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Immobilized Metal-Ion Affinity Chromatography: Status and Trends

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Abstract: The correct folding of solubilized recombinant proteins is of key importance for their production in industry. On-column refolding of proteins is mainly achieved by three methods: size-exclusion chromatography, ion exchange chromatography and affinity chromatography using immobilized metal chelates. The principles of these methods were first laid down in the 1990s, but many recent improvements have been made to these processes. Immobilized metal-ion affinity chromatography (IMAC) represents a relatively new separation technique that is primarily appropriate for the purification of proteins with natural surface-exposed histidine residues and for recombinant proteins with engineered histidine tags or histidine clusters. Because the method has gained broad popularity in recent years, the main recent developments in the field of new sorbents, techniques and possible applications are discussed in this article.

Keywords: IMAC, purification, applications, scale-up, chromatography

INTRODUCTION

In 1975 Porath et al. introduced a new type of chromatography under the name of immobilized metal chelate affinity chromatography (IMAC) (1). In this short publication, the authors described the use of immobilized zinc and copper metal-ions for the fractionation of proteins from human serum. While the basis of the method was actually introduced by Helfferich in 1961 (2) for the separation of small molecules, and Everson and Parker (3) were the

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first who adapted immobilization of chelating compounds to the separation of metalloproteins, the method became popular through the research work of Porath (4–7) and Sulkowski (8–11), who laid the foundations of this technique, mainly on its use for the separation and isolation of proteins.

In their first paper, Porath et al. introduced the means to fractionate proteins on solid supports based on their differential affinity towards immobilized metal-ions (1). That article, as well as the significant number of papers that followed, was devoted to the development of this method as a group-specific affinity separation principle (5–7).

Hundreds of articles have since been published, initially describing the use of IMAC techniques for separating proteins and peptides with naturally present, exposed histidine residues, which is the primarily main responsible unit for binding to immobilized metal-ions. However, the work of Hochuli et al. (12, 13) 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 histidine groups. Consequently, IMAC emerged as one of the major preparative methodologies used for protein purification, ranging from bench (12, 14–19) to pilot/industrial scale quantities (20–27).

Many reviews on IMAC have been published so far (5, 28–33); 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 tries to go deeper into this new dimension of IMAC-based techniques and their implications in life sciences (33).

Whereas many of the recently devised metal affinity-based methodologies in protein chemistry are based on IMAC, a number of them are not. 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.

IMAC: THE BASIS

Principles and Procedures

In IMAC the adsorption of proteins is based on the coordination between an immobilized metal-ion and an electron donor groups from the protein surface. In this kind of chromatography, use is made of a sorbent, or matrix, to which some metal-chelating groups are covalently attached. When the metal-ions are added (loaded), the multidentate chelating groups and metal-ions form complexes in which the metal-ions are secured for subsequent interaction with the compounds to be resolved. To this end, these metal-ions in the complexes must have free coordination sites in order to allow the interaction to the

proteins. These interactions between metal-ions and proteins are extremely complex in nature. They can associate the combined effect of electrostatic (or ionic), hydrophobic, and/or donor-acceptor (coordination) interactions (34).

Protein-Metal Binding

There are several studies that indicate that histidine is the amino acid with the strongest affinity for metal-ions (4, 28, 35–41). Interactions between immobilized metal-ions and proteins have been studied by three model systems: amino acids (42), short synthetic peptides (43), and proteins (8), having been demonstrated that the number of histidine residues in the peptide is of primary importance in deciding the overall affinity for immobilized metal-ions. Aromatic amino acids and the amino-terminus of the peptide also have some contributions (41).

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). The retention strength for these three amino acids is the highest, having been demonstrated that most of the remaining amino acids show essentially no retention (37, 41, 44). Yip et al. (41) correlated the retentions of a large number of synthetic, biologically active peptides in Ni(II), Zn(II), and Cu(II) chelated in iminodiacetic acid (IDA), with their amino acid profiles in order to evaluate the adsorption properties of amino acids involved in the retention of the peptides, concluding that histidine is the amino acid with the largest positive retention. They also concluded that amino acids with aromatic side chains (tryptophan, phenylalanine, and tyrosine) appear to contribute to retention, if they are in the vicinity of accessible histidine residues (9, 28).

Other investigations revealed that the retention behavior of some proteins (arginine, lysine, asparagines, glutamine, and methionine) is largely governed by exposed histidine residues on the proteins surface (4, 28, 45, 46). Cysteine also displays strong metal affinity, although to a somewhat lesser extent than histidine (28, 40, 46), mainly because there are a limited number of cysteine residues on the surfaces of proteins that leave histidine residues as the major targets for intermediate metal-ions (29, 30). The metal affinity of histidine and cysteine can be largely attributed to their functional groups, in particular the imidazole and the thiol groups, respectively (32, 47, 48). Ueda et al. (32) present in their fantastic review a general rule that can be used to predict what kind of metal-ions will lead to its retention in IMAC, and the retention strengths of the main amino acids involved in protein adsorption.

It has been widely demonstrated that the retention of proteins is mainly due to the metal affinity of their individual amino acids, but this is not the single factor that affects retention, other factors also contribute profoundly toward their metal affinity, including their amino acid sequences, folding, and surface properties (32, 47, 49). Volz et al. (50), compared several

peptides with variation of histidine positions, concluding that peptides containing two histidine residues showed high affinity to Cu(II) and Ni(II) ions; and, that sequences which contain two or more histidine residues in close proximity, i.e., HxH and HxxH, are general motifs for Ni(II) ion binding, but cysteine-containing motifs such as CxxC were capable of binding Cu(II) but not Ni(II) ions.

The selectivity of hard and intermediate metal-ions to peptides is also very different because they interact with different side chains on the protein surface (29). 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-ions. Carboxyl and phosphate functional groups are the main targets for hard metal-ions such as Fe(III) and Mg(II). The distinction between the two types has been exploited to achieve impressive separations and even single-step purifications of proteins from complex biological mixtures (29, 47, 48, 51–54).

Metal-Ions

Metal-ions are classified by most authors in the IMAC field according to the principles of hard and soft acids and bases (HSAB) described by Pearson (55), who states that when two atoms form a bond, one atom acts as a Lewis acid and the other as a Lewis base. According to this theory metal-ions can be divided into three categories (hard, "borderline" or intermediate, and soft) based on their preferential reactivity towards nucleophiles. To the group of hard metal-ions belong Fe(III), Al(III), Ca(II), Mg(II), and K(I), which show a preference for oxygen, aliphatic nitrogen and phosphorus. Soft metal-ions such as Cu(I), Hg(II), Ag(I), etc., prefer sulfurs; and "borderline" metal-ions (Cu(II), Ni(II), Zn(II), Co(II)) coordinate aromatic nitrogen, oxygen and sulfur (29). The most commonly used metal-ions are the transition ones, Cu(II), Ni(II), Zn(II), Co(II), and Fe(III), especially Ni(II), which provides a coordination number of six, electrochemical stability under chromatographic conditions, borderline polarizability and redox stability (32).

Electron-donor atoms (N, S, O) present in the chelating compounds that are attached to the chromatographic support are capable of coordinating metal-ions (electron-pair acceptors) forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds. The remaining metal coordination sites are normally occupied by water molecules and can be exchanged with suitable electron-donor groups from the protein.

