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Synthesis and Characterization of Novel Adsorbents with Different Ligand Composition for Bilirubin Conjugated with Albumin

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Abstract: The chemical composition and conformation of ligand immobilized on bilirubin adsorbent have been proposed as the key factors to determine the affinity and specificity for bilirubin in patients' plasma. However, up to now, the effects of ligand composition on bilirubin removal are not clear. In this study, we selected human serum albumin (HSA), long carbolic chain and amino groups as the functional groups of ligand immobilized on Sepharose CL-4B to discuss the effects. The results showed that amino groups and hydrophobic carbonic chains could be very important factors to adsorb bilirubin conjugated with albumin. The contribution of HSA to the adsorption may be owing to its dimensional obstruction for macromolecules approaching, thereby nonspecific adsorption was decreased in plasma. The adsorption capacity for bilirubin increased obviously with the rise of initial concentration of bilirubin in plasma. The adsorption capacity was 7.52 mg/mL gel for bilirubin in albumin phosphate buffer (pH 7.0) with bilirubin concentration of 377 mg/L, while it only got to 3.64 mg/mL in human plasma at the same concentration. The adsorption

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microenvironment with pH 7.0–8.0, higher temperature (30–40°C) and lower ionic strength were beneficial to the bilirubin adsorption.

Keywords: Bilirubin removal, ligand, HSA, bilirubin albumin conjugates

INTRODUCTION

Bilirubin, a yellow-orange bile pigment, is formed as a result of the catabolism of hemoglobin from aged red blood cells. In normal case, the free bilirubin is transported to the liver as a complex with albumin where it is normally conjugated and excreted into bile (1). When some pathological changes happened in liver, for example, patients suffering from serious hepatitis, dysfunction in the metabolism of bilirubin will occur and may cause jaundice. High-concentration bilirubin deposits in tissues, especially in the brain, and then may cause permanent brain damage or death in more severe cases (2). Therefore it is very important to remove bilirubin directly from plasma of patients suffering from hyperbilirubinemia for saving patients' lives.

Up to now, there are many studies for bilirubin removal directly from plasma including hemoperfusion, which is a circulation of blood through an extracorporeal unit containing an adsorbent system for bilirubin. But in that treatment, because of the use of microbeads adsorbents, which leads to the difficulty of mass transfer and the long time for getting to equilibrium, the curative effect is not good.

The structure of bilirubin adsorbent includes mainly two parts: (1) ligand composed by functional groups; (2) support matrix. The chemical composition and conformation of ligand determine the affinity and specificity of adsorbent for object. However the effectiveness of ligand and physical characteristics of adsorbents are also markedly dependent on the framework of support matrix. In real clinical therapy, the complexity of plasma demands that adsorbents should have higher affinity and specificity, so the selection of functional groups is more important. During the 1980s and 1990s, human albumin (3), polylysine (4), oligo-peptide (5), Cibacron Blue F3GA (6), phenyl, etc., were immobilized, respectively, on different matrixes as functional groups to prepare bilirubin adsorbents. The results of those studies showed that the adsorbents modified by different functional groups can all remove bilirubin in certain extents, but the obvious adsorption action is only limited to free bilirubin from aqueous media. When the adsorbents were applied to patients' plasma, the adsorption capacity reduced remarkably.

In order to improve the characteristics of bilirubin adsorbent effectively, a proposal was put forward, which is synthesizing several bilirubin adsorbents with different ligand to realize the effects of ligand composition on the adsorption capacity and selectivity in detail. In this study, we selected Sepharose CL-4B as the support matrix on which the ligands with different functional groups such as amino carbonic chain and HSA were immobilized,

respectively. It is generally accepted that bilirubin exists in the serum in two forms: direct and indirect. The direct reacting type is thought to be bilirubin conjugated with glucuronic acid, rendering it water soluble, while the indirect type is bound to albumin (5, 7). It is reported that some adsorbents like activated carbon can remove free bilirubin only from the aqueous phase, and the removal efficiency is limited by the tight binding of bilirubin to albumin in plasma (8). Therefore bilirubin conjugated with albumin was selected as this study object. The effects of different functional groups and some experimental factors such as pH, temperature, and ionic strength on bilirubin adsorption were investigated by batch methods.

