1. Introduction

Immobilized metal affinity chromatography (IMAC) was initially developed to purify native proteins with an intrinsic affinity to metal ions.\(^1\) This is achieved by manipulating the coordination chemistry of metal ions, which are immobilized on a solid resin, and the ligand groups on biomolecules with metal-binding ability.\(^2\) IMAC has been used for decades, in
particular to purify recombinant proteins with a polyhistidine tag (His-tag) encoded at either the N- or C- terminus of the target proteins. This technology represents a breakthrough in the purification of proteins and other biomolecules. Bacterial metabolites or metallo-antibiotics, which are compounds that demand metal ions for full functionality as antibacterials and antivirals and exhibit antitumor activities, can be extracted from bacteria and plants because IMAC has the ability to enrich metal-binding molecules. This is exemplified by Cu(II)- or Fe(III)-IMAC, which show success in retaining antibiotics including quinolones, β-lactams, tetracyclines, and aminoglycosides. Besides, multidimensional IMAC, which is composed of serial IMAC columns functionalized with different metal ions, may allow the extraction of multiple components in a single sample entity to facilitate drug discovery processes. For example, using two in-series columns Yb(III)- and Cu(II)-IMAC, bleomycin and desferrioxamine B, two clinically used anticancer agents, have been isolated from *Streptomyces verticillus* fermentation culture with an improved yield, which has reduced environmental and financial costs in processing pharmaceutics and drug discovery.

IMAC has been applied extensively to detect proteins that possess intrinsic metal-binding abilities, facilitating further understanding of the roles of metals in life processes as well as elucidating the mechanisms of action of metalloagents in biological inorganic chemistry. Metalloproteins or metal-binding proteins account for one-quarter to one-third of the proteins in proteomes. Although the identification of metalloproteins and their specific roles in life processes is challenging and warrants further exploration, metalloproteins have been found to be crucial for regulating metal homeostasis in living organisms. Moreover, the use of metallodrugs for therapeutic purposes [e.g., Pt(II)-based anticancer drugs] has received considerable attention. To accelerate the rational design of more metal-based therapeutics and diagnostic agents [e.g., Gd(III)-based magnetic resonance imaging contrasting agents], it is crucial to understand the mechanisms of action of metallodrugs, whereas the identification of potential drug targets at a proteome-wide scale by various approaches including IMAC will provide invaluable information.

In addition, IMAC has been further extended to isolate phosphoproteins and enrich substoichiometric phosphopeptides from proteolytically digested proteins before analysis by mass spectrometry (MS). IMAC combined with MS allows the investigation of phosphoproteomes, which is critical in understanding how cells function as a result of the involvement of phosphorylated proteins in multiple cellular processes: for example, cell signaling, cell–cell communications, and regulating cell division.
This chapter summarizes advances of IMAC in identifying metalloproteomes for a deeper understanding of the mechanisms of action of metallodrugs and the toxicity of certain metal ions. Practical guidance and improvements for the study of protein phosphorylation will also be discussed.

2. PRINCIPLES AND DESIGN OF IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY COLUMNS

The separation principle of IMAC is to use the differential affinities of proteins (or other biomolecules) for immobilized metal ions that influence the separation efficiency. In general, a polydentate metal–chelating group is covalently linked onto the solid resin of IMAC, which binds to a metal ion [e.g., Ni(II), Co(II), Cu(II), Mn(II), Zn(II), Fe(III) and Ga(III)]. Upon interaction with the analyte molecules in the sample, the water molecules in the metal coordination site would undergo an exchange with the surface-exposed amino acid residues of the protein targets or other metal-coordinating molecules. Because the interaction between the metal ions and the residues of proteins is reversible, it can be manipulated to allow the proteins to be adsorbed and desorbed under different conditions. The availability of coordination sites in the metal center for the retention of analyte molecules from cell culture and cellular extract has to be considered when choosing the chelator moieties. Nitrilotriacetate (NTA) and iminodiacetate (IDA) are the most commonly used metal chelators in IMAC, because there are two or three vacant sites available when the octahedral complexes of metal–NTA and metal–IDA, respectively, are formed.