Chelating Ligands

As a chromatographic technique, the IMAC procedure relies on the ability of immobilized metal-ion chelate complexes to interact with the side-chain

moieties of specific amino acids accessible at the surface of proteins via coordinative interactions. For chelating agent, multidentates are most popularly used in research works and commercial chromatographic products. Four different types of dentates, bidentate (e.g., aminohydroxamic acid, salicylaldehyde, 8-hydroxyquinoline (8-HQ), etc.), tridentate (e.g., iminodiacetic acid (IDA), dipicolylamine (DPA), *ortho*-phosphoserine (OPS), N-(2-pyridylmethyl) aminoacetate, 2,6-diamino methylpyridine, etc.), tetradentate (e.g., nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp), etc.), and pentadentate (e.g., N, N, N′-tris-carboximethyl ethylene diamine (TED), etc.), have been thoroughly investigated since immobilized metal affinity chromatography was exploited (29, 30, 32, 56). To date, most of the investigations have focused on the use of tri- or tetradentate ligands, but there are also a number of other chelating ligands (57–70) that have also been synthesized and used successfully for separation of proteins (Table 1).

According to the molecular structure and chelating mechanism of multidentates (Figure 1), the order for affording a stronger immobilization with the metal-ions should be pentadentate > tetradentate > tridentate (bidentates are not shown because of their rare usage) (5, 29, 30). This linking behavior induces a better stability of the chelate complex, which implies a lower metal-ion leakage. But, on the other hand, the stronger the metal-ligand strength is, the weaker the protein adsorption is also (30). Therefore, the order for biomolecule adsorption strength is tridentate > tetradentate > pentadentate (5, 30).

IDA is by far the most widely used chelating compound. It is commercially available from many producers due to its lower price and convenient availability. NTA and CM-Asp chelating compounds are also commercially available (Table 2) (30, 32).

Triazine dyes such as Cibacron blue (CB), Cibacron red (CR) Procion brown (PB), etc. are another type of chelating agent adopted for immobilized metal affinity method (57, 71–76) (Figure 2), but the immobilized metal-ions using these dye chelators are not as stable as using the dentate chelators (56). It may also be taken into account that the triazine dyes chelator utilization percentage (immobilized metal-ion capacity divided by the chelators' capacity) is lower. However, in some cases such as using CR 3BA (76), a utilization percentage higher than 100% could be achieved because two sites on one CR 3BA molecule are available for metal-ion chelation. Another point that should be taken into account is that dye ligands may also be used as affinity ligands themselves, which implies that part of biomolecule adsorption onto this kind of immobilized metal affinity matrices may be through dye affinity adsorption, not totally by metal-ion interaction (71–75).

Other electron-donating molecules may be used as the chelating agents, but their commercial availability and whether they can offer better immobilization and adsorption properties should be thoroughly evaluated. A successful example is to employ imidazole, an electron donor commonly used as the

Table 1. Some chelating compounds in use for immobilization in IMAC

Chelating compound	Coordination	Metal-ions	Reference
Salicylaldehyde	Bidentate	Cu(II)	(62)
8-hydroxyquinoline (im-8-HQ)	Bidentate	Al(III), Ca(II), Fe(III), Yb(III)	(63)
N-methacryloyl-(L)-histidine methyl ester (MAH)	Bidentate	Fe(III)	(57)
Dipicolylamine (DPA)	Tridentate	Ni(II), Zn(II)	(6, 65)
Iminodiacetic acid (IDA)	Tridentate	Cu(II), Ni(II), Zn(II), Ga(III)	(1, 5, 66, 64)
O-phosphoserine (im-OPS)	Tridentate	Al(III), Ca(II), Fe(III), Yb(III)	(70)
Tris(2-aminoethyl)amine (TREN)	Tridentate	Cu(II)	(58)
1,4,7-triazocyclononane (tacn)	Tridentate	Cu(II), Cr(III), Mn(II), Co(II), Zn(II), Ni(II)	(60)
Nitrilotriacetic acid (NTA)	Tetradentate	Cu(II), Ni(II), Zn(II)	(12, 13, 63, 66)
Carboxymethylated aspartic acid (CM-Asp)	Tetradentate	Ca(II), Co(II)	(6, 21, 126)
N-methacryloyl-(L)-cysteine methyl ester (MAC)	—	Fe(III)	(69)

ligand exchanger in the elution stage of the immobilized metal affinity chromatography, as the chelator to immobilize copper ions (56).

MATRICES

Selection of the supporting matrix is the first important consideration in affinity systems. To be adequate for IMAC the matrix must show the following characteristics (32, 56, 77):

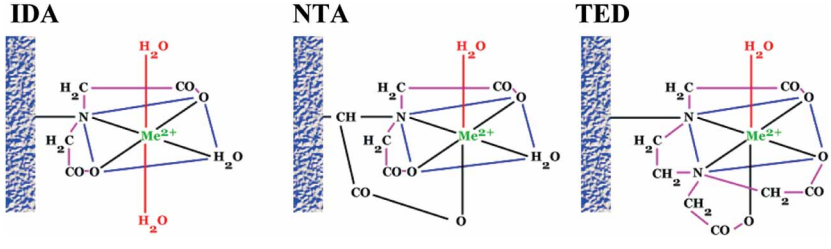

Figure 1. Putative structures of some representative dentate chelators in complex with metal-ions for the immobilized metal affinity method.

Table 2. Some chelating compounds in use for immobilization in IMAC

Ligand	Commercial source
Iminodiacetic acid (IDA)	Amersham Biosciences (Uppsala) Pierce (Rockford, IL) Sigma (St. Louis, MO) Merck (Darmstadt) Affiland (Belgium) Bioprocessing (Princeton, NJ) TosoHaas (Montgomeryville, PA) Serva Electrophoresis (Germany)
Nitrilotriacetic acid (NTA)	Qiagen (Chatsworth, CA)
Carboxymethylated aspartic acid (CM-Asp)	Clontech (Palo Alto, CA)
8-Hydroxy-quinoline (8-HQ)	Iontosorb (Czech Republic)

1. Easy to derivatize.
2. High hydrophilic character and extremely low non-specific adsorption.
3. High porosity to allow high amount of ligand immobilization.
4. Fairly large pore size and a narrow pore size distribution.

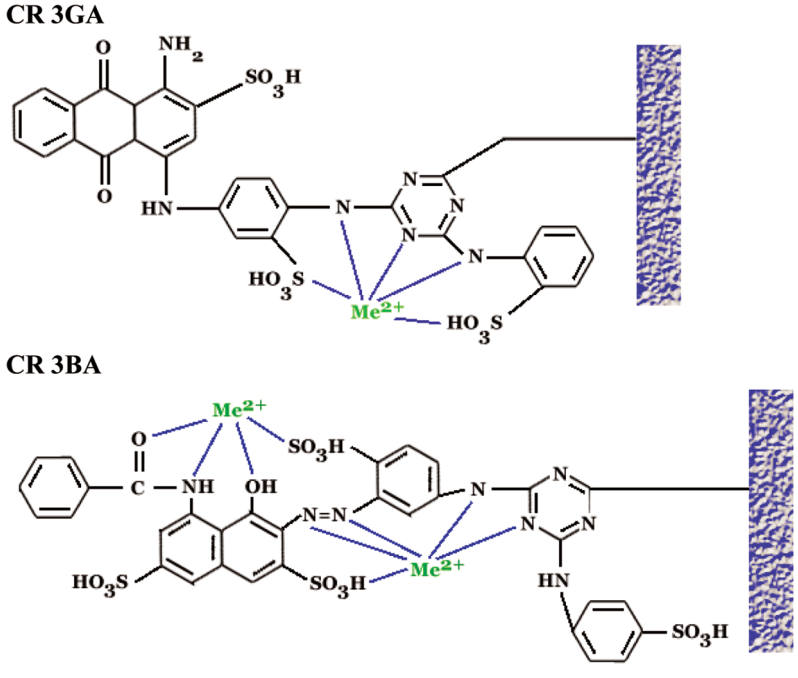

Figure 2. Putative structures of some triazine dye chelators in complex with metal-ions for the immobilized metal affinity method.

