



Review

Current and prospective applications of metal ion–protein binding

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Abstract

Since immobilized metal ion affinity chromatography (IMAC) was first introduced, several variants of this method and many other metal affinity-based techniques have been devised. IMAC quickly established itself as a highly reliable purification procedure, showing rapid expansion in the number of preparative and analytical applications while not remaining confined to protein separation. It was soon applied to protein refolding (matrix-assisted refolding), evaluation of protein folding status, protein surface topography studies and biosensor development. In this review, applications in protein processing are described of IMAC as well as other metal affinity-based technologies.

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1. Introduction

In the mid 1970s, Porath et al. [1] introduced a new type of chromatography which was first designated “metal chelate chromatography”, but was later termed “immobilized metal (ion) affinity chromatography” (IMAC). While the basis of this method was not new [2], Porath et al. [1] focused on its use for the separation and isolation of proteins. The technique is based on differences in the affinity of proteins for metal ions bound to a metal-chelating substance which is immobilized on a chromatographic support (resin) [3]. This “bio-affinity” feature soon caught attention of biochemists and biologists who employed IMAC and enhanced it to suit their particular applications. The best known improvement is the application of histidine tags for separation of recombinant polypeptides [4–14], which consists of the insertion of a short tail of histidine residues on either the N- or C-terminus of a protein or peptide [15]. The use of such histidine and other metal affinity tags in IMAC [19–24] proved to be a powerful tool for protein recovery, especially when high production and maximum yield of the proteins of interest were essential [25]. Consequently, IMAC emerged as one of the major preparative methodologies used for protein purification, ranging from bench [4–14] to pilot/industrial scale [16–20,22–32].

Many reviews on IMAC have been published so far [33–41] some of them dealt with new IMAC applications and even introduced new possible territories where IMAC could be exploited. In light of the new IMAC applications this review tried to go deeper into this new dimension of IMAC based techniques and their implications in life sciences.

Whereas many of the recently devised metal affinity-based methodologies in protein chemistry are based on IMAC, a number of them are not. The latter range from protein purity analysis [42] and protein refolding through matrix-assisted refolding [43] to more complex procedures such as the Biacore analysis of His-tagged proteins using a chelating sensor chip [44] and use of a metal-chelating microscopy tip for single molecule force measurement by atomic force microscopy [45]. This wide range of applications can also be observed within the IMAC realm, where this type of chromatography can be employed not just for protein or peptide purification, but also for separation of His₆-tagged oligonucleotides [46], inactivation of viruses [47] and endotoxin removal from protein preparations [48]. It would be safe to state that although IMAC comprises a vast area of the metal affinity applications field, it is not the only methodology based on metal–protein interactions. Many other methods make use of the same principle and hence play an important role in this developing area of protein biochemistry.

2. Immobilized metal ion affinity chromatography: utilization of differences in protein–metal ion affinity

2.1. Principles and procedures

Binding of proteins (or peptides) to metal ions is based on interaction between an electron-donating group present on a protein surface and a metal ion presenting one or more accessible coordination sites. In IMAC, use is made of a sorbent, or matrix, to which metal-chelating groups are covalently attached

(Fig. 1). When metal ions are added (loaded), the multidentate chelators and metal ions form complexes in which the metal ions are secured for subsequent interaction with the compounds to be resolved. To this end, the metal ions in the complexes must have free coordination sites in order that solvent or solute molecules can bind to them. Following interaction of proteins and metal ions, the bound proteins can be released using a displacer (e.g. imidazole). The strength of the metal–protein bond varies from protein to protein, and in many cases this differential can be exploited to yield effective separation and isolation of specific proteins [3].

2.2. Commonly used metal ions

Differences in affinity of proteins for a metal can be explained, at least in part, by the principles of hard and soft acids and bases (HSAB) as described by Pearson [49]. This theory states that when two

atoms form a bond, one atom acts as a Lewis acid and the other as a Lewis base. The strength of the bond is governed by the intrinsic “hardness” or “softness” ratings of the atoms involved. The HSAB dictates that bonds between atoms with a similar rating, e.g. a hard acid combined with a hard base, are the strongest. Following the HSAB concept, metal ions such as K^+ , Ca^{2+} , Mg^{2+} and Fe^{3+} are classified as hard Lewis acids, whereas monovalent metal ions such as Ag^+ and Cu^+ are categorized as soft Lewis acids. The transition metal ions, Co^{2+} , Zn^{2+} , Cu^{2+} and Ni^{2+} , are considered “borderline acids.” These metal ions are most often employed in IMAC, in particular $Ni(II)$, which provides a coordination number of six, electrochemical stability under chromatographic conditions, borderline polarizability and redox stability. The chelated metal ions show variations in affinity toward proteins, which can be predicted using HSAB. The latter postulates that there are three major types of ligands. Those containing oxygen (e.g. carboxylate), aliphatic nitrogen (e.g. asparagine and glutamine) and phosphor (e.g. phosphorylated amino acids) are classified as hard Lewis bases. Ligands containing sulfur (e.g. cysteine) are classified as soft bases, those containing aromatic nitrogen (e.g. histidine and tryptophan) are considered borderline bases. The borderline acids, containing Co^{2+} , Zn^{2+} , Cu^{2+} and Ni^{2+} , coordinate favorably with aromatic nitrogen atoms (borderline bases) and also with sulfur atoms (soft bases) [34]. The retention strength of the borderline metal cations, as chelated by iminodiacetate (IDA), follows the order $Cu(II) > Ni(II) > Zn(II) \sim Co(II)$. It may be noted that use of chelated metal ions displaying the highest protein retention does not necessarily translate into the best protein separation, since very high retention could also lead to increased adsorption of impurities [33].

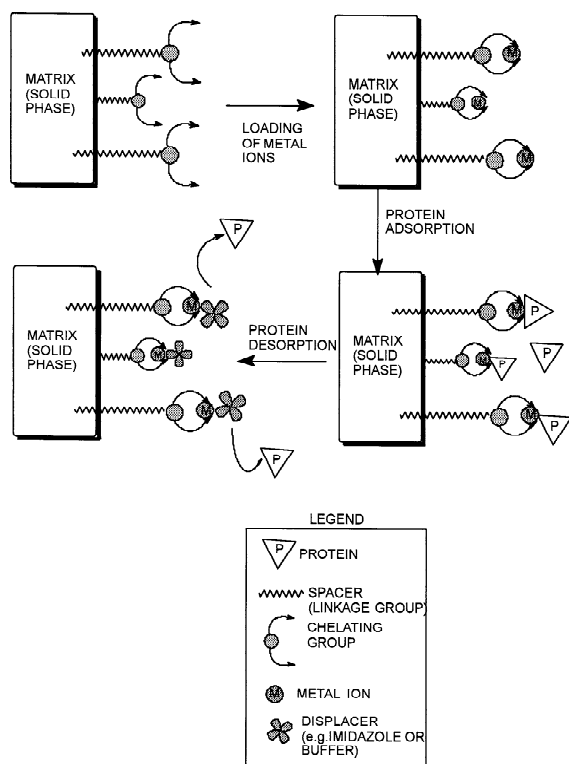


Fig. 1. Diagram of the mechanisms involved in protein adsorption and desorption in IMAC.

2.3. Factors contributing to protein–metal binding

As indicated by several studies, histidine is the amino acid with the strongest affinity for metal ions [3,33,38,50–57]. It is widely accepted that histidine, and also tryptophan and cysteine residues, as a result of strong interactions with metal ions, are key players in the binding of proteins in IMAC [1]. These three amino acids have much stronger re-

tentions, for example, than glutamate and aspartate residues, which show essentially no retention [56,57]. Yip et al. [50] have correlated the retentions of a large number of synthetic, biologically active peptides on Ni(II), Zn(II) and Cu(II) chelated in IDA, with their amino acid profiles in order to evaluate the adsorption properties of amino acids involved in the retention of the peptides. Other investigations revealed that the retention behavior of proteins is largely governed by exposed histidine residues on the protein surface [52,58].

