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Review

Exploiting immobilized metal affinity membranes for the isolation or purification of therapeutically relevant species

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Abstract

Increasing reports regarding the isolation or purification of biospecies for therapeutic purpose using the immobilized metal affinity chromatography have been presented in recent years. At the same time, membrane chromatography technique has also gained more and more attention for their advantage in speeding the separation process. The immobilized metal affinity membrane technique developed by combining these two techniques may provide an alternative potential tool for separating the therapeutically relevant biospecies. In this review paper, the features of the immobilized metal affinity membranes are discussed and concentrated on three subtopics: membrane matrices, immobilized metal affinity method, and membrane module designs. Several examples of practically applying the immobilized metal affinity membranes on the purification of potential therapeutics reported in the literature are subsequently presented. Lastly, this review also provides an overall evaluation on the possible advantages and problems existing in this technique to point out opportunities and further improvements for more applied development of the immobilized metal affinity membranes.

Keywords: Reviews; Immobilized metal affinity membranes; Purification

Contents

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1. Introduction

In recent years, developments in biotechnology are unprecedentedly rapid and promising achievements are especially found in biopharmaceutical and medical applications. Subsequently, reliable and efficient downstream bioprocessing for new biopharmaceutical and medical products, such as the isolation and purification of therapeutically relevant species (e.g., interferons, vaccines, antibodies, DNA, polypeptides, therapeutic proteins, polynucleotides, etc.) is strongly demanded. Various types of column chromatographic techniques have been widely and successfully adopted for the separation of biospecies, among which affinity chromatography usually exhibits a better performance in raising the product purity owing to its feature in high specificity with the target species. The comparison between affinity chromatography and other types of column chromatographic techniques [1] is listed in Table 1. However, certain limitations, such as high cost and inherent chromatographic problems, still prevent the popular use of affinity chromatography. For the cost problem, it is generally difficult to reduce the high cost for bio-specific affinity technique (such as immunoaffinity chromatography), and subsequently, cheaper group-specific affinity methods become a good alternative. As to the chromatographic problems, they include time-consuming and high-pressure packing, high pressure drop in the column, and slow intra-bead diffusion of solutes, and are almost inevitable in all the chromatographic systems with long columns and porous beads. To resolve these problems, membrane chromatography was first developed in 1988 [2], and it has soon become one of the significant chromatographic inventions.

Membrane chromatography is superior to the conventional chromatographic technique in the following concerns [2–7]. The macropores inside the membrane (usually suitable for microfiltration purpose) allow the convective flow of solute through the membrane. The intra-bead diffusion does not exist in membrane chromatographic systems. Moreover, the small membrane thickness could result in a small or negligible pressure drop and allow high flow-rate operations for flow processes. Besides, the membrane processes could also offer some other advantages over the conventional chromatographic systems, such as no bed compaction and easier scale up.

In addition to affinity mode, using membranes as solid supports also applies to other adsorption modes such as ion-exchange and hydrophobic interaction/reversed-phase. These membrane chromatographic systems with different adsorption modes are together called “adsorptive membranes” as well. Up to now, efforts from plenty of researches have focused on the preparation, properties, and applications of affinity or other adsorptive membranes. Several review papers summarized the developments in this topic at different stages. In 1995, Roper and Lightfoot [3] presented a detailed review on adsorptive membrane technology. They not only reported the details regarding membrane materials, geometries, devices, operations, and applications, but also emphasized on the performance in thermodynamics and mass transport effectiveness. The review by Thommes and Kula [4] of the same year discussed the fluid dynamics of affinity membrane processes, such as axial diffusion, convection, and binding rate. In 1998, the Charcosset review [5] focused on the membrane matrices and ligand performance, including chemical structures of matrices, activation procedures, and various interaction modes. Extensive listings for affinity, ion-exchange, hydrophobic interaction/reversed-phase, and mixed-mode membrane chromatographic systems were provided in this article. In 2000, Klein [6] gave a broader review on affinity membrane technique. The content contained previous review articles, affinity modification, membrane preparation, module design, kinetics, and applications. Some practical problems for membrane operations were also indicated in that paper. In 2001, Zou et al. [7] published a review paper emphasizing on detailed preparation methods for a variety of affinity membranes. They listed some updated informations such as membrane substrates and geometries, activation methods, types of spacer arms, applications on protein purification, etc. Zou et al. also gave several examples for the practical applications using affinity membranes in their lab. In 2002, Ghosh [8] reviewed the update development in the application of membrane chromatography on protein separation. The author summarized over 100 papers related to this technique and reported

Table 1
Comparison between different types of column chromatographic techniques [1]

Property	Affinity		Ion exchange	Hydrophobic interaction/ Reversed-phase
	Group-specific	Bio-specific		
Adsorption capacity	Medium–high	Low	High	Medium–high
Selectivity	Medium–high	High	Low–medium	Low–medium
Recovery	High	Medium	High	Medium
Loading condition	Mild	Mild	Mild	Sometimes harsh
Elution condition	Mild	Harsh	Mild	Mild
Regeneration	Complete	Sometimes incomplete	Complete	Incomplete
Cost	Low	High	Low	Low

some important results: flat sheet membranes are the most widely used shape; affinity interaction is the most popular mode (usage is greater than 50% based on the literature); serum antibodies, enzymes, and monoclonal antibodies are the three largest application categories. That review paper lastly suggested that the scope for future work in this area could concentrate on improved process and equipment design, development of new membranes, screening of binding properties of existing membranes, and proper system selection.

This review paper will particularly focus on the possible therapeutic applications of immobilized metal affinity membrane technique, partly owing to its great potential in lower expense, higher adsorption capacity, and better reusability (referred to the characteristics of the group-specific affinity method listed in Table 1) and also because the authors have been working on this topic in recent years. This review contains a general review for immobilized metal affinity membrane technique, several examples for the related application on the isolation or purification of therapeutically relative species, and an overall evaluation on the possible problems existing in this technique.

2. Membrane matrices for affinity membranes

2.1. Matrix characteristics

The matrix selection is the first important consideration in affinity systems. An ideal supporting membrane matrix for affinity separations should hold the characteristics in the conventional chromatographic matrices. The required characteristics [3,5,7,9] include: (1) high hydrophilicity and low nonspecific adsorption (which may be due to charged or hydrophobic groups on matrix surface); (2) high specific surface area (to allow great amount of ligand immobilization and high adsorption capacity); (3) fairly large pore size (to allow the target biospecies easily flow through) and a narrow pore size distribution; (4) high chemical, thermal, and mechanical stabilities (under a wide range of conditions such as high and low pH values, high and low temperatures, in situations which require organic solvents, detergents and disruptive eluents); (5) sufficient surface functional groups (e.g., hydroxyl, carboxyl, amide, etc.) for further derivatization and immobilization of ligands. These characteristics are usually dependent on the base membrane material, preparation method, and membrane geometry.

2.2. Types of membrane materials

In general, membrane materials could be divided into two categories: inorganic and organic [3,7]. 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. The inorganic substrates found in the literature are titanium oxide modified to form anion exchange membranes [10] and glass hollow fibers used for immobilized metal affinity membranes [11]. Organic membranes are commonly made of natural or synthetic polymer. The materials include cellulose (cellulose acetate, cellulose nitrate, cellulose ester, regenerated cellulose, etc.), hydrocarbon polymers (polyethylene, polypropylene, etc.), aromatic copolymers (polycarbonate, polysulfone, polyethersulfone, etc.), aliphatic polyamides (nylon-6, nylon-66, etc.), polyvinylalcohol, synthetic copolymer, and so on. These organic materials and their properties have been thoroughly evaluated in several of previous review papers [3,5–7].

2.3. Selection of ligand immobilization conditions

Same as for conventional chromatographic matrices, there are many methods for immobilization of ligand molecules onto the membrane matrix [3,5–7] and the correct selection of immobilization conditions mainly depends on both the matrix and the ligand. In the following are listed several items which should be noted for finding good immobilization method [9]. First of all, immobilization should be tried not through the active site of the ligand molecule to prevent any interference on the specific binding between the immobilized ligand and the target biospecies. Secondly, experimental conditions for coupling steps should be carefully chosen to avoid the loss of ligand activity or functionality.

