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Adsorption of bilirubin with polylysine carrying chitosan-coated nylon affinity membranes

Wei Shi [a,b,*], Fengbao Zhang [a], Guoliang Zhang [a]

[a] *School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, PR China*
[b] *Biomedical Engineering Research Center, Medical College, Xiamen University, Xiamen 361005, PR China*

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Abstract

Microporous polyamide membranes were activated by bisoxirane and subsequently bound with chitosan (CS) to amplify reactive groups. Then polylysine (PLL) as ligand was immobilized onto the CS-coated nylon membranes. The contents of CS and PLL of PLL-attached membranes were 93.2 and 90.4 mg/g nylon membrane, respectively. Such PLL-attached membranes were used to adsorb bilirubin from the bilirubin–phosphate solution and bilirubin–albumin solution. The adsorption mechanism of bilirubin and the effects of temperature, initial concentration of bilirubin, albumin concentration and ionic strength on adsorption were investigated by batch experiments. The results showed that the adsorption capacity increased with increasing the temperature while decreased with increasing the NaCl concentration and albumin concentration, and the adsorption isotherm fitted the Freundlich model well. The result of dynamic experiment showed PLL-attached membranes can well remove the bilirubin from the bilirubin–albumin solution.

Keywords: Chitosan; Bilirubin; Affinity membrane; Poly-L-lysine; Adsorption

1. Introduction

Bilirubin, a natural breakdown product of heme, is normally conjugated with albumin to form a water-soluble complex [1,2], and its accumulation in tissues causes jaundice [3]. Many attempts have been made to remove the bilirubin directly from plasma of patients suffering from hyperbilirubinemia such as phototherapy, hemodialysis and hemoperfusion. Phototherapy renders the normally occurring bilirubin into water-soluble forms, referred to as photobilirubin, which can be excreted more easily [4], but it is generally applicable for mild cases of hyperbilirubinemia. Hyperbilirubinemia can also be treated by hemoperfusion, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for bilirubin [5]. We prepared the albumin-fixed membrane, and it was used to remove the bilirubin from the bilirubin–albumin solution. The experiment results showed the transfer rate of bilirubin was obviously enhanced after fixing albumin into the high-flux asymmetric membrane [6].

Recently, a new technology in affinity separation, the membrane affinity chromatography has proven its efficiency and time stability. It introduces a different approach to exploit the biospecific interactions between a ligate and a ligand for biomedical applications, using porous structures with flat-sheet and hollow fiber forms. Affinity membranes operate in convective mode, which can significantly reduce diffusion and pressure drop limitations commonly encountered in column chromatography [7]. One of the most important factors in membrane affinity chromatography is the identification of suitable membranes. The selection of the membrane material and its preparation constitute dominant factors affecting the chromatographic performance [8]. 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 [9]. Another disadvantage of this matrix is nonspecific adsorption of protein [10]. In order to solve these problems, nylon membranes

* Corresponding author. Tel.: +86 592 2185299; fax: +86 592 2185299.
E-mail address: shiwei@xmu.edu.cn (W. Shi).

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are bound with polyhydroxyl-containing material, such as polyglucose, dextran, starch, cellulose and hydroxyethylcellulose (HEC) to increase reactive sites and reduce nonspecific adsorption [11]. In our previous work [12,13], HEC-coated nylon membranes were prepared, and polylysine (PLL) as ligand was immobilized onto the HEC-nylon membranes by epibromohydrin and 1,1′-carbonyldiimidazole (CDI) activation, respectively. CDI is known to show high reactivity with various functional groups, such as amino, carboxyl and hydroxyl groups. The content of PLL of affinity membrane activated by CDI was obviously higher than that activated by epibromohydrin [13]. PLL-HEC affinity membranes were used to adsorb bilirubin from the bilirubin–albumin solution. The results showed PLL-attached membranes can well remove the bilirubin from the bilirubin–albumin solutions. However, HEC-coated nylon membrane can only provide hydroxyl groups, and the content of reactive groups was still low. Lately Avramescu et al. employed ethylene vinyl alcohol (EVAL) adsorptive membranes with bovine serum albumin (BSA) as bioligand for affinity supports for bilirubin retention [14]. BSA as model ligand was coupled on the membrane by trichloro-*s*-triazine (sTT) activation. Although sTT can easily be attached to the membranes containing amino and hydroxyl groups at room temperature, residual chlorine atoms on the membrane after sTT activation easily led to nonspecific adsorption. Due to a mass of active groups existing in the EVAL membrane the higher of content of BSA immobilized on the EVAL membrane was obtained. However, the bilirubin adsorption capacities of the BSA-immobilized EVAL adsorber membranes were not greater than that of PLL-attached nylon membrane we prepared [12,13]. It is mainly because the immobilized PLL exhibits an overwhelmingly higher capacity than BSA.