Cysteine also displays strong metal affinity, although to a somewhat lesser extent than histidine [33,38,59]. The metal affinity of these two amino acids can be largely attributed to their functional groups, in particular the imidazole and thiol groups, of histidine and cysteine, respectively. In the case of histidine, other functional groups, such as carboxyl and α -amino groups, also play a role in the metal-binding process [60,61]. Other amino acids may also have substantial metal affinity, including tryptophan, phenylalanine and tyrosine acting directly via their aromatic side chains [33] and arginine, lysine, asparagine, glutamine and methionine, acting indirectly via individual or combined effects on histidine accessibility [33,62]. Although the retention of proteins or peptides is primarily due to the metal affinities of their individual amino acids, other factors also contribute profoundly toward their metal affinity, including their amino acid sequences, folding, and surface properties. In view of the latter, the retention behavior of proteins in IMAC is not easily predictable [33].

In interactions between immobilized Cu(II), Ni(II), Co(II) or Zn(II) ions and amino acid residues on protein surfaces, imidazolyl, thiol and indolyl functional groups are the main targets for the metal

Table 2

Individual contributions of amino acids involved in protein retention

| Functional group | Retention strength |
|-------------------------------------|--------------------|
| Histidine | ++++ |
| Cysteine ^a | ++++ |
| Aspartic acid, glutamic acid | – |
| Lysine, arginine | + |
| Tryptophan, tyrosine, phenylalanine | + |
| N-Terminus | ++ |

^a Cysteine is the reduced form.

ions. Carboxyl and phosphate functional groups are the main targets for hard metal ions such as Fe(III) and Mg(II). A well-accepted concept is that the spatial distribution of histidine residues over a protein surface and their accessibility would influence the retention behavior of the protein molecule [33]. Several high metal affinity peptides have been described in the literature [63,64] as well as non-contiguous metal-binding motifs such as those found in prolactin [65–70], and growth hormone [65,71–73]. It is notable that the possession of a metal-binding motif is not always a guarantee for successful IMAC, since there are a number of enzymes with metal affinity that bind to the chelating matrix via a site that is not the metal-binding catalytic site [3,33]. Some metal-binding sections of a protein, even when exposed, may contribute very little to the net retention strength of the protein in IMAC [33].

When the amino acid composition of a protein, or peptide, of interest is known, the rule presented in Table 1 can be used to predict what kind of metal ions will lead to its retention in IMAC [3]. The retention strengths of the main amino acids involved in protein adsorption in IMAC are shown in Table 2 [33].

Table 1

Protein metal affinity prediction based on accessible histidine and tryptophan residues on the protein surface

| Occurrence of accessible histidine or tryptophan residues on protein surface | Metal ions providing retention |
|--|--------------------------------|
| No His/Trp | – |
| 1 His | Cu(II) |
| >1 His | Cu(II), Ni(II) |
| His clusters | Cu(II), Ni(II), Zn(II), Co(II) |
| Several Trp, no His | Cu(II) |

2.4. Metal chelators and chelating polymeric matrices

The majority of the chelating groups used in IMAC are multidentate chelating compounds providing the strength of the complex formed by the protein, metal ion and chelating group. The composition of the eluent buffer employed varies greatly when one is seeking optimal conditions for a given protein separation, and in many cases, is the main factor of the specificity reached in some IMAC based purification protocols. These chelating substances are attached on the sorbent surface via spacers (linkage groups) which can vary in length and composition. The final structure formed after the metal ion is chelated by the chelating group must allow some free coordination sites in the metal ion for the adsorption or binding of proteins or solvent molecules. Differences in the number of free coordination sites may in part explain why some chelating substances (IDA, Iminodiacetic acid; NTA, Nitrilotriacetic acid, etc.) display different selectivities and adsorption activities towards a given target protein. In the three-dentate IDA, the metal will bind to the nitrogen atom and the two carboxylate oxygens, leaving three sites for protein or solvent molecules (using Ni^{2+}). Likewise the tetradentate NTA is supposed to bind the metal ion with an extra carboxylate oxygen; this could give it a superior metal chelating strength, but on the other hand a weaker protein retention power [3]. Another feature of the tetradentate chelator would be a minor risk of metal leaching [74]. Fig. 2 shows a model of the most commonly used chelators, IDA and NTA bound to Ni (II) atoms.

IDA is the standard, most commonly used metal-chelating ligand for immobilization of metal ions in IMAC supports [38,75]. Many other chelators (adsorbents) employed in IMAC have been designed over the past decades, each having their own advantages and limitations. They are predominantly carboxymethylated amines such as tetraethylene pentamine (TEPA) or carboxymethylated aspartic acid (CM-ASP) [38]. Another extensively used IMAC chelator would be NTA, quite recently developed by Hochuli et al. [76]. The development of new, potential chelating agents for metal affinity is not limited to a particular functional group, and new classes of compounds will likely be employed as

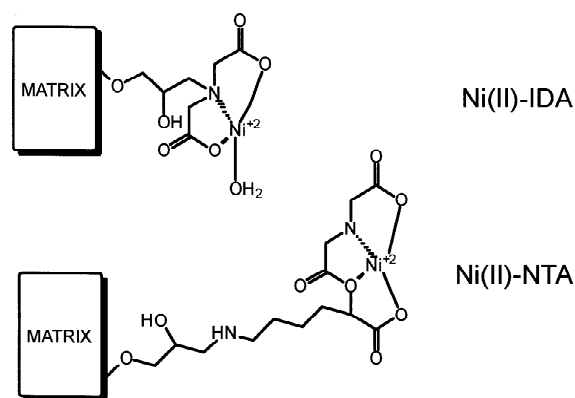


Fig. 2. Structures of two commonly used chelating resins charged with Ni(II) ions: Ni(II)-IDA and Ni(II)-NTA.

metal-anchoring molecules for IMAC in the near future. Several compounds, not chemically related to carboxymethylated amines, are used quite commonly: e.g. dye-resistant yellow 2KT [77], *O*-phosphoserine (OPS) and 8-hydroxyquinoline (8-HQ) [78]. Selecting the proper combination of chelator and metal ion is important, since the chelating resin can adversely affect protein retention. Lehr et al. [79] observed that apart from the metal employed, the chelating resin can have an important effect on protein retention, and suggested that, by using a different resin, protein retention can be altered as a result of interactions between the protein–metal complex and the chelating resin used. They succeeded in purifying histidine-tagged proteins from copper-containing medium in a single step using a chelating Sepharose matrix. This was probably due to favorable interaction between protein–copper complexes and the resin. In contrast, Ni–NTA resin displayed poor protein retention strength, likely a result of interference by the copper ions from the medium. There are a number of reports in the literature describing how the chelating matrix can influence protein purification [78,80–83]. One particular interesting case of “metal ion transfer” underscores the need for a cautious selection of the resin for IMAC: some proteins or solutes used in an IMAC run are able to disrupt the chelator–metal ion complex and can strip off the metal ion from the matrix; as a consequence they are not adsorbed by the column [84]. Fig. 3 displays the chemical

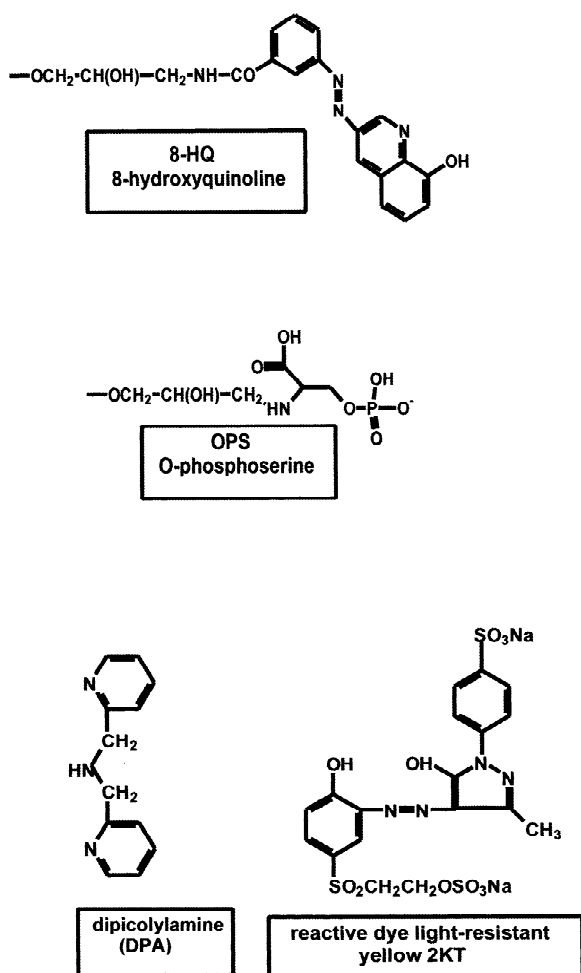


Fig. 3. Structures of newly designed chelating groups for IMAC.

structures of some more recently developed IMAC chelator groups.