EXPERIMENTAL

Materials: Sepharose CL-4B was obtained from Amersham Pharmacia Biotech AB (Sweden). N,N′-Carbonyl diimidazole (CDI) was bought from Sigma. (St. Louis. MO). Diaminodipropylamine (DADPA) was obtained from Fluka AG (Switzerland). Bilirubin and human serum albumin (HSA) were prepared from a biochemical company (Shanghai, China). Human plasma was obtained from a blood bank (Dalian, China).

Apparatus: The concentrations of bilirubin and HSA were detected by 721-spectrophotometer (Shanghai, China). The bilirubin removal was carried out by incubation in a rotary-type shaker (Shanghai, China). V-60 UV spectrophotometer was also used to quantify HSA (Jusco, Japan).

Synthesis of Adsorbents

Bilirubin is a tetrapyrrole with two propionic side groups (Fig. 1). These form internal hydrogen bonds with the distal lactam groups, converting what should be a very polar water-soluble molecule into a folded and nearly insoluble molecule (9). According to the molecular structure of bilirubin, different functional groups were immobilized on matrix, respectively, thereby the bilirubin adsorbents with different ligands were synthesized (Fig. 2). Detailed synthesis methods are as follows. Firstly, Sepharose CL-4B (10 mL) was washed by acetone to remove free water, and then activated by 1 g CDI in acetone by

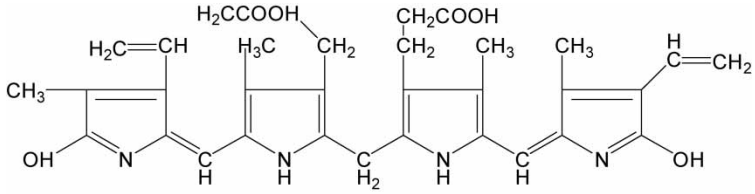

Figure 1. Molecular structure of bilirubin.

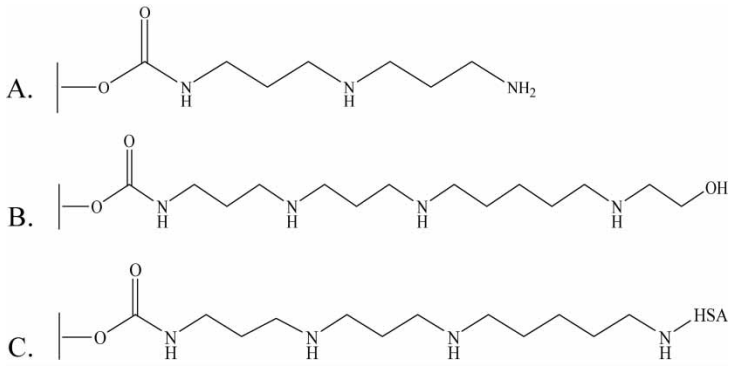

Figure 2. Ligand composition of three adsorbents. Adsorbent A: with two–H groups, one NH_2 group and six $-CH_2$ groups. Adsorbent B: with four –NH groups and twelve $-CH_2$ groups. Adsorbent C: with four –NH groups, eleven $-CH_2$ groups and HSA.

stirring at 37°C, 175 rpm for about 1 h. After the activation, the gel was washed by acetone again and was added into 10 mL acetone with 2 mL DADPA, stirring at 37°C, 175 rpm for 2 h, then adsorbent A was synthesized. Secondly, a certain amount of adsorbent A was taken and reacted with 2.5% glutaraldehyde in 0.1 mol/L borate buffer pH8.2 for 2 h. The gel was washed to remove the residual glutaraldehyde and then coupled with 2% amido-ethanol in phosphate buffer pH 7.0 at 37°C, 175 rpm for 4–5 h. The unstable Schiff bases produced in the reaction were deoxidized rapidly by sodium borohydride. Up to now, adsorbent B was prepared. Lastly, taking some asorbent A again, after the glutaraldehyde reaction, HSA was immobilized onto the adsorbent by adding HSA phosphate buffer pH 7.0, the residual activated groups on gel after HSA coupling were blocked by the addition of 2% amido-ethanol solution. The other conditions and steps were the same to the preparation of adsorbent B. The immobilized concetration of HSA was determinded by V-60 UV spectrophotometer at 280 nm. So by that means three adsorbents have the same density of functional groups and the following experimental results will be comparable.

