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Bilirubin removal from human plasma by Cibacron Blue F3GA using immobilized microporous affinity membranous capillary method

Lei Zhang, Gu Jin *

Department of Chemistry, University of Science and Technology of China, Hefei 230026, PR China

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Abstract

A novel affinity sorbent system for direct bilirubin removal from human plasma was developed. These new adsorbents comprise Cibacron Blue F3GA as the specific ligand, and microporous membranous poly(tetrafluoroethylene) capillary (modified by coating with a hydrophilic layer of poly(vinyl alcohol) after activation) as the carrier matrix. The affinity adsorbents carrying 126.5 μmol Cibacron Blue F3GA/g polymer was then used to remove bilirubin in a flow-injection system. Non-specific adsorption on the poly(vinyl alcohol) coated capillary remains low, and higher affinity adsorption capacity, of up to 76.2 mg/g polymer was obtained after dye immobilization. The bilirubin adsorption capacity of the affinity capillary decreased with increase in the recirculation rate of plasma. The adsorption capacity increased with increase the temperature while decreased with increase the ionic strength. The maximum adsorption was only observed in neutral solution (pH 6–7). The adsorption isotherm fitted the Langmuir model well. These new adsorbents have higher velocity of mass transfer, better adsorption capacity, less fouling, longer service life and good reusability. The results of blood tests suggested the dye affinity capillary has good blood compatibility.

Keywords: Bilirubin removal; Cibacron Blue F3GA; Microporous membranous capillary; Bilirubin; Blood compatibility

1. Introduction

Bilirubin (BR, Fig. 1) is a tetrapyrrole dicarboxylic acid formed in the normal metabolism of heme proteins in senescent red blood cells, and is normally conjugated with albumin to form a water-soluble complex [1,2]. As we all know, free bilirubin is a kind of lipophilic endotoxin. High concentration of free bilirubin can result in hyperbilirubinemia, especially among newborn infants, and may cause jaundice, hepatic or biliary tract dysfunction and permanent brain damage [3,4].

Many techniques have been used for the bilirubin removal from plasma of patients suffering from hyperbilirubinemia. Phototherapy is one of the most commonly used treatments for mild cases [5]. However, severe cases must be treated by more drastic methods, such as hemodialysis and hemoperfusion. Now, hemoperfusion, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for bilirubin, has become the most promising technique [3,6]. Successful hemoperfusion requires the adsorbents to be specific; have high adsorption capacity and good blood compatibility; not be poisonous. Affinity chromatography is an effective and widely used method for purification and separation of biomolecules, and is based on highly specific molecular recognition. In recent years, it has developed into a powerful tool for the removal of toxins from human plasma [7]. In this technique, a ligand having specific recognition capability is immobilized on a suitable insoluble supporting matrix (or carrier). A wide variety of functional molecules may be used as ligands, which include enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, and oligonucleotides. These ligands are extremely specific in most cases. However, they are expensive, and it is difficult to maintain their original biological activity while immobilizing certain ligands on the supporting matrix. There are many precautions required in their use and storage,

* Corresponding author.
E-mail address: gjin@ustc.edu.cn (G. Jin).

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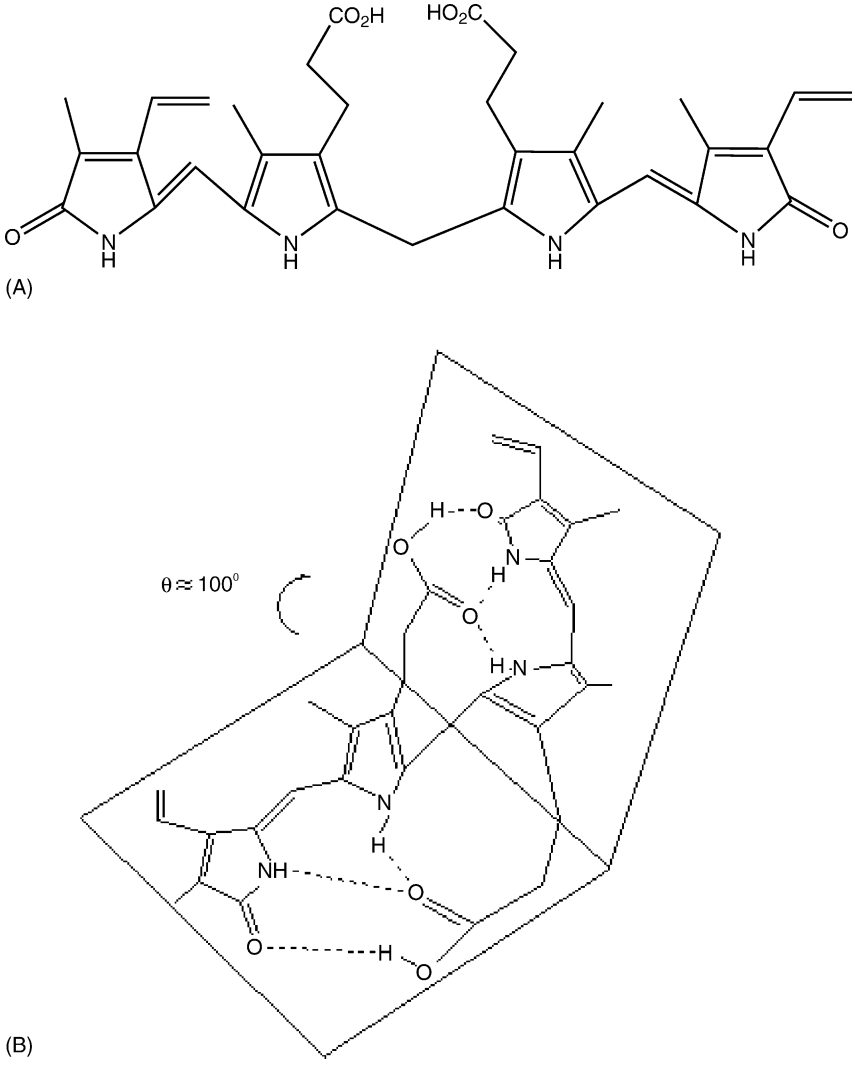

Fig. 1. Chemical Structure of bilirubin: (A) linear (B) ridge-title with intramolecular hydrogen bonding.

too. Although reactive dyes are all synthetic in nature, they are still classified as affinity ligands because they are commercially available, inexpensive, and can be easily immobilized [8]. Starting from this point, we selected Cibacron Blue F3GA (CB F3GA) as the affinity ligand in study.