The first IMAC supports were manufactured by immobilization of chelating agents on the surface of a relatively inert, appropriately activated polymeric surface (e.g. agarose). Usually IDA was covalently coupled to the selected resin [85]. Many other IMAC supports have been developed in the last decades, each with their individual advantages and limitations [3]. A number of commercially available IMAC resins are presented in Table 3.

The polymeric support has to meet some physico-chemical characteristics in order to be suitable for IMAC, the ideal support would:

- (i) Be easy to derivatize;
- (ii) Not exhibit non-specific adsorption;
- (iii) Display good physical, mechanical and chemical stability;
- (iv) Possess high porosity to provide easy ligand accessibility;
- (v) Allow use of high flow-rates;
- (vi) Be stable to eluents including, for instance, denaturing compounds;
- (vii) Permit regeneration of columns without degeneration of the matrix;
- (viii) Provide a stable gel bed with no shrinking or swelling during the chromatographic run.

2.5. Factors governing adsorption and desorption of proteins

Although the separation of proteins is mainly based on forces acting between their amino acid residues and the metal ions, many other factors influence this complex formation, including the chelate structure, type of metal ion, protein structure, pH, type of buffers, ionic strength and detergents [3].

2.5.1. Chelate structure and metal ions

As already mentioned, the number of linkages between a metal ion and an immobilized metal chelator governs the total affinity of the chelator–metal ion complex for proteins or peptides [34]. The chelator should have a strong affinity for the metal ion (to prevent, for example, the metal ion transfer effect) [84], but should also allow some free coordination sites to enable binding of the protein to the metal ion [33]. It appears that the stability of the chelator–metal ion structure depends on the type of metal ion and the composition of the eluent buffer used [3]. A number of chelate characteristics can influence the selectivity of metal–protein binding: complex coordination geometry, charge, steric bulk and chirality [33].

2.5.2. Mobile phase: pH, buffers and ionic strength

The selectivity of IMAC for a protein also depends on the composition of the mobile phase. Increasing the ionic strength of buffers can lead to suppression of secondary, electrostatically undesirable interactions while augmenting protein binding to

Table 3
Commercially available chromatographic resins for IMAC

| Manufacturer | Designation | Support description | Metal/chelating group |
|---|--|---|--|
| Chemicell, Berlin, Germany | ChromaCELL–metal | Cellulose | Ni ²⁺ , Co ²⁺ , Cu ²⁺ or Zn ²⁺ phosphonic acid group |
| Iontosorb, Czech Republic www.iontosorb.cz/iontos00.htm | Iontosorb OXI | Cellulose beads | 8-Hydroxyquinoline immobilized |
| | Iontosorb SALICYL | Cellulose beads | Salicylic acid |
| | Iontosorb DETA | Cellulose beads | Diethylenetriamine |
| | Iontosorb DTTA | Cellulose beads | Diethylenetriamine- tetraacetic acid |
| Amersham Biosciences (former Pharmacia Biotech, Uppsala, Sweden) | Chelating Sepharose Fast Flow | Cross-linked agarose | IDA |
| | Chelating Superose | Cross-linked agarose | IDA |
| | HiTrap Chelating | Highly cross-linked agarose, prepacked columns | IDA |
| Pierce, Rockford, IL, USA | Immobilized iminodiacetic acid gel | Cross-linked agarose | IDA |
| | Immobilized iminodiacetic acid gel | TSK HW-65F | IDA |
| Sigma, St. Louis, MO, USA | Iminodiacetic acid–agarose | Agarose | IDA |
| | Iminodiacetic acid–epoxy activated Sepharose 6B | Cross-linked agarose | IDA |
| Tosoh, Japan | TSKgel Chelate-5PWb | Polymer resin | |
| Qiagen, Chatsworth, USA | Ni–NTA agarose | Cross-linked agarose | Ni ²⁺ –NTA |
| | Ni–NTA superflow | Highly cross-linked agarose | Ni ²⁺ –NTA |
| | Ni–NTA magnetic agarose beads | Cross-linked agarose beads containing magnetic particles | Ni ²⁺ –NTA |
| | Ni–NTA spin column | Silica particles | Ni ²⁺ –NTA |
| Invitrogen, CA, USA | ProBond Resin | Agarose | |
| Merck, Darmstadt, Germany | Fractogel EMD IMAC | Polymer support with polymers branching from the polymer backbone | IDA |
| Clontech Laboratories | Talon resin | Cross-linked agarose | Co ²⁺ –tetradentate chelator ^a |
| | Talon superflow resin | Cross-linked agarose | |
| | Talon cell thru | Cross-linked agarose | |
| | Talon spin columns | Silica | |
| Applied Biosystems (former PerSeptive Biosystems) | Poros MC | Hydrophilized polystyrene-divinyl- benzene particles | |
| Affiland, Belgium | Iminodiacetic acid–agarose | Cross-linked agarose | IDA |
| | PDC | – ^a | Pentadentate chelator ^a |
| | PMIB | – ^a | Pentadentate chelator ^a |
| Serva Electrophoresis, Germany www.serva.de | Serdolit/Chelite/CHE | Macroporous polystyrene- based resin | IDA |
| | Serdolit/Chelite/P | Macroporous polystyrene- based resin | Aminomethylphosphonic acid group |

^a Non available data.

the chelate complexes, since decreasing the salt concentration can even result in a poorer protein adsorption [33,38]. Sodium chloride (at 0.1–1.0 M) is commonly included in IMAC buffers to suppress ionic interactions between sample and matrix, as well as between proteins [3]. The effect of electrolytes on protein retention is related to the affinity of the metal ion for its solvated water molecules. Weakening of the forces between the metal ions and water molecules by increasing the ionic strength of the buffers, favors the protein adsorption processes. The use of high pH values (7 or 8) and high ionic strength makes IMAC different from other ion-exchange chromatography. IMAC best resembles hydrophobic interaction chromatography (HIC) because of the use of high salt concentration buffers [38]. If desirable, one could easily elute the target protein by applying a continuous decreasing pH gradient rather than an increasing imidazole gradient. Some elution protocols involving the simultaneous use of both imidazole addition and pH decreasing gradient in order to achieve a better chromatographic resolution are also described [86,87]. Alternatively, the protein can be eluted by a chelating compound such as EDTA, but the elution will elute all proteins retained along with the metal ion used, thus diminishing the resolution and selectivity [3].

Adsorption of protein in IMAC is carried out at a given pH at which the electron donor groups on the protein surface are partially unprotonated. It is common practice to induce protein adsorption on the IMAC support under a weakly alkaline pH in the presence of high ionic strength buffers. Phosphate and acetate buffers are most commonly used in IMAC. The role of the pH is fairly complex in the elution and adsorption of proteins, because it influences a number of properties, including the nucleophilic behavior of the buffer components, the electron-donor acceptor properties of the solutes and metal stability. The pH range 6–8 favors retention of histidine and cysteine residues; at a more alkaline range, coordinations with amino functional groups are favored, thus decreasing selectivity [38].

2.5.3. Additives and protein displacers

Detergents can be utilized in IMAC as selectivity enhancers [3]. Tween 80 at a concentration of 0.01% (v/v) has been used in the purification of recombi-

nant human prolactin [88,89] and primate prolactin [90], and proved to be useful for diminishing undesirable interactions. Organic solvent was added to the buffer to aid in the removal of endotoxins without deleterious effects on the correct folding of proteins [48], or to strip off colored contaminants, as used in the isolation of albumin from a Cohn extract [3]. Elution of the protein can also be achieved, although with poor resolution, by extraction of the metal ions using a strong chelator (e.g. EDTA). Another method to achieve good resolution, and hence selective elution, is the incorporation in the eluent of compounds known to present a higher affinity for the adsorption sites than the proteins. Addition of histidine or imidazole has been used in gradient mode elution as an effective way for selective protein elution [38]. Recently, improved displacers have been devised, including a polymeric imidazole compound which showed superior eluting strength compared to monomeric imidazole [91]. IMAC is compatible with most buffer constituents required to maintain protein integrity (e.g. protease inhibitors, glycerol), prevent protein aggregation (e.g. 2-mercaptoethanol) or merely improve resolution (e.g. NP-40). In Table 4, a list is presented of substances showing various degrees of compatibility with IMAC [58,92,93].

