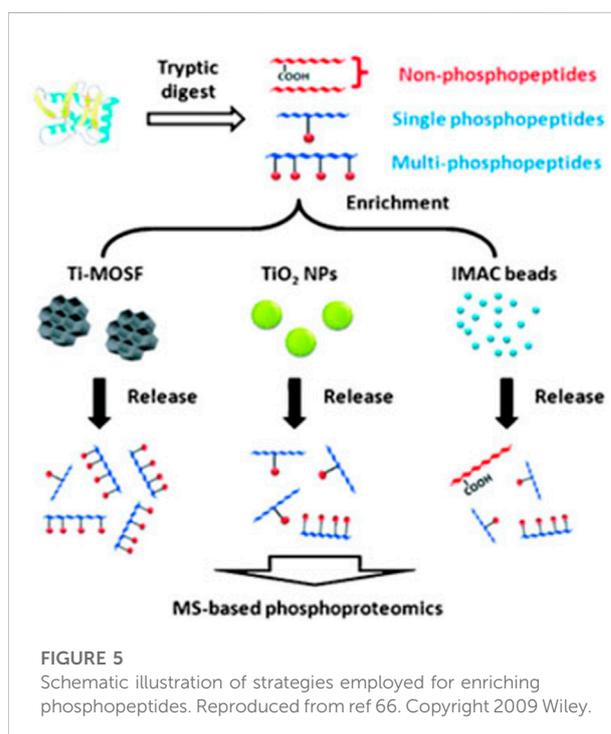
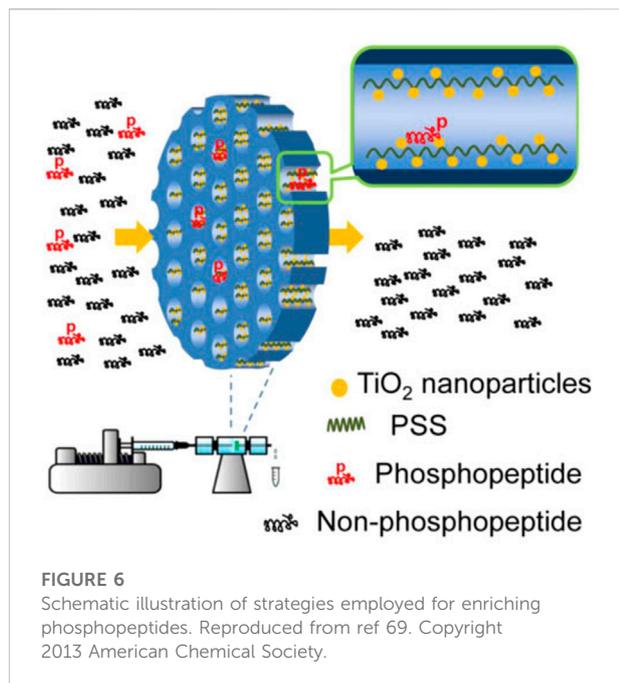


FIGURE 5

Schematic illustration of strategies employed for enriching phosphopeptides. Reproduced from ref 66. Copyright 2009 Wiley.

$\text{Fe}_3\text{O}_4@m\text{SiO}_2@m\text{SiO}_2$ microspheres are a viable contender for selectively separating and enriching endogenous peptides from difficult biological samples due to their simple and low-cost production as well as the convenient and efficient enrichment procedure (Zhu et al., 2012).

Phosphate-imprinted mesoporous silica nanoparticles (MSNs) as a sorbent for phosphopeptide selective enrichment and an offline combination with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) for very efficient protein phosphorylation analysis (Rahi et al., 2016). To make the molecule, a new technology called dual-template docking guided molecular imprinting was applied (DTD-OMI). High phosphopeptide selectivity, interference tolerance, rapid binding equilibrium, and substantial binding capacity were among the attributes of the molecularly imprinted