In affinity system, the supporting matrix is usually polymeric material in bead or membrane form. Rad et al. developed magnetic poly(2-hydroxyethyl methacrylate) beads as adsorbents [9]. Zhen et al. used cross-linked β-cyclodextrin polymer microbeads for bilirubin adsorption from aqueous solutions [10]. Brown prepared oligopeptide functionalized polyacrylamide beads as affinity sorbent system for bilirubin removal [11]. Avramescu et al. employed ethylene vinyl alcohol adsorptive membranes with bovine serum albumin as bioligand for affinity supports for bilirubin retention [7]. Wei et al. used quaternary ammonium salt immobilized cellulose membranes for bilirubin removal [12]. Shi employed polylysine immobilized affinity nylon membrane for bilirubin adsorption [13]. But the bead adsorbents have a number of drawbacks, such as the compressibility of the column packaging materials (i.e. beads), the fouling, and particularly the slow flow rate through the packed column [14]. In order to solve these problems the bead diameter has been reduced, but such carriers require complicated packing procedure and exorbitant high pressure equipments, which may bring difficulty in mass-transfer and prolong the therapeutic time in hemoperfusion. So using of bead adsorbents in clinical application is limited [15]. In contrast to bead adsorbents, membrane adsorbents bring the solute close proximity to bound ligands through convective transport, which can reduce the mass-transfer resistance and enable lower pressure drops and higher flow rates [16]. However, membrane adsorbents also have their own serious disadvantages. Usually, the affinity hollow fiber membranes are packed in housing. Compared to bead adsorbents, hollow fiber membranes are inefficiently packed because of a low membrane packing density. This leads to very large ‘interstitial’ volumes and creates large internal mixing volumes. Although hollow fiber membranes

permit very high flow and capture rates, using of buffers and eluates is inefficient. Due to its rapid development, flat sheet affinity membranes have become the first consideration for efficient packing. But stacked sheet designs increase pressure drops which lessens the advantage of membrane devices compared to bead columns [17]. Besides, stacked sheet membrane devices have to maintain a large membrane volume for fast perfusion, so some auxiliary equipment is required to equal distribute solution into membrane, and to minimize the interstitial volume that may cause mixing of solutions.

In recent studies, we focus on the preparation and application of a new form of adsorbent in order to overcome the shortcomings mentioned above. The present work developed a novel affinity sorbents for direct bilirubin removal from human plasma. These new adsorbents comprise Cibacron Blue F3GA as the specific ligand, and microporous membranous poly(tetrafluoroethylene) (PTFE) capillary (MPTFE capillary) as the carrier matrix. In order to prevent non-specific interactions between the hydrophobic MPTFE surface and protein or bilirubin molecules, and also to attach the ligand (i.e., Cibacron Blue F3GA) to the matrix, these capillaries are coated with a hydrophilic layer of poly(vinyl alcohol) (PVA) after activation. And the performance of the affinity MPTFE capillary in human blood was evaluated by blood compatibility tests. These new adsorbents have the advantage of both membrane and micro-column, and have higher velocity of mass transfer, better adsorption capacity, less fouling, longer service life and can be connected to recirculation flow system directly without any auxiliary equipment. The similar method has not been reported before.

2. Experimental

2.1. Chemicals and apparatus

Microporous membranous PTFE capillaries provided by Professor K. Watanabe, were used for the present study. These capillaries have an internal diameter of 2 mm, a wall thickness of approximately 0.5 mm and an average surface aperture of 1 μm. Scanning electron microscopy of MPTFE capillary inner walls has been presented elsewhere [18]. PVA (average M_r 14,000, 100% hydrolysed) was purchased from the chemical reagent company of Shanghai, China. Cibacron Blue F3GA, HSA (human serum albumin) and BSA (bovine serum albumin) were purchased from Sigma. The blood samples having different bilirubin initial concentrations were obtained from FeiDong People Hospital (Hefei, China). Reagents such as terephthaldehyde, sodium chloride, sodium carbonate, hydrochloride acid, etc. are all analytical reagent grade. The fresh piranha solution is composed of concentrated sulfuric acid and hydrogen peroxide (1:1, v/v).

A flow injection system (Model FI-2100, Beijing Haiguang Instrument Co., China) was used for the feeding of human plasma. The concentration of bilirubin and albumin in the plasma samples were determined by 7060 automated analyzer (HITACHI, Japan). BPC (Blood Platelet count) was determined by ABBOTT CELL-DYN 1600 Hematology Analyzer (ABBOTT, USA).

2.2. Preparation of affinity MPTFE capillaries

2.2.1. Activation of the unmodified MPTFE capillaries

MPTFE capillaries were firstly activated by fresh piranha solution [19]. The capillaries were incubated in a solution of 20 ml fresh piranha solution at room temperature for 40 min. After that, the capillaries were washed several times with distilled water.

