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Surface-modified anodic aluminum oxide membrane with hydroxyethyl celluloses as a matrix for bilirubin removal

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ABSTRACT

Microporous anodic aluminum oxide (AAO) membranes were modified by 3-glycidoxypropyltrimethoxysilane to produce terminal epoxy groups. These were used to covalently link hydroxyethyl celluloses (HEC) to amplify reactive groups of AAO membrane. The hydroxyl groups of HEC-AAO composite membrane were further modified with 1,4-butanediol diglycidyl ether to link arginine as an affinity ligand. The contents of HEC and arginine of arginine-immobilized HEC-AAO membrane were 52.1 and 19.7 mg/g membrane, respectively. As biomedical adsorbents, the arginine-immobilized HEC-AAO membranes were tested for bilirubin removal. The non-specific bilirubin adsorption on the unmodified HEC-AAO composite membranes was 0.8 mg/g membrane. Higher bilirubin adsorption values, up to 52.6 mg/g membrane, were obtained with the arginine-immobilized HEC-AAO membranes. Elution of bilirubin showed desorption ratio was up to 85% using 0.3 M NaSCN solution as the desorption agent. Comparisons equilibrium and dynamic capacities showed that dynamic capacities were lower than the equilibrium capacities. In addition, the adsorption mechanism of bilirubin and the effects of temperature, initial concentration of bilirubin, albumin concentration and ionic strength on adsorption were also investigated.

1. Introduction

Affinity chromatography represents a unique method in separation technology, as it is the only one that performs separation and purification of biomolecules on the basis of their specific interactions with other biomolecules or analogs of biomolecules [1,2]. The high specificity of affinity chromatography is due to the strong interactions between the ligand and the biomolecule of interest. As a new technology in affinity separation, affinity membrane chromatography possesses shorter diffusion time than those obtained in conventional affinity chromatography, because the interactions between molecules and active sites on the membrane occur in convective through pores, rather than in stagnant fluid inside the pores of an adsorbent particle [3–5]. For this reason, affinity membrane has the potential to maintain high efficiencies both at high flow rates and for the use of large biomolecules with small diffusivities [6,7]. In recent years, affinity membrane has been an effective method to purify proteins, peptides, and nucleic acids [6–9].

Besides purification of bioproducts, affinity membrane has also exhibited the potential application in blood detoxification, especially for the removal of toxins, bilirubin, from human plasma [10–12]. Bilirubin, one of the common metabolites of hemoglobin, is a pathogenic substance, and is released into blood due to the normal or abnormal destruction of red blood cells [13]. It deposits in tissue, especially in the brain. At high bilirubin concentration, jaundice results, indicating hepatic or biliary tract dysfunction [14]. The elevated level of bilirubin in blood (i.e. hyperbilirubinemia) may also cause permanent brain damage or death in more severe cases [14]. To prevent this from happening, many techniques have been employed for the removal of bilirubin from plasma of patients suffering from hyperbilirubinemia such as hemoperfusion, hemodialysis and affinity membrane [15]. Of these, affinity membrane exhibits short treatment time and excellent selectivity for toxins due to elimination of diffusional resistance and high specific binding [10].

An ideal membrane for blood detoxification requires high hydrophilicity and thus low nonspecific protein adsorption, good uniformities including narrow pore size and porosity distribution, high biological, chemical and mechanical resistance, good biocompatibility and a high density of reactive groups that can bind ligands with a specific binding capacity for the targeted ligate [10]. Although most research articles on membrane chromatography

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systems deal with organic membranes which offer a large choice of functional chemical groups for ligand grafting [1,6,9], inorganic membranes also have attractive properties, such as excellent uniformities, good biocompatibility, high chemical and physical stability, which make them good candidates for affinity chromatography applications [2,16]. Unfortunately the surface of inorganic membranes is less reactive than organic ones and has to be activated to enable ligand grafting. In our previous work, the inorganic membrane, anodic aluminum oxide (AAO) membrane, was chosen as a matrix. To amplify reactive groups of AAO membrane, silica and poly(pyrrole-3-carboxylic acid) were coated onto the AAO membrane by sol–gel and vapor deposition polymerization methods, respectively. The obtained silica-AAO and poly(pyrrole-3-carboxylic acid)-AAO composite membranes exhibited excellent uniformities, high density of reactive groups, and low nonspecific adsorption [10,16].

