



ISSN: 0975-766X  
CODEN: IJPTFI  
Review Article

Available through Online  
www.ijptonline.com

## IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Santosh Kumar Vobbilireddi, Rajeesha Surapaneni, Telu Visalakshi, Vijay Kotra\*  
University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Guntur.

Email: vijai.kotra@gmail.com

Received on 08-12-2011

Accepted on 23-12-2011

### Abstract

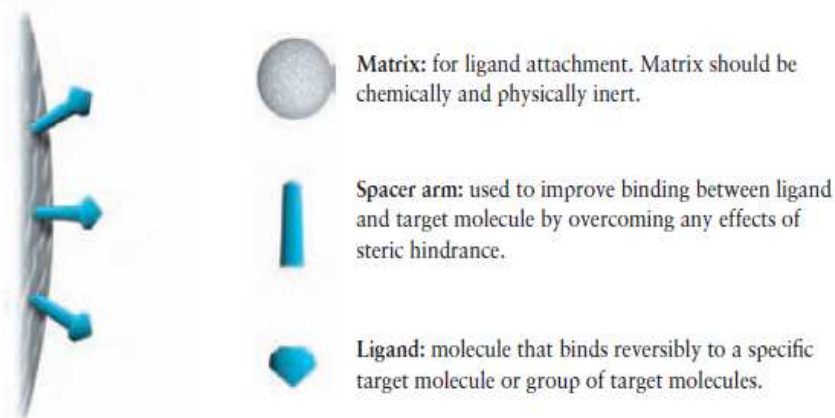
Immobilized-Metal Affinity Chromatography (IMAC) is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins, making use of coordinative binding of some amino acid residues exposed on the surface. Development of IMAC technique was accelerated by the fast maturation of recombinant techniques and by the invention of an improved chelating ligand. IMAC had originally been developed as a group separation method for metallo- and histidine-containing proteins. Today, these features are made use of in proteome-wide studies where the reduction of the complexity of the system (the proteome) is indispensable for sensitive analyses of low-abundance proteins. The IMAC principle offers wide range of applications for research in general and for production of His-tagged proteins in particular. Its robustness and versatility are the reasons why IMAC has become one of the most broadly used chromatographic methods. Modifications in production procedures for both resin and ligand materials as well as optimized application protocols led to a significant improvement of IMAC performance.

**Keywords:** Immobilized metal affinity chromatography; Immobilized ions; Ligands; Protein purification; Histidine tags.

### AFFINITY CHROMATOGRAPHY:

It is a method of separating biochemical mixtures and is based on a highly specific biological interaction such as that between antigen and antibody, enzyme & substrate or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography that sensibly binds to a known subset

Vijay. K \* et al. /International Journal Of Pharmacy&Technology  
of molecules. The method was discovered & developed by Pedro Cutreasas and Meir Wilchece.  
(<https://www.gelifesciences.com/protein-purification>)



**Fig.1: Terms in affinity chromatography**

**Uses:**

Affinity chromatography can be used to

1. Purify & concentrate a substance from a mixture into a buffering solution.
2. Reduce the amount of a substance in a mixture.
3. Discern the biological compounds bind to a particular substance such as drugs.
4. Purify and concentrate an enzyme solution.

**IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC):**

Immobilized-Metal Affinity Chromatography (IMAC) is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins, making use of coordinative binding of some amino acid residues exposed on the surface. As with other forms of affinity chromatography, IMAC is used in cases where rapid purification and substantial purity of the product are necessary, although compared to other affinity separation technologies it cannot be classified as highly specific, but only moderately so.