In this paper, nylon membranes were activated by bisoxirane, and then chitosan (CS) was coupled on the activated membranes to improve the hydrophilicity and increase reactive sites. These membranes possess a large number of reactive groups of –OH and $–NH_2$. PLL as ligand was immobilized onto the CS-coated nylon membranes via CDI activation. Such PLL-CS affinity membranes were used to adsorb bilirubin. The adsorption isotherm and the effects of temperature, initial concentration of bilirubin, albumin concentration and ionic strength on adsorption were investigated.

2. Experimental

2.1. Chemicals and apparatus

Nylon membranes (47 mm diameter, 0.45 μm pore size) were obtained from Whatman (England). Chitosan (Mrv ~400,000) (CS) and 1,4-butanediol diglycidyl ether (bisoxirane) were provided by Fluka (Swizerland). Poly-L-lysine (Mr ~242,000) (PLL), sodium cyanoborohydride and 1,1′-carbonyldiimidazole were purchased from Sigma (Germany). Bovine serum albumin was obtained from Beijing Chemical Reagent Company (China). Bilirubin was purchased from Shanghai Weihui Chemical Factory (China). The other reagents used were bought in China.

A peristaltic pump (Model BT-100, Shanghai, China) was used for the feeding of bilirubin solutions. The concentration of bilirubin and albumin-conjugated bilirubin were determined using a 752N UV–vis spectrophotometer (Shanghai Precision Instruments Co. Ltd., Shanghai, China). The membrane cartridge (donated amicably from the Dalian Chemical and Physical Institute, China) was used to load the membrane stack.

2.2. Affinity-membrane preparation

2.2.1. Preparation of CS-bound membrane

Nylon membrane disks (47 mm diameter) were shaken in 1 M HCl for 24 h at room temperature. The membranes were washed several times with water at room temperature. After partial hydrolysis of amide bonds, the membranes were shaken for 15 h at 353 K in a solution of 9 mL bisoxirane–1 mL ethanol–1 mL 25 mM Na_2CO_3, pH 11 [10]. 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 CS solution (prepared by dissolving 0.15 g CS in 10 mL 1 vol.% acetic acid solution) for 1 h at room temperature. The CS solution was then sucked through the membranes, which were subsequently incubated in an oven at 353 K for 1 h. Noncovalently bound CS was removed by washing the membranes with 1 vol.% acetic acid solution and deionized water. The amount of CS bound on the membranes was determined by the ninhydrin method.

Reaction scheme for the preparation of CS-coated nylon membrane is shown in Fig. 1.

2.2.2. Immobilization of PLL

The CS-coated membranes were shaken in a solution of 20 mg CDI per 10 mL acetone at room temperature for 1 h. After activation, the membranes were washed three times for 20 min with acetone at room temperature and then drying in vacuum.

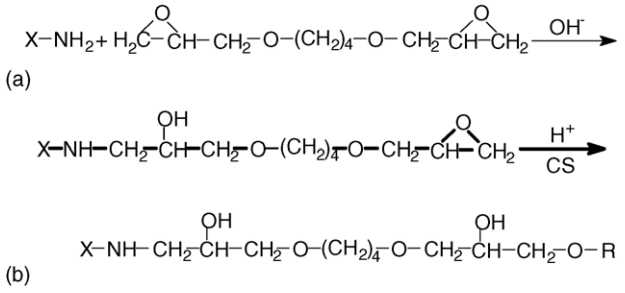

Fig. 1. Procedure for preparing CS-coated nylon membrane. (a) Nylon membrane; (b) CS-coated membrane.

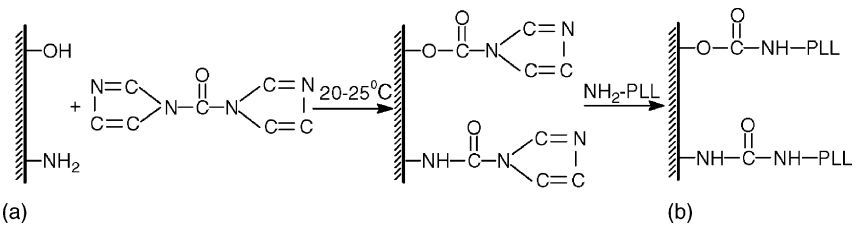

Fig. 2. Activation of the CS-coated nylon membrane with CDI and covalent immobilization of PLL. (a) CS-coated nylon membrane; (b) PLL-attached membrane.