Metal ions, especially transition metal ions, have been covalently linked to the resin of IMAC to identify different metal-binding proteins. The stability of the metal complexes, in particular first-row transition metal complexes, follows a trend known as the Irving–Williams series: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II), which suggests that the most stable complex should be formed by Cu(II) ions and a chelator ligand. Sometimes the target molecules compete with the chelator moiety for the metal ions, possibly removing the immobilized metal ions from the solid resin. Thus, the stability constants of metal–NTA complexes should be noted. The stability constants of the commonly used metal–NTA complexes for IMAC are shown in Fig. 9.1, in which the metal ions are also grouped according to their roles in IMAC analysis (for metalloproteomic or phosphoproteomic studies) discussed in this chapter.
On the other hand, affinities between the interacting amino acids and the immobilized metal ions can be predicted based on the hard–soft-acid–base theory. For instance, the hard metal ions Fe(III) and Ti(IV) show a higher preference toward hard amino acid residues such as the oxygen from aspartate and glutamate residues, whereas borderline metal ions Ni(II) and Cu(II) show considerable affinities toward borderline amino acids such as nitrogen from histidine and soft ligand sulfur from cysteine. Moreover, the coordination number of the immobilized metal ions and the geometry preference should also be taken into account when interpreting the results of IMAC analyses.

Fig. 9.2 illustrates a typical experimental procedure involved in IMAC analyses. Protein mixtures are loaded onto a metal–immobilized column and unbound molecules are washed out. Metal–binding proteins are captured on the IMAC column and then subjected to successive washing steps. Protein targets can be eluted by supplementing a buffer containing a chelate ligand, e.g., imidazole and ethylenediamine tetraacetic acid (EDTA), which displaces the protein targets or elute the bound molecules in concert with removal of the immobilized metal ions from the resin matrix. The metal-binding proteins are then identified through separation by two-dimensional gel electrophoresis (2-DE) according to the isoelectric points (in the first dimension) and the molecular weights (in the second dimension) before peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. Alternatively, on-column trypsin digestion can be performed to analyze the metal–binding peptides.
Figure 9.2 Overview of an immobilized metal affinity chromatography (IMAC) experiment for metalloproteomic analysis. Polydentate metal chelator moiety exemplified by nitrilotriacetate (NTA) is covalently conjugated to the column resin, which is coordinated to a metal ion. The sample is first loaded onto the IMAC column. Proteins bound to the immobilized metal ions are retained on the IMAC column whereas unbound molecules are removed after a few steps of washing. The bound molecules are digested by trypsin on the IMAC column and are recovered based on competitive exchange with a chelator, which are then subjected to mass spectrometry (MS)-based analysis. Alternatively, metal-binding proteins are subsequently eluted and separated by two-dimensional gel electrophoresis (2-DE) and then subjected to trypsin digestion before analysis by peptide mass fingerprinting [matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS)]. m/z, mass to charge ratio.

Further after unbound molecules are washed out, which provides information about the amino acid composition (or binding motifs) in the metal coordination sphere.

3. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY FOR METALLOPROTEOMIC STUDY

IMAC has been regarded as one of the most convenient methods for purifying recombinant proteins, e.g., using an Ni-bound NTA chelating group for the His-tagged proteins, and has been extensively used to purify
proteins from complex biological samples, such as superoxide dismutase, serine carboxypeptidases, and interferon. This technology has facilitated the functional characterization of proteins in biomedical research. Moreover, when the Ni(II) on the resin of IMAC column is replaced by other metal ions, different metalloproteomes in bacteria, mammalian cells, and plant tissues have been identified. To track the metal-binding proteins in samples, any bound metal ions from proteins can be removed by loading the samples onto the chelate resin of the IMAC column before IMAC analysis. A drawback of IMAC analysis is that the metal-binding events cannot be examined in situ. Besides, proteins that possess strong binding affinities (i.e., stronger than NTA and/or IDA) to the metal ions are usually present in their metal-bound form in cell lysate, which will not be captured by IMAC. Nonetheless, IMAC has been widely used as a prefractionation technique in the field of proteomics. Some IMAC-based metalloproteomic research will be summarized in the following sections.

3.1 Mechanism of Toxicity of Plutonium(IV) to Humans

Heavy metal (e.g., arsenic, cadmium, plutonium, lead and mercury) pollution to the environment often occurs as a result of waste from power plants, pesticides, fertilizers, industrialization, and other human activities. In general, these heavy metals possess deleterious effects on human health, such as renal dysfunction, severe damage to the nervous system, and even the development of cancer and its progression. To understand the toxic mechanism of heavy metals, it is crucial to identify proteins that interact with heavy metal ions, further analyze those proteins by protein–protein interactions, and validate the disruption of protein functions before the mechanism of toxicity.