On the other hand, spacer arms (e.g., alkylamine, diamine, polypeptide, polyamine, polyether, amino acid, etc. [5,7]) are frequently imposed between the supporting matrix and the ligand. 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 nonspecific adsorption [7]. Moreover, there usually exists an optimal spacer arm length to allow the accessibility between the immobilized ligand and the target biomolecules but without causing significant free arm folding [5,7,12,13]. The final point to be noted for ligand immobilization is that the immobilized ligand has to be stable during the adsorption operations and in the case of repeated usage. In some review papers [3,5–7], the most commonly used membrane activation and ligand immobilization methods for affinity membranes have been listed.

2.4. Various affinity modes

When employing membranes as solid supports, various affinity modes have been adopted in the literature (over 100 papers are available) [3,5–7] and their relative adsorption properties and separation efficiencies have been extensively investigated. The ligand type is generally used to categorize the affinity mode, which includes immobilized metal affinity, dye affinity, immunoaffinity, and others. The first two belong to group-specific affinity type, whereas the third mode is bio-specific. The comparison between group-specific and bio-specific membrane modes on operating conditions, selectivity, reusability, and cost could directly refer to those for affinity chromatographic systems listed in Table 1. Although bio-specific methods could offer a better selectivity, group-specific methods have gained more and more attention because they are more superior in other aspects for practical applications.

2.5. Matrix characteristics of the affinity membranes reported in the literature

In summary of the data reported in previous literature, the employed base matrices for affinity membranes include dialysis membranes with MWCO (molecular mass cut-off) of several thousands, ultrafiltration membranes with MWCO of several hundreds of thousands, and microfiltration membranes with pore sizes ranging from 0.1 to 50 μm (most are in this category). The pore sizes of these affinity membrane materials are sufficiently large to allow rapid diffusion or convection of biospecies to the binding sites on the internal surfaces. In addition, the adsorption capacities for most affinity membranes are comparable to the corresponding capacities for affinity chromatographic beads [6]. However, most membrane materials show a high tendency for nonspecific binding [6]. This should be precluded by using suitable agents to block the remained functional groups on membrane surfaces after ligand immobilization.

3. Immobilized metal affinity membranes

The immobilized metal affinity membranes (as listed in Table 2) [3,5–7,11,13–31] are one of the most popular affinity membranes (others include dye affinity and Protein A/G affinity membranes). By far, there are about 20 published research papers regarding this topic. Their designs basically follow the immobilized metal affinity chromatographic systems developed since 1970s, and hence the properties and applications are very similar.

Immobilized metal affinity method generally adopts the chelators coupled on the supporting matrix to immobilize metal ions (as electron-pair acceptors), which could specifically interact with the exposed electron-donating amino acid residues (such as histidine, cysteine, tryptophan, tyrosine, aspartic acid, or glutamic acid) on biomolecule surface through nonbonding lone pair electron coordination [1,6,11,13–37]. In determining the immobilized metal ion capacity, the functional groups on the base membrane material, the nature of chelating agent, immobilization method, types of metal ions, and metal ion concentration all play an important role. Among these factors, chelating agent and metal ions have particularly significant influences and are most commonly evaluated in the related chromatographic literature.

3.1. Chelating agents

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, etc.), tridentate (e.g., iminodiacetic acid (IDA), dipicolylamine, *ortho*-phosphoserine, *N*-(2-pyridylmethyl) aminoacetate, 2,6-diaminomethylpyridine, etc.), tetradentate (e.g., nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp), etc.), and pentadentate (e.g., *N*, *N*, *N*′-tris-carboxymethyl ethylene diamine (TED), etc.), have been thoroughly investigated since immobilized metal affinity chromatography was exploited [1,32]. To connect the reactive groups on the membrane matrices (such as hydroxyl, amine, etc.) and dentate chelators, epoxide activation agents such as epichlorohydrin, epibromohydrin, and bioxiranes are usually utilized. According to the molecular structure and chelating mechanism of multidentates (see Fig. 1, bidentates are not shown because of their rare usage), the order for affording a stronger immobilization with the metal ions should be pentadentate>tetradentate>tridentate [1,32,33]. The strong chelation for tetradentate and pentadentate could induce a better stability of chelate complex and subsequently a lower metal ion leakage. But on the other hand, the number of coordination site on metal ions left for biomolecule binding will be less and hence may cause a weaker adsorption. Therefore, the order for biomolecule adsorption strength is tridentate>tetradentate>pentadentate [32,33].

Table 2
Characteristics of the immobilized metal affinity membranes reported in the literature

Membrane material	Chelating agent	Chelating agent capacity	Metal ion	Metal ion capacity	Biospecies adsorbed or isolated	Adsorption capacity	Refs.
Glycidyl methacrylate-grafted cellulose membranes	IDA	–	Zn^{2+}	650 μmol/g	Urokinase	–	[14]
Glycidyl methacrylate-grafted polyethylene hollow fibers	IDA	1700–1800 μmol/g base polymer	Cu^{2+}	1100–1200 μmol/g base polymer (180 μmol/ml)	BSA, L-histidyl-L-leucine	BSA 0.26 μmol/ml L-Histidyl-L-leucine 78 μmol/ml	[15]
Epoxidized polysulfone membranes	IDA	39 μmol/g	Cu^{2+}	41 μmol/g	Histidine, alanine, threonine, phenylalanine	Histidine 19.6 μmol/g	[16]
Hydrophilic copolymer membranes from Sartorius (170–190 μm thick)	IDA	–	Cu^{2+} Ni^{2+} Zn^{2+} Co^{2+}	Cu^{2+} 65.75 μmol/cm^2 Ni^{2+} 59.25 μmol/cm^2	α-Chymotrypsinogen, β-lactoglobulin, lysozyme, myoglobin, cytochrome c from tuna heart and horse heart, recombinant fusion protein, HSA	HSA 1.13 mg/cm^2 (for Cu^{2+}) HSA 0.81 mg/cm^2 (for Ni^{2+})	[17]
Modified glass hollow fibers	IDA	–	Cu^{2+}	–	α-Chymotrypsinogen A, cytochrome c, lysozyme, ribonuclease A	α-Chymotrypsinogen A 3.93 mg/ml Cytochrome c 38.2 mg/ml Lysozyme 69.7 mg/ml Ribonuclease A 29.2 mg/ml	[11]
Glycidy-4-oxoheptyllether-modified polysulfone membranes	IDA	–	Cu^{2+} Ni^{2+} Zn^{2+}	Cu^{2+} 0.48–0.75 mg/ml Ni^{2+} 0.47–0.72 mg/ml Zn^{2+} 0.47–0.73 mg/ml	Histidine, tryptophan	Histidine 4.78–5.44 μmol/ml (for Cu^{2+}) Tryptophan 4.79–5.44 μmol/ml (for Cu^{2+})	[18]
Polysulfone membranes from Sartorius	IDA	–	Cu^{2+} Ni^{2+} Zn^{2+}	Cu^{2+} 0.89–0.92 mg/ml Ni^{2+} 0.88–0.93 mg/ml Zn^{2+} 0.89–0.92 mg/ml	Histidine, tryptophan	Histidine 10.93–11.42 μmol/ml (for Cu^{2+}) Tryptophan 10.71–11.04 μmol/ml (for Cu^{2+})	[18]
Hydroxyethyl cellulose-coated nylon membranes (150 μm thick)	IDA	–	Cu^{2+}	0.17 μmol/cm^2	Lysozyme, concanavalin A, ovalbumin	Lysozyme 321 μg/cm^2 Concanavalin A 451 μg/cm^2 Ovalbumin 419 μg/cm^2	[19]
Cellulose acetate membranes	IDA	–	Cu^{2+}	–	BSA, γ-globulin	–	[20]
Surface-modified polyethylene hollow fibers	IDA	–	Cu^{2+}	1500 μmol/ml	Lysozyme, histidine, hemoglobin	Lysozyme 1–8.5 μmol/ml Histidine 550 μmol/ml	[21]
Polyglycidyl methacrylate-grafted cellulose membranes	IDA	15–150 μmol/g	Cu^{2+} Ni^{2+}	–	BSA, HSA, IgG, bovine liver catalase	BSA 39.09 mg/ml	[22]
Microporous sheets with amine functional groups from Arbor Tech	IDA	–	Cu^{2+}	800 μg/g	Lysozyme, chymotrypsin	–	[23]
Polyglycidyl methacrylate-grafted cellulose membranes	IDA	–	Cu^{2+}	15.8–114.2 μmol/g	Bovine liver catalase	–	[24]
Polyvinyldiene fluoride-based membranes (140 μm thick)	IDA	–	Cu^{2+}	0.42–0.53 μmol/cm^2	Lysozyme, heptocyte growth factor	Lysozyme 0.055–0.085 μmol/cm^2	[13]

