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Purification of hepatocyte growth factor using polyvinyldiene fluoride-based immobilized metal affinity membranes: equilibrium adsorption study

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Abstract

Polyvinyldiene fluoride (PVDF)-based affinity membranes with immobilized copper ions were developed in this study. The resulting membranes were tested for their adsorption properties using a model protein, lysozyme, in batch mode. First, different lengths of diamine were utilized as spacer arms to immobilize the metal ions onto the membranes. It was found that the application of 1,8-diaminooctane as the spacer arm led to the highest adsorption capacity. Moreover, the effects of pH and salt concentration were investigated to distinguish the proportion of specific and nonspecific interactions. A big fraction of lysozyme adsorption capacity for the immobilized metal affinity membranes was considered to come from nonspecific electrostatic interactions, which could be reduced by increasing salt concentration. Lastly, the purification of hepatocyte growth factor (HGF) from insect cell supernatant was performed using the immobilized metal affinity membranes in batch mode. HGF was found in the elution condition using EDTA, indicating the successful purification of HGF.

Keywords: Polyvinyldiene fluoride-based metal affinity membranes; Equilibrium adsorption studies; Hepatocyte growth factor

1. Introduction

Techniques of immobilized metal affinity chromatography have been widely recognized and applied to the purification and recovery of biomolecules containing surface-exposed amino acids such as histidine and cysteine. In the past years, the number of research studies regarding new metal-chelating agents, different solid supports and special applications has greatly increased. One topic in recent developments is to adopt microporous membranes as stationary phases [1–14], instead of using porous particles in packed columns, for achieving better mass-transfer performance. In addition to reducing the diffusion limitations commonly encountered in traditional column processes, lower pressure drops, higher flow-rates and hence better processing efficiencies can be accomplished in membrane systems [11,15–17]. Research on immobilized metal affinity membranes includes the development of new membrane materials and surface modification methods, evaluation of batch adsorption properties and break-

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through curves, and so on. However, investigations concerning the practical applications on the purification of therapeutic biomolecules require more extensive studies.

The purification of hepatocyte growth factor (HGF), an important protein contributing to embryogenesis, wound repair and tumor invasion [18,19], is of interest. HGF has been found to bind with immobilized copper, and its purification using traditional column process has been proved successful [18]. Therefore, the purification of HGF using immobilized metal affinity membranes should be probable and needs further investigation. In this work, flat-sheet hydrophilic PVDF (polyvinyldiene fluoride) membranes were activated and coupled with the chelating agent, iminodiacetic acid (IDA), using the spacer arm and hence immobilized with copper ions. The resulting affinity membranes were tested for their adsorption properties by adsorbing lysozyme in batch mode. Finally, the batch purification of recombinant HGF from insect cell supernatant was conducted. Different elution conditions were performed to find a suitable elution method.

2. Materials and methods

2.1. Materials

The PVDF-based Immobilon AV (IAV) membranes of Millipore (Bedford, MA, USA), with an average pore size of 0.65 μm and a thickness of 140 μm, were adopted as solid supports. Chicken egg white lysozyme (L6876) was purchased from Sigma (St. Louis, MO, USA). Chemicals such as 1,6-diaminohexane, 1,8-diaminooctane, 1,10-diaminodecane, IDA, epichlorohydrin, and $CuSO_4 \cdot 5H_2O$ were obtained from Acros Organics (Geel, Belgium), Tedia (Fairfield, OH, USA) and TCI (Tokyo, Japan).

2.2. Copper ion immobilization

The commercial IAV membranes have been chemically activated for immobilizing ligands with amine groups [20]. Prior to the reactions, the IAV membranes were cut into 1 cm×1 cm pieces. Diamine of 8.6 mmol was dissolved in 5 ml of 0.5 *M* phosphate buffer, pH 7.3 and reacted with one piece of dry IAV membrane. The reaction was conducted at room temperature for 12 h. After reaction, the membrane was thoroughly rinsed to remove the unreacted diamine. The diamines tested in this work included 1,6-diaminohexane, 1,8-diaminooctane, and 1,10-diaminodecane.

The diamine-bound membrane was subsequently immersed in a mixed solution of 0.46 ml epichlorohydrin, 4.6 ml of 2 *M* NaOH, and 0.017 g $NaBH_4$ at room temperature for 2 h. Next, 2.3 ml epichlorohydrin and 4.6 ml of 2 *M* NaOH were added to the membrane mixture and the reaction was conducted for 12 h. After washing with deionized water, the epichlorohydrin-conjugated membrane was reacted with a mixed solution of 8.6 mmol IDA, 11.44 ml of 2 *M* Na_2CO_3, and 0.014 g $NaBH_4$ under 60°C for 12 h. After reaction, the membrane was sequentially washed with deionized water, 5% acetic acid, and deionized water.

To immobilize metal ions, the IDA-coupled membrane was incubated with 2 ml of 0.1 *M* $CuSO_4$ solution for 10 min. This membrane was then washed with the buffer of 1 *M* KCl in 50 m*M* Tris–HCl, pH 4 until no copper ions were eluted, and lastly, was rinsed with deionized water. The reaction steps are depicted in Fig. 1.