Bilirubin Removal from Albumin Solution and Plasma

Preparation of bilirubin albumin solution: a certain amount of bilirubin (ranging between 100 mg/L and 500 mg/L) was dissolved in 1 mL dimethylsulfoxide (DMSO), and after the addition of 0.05 M Na_2CO_3 (2 mL), the solution was transferred to an ampoule. The solution was neutralized by the addition of 2 mL 0.1 M HCl. The 100 mL albumin solution contained different concentration HSA in phosphate buffer (0.15 M, pH 7.4) was prepared according to themolar ratio 1:1.5 between bilirubin and albumin

which can ensure the form of bilirubin albumin conjugates. The albumin solution was replenished to the above-mentioned 100 mL ampoule. Since bilirubin is destroyed easily by direct exposure to sunlight or any other ultraviolet source, all adsorption batch experiments were carried out in the dark. The bilirubin albumin solution was stored at 4°C and should be used within 3 days.

The preparation method of bilirubin plasma was similar to that of bilirubin albumin solution, only human plasma replaced the albumin solution.

Bilirubin removal experiment: 0.1 mL adsorbent was added into the prepared bilirubin albumin solution or plasma (4 mL), and then incubated at 37°C in a 175 rpm rotary-type shaker for 2 h. The adsorption capability was determined by the change value between the initial and residual amount of bilirubin in solution or plasma. The bilirubin concentration was quantified by the Van den Bergh (10) method: 0.25 mL diazoreagent was added to 0.1 mL bilirubin solution. After stabilizing for 10 min, the reddish-violet supernatant was assayed by spectrophotometer at 560 nm against a reagent blank.

Adsorption Capacities for Bilirubin and Albumin from Bilirubin Albumin Solution

We prepared 100 mL bilirubin albumin conjugates solution containing 300 mg/L bilirubin and 16.9 g/L HSA according to the before mentioned methods. The same amount (1 mL) of adsorbent B and C were added to 4 mL bilirubin albumin solution respectively, incubating at 37°C in a dark 175 rpm rotary-type shaker for 2 h. After separation in a centrifuge, the amounts of residual bilirubin and HSA were determined by Van den Bergh and Bradford method, respectively.

RESULTS AND DISCUSSION

In this study, we attempted to realize the contribution of different ligand composition to the adsorption capacity of adsorbent for bilirubin conjugated with albumin. According to the molecular structure of bilirubin, the hydrogen bond and hydrophobic action may be both conduce to enhance the adsorption capacity, so the compounds containing amino groups and long hydrophobic carbon chain were selected as functional groups. In addition, HSA is the affinity ligand of bilirubin in nature, so HSA is also introduced as a functional group.

Effects of Ligand Composition on Bilirubin Adsorption

Ligand composition is the chief factor that influences the adsorption characteristics of adsorbents. Figure 3 gives the adsorption capacity and time

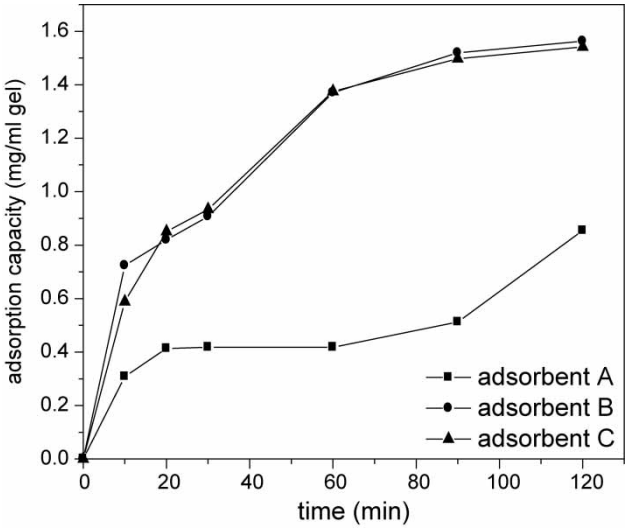

Figure 3. Effects of ligand composition on adsorption. Bilirubin initial concentration: 100 mg/L; temperature: 35°C; HSA density: 12.23 mg/mL gel; medium: bilirubin albumin phosphate solution, ionic strength 0.15 M, pH 7.4.