5. Functional surface groups (hydroxyl, carboxyl, amide, etc.).
6. Physically, chemically, thermally, and mechanically stable under a wide range of conditions.
7. Allow the use of high flow rates.
8. Permit regeneration of columns without degeneration of the matrix.
9. Provide a stable bed with no shrinking or swelling during the chromatographic run.

Classical stationary phases are based on soft-gel matrices, such as agarose, beaded cellulose or cross-linked dextran, which are compressible and restricted to low flow rate in chromatography operations, so their use in large-scale industry is limited (30). In order to meet the requirements of high efficiency and fast flow operation in chromatographic separations, efforts to develop some new rigid chromatographic matrices have been continued in the last three decades (78–85).

One of the inorganic adsorbents studied is porous silica, which is one of the ideal rigid supports, because it can be made with large pores and narrow pore size distribution to efficiently increase the macromolecular transfer inside the matrix (67). However, silica is difficult to be used directly for protein chromatography because of its complex non-specific adsorption of proteins by hydrophobic interactions. Moreover, its poor stability in basic conditions (pH $>$ 8.0) also limits its application in protein chromatography. Therefore, some surface modification is required to extend the application of silica beads as a chromatographic support for proteins. In order to increase the surface hydrophilic character and to eliminate the non-specific adsorption of silica-based matrices, many surface modification techniques have been developed (86, 87). Among them the most often used technique in coating silica-based matrices is the adsorption of hydrophilic polymers onto the support surface. It was agarose (30, 87, 88), dextran (86–89) and chitosan (67, 90, 91). The hydrophilic polymer layer not only reduces the irreversible adsorption by increasing the surface hydrophilicity, but it also increases the chemical stability and loading capacity of the coated silica. However, when silica is used as an affinity chromatographic support, two aspects should be considered. First, many non-specific interactions between the silica gel surface and biomolecules should be minimized. Second, the surface porous structure should be maintained and controlled since the original porous structure of the gel might disappear or be totally changed after the coating step (68). A number of commercially available coated IMAC matrices are presented in Table 3.

LIGAND IMMOBILIZATION CONDITIONS

There are many methods for immobilization of ligand molecules onto the matrices and the correct selection of immobilization conditions mainly depends on both the matrix and the ligand (56, 77).

Table 3. Commercially available chromatographic resins for IMAC

Manufacturer	Designation	Support	Ligand
Affiland	PDC Sepharose CL-4B Matrix	Cross-linked agarose	Pentadentate chelator
	PDC Sepharose 4 Fast Flow Matrix	Cross-linked agarose	Pentadentate chelator
Agarose Bead Technologies	Chelating Agarose Beads	Agarose	Iminodiacetic acid
Amersham Biosciences	Chelating Sepharose Fast Flow	Highly cross-linked agarose	Ni-IDA
	Streamline Chelating	Macroporous cross-linked 6% agarose + crystalline quartz core	Iminodiacetic acid
Bio-Rad Laboratories	Profinity IMAC Resins	UNOsphere beads	Iminodiacetic acid
Clontech	BD TALON Metal Affinity Resin	Cross-linked agarose	Carboxymethylated aspartic acid
	BD TALON Superflow Metal Affinity Resin	Cross-linked agarose	Carboxymethylated aspartic acid
	BD TALON CellThru	Cross-linked agarose	Carboxymethylated aspartic acid
	BD TALONspin Columns	Silica	Carboxymethylated aspartic acid
Invitrogen	Ni-NTA Agarose	Agarose	Ni-NTA
IONTOSORB-Bead Cellulose Derivatives	Iontosorb OXIN	Modified cellulose	8-Hydroxyquinoline
	Iontosorb SALICYL	Modified cellulose	Salicylic acid
	Iontosorb DETA	Modified cellulose	Diethylenetriamine
	Iontosorb DTTA	Modified cellulose	Diethylenetriaminete traacetic acid
	Iontosorb EDTA	Modified cellulose	Ethylenediaminete traacetic acid
	Iontosorb IDA	Modified cellulose	Iminodiacetic acid
Millipore	ProSep Chelating	Porous glass	Iminodiacetic acid

(*continued*)

Table 3. Continued

Manufacturer	Designation	Support	Ligand
Novagen	PreACT-Fracto-gel AZL	Polymethacrylate polymer	Linear polyelectrolyte chains ("tentacles")
Pierce	Immobilized Iminodiacetic Acid Gel	Agarose	Iminodiacetic acid
Prochem	Proteus Metal Chelate	Agarose	Ni-IDA
Quiagen	NTA Superflow	Highly cross-linked agarose	NTA
	Ni-NTA Magnetic Agarose Beads	Agarose	Ni-NTA
	Ni-NTA Superflow	Highly cross-linked agarose	Ni-NTA
	Ni-NTA Silica	Macroporous silica	Ni-NTA
Serva Electrophoresis	SERDOLIT Chelite CHE	Macroporous polystyrene	Iminodiacetic acid
	SERDOLIT Chelite P	Macroporous polystyrene + DVB	Aminomethyl phosphonic acid
Tosoh Bioscience GmbH	Toyopearl AF-Chelate-650M	Methacrylic polymer	Iminodiacetic acid
UpFront Chromatography	FastLine EBA adsorbents	Inert tungsten carbide phase + agarose bead	

First, immobilization should be attempted through the least critical region (not from the active site) of the ligand molecule, to ensure minimal interference on the specific interaction between the immobilized ligand and the metal-ion, and hence, the proteins. The active sites of biological molecules are often located deep within the three-dimensional structure of the molecule, which may cause an important steric hindrance between complementary ligand and proteins. Secondly, experimental conditions for coupling steps should be carefully chosen to avoid the loss of ligand activity or functionality.

In these circumstances, spacer arms, usually short alkyl chains, are frequently imposed between the matrix and the ligand to ensure accessibility. An ideal spacer arm must be bifunctional to be able to react with both membranes and ligands, but should not have any active center to cause extra non-specific adsorption (56). Moreover, there usually exists an

optimal spacer arm length to allow the accessibility between the immobilized ligand and the proteins without causing significant free arm folding (56, 92, 93). The final point to be noted for ligand immobilization is that the immobilized ligand has to be stable during the adsorption operations and in case of repeated usage.

Two alternative procedures may be followed (Figure 3). The matrix is first activated with an activation agent, and then the spacer arm is attached covalently to the matrix through the active points. The ligand is then reacted with the other end of the spacer molecules. Alternatively, the ligand-spacer arm conjugate is first synthesized and then attached to the carrier in one single step. Zou et al. (93) describe extensively the activation methods reported next here.