3. Immobilized metal ion affinity chromatography as a preparative technique

3.1. Purification by immobilized metal ion affinity chromatography: pros and cons

IMAC is primarily based on affinity adsorption and therefore possesses advantages as well as disadvantages associated with this type of separation technology. Notably, the main feature of affinity chromatography is the specificity of the interaction between the protein of interest and the ligand (e.g. chelated metal ion, antibody, dye etc.). However, when compared to other types of affinity chromatography, in particular immunoaffinity chromatography, IMAC exhibits some noteworthy advantages, as described in Table 5 and discussed below [33].

Purification of proteins by IMAC is based on their affinity for metal ions. Many proteins, however, lack

Table 4
Compatibility of IMAC and various ions/reagents

| Ions/reagents | Compatible | Non-compatible |
|---|------------|----------------|
| Na ⁺ | X | |
| K ⁺ | X | |
| NH ₄ ⁺ | X | |
| TRIS: Tris (hydroxymethyl) aminomethane | X | |
| Mg ²⁺ | X | |
| Cl ⁻ | X | |
| Acetate | X | |
| CO ₃ ²⁻ | X | |
| SO ₄ ²⁻ | X | |
| PO ₄ ³⁻ | X | |
| Glycine | X | |
| HEPES: <i>N</i> -2-Hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid | X | |
| MOPS: 3-(<i>N</i> -Morpholino) propanesulfonic acid | X | |
| Imidazole | X | |
| Ethanol | X | |
| Glycerol | X | |
| 2-Mercaptoethanol | X | |
| Urea | X | |
| Guanidinium | X | |
| Cetyltrimethylammonium bromide | X | |
| Sodium <i>N</i> -laurylsarcosine | X | |
| Sodium laurylsulfate | X | |
| Sodium deoxycholate | X | |
| Triton X-100 | X | |
| Tween 20 | X | |
| Tween 80 | X | |
| NP-40 | X | |
| CHAPS: (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) | X | |
| DTT: Dithiothreitol | | X |
| DTE: Dithioerythritol | | X |
| EDTA: Ethylenediaminetetraacetic Acid | | X |
| EGTA: Ethylene Glycol-bis(β-aminoethyl Ether) | | X |

metal affinity, a disadvantage which in the case of recombinant proteins can be overcome by using an oligohistidine tag inserted at their N- or C-terminus [76], where it likely is freely exposed on the protein surface [58]. Using this technology, large amounts of

recombinant protein can be recovered, most often in a single chromatographic step. Contamination by *E. coli* proteins is usually low since such proteins in general lack metal affinity, although some *E. coli* proteins which show metal affinity, e.g. superoxide

Table 5
Comparisons between IMAC and standard affinity chromatography

| Feature | Metal affinity | “Bio-specific/bio-affinity” |
|---|----------------|-----------------------------|
| Ligand stability | High | Low |
| Protein loading | High | Low |
| Elution conditions | Mild | Often extreme |
| Ligand recovery after column regeneration | Complete | Generally incomplete |
| Selectivity | Low-medium | High |
| Cost | Low | High |

dismutase, could interfere with the purification process [58].

Since there is no need for extreme pHs in the mobile phase, elution conditions are usually mild. High imidazole and salt concentrations, however, could create a problem, in particular if the isolated protein is required for structural analysis (e.g. crystallography).

The most outstanding features of the use of histidine tail tags are the highly selective binding between the tag and the chelated metal ion and the relatively low interference of other compounds during a purification run [58]. IMAC has some unique, attractive characteristics:

(i) IMAC often allows single step purification [94];

(ii) The protein loading capacity is relatively high when compared with other affinity chromatographic techniques (0.1–10 $\mu\text{M}/\text{ml}$ gel) [33,94];

(iii) The metal ions can be easily removed from the resin with a strong chelating agent such as EDTA or EGTA. Different metal ions can therefore be tested, using the same chelating resin, to determine the best ligand for separation of a protein of interest;

(iv) IMAC up-scaling is fairly easy and reproducible, also for industrial applications [94];

(v) IMAC is useful for concentrating dilute protein solutions [94];

(vi) IMAC is compatible with a number of buffers containing high ionic force or chaotropic components [94];

(vii) IMAC in general does not adversely affect the structure of proteins. A few cases have been reported in which metalloenzymes had their essential metal ion stripped off. A case has also been reported where a protein was damaged by IMAC on a Cu(II)–IDA column, but the damage was triggered by reducing agents which caused oxidative proteolysis catalysed by Cu(II) [95];

(viii) Upon passage through a non-charged IMAC column, solutions become transiently sterile since all metal ions essential for bacterial growth are removed by chelation [94];

(ix) An IMAC resin can be regenerated several hundreds of times without loss of chromatographic characteristics [34]. IMAC gels are also extremely sturdy provided that pH values below 4 are not used [94].

Some potential IMAC disadvantages are:

(i) Metal ion transfer (MIT) phenomena leading to protein loss [74,84,94,96] and low protein yields are not the only concerns about this subject. Another problem would be if the target protein retains the capturing metal ion within its structure. In this case the trapped ion could hinder or even abolish the protein's bioactivity, but MIT could also be carried out to charge a given protein with a desired metal [74,96]. The metal ion sequestered by the protein could also be hazardous if the protein is intended for therapeutics since some metals commonly used in IMAC are considered carcinogens. Metal ions Co(II) or Ni(II), widely used in IMAC, are known carcinogenic agents, although they are considered weak mutagens when compared with arsenic or hexavalent chromium. Nickel has been linked to the anomalous expression of a very large number of genes related to cancer induction [97]. One could strip off the undesired metal from the protein using a metal-free chelating column packed with a strong chelating adsorbent such as TED [tris(carboxymethyl)-ethylenediamine] [40,98].

(ii) Metal ion leakage from the resin leading to metal ion contamination of the final product, a problem which would be especially important in the use of IMAC for the preparation of therapeutics [42]. Apart from the obvious threat of metal contamination of the final product, a major problem would be the shelf life of the therapeutic protein since metal ions are known to trigger not only oxidative reactions as catalysts [94,95,99,100] but also disrupt stabilization of lyophilized protein preparations [100], although metallic ions have also been used as additives along with sugars to enhance protein stability upon lyophilization [100–102]. Some authors, aware of the stabilizing effects of metal ions on proteins, have proposed their use to enhance protein stability [77]. In cases when the metal is a destabilizing factor, chelating agents can be added for stability improvement [103]. Protein samples tainted with metals should also raise some concern, since metal displacement can occur during an IMAC step [40,104], and metal toxicity is undesirable when it comes to therapeutic proteins, but purification protocols usually have multiple steps after IMAC and this subsequent polishing generally yields a metal free final preparation [42]. In case one would like to rest

assured that all metal traces were banished, one could rely on the same post treatment recommended for MIT prone samples and carry out an IMAC with an uncharged TED column to capture the contaminating metal ions from the protein mixture [40,98]. Another elegant way to circumvent the metal leaching problem is to use either chelating or metal immobilizing supports with strong ion binding constants that prevent such metal loss [105].

(iii) Use of oxidative and catalytic conditions during a run, when the immobilized metal ions are prone to undergo redox reactions [94,95,99].