relationship curves of three absorbent with different ligand that are obtained at the initial concentration of 100 mg/L bilirubin albumin solution. It showed that the adsorption capacity for bilirubin of absorbent A was remarkably lower than that of adsorbent B or C, however, the adsorption ability of adsorbent B and C had no obvious difference. Compared with the structures of other adsorbents, the most distinct difference of adsorbent A is that it bound a shorter ligand chain. So this should be the primary reason for the difference of adsorption capacity among them. This result demonstrated that when the ligand chain is lengthened, the electric forces between $-NH_3^+$ on adsorbents and $-COO^-$ of bilirubin, hydrogen bonds, and hydrophobic characteristics all are strengthened. At the same time, the increasing of ligand chain length may afford more active sites so that an arm may adsorb more bilirubin molecules. However, when macromolecule HSA attached covalently at the terminal of the ligand chain, the adsorption capacity of adsorbent C is only almost equal to that of absorbent B. This result showed primarily that HSA didn't contribute to increase of adsorption capacity of absorbent for bilirubin conjugated with albumin in phosphate buffer.

Effects of HSA Immobilizing on Bilirubin Adsorption

In order to realize the effect of HSA on bilirubin adsorption in detail, a series of adsorbent C with different HSA density were prepared. Figure 4 gives the

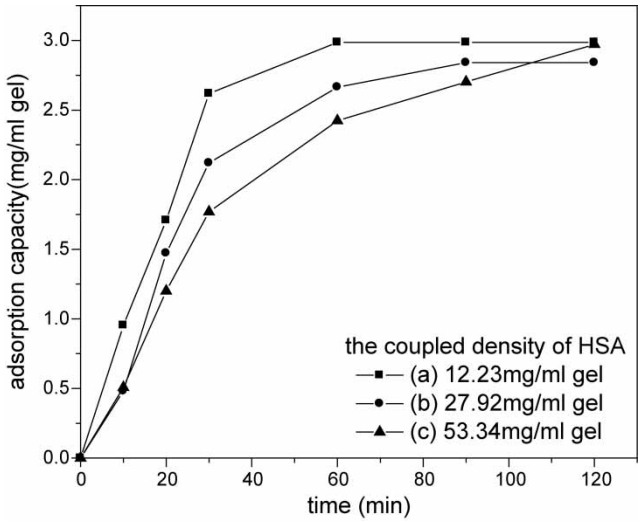

Figure 4. Effects of immobilized HSA density on adsorption. Bilirubin initial concentration: 350 mg/L; temperature: 35°C; medium: bilirubin albumin phosphate solution, ionic strength 0.15 M, pH 7.4.

adsorption relationship curves between the HSA density and the bilirubin adsorption capacity in bilirubin albumin solution. The data showed that the adsorption rate reduced obviously with increasing HSA density, although the ultimate adsorption capacities for bilirubin were almost a same value, the time that gets to adsorption equilibrium was postponed. The result further revealed that although HSA can conjugate with free bilirubin specifically, the adsorbent of coupled HSA can't adsorb the bilirubin conjugated with albumin effectively, and the adsorption action of absorbent C for bilirubin relies on the spacer molecules between the matrix and the HSA. It is speculated that HSA coupled may be leads to the dimensional obstruction between bilirubin albumin conjugates and the spacer thereby the adsorption rate slower.

A group of experiments were carried out to confirm the effects of ligand terminal HSA on weakening nonspecific adsorption for biomacromolecules in plasma ulteriorly. Figure 5 displays that in human plasma the adsorption capacity of adsorbent C for bilirubin is much higher than that of adsorbent B. From above results, the conjecture that seemed most feasible is that the existence of HSA decreased the partial nonspecific adsorption owing to its dimensional obstruction for macromolecules in plasma. However, another problem will be presented that in which form the bilirubin conjugated with albumin was adsorbed. The following experiment is arranged to understand this adsorption process.