Plutonium has been known to be carcinogenic; it can enter the human body via different routes and is accumulated inside the body. Exposure to plutonium is largely attributed to nuclear power plants. Although Fe(III)-binding proteins human transferrin and ferritin have been found to be associated with Pu(IV), the specific biochemical interactions of Pu(IV) are not fully understood. The causes of diseases relating to plutonium exposure and the potential therapy for treating these diseases remain vague. Using Pu(IV)-NTA IMAC in combination with 2-DE and MS, seven Pu(IV)-binding proteins were identified in mammalian cells. Bioinformatic analysis of the subcellular localization and functions of the putative Pu(IV)-binding proteins revealed that Pu(IV) ions were predominantly accumulated in the cytoplasm of cells whereas the protein targets (e.g., 78 kDa
Table 9.1 Selected Metalloproteomic Studies Using Immobilized Metal Affinity Chromatography (IMAC)

<table>
<thead>
<tr>
<th>IMAC</th>
<th>Sample Type</th>
<th>Results</th>
<th>Comments</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Pu(IV)-NTA</td>
<td>PC12 cells (from rat adrenal gland)</td>
<td>7 potential Pu(IV)-binding cytosolic proteins were identified that were involved in antiapoptosis</td>
<td>Pu(IV) ions were retained on an IMAC column, which facilitated the subsequent analysis without concerning the radioactivity of samples</td>
<td>36</td>
</tr>
<tr>
<td>Cu(II)-IDA</td>
<td>Rice (Oryza sativa L.), B1139 (Cu-tolerant) and B1195 (Cu-sensitive)</td>
<td>27 differentially expressed Cu(II)-binding proteins were found, which elucidates the mechanism of heavy metal stress response and tolerance in plants</td>
<td>Samples were pretreated with a metal-unloaded IMAC column to remove any bound metals on proteins</td>
<td>27</td>
</tr>
<tr>
<td>Bi(III)-NTA</td>
<td>Helicobacter pylori</td>
<td>Proteins involved in various cellular processes were indicated to be potential drug targets, which corroborated the multitargeting mechanism of action of bismuth-based antimicrobials</td>
<td>IMAC served as an excellent method to characterize the biocoordination chemistry of metal in biological samples</td>
<td>49,50</td>
</tr>
<tr>
<td>Co(II)-IDA and Ni(II)-IDA</td>
<td>Streptococcus pneumoniae</td>
<td>IMAC identified Co(II)-binding proteins and Ni(II)-binding proteins that offered insight into metal homeostasis and metal-linked bacterial virulence</td>
<td>IMAC column without metal ions was used as a control to exclude nonspecific binding</td>
<td>25</td>
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glucose-regulated protein precursor and α-crystalline B chain) were correlated with antiapoptotic processes, which suggests that the carcinogenicity of Pu(IV) is attributed to its involvement in cancer development. Moreover, most identified Pu(IV)-binding proteins exhibit metal-binding ability including calcium, magnesium, or divalent transition metal ions; this implies that interaction with Pu(IV) may perturb the coordination of these metals to metalloproteins or may disturb the homeostasis of these metals. Although characterization of Pu(IV)–protein interactions warrants further investigation, this study postulates the mechanism of carcinogenesis of Pu(IV).

3.2 Copper Tolerance in Plants

Minerals, including nonmetal elements such as carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus and metal ions such as potassium, magnesium, and calcium, are essential for the growth and development of plants. These elements are considered to be macroelements, which indicates that they are needed in relatively large quantities. On the other hand, trace elements refer to the mineral nutrients that are required in small amounts, such as chlorine, copper, zinc, and molybdenum. The availability of nutrients varies significantly, depending on abiotic factors such as climate and soil properties as well as all living organisms in the same habitats. Excess metal ions may induce the production of reactive oxidative species, resulting in perturbation of the metal homeostasis. Hence, mineral elements, in particular metal nutrients, have to be tightly regulated because of the potential toxicity exerted by the accumulation of excess metal ions.

IMAC is renowned for examining the changes in metalloproteomes in response to different stress conditions by analyzing the gel images of 2–DE after IMAC enrichment (e.g., appearance or disappearance of certain protein spots, increase or decrease in intensities of protein spots) (Fig. 9.3). Comparative proteomic analysis of copper proteomes in the Cu(II)-sensitive and Cu(II)-tolerant varieties of rice (Oryza sativa L.) by Cu(II)-IDA IMAC and 2–DE enabled the identification of molecular targets. By comparing the intensities of the protein spots on 2-DE gel, more than 20 proteins were found to be expressed differently (either upregulated or downregulated) under copper stress. Among them, 16 proteins have not been previously reported to be involved in copper binding in animals or plants. Functional analysis of the novel copper proteomes indicated that oxidative defense, detoxification, and pathogenesis may be disturbed upon exposure to high copper concentrations, whereas putative cold-shock domain proteins (also known to be RNA chaperones) and proteins involved in translation
Figure 9.3 Comparative proteomic analysis of metal-binding proteins using immobilized metal affinity chromatography (IMAC). Metal-binding proteins of the control and the treated samples are enriched by IMAC, followed by separation in two-dimensional gel electrophoresis (2-DE) and protein identification by mass spectrometry (MS)-based analysis. The difference in expression of metalloproteomes is quantified by comparing the intensities of the protein spots. Further analysis by bioinformatics provides more comprehensive information on the metalloproteomes, such as the interacting partners and the geometry and preferences of amino acid residues at the metal-binding sites. m/z, mass to charge ratio.
initiation were also implicated to be linked to excess copper stress response and tolerance in plants, shedding light on the molecular mechanism of actions of the metal.