In this paper, hydroxyethyl celluloses (HEC) coated AAO membrane was fabricated and used as affinity matrix, for the first time. AAO membranes were activated by 3-glycidoxypropyltrimethoxysilane (GPTMS), and then HEC was coupled on the activated membranes to increase reactive sites. The obtained HEC-AAO composite membrane exhibited excellent uniformities, high density of reactive groups, and low nonspecific adsorption. Arginine as model ligand for affinity adsorption of bilirubin was immobilized on the composite membrane to obtain arginine-immobilized affinity membrane. The preparation method, characteristics and applicability of arginine-immobilized affinity membrane were presented and discussed in detail.

2. Experimental

2.1. Chemicals and apparatus

AAO membranes (60 μm thickness, 0.2 μm pore size and 47 mm diameter) were purchased from Whatman. HEC (Cellosize WP 40) and 1,4-butanediol diglycidyl ether (bisoxirane) were provided by Fluka (Switzerland). Bilirubin was purchased from Shanghai Zhijie Biotechnology Company (China). Bovine serum albumin (BSA) was obtained from Sigma. GPTMS was obtained from Danyang Organosilane Material Chemical Company (China). Ninhydrin·H_2O and L-arginine were the products of Sinopharm Chemical Reagent Company (China). All other chemicals were of analytical grade and used without further purification. All solutions were prepared using deionized Milli-Q water (Millipore).

The concentrations of bilirubin, HEC and arginine were determined using a Beckman coulter DU 800 Nucleic acid/protein analyzer (Beckman Coulter Inc., USA). The membrane cartridge (donated amicably from the Dalian Chemical and Physical Institute, China) was used to load the membrane stack. A peristaltic pump (Model BT600-2J, China) was used for the feeding of bilirubin solutions. The morphologies of the membranes were visualized with the field-emission scanning electron microscope (FE-SEM) (LEO, Germany).

2.2. Preparation of affinity membrane

2.2.1. Activation of AAO membrane

The AAO membrane was activated with GPTMS, as reported in our previous work [17]. Briefly, ten AAO membrane disks were immersed in a solution of 5 ml GPTMS–30 ml ethanol–2 ml sodium acetate buffer solution (50 mM, pH 5.0). The polytetrafluoroethylene device containing the above solution was vacuumized for 5 min to remove air from the pores of the membranes. The membranes remained in this solution for another 15 min under ambient pressure before being rinsed with ethanol. The membranes were then cured by heating in vacuum at 100 °C for 1 h.

2.2.2. Preparation of HEC-AAO composite membrane

The activated membranes were shaken in 2% (w/w) HEC solution, pH 9 (adjusted by $NaHCO_3$), for 30 min at room temperature. The HEC solution was then sucked through the membranes, which were subsequently incubated in an oven at 90 °C for 1.5 h. Non-covalently bound HEC was removed by washing the membranes with 0.1 M NaOH and deionized water. The amount of HEC bound on the membranes was determined by the phenol–sulfuric acid method [18]. The reaction for the preparation of HEC-AAO composite membranes was illustrated in Scheme 1.

2.2.3. Immobilization of arginine

The HEC-AAO composite membranes were shaken for 15 h at 80 °C in a solution of 9 ml bisoxirane-1 ml ethanol-1 ml 25 mM Na_2CO_3, pH 11 [19]. After activation, the membranes were washed three times with water at room temperature. Bisoxirane-activated membranes were stored in a dry atmosphere until further use to avoid hydrolysis of the epoxy groups.

The activated membranes were shaken in 10 ml of 0.5 M arginine solution in 10 mM PBS at pH 7.4 for 3 h. Afterwards the membranes were washed in 1 M NaCl and water extensively. The amount of arginine immobilized on the HEC-AAO composite membrane was assayed by the ninhydrin method.

2.3. Bilirubin adsorption experiments

The arginine-immobilized HEC-AAO membranes were tested for the adsorption of bilirubin in 10 mM phosphate buffer (pH 7.4) and in bilirubin–BSA solution by batch experiment. Because bilirubin is easily destroyed by exposure to direct sunlight or any other source of ultraviolet light, include fluorescent lighting, all adsorption experiments are carried out in a dark room. The amounts of bilirubin adsorbed were determined with Eq. (1).