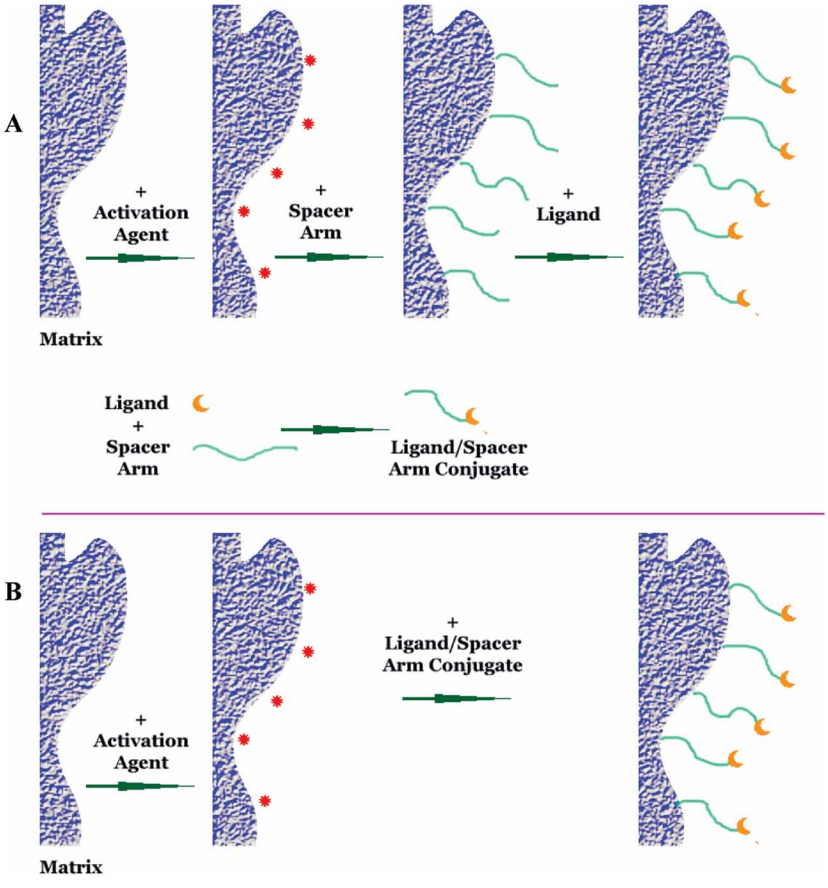

Figure 3. Strategies for coupling of ligands to the support matrix: (A) coupling through spacer arms; (B) coupling through spacer arm-ligand conjugates.

Cyanogen bromide activation has become one of the most popular methods. Activation is reasonably simple and involves attachment of the ligand via primary aromatic or aliphatic amino groups. However, cyanogen bromide has been reported to be strongly toxic and carcinogenic. Less toxic and easier to obtain than cyanogen bromide are epoxide reagents. Reaction of OH groups with bisoxiranes (such as 1,4-butanediol diglycidyl ether), epichlorohydrin, or epoxy bromopropane will produce activated supports with oxiran groups (94). Because the activation is simple, it plays an increasingly important role in the activation of the separation media. Another popular activation technique for the immobilization of proteins is the oxidation of polysaccharide supports. Reactive vicinal *cis*-hydroxyl groups within the matrix can be modified by treatment with sodium peroxidate. The result of this oxidation produces aldehydes that are easily converted into secondary amines by reductive amination or into hydrazides by reaction with dihydrazides. The reducing agent usually is a freshly prepared solution of sodium borohydride.

Spacer arms of ligands are attached via primary amine groups. A great disadvantage of this technique is that undissolved salts and other compounds produced during the oxidation and reduction steps may clog or destroy the micropores. To couple either spacer arms of protein ligands to any substrate that has free amino groups, carbodiimide condensation can be used. Such condensation reaction forms a peptide bond between three amino groups on the support surface and free carboxy groups on the ligand. When glass and/or silica are used as substrates, carbonylation with 1,1′-carbonyl diimidazole can be performed. This reaction is safe, simple, and effective and hence is becoming increasingly popular. The most popular affinity reaction group is succinimide ester, which allows a rapid and reliable attachment of protein ligands to a support matrix via amino groups.

Operating Conditions

Designing or optimizing an IMAC process on preparative scale demands a thorough understanding of all the fundamental mechanisms that govern the various interactions involved. Knowledge of these aspects can provide a rationale for selecting a suitable chelating ligand and establishing appropriate chromatographic conditions for purification (94). As such, the interactions between the immobilized metal-ions and proteins are extremely complex in nature. The protein retention on immobilized metal-ion affinity matrices is the combined effect of:

1. electrostatic (or ionic) interaction between the charged biomolecules and (1a) positively charged metal-ions, (1b) the negatively charged sites remaining on the matrix surface (such as the unreacted functional groups from the basic membrane materials, the residual carboxyl

groups for dentate chelators, or the residual SO_3^- groups for triazine dye chelators) due to the incomplete chelator coupling of metal-ion immobilization;
2. hydrophobic interaction between biomolecules and the hydrophobic sites on the matrix surface, and/or
3. donor-acceptor (coordination) interactions of the exposed amino acid residues (such as the imidazole groups of the histidine residues) on the biomolecule surface with the immobilized metal-ions (56, 76, 92, 94).

The effects of all these binding mechanisms can be evaluated by varying the pH and salt concentration of the adsorption solution. The dominance of a particular type of interaction over others is primarily governed by a number of variables such as the nature of the chelating ligand, metal-ion, surface amino-acid composition, and the surrounding chemical environment (i.e., nature of buffer salts, pH, and ionic strength) (6, 8, 28, 32, 40).

Quite a few fundamental studies have indicated that ionic strength and pH of the medium play a very significant role in governing the mechanism and extent of the interactions between the immobilized metal-ions and the proteins. The presence of a kosmotrope (or water structuring) salt in the adsorption buffer has a profound effect on protein adsorption on immobilized metal-ion affinity gels. This kind of gels behaves as weak ion exchangers under low ionic conditions (95, 96) primarily due to the dominance of electrostatic interactions. However, at higher ionic strengths these interactions are limited or eliminated and protein adsorption becomes more selective (41, 60, 70, 76, 89, 92, 96–102). Furthermore, the protein adsorption is controlled by the pH of the medium as protons affect the net charge on immobilized metal-ions as well as the surface properties of the proteins resulting in an altered retention behavior (5, 41, 49, 57, 60, 67, 68, 70, 75, 76, 89, 92, 96–104).

Nevertheless, most of the information available on this subject is qualitative in nature, and the quantitative data in terms of sorption capacities and binding affinity is quite limited (41, 60, 76, 92, 94, 97–99, 102). Moreover, there is no consensus on which theory/model should be used to explain the adsorption interactions in IMAC. The paucity of the relevant quantitative information results in a low predictability of IMAC separations making the optimization and scale-up a hard task. Traditionally, the classical Langmuir isotherm model is applied to account for these interactions as the first approach (26, 68, 74, 98, 105–107), assuming a single site of interaction between the protein and the metal ion, and neglecting any site exclusion effects. However, several studies have connoted the inadequacy of the simple Langmuir model in describing the heterogeneity and cooperativity in protein adsorption on IMA matrices as evidenced by curvilinear Scatchard plots (25, 34, 98, 106). Consequently, several other isotherm models such as Temkin, bi-Langmuir, Langmuir (multilayer) and Langmuir–Freundlich have also been considered in recent years (74, 94, 106, 108).

In summary, a pH value higher than the pK_a of surface exposed electron-donating amino acids (e.g., about 6–7 for the exposed histidine residue of protein (5, 28) is required for successful biomolecule adsorption (76, 92, 108). Moreover, to enhance the binding between biomolecule and the immobilized cationic metal-ions, a pH value higher than the isoelectric point or p*I* of the biomolecule is preferred (57, 60, 67, 76, 89, 92, 108, 109). Non-specific binding may occur due to incomplete chelator coupling or metal-ion immobilization (56). To reduce these non-specific binding effects, relatively high-ionic-strength buffers (usually 0.1–1.0 M salt concentration (32, 56) could be used at the adsorption or washing stage (but the buffer itself should not coordinatively bind to the immobilized metal-ions) (30, 32, 56). In addition, the use of these relatively high-ionic-strength buffers may also help remove some weakly bound impurity bio-species containing certain surface-exposed amino acids out of the affinity matrices. As for elution, a pH value lower than the pK_a of surface exposed electron-donating amino acids (protonation method) or a high salt concentration is often employed. If harsher conditions are needed, a high displacement agent (e.g., imidazole, ligand exchange method) or stronger metal-chelating agent (e.g., EDTA, the chelate annihilation method) could be tried (5, 8). 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 (10). Guidance and recommendation of choosing suitable buffers for different stages can be obtained from some review articles in this topic (6, 29).