3.3 Molecular Mechanism of Bismuth(III)–Based Antiulcer Drugs

Bismuth-based antimicrobials have been used to treat bacterial infections for over 100 years, in particular for the eradication of *Helicobacter pylori* when used with antibiotics as triple or quadruple therapy. Incorporation of bismuth-based drugs into the therapies slowed the relapse rate of *H. pylori* infection in addition to the synergy observed for clinically used bismuth drugs with clarithromycin and tetracycline against antibiotic-resistant *H. pylori* strains. Until now, resistance to bismuth drugs has not been reported for *H. pylori* and other pathogenic microbes. Sustained susceptibility of pathogens to bismuth drugs and the low toxicity of bismuth compounds in humans attributable to the glutathione and multidrug resistance protein transporter-mediated disposition in human hosts aroused further exploration of bismuth-based compounds for antimicrobial and other purposes.

It is believed that proteins and enzymes are the targets of bismuth drugs. To understand the molecular mechanisms of action of commonly prescribed bismuth drugs for accelerating novel drug design, it is important to identify the proteins that bind to the drugs or are differentially expressed. Comparative proteome analysis of *H. pylori*, with and without the clinically used bismuth-based antiulcer drug colloidal bismuth subcitrate, based on 2-DE and peptide mass fingerprinting, revealed several bismuth-binding proteins. Using immobilized-bismuth affinity chromatography (Bi-IMAC), proteins that were significantly upregulated or downregulated were identified from *H. pylori*, including chaperonin HspA and HspB, neutrophil-activating protein NapA, as well as three enzymes (alkyl hydroperoxide reductase TsaA, fumarase, and the urease subunit proteins UreB) and a translational factor (Ef-Tu). This study suggests that bismuth may act against *H. pylori* via multiple functions such as the inhibition of proteases, modulation of cellular oxidative stress, and interference with nickel homeostasis.

A study revealed more than 300 bismuth-binding peptides in *H. pylori* based on Bi-IMAC. Bismuth exhibited much higher selectivity toward peptides enriched with cysteine and histidine residues with CXnC, CXnH, and HXnH as the dominated motifs (Fig. 9.4). Bioinformatic analysis of the
Figure 9.4 Analysis of bismuth-binding proteins/motifs in *Helicobacter pylori* based on immobilized-bismuth affinity chromatography and mass spectrometry as well as the protein–protein interaction network from bioinformatics. Binding sites consisting of cysteine and histidine, with the motifs sequences of CX\(_n\)C, CX\(_n\)H, and HX\(_n\)H, are predominantly identified. The protein–protein interaction network reveals that the metallodrug disturbs multiple cellular processes, elucidating the possible mechanisms of action of the antimicrobial drug. CBS, colloidal bismuth subcitrate. Adapted from Wang, Y.; Tsang, C.-N.; Xu, F.; Kong, P.-W.; Hu, L.; Wang, J.; et al. Bio-coordination of Bismuth in *Helicobacter pylori* Revealed by Immobilized Metal Affinity Chromatography. Chem. Commun. 2015, 51, 16479–16482 © 2016 Royal Society of Chemistry.
Bi-binding proteins disclosed that bismuth perturbs numerous metabolic processes that are critical to the pathogen, including the defense mechanism against oxidative stress, transcription and translation processes, and protein folding. Importantly, proteins involved in transition metal ion binding (e.g., FKBP-type peptidyl-prolyl cis-trans isomerase SlyD, metallo-GTPase HypB, ribosomal proteins RpmE and RpmJ, urease subunit α UreA and urease subunit β UreB, urease accessory subunit UreG) were found to bind bismuth, which implied the potential functional disruption of the metal homeostasis in the pathogen. Indeed, an in vitro study has shown that bismuth inhibited the GTPase activity of \( HpTufA \) and \( HpHypB \).\(^{51,52} \) The study further confirmed the multitargeting mechanism of bismuth drugs through binding to important metalloenzymes and metalloproteins, thus disrupting multiple biological processes that are essential for the survival and pathogenesis of the pathogen.