An amount of 100 mg PLL was dissolved in 10 mL $NaHCO_3$ solution, pH 8.4. One CDI-activated CS-coated membrane was given in reaction solution and shaken overnight at room temperature. Afterwards the membranes were washed in 1 M NaCl and water extensively. The amount of PLL immobilized on the CS-coated membrane was assayed by the ninhydrin method [15].

CS-coated membranes possess a large number of reactive groups of –OH and $–NH_2$. Reaction scheme for the preparation of affinity membrane based on CS-coated membrane is shown in Fig. 2.

2.3. Batch experiments of bilirubin adsorption

The PLL-attached membranes were tested for the adsorption of bilirubin in 0.066 M phosphate buffer (pH 7.4) and in bilirubin–albumin 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 100 mg membrane was shaken in 20 mL bilirubin solution at different temperatures (i.e. 25 and 37 °C), and then the 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 50 mg membrane was shaken in 5 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 0.05, 0.1, 0.2 and 0.4 M).

2.4. Dynamic experiments of the membrane stack

The dynamic experiments were carried out in the cartridge to investigate the breakthrough performance. It was impelled by peristaltic pump with two different flow rate (i.e. 2 and 4 mL/min) to flow through the membrane stack (containing 10 overlapped membranes). Then the amounts of bilirubin through the membrane cartridge were measured in succession with a spectrophotometer.

2.5. Regeneration of the membranes

The bilirubin-saturated membrane was regenerated with BSA and sodium hydroxide. The bilirubin saturated membrane was eluted by recirculating the BSA solution. Then the absorbed BSA on the membrane was eluted with 0.5 N NaSCN eluant, and the membranes regenerated successively with 6 M urea, 1% Tween 80 and distilled water. The elution process by the alkaline solution included immersing the bilirubin absorbed membranes in 0.1 N NaOH aqueous solution, followed by the procedure of washing with large volume of distilled water and phosphate buffer (pH 7.4). The regenerated membranes were then reused for bilirubin equilibrium adsorption.

3. Result and discussion

3.1. Contents of CS and PLL on affinity membrane

The amount of CS coupled on the membrane was 93.2 mg/g nylon membrane. These CS-coated membranes possess a large number of reactive groups of –OH and $–NH_2$. The content of PLL reached 90.4 mg/g nylon membranes. The content of PLL was great higher than that of HEC-coated nylon affinity membranes [12,13,15].

3.2. Effects of bilirubin initial concentration

Fig. 3 showed the non-specific and specific adsorption of bilirubin onto the unmodified and PLL-attached membranes. The non-specific bilirubin adsorption on the membranes is quite low, and the amount was about 0.6 mg bilirubin/g unmodified membrane only. While much higher binding capacity, up to 40.7 mg bilirubin/g membrane was obtained

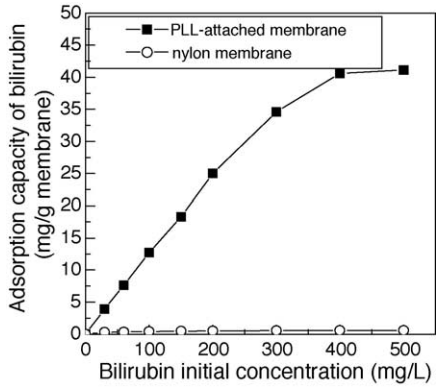

Fig. 3. The effect of bilirubin initial concentration on the adsorption capacities. Bilirubin solution volume: 5 mL; membrane weight: 0.050 g; temperature: 37 °C.

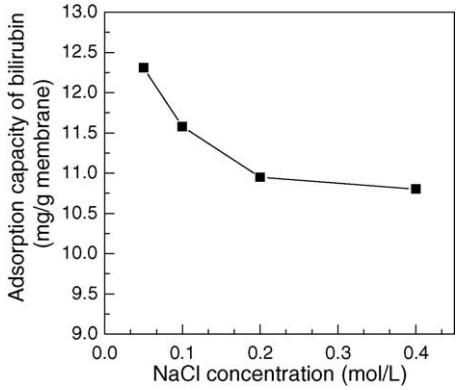

Fig. 5. Effect of NaCl concentration on the bilirubin adsorption capacities on PLL-attached membrane. Temperature: 37 °C; bilirubin solution volume: 5 mL; bilirubin initial concentration: 100 mg/L; membrane weight: 0.050 g.

after hydrophilic process in case of the PLL immobilization. The specific bilirubin adsorption increased with the increasing of bilirubin initial concentration at the given concentration range. The bilirubin adsorption capacities of the PLL-CS affinity membranes were greater than the literature data [12–14].

