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New affinity nylon membrane used for adsorption of γ-globulin

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Abstract

Microporous polyamide membranes were first modified by acid hydrolysis and subsequently bound with hydroxyethylcellulose to amplify reactive groups and reduce nonspecific interactions with proteins. Then 1,6-diaminohexane as space arm and phenylalanine as ligand were immobilized onto the nylon membranes by *s*-triazine trichloride activation. Affinity membranes thus obtained were set in a stack and used to adsorb γ-globulin. The adsorption capacity (q_m) of the affinity membrane is 53 μg γ-globulin per m^2 membrane and the desorption constant (K_d) is $2.35 \cdot 10^{-6}$ mol/l. The effects of feed, washing and elution rates on adsorption and desorption behavior were investigated. The results showed that affinity purification through these membranes could not be operated at very high flow-rates. A stack of 20 membranes with 47 mm diameter can adsorb 7.8 mg γ-globulin with a purity of 91.6% from 4 ml of human plasma in a single-pass mode.

Keywords: Membranes; Affinity membranes; Stacking; Globulin; Proteins

1. Introduction

In recent years alternatives to conventional gel beads used in chromatography, such as agarose, dextran, cellulose, silica, and controlled pore glass beads, have been proposed and marketed. Microporous membranes as a support for chromatography have the advantages of convective flow, short diffusion path length and a low pressure drop [1], and thus slow intraparticle diffusion for larger particles or lower axial velocities and high-pressure drops for small particles in packed bed system can be overcome [2]. Charcosset [3] reviewed the applications of membrane chromatography to the separation of a wide range of compounds. Membrane separation has been combined with affinity purification (affinity membrane chromatography) in the 1990s. This technique produced high purity proteins in very short times and reduced the possibility of denaturation of biomolecules during the separation process.

In affinity chromatography, membrane matrices that have been often investigated are cellulose, polyamide, polysulphone, polyethylene and filter paper, etc. Some other membrane matrices used in affinity separation are hydroxyethylmethacrylate [4–6], chitosan and chitin [7], polyvinyl alcohol [8] and glass hollow fiber [9], etc. An ideal membrane for protein separation must fulfil the following requirements: high hydrophilicity and low nonspecific adsorption; fairly large pore size and a narrow pore size

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distribution; chemical and mechanical resistance as well as enough reactive groups [10].

Nylon membrane offers narrow pore size distribution and good mechanical rigidity. However, nylon membrane has a low concentration of primary amino groups leading to low ligand density. Another disadvantage of this matrix is nonspecific adsorption of protein [10]. There are two methods to solve these problems: hydrating nylon membrane to increase the number of primary amino groups on the membranes [11]; binding with polyhydroxyl-containing material, such as polyglucose, dextran, starch, cellulose [11] and hydroxyethylcellulose [12,13] to increase reactive sites and reduce nonspecific adsorption.

In this work nylon membrane was first hydrated under conditions which maintained its mechanical integrity. After hydrolysis, the membranes were activated with *s*-triazine trichloride and then bound with hydroxyethylcellulose. The hydroxyethylcellulose-bound membranes were activated again with *s*-triazine trichloride and then bound with 1,6-diaminohexane which is the space arm. Following the work of Kim and Kiyohara et al. [14,15] phenylalanine was chosen as the ligand to adsorb γ-globulin. After the activation of the amino groups by *s*-triazine trichloride, phenylalanine was immobilized onto the membranes. Finally, the residual chlorine atoms on the membranes were blocked with 2-mercaptoethanol. In Fig. 1 the procedure for the preparation of this affinity membrane is shown.

We have designed and manufactured a stack for setting flat affinity membranes, which can be used for analytical and preparative purification of protein [16]. The objective of this work was to study adsorption and desorption behavior on the nylon affinity membrane stack.

2. Experimental

All experiments were performed at room temperature.