2.2.2. Coating of MPTFE capillaries with PVA

MPTFE capillaries were coated with PVA by a two-step procedure. In the first step, PVA was deposited on the surface of capillary by a simple adsorption process carried out in an aqueous medium. The initial PVA concentration was 40 mg/ml. The solution (MPTFE capillaries added) was stirred for 2 h with a magnetic stirrer at 200 rpm at room temperature.

In the second step, PVA molecules adsorbed on the MPTFE capillaries were chemically cross- linked to give a stable PVA coating on the surface. After adsorption of PVA from solution, the final acid concentration of the medium was adjusted to 0.1 M by adding HCl. A 10-mg amount of terephthaldehyde was dissolved in 10 ml of water and this solution was added to the previous medium. Then stop stirring the medium and increase the temperature to 80 °C. Cross-linking was completed in 2 h. The capillaries were washed several times with hot distilled water. The PVA-coated capillary was stored in distilled water.

2.2.3. Immobilization of Cibacron Blue F3GA

CB F3GA was immobilized onto the capillaries by the methods of Xia and Zhang [20]. Briefly, a 0.6 g amount of CB F3GA was dissolved in 20 ml of water, and then the PVA-coated capillaries were immersed in the dye solution for 60 min at 60 °C after which 1 g of NaCl was added. 30 min later, 0.5 g of Na_2CO_3 were added to adjust the pH value of the (to about pH 10) solution. The reaction then took place in the following 4 h. The capillaries were washed with distilled water and methanol several times until all the unbound dye was removed. The dye immobilized capillary was stored in phosphate solutions (pH 7.0) containing 0.02 wt.% sodium azide at 4 °C to prevent microbial contamination.

The dye content of the capillary was determined spectrophotometrically by first hydrolyzing the capillaries in 12 M hydrochloric acid aqueous solution, at 80 °C, for 30 min. The solution was then diluted to 6 M with distilled water, and neutralized with 6 M NaOH aqueous solution. Then the concentration was determined spectrophotometrically at its maximum absorbance wavelength (λ_{max} = 610 nm) [21]. The leakage of the CB F3GA from the capillaries was followed by treating the capillaries with fresh human plasma samples for 24 h at room temperature. CB F3GA released after this

treatment was measured in the liquid phase spectrophotometrically at 630 nm.

2.3. Bilirubin removal from human plasma

The adsorption studies were carried out in a flow injection system (Fig. 2). The affinity MPTFE capillary was connected to the flow injection system directly without any auxiliary equipment. Because the modified capillary was still soft, we added a bushing for protection of the capillary. And the bushing also played a role of rivet for immobilization of the capillary. Recirculation of human plasma was achieved by changing rotation direction of pump. The affinity capillary was equipped with a water bath apparatus for temperature control. This system was controlled by computer, so automatic operation could be achieved.

Since bilirubin will be destroyed by exposure to direct sunlight or any other source of ultraviolet light, including fluorescent light, all adsorption experiments were carried out in a dark room. The amounts of bilirubin removed from the human plasma (or adsorption capacity) were described by the following equation:

$$T = \frac{(C_0 - C)V}{m} \quad (1)$$

where T is the amount of bilirubin adsorbed onto unit mass of the capillary (mg/g polymer); C_0 and C are the concentrations of the bilirubin in the initial (before adsorption) and in the final solution (after adsorption) (mg/l), respectively; V is the volume of the human plasma; and m is the mass of the capillary (g). The concentration of bilirubin and albumin in the plasma samples were determined by using HITACHI 7060 automated analyzer.

In a typical flow injection system, 50 ml of the plasma was recirculated through the modified capillary for 2 h. In the first group of experiments, the flow rate of the plasma (bilirubin content of 212 mg/l) varied between 0.5 and 2.5 ml/min. The temperature was kept constant at 25 °C.

The second group of experiments, bilirubin adsorption from human plasma containing 212 mg/l was studied at different temperatures (4, 25 and 37 °C), pH values (3–9.5) and ionic strengths (adjusted by adding NaCl to the plasma). Adsorption rates were obtained both in the continuous recirculation flow injection system. Human plasma samples containing different amounts of bilirubin were used in these experiments. Changes of the bilirubin concentration with time were followed to obtain the adsorption rate curves. The flow rate was 1 ml/min. These studies were performed at a constant temperature of 25 °C.

In the last group, in order to observing the interrelation between albumin and bilirubin adsorptions, bilirubin adsorption from human plasma containing different amounts of human serum albumin (38.9–49.2 mg/ml, adjusted by adding HSA to the plasma) was studied at 25 °C.

2.4. Regeneration of the modified capillary

The bilirubin-saturated capillary was regenerated by using BSA and sodium hydroxide. The bilirubin-saturated capillary was eluted by recirculating the BSA (300 mg/l) solution. Then the absorbed BSA on the capillary was eluted with 5 M NaSCN eluant, and the capillary was regenerated subsequently by using 1% Tween 80 solution and distilled water. The elution process by the alkaline solution included immersing the bilirubin absorbed capillary in 0.1 M NaOH aqueous solution, followed by the procedure of washing with large volume of distilled water and phosphate buffer (pH 7.4). And we also used a method combining the above two. The regenerated capillary was then reused for bilirubin adsorption.