APPLICATIONS

Proteins Purification

Purification of Naturally Occurring Metal-Binding Proteins

Histidine residues are found in most of the natural protein amino acid sequences, but as a consequence of their mild hydrophobicity, only a few of them are located on the protein surface (30). In view of this and due to their importance in metal-ion adsorption processes, just a low number of naturally occurring proteins are potentially suitable for purification by this type of chromatography. It must also be taken into account that these histidine residues must be accessible to metal-ions and their bulky chelating compounds also. These two conditions depend on proximity, orientation and spatial accessibilities of the histidines. Multipoint binding of different histidines can be achieved (49, 106–108). However, some other characteristics, as the cooperation between neighboring amino acid side groups or the local conformations, are of high importance in protein retention (30).

Consequently, IMAC has been used as a sensitive tool in the determination of proteins topography with respect to histidine and their surroundings (4, 110).

As a general rule, Garberc-Porekar and Menart (30) stated that "the protein shows the highest affinity for the metal surface arrangement which best matches its own distribution of functional histidines," being possible to adjacent histidines to bind to the same or to 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) (30).

Many proteins showing metal affinity have been successfully purified by IMAC without prior modification, including:

1. Bromelain (47)
2. Immunoglobulins (58, 111–113)
3. Lysozyme (59, 74, 85, 96, 97, 99, 114)
4. Human serum proteins (5, 23, 60, 70, 115–117)
5. Superoxide dismutase (118–120)
6. Catalase (36, 57, 121)
7. Human growth hormone (122)
8. Multimeric thermophilic enzymes from *E. coli* (123)
9. α-amylase (80, 124)
10. Metalloproteinase (125)
11. Bovine serum albumin (BSA) (67, 68, 74, 85, 103)
12. Lactate dehydrogenase (LDH) (126)

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 (32).

Purification of Engineered Metal-Binding Proteins

IMAC methodology has been widely used for protein recovery and purification since the research works of Hochuli et al. (12, 13), who stated the use of genetically engineered affinity tags attached to C- or N-terminals of recombinant proteins to improve the resolution of the first separations performed with IMAC.

Throughout all these years, many different histidine tags have been employed, from very short ones to rather long extensions attached to various model proteins. However, nowadays the most-used histidine tags consist of six consecutive histidine residues (30).

For several years there have been on the market some commercial expression vectors containing nucleotides coding for His6, His10, and some other fusions, but His10 tags, even though demonstrated efficient (127), have never received as much attention as His6 (12, 13, 84, 128–138).

The principle of polyhistidine tags is based on the premise that multiplicity of histidines may increase binding, but it may be remembered that very high affinity is not always advantageous in chromatographic multi-stage processes since it leads to very long retention volumes (28). Consequently, an ideal affinity tag should enable effective but not too strong a binding, and allow elution of the desired protein under mild, non-destructive conditions.

For example, in the case of *E. coli*, many host proteins strongly bind to Cu(II) or Ni(II) charged IMAC matrices, being thus eluted with the target proteins (30). For that, the latest approaches have focused on elution of the target protein in a "contaminant-free" window by selecting improved histidine tags. This has been done by a phage-displayed library, showing that His-His (His2) tags possess better chromatographic characteristics than those of the most commonly used His6 tag (105). Some other approaches have focused on the use of Zn(II) ions, which have shown better results than Cu(II) or Ni(II) ions due to their relatively low binding affinity for host cell proteins (139), and on the usage of "tailor-made" chelating supports with very short spacer arms and low surface density of chelating groups (140). Another similar situation occurs with oligomeric proteins, such as for example trimeric TNF-α, because interactions with the matrix are multiplied, making more difficult the selection of a proper affinity tag (141).

Histidine-tagged proteins can be successfully produced in prokaryotic and eukaryotic organisms, intracellularly or as secreted proteins (18), seeming to be compatible with all expression systems used today. However, it is necessary to experimentally determine where the His tag should be added, in the C- or in the N-terminus (30), although the addition of His tags to the N-terminus of the protein appears to be universal, if judged from the huge number of cases reported (142–145).

In most cases, the His tag does not need to be removed for some applications of the purified proteins. However, when the recombinant protein is intended for structural or physiological studies of pharmaceutical use, the His tag must be removed to obtain the protein with the correct amino acid sequence, thus avoiding unpredictable properties (144). Histidine tagging and IMAC have become a routine for easy first-time isolation of newly expressed proteins.

SOME OTHER APPLICATIONS OF IMAC

IMAC and Protein Refolding: Matrix-Assisted Refolding

Inclusion bodies produced in *Escherichia coli* are composed of densely packed denatured protein molecules in the form of particles. Refolding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery and accounts for the major cost in production of recombinant proteins from *E. coli* (145). Inclusion body proteins are empty of biological

activity and need elaborate solubilization, refolding and purification procedures to recover functionally active product (145). In general, inclusion bodies are solubilized by the use of a high concentration of denaturants along with a reducing agent, these high concentrations of chaotropic reagents results in the loss of secondary structure leading to the random coil formation of the protein structure and exposure of the hydrophobic surface (145). Solubilized proteins are then refolded by slow removal of the denaturant in the presence of oxidizing agent. Loss of secondary structure during solubilization and the interaction among the denatured protein molecules during refolding resulting in their aggregation are considered to be the main reasons for the poor recovery of bioactive proteins from the inclusion bodies.

Many times, the overall yield of bioactive protein form inclusion bodies is around 15–25% of the total protein and accounts for the major cost in production of recombinant protein from *E. coli* (145). Thus, a major bioprocess engineering challenge has been to convert this inactive and insoluble protein more efficiently into soluble and correctly folded product.

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 (Figure 4). The "matrix-assisted refolding" method can be briefly described as follows (32, 146):

1. Production of tagged protein in inclusion bodies
2. Solubilization
3. Denaturation
4. Immobilization on a charged chelating resin
5. Renaturation or refolding
   a. applying a linear gradient spanning denaturing to renaturing conditions (134, 147–153),
   b. applying an iterative refolding: technique based on repeated cycles of renaturation and denaturation with a decreasing concentration of the denaturing agent in each consecutive cycle (154–155).
6. Elution in its native and soluble form.

Although IMAC-based matrix-assisted refolding has distinct advantages over standard refolding techniques (32), it is necessary to take into account some disadvantages:

IMAC refolding is mostly limited to proteins where the inclusion of polyhistidine-tag does not interfere with the formation of the native configuration (146). It is possible, however, to refold bovine prion protein on Ni-NTA agarose utilizing the metal-binding properties of octa-repeat sequences (151); thus, a tag-less native protein could be produced.