2.1. Materials and equipment

Nylon 66 microporous membranes (specific area: 435 m^2/g; pore size: 1 μm) were obtained from Qingjiang Chemical Plant (China). *s*-Triazine trichloride ($CyCl_3$) and bovine γ-globulin (BGG, 99% purity) were bought from Sigma (Steinheim, Germany). Hydroxyethylcellulose (HEC) and 2-mercaptoethanol were purchased from Fluka (Buchs, Switzerland). Phenylalanine (Phe) was obtained from Shanghai Lizhu Dong Feng Biotechnology (China). Human plasma was a gift from the First Teaching Hospital of Dalian Medical University (China). The other reagents used were bought in China. All reagents were of analytical grade. Affinity separation was performed on the Automated Econo System (Bio-Rad, USA). Model HP 3394A integrator (Hewlett-Packard, USA) was used in data collection. Model 752C UV–visible spectrophotometer was used in determining the concentration of the protein solution. The Camag scanner system in connection with an IBM computer provided with CATS 3.17 software (Camag, Switzerland) was used in scanning the electrophoresis photograph.

2.2. Methods

2.2.1. Synthesis of affinity nylon membrane

Membranes with 47-mm diameter were shaken in 1 *M* HCl for 72 h at room temperature. After partial hydrolysis of amide bonds the membranes were

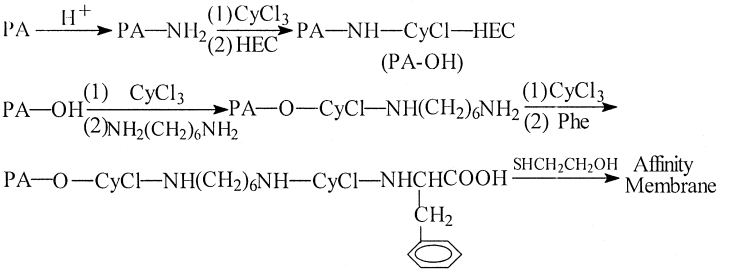

Fig. 1. Procedure for preparing affinity membrane.

activated with $CyCl_3$ according to a procedure described by Smith et al. [17]. Activated membranes were shaken in 2% (w/w) HEC solution (pH 6–7, adjusted by $NaHCO_3$) at 45°C for 4 h and then washed with 0.1 *M* NaOH and water. The amount of HEC bound on membranes was determined by the phenol–sulfuric acid method [18]. The HEC-bound membranes were activated with $CyCl_3$ again. The activated HEC-bound membranes were shaken in 2% 1,6-diaminohexane (DAH) solution at 45°C for 12 h. After being washed with water and then activated with $CyCl_3$ the membranes were shaken in 50 m*M* acetate buffer, pH 6.2, containing 2 mg/ml Phe for 11 h at 45°C. The ligand density was 0.23 $\mu mol/m^2$, which was determined by detecting the difference in concentration before and after reaction through the ninhydrin method. Finally, the affinity membranes were shaken in 1 *M* 2-mercaptoethanol (pH 6~7, adjusted with Na_2CO_3) for 10 h at 45°C to block the residual chlorine atoms. Then the membranes were washed with water and dried in vacuo and stored for later use.

2.2.2. Static adsorption experiments

The nonspecific adsorption on HEC-bound membranes and original membranes and the adsorption isotherm on affinity membranes were measured by a batchwise method. Membranes (0.05 g) were equilibrated by shaking in 30 m*M* sodium phosphate buffer, pH 7.4 (equilibrium buffer) for 10 min. Then the membranes were shaken in 5 ml of BGG dissolved in the equilibrium buffer for 24 h at room temperature. The absorbance of the supernatants was measured at 280 nm. The amount of protein adsorbed was calculated from the concentration difference of the supernatants with and without membranes. The adsorption isotherm was plotted as the adsorption capacity vs. the final concentration of protein in the solution.

2.2.3. Dynamic adsorption of bovine γ-globulin

2.2.3.1. Determination of the relationship between flow-rate and pressure drop of membranes

Twenty sheets of original and HEC-bound membrane with a diameter of 47 mm were set into two stacks. Pressure drops were measured by pumping bidistilled water through the stacks at different flow-rates.