Table 2 (*Continued*)

Membrane material	Chelating agent	Chelating agent capacity	Metal ion	Metal ion capacity	Biospecies adsorbed or isolated	Adsorption capacity	Refs.
Regenerated cellulose membranes (160 μm thick)	IDA TED	IDA 1.28 μmol/cm^2 TED 1.11 μmol/cm^2	Cu^{2+}	1.22 μmol/cm^2 (for IDA) 0.62 μmol/cm^2 (for TED)	Lysozyme, γ-globulin, BSA	Lysozyme 0.0244 μmol/cm^2 (for IDA) 0.0086 μmol/cm^2 (for TED) γ-Globulin 0.001 μmol/cm^2 (for IDA) 0.0009 μmol/cm^2 (for TED) BSA 0.0015 μmol/cm^2 (for IDA) 0.0016 μmol/cm^2 (for TED)	[25]
Regenerated cellulose membranes (160 μm thick, 47 mm diameter)	IDA	–	Cu^{2+} Ni^{2+} Zn^{2+} Co^{2+} Ca^{2+} Fe^{2+} Al^{2+}	Cu^{2+} 33.4 μmol/disk	Penicillin G acylase	–	[26]
Poly(2-hydroxyethyl methacrylate) membranes (ca. 600 μm thick)	CB F3GA	1.07 μmol/cm^2	Fe^{3+}	47.3 μg/cm^2 (8.48×10^{-4} μmol/cm^2)	Glucose oxidase, catalase, BSA	Glucose oxidase 8.7×10^{-7} μmol/cm^2 Catalase 2.15×10^{-7} μmol/cm^2 BSA 2.21×10^{-7} μmol/cm^2	[27]
Poly(2-hydroxyethyl methacrylate) membranes (ca. 600 μm thick)	CB F3GA	1.07 μmol/cm^2	Cu^{2+}	21.6 μg/cm^2	Lysozyme	Lysozyme 165.1 μg/cm^2	[28]
Polyamide hollow fibers	CB F3GA	35.8 μmol/g	Zn^{2+}	250 μmol/g	Lysozyme, BSA	Lysozyme 144.2 mg/g (10.3 μmol/g) BSA 162 mg/g (2.4 μmol/g)	[29]
Poly(2-hydroxyethyl methacrylate)/chitosan interpenetration networks membranes (ca. 600 μm thick)	PB MX 5BR	0.361 μmol/ml	Fe^{3+} Cu^{2+}	Fe^{3+} 179 μmol/ml Cu^{2+} 234 μmol/ml	Lysozyme	147.4 mg/ml (for Fe^{3+}) 127.8 mg/ml (for Cu^{2+})	[30]
Regenerated cellulose membranes (160 μm thick)	CB 3GA CR 3BA	CB 3GA 0.52 μmol/cm^2 CR 3BA 0.5 μmol/cm^2	Cu^{2+}	0.27 μmol/cm^2 (for CB 3GA) 0.7 μmol/cm^2 (for CR 3BA)	Lysozyme, γ-globulin	Lysozyme 0.0103 μmol/cm^2 (for CB 3GA) 0.0165 μmol/cm^2 (for CR 3BA) γ-Globulin 0.001 μmol/cm^2 (for CB 3GA) 0.0029 μmol/cm^2 (for CR 3BA)	[25]
Cellulose membranes (100 μm thick)	Imidazole	–	Cu^{2+}	388 μg/cm^2	Human albumin, γ-globulin, fibrinogen, IgG	Human albumin 92.5 μg/cm^2 γ-Globulin 174 μg/cm^2 Fibrinogen 76 μg/cm^2 IgG 275.5 μg/cm^2	[31]

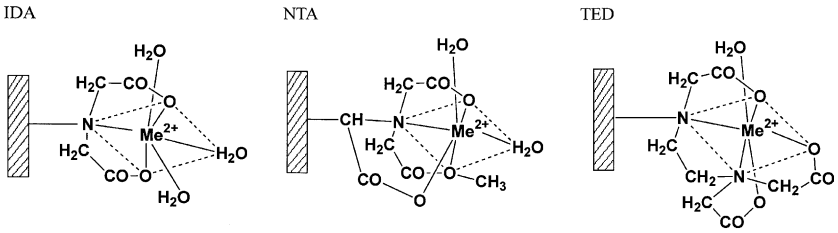

Fig. 1. Putative structures of some representative dentate chelators in complex with metal ions for the immobilized metal affinity method.

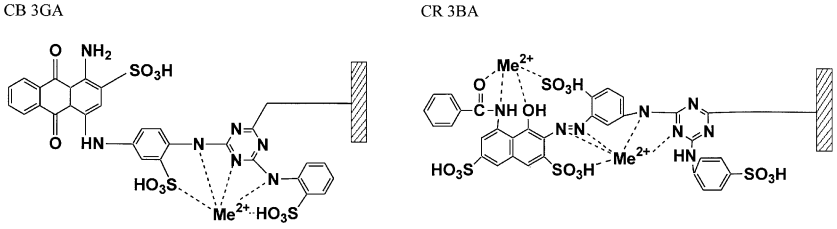

Fig. 2. Putative structures of some triazine dye chelators in complex with metal ions for the immobilized metal affinity method.

By far, the tridentate IDA is the most popular chelator adopted in the literature and the commercial products due to its lower price and convenient availability [32]. The immobilized metal affinity adsorbents using the tetradentates NTA and CM-Asp are also commercially available from some producers. As to the pentadentate TED, its chemical is not easily obtained from commercial sources and a two-step reaction is usually required in the lab to couple TED on the matrices. Nevertheless, this two-step reaction may lead to a failure in forming a stable metal chelate [25].

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 [25,27–30]. Their chelating mechanism is presented in Fig. 2. The immobilized metal ions using these dye chelators are not as stable as using the dentate chelators and their chelator utilization percentage (defined as the immobilized metal ion capacity divided by the chelator capacity) is usually lower. However, in some cases such as using CR 3BA [25], a utilization percentage higher than 100% could be achieved (refer to Table 2) because two sites on one CR 3BA molecule are available for metal ion chelation (see Fig. 2). Another point should be noted for the employment of dye chelators that the dye itself could also be used as affinity ligand. Accordingly, part of biomolecule adsorption onto this kind of immobilized metal affinity membranes may be through dye affinity adsorption, not totally by metal ion interaction [27–30].

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 doner commonly used as the ligand exchanger in the elution stage of the immobilized metal affinity chromatography, as the chelator to immobilize copper ions onto the cellulose-based dialysis membranes [31].

On the other hand, chelating agents may not be needed if the membrane matrices contain functional groups suitable for direct metal ion immobilization. For example, silver ions could be directly immobilized onto the sulfonated polystyrene-grafted polyethylene hollow fibers via ion-exchange interaction with sulfonic acid groups [38]. These silver ion-immobilized membranes were successfully applied for the purification of docosahexaenoic acid ethyl ester from bonito oil ethyl ester solution.