Because 9 cm×9 cm membrane pieces would be adopted for purifying HGF from cell culture supernatant later in this work, the ligand immobilizations onto 9 cm×9 cm membranes were conducted as well. Only 1,8-diaminooctane was used as the spacer arm in these cases. The same immobilization procedures as above were followed, except that the volumes of chemical solutions were changed. For membrane coupling with epichlorohydrin and IDA, the solution volumes were increased 10 times while chemical concentrations remained unaltered. For copper ion immobilization, 30 ml of $CuSO_4$ solution was used.

2.3. Determination of copper ion capacity

A 1 cm×1 cm piece of immobilized metal affinity membrane was suspended in 5 ml of 50 m*M* EDTA solution for 10 min. The released copper concentration in EDTA solution was detected using an atomic absorption spectrophotometer (Z6100,

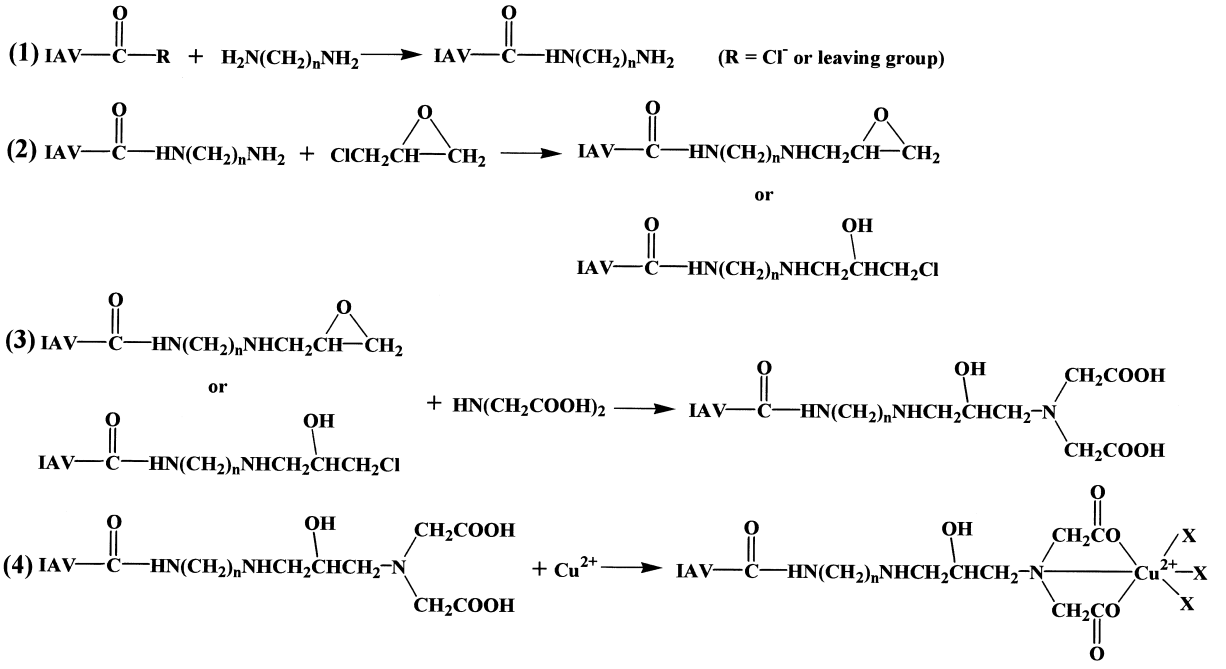

Fig. 1. Schematic diagram for the immobilization of copper ions onto the IAV membrane.

Hitachi, Tokyo, Japan). The immobilized copper ion capacity equals the released amount.

2.4. Batch adsorption of lysozyme

Tris–HCl (50 m*M*) was used as loading buffer for lysozyme adsorption. The pH values tested in this study were 8, 7, and 6, while 0.1 *M* and 0.5 *M* KCl in the loading buffer of pH 7 were adopted to investigate the effect of salt concentration on adsorption. The elution buffer for removing the adsorbed lysozyme was 1 *M* KCl in 50 m*M* Tris–HCl, pH 4. All the buffers were filtered through 0.2-μm cellulose acetate membranes (MFS, Osaka, Japan). Lysozyme solutions were prepared with the loading buffer and filtered by 0.2-μm cellulose acetate filters (MFS). All solutions were degassed prior to use.

In the batch adsorption experiment, a piece of air-dried affinity membrane was incubated with a lysozyme solution of a certain concentration at room temperature for 12 h. A 2-ml volume of solution was used for the 1 cm×1 cm membrane piece, and 30 ml for the 9 cm×9 cm piece. After adsorption, the membrane was rinsed with loading buffer and then air-dried. Next, the membrane was washed twice with elution buffer to remove the bound protein. Lysozyme concentration in solution was measured using a UV–Vis spectrophotomer (UV-1601, Shimadzu, Auburn, Australia) at 280 nm. The extinction coefficient ($E^{280\ nm}_{1\ mg/ml}$) for lysozyme is 2.64 [21]. Prior to each batch, the immobilized metal affinity membrane was placed in 2 ml (for a 1 cm×1 cm piece) or 30 ml (for a 9 cm×9 cm piece) of 0.1 *M* $CuSO_4$ solution for 15 min to avoid the experimental error from the leakage of immobilized copper ions. For each investigation, two membrane pieces formed from the same immobilization method were employed to study the reproducibility of the adsorption data.