If redox systems are required for proper refolding they have to be adjusted very carefully in IMAC to avoid the reduction of metal-ions. If reducing

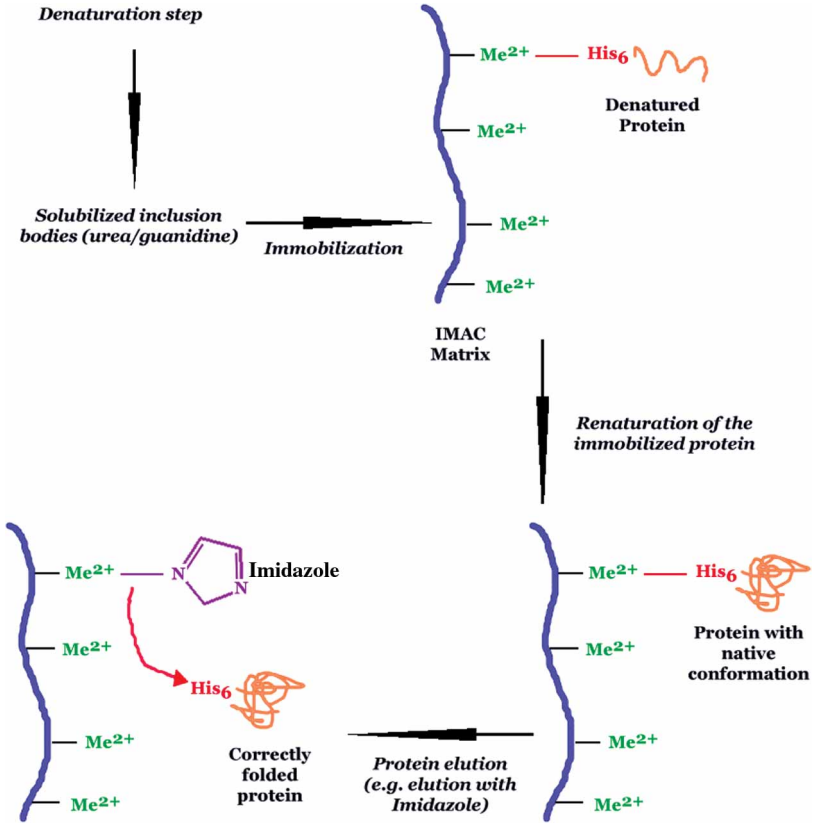

Figure 4. Schematic representation of matrix-assisted refolding on an IMAC support.

agents are necessary for solubilization of inclusion bodies a buffer exchange has to be performed before adsorption.

There are unspecific bindings through ionic interactions. Higher salt concentration leads to an increase in hydrophobic forces favoring the aggregation of folding intermediates; therefore, binding, washing, and refolding conditions require distinct optimization.

Ionic exchange chromatography refolding of polylysine or polyarginine-tagged proteins can be used as an alternative if the limitations of IMAC cannot be overcome (146). Some applications of IMAC in refolding processes are summarized in Table 4.

IMAC 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

Table 4. Refolding using immobilized metal affinity chromatography

Proteins	Media	Reference
Ykt6p SNARE	Ni-Chelating Sepharose Fast Flow	(150)
Aequorin	Ni-NTA agarose	(147)
Glutamyl-tRNA reductase	Ni-Chelating Sepharose Fast Flow	(149)
Trypanosoma brucei exopolyphosphatase	Ni-Chelating Sepharose Fast Flow	(134)
Polyhydroxyalkanoate synthase	Ni-NTA agarose	(148)
Human prion proteins	Ni-NTA agarose	(152)
Bovine prion protein	Ni-NTA agarose	(151)
EC-SOD	Ni-Chelating Sepharose Fast Flow	(153)
α-Tocopherol transfer protein	HiTrap Chelating Column	(154)
β-Glucosidase Zm-p60.1	Ni-NTA Sepharose Superflow	(155)
Toc75 and LHC2 membrane proteins	Ni-Chelating Sepharose Fast Flow	(218)
Mycobacterium tuberculosis antigens	Ni- HiTrap Chelating Column	(219)

involved in their metal-ion affinity (41, 50, 97, 99, 102, 109, 156) surface topography of histidine residues (4, 110) and analysis of protein-metal-ion interaction (34, 49, 60, 70, 74, 94, 98, 106–108, 157, 158).

An IMAC-derived technique designated "immobilized metal-ion affinity partitioning" (IMAP) has been applied to probe metal affinity domains and distinguish different surface features among structural related proteins (159, 160). The immobilized metal-ion affinity concept was extended to phase partitioning by grafting a chelator, iminodiacetate onto poly(ethylene glycol) (PEG) (115). This technique has been successfully used for the separation of human cord blood mononuclear cells, as a function of the nature and concentration of the ligand used (115).

Another IMAC-derived technique is "immobilized metal-ion affinity capillary electrophoresis" (IMACE) (161–163). This technique developed by Haupt et al. (161) extends the principle of IMA to capillary electrophoresis, employing ligands (IDA-Me(II)) covalently coupled to PEG, which serves as a soluble and replaceable polymer matrix.

IMAC and Processing of Phosphoproteins and Phosphopeptides

Phosphorylation is one of the more abundant posttranslational modifications of proteins, being implicated in cellular functions ranging from signal transduction and gene expression to the regulation of growth and involvement in disease mechanisms (164). IMAC is widely used for the selective enrichment of phosphopeptides from proteolytic digest mixtures containing both phosphorylated and nonphosphorylated components (165). To enrich and

separate phosphorylated proteins and peptides by IMAC, Fe(III) of Ga(III) has to be used as chelating cations, as these systems have the advantage of identifying both the protein and the phosphorylation site (166).

Although Ga(III) has been noted to have distinct advantages in selectivity and ease of elution (165), Fe(III) loaded columns remain the most commonly used form of IMAC for phosphopeptides selection (167–170), in particular, has been used to selectively purify and concentrate phosphorylated proteins and peptides (142, 165, 166, 171–177). In order to evaluate the effectiveness of separation method, IMAC is often carried out in conjunction with mass spectroscopy (164–166, 168, 170–172,174, 177) and other techniques, such as reversed-phase high-performance liquid chromatography (173) and capillary electrophoresis (142, 167, 171, 175).

ADVANTAGES AND DISADVANTAGES

Based on affinity chromatography, IMAC possesses the advantages and disadvantages of this type of separation technology, but with some important differences such as higher ligand stability and protein loading capacity, and lower costs (32).

Some Special Advantages of IMAC

IMAC purification of proteins takes advantage of their affinity for metal-ions, but it must be taken into account that many proteins do not have metal affinity, which is a disadvantage to overcome. A solution to this problem is the use of oligo-histidine tags inserted at the N- or C-terminus of recombinant proteins (13), where it likely is freely exposed on the proteins' surface (45).

This technique has been used in the production of recombinant proteins by the intracellular expression of heterologous genes in genetically engineered *E. coli* strains, but in most of the cases these recombinant proteins are deposited as insoluble aggregates of unfolded proteins in inclusion bodies, being thus necessary to use denaturation and refolding/renaturation techniques for their production as pure and biologically active proteins (30). In this situation there are two main advantages in using IMAC chromatography. The first one is the possibility of efficiently separating His-tagged proteins in the presence of denaturing concentrations of urea and guanidine-HCl, and the second is that this purification and the subsequent refolding can be done in a single step (30).

Another possibility when using His tags for the separation of proteins is the non-specifically binding of protein and non-protein contaminants to the chromatographic matrices or to the tagged protein itself. In this respect, IMAC matrices have the advantage of enabling the use of relatively harsh conditions. Thus, with membrane proteins, detergents can be used (127).

A great advantage of this kind of chromatography is that IMAC adsorbents can be easily regenerated in two single steps. In the first one, the chelated metal-ions are removed from the matrix by using strong chelating agents, being then, their metal-ion capacity, regenerated, by incubating the affinity matrices in a metal-ion solution. If necessary, after the first step, the resin can undergo very stringent washing and sterilization procedures, which are impossible with most affinity adsorbents (29). In general, the reusability of the immobilized metal affinity matrices for multiple experiments is quite excellent (71, 75–77,92, 100, 104, 178).