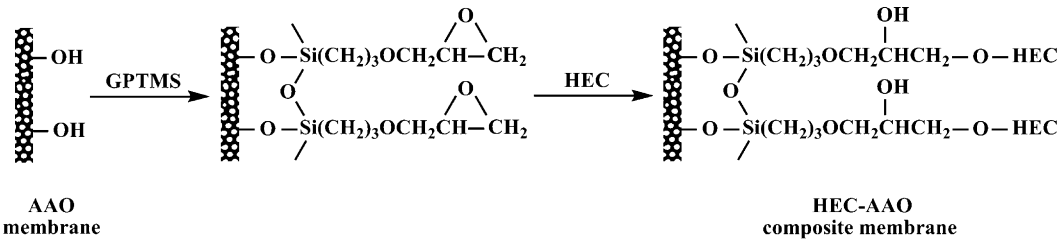
$$q = \frac{(c_i - c_t)V_s}{m} \quad (1)$$

where q is the amount of bilirubin adsorbed onto unit mass of the membrane (mg/g); c_i and c_t are the concentrations of the bilirubin in the initial and in the aqueous phase after adsorption, respectively (mg/l); V_s is the volume of the bilirubin solution (l); and m is the mass of the membrane (g). The concentration of the solution of the free bilirubin was detected by spectrophotometry at the wavelength of 438 nm and the ones containing bilirubin–albumin complex, at 460 nm.

Some factors that affect the adsorption processes were studied in the present paper. An amount of 40 mg membrane was shaken in 25 ml bilirubin solution at different temperatures (i.e. 4, 25 and 37 °C), and then the bilirubin concentration of the solution was examined at certain time intervals to study the effect of temperature and the equilibrium time at different temperature; An amount of 40 mg membrane was shaken in 25 ml bilirubin solution at 37 °C to study the adsorption isotherm of bilirubin and the effect of initial concentration of bilirubin on adsorption; and the effect of ionic strength was investigated in the bilirubin solution containing NaCl (the concentrations were 0.1, 0.2, 0.3 and 0.4 M).

The dynamic experiments were carried out in the cartridge to investigate the effect of flow rate on adsorption. The bilirubin solution (100 ml) was impelled by peristaltic pump to flow through the membrane stack (containing five overlapped membranes) at different flow rate (0.5, 1, 2, 3, 4 ml/min).

Bilirubin removal from human plasma with arginine-immobilized HEC-AAO membranes was also studied in the cartridge. The blood samples were obtained from patients with



Scheme 1. Procedure for preparation of the HEC-AAO composite membrane.

hyperbilirubinemia. Plasma was separated by centrifugation at 3000 × *g* for 20 min at room temperature. The plasma was filtered using 0.45 μm syringe filters [20], and then was impelled by peristaltic pump with flow rate of 2 ml/min to flow through the membrane stack (containing five overlapped membranes). The amounts of bilirubin through the membrane cartridge were measured in succession with a spectrophotometer.

2.4. Regeneration of the membranes

The bilirubin saturated membrane was eluted by recirculating the 0.3 M NaCl, 0.5 M NaCl or 0.3 M NaSCN solution, each in 0.01 M Tris buffer (pH7.4). The desorption ratio of the bilirubin from the arginine-immobilized HEC-AAO membranes was evaluated by comparing the amounts of bilirubin eluted and adsorbed.

3. Results and discussion

3.1. Characteristics of the membranes

The morphologies of AAO membrane and HEC-AAO composite membrane were observed by field-emission scanning electron microscope (FE-SEM) and were illustrated in Fig. 1. The AAO membrane has a precise, non-deformable honeycomb pore structure (Fig. 1A) with no lateral cross over between individual pores. After coating HEC onto the AAO membrane, the pore size of the membrane has not apparently changed (Fig. 1B). Moreover, the HEC-AAO composite membrane also exhibited homogeneous surfaces. The precise pore structure and narrow pore size distribution of the HEC-AAO composite membrane might significantly reduce diffusion resistance, possess high flow rates, and ensure a high level of affinity adsorption performance in purification of bioproducts [10,16].

The HEC content of HEC-AAO composite membrane was ~52.1 mg/g membrane. Since there are plentiful reactive groups (—OH) on the HEC molecule, the compatibility with the usual ligands was greatly enhanced by coating HEC on the AAO membrane. The amount of arginine immobilized on affinity membrane can be determined spectrophotometrically at ~570 nm, and the arginine content was 19.7 mg/g membrane.