2.5. Blood compatibility

2.5.1. Plasma recalcification time (PRT)

The human whole blood containing 10% ACD (acid–citrate–dextrose) was centrifuged at 3000 rpm for 10 min to separate the blood cells, and the remaining platelet-poor plasma (PPP) was used for the PRT experiments [22]. The PPP (0.3 ml) was placed on the sample attached to a round watch glass, preswelled with distilled water (2 ml), and incubated statically at 37 °C. A 0.025 M $CaCl_2$ aqueous solution (0.3 ml) was then added to the PPP and the plasma solution was monitored for clotting by manually dipping a stainless-

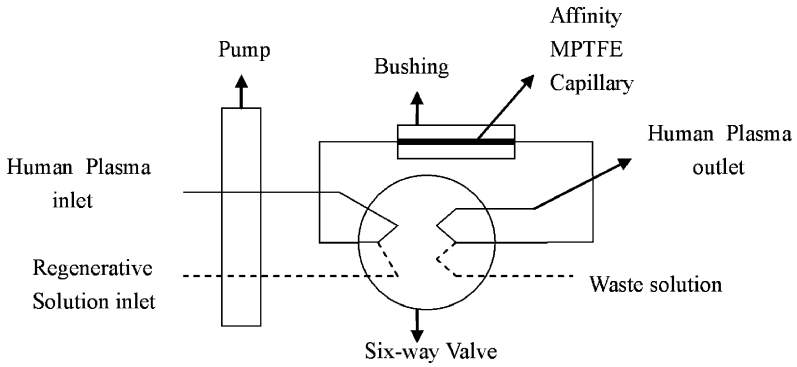

Fig. 2. Flow injection system for bilirubin removal.

steel wire hook coated with silicone into the solution to detect fibrin threads. Clotting times were recorded at the first signs of any fibrin formation on the hook. The experiment was repeated six times and a mean value was calculated.

2.5.2. Analysis of hemolysis

The analysis of hemolysis was performed by using the unmodified capillary, the PVA coated capillary and the dye-affinity capillary. Hemolytic activity was assessed by determining hemoglobin release under static conditions using the two-phase ISO/TR 7405–1984 (f) hemolysis test [23]. Blood testing solution was prepared by using 4 ml fresh human whole blood with an ACD medium and was diluted with 5 ml of 0.9% saline. In the first phase, each sample was incubated for 30 min in pure saline and then diluted fresh human whole blood was added and incubation went on for another 60 min. In the second phase, the immersion liquid was centrifuged at 750 rpm for 5 min and the topical density of the supernatant was read by a spectrophotometer. The positive reference (100% lysis) was blood/water mixture and the negative reference (0% lysis) a blood/ saline mixture.

2.5.3. Platelet adhesion test

The platelet-rich plasma (PRP) was prepared by centrifugation (1200 rpm, 15 min) of citrated human whole blood donated from healthy volunteers. It was a mixture of 3.0 ml of a 3.8 wt.% aqueous sodium citrate solution and 27 ml of fresh blood. The PRP (0.7 ml) was kept in contact with the capillary for 60 min at 37 °C. After that, the BPC (blood platelet count) of plasma was determined by ABBOTT CELL-DYN 1600 Hematology Analyzer.

3. Results and discussion

3.1. Preparation of Affinity Capillaries

In this study, we attempted to prepare a novel sorbent for bilirubin removal from human plasma with hyperbilirubinemia. CB F3GA was used as the affinity ligand for specific binding of bilirubin molecules. MPTFE capillary was selected as the carrier matrix. MPTFE capillary is a new type of functional material, which has good chemical and mechanical stability, fairly large pore size on surface and large porosity as described in our previous papers [18]. MPTFE capillary has been used in the field of analysis [18,24,25].

As we know, PTFE is an extremely inert and hydrophobic matrix, which has poor blood compatibility and is not suitable for cell growth, so it must be modified before used in field of biological or medical treatment. Modification of the MPTFE capillary is a very difficult job, not only because of its chemical stability, but also because of the shape of capillary. At present, the modification of PTFE has been performed by methods of irradiation (i.e., γ-radial, laser and plasma) [26–28], but these methods need special equipment and destroy the surface structure. Now, wet chemical methods can result in a sufficient number of free functional groups without damaging the structural integrity of the PTFE polymer. LÖhbach first modified PTFE vessels with fresh piranha solution [19]. Watanabe modified the PTFE capillary with NaOH solution [24,25]. However, these wet chemical methods are not sufficient because the surface of PTFE remains hydrophobic and surface density of the active site is quite low.

In this paper, the PVA was coupled to the surface of PTFE for the first time, which can be an intermediate for further attachment to an adhesion molecule. After this, CB F3GA was covalently coupled to the MPTFE capillary via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the PVA which was coated on the capillary under alkaline conditions. Because few contaminants will be immobilized on the supporting matrix and proper washing of the matrix can remove absorbed contaminants, purification of the dye before immobilization is not necessary [29]. The average density of CB F3GA attached on the capillary is 126.5 μmol/g polymer. CB F3GA leakage was investigated in human plasma. CB F3GA leakage was not apparently observed from any of the dye-attached capillary even after a long period of time (more than 1 month). This suggested CB F3GA attached PVA coating should be quite firm.

These new adsorbents have the advantage of both membrane and micro-column, and have higher velocity of mass transfer, better adsorption capacity, less fouling, longer service life. And this system is inexpensive and easy to operate.

3.2. Capillary performance

3.2.1. Effects of flow-rate on adsorption

In these experiments, the volumetric recirculation rate of the human plasma varied between 0.5 and 2.5 ml/min; other parameters were kept constant. The adsorption capacities at different flow-rates are given in Fig. 3.

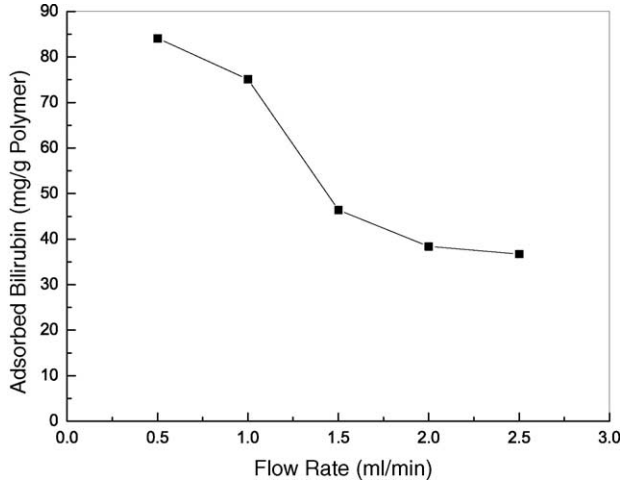

Fig. 3. Bilirubin adsorption at different flow-rates. Bilirubin initial concentration: 212 mg/l; temperature: 25 °C; and total plasma volume: 50 ml.