2.5. Purification of HGF

The procedures for recombinant HGF production in the literature [19] were followed. Insect cell adopted in this work was Sf9 and the medium was TNM-FH (medium originally developed for the culture of *Trichoplusia ni* cells, bought from Sigma) with 10% fetal bovine serum (FBS; Life Tech-

nologies, Grand Island, NY, USA), pH 6.1–6.2. The HGF-containing supernatant 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.). A 50-ml volume of supernatant was incubated with two 9 cm×9 cm pieces of immobilized metal affinity membranes at room temperature for 12 h. After the membranes were washed with 50 m*M* Tris–HCl buffer of pH 7, conditions with harsher elution strength were tested sequentially: 0.1 *M* KCl in 50 m*M* Tris–HCl buffer of pH 7, 0.5 *M* KCl in 50 m*M* Tris–HCl of pH 7, 1 *M* KCl in 50 m*M* Tris–HCl of pH 7, 1 *M* KCl in 50 m*M* Tris–HCl of pH 4, 1 *M* KCl in 50 m*M* EDTA of pH 7. Each elution volume was 50 ml and each elution step was conducted for 2 h.

2.6. Western blotting analysis

After being subjected to 10% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), proteins were transferred electrophoretically to an Immobilon P transfer paper (Millipore). The transfer paper was incubated with HGF antiserum (1:2000) and was visualized by successive incubation with alkaline phosphatase conjugated goat anti-mouse IgG antibody (Jackson ImmunoReasearch, West Grove, PA, USA) (1:10 000). Finally, the transfer paper was color-developed in 10 ml of alkaline phosphatase buffer containing 66 μl of NBT (nitroblue tetrazolium) and 33 μl of BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock solution. The detailed procedures for Western blotting were described elsewhere [19].

3. Results and discussion

3.1. Immobilized copper ion capacity

The amounts of immobilized copper ions on different membranes were measured and the data are listed in Table 1. The copper ion capacity was considerable only under the use of diamine spacer arms and epichlorohydrin for immobilization of copper ions. Although IDA could be directly coupled to the IAV membranes, the relevant immobilized copper ions were a lot fewer. Among the application of diamine spacer arms, 1,6- or 1,8-diamine resulted in a greater copper ion capacity than 1,10-diamine. This is a possible consequence of arm folding with longer spacer arms, which may cause a difficulty for metal ions to touch the IDA on the free end of arm and lead to a reduction in immobilization capacity. The immobilized metal ion capacity for polymeric membranes strongly depends on the active sites on the base polymer, membrane shape, immobilization method, type of metal ions, metal ion concentration, etc. In comparison with the data reported in the literature, the highest copper ion capacity obtained in this study (0.53 $\mu mol/cm^2$ frontal area or 37.9 $\mu mol/cm^3$ membrane volume) is 5–40 times lower than those for grafted polyethylene hollow fibers (operated in static mode) [1,10] and 100-fold less

Table 1
Copper ion capacities and lysozyme adsorption properties (at pH 7) for different 1 cm×1 cm membranes

Membrane	Cu^{2+} capacity ($\mu mol/cm^2$)	q_m (mg/cm^2)	K_d (mg/ml)	Cu^{2+} utilization (%)
IAV membrane+Cu^{2+}	–	–	–	–
IAV membrane+IDA+Cu^{2+}	0.0087±0.0025	0.023±0.0055 0.018±0.0080	0.047±0.054 0.18±0.26	18.5 14.5
IAV membrane+1,6-diaminohexane+epichlorohydrin+IDA+Cu^{2+}	0.53±0.0079	1.08±0.29 1.04±0.31	0.13±0.099 0.12±0.11	14.2 13.7
IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA+Cu^{2+}	0.53±0.0039	1.22±0.31 1.07±0.35	0.15±0.10 0.11±0.10	16.1 14.1
IAV membrane+1,10-diaminodecane+epichlorohydrin+IDA+Cu^{2+}	0.42±0.0020	0.86±0.28 0.79±0.26	0.12±0.12 0.074±0.081	14.3 13.1

–: Negligible or not applied.

than that for Satorius product (operated in flow mode at 1 ml/min) [3], but it is about threefold of those for hydroxyethyl cellulose-modified polyamide membranes (in static mode) [6] and polysulfone membranes (in flow mode at 5300 $l/m^2/day$) [7].