3.4 Potential Roles of Cobalt(II) and Nickel(II) in Gram-Positive Bacteria

Pathogenic microbes require metal ion as nutrients to stabilize the structures and for the full functions of metalloproteins.\(^53\) Transition metal ions in particular serve as cofactors to activate metalloenzymes that are critical for the survival and pathogenesis of the microbes. On the other hand, toxicity can be induced at high levels of metals in the niche. Pathogenic microbes therefore employ a highly sophisticated system of proteins that regulates the availability of metal nutrients and circumvents the toxicity of metals.\(^54\) By capturing the metal-binding proteins through IMAC and MS-based analysis, 208 Ni(II)-binding proteins and 223 Co(II)-binding proteins from \( Streptococcus pneumonia\) were identified.\(^25\) Although a certain extent of overlapping in the Co(II)- and Ni(II)-binding proteins was observed, distinctive functions of the identified proteins and selectivity on the amino acid residues were revealed. Gene ontology enrichment analysis suggested that Co(II)-binding proteins were involved in structural stabilization of ribosome and interaction with RNA, whereas proteins with ligase or oxidoreductase activity were found to bind Ni(II) ions. Moreover, lysine was exclusively recognized in peptides for Co(II) binding whereas valine and aspartic acid were more frequently associated with Ni(II) ions, despite similar preference in histidine and methionine toward Co(II) and Ni(II) coordination. This metalloproteomic study by IMAC provides a molecular basis for metal homeostasis, and the mechanisms of toxicity of metals to the bacterium are likely caused by the inhibition of essential enzymes.
Posttranslational modifications (PTMs) of proteins, consisting of phosphorylation, acetylation, acylation, alkylation, glycosylation, sumoylation, oxidation, and ubiquitination, can be considered covalent modifications of the side chains of specific amino acids that modulate the functions, cellular localization, and activities of proteins by altering the tertiary and quaternary structures of biomolecules to regulate diverse cellular processes. Approximately 5% of the genomes of eukaryotes encode enzymes that participate in PTMs in proteomes, whereas about 75% of common amino acids may undergo these diversified modifications.

One extensively studied PTM is protein phosphorylation, which involves histidine and aspartic acid in bacterial and fungal proteomes in addition to serine, threonine, and tyrosine in mammalian proteins. Enzymes such as kinases and phosphatases are responsible for the phosphorylation and dephosphorylation of proteins, respectively. Phosphorylation introduces a negatively charged phosphate group to proteins, leading to conformational changes in the local microenvironment of proteins, subsequently resulting in reorganization of a domain that initiates cellular signaling pathways.

Owing to the heterogeneity and scarcity of the modified proteins (about 1%) as well as dynamic modification processes in vivo, enormous effort has been made to detect and enrich specific modifications in cells and cell lysates. To capture phosphoproteomes, different approaches such as strong cation exchange chromatography, strong anion exchange chromatography, and antibody-based immunoprecipitation have been established to separate phosphorylated peptides from nonphosphorylated ones. In addition, considering the nature of phosphate anions, hard metal ions including Fe(III), Ga(III), Zr(IV), and Ti(IV) are loaded to IMAC resin to study phosphoproteomes. The antibody-based method (immunoprecipitation) preferentially isolates targets based on the recognition of the specific amino acid sequences by antibodies, whereas the IMAC technique may be more applicable to phosphopeptides with higher abundance. IMAC is one of the widely used techniques for proteomic analysis because the column resin shows high chemical stability and because of the relatively low cost, which is compatible with commonly used detergents such as Brij35, Tween-20, Tween-80, and Triton-X-100 compared with immunoaffinity-based methods. Moreover, the column resin exhibits high tolerance toward
a wide range of reducing agents, e.g., dithiothreitol, dithioerythritol, and β-mercaptoethanol.

Ti(IV) is highly acidic, with a $pK_a$ of about 0.5; therefore, it signifies a higher affinity toward oxygen, i.e., phosphoproteomes compared with Fe(III) and Ga(III) ions. Besides, differences in redox potential result in the deviation in identified phosphoproteomes when applying different metal ions in IMAC analysis. In contrast, nonspecific capture of peptides with amino acids such as glutamic acid and aspartic acid on the solid resin of IMAC has been reported as a result of the coordination of the carboxylate (R–COO$^-$) with immobilized metal ions such as Ti(IV) ions. Furthermore, hydrophobic interaction with the resin has been noted. To improve specificity, the acidic residues can be modified by methyl esterification before IMAC enrichment; incorporation of organic solvents during sample loading will also minimize nonspecific interaction with the column matrix. Alternatively, metal–oxide affinity chromatography, such as Al(OH)$_3$, ZrO$_2$, and TiO$_2$, can be used.