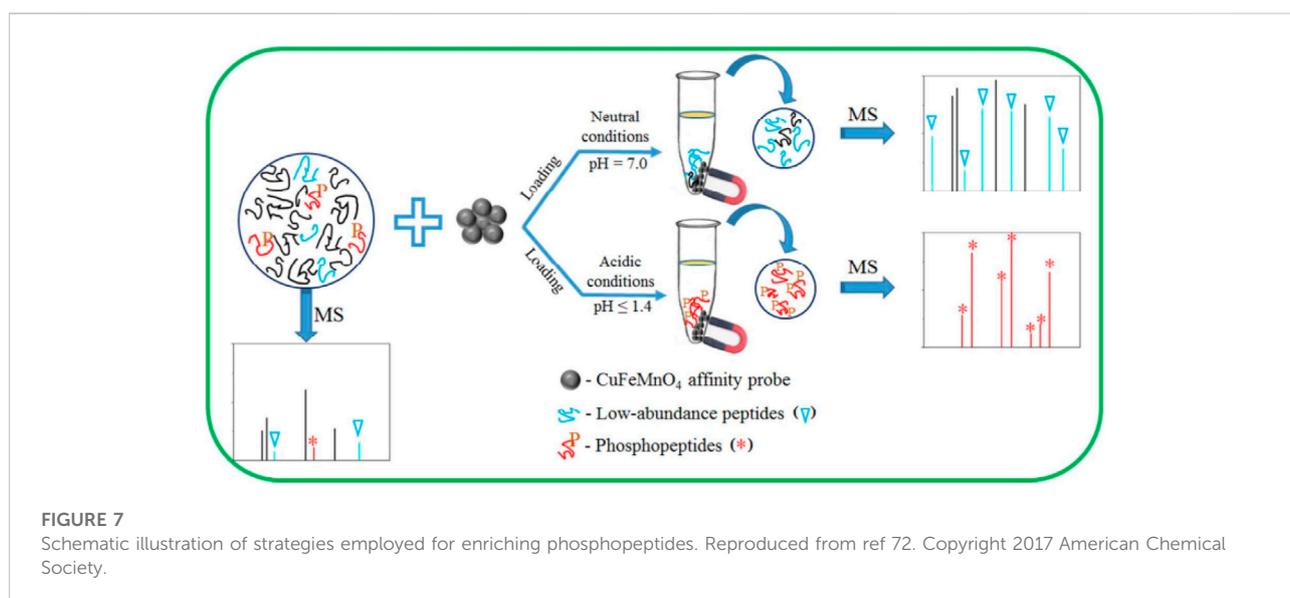
mesoporous material produced, making it an ideal sorbent for selective phosphopeptide enrichment.

Macroporous silica with open big pores outperforms mesoporous silica in terms of MOs loading and subsequent phosphorylated protein/peptide binding to affinity composites. For chromatography, a mechanically stable silica microparticle with macrosized interior pores (1.6 μm particles with 100 nm pores) was developed (Mann et al., 2013). Concanavalin A (Con A) and Aleuriaurantia lectin (AAL) were used to derivatize the material, and the binding capabilities were examined using conventional

glycoproteins. Con A was able to bind 75 g of a common glycoprotein in a 50 1 mm column, demonstrating good binding capacity for microaffinity enrichment. In a 50 1 mm column, Con A was able to bind 75 g of a common glycoprotein, exhibiting good binding capacity for microaffinity enrichment (Mann et al., 2013). Importantly, in some mesoporous materials with lengthy channels, the open macroporous structure can allow effective mass transmission while avoiding the so-called “shadow effect.” Figure 5 shown to make Al-MOSF and Ti-MOSF, Al₂O₃ and TiO₂ were placed onto macroporous ordered silica foam, respectively (MOSF) (Wan et al., 2009).

Hydrogel particles

Using cellulose as a matrix, a new simple synthetic technique for producing magnetic cellulose or nanocomposite microspheres was successfully developed. The cellulose matrix’s micro-nano porous architectures were critical in *in situ* synthesising TiO₂ nanoparticles while preserving TiO₂’s basic structure and character, enabling for enrichment in a hostile environment (Montoya et al., 2011). Due to their strong capture capability, the novel magnetic cellulose/TiO₂ nanocomposite microspheres (MCTiMs) showed high effectiveness for selective extraction of trace phosphopeptides from tryptic digest of alp-casein and human serum samples. With a molar ratio of 1:1000, MCTiMs were used to preferentially concentrate phosphopeptides from alpha-casein and BSA mixtures, which was significantly better than commercial TiO₂ nanoparticles (Kupcik et al., 2019). The Figure 6 shown TiO₂ nanoparticles implanted in the magnetic cellulose microspheres had high specific surface areas and a high adsorbing nature, resulting in a strong Lewis acid-base interaction, according to our findings. As a result, MCTiMs will



be a great choice for phosphopeptide enrichment in complex systems using MS (Tan et al., 2013).