3.2. Metal ions

In addition to chelating agents, suitable metal ions should be selected to ensure a stable immobilization and a higher adsorption with the target biospecies. According to their reactivity to different nucleophiles, metal ions could be divided into three subcategories: soft, hard, and intermediate [39]. Soft Lewis metal ions (such as Hg^{2+}, Cd^{2+}, etc.) has a better reactivity with sulfur atom, whereas hard Lewis metal ions (such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}, etc.) prefer oxygen-rich groups (e.g., aspartic acid, glutamic acid, or phosphate groups) [32]. The intermediate type (such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, etc.) includes mostly the first transition metal series and could couple with sulfur-, oxygen-, and nitrogen-containing amino acids. Intermediate metal ions are by far the most commonly adopted for immobilized metal affinity method [1,32]. When applying IDA as the chelator, the affinities to the retained biomolecules are usually in the following order: $Cu^{2+}>Ni^{2+}>Zn^{2+}\geqslant Co^{2+}$ [32]. However, in practical applications, the selectivity to the desired biospecies for the immobilized metal ions should be more crucial. Up till now, Cu^{2+} and Ni^{2+} are the most commonly used metal ions for immobilized metal affin-

ity method in the literature and the commercial chromatographic systems. The amino acid side chain interaction with the chromatographic adsorbents immobilized with these two metal ions could refer to the literature [34].

3.3. Selection of operating conditions

The mechanisms for biospecies binding onto the immobilized metal affinity membranes were thoroughly evaluated in our previous studies [13,25]. They can be categorized into four types of interactions [13,19,25,33,35]: (1) the affinity binding provided by the electron-donating groups of the exposed amino acid residues (such as the imidazole groups of the histidine residues) on the biomolecule surface with the immobilized metal ions; (2) the electrostatic interaction between the charged biomolecules and positively charged metal ions; (3) the electrostatic interaction between the charged biomolecules and the negatively charged sites remaining on the membrane 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) owing to the incomplete chelator coupling or metal ion immobilization; and (4) the hydrophobic interaction between biomolecules and the hydrophobic sites on the membrane surface. The first two interactions contribute to the specific bindings, whereas the latter two are nonspecific bindings. The effects of these binding mechanisms can be evaluated by varying the pH and salt concentration of the adsorption solution. To further quantitatively measure the proportion of nonspecific binding (usually nonspecific electrostatic interaction), a detailed investigation on the degree of conversion for each coupling step in the preparation process of immobilized metal affinity membranes is needed.

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 [36]) is required for successful biomolecule adsorption [13,25,35]. Moreover, to enhance the binding between biomolecule and the immobilized cationic metal ions, a pH value higher than the pI of biomolecule is preferred [13,25,35]. Nonspecific binding may occur owing to incomplete chelator coupling or metal ion immobilization as mentioned above. To reduce these nonspecific binding effects, relatively high-ionic-strength buffers (usually 0.1–1 *M* salt concentration) could be used at the adsorption or washing stage (but the buffer itself should not coordinatively bind to the immobilized metal ions) [32]. In addition, the use of these relatively high-ionic-strength buffers may also help remove some weakly bound impurity biospecies 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 a harsher condition is needed, displacement agent (e.g., imidazole, ligand exchange method) or stronger metal-chelating agent (e.g., EDTA, chelate annihilation method) could be tried [32,37]. Guidance and recommendation of choosing suitable buffers for different stages can be obtained from some review articles in this topic [1,34].

3.4. Metal ion leakage and regeneration

The effects of metal ion leakage and resulted toxicity during adsorption and elution are also significant issues to be evaluated [1,13,25,32]. The reasons for metal ion leakage at different stages are not the same. At 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 [25], or a post trap for leaching metal ions should be adopted [1]. To regenerate the immobilized metal ion capacity, an incubation of the affinity membranes in the metal ion solution is suggested. In general, the reusability of the immobilized metal affinity membranes for multiple experiments is quite excellent [13,17,24–29].

3.5. The immobilized metal affinity membranes reported in the literature and commercial products

Table 2 lists the immobilized metal affinity membranes reported in the literature. In summary, most of the membranes employ IDA and copper ions [5,7,11,13–26] and high-percentage chelator utilization could be achieved. If a broader definition for the terminology “membrane” is adopted, commercial products of immobilized metal affinity membranes may include Sartobind IDA (Sartorius) [5,17,32], Ni-NTA HisSorb Strips (Qiagen) [32], SwellGel™ Ni Chelated Discs (Pierce) [32], Empore™ Chelating Disks (3M), etc. Except for the Sartobind product, most products are designed only for the purposes of small-amount protein isolation and analysis or trace metal analysis, not really for larger-scale biomolecule separation. However, the Sartobind product is no more marketed [32] and there are no commercial immobilized metal affinity membranes available at the present.

4. Membrane shapes and module designs

When employed for practical applications, affinity membranes are usually housed in a module to allow the perfusive operation of solution. Batch operation mode is not economical, although the use of this mode may achieve the desired adsorption and recovery. The design of membrane module

depends on the membrane shape. Various membrane shapes and module designs have been adopted in different adsorptive membrane processes. In general, the forms in flat sheets and hollow fibers are most frequently adopted [3,7,8,40–42] because they are inexpensive and conveniently available. Their features, advantages, and inherent problems will be discussed in the following subsections.

4.1. Flat sheets

For flat-sheet membranes (usually in disc shape), there are plenty of inexpensive products (made for microfiltration, ultrafiltration, or dialysis usage) with various sizes and materials readily available in the market [40,41]. Their internal pore structures are often more homogeneous than other membrane shapes. In addition, flat sheets can be simply scaled up by stacking several membrane sheets together [3,5–8,43] or using wider membrane sheets. These characteristics and advantages make the flat-sheet shape become the most popular in the affinity membrane technique.

4.1.1. Disc holder

Various module designs result in different flow operation conditions and distinct separation efficiencies. Possible module designs for flat-sheet membranes include disc holder, plate-and-frame, spiral-wound, and others. Disc holder (refer to Fig. 3) is the simplest and usually the cheapest way to mount the membrane discs inside for flow operations [40,41]. Only dead-end mode can be applied to the operation of disc holder. In this design, o-ring or gaskets are usually employed to ensure multiple membranes stacked compressively and to prevent the fluid leaking from disc edge [3,7,44]. Flow maldistribution is another problem requires consideration because it occurs more significantly in a short and wide bed such as the flat-sheet membrane shape [3,7,8,40–42]. Accordingly, the use of flow distributor is necessary for achieving better transport property [7,43]. On the other hand, due to the use of dead-end operation, crude solutions have to be clarified before being fed into the holder. Otherwise, large substances in the solutions will clog the pores and foul the membranes [3,6]. In addition, both intra-membrane mixing (such as axial dispersion) and extra-membrane mixing (such as dead-volume mixing) effects are found to be primary contributors for band broadening or separation inefficiency in several research works [3,6,44–46]. The axial dispersion effect could be estimated using the dimensionless transport group, axial Peclet number, and be diminished by adjusting the operation conditions [43,44], whereas the dead-volume effect is inbuilt in the design of the whole operation system including disc holder, tubing, and detection instrument [44,46,47].

The disc holder design for larger-scale separations is not difficult since it could be set up in wider or multi-sheet-stacked arrangement. In addition to maintaining a large membrane volume for adsorption and separation purpose, stacking membranes can also facilitate high flow-rates, diminish the effects of porosity and thickness variations, and minimize the interstitial void volume that may cause dispersion of solutions [6,8,43]. However, stacked-sheet designs will increase pressure drops, which lessens the advantage of membrane devices compared to bead columns [6], and the disc edge leaking problem may become more severe [3,40,41].

4.1.2. Plate-and-frame module

Usually used in the filtration unit, cross-flow designs are capable to reduce the membrane fouling problem. When applying in the adsorption processes, cross-flow devices could offer both filtration and adsorption effectiveness at the same time [3,6,40–42]. Therefore, isolation or purification of target biospecies from crude solutions or suspensions and further usage as bioreactor all become possible in cross-flow flat-sheet membrane modules. Various modules, such as plate-and-frame, spiral-wound, and others, have been designed for the cross-flow operations of flat-sheet membranes.