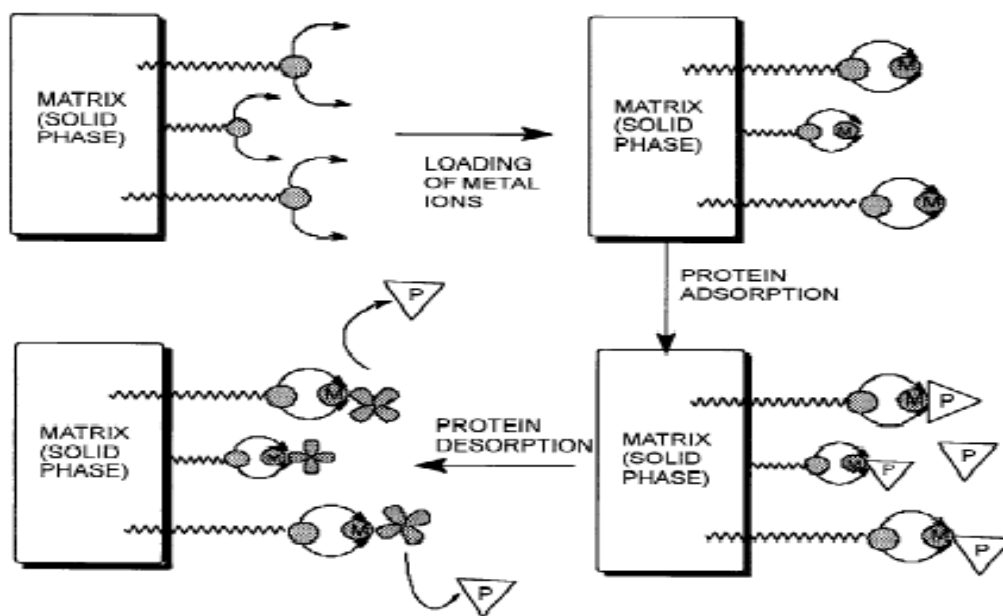
On the other hand, IMAC holds a number of advantages over biospecific affinity chromatographic techniques, which have a similar order of affinity constants and exploit affinities between enzymes and their cofactors or inhibitors, receptors and their ligands or between antigens and antibodies. The benefits of IMAC—ligand stability, high protein loading, mild elution conditions, simple regeneration and low cost<sup>1</sup> are decisive when developing large-scale purification procedures for industrial applications.

Everson and Parker<sup>2</sup> were the first who adapted immobilization of chelating compounds to the separation of metalloproteins. The method became popular through the research work of Porath<sup>3,4</sup> and Sulkowski who laid the foundations of the technique that is widely used today and applicable for a variety of purposes, including analytical and preparative purification of proteins, as well as being a valuable tool for studying surface accessibility of certain amino acid residues. Initially, IMAC techniques were used for separating proteins and peptides with naturally present, exposed histidine residues, which are primarily responsible for binding to immobilized metal ions. However, the work of Hochuli et al pioneered the efficient purification of recombinant proteins with engineered histidine affinity handles attached to the N- or C-terminus, especially in combination with the Ni (II)-nitrilotriacetic acid (Ni-NTA) matrix, which selectively binds adjacent histidines. Since numerous neighboring histidine residues are uncommon among naturally occurring proteins, such oligo-histidine affinity handles form the basis for high selectivity and efficiency, often providing a one-step isolation of proteins at over 90% purity.

Another distinct advantage of this kind of IMAC over biospecific affinity techniques is its applicability under denaturing conditions. This is often necessary when recombinant proteins are highly expressed in *E. coli* in the form of inclusion bodies. When appropriate cleavage sites are engineered between the affinity tags and proteins, with the purpose of enabling effective and precise tag removal after the main isolation step, IMAC seems to be an ideal solution for many applications. However, for the production of therapeutic proteins in substantial quantities, multiple operational cycles with high reproducibility are required. To this end, the principles of the method have been studied intensively, and numerous modifications have been made for specific purposes.