3.2. Batch adsorption of lysozyme

3.2.1. Adsorption at pH 7

The target protein to purify from cell supernatant in this study is HGF, which has a molecular mass of 91 kDa (for pro-HGF) [19] and an isoelectric point (p*I*) of ca. 9.5 [22]. In the cell culture supernatant of pH 6.8–7.0, HGF would be cationic and expectantly be adsorbed onto the immobilized metal affinity membranes and separated. It is hard to use pure HGF to investigate the related adsorption properties of the immobilized metal affinity membranes developed in this work. Therefore, lysozyme, which has a molecular mass of 14.3 kDa and a p*I* of ca. 11, was adopted as a model protein for the evaluation of adsorption properties of affinity membranes because it is also positively charged at the adsorption pH of 7.

For batch protein adsorption, two methods can be used to determine the amount of adsorbed protein. The first method is to evaluate the difference between the protein amount originally in the solution and that remained after adsorption. The second method is to calculate the eluted protein amount by multiplying the eluted protein concentration with the elution solution volume. As the elution is complete, the amount of adsorbed protein should be equal to the eluted amount. In this work, it was found (data not shown) that, for feed lysozyme concentrations greater than 0.5 mg/ml, the values of adsorbed amount determined by these two methods were in a close agreement (less than 10% of difference). For feed concentrations less than 0.5 mg/ml, the difference became more significant. Considering the effects of other interfering factors (the reason will be described later), the second method was adopted to determine the adsorbed amount of lysozyme.

The isotherm results for lysozyme adsorption onto 1 cm×1 cm immobilized metal affinity membranes at pH 7 are shown in Fig. 2. These data were fitted by the Langmuir isotherm model, $q=q_m C/(K_d+C)$, where q_m represents the saturation capacity of protein and K_d is the dissociation equilibrium constant.

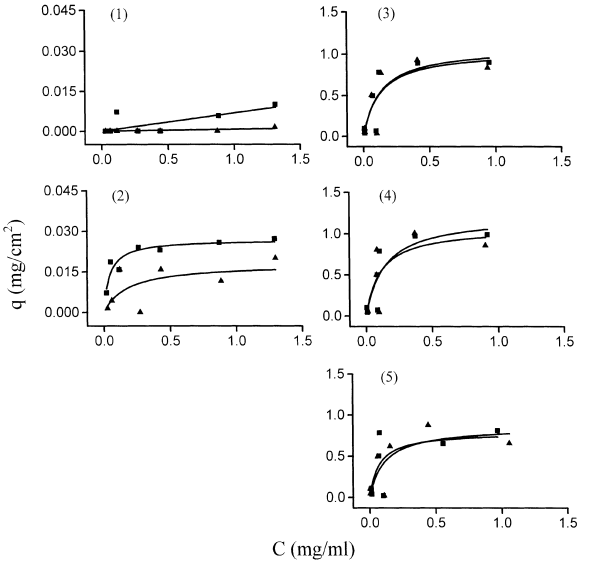

Fig. 2. Adsorption isotherms of lysozyme (at pH 7) for different 1 cm×1 cm membranes. (1) IAV membrane+Cu^{2+}. (2) IAV membrane+IDA+Cu^{2+}. (3) IAV membrane+1,6-diaminohexane+epichlorohydrin+IDA+Cu^{2+}. (4) IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA+Cu^{2+}. (5) IAV membrane+1,10-diaminodecane+epichlorohydrin+IDA+Cu^{2+}. Two trials: ■ and ▲, model results: ——.

The fitted isotherm parameters are listed in Table 1. The results illustrate that the use of 1,8-diaminooctane as spacer arm led to the highest saturation capacity, whereas the dissociation equilibrium constants were close in most cases. It may imply that the length of spacer arm did not alter the binding strength between lysozyme and immobilized copper ions, but the reduction in copper capacity for 1,10-diamine contributed to a decrease of lysozyme adsorption. In comparison with the saturation capacity of lysozyme reported in the literature, the highest capacity obtained in this work (1.22 mg/cm^2 frontal area or 0.085 $\mu mol/cm^2$ or 6.09 $\mu mol/cm^3$ membrane volume) is similar to those for grafted polyethylene hollow fibers (operated in static mode) [10] and inorganic glass hollow fibers (in static mode) [5], and is fourfold of that for hydroxyethyl cellulose-coated polyamide membranes (operated in flow mode at 2 ml/min with recirculation) [6].

The utilization percentage of immobilized copper ions in Table 1 was determined by dividing the saturation capacity of lysozyme by the immobilized

copper capacity. In all the cases, about 13–19% copper ion capacity was utilized for lysozyme binding. Compared to the results reported in the literature, this percentage is similar to that for hydroxyethyl cellulose-modified polyamide membranes [6], and is 5–300 times higher than the result for grafted polyethylene hollow fibers [10].

It is worth noting that the leakage of copper ions during adsorption and elution was detected. The released copper results for a feed lysozyme concentration of 1.31 mg/ml are listed in Table 2. The copper leakage at adsorption stage was more serious than at elution. At adsorption stage, the unstably immobilized copper ions may be tightly captured by lysozyme molecules in solution and hence released from the membranes. On the other hand, at elution stage, the unstably immobilized copper ions were possibly displaced by salt ions in the elution buffer. When protein capture was stronger than salt displacement, copper leakage would be more severe at adsorption stage. While fewer copper ions were released in the solution, the interference to detection of protein concentration would be lower. Consequently, the method to determine the adsorbed amount by using the elution results should be more accurate and was subsequently adopted through this study.