3.3. Effect of temperature

The bilirubin adsorption curves obtained at 25 and 37 °C are shown in Fig. 4. The amount of adsorbed bilirubin per unit amount of the sorbent increases with increasing temperature. In general, adsorption decreases as temperature increases, but in bilirubin case, it was different. One hypothesis is that a conformational change takes place in the bilirubin molecule [12,13,16,17]. 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 attached PLL molecules.

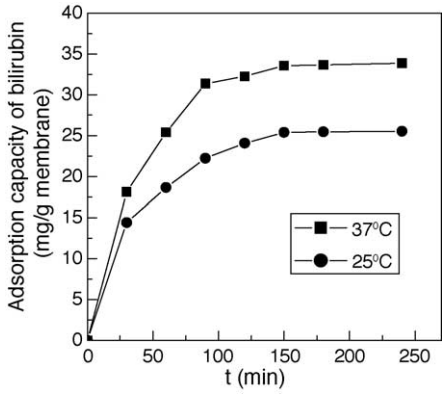

Fig. 4. Effect of temperature on the bilirubin adsorption capacities on PLL-attached membrane. Bilirubin solution volume: 20 mL; bilirubin initial concentration: 100 mg/L; membrane weight: 0.10 g.

3.4. Effect of ionic strength

The effect of the ionic strength on bilirubin adsorption is presented in Fig. 5, which shows that the adsorption capacity decreases with increasing NaCl concentration in bilirubin solution. The binding of bilirubin to PLL 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. When the NaCl concentration changes from 0.05 to 0.4 M, the adsorption of bilirubin decreases by 12.3%. The decrease in the adsorption capacity as the ionic strength increases can be attributed to weaken the electrostatic interaction between the PLL-attached membranes and bilirubin molecules.

3.5. Analysis of the adsorption mechanism

Freundlich adsorption isotherms are applied for the description of the adsorption mechanism for bilirubin on PLL-attached membrane. The isotherms can be described as follows:

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

where q^* is adsorption capacity (mg) of bilirubin at equilibrium; m is the weight of the membrane in grams; c^* is the equilibrium concentration of bilirubin; n and K are the physical constants of Freundlich adsorption isotherm.

Eq. (2) can be transformed into Eq. (3)

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

Fig. 6 shows the linear relationship of the Freundlich isotherm for the adsorption of bilirubin with PLL-attached membranes. This indicates that the adsorption mechanism is a monolayer adsorption.

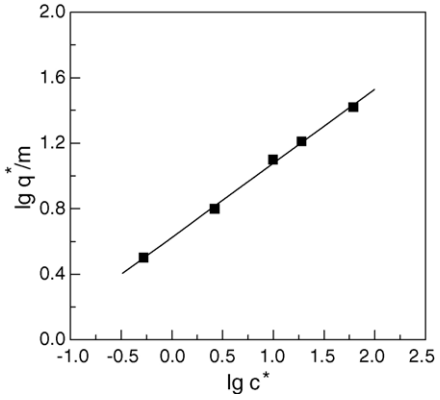

Fig. 6. Freundlich adsorption isotherm of PLL for bilirubin. Temperature: 37 °C; bilirubin solution volume: 5 mL; membrane weight: 0.050 g.

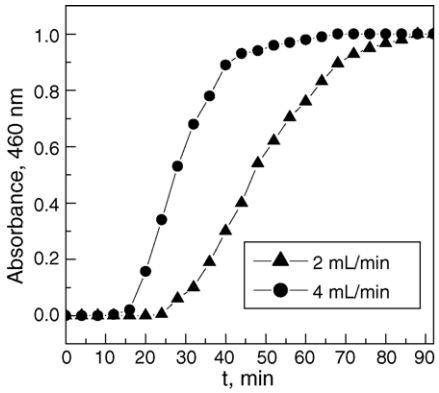

Fig. 8. Breakthrough curves at different flux. Temperature: 37 °C; bilirubin initial concentration: 200 mg/L in bilirubin–BSA solution; affinity stack: 10 membrane disks.