With the increase of the flow-rate from 0.5 to 2.5 ml/min, the adsorption capacity decreased significantly from 84.1 to 36.7 mg bilirubin /g polymer. This change may be due to the decrease of residence time in the capillary, which does not give enough time for the bilirubin molecules to interact with the sorbent. Thus low adsorption capacities were observed at high flow-rates. When flow-rates were lower than 1.0 ml/min we encountered some technical problems in our experiment apparatus; therefore we carried out all other adsorption tests at a flow-rate of 1.0 ml/min.

3.2.2. *Effects of temperature on adsorption*

In these experiments, bilirubin adsorption studies were performed at 4, 25 or 37 °C. The effect of temperature on the adsorption of bilirubin by CB F3GA-attached capillary is shown in Fig. 4. As seen here, adsorption capacity of bilirubin of the sorbent increased with increasing temperature. We obtained the maximum bilirubin adsorption (94.5 mg bilirubin/g polymer) at 37 °C. Since this is human body temperature, we did not try to work at higher temperatures.

The result was similar to that of bead sorbents or membrane sorbents [3,7,9,10,12,13,20]. It is well known that adsorption is an exothermic process, so adsorption capacity usually decreases with increasing temperature. A hypothesis for the phenomenon in our experiments is that a conformational change takes place in the bilirubin molecule. 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 CB F3GA molecules [3,6,20]. Another possible reason is as follows: In this conformation (Fig. 1B) intramolecular hydrogen bond makes bilirubin molecule itself wrapped with hydrophobic groups [30], so bilirubin adopts the shape of a half-opened book or a ridge-title. However, the adsorption process needs an effective dissolution and diffusion process from the bulk solution to the solid phase (i.e. the sorbent). As we known, the hydrogen bonds are weaker at higher temperature. With the increase of temperature the hydrogen bonds in the molecular structure became weaker and the solubility of bilirubin increased, which resulted in the increasing of binding capacity on the capillary.

3.2.3. *Adsorption rate*

Fig. 5 gives the adsorption rate curves which were obtained by changing the concentration of bilirubin in the plasma with time in the flow injection system. As seen here, adsorption equilibrium is achieved in about 50 min. Adsorption rates increased with increasing bilirubin concentration. The reason for this result may be that the higher the bilirubin concentration is, the higher driving force bilirubin concentration difference between the human plasma and the solid (i.e., the capillary) phases brings.

3.2.4. *Effects of pH*

Fig. 6 provided the effects of pH on bilirubin adsorption. The adsorption capacity increased firstly under acidic conditions and then decreased under alkaline conditions with the increase of pH. In acidic solution bilirubin is polar because its amino groups are protonated but in basic solution acidic groups of bilirubin are strongly dissociated and this compound is again polar. Only in neutral solution (pH 6–7) bilirubin has the smallest polarity and has the biggest affinity to the dye-immobilized capillary. And with the increase of pH, the hydrogen bonds in the molecular structure were destroyed gradually and the solubility of bilirubin increased gradually, which resulted in the increase of binding capacity on the capillary. In addition, this phenomenon also can be explained by electrostatic interactions: both bilirubin and dye molecules tend to be positively charged in acidic solution and to be negatively charged in basic solution, so a strong electrostatic repulsion effects occurred between bilirubin and dye molecules, which resulted in low adsorption capacity in strong acidic or basic solution. But only

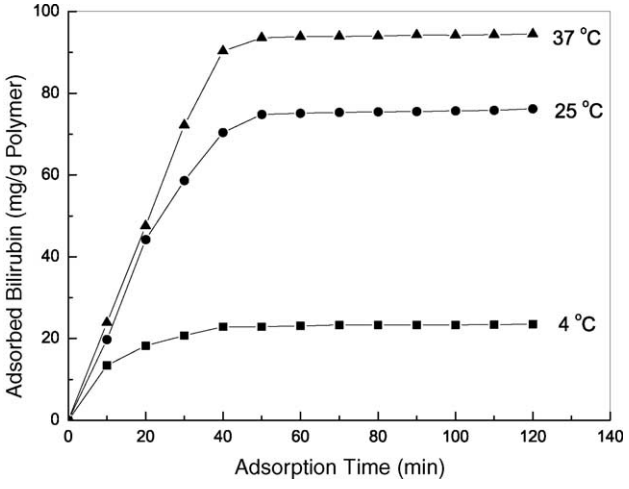

Fig. 4. Effects of time on bilirubin adsorption at different temperatures. Bilirubin initial concentration: 212 mg/l; flow-rate: 1.0 ml/min; and total plasma volume: 50 ml.

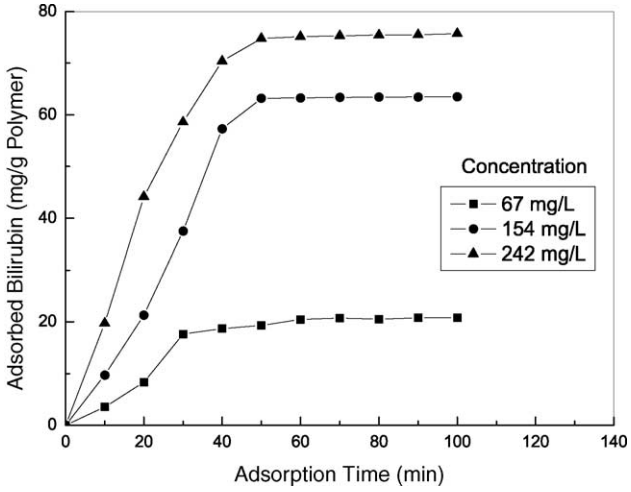

Fig. 5. Effects of time on bilirubin adsorption at different concentrations. Flow-rate: 1.0 ml/min; temperature: 25 °C; and total plasma volume: 50 ml.