In addition, two 9 cm×9 cm pieces of immobilized metal affinity membranes (using 1,8-diaminooctane as the spacer arm) formed from the same immobilization method, I1 and I2, were also employed for lysozyme adsorption. Their isotherm results are presented in Fig. 3. The fitted parameter values are: $q_m = 1.02 \pm 0.09$ mg/cm^2 and $K_d = 0.35 \pm 0.15$ mg/ml for I1; $q_m = 0.69 \pm 0.02$ mg/cm^2 and $K_d = 0.16 \pm 0.02$ mg/ml for I2. The saturation capacity for I1 is close to those for 1 cm×1 cm membranes shown in Table 1, but the capacity for I2 is about 70% of I1. On the other hand, the K_d value for I2 is close to those for 1 cm×1 cm membranes, while that for I1 is twice. The results for these two membranes are not perfectly matched, possibly re-

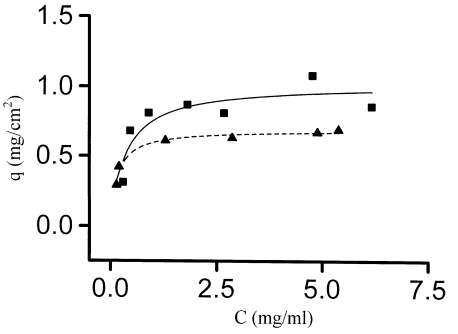

Fig. 3. Adsorption isotherms of lysozyme (at pH 7) for 9 cm×9 cm immobilized copper ion affinity membranes using 1,8-diaminooctane as the spacer arm. Two trials: I1, ■ and ——; I2, ▲ and - - -.

Table 2
Copper ion leakage for different 1 cm×1 cm membranes

Membrane	Cu^{2+} leakage at adsorption[a] (ppm)	Cu^{2+} leakage at first elution[b] (ppm)	Cu^{2+} leakage at second elution[b] (ppm)
IAV membrane+Cu^{2+}	0	0	0
	0	0	0
IAV membrane+IDA+Cu^{2+}	0.047	0	0
	0	0	0
IAV membrane+1,6-diaminohexane+epichlorohydrin+IDA+Cu^{2+}	8.07	0.76	0.048
	7.72	0.25	0
IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA+Cu^{2+}	8.68	1.03	0.25
	8.65	0.18	0
IAV membrane+1,10-diaminodecane+epichlorohydrin+IDA+Cu^{2+}	5.76	0.21	0
	8.21	0.27	0

[a] $C_0 = 1.31$ mg/ml; 2 ml, pH 7.
[b] $C_0 = 1.31$ mg/ml; 4 ml, 1 *M* KCl, pH 4.

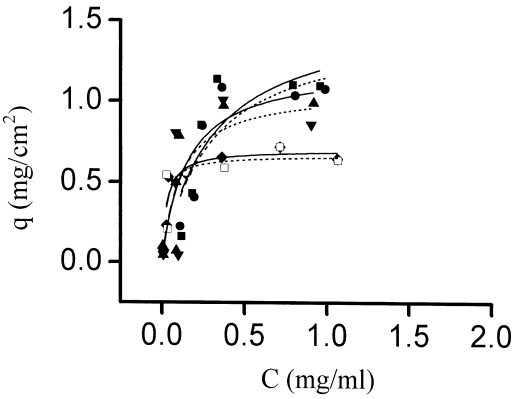

Fig. 4. Adsorption isotherms of lysozyme with varying pH for 1 cm×1 cm immobilized copper ion affinity membranes using 1,8-diaminooctane as the spacer arm. Two trials: ■ (pH 8), ▲ (pH 7), ♦ (pH 6) and ——; ● (pH 8), ▼ (pH 7), □ (pH 6) and - - -.

sulted from the error in the immobilization procedures. However, considering that the results for 9 cm×9 cm and 1 cm×1 cm membranes are in the same order of magnitude, bigger membrane size is demonstrated to have minor influence on the lysozyme adsorption properties.

3.2.2. pH effect

The results of lysozyme adsorption for varying pH values are presented in Fig. 4 and Table 3. Lysozyme adsorption increased with increasing pH. To explain this pH effect, the possible binding forces for lysozyme to adsorb onto the immobilized metal affinity membranes should be expressed. There are three possible binding interactions: (1) the specific binding provided by the electron-donating capacity

Table 3
Lysozyme adsorption capacities with varying pH for 1 cm×1 cm immobilized copper ion affinity membranes using 1,8-diaminooctane as the spacer arm

pH	q_m (mg/cm²)
8	1.56±0.47
	1.47±0.39
7	1.22±0.31
	1.07±0.35
6	0.70±0.060
	0.67±0.087