3.2. Dependence of flow rate through affinity membrane on pressure drop

The most significant feature of the affinity membranes is the fast flow rate and low pressure drop, in comparison with the slow flow rate and high pressure drop of the affinity column chromatography. Fig. 2 presents the relationship between the pressure drop and flow rate of water through the arginine-immobilized HEC-AAO membranes. As shown in this figure, the flow rate increases almost linearly with the increase of pressure drop. It indicated that the arginine-immobilized HEC-AAO membranes are difficult to be compressed at high pressure drops [21]. One can see from Fig. 2 that relatively high flow rate are achieved at very low pressure

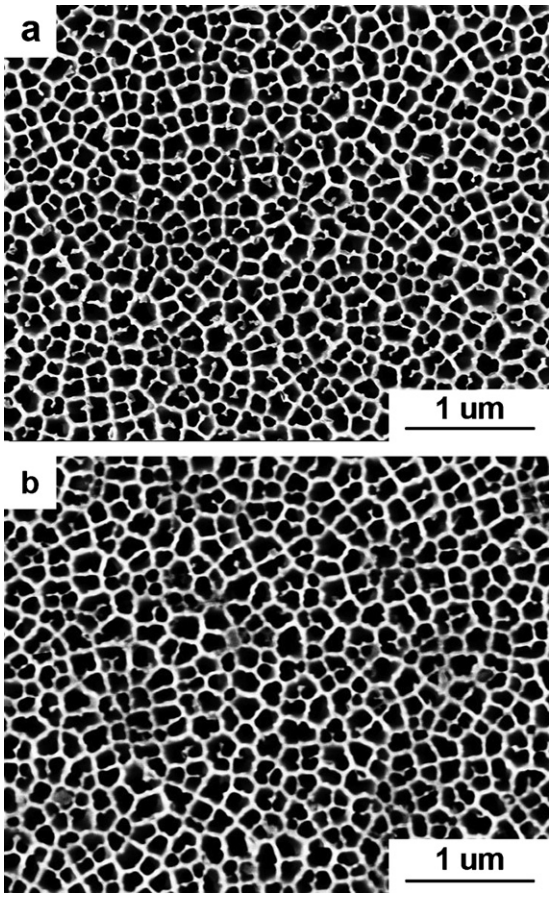


Fig. 1. SEM images of surface of original AAO membrane (a) and HEC-AAO composite membrane (b).

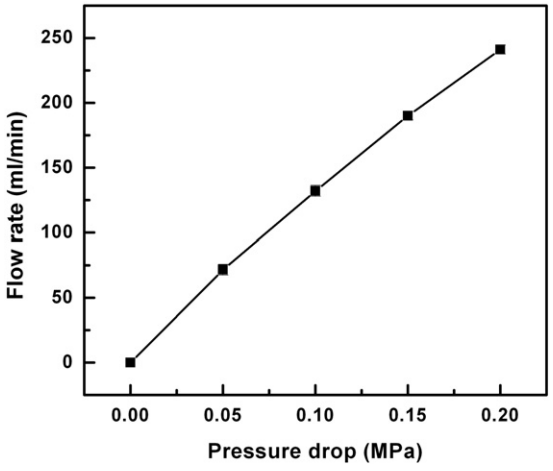


Fig. 2. Relationship between the pressure drop and flow rate of pure water through the arginine-immobilized HEC-AAO membranes.

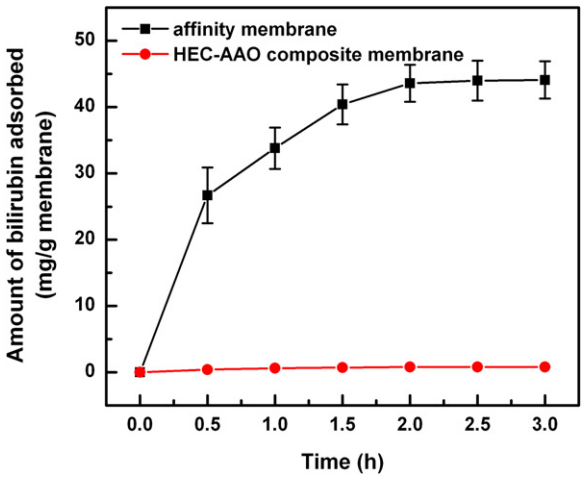


Fig. 3. Adsorption rates of bilirubin on the membranes. Initial bilirubin concentration: 200 mg/l; temperature: 37 °C; medium: phosphate buffer (pH 7.4, 10 mM); bilirubin solution volume: 25 ml.