3.6. Effect of concentration of BSA

The unconjugated bilirubin binds with albumin in the human body. Albumin is the natural carrier of bilirubin in the blood. It is composed of 584 amino acid residues with a molecular weight of approximately 66,000. Each BSA molecule may have as many as 12 binding sites for bilirubin, but only two sites bind bilirubin molecules tightly [18,19]. For successful use in removal of bilirubin, a ligand should be capable of competing with at least the weak binding sites on albumin for the unconjugated bilirubin.

Adsorption experiments were performed by adding the PLL-attached membrane to previously prepared bilirubin solutions, with an initial concentration of 100 mg/L, containing various concentrations of BSA. The effect of concentration of BSA on bilirubin adsorption was shown in Fig. 7. The experimental results indicate that the adsorption capacity of the PLL-attached membrane for bilirubin was greatly influenced by the BSA concentration and the adsorption capacity for bilirubin decreases with an increase in BSA concentration. For BSA concentration greater than 20 g/L, the effect of BSA concentration on bilirubin adsorption is insignificant. Thus, PLL-attached membrane can well compete for those bilirubin molecules which are weakly bound to BSA.

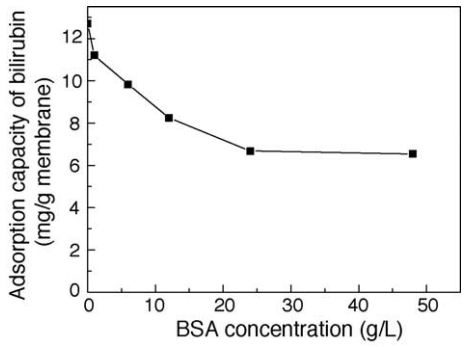

Fig. 7. The effect of BSA concentration on the adsorption capacities on PLL-attached membrane. Membrane weight: 0.050 g; temperature: 37 °C.

3.7. Effect of feed rate on adsorption

We adopted a 1:1 molar ratio of BSA bound bilirubin to observe the adsorption performance of the membranes. Bilirubin–BSA solution (200 mg bilirubin/L) was loaded onto the affinity stack of 10 membranes with 47 mm diameter in a single-pass mode at constant flow-rate. The effect of *flux* on the adsorption of bilirubin was studied and the breakthrough curves are shown in Fig. 8. From the figure, we notice that the breakthrough curves became sharper and equilibrium was reached faster with increasing the flux, but the amount of bilirubin adsorbed decreased. A reasonable explanation is that local equilibration can be achieved and ligands on the membrane surface and in pores can be utilized efficiently at low flux, while at high flux, the retarding time was so short that bilirubin had not enough time to make contact with the ligands and utilization ration of the ligands decreased. It also shows a high rate for bilirubin removal 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, and the ligands on the membranes could be made the most usage by recirculating the plasma with certain flow rates.

3.8. Regeneration and reuse of the membranes

The membranes have very preferable mechanical and chemical properties. Both BSA and NaOH were used to regenerate the membrane, and the regenerated membrane seems to have a comparative performance of adsorption capacity approximate to the PLL-attached membrane (Table 1). The bilirubin adsorption was still remaining a relatively high level. And the physical character of the membrane keeps nearly unchanged.

Table 1
Regeneration of the membrane and reutilization for bilirubin adsorption

	BSA regenerated membranes	Caustic alkaline regenerated membranes
Recovery of eluant (%)	85.3	
Adsorption capacity for bilirubin (mg/g membrane)	27.65	28.65
Reduction ratio of the adsorption capacity (%)	14.3	11.2

Membranes were obtained from the equilibrium adsorbed membranes at 37 °C in the batch experiments for effect of temperature; the adsorption temperature was 37 °C for the regenerated membranes.

4. Conclusions

Activated membranes for covalent immobilization of CS were obtained by reaction of microfilitration nylon membranes with bisoxirane. CS-coated nylon membranes were functionalized with polylysine by CDI activation. The affinity membranes were used to adsorb the bilirubin from the bilirubin–phosphate buffer solutions and bilirubin–albumin solutions. The batch experiments results showed that the mechanism of adsorption of bilirubin with PLL-attached membranes was a monolayer adsorption and adsorption capacity increased with increasing the temperature while decreased with increasing the NaCl concentration and albumin concentration. Breakthrough curves revealed that theses membranes can well remove the bilirubin from the bilirubin–albumin solutions and high flux was not suitable for affinity membrane chromatography. Dynamic experiments also indicated a higher bilirubin removal rate compared to the batch-wise experiments. Regeneration of the membrane suggested good mechanical and chemical stability.

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