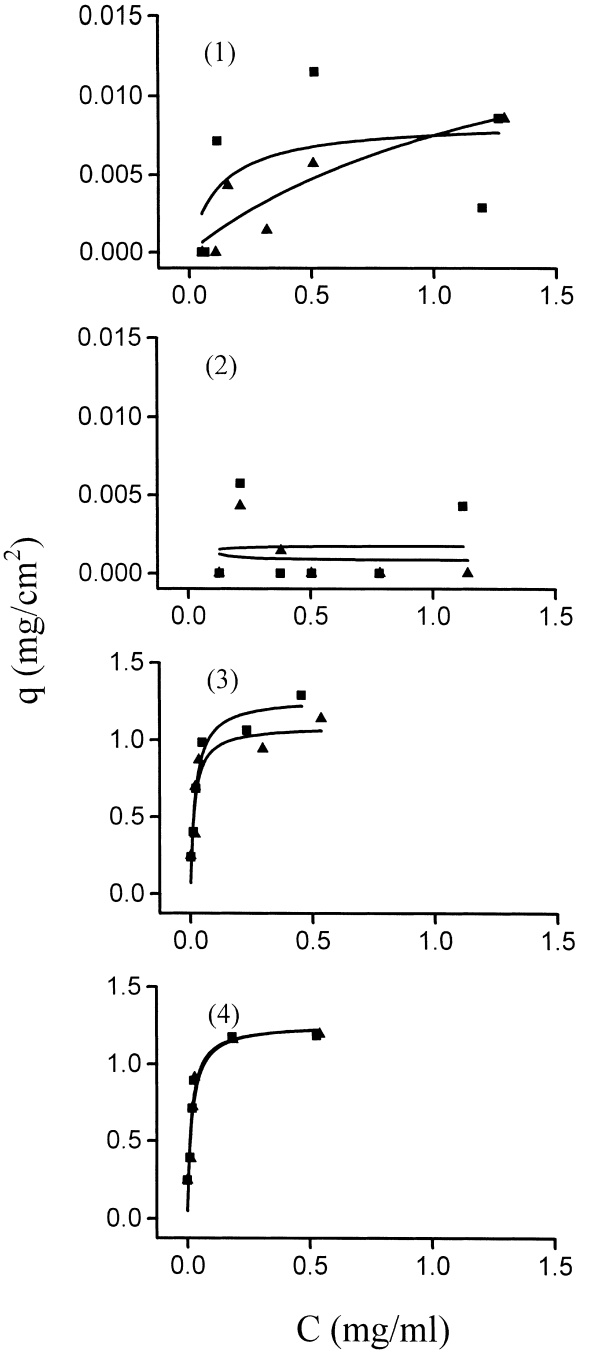

Fig. 5. Nonspecific binding isotherms of lysozyme (at pH 7) for different 1 cm×1 cm membranes. (1) IAV membrane. (2) IAV membrane+1,8-diaminooctane. (3) IAV membrane+1,8-diaminooctane+epichlorohydrin. (4) IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA. Two trials: ■ and ▲, model results: ——.

of the imidazole group of the single exposed histidine residue on the lysozyme surface with immobilized copper ions [6,23]; (2) the nonspecific binding caused by the electrostatic interaction between the cationic lysozyme and the negatively charged sites on the membrane surface such as the residual carboxyl groups of immobilized IDA [23]; and (3) the nonspecific binding from the hydrophobic interaction between lysozyme and the hydrophobic sites on the membrane surface [23].

With higher pH, the partial deprotonation of the histidine residue of lysozyme will be promoted, which increases the possibility of its specific binding with the immobilized positively charged copper ions [23]. It should be noted that the pK_a value of the side chain of histidine residue on lysozyme is in the range of 5.5 to 8.5 [23]. The pH values below pK_a will lead to a reduced adsorption capacity due to less specific binding. This is why only the pH values above 5.5 were adopted in this study and also the reason to adopt a lower pH for lysozyme elution.

To further investigate the effects of nonspecific binding, the 1 cm×1 cm membranes from each intermediate reaction step before complete immobilization were employed. Their related adsorption isotherms at pH 7 are presented in Fig. 5, and the fitted parameters are listed in Table 4. When the immobilization procedure was terminated at epichlorohydrin or IDA, the adsorption of lysozyme onto the resulting membrane was significant. This considerable adsorption is regarded mainly from the electrostatic interaction between cationic lysozyme and the residual negatively charged groups such as hydroxyl or carboxyl groups, although hydrophobic binding may also have some effect on it. In this study, the percentage of unbound epichlorohydrin or IDA was not analyzed. According to the results reported in the literature, for polysulfone-based membranes, only 7% of epoxy groups were substituted by IDA but 100% IDA chelated copper ions [4]; for grafted polyethylene hollow fibers, 60–70% of IDA was bound with copper ions [1]. If the unbound percentage of this work were as much as those reported in the literature, the proportion of nonspecific binding would be high.

Table 5 presents the pH effects on the nonspecific bindings. With increasing pH (above 7), the lysozyme adsorption capacity (at high solution concentration) was almost identical. To further understand which nonspecific binding mechanism is dominating, the two different nonspecific interactions are compared as follows. The electrostatic interaction may hardly be changed by a higher pH, because lysozyme will become less cationic but the residual negatively charged groups on the membranes will be more anionic. On the other hand, with a pI around 11, lysozyme will possess a higher degree of neutrality with higher pH and its hydrophobic interaction with the membrane surface may be enhanced [23]. The tendency of the results in Table 5 is more like the consequence for electrostatic interaction, which implies again that the main nonspecific binding came from the electrostatic interaction.