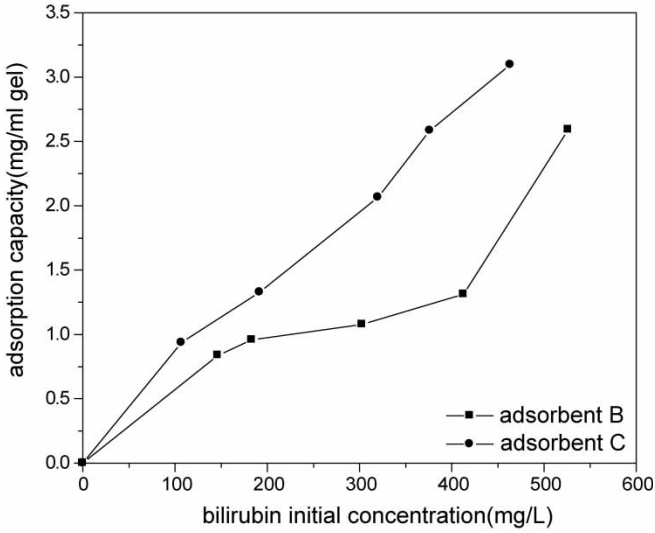

Figure 5. Compare of adsorption capability of adsorbent B and C for bilirubin from human plasma. Bilirubin initial concentration: 100–500 mg/L; temperature: 35°C; HSA density: 45.62 mg/ml gel; reaction time: 2.0 h.

Adsorption Mechanism of Adsorbent for Bilirubin Conjugated with Albumin

According to the correlative literature, each albumin molecule can bind two bilirubin molecules at least (11). It means that if the bilirubin is adsorbed in the form of bilirubin albumin conjugates, the adsorbed molar ratio between albumin and bilirubin should be not more than 1:2. In this study, the molar ratio between HSA and bilirubin was prepared as 1:2. From the Table 1, we can see that the adsorption capacity for albumin is 0.37 μmol/mL gel which is much lower than the adsorption capacity for bilirubin that is

Table 1. The adsorption capacities of adsorbent B and C for bilirubin and albumin from albumin solution bilirubin albumin solution containing 300 mg/L bilirubin and 16.9 g/L HSA (molar ratio of bilirubin to albumin 1:2), adsorbent C with HSA density of 12.23 mg/mL gel; temperature: 35°C; reaction time: 2 h

	Adsorption capacity for albumin		Adsorption capacity for bilirubin	
Adsorbent	mg/mL gel	μmol/mL gel	mg/mL gel	μmol/mL gel
B	24.40	0.37	6.52	11.13
C	8.40	0.13	7.19	12.27

11.13 μmol/mL gel to adsorbent B, and the similar thing happened to adsorbent C. By this token, whatever adsorbent B or C, the adsorbed molar ratio between albumin and bilirubin was far lower than 1:2, namely the bilirubin was not adsorbed in the form of bilirubin albumin conjugates. In fact, purified human serum albumin has a single high-affinity binding site for bilirubin with an affinity constant of 7×10^7 M, and two or more secondary sites binding constants of about 5×10^5 M at 37°C (12). Therefore we conjectured the adsorption process as follows. There should be a balance between free bilirubin and bilirubin albumin conjugates in solution. So when adsorbent was put into solution, free bilirubin was immediately adsorbed and then the equilibrium was broken. Free bilirubin was adsorbed continuously to the adsorbent until the new equilibrium attained once again. That is the synthesized adsorbent B or C should have higher affinity constants to bilirubin, so bilirubin was released from bilirubin albumin conjugates continually and was adsorbed.

From Table 1, we also observed that adsorbent B adsorbed much more albumin than that of adsorbent C, which verified further that the terminal HSA reduced the nonspecific adsorption indeed.

Effects of Initial Concentration of Bilirubin on Adsorption

Figure 6 showed the effect of initial concentration of bilirubin on the adsorption capacity of adsorbent C. The adsorbent immobilized with HSA was added