Plate-and-frame module can be arranged in either a simple or complicated way. The complicated plate-and-frame membrane module could be referred to the design adopted in the filtration field [48]. A simple device was recently developed in our laboratory (see Fig. 3) [41] and it allows the feed solution to pass the membrane front surface in both parallel and

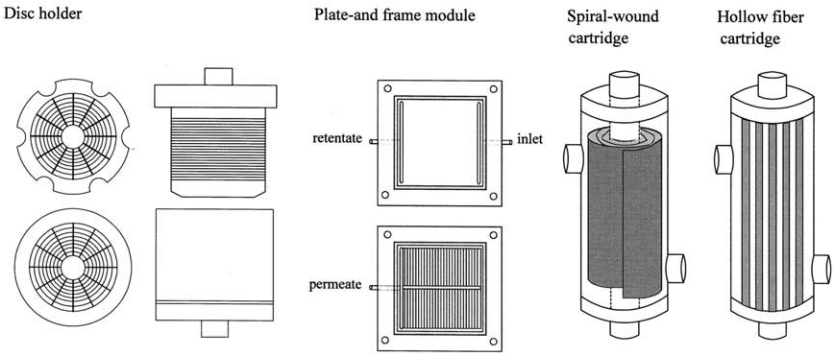

Fig. 3. Schematic diagrams for some typical membrane modules.

vertical directions. If suspension is fed into this module, the substances larger than the membrane pores will directly pass out parallelly (as the retentate) and the molecules smaller than pore dimension will penetrate through the pores (as the permeate) [41,42]. Only the permeate molecules could be adsorbed onto the internal membrane pore surface. With this design, the replacement of membranes, the module cleaning, and the scale-up (membrane stacks) are as convenient as for those for the disc holder design. To evenly distribute the inlet fluid, both multi-channel inlet and built-in flow distributor are preferred. In addition, the pressure drops are low in this design with the use of affinity membranes [41].

The possible problems for the plate-and-frame module include fluid leakage from membrane edge and the mixing effect caused by extra-membrane spacing in the module. Moreover, it may be worthy to note that part of the substances passing through the membranes parallel to the retentate outlet can still be adsorbed onto the membrane frontal surface [41]. This may cause the membrane fouling and decrease the separation efficiency. A solution of this problem may be employing a filtration membrane placed in front of the affinity membranes to strengthen the filtration function of this design. However, some other effects such as poorer adsorption and more dispersion may occur subsequently [41]. If the feed is a clarified solution and the filtration function is not necessary, the cross-flow design will spilt the outlet solution into two streams and may cause dilution effect. In this case, the dead-end operation mode is suggested.

4.1.3. Spiral-wound cartridge

The spiral-wound design for affinity membrane process (shown in Fig. 3) is to wind a single flat-sheet membrane around a rigid and permeable core cylinder and use a outer sleeve for protection [3,6,8,12,14]. An operation mode is to radially distribute the influent solution to the peripheral surface of the cartridge. After passing through the membrane matrix, the solution will flow along the central core and leave the cartridge [12,14]. Another mode is to feed the solution into the central core. The retentate will flow axially through the cylinder, whilst the permeate can pass into the membrane in the radial direction and the target species could then be adsorbed. In the first mode, pore clogging and membrane fouling are possible. As to the second mode, the cylindrical core is used as a filtration unit and its material and pore properties should be correctly selected for the separation system. Since the membrane sheet is wound to form multi-layers [3], the advantages for stacked membranes such as allowing high flow-rates and the minimal effects of porosity and thickness variations will be remained. In addition, the membrane-sheet edge leakage problem does not exist in the radial-flow designs such as spiral-wound cartridge. As to the possible problems for the spiral-wound design, there included extra-membrane mixing effect, difficult membrane replacement and module cleaning, and the flow complexity caused by the decline in transmembrane pressure drop along the radial direction.

4.1.4. Others

Some other membrane configurations are also feasible, e.g., tubular module, polymer rod column, fan-folded or pleated sheet designs [3,5,6,49,50]. The tubular module and polymer rod column have been practically adopted for adsorptive membrane technique. However, the fan-folded or pleated flat-sheet membrane design is only available for filtration purpose [6], but not effectively adopted in the adsorptive membrane process yet.

4.2. Hollow fibers

The membrane form in hollow fibers is usually considered to have high specific surface area [8]. Since high specific area could lead to high relative adsorption capacity, hollow fiber becomes one of the most popular shapes adopted in affinity membrane technique [40,41]. Moreover, commercial hollow fiber products are versatile and readily available.

The housing for hollow fibers is typically a tube-and-shell-like cartridge with a bundle of hollow fibers mounted inside (see Fig. 3) [3,6,7,38]. In this design, cross-flow operation is feasible and an effective separation for crude solutions or suspensions could be performed. Since the hollow fiber cartridge is a radial-flow design, no lateral leaking problem should be concerned. The design using affinity hollow fibers, however, may suffer from the problem of nonuniform ligand distribution if the ligand is immobilized onto the fibers already entrapped in a cartridge [41,42,51,52]. Besides, it can also lead to a difficulty in assembling the fibers into a cartridge after a uniform ligand immobilization onto individual fibers [41,42]. Moreover, the hollow fibers require large inside diameters and should be packed at a low fiber density to avoid the plugging of big biomolecules. However, this will cause large extra-membrane volumes and create large mixing effects [6].

5. Review of applications on isolation or purification of therapeutically relevant species using immobilized metal affinity membranes

Since the basic purpose of using membrane matrices is simply to reduce pressure and processing time, the affinity membranes are not expected to provide completely new separations, but only intended to speed the separations for many known processes [6]. Therefore, the applications of affinity membranes are very similar to affinity chromatography with packed columns. As discussed in Section 3 that the immobilized metal affinity method is developed for the separation of biomolecules with certain surface-exposed amino acid residues such as histidine, the immobilized metal affinity membranes have been found to be applied in isolating or purifying enzymes, albumins, immunoglobulins, hemoglobin, ribonuclease, growth factors, etc. (refer to Table 2) [5,7,11,13–31]. In addition, polyhistidine tags (such as His6) are usually used for those biospecies with-

out directly accessible surface-exposed special residues and the resulted immobilized metal affinity isolation is very efficient (e.g., high capacity or binding strength) [32,53]. A successful example by Reif et al. [17] is the isolation of His6-tagged *Eco*RV, a recombinant fusion protein and an *E. coli* restriction endonuclease, using an IDA-chelating immobilized metal affinity membrane adsorber from Sartorius with Ni^{2+} immobilized. Consequently, great possibility exists in using this method for the purification of potential therapeutics or biopharmaceutic molecules. In the subsequent subsections, several exhaustive immobilized metal affinity membrane examples in the applications of isolating and purifying therapeutically relevant biospecies from the previous literature are presented.

5.1. Urokinase

Urokinase (UK) is a plasminogen-activating enzyme and can effectively catalyze the conversion of plasminogen to plasmin, which can lyse the fibrin clots associated with vascular blockage [6]. Hou and Zaniewski [14] purified crude UK from human urine through a two-step adsorptive membrane process, strong cation exchange (sulfonyl) membranes and IDA-Zn^{2+} glycidyl methacrylate-grafted cellulose membranes in series. In the first step, the crude UK (with a p*I* of ca. 9) was loaded and adsorbed to the cation exchange membrane cartridge (spiral-wound, 100 ml) at pH 4.5 under a flow-rate of 100 ml/min, cationic impurities were washed out with 0.4 *M* NaCl, and lastly the UK was eluted with 1 *M* NaCl. The purification factor was 6.5. In the second step hiring the IDA-Zn^{2+} membrane cartridge (spiral-wound, 100 ml), the UK solution eluted from the previous step was loaded and adsorbed onto the affinity membrane at pH 8.2 under a flow-rate of 50 ml/min and then eluted using 0.05 *M* imidazole. A further 3.3-fold purification was achieved. The overall UK recovery was 80%.