An overview of IMAC-based phosphoproteomic analysis is illustrated in Fig. 9.5. Because of the small sample volume, and to reduce the sample loss, IMAC columns are often packed in commercial pipette tips (e.g., GELoader tip). With reference to the preparation of a StageTip, enrichment is performed by centrifugation. In general, samples containing phosphoproteins or phosphopeptides are first alkylated via iodoacetamide or esterified via methanol, followed by a desalting step using a C18 reverse-phase column. To minimize nonspecific binding to the IMAC and increase the specificity of the method toward phosphopeptides, the samples are loaded onto an IMAC column in buffers at pH < 3.0 (e.g., 0.1% trifluoroacetic acid and 50% acetonitrile). Under such a condition, acidic peptides in the sample will be neutralized whereas phosphopeptides will retain their negative charge and binding affinities toward the IMAC resins. The phosphopeptides are eluted from IMAC resin using alkaline buffers (e.g., 10% v/v ammonium solution at pH 10–11) or with chelate ligands (e.g., EDTA or ascorbic acid) or buffers containing a high concentration of phosphate.

To overcome the drawback of an IMAC-based phosphoproteomic study, several improvements to IMAC enrichment techniques have been made through the rational design of the experimental setup. First, to improve the matrix material to reduce nonspecific adsorption, monodisperse microspheres made of polystyrene with a uniform size (about 13 μm) and column packing have been made to provide higher chemical stability and column
Figure 9.5 Overview of phosphopeptide enrichment using immobilized metal affinity chromatography (IMAC). Cells are first lysed and esterified by methanol or alkylated by iodoacetamide. The samples are then digested by proteases and desalted by C18 column. Enrichment by IMAC is achieved through centrifugation, after which mass spectrometry (MS) technique is used for qualitative and quantitative analysis. LC, liquid chromatography.

Efficiency as well as a larger surface area of hydrophilic matrix to interact with phosphopeptides.\textsuperscript{69,70} Besides, graphene has been suggested as a matrix material for IMAC, considering the possibility of synthesizing functionalized graphene material with large surface areas.\textsuperscript{71} Second, the orientation of the metal–chelator complexes in the column resin can be optimized by incorporating a flexible linker between the microsphere matrix and the chelator moiety, generating poly(glycidyl methacrylate–co–trimethylolpropane trimethacrylate) microspheres that offer spatial separation between the immobilized Ti(IV) ions and the microsphere matrix.\textsuperscript{70} Third, conventional NTA and IDA chelator moieties can be replaced to enhance metal immobilization. Oxidative self-polymerization of dopamine to form hydrophilic polydopamine has been proposed, owing to its excellent biocompatibility, serving as a potential ligand to coordinate Fe(III) or Ti(IV).\textsuperscript{71,72} Moreover, the use of phosphonate group as the ligand group to bind Ti(IV) ions, with two oxygen atoms from two phosphonate groups coordinating each Ti(IV) ion, has been recommended.\textsuperscript{66} Fourth, the chemical stability of the matrix material is crucial for phosphopeptide enrichment because of the necessity in sample loading and elution under acidic pH (pH < 3.0) to preserve the
negative charge of phosphorylated peptides while protonating the peptides with glutamate or aspartate residues. Trifluoroacetic acid (6% v/v) may be used to provide an acidic condition to restrain nonspecific interaction between acidic amino acid residues and the column resin.

Furthermore, it has been documented that the detection of multiphosphorylated peptides may be interfered by monophosphorylated peptides because of charge differences that contribute to differentiated electrostatic interactions between positively charged metal ions and negatively charged phosphate groups. Moreover, monophosphorylated proteins and multiphosphorylated proteins possess discriminated ionization efficiency, resulting in suppression of signals in MS for multiphosphorylated peptides and acidophilic phosphopeptides by monophosphoproteomes. Multidimensional IMAC, consisting of two or more IMAC columns in sequence, offers a feasible solution for the separation of monophosphorylated and multiphosphorylated peptides in samples to enhance capture efficiency. Sequential elution from IMAC, in which multiphosphorylated peptides are first separated from the monophosphorylated and nonphosphorylated peptides in general, may greatly improve efficiency in identifying phosphorylation sites, especially when analyzing samples with low amounts of sample input. It has been reported that loading samples to Ga(III)-IMAC and subsequently to Fe(III)-IMAC demonstrated a higher efficiency in enrichment. Almost all of the tested multiphosphorylated peptides (>90%) were isolated by Ga(III)-IMAC, predominantly consisting of peptides with acidophilic phosphorylation sites (phosphorylation sites consisting of aspartic acid or glutamic acid). On the other hand, Fe(III)-IMAC exhibited a higher preference toward proline-directed or basophilic phosphorylation sites (sites with arginine or lysine).