3.2. Purification of proteins fused with poly-histidine tags and other engineered metal-binding sites

A reliable and easy way to obtain a highly purified recombinant protein is to synthesize the protein fused to a metal affinity site and then purify it using IMAC [106]. The use of a poly-histidine tag as a metal affinity site, applied at the C- or N-terminus of the protein to be purified, proved to be a highly effective approach, usually enabling purification of the protein by IMAC in a one-step procedure [79,92,107–110]. Consequently, this methodology has been widely used for protein recovery and purification. Proteins such as human glutathione *S*-transferase P1-1 [111], murine interleukin 12 [79], cytochrome *b5* [20], green fluorescent protein [109], chicken lactate dehydrogenase [92], mitochondrial ADP/ATP carrier protein [27], HTLV-I surface envelope glycoprotein fragment [110], and many others, have been successfully purified making use of poly-histidine tagging. High affinity for metal ions can also be achieved using other metal-binding peptide tags or via engineering of metal affinity sites on protein structures [33,112,113]. Such tags/sites were devised to suit specific demands not met by regular histidine tags. Pasquinelli et al. [114] identified several fusion tags that shifted the elution of the target protein to the low background region of the Zn(II)–IDA elution profile, thus facilitating purification under mild conditions. Gaberc-Porekar et al. [115] developed a new histidine tag which enabled the purification of a multimeric protein (TNF- α). Enzelberger et al. [22] indicated the need for newly designed metal affinity tags, offering more choices of selectivity; a new

affinity tag (His–Asn–Arg–Tyr–Gly–Cys–Gly–Cys–Cys) was produced which exhibited a stronger metal-binding capacity and higher yields when compared to the regular hexa-histidine tag. Schmidt et al. [116] engineered a Zn(II)-binding site from the active center of human carbonic anhydrase II into a retinol-binding protein, giving it selectivity for Zn(II) when compared to Cu(II) and Ni(II), an advantage which cannot be obtained with regular oligo-histidine tags which exhibited a less rigid conformation when compared with the engineered zinc-binding site. The design of synthetic tags with an affinity for Cu(II) ions higher than that of hexa-histidine tags has also been described [117].

3.3. Purification of naturally occurring metal-binding proteins

In general, histidine residues in proteins are relatively rare, amounting to about 2% of the amino acid content of globular proteins; in addition, only half of them are exposed on protein surfaces [33]. In view of this, only a low number of naturally occurring proteins would present some metal affinity and hence be potentially suitable for purification by this type of chromatography. Natural possession of metal affinity by a protein poses a great advantage for its purification, since there is no need for inclusion of an affinity tag or metal-binding scaffold engineering. As previously mentioned, these naturally occurring metal affinity domains have selectivity for certain metals, a characteristic not shared by hexa-histidine tags which are able to bind to most divalent metal ions, thereby increasing the selectivity of the separation obtained via IMAC [118]. Many proteins showing metal affinity have been successfully purified by IMAC without prior modification, including growth hormone and prolactin [85,89,118–120], two pituitary protein hormones with zinc affinity domains [66–73]. Prolactin also exhibits a relatively high affinity for Ni(II), and Ni(II)-based IMAC of prolactin proved to be superior to other chromatographic techniques previously utilized [89]. Anguenot et al. [121] have described the purification of tomato sucrose synthase isoforms by Fe(III)-IMAC; Boden et al. [122] reported a one-step IMAC purification for goat immunoglobulins. Other proteins such as human protein C [29], calcium-binding

proteins [123], recombinant human interferon γ [42] and pore-forming protein (perforin) [124] have also been purified or fractionated by IMAC without prior modification. It may be noted that the ability of a protein to bind a metal ion in solution is not necessarily a guarantee that it can be purified by IMAC. Thus it was found for many metalloenzymes that the sites, which bind metals for catalytic function, were not available for use in IMAC, turning this potential binding site useless with regard to application of this type of separation technology [3].

3.4. Immobilized metal ion affinity chromatography: up-scaling and industrial use

Metal affinity chromatography has compelling properties encouraging its application in large scale protein processing. Among the major advantages of the use of IMAC for protein purification at an industrial scale is the ease with which its bench level protocols can be scaled up and the high reproducibility of this technology [94]. A potential drawback of IMAC, however, would be metal ion leakage from its resins leading to metal contamination of the final product [38,94]. This shortcoming could be circumvented by the use of an additional chelating gel column to capture the metal ion contaminants [38]. This extra step, besides the additional costs to the process, would bring other concerns such as environmental problems associated with the disposal of the metal residues [38].

An alternative to regular IMAC, which basically is packed-bed chromatography, is “expanded bed adsorption” (EBA) chromatography. This technique allows separation of soluble target proteins in whole mammalian cell culture broth from the crude extract in a single step, by adsorption to chelator–metal ion complexes on resin particles packed in a column (e.g. metal affinity adsorption). By applying an upward flow of the broth the sorbent gel is expanded, creating a void space between the sorbent resin beads which allows cell debris, whole cells and other matter to flow through the EBA column as the protein is adsorbed [125,126]. Thus the adsorption of the protein of interest to the adsorbent gel in a “fluidized” bed eliminates the need for an extra step for particle removal. During elution of the protein, the liquid flow is reversed, leading to packing of the

resin bed. Chromatography using fluidized beds can be scaled up and there are several reports describing applications of expanded bed adsorption chromatography in large-scale protein separation [127–131]. Use of EBA for purification of His-tagged proteins [132–134] and for non-modified protein [135] has been reported. Streamline expanded bed adsorption is a technique based on fluidized bed chromatography, in which the expanded bed has been stabilized; this renders features to the method of regular packed bed chromatography, in terms of adsorption and flow-rates, but still allows processing of unclarified extracts [129]. Another protein separation system, based on metal affinity of proteins and often used in industrial scale methodologies, is the “aqueous two-phase system,” also known as “affinity partitioning of protein” [33]. This methodology is based on the partitioning of proteins of a given mixture between two aqueous phases, one of them a PEG-based metal chelate phase which selectively pulls metal-binding proteins into this phase. The affinity partitioning of proteins and peptides which makes use of metal-chelating polymers is termed “immobilized metal affinity partitioning” (IMAP) and has been described in several studies on protein isolation [77,136–139]. Another non-chromatographic purification approach, described by O’Brien et al. [140], is based on the use of magnetic chelator particles charged with Cu(II) ions for the recovery of His-tagged proteins, e.g. T4 lysozyme. As indicated by the authors, the high recovery yield and the avoidance of a clarification step are major advantages of this method when compared to standard IMAC. As pointed out in a comprehensive review by Wong et al. [38], many parameters, including pH, buffer ionic force, flow-rate and protein structure are major players in large-scale IMAC. A better understanding of their roles could lead to improvements in the up-scaling of IMAC and in its industrial application. Other important chromatographic parameters, including adsorption rate constants, mass transfer coefficients and equilibrium parameters also need attention [38]. To date, there are only a few reports on the use of IMAC for purification of proteins to be used in clinical therapy [141]. This is likely due to concern over potential metal ion leakage interfering with the very stringent purity requirements of therapeutics [115], although data are available showing

that metal ion tainting of IMAC-purified products is not likely to occur [42].

3.5. Use of immobilized metal ion affinity chromatography in conjunction with other chromatographic techniques

IMAC is frequently carried out with high ionic strength running buffers. As such, IMAC could supplement other chromatographic procedures where a high salt concentration is needed or does not interfere with the separation, such as HIC [34]. Another possible combination described in the literature is the development of a new class of chromatographic methods termed “adsorptive size exclusion,” where size exclusion chromatography is combined with adsorption chromatography such as IMAC or HIC [129]. Use of sequential IMAC columns, charged with different metal ions, would make it possible to retain a variety of target proteins on the basis of their particular metal affinity characteristics [34]. Another methodology devised to improve resolution is the use of IMAC followed by affinity chromatography employing anti-His-tag monoclonal antibodies [142].

4. Metal affinity tags: advantages and applications

Application of metal affinity tags as well as other tags, e.g. based on avidin affinity, such as the biotin acceptor peptide [143], have proven highly advantageous to protein purification:

(i) Identification of fusion proteins, incorporating a metal affinity peptide tag (e.g. hexa-histidine), has become possible with the use of antibodies directed against the tag in e.g. an immunodetection assay [16]. It allowed step-by-step monitoring of a fusion protein, by western blots or enzyme-linked immunosorbent assay (ELISA), during procedures aimed at optimizing the expression of the protein [144]. McMahan and Burgess [145] synthesized a bifunctional compound for the detection of nitrocellulose-bound his-tagged proteins. It has a biotin as one functional group and a Ni(II)-charged nitrilotriacetic acid as the other. Following binding of the latter to a histidine tag, the biotin group can be detected using a

streptavidin–horseradish peroxidase conjugate; its emitted chemiluminescence can be detected using X-ray film. Using this technique, as little as 0.11 pmol of polyhistidine-tagged *Escherichia coli* RNA polymerase σ^{70} subunit could be detected on nitrocellulose membranes [145]. Similarly, Ni–NTA–alkaline phosphatase conjugate (commercially available from Qiagen, Hilden, Germany) was used to analyze polyhistidyl peptides on bacterial surfaces [146]. Another labeling system used for visualization of his-tagged protein structures was developed by Buchel et al. [147] and is based on the use of Ni(II)–NTA gold clusters for imaging via electron microscopy.