5.2. Human serum albumin

Human serum albumin (HSA) is a protein commonly used for therapeutic purpose, such as shock, heavy loss of blood, etc. [7,22]. Yang et al. [22] prepared immobilized metal affinity membranes by coupling IDA and Ni^{2+} onto the glycidyl methacrylate-grafted cellulose composite membranes. The membrane cartridge they adopted was 6×16 mm I.D. and 22 pieces of affinity membranes were stacked inside for the purification of HSA from a commercially available solution (obtained from Behring). The flow-rate was 1.5 ml/min and the back pressure was low, only 0.117 MPa. The elution was conducted by using 1 *M* NaCl in 20 m*M* phosphate buffer, pH 7.8, 1 *M* NaCl in 20 m*M* phosphate buffer, pH 6.0, and 5 m*M* acetate buffer, pH 4.5, sequentially. Their results showed that the purification efficiency of immobilized metal affinity membranes were comparable with similar agarose-bead-packed column, but the membrane chromatography exhibited a four to five times faster performance than the packed column.

5.3. Hepatocyte growth factor

Hepatocyte growth factor (HGF), with a molecular mass of 91 kDa (for pro-HGF) [54] and a p*I* of ca. 9.5 [55], is an important protein contributing to embryogenesis, wound repair and tumor invasion [54,56]. HGF has been found to bind with immobilized copper, and its purification using the column process has been proved successful in the literature [56]. Consequently, employing the immobilized metal affinity membranes for HGF purification should be probable and has been confirmed in our laboratory [13].

In that study of HGF purification [13], we prepared the immobilized metal affinity membranes by coupling diamine-epichlorohydrin–IDA-Cu^{2+} onto the flat-sheet hydrophilic PVDF (polyvinyldiene fluoride)-based Immobilon AV membranes (Millipore). The recombinant HGF-containing Sf9 insect cell supernatant (the medium was TNM-FH with 10% fetal bovine serum, pH 6.1–6.2) for purification had a pH around 6.8–7.0 and a salt concentration of 6.5 mg/ml (including $CaCl_2$, $MgCl_2$, $MgSO_4$, KCl, etc.). The HGF purification was conducted in the batch mode. Fifty ml of supernatant were incubated with two pieces of 9×9 cm immobilized metal affinity membranes at room temperature for 12 h.

The results were analyzed by Western blotting [54] (as presented in Fig. 4). The original insect cell supernatant (lane 6) included HGF and other biospecies. The darkest mark (around 91 kDa) is pro-HGF, and a light band in 64 kDa is the α-chain of recombinant HGF. After adsorption by the immobilized metal affinity membranes, most biospecies remained in the solution (lane 7). Lanes 1–5 represent different elution results. Clear protein bands are only shown in lane 5, where the harsh elution condition using EDTA was

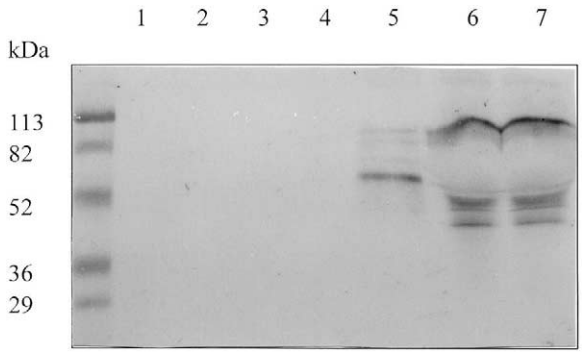

Fig. 4. Western blotting analysis for HGF purification using IDA-Cu^{2+} PVDF-based membranes in the batch mode. Lanes: (1) elution solution using 0.1 *M* KC1 in 50 m*M* Tris–HC1, pH 7; (2) elution solution using 0.5 *M* KC1 in 50 m*M* Tris–HC1, pH 7; (3) elution solution using 1 *M* KC1 in 50 m*M* Iris HC1, pH 7; (4) elution solution using 1 *M* KC1 in 50 m*M* Tris–HCI, pH 4; (5) elution solution using 1 *M* KC1 in 50 m*M* EDTA, pH 7; (6) original insect cell supernatant; (7) supernatant after adsorption.

adopted. In this EDTA elution solution, the overall number of biospecies is significantly reduced, and the species of largest portion (the darkest mark in lane 5) is the α-chain of recombinant HGF. Consequently, HGF has been successfully isolated from insect cell supernatant using the IDA-Cu^{2+} immobilized metal affinity membranes with the use of severe elution condition. This elution phenomenon is similar to that reported in the literature using immobilized copper affinity chromatography [56] and has indicated the possibility of a multi-site interaction of HGF with immobilized Cu^{2+}.

5.4. Penicillin G acylase

Penicillin G acylase (PGA) is an important biocatalyst which could hydrolyze penicillin G to 6-aminopenicillanic acid (6-APA) for further production of semi-synthetic penicillins. Since PGA has plenty of specific amino acid residues (including 13 histidines, 28 tryptophans, 43 aspartic acids, and 36 glutamic acids), some of them are very possibly exposed on the surface of molecules. Consequently, PGA purification using immobilized metal affinity membranes should be feasible. The immobilized metal affinity membranes adopted in our laboratory were IDA-Cu^{2+} regenerated cellulose-based membranes [26]. An optimal metal ion capacity was achieved by varying different reaction conditions and metal ions.

Crude PGA extract was the supernatant from the lysis of cells in the fermentation broth from *E. coli* cultivation. Protein concentration was measured with the Bio-Rad Protein Assay using BSA as standard. PGA activity was determined using the colorimetric method proposed by Balasingham and one unit (IU) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol 6-APA/min at 37 °C, pH 8.0. The effects of various conditions on PGA adsorption, such as temperature, pH value, and salt concentration, were evaluated in the batch experiments. It was found that the optimal PGA adsorption capacity was achieved at 4 °C, pH 8.5, and with the addition of 0.5 *M* NaCl. On the other hand, the elution effects of using NH_4Cl or imidazole at various concentrations were also tested. With regard to both efficiencies of recovery and purification factor, 1.0 *M* NH_4Cl was selected as the best eluent. Moreover, 0.02 *M* NH_4Cl was suggested to adopt in the washing stage because it could help elute other impurity protein out of the affinity membranes, without the loss of PGA.

For flow experiments, 10 pieces of 47-mm diameter affinity membranes (160 μm thick) were stacked and housed in an acrylic disc holder. One hundred ml of crude PGA extract in the optimal loading buffer (0.5 *M* NaCl, 10 m*M* phosphate buffer, pH 8.5) were loaded to the holder at 4 °C under a flow-rate of 1.2 ml/min. Some impurity protein was washed out with 72 ml of washing buffer (0.02 *M* NH_4Cl, 0.5 *M* NaCl, 10 m*M* phosphate buffer, pH 6.8), and then bound PGA was eluted with 63 ml of elution buffer (1 *M* NH_4Cl, 0.5 *M* NaCl, 10 m*M* phosphate buffer, pH 6.8). The results are displayed in Fig. 5. A purification factor of 9.11 in specific activity and a PGA recovery of 90.25% were attained in this membrane chromatographic process, which are comparable with the results reported by Fitton and Santarelli [57] using Cu^{2+}-chelating Sepharose packed column (a purification factor of 4.64 in specific activity and a PGA recovery of 100% with one-step NH_4Cl elution; a purification factor of 12.36 and a PGA recovery of 97% with three-step NH_4Cl elution).