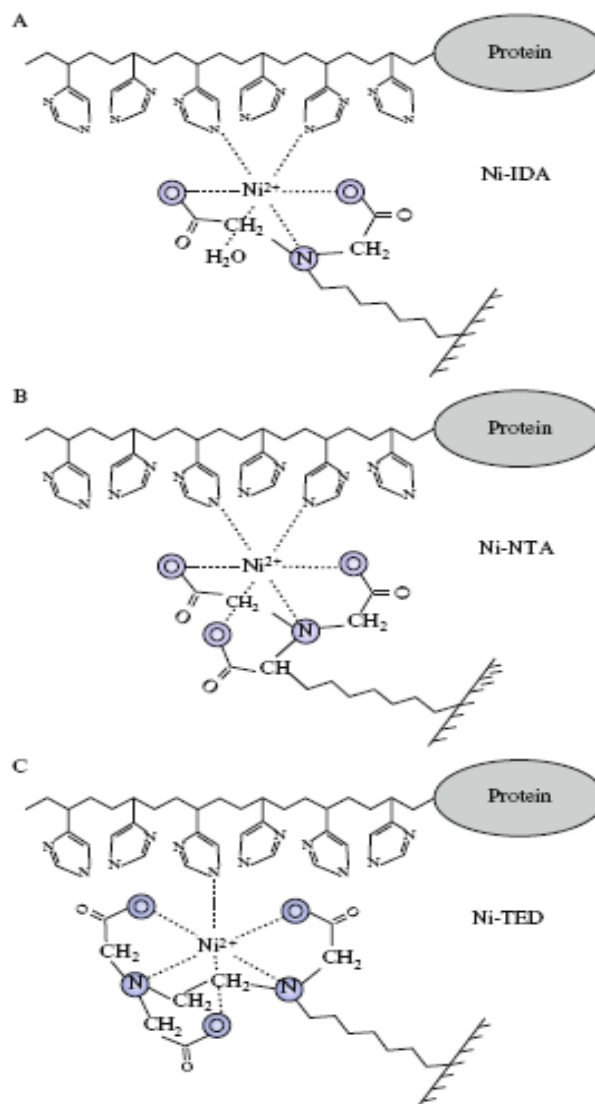
## OVERVIEW ON IMMOBILIZED IONS AND LIGANDS:

Development of IMAC technique was accelerated by the fast maturation of recombinant techniques and modern molecular biology in the late 1970s and by the invention of an improved chelating ligand, nitrilotriacetic acid (NTA) in the 1980s. In the meantime, the purification of recombinant proteins genetically modified on the DNA template level in order to generate oligohistidine extended (His-tagged) polypeptides using NTA based supports represents the most important application of IMAC<sup>5</sup>. The principal mechanism of the interaction of a His-tagged protein to an immobilized metal ion is presented in Fig.2



**Fig.2 : Mechanism of action**

A possible model is that of an interaction of a metal ion with histidine residues  $n$  and  $n+2$  of a His tag. This is confirmed by the fact that IMAC ligands can bind to His tags consisting of consecutive histidine residues as well as to alternating tags. The NTA ligand coordinates the  $Ni^{2+}$  with four valences (tetradentate, coordination number 4) highlighted spherically in Fig.3 and two valences are available for interaction with imidazole rings of histidine residues.



**Fig.3: Model of the interaction between residues in the His tag and the metal ion in tri- (IDA), tetra- (NTA), and pentadentate IMAC ligands (TED).**

Choice of the metal ligand is through variation of the structure of the chelating compound, by variation of the spacer arms, ligand density, concentration of salts and competing agents, etc. For example, in the case of human growth hormone, reduction of ligand IDA–Cu (II) density on chelating sorbent resulted in higher protein purity and increased yield. The apparent affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination. In the case of the iminodiacetic acid (IDA) chelator, the affinities of many retained proteins and their respective retention times are in the following order: Cu (II) > Ni (II) > Zn (II) ≥ Co (II). In contrast to these

currently most commonly used metal ions, which have a preference for extra-nitrogen-containing amino acids, hard Lewis metal ions, such as Al(III), Ca(II), Fe(III), Yb(III), prefer oxygen-rich groups of aspartic and glutamic acid or phosphate groups and this provides an opportunity to engineer new affinity handles, based on glutamic- or aspartic-acid-rich affinity tails. IDA is by far the most widely used chelating compound.

Recently, an interesting application of IDA–Cu (II) polysulfone hollow-fibre membrane for fractionation of the commercial pectic enzyme has been reported. Pectinlyase, useful for large-scale fruit-juice clarification, passed through the membrane, while pectinesterase, responsible for undesirable methanol production, was retained. Cibacron Blue F3GA was covalently bound to micro porous poly (2-hydroxyethyl methacrylate) membrane and charged with Fe (III) ions to study adsorption or desorption behavior of various pure proteins. The same type of membranes was used for immobilization of glucose oxidase and the potential for the construction of glucose biosensors was demonstrated. Example of novel IMAC stationary phases are various metallo protoporphyrins, covalently bound to silica supports, which have demonstrated a fundamental advantage of stable metal binding over the normally used stationary phases with weakly anchored metal ions<sup>6</sup>.