2.2.3.2. Effects of feed, washing and elution rates on the dynamic adsorption behavior of BGG

Two sheets of affinity membrane with a diameter of 47 mm were set into a stack. The sequence of affinity purification was equilibration, loading, washing, elution and regeneration. The stack was first equilibrated with the equilibrium buffer. Then the BGG solution was fed in until adsorption was completed. After adsorption the stack was washed with the equilibrium buffer until A_{280} was <0.02. The protein adsorbed was eluted with 50% ethylene glycol in 1 *M* sodium chloride solution. Then the stack was washed with bidistilled water and equilibrated with equilibrium buffer again. To determine the effect of feed-rate on adsorption, 20 ml of 0.17 mg/ml BGG solution was flushed through the stack at different feed-rates. This was followed by washing at a flow-rate of 10 ml/min, and elution at 1 ml/min. To detect the effect of washing rate on desorption, 20 ml of 0.3 mg/ml BGG solution was flushed through the stack at 2 ml/min. Then the stack was washed at different rates and eluted at 0.5 ml/min. To examine the effect of elution rate on desorption, 27 ml of 0.1 mg/ml BGG solution was loaded onto the stack at a rate of 2 ml/min. Then the stack was washed for 18 min at 2 ml/min. The elution rates were 0.5, 1, 2 and 3 ml/min.

2.2.3.3. Separation of γ-globulin from human plasma

Human plasma (4 ml) was diluted to 20 ml with equilibrium buffer, then loaded onto the affinity stack of 20 membranes with 47-mm diameter in a single-pass mode at a flow-rate of 1 ml/min. Then the stack was washed and eluted at a rate of 1 ml/min. The eluted protein was dialyzed overnight against 0.0625 *M* Tris buffer, pH 6.8. The amount of protein obtained was determined by the Coomassie Brilliant Blue method [19]. The purity of human γ-globulin (HGG) was assayed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [20] using 10% separating gel (9×7.5 cm) and 6% stacking gel. Gels were stained with 0.25% (w/v) Coomassie Brilliant R 250 in acetic acid–methanol–water (1:5:5, v/v/v) and destained

in ethanol–acetic acid–water (1:3:6, v/v/v). Electrophoresis was run for 1.5 h with a voltage of 110 V. Lysozyme, bovine serum albumin (BSA) and HGG were used as standards. The electrophoresis photograph was scanned at 550 nm.

3. Results and discussion

3.1. Hydraulic permeability and nonspecific adsorption of HEC-bound membranes

The amount of HEC bound on membranes is 36.9 μmol glucose/g membrane. HEC bound on nylon membranes can reduce the nonspecific adsorption of BGG to the membranes from 10.5 to 0.8 $\mu g/m^2$ membrane when the starting concentration of BGG was 0.33 mg/ml according to batchwise method. To investigate the change of pore size after binding HEC, 20 original and HEC-bound membranes with 1 μm pore diameter was set into two stacks. The relationship between the flow-rate and the pressure drop through these stacks is plotted in Fig. 2. The pressure drops of the HEC-bound membranes were higher than those of the original membranes at the same flow-rate, which indicates the pore diameter of the membranes was reduced after binding of HEC. The reduction of hydraulic permeability of the membranes indicates that HEC was bound to the membranes and could enter into the pores.

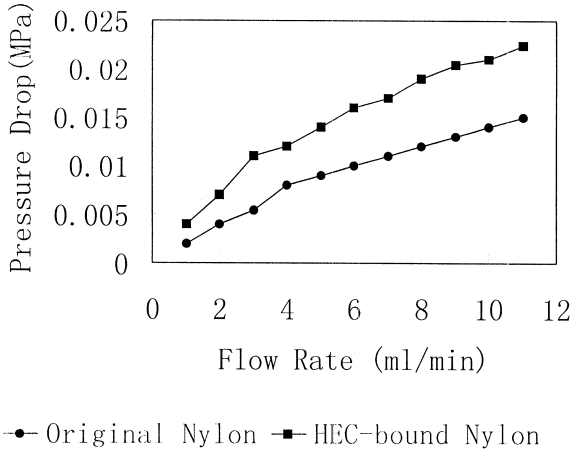

Fig. 2. Permeability of nylon membrane stack of 20 membranes with a diameter of 47 mm and a pore size of 1 μm.