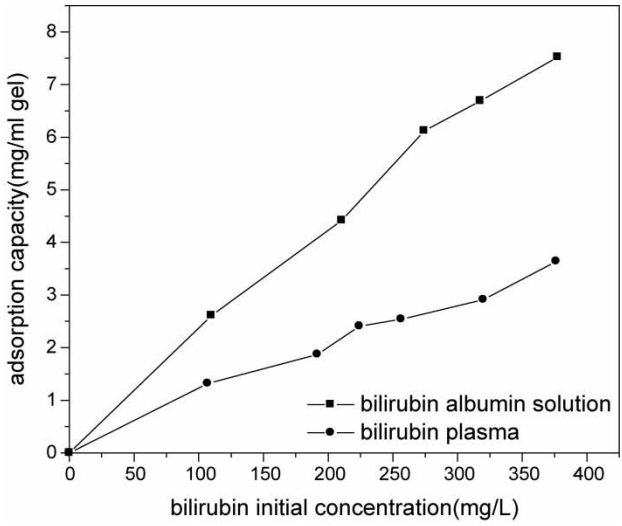

Figure 6. Effects of bilirubin initial concentration on adsorption capacity of absorbent C. Bilirubin initial concentration: 100–400 mg/L; temperature: 35°C; HSA density: 45.62 mg/mL gel; reaction time: 2.0 h.

respectively to bilirubin albumin solution and bilirubin plasma. The initial concentration ranged from 100 mg/L to 400 mg/L. The bilirubin albumin solution was prepared with phosphate buffer (0.15 M, pH7.4) in order to ensure the comparability of ionic strength and pH value with human plasma. The experimental results revealed that the adsorption capacity increases with increasing the initial concentration of bilirubin, and does not reach a plateau yet, at which we may assume that not all of the active sites are occupied by bilirubin molecules, and there are also available sites.

Note that the adsorption capacity can reach 7.52 mg/mL gel when the initial concentration of bilirubin is 377 mg/L in bilirubin albumin solution, while the adsorption capacity reduces to 3.64 mg/mL gel at the same concentration in real plasma. The phenomenon is probably conduced by the complexity of human plasma. Although the dimensional obstruction of HSA immobilized on the adsorption C can block the partial interaction between macromolecules and active sites, the existence of small hydrophobic molecules in plasma such as aroma amino acid and so on may still occupy a few active sites available for bilirubin adsorption, so that the adsorption capability is reduced.

Effects of Microenvironment on Adsorption Capacity

Bilirubin is removed mainly through electric forces between $-NH_3^+$ on the arm and $-COOH$ of bilirubin as well as hydrophobic arm, so when the factors affecting the electric forces and hydrophobic interaction such as ionic strength, pH and temperature of adsorption system are changed, then the adsorption is affected simultaneously (Figs. 7, 8, and 9).

The effect of ionic strength on bilirubin adsorption was studied using four different bilirubin albumin solutions containing 0.05 mol/L, 0.10 mol/L, 0.15 mol/L and 0.20 mol/L NaCl, respectively. Figure 7 showed that the adsorption capacity cuts down with an increasing in ionic strength. Actually, the average ionic strength of human blood is about 0.15 mol/L, and under this ionic strength the adsorption capacity is 63% of the capacity in 0.05 mg/L ionic strength. The probable reason is that the carboxyl groups of bilirubin are electronegative, which can interact with anti-ions around it in solution to form an 'ionic atmosphere'. With the increasing of ionic strength, the concentration of ionic atmosphere also goes up, which will weaken or destroy the interaction between bilirubin and adsorbents, so the adsorption capacity decreases.

The bilirubin albumin solutions with different pH (6.5, 7.0, 7.5, 8.0, and 9.0) were examined. Figure 8 demonstrated that the adsorption capacity ascended in the beginning at lower pH and a maximum adsorption capacity was obtained at about pH7.0, but it descended subsequently at upper pH. Under the circumstance of acidic or alkali solution, the adsorption capacity decreased both a little.

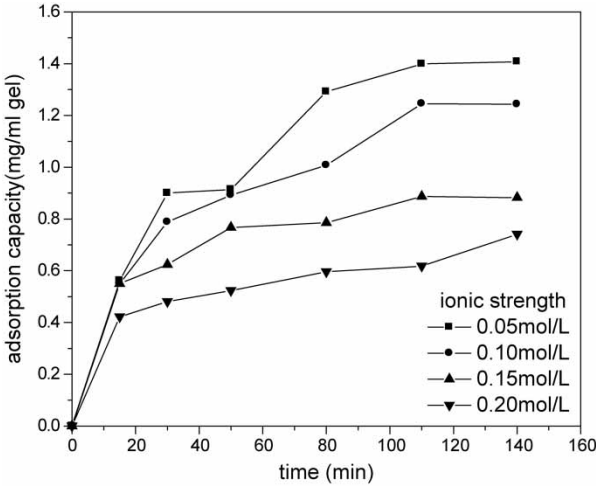

Figure 7. Effects of ionic strength on adsorption. Bilirubin initial concentration: 120 mg/L; temperature: 35°C; HSA density: 12.23 mg/mL gel; medium: bilirubin albumin phosphate solution, ionic strength 0.15 M, pH 7.4; reaction time: 2.0 h.