#### **DETECTION AND IMMOBILIZATION:**

In efforts to make use of the specificity and high affinity of His-tagged proteins to immobilized metal ions, IMAC ligands have been employed for applications such as protein: protein interactions where proteins need to be stably immobilized on surfaces. Two applications — ELISA as a diagnostic tool and chip-based technologies for functional investigations — shall be briefly described. Ni-NTA ligands attached to surfaces of microtiter plates are used to immobilize His-tagged antigens in its soluble and structurally intact form for serological studies. The directed immobilization via the His tag can be an advantage to standard ELISA where proteins are randomly adsorbed to plastic surfaces which destroys the protein structure and hides part of the protein surface and possible antibody-binding sites. In contrast, IMAC based ELISA allows the screening of conformation-dependent monoclonal antibodies and immunosorbent assays with increased sensitivity<sup>7</sup>.

Immobilization of His-tagged proteins on chip surfaces for interaction studies with other molecules, for example by SPR, is a widely used protein characterization method. The factor “stability” is important to reduce “bleeding” of the immobilized molecule, and therefore, due to its favorable binding features described above the NTA ligand is frequently used for immobilization applications. However, interaction of biotinylated proteins to supports coated with streptavidin is still considerably stronger. A significant improvement in the stability of functional immobilization of His-tagged proteins on glass-type surfaces even at low concentration was achieved by the concept of multivalent chelator heads where a single ligand molecule carries three NTA moieties. This development represents a valid alternative to streptavidin/biotin-based protein immobilization and allows the use of His-tagged proteins without the need for biotin-labeling of proteins following purification.

IMAC ligands have also been used successfully as reporter in immunoblot type of applications replacing an antibody. His-tagged proteins transferred to nitrocellulose membrane (Western or dot blot) can be detected by probing with Ni-NTA conjugated to alkaline phosphatase or horseradish peroxidase reporter enzymes in a chromogenic or chemiluminescence reaction or to quantum dots for fluorescent detection . This represents an attractive fast and economic alternative to antibody-based detection reactions in cases where the high specificity of an antibody is not required. The specificity of NTA conjugate-based detection has been increased by generation of tris-NTA conjugates<sup>8</sup>.

### **PROTEIN PURIFICATION:**

IMAC had originally been developed as a group separation method for metallo- and histidine-containing proteins. Today, these features are made use of in proteome-wide studies where the reduction of the complexity of the system (the proteome) is indispensable for sensitive analyses of low-abundance proteins. Consequently, pre separation methods such as liquid, reverse-phase, ion-exchange, and affinity chromatography—such as IMAC—have gradually been used in proteomics to enrich proteins that may otherwise be lost in detection<sup>9</sup>. The application of IMAC in proteomics has recently been reviewed and is focused on the enrichment of phosphoproteins and phosphopeptides and on metal-binding proteins. In the enrichment step, a complex sample such as a cell lysate or

blood is passed over the IMAC matrix, washed and the fraction of interest eluted by variation of pH or with high concentrations of imidazole. This fraction is then analyzed by mass spectrometry (MS) or fractionated further by two dimensional gel electrophoresis followed by MS or by additional liquid chromatography coupled to MS (LC-MS).

IMAC can also be used to bind and separate at least mono- and dinucleotides based on a complex phenomenon accounted for by differential interplay of affinities of the potential binding sites (oxygen in the phosphate group, nitrogen and oxygen on the bases, hydroxyl groups on the ribose) to the immobilized metal. A quite different group-specific separation application of IMAC is represented by the affinity of antibodies to immobilized metal ions. As the molecular basis for this interaction an endogenous metal-binding site on the heavy chain and an arrangement of histidine residues on the antibody have been discussed. Adsorption of immunoglobulins from different sources on IMAC matrices has been reported by many authors (human IgG, humanized murine IgG, goat IgG. Antibody purification has been successfully performed using various IMAC formats including gels, methacrylate polymer, and membranous hollow fibers. The mild elution of the protein with salts, costs, and the robustness of IMAC matrices have been identified as advantageous over traditional protein A or G chromatography.