3.2. Adsorption isotherm

The equilibrium adsorption of a protein on an affinity sorbent is often described simply by the Langmuir equation (Eq. (1)), with the assumption that a single-site, homogeneous interaction occurs between the protein and the ligand, and that nonspecific interactions promoted by the support are absent [21].

$$q^* = q_m - K_d \frac{q^*}{c^*} \quad (1)$$

where K_d represents the dissociation constant, c^* and q^* the equilibrium concentration and equilibrium adsorption capacity, respectively, and q_m is maximum adsorption capacity.

The adsorption isotherm, obtained from batch experiments, is presented in Fig. 3. The corresponding Scatchard plot gave rise to a linear plot,

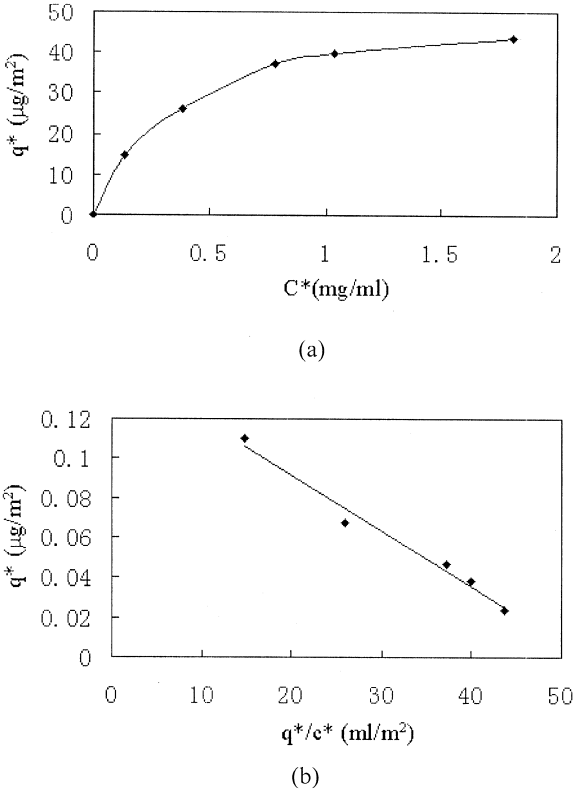

Fig. 3. (a) Adsorption isotherm of γ-globulin on affinity membrane; (b) Scatchard plot analysis derived from adsorption data.

indicating that the Langmuir model could be applied in this system. According to Fig. 3, one obtains $q_m = 53\ \mu g/m^2$, and $K_d = 2.35 \cdot 10^{-6}$ mol/l.

3.3. Effect of feed-rate on adsorption

The experimental result of the relationship between flow-rate and pressure drop shows that the pressure drop is still very low even at high flow-rate, thus high flow-rate can be used in membrane chromatography in theory. But in affinity membrane chromatography, the flow-rate is limited by association kinetics between the solute protein and the immobilized ligand [2]. The effect of flow-rate on the adsorption of BGG was studied and the results are presented in Fig. 4 and Table 1.

In Table 1 the time when the concentration of protein out of the stack is 50% of the original protein concentration ($t_{50\%}$) reflects the rate of achieving equilibration. The breakthrough curves became sharper and equilibrium was reached faster with increasing feed-rate, but the amount of protein adsorbed decreased. A reasonable explanation is that local equilibration can be achieved [2] and ligands on the membrane surface and in pores can be utilized efficiently at low flow-rate, while at high flow-rate, the retarding time was so short that protein had not enough time to make contact with the ligands and the utilization ratio of the ligands decreased. Thus high feed-rate is not suitable for affinity membrane chromatography.