An integrated strategy to analyze different PTMs sequentially in a single sample flow has been proposed, enabling the analysis of protein phosphorylation, ubiquitination, and acetylation in succession. The technique used Fe(III)-NTA-based IMAC to enrich phosphoproteomes and antibodies to isolate ubiquitinated and acetylated proteins. Taking advantage of volatile solvents used in phosphopeptide enrichment, it is convenient first to sequester phosphorylated proteins before examining other PTMs by immunoaffinity-based techniques. The serial analysis of several PTMs offers a methodology for understanding signal transduction pathways systemically.

4.1 Development of Immunotherapies

The immune system is sophisticated, constituting of a diverse network of pathways and processes that may affect immune responses. Antigen molecules
(whether self or foreign) are recognized by receptors on the cell surface, after which immune responses are elicited. Balance in the costimulatory and inhibitory signals thus regulates the amplitudes of immune responses. This is exemplified by the actions of cytotoxic T-lymphocyte–associated protein 4 (CTLA4) and cluster of differentiation 28 (CD28). CTLA4 is a receptor protein that suppresses immune response in T-lymphocytes (also called T-cells) whereas CD28 counteracts its action by generating costimulatory signals to activate T-cells. The exact mechanism of T-cell activation–deactivation by CD28 and CTLA4 is obscure; however, it is believed that protein phosphatases SHP2 and PP2A are involved against the actions of kinases initiated by CD28.

Cancer immunotherapies rely on the presentation of cancer-specific antigens to specific receptors to stimulate cytotoxic T-cells to eliminate tumor cells selectively in cancer treatment. Development of immunotherapies has become increasingly popular owing to the potential discrimination between cancerous cells and normal healthy cells. Dysregulation of phosphorylation and dephosphorylation of certain proteins have been revealed to be correlated with some disease conditions, especially in malignant transformation, and have a crucial role in signal transduction in oncogenes and abnormal cell differentiation and growth. In addition, alterations in phosphorylation of proteins are implicated as serving as characteristic biomarkers for cancer progression. Phosphopeptides from various types of cancers, such as ovarian cancer, colorectal cancer, skin cancer, and even leukemia, have been determined. Tumor-specific phosphopeptides have therefore been considered excellent targets for immunotherapies. Methodologies that can effectively segregate and identify phosphopeptides from mammalian cell lines and from clinical samples are urgently demanded.

A study showed that Fe(III)-IDA- and Fe(III)-NTA-based IMAC workflow using a fused silica microcapillary column can be used to enrich a subfemtomolar level of human leukocyte antigen (HLA) class I–associated phosphopeptides from ovarian cancer cells and colorectal cancer tissue. Over 150 and 80 HLA-associated phosphopeptides were uniquely identified in cancerous cell lines and tissue from a cancer patient, respectively. A combination of collision-activated dissociation and electron transfer dissociation for peptide fragmentation has been suggested to preserve the labile phosphorylation sites. The authors also affirmed the use of anhydrous methanol and anhydrous acetyl chloride to esterify acid residues; phosphopeptide standards were included to monitor the capture efficiency and sample recoveries of the proposed protocol. The efficiency of the two chelator moieties (IDA and NTA) was compared, with a higher selectivity when...
Fe(III)-NTA-based setup was used. This implied that NTA might be superior to IDA for capturing phosphopeptides. This protocol will facilitate the enrichment of low-input samples ($10^{-6}$ to $10^{-9}$ g of peptides), enabling the investigation of phosphoproteomes in clinical samples.

4.2 Proteomic Study of Drug Responses

Kinase inhibitors such as SU11274 and staurosporine are clinically effective against various kinds of cancers. The former is an inhibitor of c-Met tyrosine kinase, which competes with the substrate adenosine triphosphate (ATP) to interact with c-Met kinase, whereas the latter originated naturally from the bacterium *Streptomyces staurosporeus* and inhibits binding of ATP to protein kinase C (PKC). c-Met and PKC signaling are highly linked with gastric carcinomas.\textsuperscript{80,81} For instance, c-Met inhibitors may interfere with the signaling in $\beta$-catenin, apoptosis, and the mitogen-activated protein kinase/Erk pathways, disturbing the growth and survival of cancer cells.