Some other unique characteristics in IMAC chromatography are:

1. Often allows single-step purification procedures (31).
2. Taking advantage of the IMAC matrix regeneration properties, metal-ions can be easily removed from the resin and changed to other ones, by using a strong chelating agent (32), allowing to investigate how the different metal-ions affect the adsorption process without changing the matrix
3. Has high protein loading capacities if compared to other affinity chromatographic techniques (31, 32).
4. Is useful for concentrating dilute protein solutions (31).
5. Is compatible with a number of buffers containing high ionic strength or chaotropic components (31).
6. Generally does not affect the structure of proteins (32).
7. The use of a non-charged IMAC column allows solutions to become transiently sterile since all metal-ions essential for bacterial growth are removed by chelation (31).

SOME POTENTIAL IMAC DISADVANTAGES

One important disadvantage of IMAC chromatography is the presence of metal-ions contaminating the purified protein solution, because they may whether destabilize or stabilize the protein. Negative counter-ions may significantly affect protein stability either positive or negatively, thus the contribution of metal-ions to protein stability must be carefully interpreted.

Metal-ions are known to trigger not only oxidative reactions as catalysts (31, 179–181) but also disrupt stabilization of lyophilized protein preparations (181), which affects the shelf life of the therapeutic proteins. On the other hand, metallic ions have also been used as additives along with sugars to enhance protein stability upon lyophilization (181). Moreover, in the case of pharmaceutical proteins, it is necessary to have special caution, because metal-ions trapped inside the protein could result in higher uptake by the target cells, through internalization mechanisms via receptors, especially when long-term therapies are required (30).

Taking this information into account, metal-ion transfer (MIT) and the metal-ion leakage can be considered as potential IMAC disadvantages. The effects of these two phenomena and their resulted toxicity during adsorption and elution are also significant issues to be evaluated (29, 30, 32, 76, 92).

Metal-ion transfer is a phenomenon that mainly leads to protein loss (32, 182) and low protein yields. Furthermore, if the target protein retains the capturing metal-ion within its structure it could hinder or even abolish the protein's bioactivity. It is of key importance to consider that some commonly used metal-ions are considered carcinogens, so the protein could also be hazardous if it is intended for therapeutics. Metal-ions Co(II) or Ni(II), widely used in IMAC, are known carcinogenic human agents, although they are considered weak mutagens when compared with arsenic or hexavalent chromium (183).

Metal-ion leakage from the resin leading to metal-ion contamination of the final product is a problem that would be especially important in the use of IMAC for the preparation of therapeutics (32). The reasons for metal-ion leakage at different stages are not the same. At the adsorption stage, the unstably immobilized metal-ions may be tightly captured by the biomolecules and released to the solution. On the other hand, they are possibly displaced by salt ions in the elution buffer at elution stage. A higher salt concentration at the elution stage is more effective for gaining high recoveries, but it may cause more severe metal-ion leakage. Reduction in salt concentration could diminish the metal-ion leakage, but the adsorbed biomolecule may not be able to completely elute out of the matrices. Consequently, an appropriate salt concentration in the elution buffer should be carefully selected (76), or a post trap for leaching metal-ions should be adopted (29).

In order to strip off the undesired metal from the protein and solve this problem, it is possible to use a metal-free chelating column packed with a strong chelating adsorbent such as TED (8, 31), or to add a chelating agent, such as EDTA, to the collecting vials (8).

Scale-up and Industrial Use

Metal affinity chromatography has crucial 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 technique (31). A potential drawback of IMAC, however, would be metal-ion leakage from its resins, leading to metal contamination of the final product (40). This could be solved by the use of an additional non-charged chelating column to capture the metal-ion contaminants (8, 31). 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 resin (40).

The design, optimization and scale-up of a chromatographic process using IMAC demands a thorough understanding to be developed regarding the fundamental factors governing the various interactions between immobilized metal-ions and proteins (34). Consequently, there is an immediate need to find a theory that is able to account for these interactions most efficiently in a qualitative as well as a quantitative manner. This model/theory could then, be used for prediction purposes and designing large-scale separation without the need of exhaustive experimentation (94). Nevertheless, other important chromatographic parameters, including adsorption rate constants, mass transfer coefficients and equilibrium parameters also need attention (40, 184).

To date, there are only a few reports on the use of IMAC for purification of proteins to be used in clinical therapy, likely due to concern over potential metal-ion leakage interfering with the very stringent purity requirements of therapeutics (141), although data are available showing that metal-ion tainting of IMAC-purified products is not likely to occur (185).

Immobilized Metal Affinity Membranes (IMAM): An Alternative

As explained here, current chromatographic problems in packed-bed include time-consuming and high-pressure packing, high-pressure drop in the columns, and slow intra-bead diffusion of solutes, and are almost inevitable in all the chromatographic systems with long columns and porous beads. To minimize all these problems, in 1988 an alternative to traditional chromatography based on the use of membranes as chromatographic matrices was developed (186), and it soon became one of the significant chromatographic inventions.

Membrane chromatography is superior to the conventional chromatographic technique because the macropores inside the membrane allow the convective flow of solute through the membrane, the intra-bead diffusion does not exist, and the small membrane thickness could result in a small or negligible pressure drop and allow high flow-rate operations for flow processes (56, 93, 186). Besides, the membrane processes could also offer some other advantages such as no bed compaction and easier scale-up. Among the most popular affinity membrane techniques are the immobilized metal affinity membranes (IMAMs) (71, 73, 75, 76, 92, 93, 100, 104, 188–199).

IMAMs design basically follows the immobilized metal affinity chromatographic systems developed since the 1970s. Hence the properties and applications are very similar.

As in IMAC, the matrix selection is the first important consideration, and the ideal membrane should, as well, hold the characteristics in the conventional chromatographic matrices. These characteristics are usually dependent on the base material, preparation method, and membrane geometry.

In general, membrane materials could be divided into two categories: inorganic and organic (93, 187). Inorganic materials usually show better

performance in mechanical strength, thermal stability, and chemical resistance than organic materials. But on the other hand, their pore properties, cost, capability for surface modification may not be competitive. Accordingly, inorganic materials are infrequently adopted as the affinity membrane supports. Organic membranes are commonly made of natural or synthetic polymer. The base materials for IMAMs reported in the literature (56, 92, 101–104, 188–197) include cellulose (cellulose acetate, cellulose nitrate, cellulose ester, regenerated cellulose, etc.), aliphatic polyamides (nylon-6, nylon-66, etc.), aromatic copolymers (polycarbonate, polysulfone, polyethersulfone, etc.), hydrocarbon polymers (polyethylene, polypropylene, etc.), polyvinylalcohol, glass hollow fiber, synthetic copolymer, etc.

Purification of Therapeutically Relevant Species Using IMAM

According to Liu et al. (100) the basic purpose of using membrane matrices is "simply to reduce pressure and processing time," thus "the affinity membranes are not expected to provide completely new separations, but only intended to speed the separations for many known processes." Therefore, the applications of affinity membranes are very similar to affinity chromatography with packed columns.