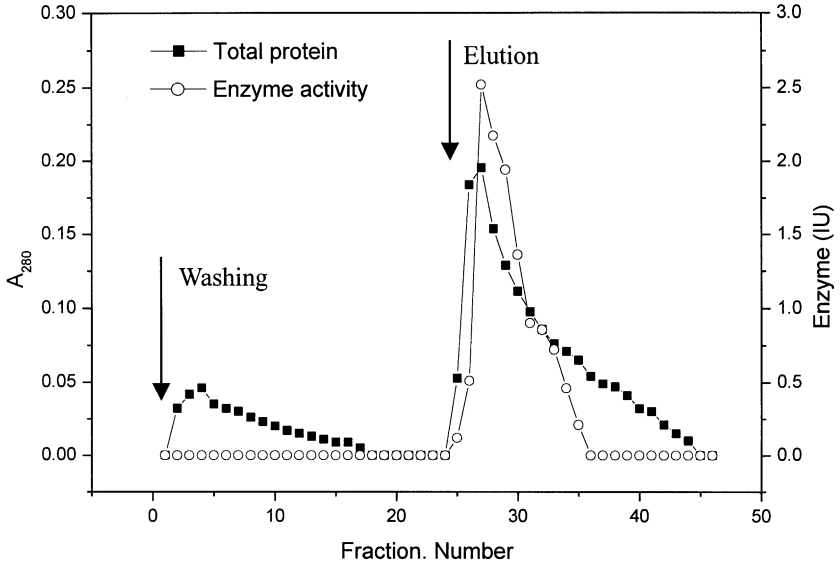

Fig. 5. PGA purification results using a 47-mm diameter membrane disc holder with 10 pieces of IDA-Cu^{2+} regenerated cellulose-based membranes at a flow-rate of 1.2 ml/min. Loading protein: 100 ml, 0.245 mg/ml, 0.1305 IU/ml activity, 0.533 specific activity. Loading buffer: 0.5 *M* NaC1, 10 m*M* phosphate, pH 8.5. Washing buffer: 0.02 *M* NH_4C1, 0.5 *M* NaCl, 10 m*M* phosphate, pH 6.8. Elution buffer: 1 *M* NH_4C1, 0.5 *M* NaCl, 10 m*M* phosphate, pH 6.8. Washing started from fraction 1 and elution from fraction 25.

6. Challenges for the practical therapeutic applications of immobilized metal affinity membrane technique

The main benefit using the immobilized metal affinity membrane technology is to reserve the characteristics of the immobilized metal affinity method but accelerate the separation process by hiring the membrane chromatography to minimize the mass transfer limitations. When the process time is shortened, most of the biological activity of the target species will be retained [3,6]. Since there have been many reports on the application of isolation of potential therapeutics using the immobilized metal affinity columns, similar procedures should be able to directly applied to the immobilized metal affinity membranes and similar efficiencies are expected. Most of the advantages for the immobilized metal affinity method and the membrane chromatography have been indicated in the previous sections, which will not be repeated again.

For isolation or purification of therapeutically relevant biospecies, a high level of purification is certainly required. Since the immobilized metal affinity method is a group-specific affinity method, it may not be easy to meet this criterion. One of the improved methods is to enhance the affinity strength between the immobilized metal ions and the surface-exposed special amino acid residues of the target biomolecules. The use of polyhistidine tags is a possible method [32]. However, the removal of histidine tags would be a crucial problem for practical medical application and mass production [32]. In addition, when the affinity binding is stronger, the required elution condition will be relatively harsher. Harsh elution conditions may affect the biological activity of target species, or cause a more severe metal ion leakage (metal toxicity problem [32]). All of these problems need to be carefully resolved.

Another possible alternative to increase the product purity is to employ the mixed-mode or 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 [42,58], or connecting multiple modules in tandem but with a certain kind of adsorptive membrane in each module [42]. This design is similar to mixed-bed chromatography, but the use of membranes as supporting matrices can offer more controllable interaction conditions and simple membrane replacement. High separation efficiencies with the use of mixed-mode membrane chromatography were verified in the literature [42,58]. Multidimensional membrane chromatography is to use multiple adsorptive membrane modules in a sequential operation [5,14,59,60], where the eluted fraction from the first module is directly loaded to the second module, and so on. The corresponding application and purification performances have been tested in several research works [14,59,60]. Undoubtedly, the number of processing steps and the total process time would be increased in a multidimensional arrangement. With regard to the retention of biological activity, using high throughput procedure such as membrane chromatography in the multidimensional design is definitely more beneficial [6].

As to the disadvantages and problems of the immobilized metal affinity membranes, most of them for the immobilized metal affinity method (such as nonspecific binding fraction, competitive adsorption of impurities, metal ion leakage and toxicity, etc.) have been discussed in several review articles [1,32] and in the preceding sections. Here we concentrate on the possible problems existing in the membrane chromatography.

The first problem is the availability of the membrane matrices. Several commercial affinity membrane products were listed in the review paper of Roper and Lightfoot [3], such as Affinity 10 from Cuno, AbSorbent (Protein G) from Genex, MemSep (Protein A and G) from Millipore, and Sartobind (Epoxy) from Sartorius. However, most of these products are not marketed any more. In a more recent review paper reported by Ghosh [8], 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.

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 biosystem remain identical, or at least, very close. However, for some affinity systems, the above postulation cannot be held. The first example is the adsorption of two enzymes, pepsin and chymosin, onto the pepstatin A-immobilized membranes as shown in the literature [44]. In that work, chymosin (K_d=4×10^{-6} M) exhibited a higher affinity than pepsin (K_d=9×10^{-6} M), which is opposite to the results for gel beads at the same adsorption conditions [61,62] where the K_d value was 1.4×10^{-7} M for chymosin and 3.9×10^{-9} M for pepsin. Moreover, the discrepancy in the affinity strength of this enzyme system between the gel beads and the membranes is as large as 30–2300 times. The second example is the BSA adsorption onto different Cibacron blue (CB) dye affinity adsorbents at identical adsorption conditions. BSA could be greatly bound to the commercial agarose-based dye affinity beads, but no adsorption was observed for the use of membrane supports [46,63]. These examples remind us that the same separation efficiencies may 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. However, from an opposite point of view, if the totally different affinity properties could be achieved by replacing packed columns with membranes, a new separation may be expected. In the above first example, the elution order for the two enzymes can be reversed for the use of membrane supports. In the second example, a separation between BSA

and other adsorbed biospecies using CB dye affinity membranes will become possible, whilst it is quite difficult for CB dye affinity bead columns.

Beside the possible difference in affinity strength, the intrinsic adsorption kinetics of the same biosystem 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 [44,61,62]. The slow binding rates between the target species and the the immobilized ligands will result in greatly reduced dynamic adsorption capacities, except for the use of low flow-rates [6,41,46]. 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. According to our experiences in the various adsorptive membranes, slow intrinsic binding rates usually occur in the affinity mode. The ion-exchange membranes typically allow faster binding [5,42,44] and high flow-rates are accessible in their relevant processes. A commercial example is the Sartobind ion-exchange membrane adsorbers from Sartorius. High purification and high recovery can still be obtained at the flow-rates as high as 50–150 ml/min (information from the manufacturer).

On the other hand, the commonly higher extra-membrane volumes existing in most membrane modules (e.g., plate-and-frame, hollow fibers) intend to cause larger mixing effects [6], which will lead to band broadening and worsen the separation efficiencies. Detailed membrane packing information should be investigated during designing the module and housing the membranes. Another factor that may more or less influence the adsorption performance is the pore size distribution of the membrane matrices [8]. The pore property provided from the membrane manufacturer is usually the average pore size, not the pore size distribution. Two review papers [6,8] indicated that very small pores are seldom reached during solution convection through the membranes because of entrapped gases and the applied pressures required to wet these small pores and displace the gas can rarely be attained in the low-pressure operations of affinity membranes. Although some isoporous membranes are now available, they are expensive and have low porosity [8].

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? Although the scale up of most membrane modules is generally not difficult, their relevant information from bench to production scale still requires further explorations [6].

7. Conclusions and scope for future work

Affinity membrane chromatography technique is developed principally to overcome the problems of slow intrabead diffusion and high backpressure in the packed column chromatographic systems, but still keeps the required adsorption capacity and separation efficiency. The relatively faster process time of affinity membranes has been successfully demonstrated in some previous works. At present, more and more attention focuses on their practical applications and their potential for further commercialization, especially in the isolation or purification of biospecies for therapeutic purpose.