The response of SU11274 and staurosporine in human gastric cancer cells investigated by IMAC and immunoaffinity methods has allowed the profiling of PTMs of proteins as well as the analysis of changes in total proteomes.\textsuperscript{82} Total proteome analysis indicated trivial changes in total protein levels; however, considerable deviations in phosphoproteomes were discovered after treatment by the two drugs. Western blotting analysis of proteins Erk1 and Erk2 confirmed the relatively unchanged protein level but a significant reduction in the phosphorylation of Thr185/202 and Tyr187/204 in response to treatment with SU11274, which confirmed the importance of analyzing alterations in PTMs, together with the total protein level, for a better understanding of the biological responses against a drug treatment.\textsuperscript{83} Analysis of the phosphorylated proteomes by Fe(III)-IMAC uncovered as much as 7000 phosphopeptides, whereas about 1500–3500 peptides were yielded by different antibodies. Surprisingly, little overlap in identified phosphopeptides between IMAC and antibody-based methods suggested that both methods were complementary, and it might be beneficial to employ different experimental techniques to obtain complete phosphoproteome coverage. It was also discovered that compared with SU11274, staurosporine led to a larger change in the phosphoproteomes (either an increase or decrease in abundance) upon treatment of gastric cancer cells, which signifies that SU11274 and staurosporine may interrupt different cellular processes despite the similar actions of the two drugs in inhibiting ATP binding to kinases. This study offers a robust label-free quantitative strategy for the analysis of potential
5. NOVEL POLYMER-BASED METAL ION AFFINITY CAPTURE: ALTERNATIVE TO IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Polymer-based metal ion affinity capture (PolyMAC) uses soluble nanopolymers (e.g., polyamidoamine dendrimers) functionalized with metal ions to allow the chelation and subsequent isolation of phosphopeptides in a homogeneous environment. The concept was first proposed in 2005 to capture and characterize phosphorylated proteins, in which soluble dendrimers were used, and was further developed using polyamidoamine dendrimers functionalized with titanium or iron to enrich and analyze phosphopeptides.

Most phosphoproteomic approaches including IMAC have several drawbacks such as low reproducibility and selectivity, largely owing to the heterogeneity of solid-phase extraction-based isolation during phosphopeptide enrichment steps, which makes it difficult to recover low-abundance

Figure 9.6 Schematic illustration of polymer-based metal ion affinity capture for phosphoproteomics. Phosphopeptides are incubated with Fe(III)-functionalized nanopolymers (gray sphere), which are then captured by aldehyde-functionalized agarose beads (green) via amide linkage. Phosphopeptides are finally recovered in alkaline buffer.
phosphopeptides from complex samples. However, the PolyMAC approach overcomes these constraints. PolyMAC-based phosphopeptide enrichment is performed in a homogeneous environment instead of solid-phase extraction, reducing the operation time and improving the selectivity, robustness, and reproducibility of capture processes. Until now, Ti(IV) and Fe(III) have been chelated on soluble polymers to identify phosphoproteomes. As illustrated in Fig. 9.6, in the latest generation of PolyMAC, samples were first incubated with PolyMAC reagents, after which the PolyMAC-bound phosphopeptides were covalently linked to aldehyde-functionalized agarose beads via the linker molecule hydroxylamine to isolate the PolyMAC-coordinated phosphopeptides from the bulk solution. Those captured phosphorylated peptides were eventually retrieved in buffers of alkaline pH (e.g., 400 mM ammonium hydroxide). The selectivity (i.e., ratio of phosphopeptides to the total number of peptides) was doubled using PolyMAC-Fe over Fe-IMAC in the same sample set, which indicated that PolyMAC may offer a powerful and widely applicable technique for phosphoproteomics and molecular signaling.

6. CONCLUSION

IMAC serves as an important proteomic approach and has been used extensively on metalloproteomics and phosphoproteomics in past decades in addition to its wide use in the pharmaceutical industry for drug development. Combined with other techniques, IMAC facilitates the elucidation of mechanisms of action of metallodrugs and metallo-agents as well as an understanding of the role of metal ions in living organisms. Moreover, IMAC is a powerful tool for enriching and isolating phosphopeptides for phosphoproteomics. It provides an insight into the diverse signaling pathways in biological systems. Improvements to IMAC through advancements in the device designs and sample preparation procedures have been under vigorous investigation. Given its high compatibility and simplicity, IMAC coupled with various biochemical techniques as integrative methodologies will have a continuously important role in proteomic and chemical biology research.

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