Immobilized metal affinity membranes have been applied in isolating or purifying native proteins as enzymes, albumins, immunoglobulins, haemoglobin, ribonuclease, growth factors, etc. (71–73, 75, 76, 92, 100–104, 188–202), and polyhistidine-tagged (such as His6) reaching very efficient (e.g., high capacity or binding strength) isolations (30, 63). Successful examples are, the isolation of His6-tagged *Eco*RV by Reif et al. (101) using an IDA-chelating immobilized metal affinity membrane adsorber from Sartorius with Ni(II) immobilized; the purification of crude urokinase form human urine by Hou and Zaniewski (201) getting an overall urokinase recovery of 80%; the purification of anti-TNP IgG1 mouse McAbs using a Zn(II)-IDA-PEVA hollow fiber membrane system by Serpa et al. (199) getting a similar selectivity than when using a conventional agarose bead system (Zn(II)-IDA-agarose); the purification of the hepatocyte growth factor from an insect cell supernatant by using an IDA-Cu(II) immobilized metal affinity membrane by Tsai et al. (92), and so on. Consequently, great possibility exists in using this method for the purification of potential therapeutics of biopharmaceutic molecules.

Challenges for the Practical Therapeutic Applications of IMAM

The main advantage in using IMAM against IMAC is to keep its characteristics but accelerating the separation process by employing membrane chromatography in minimizing mass transfer limitations and shortening the process

time by using high flow rates, letting get better retention of biological activity (203). According to this, similar procedures to the ones applied in the isolation of potential therapeutics using IMAC should be directly applied on IMAM and similar efficiencies should be expected (56).

For isolation or purification of therapeutically relevant bio-species, a high level of purification is certainly required. Apart from the use of His tagged recombinant proteins, the product purity can be increased by employing a mixed-mode or a multidimensional (cascade-mode) design. Mixed-mode design could be set up by stacking different types of flat-sheet adsorptive membranes in the preferred arrangement in one membrane module (65, 204–206), or connecting multiple modules in tandem but with a certain kind of adsorptive membrane in each module (207). The advantage of this design against its homologous in IMAC (mixed-bed chromatography) is that the use of membranes as supporting matrices can offer more controllable interaction conditions and simple membrane replacement (56). There are several papers in which this technique allowed researchers to obtain high separation efficiencies (65, 204–207). Multidimensional membrane chromatography is to use multiple adsorptive membrane modules in a sequential operation (178, 201, 208–212), where the eluted fraction from the first module is directly loaded to the second module, and so on. Undoubtedly, the number of processing steps and the total process time would be increased in a multidimensional arrangement, so, with regard to the retention of biological activity, using membrane chromatography in the multidimensional design is definitively more beneficial (203). The corresponding application and purification performances have been tested in several research works (208–212).

Most of the disadvantages and problems of the immobilized metal affinity membranes are for the immobilized metal affinity method and these have been discussed in a previous section. Thus, here we concentrate on the possible problems existing in the membrane chromatography.

Some of the major limitations, or shall we say challenges, with membrane chromatography are:

1. Availability of the membrane matrices
2. Different affinity strength
3. Slower adsorption rates
4. Membrane pore size distribution
5. Inlet flow distribution
6. Uneven membrane thickness
7. Lower binding capacity

Availability of the membrane matrices: Several commercial affinity membrane products were listed in the reviews paper of Roper and Lightfoot (187) and of Zou et al. (93). However, most of these products are not marketed any more. In a more recent review paper reported by Ghosh (210), the listed commercially available membrane adsorbers were of

ion-exchange mode. It is obvious that, at the present stage, people need to prepare their own affinity membranes. If the required membrane materials are not commercially available, the difficulty of using affinity membrane technique will be greatly raised.

Different affinity strength: Even if the membrane matrices could be readily obtained and the ligand immobilization is simple, the subsequent practical problem is whether the purification efficiencies using affinity membranes are close to affinity columns or not. Because the main advantage of using affinity membranes resides in the speeding of separation process, we may expect that all the affinity properties of the bio-system remain identical, or at least, very close. However, there are some examples in literature (213–216) where the above postulation cannot be held. These examples remind us that the same separation efficiencies many not always be achieved when employing affinity membranes to replace the packed columns, except in the cases that the same affinity properties have been testified (56).

Slower adsorption rates: Beside the possible difference in affinity strength, the intrinsic adsorption kinetics of the same bio-system may also be different between affinity bead columns and affinity membranes. Sometimes, the adsorption rates of biospecies are slower in the membrane systems than in the column systems, as shown in the pepsin and chymosin system (214, 215). The slow binding rates between the target species and the immobilized ligands will result in greatly reduced dynamic adsorption capacities, except for the use of low flow-rates (203, 216). The advantage of expediting the separation process at high accessible flow-rates under low pressure drops for affinity membranes will accordingly be deducted by the slow-binding kinetics and low recovery to eventually become unrealistic. In summary, sufficient knowledge of the binding chemistry, thermodynamics, and kinetics are very important in making choices from various adsorbents and processes (56).

Inlet flow distribution: Flow distribution problems are not unique to membrane adsorbers alone. However, this problem can be particularly acute in membrane chromatography due to the presence of a large frontal area with respect to the bed height. During the introduction of the feed into the membrane adsorber, the solute (i.e. protein) front should ideally hit all points of the leading membrane simultaneously. This is quite difficult to achieve due to a variety of reasons. Inefficient flow distribution within the adsorber itself further distorts the solute front, thus broadening the shape of the breakthrough curve leading to reduction in efficiency of adsorbent utilization (210). Improvement of inlet flow distribution is one area where a significant amount of work needs to be done if membrane chromatography is to be competitive.

Membrane pore size distribution: The pores present in microporous and macroporous membranes are generally not the entire same diameter. There is usually a pore size distribution, which in most cases is mono-modal. The

problem of having a wide pore size distribution is that the flow of feed will preferentially take place through the larger "flow-pores" and very little material will be carried through the smaller pores. As a result, the efficiency of adsorbent utilization will be greatly reduced (210, 217).

Uneven membrane thickness: Uneven membrane thickness presents a problem similar to that with large pore size distribution. Flow is encouraged where the thickness is smaller due to the lower pressure drop. Suen and Etzel (215) have recommended the use of stacks of large numbers of sheet membranes to even out this membrane unevenness related dispersion effect.

Lower binding capacity: The lower binding capacity of membrane adsorbers can be attributed to lower surface-to-bed volume ratio as well as to flow distribution problems. The most direct approach to address the former is to develop membranes having a high specific surface area. This is not quite so easy to achieve. Increasing the specific surface area without compromising other membrane properties such as mechanical strength, hydraulic permeability and pore size distribution may prove to be tricky. With low-molecular-mass ligands, low protein binding may sometimes be attributed to steric hindrance. Adding a spacer arm between the ligand and the support can solve this problem. Another method by which the membrane-binding capacity can be increased is by coating the pores with a porous polymer. The objective is to create a three-dimensional coating that would lead to a significant increase in the binding surface (210).

Even though the recovery and purification efficiencies of affinity membranes are comparable with the affinity columns, subsequent questions of engineers' concern are whether the cost of affinity membrane matrices and modules is lower than that of beads and columns, and whether there will arise any unforeseen problems during the scale-up of the affinity membrane process.

OUTLOOK

It has been proved that immobilized metal affinity chromatography (IMAC) is a powerful protein fractionation method used to enrich metal-associated proteins and peptides. 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 interactions. However the IMAC will play an important role in proteomics development.

The progress of proteomics is mostly determined by the development of advanced and sensitive protein separation technologies. In proteomics, IMAC has been widely employed as a prefractionation method to increase the resolution in protein separation. The combination of IMAC with other protein analytical technologies has been successfully utilized to characterize metalloproteome and post-translational modifications. In the near future, newly developed IMAC integrated with other proteomic methods will

greatly contribute to the revolution of expression, cell-mapping and structural proteomics.

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