Since the electrostatic interaction is the dominant nonspecific binding mechanism and is not altered by the elevated pH, to sum up the results of specific and nonspecific interactions, the increase of lysozyme adsorption capacity onto the immobilized metal

Table 4
Nonspecific binding properties of lysozyme (at pH 7) for different 1 cm×1 cm membranes

Membrane	q_m (mg/cm^2)	K_d (mg/ml)
IAV membrane	–	–
IAV membrane+1,8-diaminooctane	0.0018±0.0022	0.020±0.37
	0.00083±0.0011	−0.041±0.19
IAV membrane+1,8-diaminooctane+epichlorohydrin	1.09±0.14	0.016±0.0082
	1.27±0.088	0.020±0.0055
IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA	1.26±0.11	0.016±0.0050
	1.26±0.13	0.018±0.0068

–: Negligible or not applied.

Table 5
Nonspecific binding capacities of lysozyme with varying pH for different 1 cm×1 cm membranes

pH	q (mg/cm^2)	
	IAV membrane+1,8-diaminooctane+epichlorohydrin	IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA
10*	1.13	1.30
	1.18	1.39
9*	1.10	1.33
	1.20	1.34
8*	1.02	1.26
	1.11	1.25
7#	1.14	1.19
	1.29	1.19

*C_0=1.31 mg/ml.
#C_0=1.14 mg/ml.

affinity membranes with increasing pH (as shown in Fig. 4) should be dominated by the specific binding.

3.2.3. Salt concentration effect

Figs. 6 and 7 display the salt concentration effects on lysozyme adsorption at pH 7 onto the immobilized metal affinity membranes and the membranes from the intermediate reaction steps terminated at epichlorohydrin and IDA. In Fig. 6, lysozyme adsorption capacity for the immobilized metal affinity membranes was not changed when 0.1 *M* KCl was added, but it was reduced when KCl concentration was raised to 0.5 *M*. However, the nonspecific binding of lysozyme was significantly decreased with increasing KCl concentration in Fig. 7.

When the salt concentration increases, the specific interaction between lysozyme and the immobilized

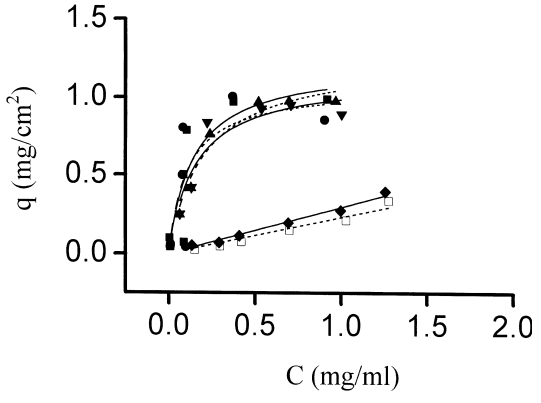

Fig. 6. Adsorption isotherms of lysozyme (at pH 7) with varying KCl concentration for 1 cm×1 cm immobilized copper ion affinity membranes using 1,8-diaminooctane as the spacer arm. Two trials: ■ (0 *M*), ▲ (0.1 *M*), ♦ (0.5 *M*) and ——; ● (0 *M*), ▼ (0.1 *M*), □ (0.5 *M*) and - - -.

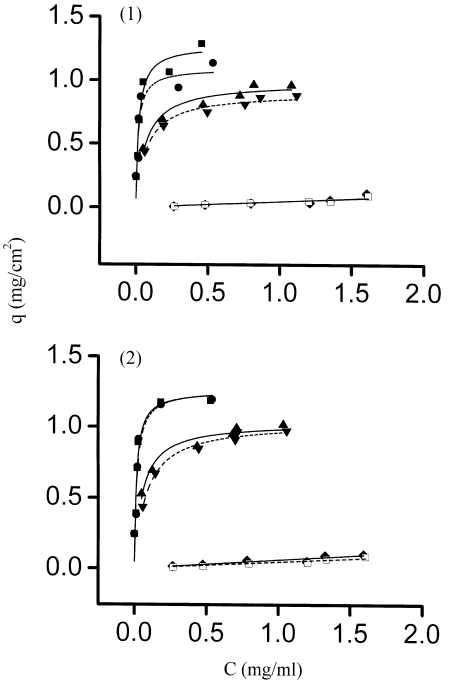

Fig. 7. Nonspecific binding isotherms of lysozyme (at pH 7) with varying KCl concentration for different 1 cm×1 cm membranes. (1) IAV membrane+1,8-diaminooctane+epichlorohydrin. (2) IAV membrane+1,8-diaminooctane+epichlorohydrin+IDA. Two trials: ■ (0 *M*), ▲ (0.1 *M*), ♦ (0.5 *M*) and ——; ● (0 *M*), ▼ (0.1 *M*), □ (0.5 *M*) and - - -.

metal ions would barely be affected [23]. On the other hand, the electrostatic attraction between the cationic lysozyme and the negatively charged sites on the membranes would be reduced with increasing salt concentration, as shown in Fig. 7, but the strength of hydrophobic interaction should be enhanced [23]. Accordingly, the reduction on lysozyme adsorption capacity for immobilized metal affinity membranes with the addition of 0.5 *M* KCl may imply that a big proportion of lysozyme adsorption capacity came from the electrostatic interaction.