We have also checked the effect of temperature on bilirubin adsorption. The bilirubin adsorption curves that gained at temperatures 37°C and 25°C, respectively, are shown in Fig. 9. The adsorption capacity for bilirubin increased with increasing temperature. The similar phenomenon also was found in Denali's experiment (13). In general, it is known that adsorption

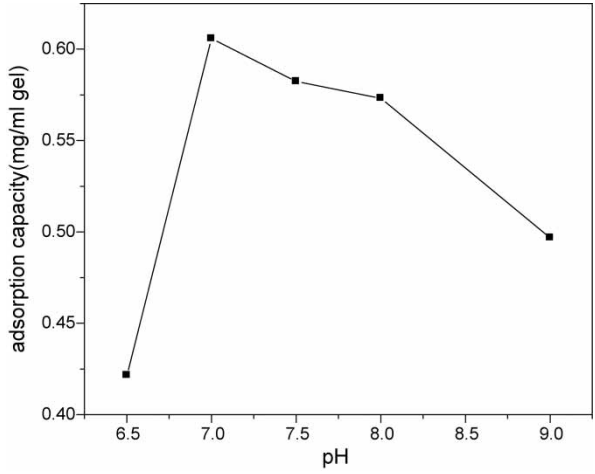

Figure 8. Effects of pH on adsorption. Bilirubin initial concentration: 60 mg/L; temperature: 35°C; HSA density: 12.23 mg/mL gel; medium: bilirubin albumin phosphate solution, ionic strength 0.15 M, pH 7.4; reaction time: 2.0 h.

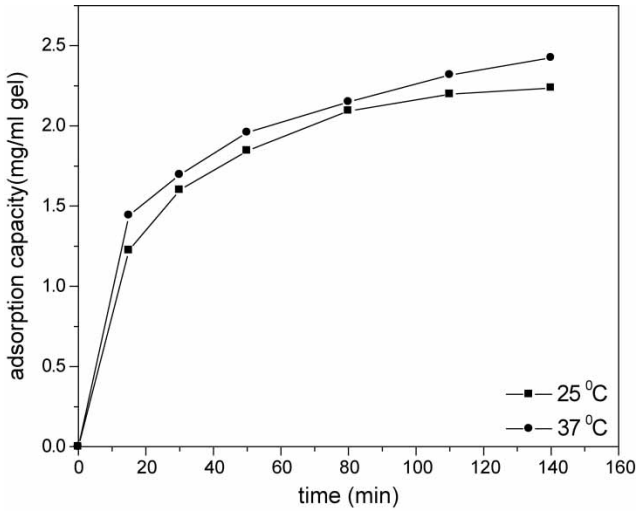

Figure 9. Effects of temperature on adsorption. Bilirubin initial concentration: 150 mg/L; HSA density: 12.23 mg/mL gel; medium: bilirubin albumin phosphate solution, ionic strength 0.15 M, pH 7.4; reaction time: 2.0 h.

capacity decreases with increasing temperature because adsorption is an exothermic process. In fact, it is a typical characteristic of hydrophobic adsorption that the adsorption capacity increases with increasing temperature. So this phenomenon further verified that the hydrophobic chains play a certain role in the adsorbing process.

CONCLUSION

This study demonstrated that ligand composition affected obviously the adsorption capacity and selectivity of absorbent for bilirubin conjugated with albumin from plasma. The amino groups and hydrophobic carbonic chain of ligand could be decisive factors to adsorb bilirubin conjugated with albumin. The HSA coupled at the terminal of ligand chain reduced the nonspecific adsorption for macromolecules and thereby enhanced the selectivity of absorbent in human plasma. The experiment results will give a very important reference to improve further the property of bilirubin adsorbent.

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