#### **Native surface histidines:**

Numerous natural proteins contain histidine residues in their amino acid sequence. However, histidines are mildly hydrophobic and only few of them are located on the protein surface. For proteins with known 3D structure, data about the number and arrangement of surface histidine residues can be obtained from protein data banks. This can also serve as a basis for forecasting their behavior in IMAC. In the coming years, with the development of proteomics, the number of proteins with known primary structure is bound to grow much faster than the number of 3D structures resolved, but structure modeling, based on the known primary amino acid sequence, will also become more useful and more accurate. However, until now, no data from systematic searches of the protein data bases regarding surface histidines have been published. For use in IMAC, protein-surface histidine residues must also be accessible to the metal ions and their bulky chelating compounds. Adjacent histidines can bind to the same or different chelating sites. Usually, one histidine is enough for weak binding to IDA-Cu(II), while more proximal



histidines are needed for efficient binding to Zn(II) and Co(II). Some interesting examples of using IMAC for proteins with naturally exposed histidine residues include human serum proteins, interferon, lactoferrin and myoglobin, tissue plasminogen activator, antibodies, and yeast alcohol dehydrogenase. In general, a positive correlation is found between the number of accessible histidines and the strength of binding. Separation of gamma-chymotrypsin, a common contaminant in commercial alpha chymotrypsin, was achieved by IMAC, due to various number of surface histidines, indicating possible industrial application.

Evolutionary variants of the lysozymes show varied affinities for IMAC matrices due to differences in the surface topography of histidines. On the other hand, albumins contain up to 16 histidine residues in their structure but only one high-affinity binding site His3 at the N-terminus. Recently, human serum proteins have been used for testing new IMAC affinity ligands.

#### **Histidine tags:**

Although the first demonstrations of IMAC were low-resolution group separations, the resolution has significantly improved with the use of genetically engineered affinity tags that can be attached to amino or carboxy terminals of the recombinant proteins. The first histidine-rich fusions were made on the basis of the high affinity of certain natural proteins containing histidine residues near the N-terminus. Recently, a natural amino acid sequence, located on the N-terminus of chicken lactate dehydrogenase, has been described which is responsible for efficient binding to Co (II) -carboxymethylaspartate IMAC<sup>10</sup>. The natural peptide contains six histidines, unevenly interleaved by other amino acid residue. Its truncated version, designated as histidine affinity tag HAT was fused to the N-terminals of three recombinant proteins to demonstrate its utility as a purification tag. In the past, numerous histidine tags were employed, from very short ones, e.g., HisTrp, utilized for isolation of sulfitolized proinsulin to rather long extensions, containing up to eight repeats of the peptide Ala-His-Gly-His-Arg-Pro, attached to various model proteins. However, today by far the most widely used histidine tags consist of 6 consecutive histidine residues. An ideal affinity tag should enable effective but not too strong a binding, and allow elution of the desired protein under mild, nondestructive conditions. In the case of recombinant *E. coli*, many host proteins strongly adhere

to the IMAC matrices, especially when charged with Cu (II) or Ni (II) ions, and are eluted with the target proteins.

Therefore, new approaches for selecting improved histidine tags have focused on elution of the target protein in the contaminant-free window.

An approach in achieving selective adsorption of engineered oligo-histidine tagged proteins with minimal interference of host cell proteins involves “tailor-made” chelating supports with very short spacer arms and low surface density of chelating groups.

Histidine tags seem to be compatible with all expression systems used today. Thus, His-tagged proteins can be successfully produced in prokaryotic and eukaryotic organisms, intracellularly or as secreted proteins. The use of long histidine tags in *E. coli* cells may reduce the accumulation level or induce the formation of inclusion bodies of otherwise soluble protein, unpublished results. However, which position is preferable for the addition of His tag, N- or C-terminus, depends on the nature and intended use of the protein, and must be determined experimentally. Addition of His tag to the N-terminus of the protein appears to be more universal, if judged from the huge number of cases reported. Most likely, N-terminal tagging is more frequently used because several efficient endoproteases are available for precise cleavage of the tag after purification. Histidine tagging and IMAC have become a routine for easy first-time isolation of newly expressed proteins. In most cases, the histidine tags neither affect protein folding, nor interfere significantly with the biological functionality.

#### **ADVANTAGES OF IMAC TECHNIQUE<sup>12</sup>:**

1. IMAC chromatography has the advantage of enabling histidine -tagged proteins to be separated efficiently in the presence of denaturing concentrations of urea or guanidine-HCl. Additionally, affinity tagging by consecutive histidines offers the possibility of efficient purification.
2. Due to the relatively high affinity of histidine tags for special IMAC matrices these can serve for the reversible immobilization of proteins.