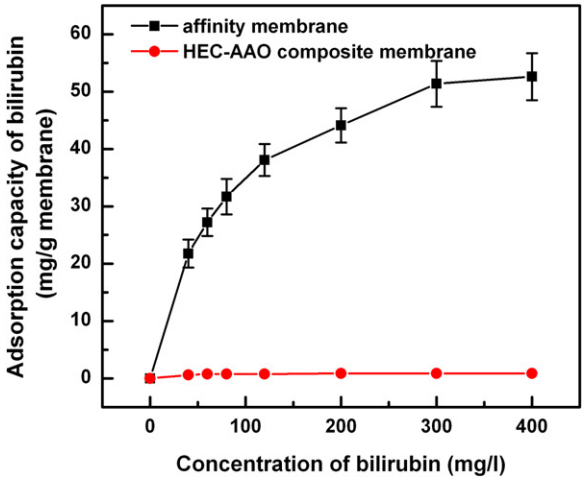


Fig. 4. Effect of bilirubin initial concentration on the adsorption capacities. Temperature: 37 °C; medium: phosphate buffer (pH 7.4, 10 mM); bilirubin solution volume: 25 ml; adsorption time: 2 h.

drops. For instance, at a pressure drop of 0.05 MPa, the flux is 4.5 ml/(min cm^2).

3.3. Adsorption kinetics

Albumin is the natural carrier of bilirubin in the blood. Bilirubin is bound reversibly to albumin in two classes of binding sites, lysine and arginine. Arginine residues are closely associated with the strong hydrophobic anion-binding sites of serum albumin [22,23]. Therefore, we chose arginine as ligand for bilirubin adsorption. The rate of adsorption is of key importance for bilirubin adsorbents to be used for blood detoxification. To minimize the operational time and cost, an ideal membrane should remove bilirubin rapidly. Fig. 3 gives the adsorption rate curves which were obtained by following the decrease of concentration of bilirubin within the solution with time. As seen here, there were relatively faster adsorption rates were observed at the beginning of adsorption process, and then adsorption equilibrium was achieved gradually in ca 2 h. It should be pointed out that there was a negligible non-specific bilirubin adsorption (i.e. the adsorption onto the HEC-AAO composite membranes) which was ca 0.8 mg/g membrane. There is no reactive binding group onto the HEC-AAO composite membranes which interacts with bilirubin molecules, hence, this adsorption may be due to diffusion of bilirubin into the pores of composite membrane and weak interactions between bilirubin and hydroxyl groups on the surface of composite membrane. On the other hand, much higher adsorption rates were observed when the arginine-immobilized HEC-AAO membranes were used.

3.4. Effect of initial concentration of bilirubin

Fig. 4 shows the effect of initial bilirubin concentration on adsorption. Note that one of the main requirements in the affinity system is the specificity of the sorbent. The non-specific interaction between the carrier matrix (here the HEC-AAO composite membranes) and the molecules to be adsorbed (here bilirubin) should be minimum in order to have a high specificity. As presented in this figure, the amount of bilirubin adsorption on the unmodified HEC-AAO composite membranes was quiet low, and the nonspecific adsorption capacity almost remained unchanged when bilirubin concentration increases. While much higher binding capacity, up to ~52.6 mg bilirubin/g membrane was achieved in the case of the arginine-immobilized HEC-AAO membranes. The specific bilirubin adsorption increased with the increasing of bilirubin initial concentration. However, this levels off at around 300 mg/l initial bilirubin concentrations in the adsorption medium.

Batch experiment provides the equilibrium parameters of the membranes for the bilirubin adsorption. Freundlich adsorption isotherms are applied for the description of the adsorption mechanism. The isotherms can be described as follows:

$$q^* = Kc^{*1/n} \tag{2}$$

Eq. (2) can be transformed as follows:

$$\log q^* = \log K + \frac{1}{n} \log c^* \tag{3}$$

where q^* is adsorption capacity of bilirubin on unit mass of the affinity membrane at equilibrium (mg/g membrane); c^* is the equilibrium concentration of bilirubin (mg/l); n and k are the physical constants of the Freundlich adsorption isotherm. From the experimental results, as shown in Fig. 5 it can be deduced that the adsorption mechanism of the arginine-immobilized HEC-AAO membranes for bilirubin is in accordance with the Freundlich adsorption isotherm which belongs to the monolayer adsorption.