(ii) Obviously, the major advantage of metal affinity or other affinity tags is that they speed up the purification process. The expressed fusion protein can be rapidly purified using a chelating resin, charged with the metal ion of choice, which captures and retains the engineered protein; the purification can usually be carried out in a single step operation [79,92,109,110,144]. The specific interaction between the target protein and the support is highly critical when the protein of interest is present in crude extract at low levels due, for example, to poor expression. There are some examples where the use of affinity tags enables the purification of poorly expressed proteins without the need of upstream regulation, since protein expression control adjustments are more cumbersome than downstream processing set-ups. Another advantage is that the metal affinity tag can most of the times be used for a variety of proteins, eliminating the necessity of elaborating individual downstream processes [144].

(iii) Metal affinity tags can be used to immobilize proteins on a surface, as required for certain assays. This approach would permit rapid screening of bioactivity or affinity towards a given analyte [144]. Nieba et al. [44] demonstrated that His tags can be used not only to facilitate purification and detection of proteins but also to ease kinetic studies through biosensor analysis. It would also be useful for immobilization of enzymes, since it has been found that such bio-affinity based immobilization procedures often yield preparations exhibiting high catalytic activity and enhanced stability against denaturation. Bio-affinity immobilization is reversible (facilitating reuse of the support matrix), orients the

enzymes favorably and permits immobilization of target enzymes from crude extracts or cell lysates [148]. The same orientation or immobilization methodology can be applied to the manufacturing of affinity columns [149] and to other processes where molecule orientation or anchoring is required, such as in atomic force microscopy [45], surface plasmon resonance (Biacore detection technology) and the scintillation proximity format [44,149]. The importance of site-specific immobilization of proteins is accentuated by the problems encountered with random immobilization of enzymes: i.e. structural deformations, inability to control and predict structure–function relationships and impaired catalytic activity [150].

(iv) The use of metal affinity tags can in many instances circumvent problems resulting from incorrect protein expression. In *E. coli* expression systems, for example, truncated forms of the target protein can be produced due to errors such as internal initiation of translation or premature terminations of protein synthesis. Since these truncated forms share many common physico-chemical characteristics with the non-modified molecule, they can lead to contamination of the final product. Inserting a metal affinity tag at a terminus of the molecule of interest could enhance isolation of only the correctly expressed proteins. To which terminus the tag would have to be added would depend on the type of truncation encountered [144].

(v) Affinity tags can be helpful in other ways than facilitating protein screening. A number of authors have reported improvements in protein stability, and protein expression levels following insertion of a metal affinity tag [21,151]. Supattapone et al. [152] have reported increased resistance of histidine-tagged PrP proteins against protease activity, resulting from a protease-resistant conformation of the tagged proteins. The protein stabilization induced by insertion of histidine or other metal affinity tags could be a result of disruption of the natural protein turnover as controlled by the N-terminus of the protein, since the N-end rule states that insertion of an N-terminal histidine before a destabilizing amino acid would increase the *in vivo* half life of the protein [153]. However, it is possible that the protein stabilization is in fact related to the increased speed of the purification process rather than to a change induced by insertion of the metal tag [58]. Fusion proteins

can have their affinity tags removed when required, usually via enzymatic cleavage by a site-specific protease [25,107,154], although in many instances the histidine tail does not impair activity or folding of the protein [111,155–158]. Metal-binding tags can be combined with other affinity tags in the same target molecule. This dual affinity approach can bring many advantages, including increased protein stability, higher purity due to increased selectivity and the option of employing different methodologies for detection, quantification, immobilization and adsorption of the target fusion protein [154].

5. Metal affinity tagging: pitfalls and limitations

Although use of metal-binding tags has exceptional advantages, it is not free of problems and in many cases other approaches may have to be considered. It is worth emphasizing, however, that the limitations associated with the tag insertion approach have not blocked its increasing application, as can be validated by the growing number of manuscripts published on the subject [144]. As use of tag technology escalated over the past few years, some important limitations have been spotted. A major problem arises if the attached peptide tag disrupts protein bioactivity or function by altering the folding of the protein [144,154]. This apparently is the case when a polyhistidine tag is introduced in L-lactate dehydrogenase at the C-terminus. The resulting fusion protein has decreased bioactivity when compared both to the wild-type enzyme or the N-terminally tagged variant [159]. Interference with antigen-binding property was also detected in histidine tag-containing antibodies where again the C-terminal position of the his-tail adversely affected the binding properties of the construct [160]. Problems associated with functionality have also been reported. Histidine tags were found to modify the interaction of a DNA-binding protein with DNA [161]. Another case concerning a DNA-binding protein [protein pi(30.5)], showed that insertion of histidine tails induced dimerization of the fused proteins [28]. Insertion of metal-binding tags can also lead to unstable or degraded proteins [162], or interfere with both the expression and assembly of a protein complex, e.g. the PsaK1 [163]. Ni(II)-induced oligomerization was observed in proteins containing 10-mer histidine tags [164]. Whereas

a new metal affinity tag (heli_{wt}-tag) has a higher metal-binding strength than the frequently used his₆-tag, the much higher imidazole concentrations, required for eluting a heli_{wt}-tagged protein, can lead to protein denaturation [22]. Following a decision to adopt tag technology in the purification of proteins, careful evaluation of the fusion protein design is required for each individual protein. The more information on the structure and folding of the protein or peptide of interest can be obtained, the more successful the use of the tag will be; ideally, the tag would produce minimal changes in the activity or structure of the protein. In the tag selection process, details such as the type of metal affinity tag, the terminus at which the tag will be inserted and the structure of the protein should be given utmost attention to ensure a successful application [144].

Other problems, associated with the use of metal affinity tags, may arise when the target protein is generated for studies of its properties that could be affected by the tag, including structural, physiological and pharmacological features. In such a case, removal of the tag following purification of the protein would be essential. In general, however, there is no need for removal of histidine tags [107,144]. Removal of the tag can be achieved by chemical cleavage or by enzymatic action. Chemical cleavage has several drawbacks such as harshness of the conditions used and the generation of toxic byproducts [107]. Enzymatic cleavage via endoproteases that recognize specific amino acid sequences within the protein, has distinct advantages over the chemical method, including greater specificity and less harsh conditions. However, enzymatic cleavage can be incorrect due to cleavage of internal, non-canonical cleavage sites and may not always be effective, as found for the his-tag removal from human prolactin by factor Xa, which was only partially successful. The latter was presumably a result of poor accessible cleavage sites displayed by the protein [107,155].

6. Beyond immobilized metal ion affinity chromatography: other applications of metal affinity

The principles of metal or other affinity chroma-

tography, applied for the separation of proteins, can also be used for other protein processing methods. For example, biomolecular recognition techniques, initially employed in affinity chromatography for detection and quantification of proteins or peptides, have been applied as the basis for several biosensor techniques [144]. Immobilized ligand-based tag technology has also been adapted for plate assays and biosensor methods [144]. In this section, the adaptation of IMAC-related methodology in other areas of protein processing is discussed.