3. When the histidine tail is extended to 10 residues, stringent washes at high imidazole concentrations result in more efficient removal of host cell proteins. IMAC columns are relatively efficient for virus removal, and reduction of 4.0 log to 6.5 log for different viruses has been demonstrated during Co-chelate chromatography of factor IX.

#### **DISADVANTAGES OF IMAC TECHNIQUE<sup>12</sup> :**

1. Several amino acids, especially histidine, lysine, cysteine, proline, arginine, and methionine, are susceptible to metal-catalyzed oxidation reactions that produce highly reactive radical intermediates which can damage a variety of proteins.

2. With every IMAC column some leaching of metal ions occurs, depending on the type of chelating compound involved and the sort of elution.

3. Since (histidine ligand chromatography), in which immobilized histidine functions as a pseudo affinity tag, can be used for selective binding of lipo polysaccharides and effective pyrogen removal, it is reasonable to assume that histidine affinity tags could also bind pyrogens. Thus, contamination of the target protein with pyrogens would occur, and this is not acceptable for pharmaceutical proteins.

#### **APPLICATIONS**

1. An immobilized metal-ion affinity chromatography (IMAC) method has been developed and validated for the separation of copper complexing ligands from soil solution. IMAC is an effective tool for the fractionation of copper complexing ligands that are capable of forming ternary complexes<sup>13</sup>.

2. Protein phosphorylation is a dynamic post-translational modification that plays a critical role in the regulation of numerous cellular events including signal transduction, gene expression, and apoptosis. Phosphopeptides are often found in low natural abundance and ionize poorly, complicating their identification by mass spectrometry. Immobilized metal affinity chromatography (IMAC) is a tool that has been developed to aid in the isolation and subsequent identification of phosphorylated molecules. A novel silica matrix for IMAC applications utilizing a proprietary nitrilotriacetic acid (NTA) analog has been developed and its use optimized after being complexed with gallium<sup>14</sup>.

3. Many of the most widely employed operations in molecular biology hinge upon the use of single stranded DNA as a probe or template. A straight forward method is to produce long single stranded DNA molecules using the polymerase chain reaction (PCR) in combination with immobilized metal affinity chromatography (IMAC)<sup>15</sup>.
4. The resolution of an  $\alpha$ -fetoprotein from serum albumin should aid the purification of a-fetoprotein from a biological fluid containing overwhelming quantities of albumin, for example, serum. Importantly, the separation of human a-fetoprotein from human serum albumin may improve and help maintain the accuracy of immunoassays for a-fetoprotein, making the Chromatography on immobilized Ni<sup>2+</sup> a valuable diagnostic tool<sup>16</sup>.
5. Screening for proteins that yield high recovery after IMAC, both after small-scale and large-scale expression, improves the selection of proteins that can be successfully purified and will yield a crystal structure.
6. Immobilized metal affinity chromatography (IMAC) is a commonly used technique for phosphoproteome analysis due to its high affinity for adsorption of phosphopeptides. Miniaturization of IMAC column is essential for the analysis of a small amount of sample. Nano scale IMAC column was prepared by chemical modification of silica monolith with iminodiacetic acid (IDA) followed by the immobilization of Fe<sup>3+</sup> ion inside the capillary. IMAC capillary column coupled with  $\mu$ RPLC-nanoESI MS was very suitable for the phosphoproteome analysis of minute sample<sup>17</sup>.
7. Addition of His<sub>6</sub> tag to the Hepatitis B virus core antigen(HBcAg), expressed in *E. coli*, enabled purification under milder denaturing conditions by Ni-NTA at high pH. Contaminating *E. coli* proteins and DNA were completely removed. This was otherwise impossible by standard sedimentation of virus-like particles in sucrose gradients.
8. A malaria-transmission-blocking vaccine candidate, based on the *Plasmodium falciparum* predominant surface protein Pfs25 with a His<sub>6</sub>-tag at the carboxyl terminus, was produced by secretion from *Saccharomyces cerevisiae* and purified on a large scale by Ni-NTA. Histidine-tagged protein exhibited higher potency and antigenicity than the original Pfs25. This indicates that in some cases vaccination with His-tagged proteins may be even advantageous.

9. Expanded-bed adsorption (EBA) techniques constitute another broad field of IMAC application and require additional properties of column matrix, e.g., higher particle density and high resistance to harsh conditions during column cleaning or sanitization.