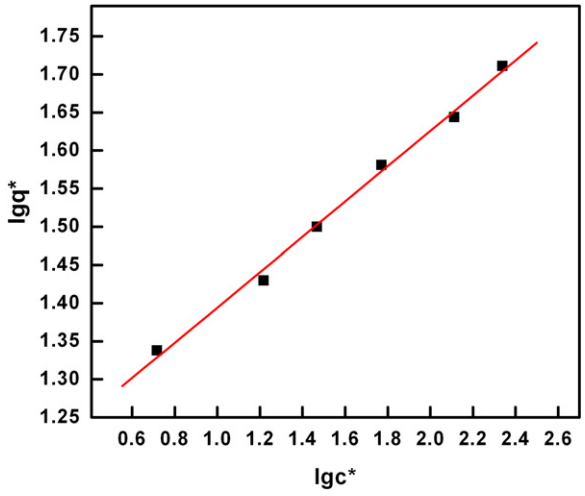


Fig. 5. Freundlich adsorption isotherm of bilirubin on the arginine-immobilized HEC-AAO membranes. Temperature: 37 °C; medium: phosphate buffer (pH 7.4, 10 mM); bilirubin solution volume: 25 ml; adsorption time: 2 h.

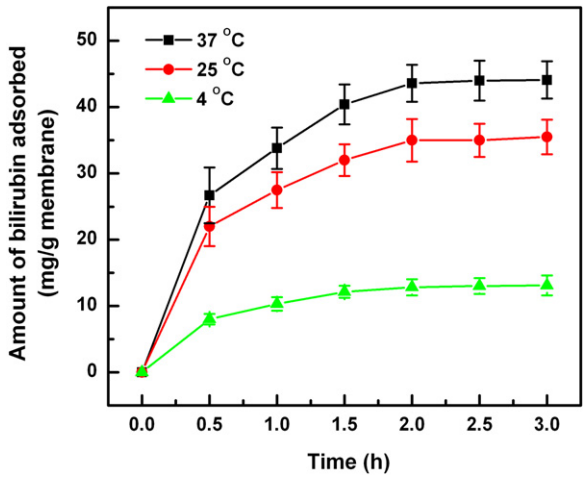


Fig. 6. Effect of temperature on bilirubin adsorption. Initial bilirubin concentration: 200 mg/l; medium: phosphate buffer (pH 7.4, 10 mM); bilirubin solution volume: 25 ml.

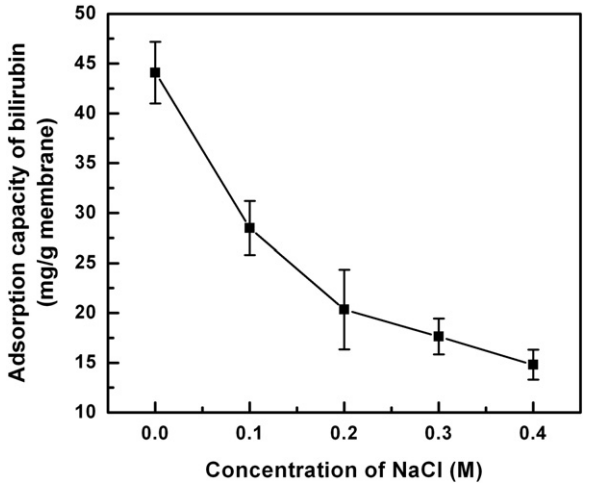


Fig. 7. Effect of NaCl concentration on adsorption capacities. Initial bilirubin concentration: 200 mg/l; temperature: 37 °C; medium: phosphate buffer (pH 7.4, 10 mM); bilirubin solution volume: 25 ml; adsorption time: 2 h.

3.5. Effect of temperature on bilirubin adsorption

The effect of temperature on the adsorption of bilirubin by the arginine-immobilized HEC-AAO membranes was performed at 4, 25 and 37 °C and was shown in Fig. 6. As seen here, the bilirubin adsorption capacity increased with increasing temperature. The maximum bilirubin adsorption was 44.1 mg/g membrane, which was obtained at 37 °C. Since this is body temperature, we did not attempt to work at higher temperatures.