6.1. Immobilized metal ion affinity chromatography and protein refolding: matrix-assisted refolding

Production of recombinant proteins in bacteria in the form of cytoplasmic inclusion bodies is associated with serious problems with regard to the proper structure and function of the proteins resulting from protein misfolding. The latter is especially a problem when the proteins are composed of multiple subunits, have several disulfide bridges, or contain prosthetic groups. In the inclusion bodies, the protein is present in an insoluble form, with reduced disulfide bonds and no or impaired bioactivity. This worst scenario of misfolded proteins in particular occurs if the bacterial synthesis of the protein is not supported by chaperones assisting in the folding of the protein. However, even when use is made of protein folding aid devices in the production of the protein (co-expression of chaperones, optimization of growth conditions), a refolding step is usually needed for maximum recovery of the desired protein in the native form [165,166]. In many cases, however, the folding step is ineffective, resulting in poor recovery and high operation costs [166]. Recently, a reliable technique, based on affinity tagging and designated “matrix-assisted refolding,” has been developed for the production of soluble and functional proteins from inclusion bodies (Figs. 4 and 5). Briefly, following production of the tagged protein in inclusion bodies, it is solubilized, denatured and then immobilized on a charged chelating resin [e.g. Ni(II)-NTA]. The subsequent renaturation step can be carried out by applying a linear gradient spanning denaturing to renaturing conditions or by iterative refolding, a technique based on repeated cycles of renaturation and denaturation with a decreasing concentration of the denaturing agent in each con-

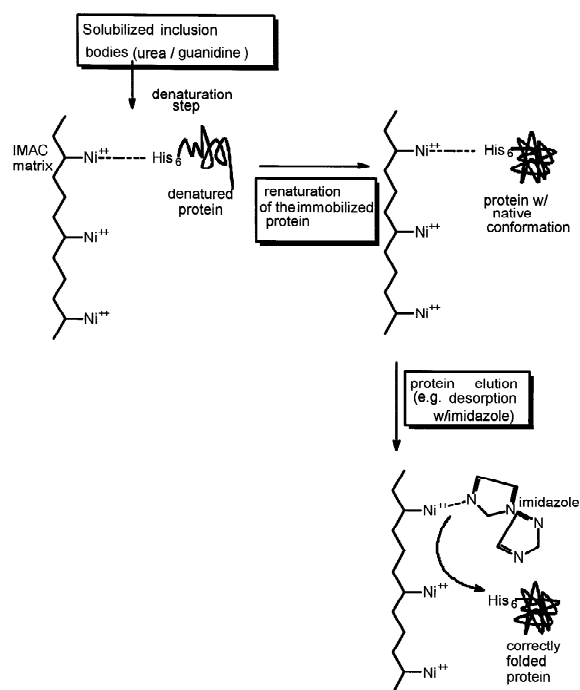


Fig. 4. Schematic representation of matrix-assisted refolding on an IMAC support.

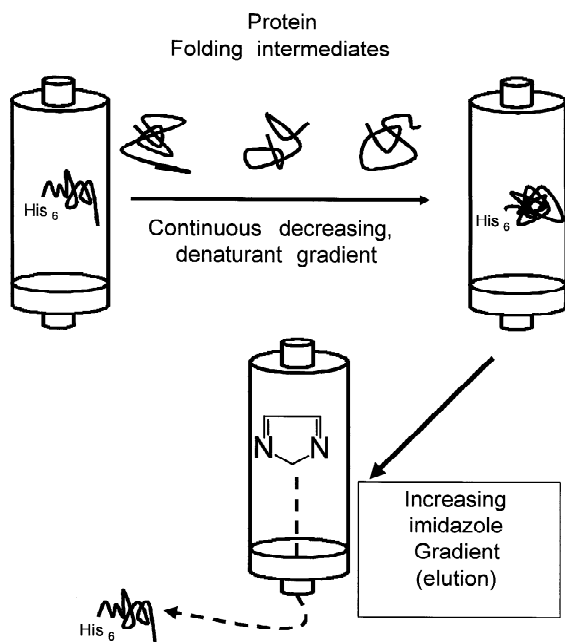


Fig. 5. Matrix-assisted refolding mechanism consisting of refolding and elution steps.

secutive cycle. After the final renaturation, the target protein is eluted in its native and soluble form [43,77]. Successful renaturation of a β -glucosidase, Zm-p60.r, was accomplished through IMAC-based, matrix-assisted refolding: the only refolding protocol that so far has produced a correctly folded Zm-p60.r protein [43]. Matrix-assisted refolding on a Ni(II)–NTA column of a mammalian prion protein has yielded a stable and aggregate-free protein preparation [167]. Other refolding protocols, employing Ni(II)–NTA as the matrix support, have been used for the renaturation of human matrix metalloproteinase-7 [168], a DNA helicase [169], a recombinant fragment of the Willebrand factor [170] and human pulmonary surfactant protein B [171]. The IMAC-based, matrix-assisted protocol for protein refolding has distinct advantages over standard refolding techniques:

(i) Matrix-assisted refolding of some proteins was successful in contrast to other techniques [37];

(ii) Protein immobilization on the matrix prevents non-specific protein–protein interactions, thus precluding undesirable aggregate formation. This is particularly important when the denaturing agent is removed [37,172];

(iii) The immobilized protein has a decreased probability of misfolding due to its limited spatial range of motion when docked to the polymeric support [170];

(iv) Functional or quantitative assays can be carried out on the refolded immobilized proteins prior to the elution step [171];

(v) Matrix-assisted refolding allows gradual renaturation of the desired protein [172]; it usually gives high recovery yields of soluble, functional protein [170];

(vi) The technique is rather simple; special skills, equipment or reagents are not required;

(vii) The immobilization of the protein via metal affinity interactions is reversible. As such, it allows use of unbound protein molecules in an assay in the event that the matrix support would interfere with the assay.

6.2. Site-specific immobilization of proteins and biosensors

IMAC in conjunction with recombinant DNA

technology allows efficient immobilization of a tagged protein to a matrix with a consistent and defined spatial orientation. The directed reversible immobilization of proteins on surfaces by use of metal complexes opens new ways for structural investigation of proteins and receptor–ligand interaction. Site-specific immobilization of proteins can be used for analysis of interactions between biomolecules, such as protein–protein, protein–lipids, protein–drugs and protein–DNA interactions, by immobilizing the protein of interest via an affinity tag on a solid-phase for subsequent interaction with the second molecule. Site-specific immobilization of proteins has a number of advantages when compared to random immobilization of proteins, including good steric accessibilities of active binding sites of the attached proteins (e.g. enzymatic activity) and increased stability. Stability can be perceived not only as an increased enzyme or protein shelf life but also as the feasibility of reutilization of the immobilized enzyme for several cycles without significant bioactivity loss [173]. One major disadvantage of random immobilization of protein on polymeric membranes is that the bioactivity of the immobilized protein is often significantly decreased [174], in some cases because the active site may be blocked upon ligation to the support [149,150].

A useful application of IMAC technology has been described [58] for isolation and characterization of protein partners, i.e. protein complexes in which one subunit contains an oligohistidine tag. This system can be used to test *in vitro* and also *in vivo* assembled protein complexes containing a histidine-tagged subunit. Immobilization of a his-tagged protein can also be used for identification of protein partners displaying affinity for a protein which is immobilized on a metal ion-charged chelating column. This methodology has the option to either elute the protein–protein complex by elution with buffers containing high concentrations of imidazole or, alternatively, the interacting partner can be selectively eluted by increasing the ionic strength of the buffers, or by addition of detergents, depending on the nature of the interaction. It was also pointed out that this method is not limited to protein–protein interactions, since it can be applied to identification of any molecule displaying affinity for the immobilized protein [58]. Immobilization of proteins on a

membrane can be used for the preparation of the so-called bio-functional membranes, entities in which a biomolecule is immobilized on polymeric matrices cast on porous membranes. They have been used in catalysis (membrane-based enzyme bioreactors), separations (affinity membranes), analysis (biosensors, metal ion-specific separations) and artificial organs [150]. One of the applications of metal affinity-based protein adsorption is in biosensor techniques. Biosensors are analytical tools in which a molecule (e.g. an enzyme), covalently and irreversibly bound through cyanogen bromide activation of the support or reversibly through the exchange of the metal ions of the IDA to an electrode surface, is utilized for the measurement of an analyte, or series of analytes, within a specimen. Sosnitzer et al. [175] described application of reversible immobilization techniques for biosensors in which enzymes such as glucose oxidase, invertase and peroxidase were immobilized on a metal ion-charged Chelating Sepharose Fast Flow resin (Pharmacia Biotech, Sweden), a set-up with analytical capability comparable to that of regular biosensor systems. Use was made of the Biacore system, a biosensor instrument using plasmon resonance detection for assessment of binding constants of protein and non-proteinaceous molecules to investigate how many histidine tags are needed in a protein for stable binding to a metal ion-charged, chelating NTA sensor chip. Two histidines are usually sufficient for stable binding to the Ni^{2+} -NTA surface [44]. In a case described by Zhu et al. [176], hexahistidine-tagged proteins were immobilized on a nickel-coated glass slide. This procedure yielded superior quality protein chips when compared to regular protein arrays (e.g. aldehyde-treated slides). Studies of the yeast proteome were performed by cloning 5800 open reading frames; the corresponding proteins were purified and spotted onto nickel-coated slides and screened for the ability to interact with proteins and phospholipids. Using this technology, many new calmodulin and phospholipid-interacting proteins were identified. Fig. 6 shows a diagram of protein printing on the glass slides. It appears that the lipid-based immobilization of proteins via metal affinity sites is a promising method for the attachment of proteins to an interface in an orderly fashion without functional or structural losses [177–179]. Metal affinity-based