3.3. Purification of HGF

The purification results of HGF analyzed by Western blotting are presented in Fig. 8. The original insect cell supernatant (lane 6) included HGF and other biomolecule species. 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 two immobilized metal affinity membranes (I1 and I2), most species 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 adopted. In this EDTA elution solution, the overall number of biomolecule species is significantly reduced, and the species of greatest portion (the darkest mark in lane 5) is the α-chain of recombinant HGF. Consequently, the purification of HGF using the immobilized metal affinity membranes was proved successful.

High salt concentration could effectively elute the adsorbed lysozyme out of the immobilized metal affinity membranes, but it could not for HGF as shown in lanes 1–4. Only severe elution condition could elute HGF. It indicates that HGF may possess a stronger affinity with the immobilized copper ions than lysozyme, so that a harsher condition to remove immobilized copper ions such as the use of EDTA was needed to elute the bound HGF. This elution phenomenon is similar to that mentioned in the literature using immobilized copper affinity chromatography [18], which pointed out the possibility of a multiple-site interaction of HGF with immobilized copper ions.

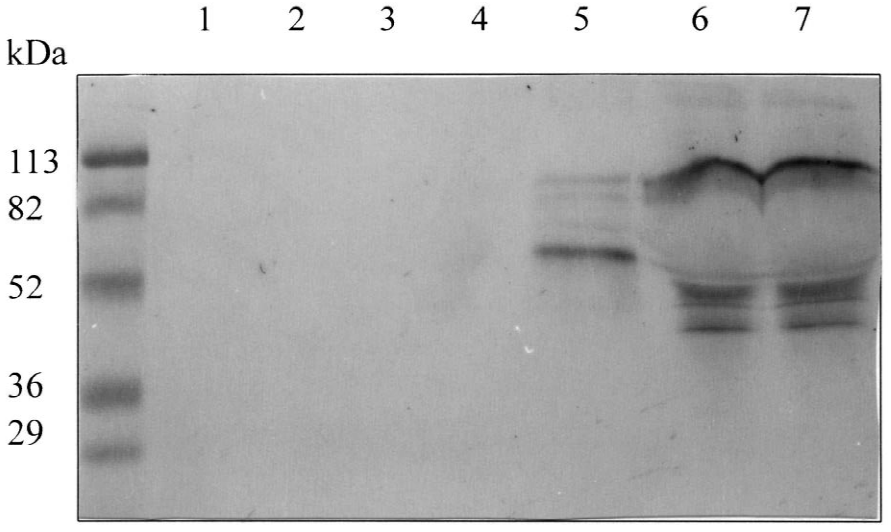

Fig. 8. Western blotting analysis for HGF purification using two 9 cm×9 cm immobilized copper ion affinity membranes with 1,8-diaminooctane as the spacer arm. Lane 1: elution solution using 0.1 *M* KCl in 50 m*M* Tris–HCl, pH 7. Lane 2: elution solution using 0.5 *M* KCl in 50 m*M* Tris–HCl, pH 7. Lane 3: elution solution using 1 *M* KCl in 50 m*M* Tris–HCl, pH 7. Lane 4: elution solution using 1 *M* KCl in 50 m*M* Tris–HCl, pH 4. Lane 5: elution solution using 1 *M* KCl in 50 m*M* EDTA, pH 7. Lane 6: original insect cell supernatant. Lane 7: supernatant after adsorption.

4. Conclusions

Microporous PVDF-based IAV membranes were coupled with IDA and immobilized with copper ions to produce the immobilized metal affinity membranes in this study. The batch adsorption of lysozyme onto the affinity membranes was then conducted to find a suitable length of diamine molecule as the spacer arm, in which 1,8-diaminooctane is selected. In addition, the effects of pH and salt concentration were investigated for distinguishing the fractions of specific and nonspecific interactions between lysozyme and the immobilized metal affinity membranes. The results imply that a big proportion of adsorption capacity came from electrostatic interactions and the use of 0.5 *M* KCl could reduce this nonspecific binding. To fundamentally solve this high nonspecific binding problem, copper ion immobilization procedures should be further improved and a method to block the unreacted epichlorohydrin and IDA is necessary.

In this study, the purification of therapeutic biomolecule HGF from insect cell supernatant using the immobilized metal affinity membranes was conducted, also in batch mode. HGF was successfully recovered when using a harsh elution condition with EDTA and it was pretty stable under this condition. Moreover, the immobilized metal affinity membranes were reused several times and the results were reproducible. Therefore, the practical application of immobilized metal affinity membranes on the purification of therapeutic biomolecules is successfully explored.

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