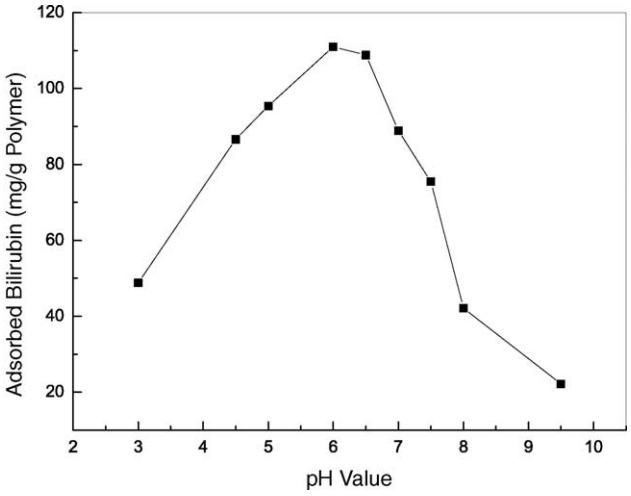

Fig. 6. Effects of pH on bilirubin adsorption. Flow rate: 1.0 ml/min; bilirubin initial concentration: 192 mg/l; temperature: 25 °C; and total plasma volume: 50 ml.

very weak electrostatic interactions exist in neutral solution [31].

3.2.5. *Effects of ionic strength*

The effect of the ionic strength on bilirubin adsorption was presented in Fig. 7. When the salt concentration changes from 0.05 to 0.5 M, the adsorption capacity decreases by about 15.8 % for MPTFE capillaries. This decrease inclines to have a notable influence in high salinity solutions. It may be because the negative carboxyl ion of bilirubin in the experimental conditions could absorb antiparticles around it to form an "ionic atmosphere". At the same time, the dye immobilized capillary was also negatively charged in the solution, which was also sources for "ionic atmosphere" [32]. The existence of ionic atmosphere can weaken or destroy the interactions between bilirubin and the adsorbent and it can also decrease the effective concentration of bilirubin in solution. The increase of the salinity in the solution intensified this process. Therefore, under the condition of high ionic strength the interactions between bilirubin and the adsorbent were interfered which led to a decrease of bilirubin adsorption on the affinity capillary.

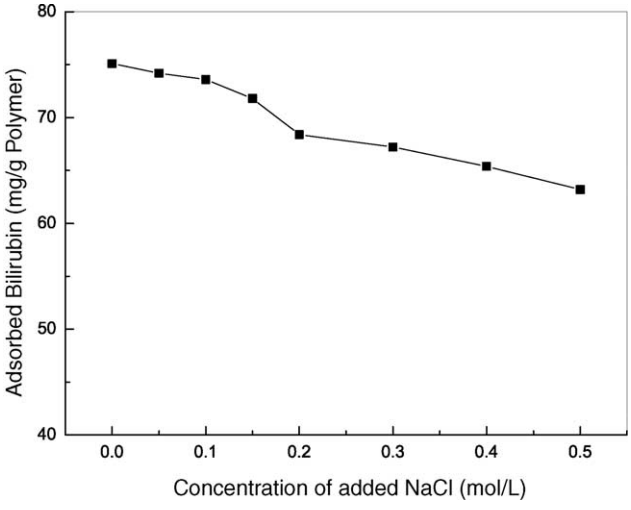

Fig. 7. Effects of ionic strength on bilirubin adsorption. Flow rate: 1.0 ml/min; bilirubin initial concentration: 192 mg/l; temperature: 25 °C; and total plasma volume: 50 ml.

3.2.6. *Adsorption capacity*

Fig. 8 showed the non-specific and specific adsorption of bilirubin onto the PVA coated and dye immobilized capillary.

The amount of bilirubin adsorption on the unmodified capillary was quite low (about 0.34 mg bilirubin/g polymer), while much higher adsorption values (up to 76.2 mg bilirubin/g polymer) were achieved in the case of the dye immobilized capillary. This confirmed the affinity capillaries have a high specificity for bilirubin removal. As can be seen in Fig. 8, bilirubin adsorption first increased significantly with the increase of the initial bilirubin concentration and then reached plateau values at around 196 mg bilirubin/l, at which point we may assume that all the active binding sites are utilized for bilirubin molecules.

Affinity adsorption is a monolayer adsorption process, which means that adsorption equilibrium is reached when all the ligand molecules are combined with the complementary target molecules. This phenomenon may be described by simple adsorption equilibrium expressions, namely Langmuir and Fruendlich equations [8]. According to related literature, most cases [20,31] fit Fruendlich equation well which used phosphate buffer as solvent for bilirubin, and Langmuir equation is adopted in those cases [3,9] which removed bilirubin from human plasma directly. That may be because phosphate molecules also were adsorbed on the sorbents [31] and biological activity of bilirubin in phosphate solution is lower than that in human plasma. It is a typical Langmuir type monolayer adsorption behavior in our case (we used human plasma), which can be described by the following Langmuir