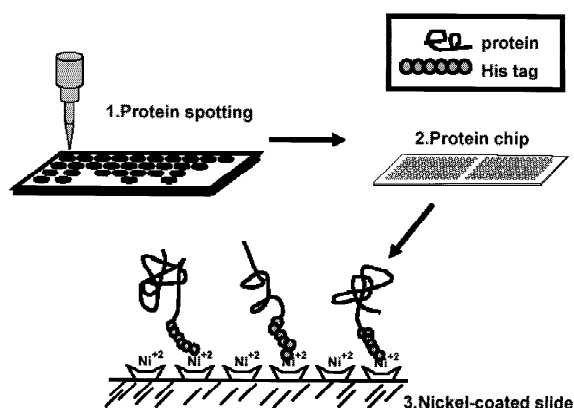


Fig. 6. Diagram showing the construction of a protein array slide: (1) a robot probe delivers precise amounts of His-tagged proteins on a nickel-coated plate at defined positions; (2,3) following immobilization of the target proteins, the slide (or chip) is ready for analysis.

immobilization of proteins is also applied in studies on the structure and function of proteins present on the surface of cell membranes. For example, Ni(II)-charged, metal-chelating lipid membranes were used by Celia et al. [180] to capture and orient His-tagged MHC molecules for subsequent investigation of their binding to the T cell receptor, using surface plasmon resonance. Similar lipid surfaces have been used to grow two-dimensional protein crystals followed by X-ray crystallography of the MHC molecule. Similarly, Wolfgang et al. [181] showed that two naturally occurring surface histidines can be utilized to bind and crystallize the protein streptavidin on an IDA–Cu(II)–lipid monolayer. The NTA/His-tag system was also used for anchoring of proteins for investigations of binding force measurement of receptor–ligand systems at the single molecule level using a metal-chelating microscopic tip for assays via atomic force microscopy [45].

Biosensing technology can also benefit from metal affinity proteins, in particular when it comes to environmental applications such as toxic metal sorbents, more specifically biosorbents. Recombinant *Staphylococcus xylosus* and *Staphylococcus carnosus* were engineered to display metal-binding peptides on their surface for use in bioremediation of heavy

metals such as Cd(II). These engineered metal-binding bacteria can be immobilized for the generation of whole-cell microbial tools for bioremediation purposes. One way could be the co-expression of a metal-binding protein with a cellulose-binding domain (CDB), the latter used as the anchor site for cell immobilization on cellulose fibers [146,182,183].

6.3. Immobilized metal ion affinity chromatography as an analytical and characterization tool in protein chemistry

IMAC has been used extensively for characterization of natural, recombinant and modified proteins, including identification of amino acid residues involved in their metal ion affinity, surface topography of histidine residues and analysis of protein–metal ion interaction [26,52,54,184–187]. For example, human growth hormone has been studied with regard to its zinc-binding property as related to the distribution of metal ion affinity-expressing amino acids on its surface [71–73]. IMAC has proven to be a reliable tool for differentiating non-modified recombinant proteins from their variants or erroneously expressed recombinant proteins [38]. In a recent publication, an IMAC-derived technique, designated “immobilized metal ion affinity partitioning” (IMAP), was applied to probe metal affinity domains and distinguish different surface features among structurally related proteins [77]. IMAC was also used for protein purity analysis [42].

Analytical applications of IMAC have led to the development of new IMAC-based methodologies such as “high-performance IMAC” (HP-IMAC) [84,188,189] and “immobilized metal ion affinity capillary electrophoresis” (IMACE) [190,191]. Other potential future applications of IMAC include IMAC-based clinical chemistry assays [192], endotoxin removal from protein preparations [48], combined use of IMAC with mass spectroscopy in proteome analysis [193–195], identification of metal affinity domains in naturally occurring peptides [196] and mapping of epitopes on a protein, based on interaction of a monoclonal antibody with immobilized histidine-tagged fragments of the protein [197].

6.4. Immobilized metal ion affinity chromatography and processing of phosphoproteins and phosphopeptides

Phosphorylation and dephosphorylation of proteins form a major mechanism in the control of protein activity and regulation of biochemical pathways in cells [198]. IMAC was found to provide a specific method for identification of phosphoproteins, based on metal affinity of phosphopeptides, and Fe(III)-IMAC in particular, has been used to selectively purify and concentrate phosphorylated proteins and peptides [121,187,198–200]. IMAC is often carried out in conjunction with mass spectroscopy and other techniques, such as reversed-phase high-performance liquid chromatography and capillary electrophoresis, to evaluate the effectiveness of the separation method [195,198,201–203].

6.5. Metal binding and protein stability

Aggregation of protein hormones, such as prolactin and growth hormone, is an important step in their concentration into secretory granules in the pituitary. Such aggregation can be induced by interaction of the proteins with metal ions, and zinc, for example, has a role in the formation and stabilization of prolactin oligomers within the secretory granules [68,77]. Proteins containing multiple accessible histidine residues on their surface can be precipitated using metal ion-charged chelators (i.e. bis-copper chelate). A precipitation technique analogous to the antibody–antigen precipitin reaction has been developed termed “metal affinity precipitation”. It involves the formation of insoluble protein complexes, bound together into a net-like structure and crosslinked via metal chelates [204]. The complexes can be resolubilized with either a strong chelator (i.e. EDTA, EGTA) or a displacer such as imidazole [205]. Affinity precipitation of proteins can also be obtained using reversibly soluble–insoluble polymers which have either a natural (chitosan) or synthetic (isopropylacrylamide) origin [205,206].

Metal binding also provides methods for protein stabilization. Thus prolactin has a higher resistance to the enzyme, kallikrein, when complexed with zinc ions than in the non-complexed state [69]; also,

prolactin is more stable when stored as oligomers in secretory granules than in the monomeric form [67,68]. Addition of Zn(II) to growth hormone, a prolactin-related protein, has led to greater stability of the hormone and this step has potential for use in pharmaceutical preparations [71]. In fact, engineered metal-binding sites can lead to enhancement of protein stability: a zinc-binding site insertion into protein α_4 , an engineered four-helix-bundle connected by three identical loops, rendered increased resistance against chemical denaturation probably due to “tightening” of the protein structure [207]. Other reports also demonstrate increased protein stabilization, e.g. increased thermal stability, conformational stability and lower proteolytic degradation as a result of insertion of engineered metal affinity chelating sites, an approach which is easy to implement and can be applied to a wide range of proteins [112,113,208,209]. It is a well-accepted fact that certain metal ions (calcium, magnesium and zinc) can stabilize a protein and are used as stabilizing excipients in pharmaceutical preparations. These metal ions stabilize the target protein through turning its structure more rigid and compact, due to stabilization of the folding [77,100].

7. Conclusions

This article reviews the rapidly increasing use of IMAC for protein purification as well as protein characterization. It also highlights use of IMAC in protein refolding, protein recognition and IMAC-derived or -related methodologies such as oriented immobilization of proteins and protein stabilization via metal affinity engineered sites. The current literature indicates clear advantages of IMAC and IMAC-derived techniques in protein or peptide processing, when compared to other methodologies, in terms of resolution, reliability, reproducibility and processing time. Protein–metal affinity has a central role in a wide variety of applications in the processing of non-modified or engineered proteins, including matrix-assisted refolding, protein partners identification, protein characterization, phosphoprotein analysis and fractionation, protein purity assessment, protein-folding evaluation, site-specific protein im-

mobilization (biosensors, functional assay plates and molecular interaction studies), protein detection and quantification, protein extraction (two phase metal affinity partitioning or IMAP) and IMAC coupled to other chromatographic and non-chromatographic protein separation techniques.

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