Table 1
Effect of feed-rate on adsorption

Feed-rate (ml/min)	$t_{50\%}$ (min)	Area of elution peak (mm^2)
1	13	632
2	5	441
4	2.7	384
6	2	292

3.4. Effect of washing rate on desorption

The effect of washing rate on the desorption was investigated and the results are presented in Table 2. The amount of protein eluted increased shows that the amount of protein washed out decreased with the increase of washing rate. At low washing rates, that protein both adsorbed by nonspecific adsorption and in dead volume had enough time to disperse into the washing buffer and could be washed out of stack completely. At a high washing rate the BGG adsorbed nonspecifically cannot be washed out and only the BGG in the dead volume can be washed out. A reasonable explanation is that though nonspecific adsorption caused by matrix modified by HEC was very small, there was fairly strong nonspecific interaction between the hydrophobic space arm and BGG. This part of BGG had no time to

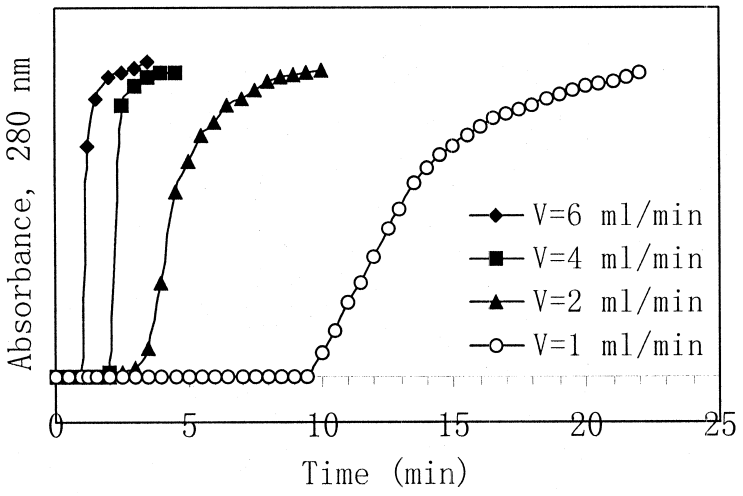

Fig. 4. Breakthrough curves at different feed-rates.

Table 2
Effect of washing rate on desorption

Washing rate (ml/min)	Washing time (min)	Amount of buffer used (ml)	Area of elution peak (mm^2)
1	33	33	138
3	10	30	196
5	6	30	333
7	4	28	715
10	2.5	25	1106
14	1.6	22	1106

dissociate from the membrane at high flow-rate, which led to the amount of protein eluted at last increased. It is concluded that the washing efficiency decreased with the increase in the washing rate. Though the separation time can be shortened and the amount of washing buffer reduced with the increase of washing rate, it is not suitable to use a high washing rate.

3.5. Effect of elution rate on desorption

The effects of elution rate on desorption are given in Table 3. The retention time (t_R) was much reduced with the increase of the elution rate and the amount of protein eluted did not change with the change of elution rate. The volume of elution was large and the symmetry of peak became worse at slow ($v=0.5$ ml/min) or fast ($v=3$ ml/min) elution rates. This phenomenon shows that the dissociation rate of the affinity complex is limiting. A suitable elution rate is 1–2 ml/min.

3.6. Separation of γ-globulin from human plasma

The separation of γ-globulin from human plasma was performed by single-pass mode on a stack with 20 membranes and the chromatogram is shown in Fig. 5. The purity of HGG was assayed by SDS-PAGE (see Fig. 6) and scanning at 550 nm. The amount of protein obtained was assayed using Coomassie Brilliant Blue method. Then it is known that 7.8 mg HGG with purity of 91.6% can be obtained from 4 ml human plasma. The adsorption capacity of this stack is about 27 mg. There is about 25–44 mg HGG in 4 ml plasma [22]. The amount of HGG obtained by the single-pass mode was not high. It is possible that the large amount of other proteins adsorbed physically or nonspecifically on the membranes, covered many affinity adsorption units. The long washing time in Fig. 5 indicates this possibility. Using recycling may increase the amount of HGG purification. In addition, because the small hybrid peak is near the HGG peak, they were collected together, which decreased the purity of protein obtained.