## Conclusion

The IMAC principle offers wide range of applications for research in general and for production of His-tagged proteins in particular. Its robustness and versatility are the reasons why IMAC has become one of the most broadly used chromatographic methods. Modifications in production procedures for both resin and ligand materials as well as optimized application protocols led to a significant improvement of IMAC performance in the recent past, well reflected by, for example, the increase of binding capacity of both NTA- and IDA-based matrices for His-tagged proteins from 5–10 to 50 mg per ml resin bv. We anticipate a continued methodological improvement and dissemination of the use of IMAC in the field of purification of recombinant proteins, for example, regarding industrial- scale production of biopharmaceuticals.

## References

1. Arnold FH. Metal-affinity separations: a new dimension in protein processing. *Bio/Technology* **1991**; 9:150–5.
2. Everson RJ, Parker HE. Zinc binding and synthesis eight-hydroxy-quinoline agarose. *Bioinorg Chem* **1974**; 4:15–20.
3. Porath J, Carlsson J, Olsson I, Belfrage G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **1975**; 258:598–9.
4. Porath J, Olin B. Immobilized metal ion affinity adsorption and immobilized metal ion affinity chromatography of biomaterials: serum protein affinities for gel-immobilized iron and nickel ions. *Biochemistry* **1983**; 22:1621–30.
5. Hochuli, E, Do beli H, and Schacher A. New metal chelate adsorbent for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **1987**; 411: 177–184.

6. Xiao J, Meyerhoff ME. Retention behavior of amino acids and peptides on protoporphyrin-silica stationary phases with varying metal ion centers. *Anal Chem* **1996**; 68:2818–25.
7. Padan E Venturi, M Miche, H, and Hunte C. Production and characterization of monoclonal antibodies directed against native epitopes of NhaH, the Na antiporter of E. coli. *FEBS Lett.* **1998** ;441: 53–58.
8. Lata, S, Gavutis, M, Tampe, R, and Piehler, J. Specific and stable fluorescence labeling of histidine-tagged proteins for dissecting multi-protein complex formation. *J. Am. Chem. Soc.* **2006**; 128: 2365–2372.
9. Lee H, Lee W C. Applications of affinity chromatography in proteomics, *Analytical Biochemistry* **2004**;324: 1–10
10. Garcia Gonzalez, L A, Rodrigo Tapia J P, Sa´nchez Lazo P, Ramos S, and Sua´rez Nieto C. DNA extraction Using Chelex resin for the oncogenic amplification analysis in head and neck tumors. *Acta Otorrinolaringol. Esp.* **2004**; 55: 139–144.
11. Chaga G, Bochkariov DE, Jokhadze GG, Hopp J, Nelson P. Natural poly histidine affinity tag for purification of recombinant proteins on cobalt(II)-carboxymethylaspartate crosslinked agarose. *J Chromatogr, A* **1999**; 864:247–56.
12. Gaberc-Porekar V, Menartr J V. Perspectives of metal-affinity chromatography. *Biochem. Biophys. Methods* **2001**; 49:335–360.
13. Paunovic et al. *J. Chromatogr. A* Evaluation of immobilized metal-ion affinity chromatography for the fractionation of natural Cu complexing ligands **2005**;1100: 176–184.
14. Jeffrey L Turner, Justin Wildsmith, Judy Boland, Jessica Moeller-Gaa, Kevin Ray, John G Dapron, Graham B I Scott. A Novel Silica-Based Immobilized Metal Affi nity Chromatography (IMAC) Technology for the Enrichment of Phosphopeptides. *Sigma elorich* **2006**.
15. Changhee Min, Gregory L Verdine. Immobilized metal affinity chromatography of DNA, *Nucleic Acids Research*, **1996**;24:3806–3810.

16. Lennart Andersson, Eugene Sulkowski and Jerker Porath. Facile Resolution of  $\alpha$ -Fetoproteins and Serum Albumins by Immobilized Metal Affinity Chromatography, *Cancer research* **1987**; 47: 3624-26.
17. S. Feng et al. Fe31 immobilized metal affinity chromatography with silica monolithic capillary column for phosphoproteome analysis, *Proteomics* **2007**; 7: 351–360.

**Corresponding Author:**

**Vijay. K\***

**Email:**[vijai.kotra@gmail.com](mailto:vijai.kotra@gmail.com)