This result is in contrast to that observed in most adsorption studies. In general, it is known that adsorption decreases with increasing temperature, because adsorption is an exothermic process. One hypothesis to explain this unexpected behavior is that conformational changes takes place in bilirubin molecule with the increase in temperature. The bilirubin molecule changed from a *cis* configuration to a *trans* configuration with increasing temperature. This would allow for lessened steric hindrance in the binding of bilirubin to the immobilized arginine molecules [24].

3.6. Effect of ionic strength

The isoelectric point of arginine is about 10.76. Thus, the amino groups of the arginine are protonated under the conditions of adsorption experiment (pH 7.4) so that these groups can form ionic linkages with the carboxyl groups of bilirubin. The ionic linkage makes an important contribution to the binding, although other kinds of interactions such as hydrophobic interaction and hydrogen bonding may play lesser roles. Fig. 7 presents the effect of the ionic strength on bilirubin adsorption, which showed that the adsorption capacity decreased with increasing NaCl concentration in the bilirubin solution. The binding of bilirubin to arginine is primarily achieved by electrostatic interactions between the positively charged functional groups of the constituent amino acids and the negatively charged carboxyl groups on the bilirubin molecule [13]. When the NaCl concentration changed from 0 to 0.4 M, the adsorption of bilirubin decreased by 66.4%. The decrease in the adsorption capacity as the ionic strength increased can be attributed to weakened electrostatic interaction between the arginine-immobilized membranes and bilirubin molecules.

3.7. Adsorption in the presence of albumin

Serum albumin, the natural carrier of bilirubin in the blood, may have as many as 12 binding sites for bilirubin, but only two of the sites bind bilirubin molecules tightly [25,26]. For successful use in blood detoxification, an adsorbent should be capable competing with at least the weak binding sites of albumin for the unconjugated bilirubin.

Here, bilirubin–BSA solution was used as simulation plasma. Adsorption experiments were performed by adding the affinity membrane to previously prepared bilirubin solutions, with an initial concentration of 200 mg/l, containing various concentrations of BSA. The effect of BSA concentration on bilirubin adsorption was shown in Fig. 8. The results indicated that the adsorption capacity of the arginine-immobilized HEC-AAO membrane for bilirubin was greatly affected by the BSA concentration and the adsorption capacity for bilirubin decreases with an increase in BSA concentration. When BSA/bilirubin molar ratio is greater than 0.5, the amount of adsorbed bilirubin becomes insensitive to the albumin concentration. Thus, arginine-immobilized HEC-AAO membrane can well compete for those bilirubin molecules which are weakly bound to albumin.

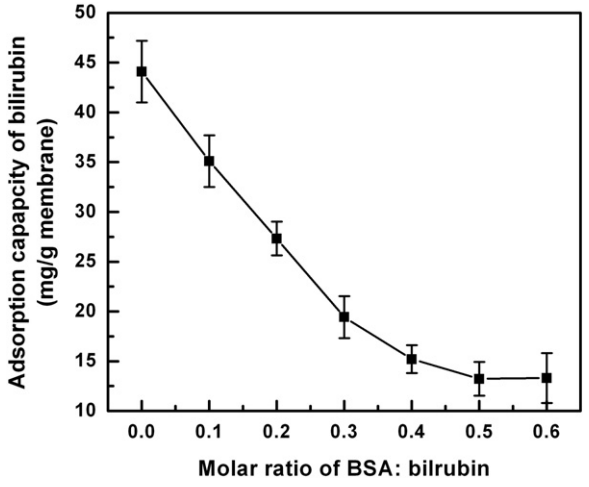


Fig. 8. The effect of BSA concentration on adsorption capacities. Initial bilirubin concentration: 200 mg/l; temperature: 37 °C; bilirubin solution volume: 25 ml; adsorption time: 2 h.

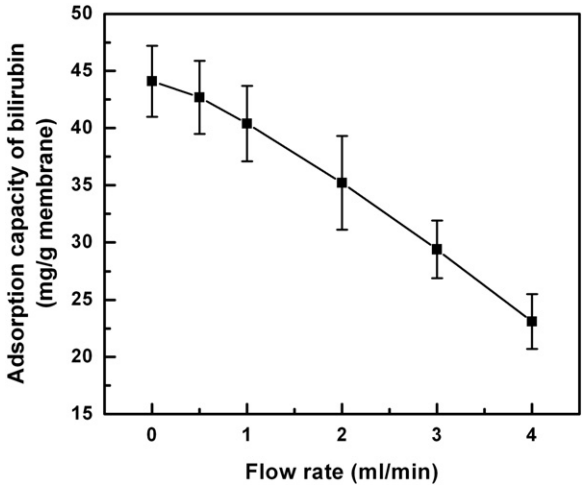


Fig. 9. Bilirubin adsorption at different flow rate. Initial bilirubin concentration: 200 mg/l; temperature: 37 °C; medium: phosphate buffer (pH 7.4, 10 mM).