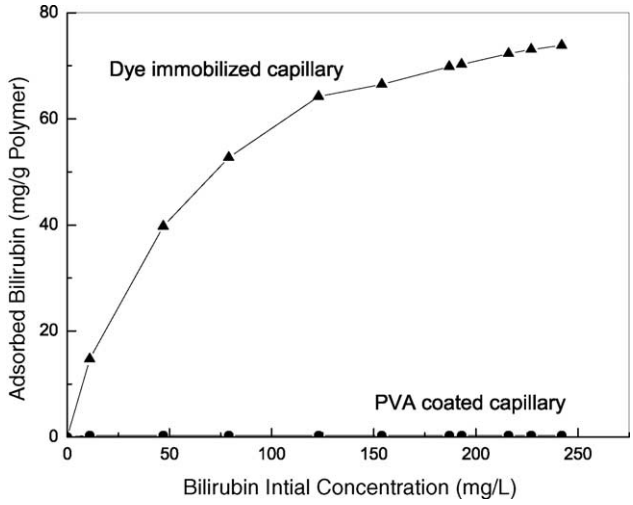

Fig. 8. Effect of bilirubin initial concentration on adsorption. Flow-rate: 1.0 ml/min; temperature, 25 °C; and total plasma volume, 50 ml.

equation:

$$Q = \frac{q_m c}{(K_d + c)} \quad (2)$$

where q is the amount of bilirubin adsorbed on the sorbent at equilibrium (mg/g); q_m is the maximum value of q (mg/g); c is the equilibrium concentration of bilirubin in the aqueous phase (mg/l); and K_d is a constant. The values of K_d and q_m for our adsorption system were found from the straight-line plot of c/q versus c by linear regression, and were 58 mg/l and 91.5 mg/g, respectively (Fig. 9). The correlation coefficient (r) of the isotherm was 0.99806, indicating that the data fits the Langmuir model well.

Different sorbents with different adsorption capacities were reported in literature for bilirubin removal. Denizli et al. presented capacities of 24.2 mg bilirubin/g with their dye-affinity microbeads, and the adsorption equilibrium time was 1 h [3,6]. Avramescu et al. obtained adsorption capacities of 30 mg bilirubin/g with BSA-affinity membrane, and the adsorption equilibrium time was 6 h [7]. Rad et al. reported 64.7 mg bilirubin/g with a HSA-affinity membrane, and showed an equilibrium time of approximate 60 min [9]. Xia et al. reported 63.4 mg bilirubin/g with a dye-affinity membrane, and showed an equilibrium time of approximate 2.5 h [20]. The maximum bilirubin adsorption we achieved with the sorbent system developed in our study was 76.2 mg bilirubin/g polymer which was quite comparable with the related literature. And comparison between our and those traditional bilirubin sorbents shows that the CB F3GA immobilized affinity MPTFE capillary has not only a high-affinity adsorption capacity towards bilirubin molecules but also a shorter adsorption equilibrium time (about 50 min).

3.2.7. Bilirubin versus albumin adsorption

As we known, CB F3GA is also a good ligand for affinity separation of albumin [33,34], and bilirubin is normally conjugated with albumin to form a water-soluble complex. So, when the human plasma flows through the CB F3GA immobilized affinity MPTFE capillary, both albumin and bilirubin were adsorbed on the capillary. In order to observe the effect of albumin adsorption on bilirubin adsorption, we changed albumin concentration (by adding HSA) in the plasma. The effect of HSA concentration on bilirubin removal was shown in Fig. 10.

As seen here, the adsorption capacity decreases with the increase of HSA concentration. This decrease can be explained by two possible reasons. One is the complex of HSA that conjugated with bilirubin have a larger molecular volume compared to bilirubin molecular, so it can decrease the diffusion of bilirubin from the bulk solution to the surface of capillary; on the other hand, larger molecular volume can reduce the opportunity of bilirubin molecules interacting with the dye molecules. Another possible reason is a competitive adsorption exists between bilirubin molecules and HSA molecules. The competitive ability of HSA molecules is improved with increasing its concentration, so the sorbents have a smaller adsorption capacity for bilirubin molecules [35]. Each albumin molecule can offer twelve sites for bilirubin adsorption, but only two sites can bind bilirubin molecule tightly. However, the mol ratio of bilirubin molecules to albumin molecules adsorbed on the affinity capillary is significantly larger than two. The result was similar to that of other related experiments [3,6,9]. Denizli et al. [6] considered that adsorption of albumin-bilirubin conjugates might occur in the sorbents, but bilirubin molecules were preferentially adsorbed by ligands in direct interaction. We believe that it is also the case in our system. There is an equilibrium between the free and albumin-conjugated bilirubin. More bilirubin molecules will be released from the albumin conjugates in order to attain this equilibrium when one removes the free form by using sorbents. This process will continuously strip bilirubin molecules from the protein conjugate until adsorp-

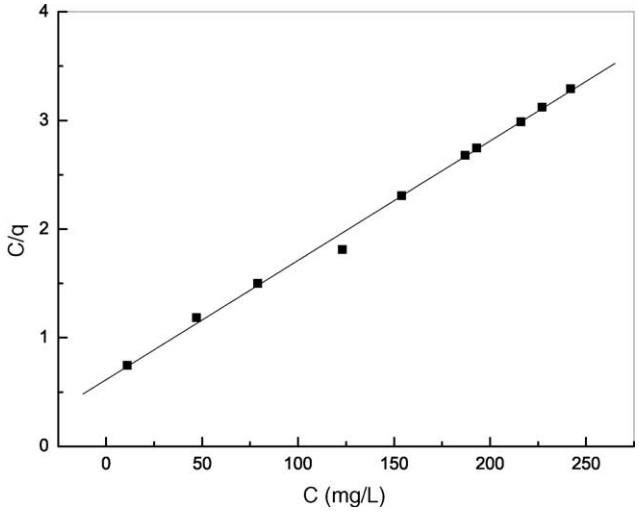

Fig. 9. The adsorption isotherm of bilirubin on CB F3GA immobilized MPTFE capillary. Flow-rate: 1.0 ml/min; temperature, 25 °C; and total plasma volume, 50 ml.