This review provides certain information on the possible advantages and disadvantages of immobilized metal affinity membranes applied to therapeutic usage, partly based on the available literature and partly based on the research experiences in our laboratory. In summary, there is still great potential for developing higher-efficiency immobilized metal affinity membrane devices, if the following issues are improved: the correct and careful designs in the adsorption properties (e.g., raising the degree of conversion in each reaction step during metal immobilization and minimizing the nonspecific binding proportion), module designs (e.g., disc holder, plate-and-frame module, spiral-wound cartridge, or hollow fiber cartridge), and operation conditions (e.g., selection of buffers, selection of flow-rate for better recovery and purification, or the use of mixed-mode or multidimensional arrangements).

References

[1] G.S. Chaga, J. Biochem. Biophys. Methods 49 (2001) 313.
[2] S. Brandt, R.A. Goffe, S.B. Kessler, J.L. O'Connor, S.E. Zale, Biotechnology 6 (1988) 779.
[3] D.K. Roper, E.N. Lightfoot, J. Chromatogr. A 702 (1995) 3.
[4] J. Thommes, M.-R. Kula, Biotechnol. Prog. 11 (1995) 357.
[5] C. Charcosset, J. Chem. Technol. Biotechnol. 71 (1998) 95.
[6] E. Klein, J. Membr. Sci. 179 (2000) 1.
[7] H. Zou, Q. Luo, D. Zhou, J. Biochem. Biophys. Methods 49 (2001) 199.
[8] R. Ghosh, J. Chromatogr. A 952 (2002) 13.
[9] A. Denizli, E. Piskin, J. Biochem. Biophys. Methods 49 (2001) 391.
[10] Y. Li, H.G. Spencer, J. Biotechnol. 26 (1992) 203.
[11] G.C. Serafica, J. Pimbley, G. Belfort, Biotechnol. Bioeng. 43 (1994) 21.
[12] K.C. Hou, R. Zaniewski, S. Roy, Biotechnol. Appl. Biochem. 13 (1991) 257.
[13] Y.-H. Tsai, M.-Y. Wang, S.-Y. Suen, J. Chromatogr. B 766 (2002) 133.
[14] K.C. Hou, R. Zaniewski, J. Chromatogr. 525 (1990) 297.
[15] H. Iwata, K. Saito, S. Furusaki, T. Sugo, J. Okamato, Biotechnol. Prog. 7 (1991) 412.
[16] K. Rodemann, E. Staude, J. Membrane Sci. 88 (1994) 271.

[17] O.-W. Reif, V. Nier, U. Bahr, R. Freitag, J. Chromatogr. A 664 (1994) 13.
[18] K. Rodemann, E. Staude, Biotechnol. Bioeng. 46 (1995) 503.
[19] T.C. Beeskow, W. Kusharyoto, F.B. Anspach, K.H. Kroner, W.-D. Deckwer, J. Chromatogr. A 715 (1995) 49.
[20] N. Kubota, Y. Nakagawa, Y. Eguchi, J. Appl. Polym. Sci. 62 (1996) 1153.
[21] S.A. Camperi, M. Grasselli, A.A. Navarro del Canizo, E.E. Smolko, O. Cascone, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 1283.
[22] L. Yang, L. Jia, H. Zou, D. Zhou, Y. Zhang, Sci. China, Ser. B: Chem. 41 (1998) 596.
[23] J. Crawford, S. Ramakrishnan, P. Periera, S. Gardner, M. Coleman, R. Beitle, Sep. Sci. Tech. 34 (1999) 2793.
[24] L. Yang, L. Jia, H. Zou, Y. Zhang, Biomed. Chromatogr. 13 (1999) 229.
[25] C.-Y. Wu, S.-Y. Suen, S.-C. Chen, J.-H. Tseng, J. Chromatogr. A 996 (2003) 53.
[26] Y.-C. Liu, C.-C. ChangChien, S.-Y. Suen, J. Chromatogr. B (2003) in press.
[27] M.Y. Arica, H.N. Testereci, A. Denizli, J. Chromatogr. A 799 (1998) 83.
[28] A. Denizli, S. Senel, M.Y. Arica, Colloids Surfaces B 11 (1998) 113.
[29] S. Senel, R. Say, Y. Arica, A. Denizli, Colloids Surfaces A 182 (2001) 161.
[30] G. Bayramoglu, B. Kaya, M.Y. Arica, Chem. Eng. Sci. 57 (2002) 2323.
[31] P.R. Hari, W. Paul, C.P. Sharma, J. Biomed. Mater. Res. 50 (2000) 110.
[32] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[33] J. Porath, B. Olin, Biochemistry 22 (1983) 1621.
[34] J. Porath, Protein Express. Purif. 3 (1992) 263.
[35] W.-Y. Chen, C.-F. Wu, C.-C. Liu, J. Colloid Interface Sci. 180 (1996) 135.
[36] F.H. Arnold, Biotechnology 9 (1991) 151.
[37] E. Sulkowski, Trends Biotechnol. 3 (1985) 1.
[38] I. Ozawa, M. Kim, K. Saito, K. Sugita, T. Baba, S. Moriyama, T. Sugo, Biotechnol. Prog. 17 (2001) 893.
[39] R.G. Pearson, J. Chem. Educ. 45 (1968) 581.
[40] S.-Y. Suen, Y.-D. Tsai, Sep. Sci. Tech. 35 (2000) 69.
[41] Y.-D. Tsai, S.-Y. Suen, Ind. Eng. Chem. Res. 40 (2001) 854.
[42] S.-Y. Lin, S.-Y. Suen, J. Membrane Sci. 204 (2002) 37.
[43] S.-Y. Suen, M.R. Etzel, Chem. Eng. Sci. 47 (1992) 1355.
[44] S.-Y. Suen, M.R. Etzel, J. Chromatogr. A 686 (1994) 179.
[45] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.
[46] S.-Y. Suen, Y.-H. Tsai, R.-L. Chen, Sep. Sci. Tech. 35 (2000) 573.
[47] J.E. Kochan, Y.-J. Wu, M.R. Etzel, Ind. Eng. Chem. Res. 35 (1996) 1150.
[48] W.S.W. Ho, K.K. Sirkar (Eds.), Membrane Handbook, Van Nostrand Reinhold, New York, 1992.
[49] S. Najarian, B.J. Bellhouse, Biotechnol. Prog. 13 (1997) 113.
[50] F. Svec, J.M.J. Frechet, J. Chromatogr. A 702 (1995) 89.
[51] C. Charcosset, Z. Su, S. Karoor, G. Daun, C.K. Colton, Biotechnol. Bioeng. 48 (1995) 415.
[52] S.-Y. Suen, H.-C. Chiu, Y.-D. Tsai, Sep. Sci. Tech. 35 (2000) 1343.
[53] C. Mateo, G. Fernandez-Lorente, B.C.C. Pessela, A. Vian, A.V. Carrascosa, J.L. Garcia, R. Fernandez-Lafuente, J.M. Guisan, J. Chromatogr. A 915 (2001) 97.
[54] M.-Y. Wang, Y.-H. Yang, H.-S. Lee, S.-Y. Lai, Biotechnol. Prog. 16 (2000) 146.
[55] E. Gherardi, J. Gray, M. Stoker, M. Perryman, R. Furlong, Proc. Natl. Acad. Sci. USA 86 (1989) 5844.
[56] N. Rahimi, S. Etchells, B. Elliott, Protein Express. Purif. 7 (1996) 329.
[57] V. Fitton, X. Santarelli, J. Chromatogr. B 754 (2001) 135.
[58] R. Freitag, H. Splitt, O.-W. Reif, J. Chromatogr. A 728 (1996) 129.
[59] B. Champluvier, M.-R. Kula, Bioseparation 2 (1992) 343.
[60] O.-W. Reif, R. Freitag, Bioseparation 4 (1994) 369.
[61] T.-J. Fu, PhD thesis, Penn. State University, University Park, PA, USA, 1992.
[62] S.-Y. Suen, M. Caracotsios, M.R. Etzel, Chem. Eng. Sci. 48 (1993) 1801.
[63] S.-Y. Suen, Y.-S. Chang, J. Chin. Inst. Chem. Eng. 29 (1998) 307.