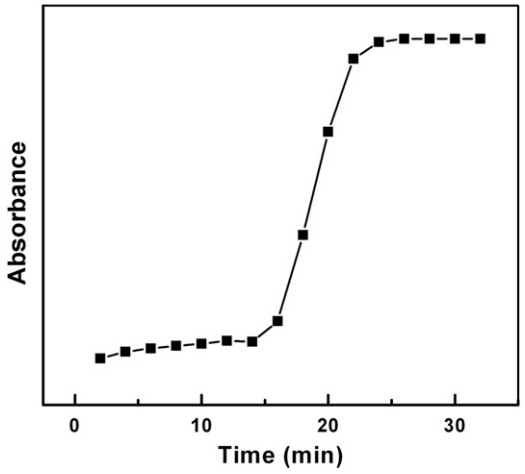


Fig. 10. Breakthrough curve of bilirubin. Initial bilirubin concentration: 216 mg/l; flow rate: 2 ml/min; temperature: 37 °C; medium: human plasma.

3.8. Effect of eluant on desorption

Desorption experiments were performed with three kinds of eluants, namely 0.3 M NaCl, 0.5 M NaCl, or 0.3 M NaSCN, all in 0.01 M Tris buffer (pH 7.4). The results showed that 63% and 72% of bound bilirubin were eluted with 0.3 and 0.5 M NaCl eluants, respectively. However, 85% of the bilirubin adsorbed on the arginine-immobilized HEC-AAO membrane could be eluted with 0.3 M NaSCN. These observations can be explained as follows: when the ionic strength is changed by increasing the NaCl concentration from 0.3 to 0.5 M, the electrostatic attraction between the positively charged affinity membrane and the negatively charged bilirubin is decreased and this facilitates desorption. The much stronger desorption which arises in the presence of NaSCN is due not only to the above effect, but also to the disorganization of the structure of water by NaSCN [27].

3.9. Effect of flow rate on bilirubin adsorption

The effect of flow rate on the adsorption of bilirubin was studied for a loading of 100 ml of 200 mg/l bilirubin solution at the flow rates of 0.5, 1, 2, 3, 4 ml/min, and the results are presented in Fig. 9. The adsorption capacity of the arginine-immobilized HEC-AAO membranes decreases from 44.1 to 23.1 mg/g membrane with the raise of the flow rate from 0 to 4 ml/min, because of the decrease in the residence time. However, the adsorption capacity is near to the equilibrium value of static adsorption for flow rates below 0.5 ml/min.

3.10. Bilirubin removal from human plasma

Based on the promising bilirubin retentions obtained for the arginine-immobilized HEC-AAO membranes, we carried out dynamic measurements of bilirubin adsorption from human plasma through a stack of membranes. Fig. 10 presents the absorbance of permeate concentrations as function of the permeate time. As shown in Fig. 10, a high rate for bilirubin removal was obtained in dynamic experiments. Because the mass transfer in dynamic experiment is convective mode, the resistance is greatly reduced. As a result, the operation for bilirubin removal could be speeded up. From the figure, we also notice that the breakthrough curves were sharp, indicating good bilirubin removal efficiency. It can be attributed to the high ligate capacity and excellent uniformities of the membranes. In real biomedical applications the filtration process can be stopped in an earlier stage when the average bilirubin concentration in the permeate is below the normal bilirubin level in the plasma.

4. Conclusions

Bilirubin adsorbent has been prepared, using HEC-AAO composite membranes as the matrix and arginine as bilirubin ligand. The HEC-AAO composite membranes which exhibited a relatively large flux, were fabricated by coating HEC on the AAO membrane by activating with GPTMS. Arginine was immobilized onto the composite membrane by activating with bisoxirane. The arginine-immobilized HEC-AAO membranes provided high binding capacity, good specific adsorption and excellent mechanical properties. These membranes may be an important alternative to the existing adsorbent in the therapy of hyperbilirubinemia.

Acknowledgments

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