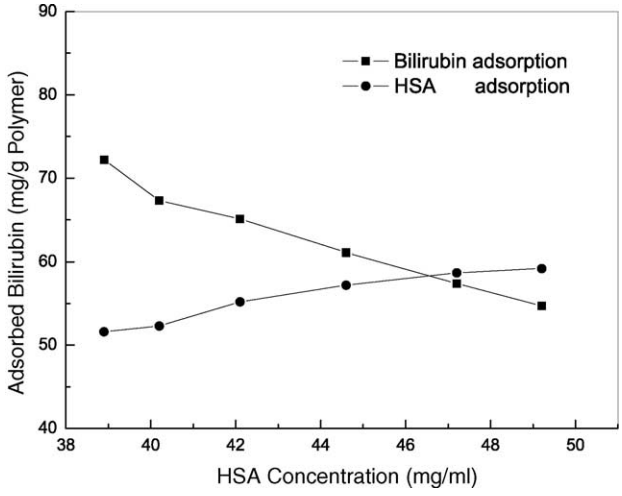

Fig. 10. Effects of HSA concentration on bilirubin adsorption. Flow rate: 1.0 ml/min; bilirubin concentration: 212 mg/l; HSA initial concentration: 38.9 mg/ml; and total plasma volume, 50 ml.

Table 1
Regeneration of the capillary and reutilization for bilirubin adsorption

Method	Adsorption capacity for bilirubin (mg/g polymer)	Reduction ratio of the adsorption capacity (%)
BSA method	64.6	15.2
NaOH method	68.5	10.1
Combined method	72.7	4.6

tion equilibrium is reached between the free bilirubin, the albumin-conjugated bilirubin and the sorbent.

Many other sorbents for bilirubin removal have good performance in phosphate solution, but very poor adsorption capacity of bilirubin in human plasma or albumin solution, which reduces the possibility of these sorbents in biomedical use. Since the normal value of HSA concentration is 40–55 mg/ml, we did not attempt to work at higher concentration. From Fig. 10, we can see the affinity capillary still maintains relatively higher adsorption capacity at high concentration solution of HSA.

3.3. Regeneration and reuse of the affinity capillary

The reusability is one of important advantages of this novel sorbents. In order to show the reusability of the dye affinity capillaries, the adsorption-desorption cycle of BSA was repeated ten times by using the same capillaries. Both BSA and NaOH solution were used to regenerate the affinity capillary (Table 1). As seen from Table 1, there was no remarkable reduction in the adsorption capacity of the affinity capillaries. The bilirubin adsorption capacity decreased only 4.6% after ten cycles.

3.4. Blood compatibility

The plasma recalcification times (PRT) of the surface-modified MPTFE capillary are shown in Table 2. We selected sample blank as reference. The PRT of unmodified MPTFE capillary surface was slightly increased by attaching the PVA coating. And the PRT was significantly prolonged on the surfaces of affinity capillary than on PVA-coated capillary ($P > 0.05$). The results suggested the dye-affinity capillary has a good anticoagulant effect. The anticoagulant mechanisms of affinity capillary need further study.

The hemolysis values for the different tested materials are compared in Table 3. No apparent hemolysis takes place on modified capillary (i.e., PVA-coated capillary and dye-affinity capillary). However, there is evidence that some extent of hemolysis occurs on the unmodified capillary. Again, this confirms that better blood compatibility can be achieved by the affinity dye (i.e., CB F3GA) immobilized on the capillary.

Table 2
Plasma recalcification time of the surface-modified PTFE capillary

Material	Recalcification time (s)						Mean value(s)
	1	2	3	4	5	6	
Blank	182	174	183	184	172	178	178.8
Unmodified capillary	92	94	95	90	87	96	92.3
PVA-coated capillary	133	135	138	129	132	132	133.2
Dye-affinity capillary	152	154	159	159	153	156	155.5

Table 3
Hemolysis of the materials

Materials	Values (%)
Unmodified capillary	4.58
PVA-coated capillary	0.28
Dye-affinity capillary	0.19

Table 4
Blood platelet count (BPC) of different materials

Materials	BPC ($\times 10^9$/l)
Blank	250
Unmodified capillary	93
PVA-coated capillary	173
Dye-affinity capillary	192

The blood platelet count (BPC) results of different samples in platelet adhesive test were presented in Table 4. As seen here, adding PRP to unmodified capillary led to a significant decrease of BPC. But the effect of the latter two materials was weak.

So, we can conclude the affinity capillary has good blood compatibility from the results of above experiments. This point is very important, because many sorbents do not have good blood compatibility though they have good adsorption capacity of bilirubin. So these sorbents are prevented from being used in biomedical and the use of them usually requires auxiliary methods such as envelope.

4. Conclusion

In this study, we developed a novel affinity sorbent system for direct bilirubin removal from human plasma. The affinity capillary sorbents were prepared by immobilizing CB F3GA onto the MPTFE capillary which is modified by PVA coating. Optimal adsorptions could be achieved at a suitable flow rate and an appropriate temperature. Comparison with other bilirubin sorbents shows that the CB F3GA immobilized affinity capillary sorbents have good affinity adsorption capacity for bilirubin removal, and a shorter adsorption equilibrium time. These new adsorbents have the advantage of both membrane and micro-column, and have higher velocity of mass transfer, better adsorption capacity, less fouling, longer service life and can be connected to recirculation flow system directly without any auxiliary equipment. And this system is inexpensive and easy to operate. The results of blood experiments suggested the dye-affinity capillary also has good blood compatibility. Therefore, these dye immobilized capillaries can replace traditional sorbents and have potential merits in clinical application for bilirubin removal.

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