4. Conclusions

Nylon membranes were hydrated at first to am-

Table 3
Effect of elution rate on desorption

Elution rate (ml/min)	t_R (min)	Amount of protein eluted (mg)	Volume of elution (ml)	Asymmtery of peaks
0.5	16.0	0.198	30	0.7
1	8.5	0.196	24	1.0
2	4.0	0.190	21	1.0
3	2.2	0.196	30	0.9

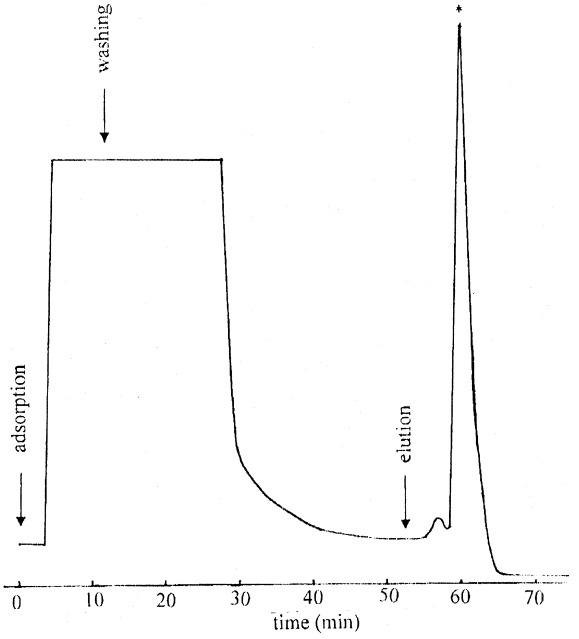

Fig. 5. Separation chromatogram of γ-globulin from human plasma on a stack of 20 membranes with 45 mm diameter. Human plasma (4 ml) was diluted to 20 ml with equilibration buffer. The flow-rate is 1 ml/min, *γ-globulin.

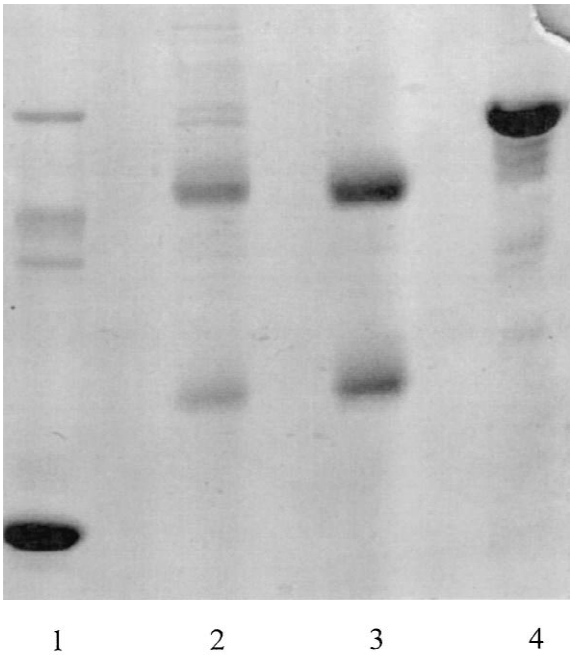

Fig. 6. SDS-PAGE assay of γ-globulin obtained from human plasma. (1) Chicken egg white lysozyme (M_r 14 300); (2) HGG (elution fraction); (3) HGG (standard, M_r: H chain 50 000; L chain 23 500); (4) BSA (M_r 68 000).

plify reactive groups and subsequently activated with $CyCl_3$ for coupling of hydroxyethylcellulose to increase the reactive sites and reduce nonspecific adsorption. Binding of HEC can decrease the pore diameter.

Phe can be immobilized onto nylon membrane by $CyCl_3$ activation. The Scatchard plot derived from adsorption isotherm indicated that the adsorption follows the Langmuir model.

The relationship between pressure and flow-rate revealed that the pressure drop of the membrane stack is very low at high flow-rate. But the effects of feed, washing and elution rates on adsorption and desorption show that high flow-rates are not suitable for affinity membrane chromatography, because the adsorption and desorption rates are limited by the association kinetics between the solute protein and the immobilized ligand.

The affinity membrane prepared can be used to purify HGG from human plasma. The purity of HGG obtained is more than 90%.

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