For the first time, magnetic cellulose/TiO₂ nanocomposite microspheres (MCTiMs) were made using a two-step process. In the first stage, magnetic cellulose microspheres (MCMs) were created by microwave heating cellulose/Fe₃O₄ colloidal microdroplets efficiently to induce the sol-gel transition (Luo et al., 2009). MCTiMs were generated in the second step by dispersing MCMs in TiO₂ precursor solution and then generating TiO₂ nanoparticles *in situ* in MCMs' micro-nano pores. Meanwhile, the amount of TiO₂ in MCTiMs may be controlled by changing the precursor solution feeding volume to make MCTiMs-1, MCTiMs-2, MCTiMs-3, and MCTiMs-5, a series of MCTiMs (Duan et al., 2015). Meanwhile, the amount of TiO₂ in MCTiMs may be controlled by changing the precursor solution feeding volume to make MCTiMs-1, MCTiMs-2, MCTiMs-3, and MCTiMs-5, a series of MCTiMs. The growth of a more compact shell layer at a higher level of TBOT resulted in an impediment for TBOT diffusing into the microspheres due to the uncontrolled creation of TiO₂ nanoparticles on the surface of microspheres. The successful application of phosphopeptide enrichment necessitates the use of microspheres with excellent magnetic response qualities. Using an external magnetic field, MCTiMs with a sensitive magnetic response can be swiftly removed from peptide solutions, allowing for convenient recycling. A tryptic digest of alpha-casein, a combination of alpha casein and BSA, and fresh human blood samples were used to demonstrate the selectivity of MCTiMs for phosphopeptides. The process and procedure for phosphopeptide enrichment utilising MCTiMs are proposed. The TiO₂ nanoparticles were firmly attached in the pores of MCTiMs, and the magnetic cellulose matrix's pore wall safeguarded their structure and reaction activity. During the incubation process, phosphopeptides diffused rapidly into the magnetic cellulose microspheres, colliding with TiO₂ nanoparticles and quickly anchoring on the TiO₂ surface *via* Lewis reaction. Enrichment was successful and effective. Based on Lewis acid-base interaction, TiO₂ with a positively charged surface can selectively adsorb phosphorylated species at acidic pH, and TiO₂ with a large surface area has a stronger binding ability on phosphopeptides (Tan et al., 2013).

Magnetic celluloseTiO₂ was developed by Duan's research team for selective phosphopeptide enrichment. Magnetic cellulose microspheres were created using Solgel (MCMs). The transition of cellulose Fe₃O₄ colloidal microdroplets is caused by microwave heating. Then MCMs were distributed in TiO₂ precursor solution and *in situ* manufactured TiO₂ nanoparticles within the porous structure of MCMs to make magnetic celluloseTiO₂nanocomposite

microspheres (MCTiMs) (Long et al., 2017). MCTiMs had their magnetic hysteresis curve reduced, but they still allowed for efficient separation under external magnetic fields. Using the Lewis acid-base method, the phosphopeptides could be efficiently bonded to the nanosphere and separated, as shown in Figure 7.

Conclusion

A wide range of affinity materials for enrichment of phosphorylated proteins/peptides based on diverse enrichment processes have been developed during the last few decades. Despite significant progress, the majority of these materials' applications are still in their infancy. The bulk of these affinity materials were tested utilising similar or even rigid assessment systems (e.g., digest mixes of standard pure phosphorylated and nonphosphorylated proteins, human serum, and nonfat milk), demanding more extensive and precise testing. It is important to note that proper enrichment conditions, such as pH and solvent composition, are also necessary for effective phosphoproteomics uses of these materials. Furthermore, because different affinity materials might have complementary enrichment capability, a mixture of affinity materials can be used when a comprehensive analysis of exceedingly complicated biosamples is required. Furthermore, the interaction mechanisms and concepts between affinity materials and target phosphorylated molecules are critical for the design of desired efficient affinity materials, though the bulk of these are unknown and require further research.

A substantial amount of research will be committed to the enhancement and production of affinity materials to meet the needs of phosphoproteomics analysis, in tandem with the development of phosphoproteomics analysis. More powerful multifunctional affinity materials are projected to come from the combination of established preparation methods and emerging technologies (e.g., nanotechnology). Understanding the basic microscopic enrichment methods and principles could be another challenge, thus further research will be done in this area to provide theoretical guidance for the design and fabrication of powerful affinity materials.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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