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Alkali Blue 6B-Derivatized Poly(EGDMA/HEMA) Microbeads for Bilirubin Removal from Human Plasma

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ALKALI BLUE 6B-DERIVATIZED POLY(EGDMA/HEMA) MICROBEADS FOR BILIRUBIN REMOVAL FROM HUMAN PLASMA

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Key Words: Bilirubin Removal, Human Plasma, Poly(EGDMA-HEMA) Microbeads, Alkali Blue

ABSTRACT

Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads were used as a specific sorbent for bilirubin removal from human plasma. Poly(EGDMA-HEMA) microbeads were produced by a modified suspension copolymerization technique. Alkali Blue 6B was covalently immobilized to the poly(EGDMA-HEMA) microbeads via condensation reactions between the aromatic amine groups of the dyes and the hydroxyl groups of the HEMA, under alkaline conditions. Bilirubin adsorption was investigated from hyperbilirubinemic human plasma on the poly(EGDMA-HEMA) microbeads containing different amounts of attached Alkali Blue 6B, (between 8.0-23.7 μmol/g). The non-specific bilirubin adsorption on the underivatized poly(EGDMA-HEMA) microbeads were 0.32 mg/g from human plasma. High adsorption rates were observed at the beginning, and the adsorption equilibrium were then gradually achieved in about 30-60 minutes. Much higher bilirubin adsorption values, up to 6.8 mg/g, were obtained with the Alkali Blue 6B-derivatized microbeads especially at 37°C. The numbers of

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(as μmols) bilirubin molecules coupled to albumin molecules on the sorbent microbeads were in the range of 10-15, which showed that bilirubin molecules were preferentially adsorbed to the sorbent beads through the ligand, i.e. Alkali Blue 6B. Bilirubin adsorption increased with increasing temperature.

INTRODUCTION

A partially functioning liver is usually unable to clear the body of bilirubin, a product of the catabolic breakdown of hemoglobin. Large concentrations of bilirubin in the blood have therefore been associated with hepatic failure, and with neurological brain damage of newborn babies suffering from hemolysing diseases [1]. Bilirubin is a yellow-orange bile pigment. The free bilirubin is toxic. It is transported to the liver as a complex with albumin where it is normally conjugated and excreted into the bile [2]. There have been many attempts including removal of bilirubin directly from plasma of patients suffering from hyperbilirubinemia by hemoperfusion treatment, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for bilirubin [3-17]. Albumin Immobilized agarose [4], activated charcoal [5], and agar [6] have been used as sorbents in hemo-perfusion columns. In most cases basic ion-exchange resins have been utilized [7,8]. It has been shown that uncharged resins can adsorb bilirubin from aqueous media [9,10]. Idezuki *et al.* have used anion exchange synthetic fibers, and clinically applied this sorbent system in a selective bilirubin separation [11]. Sideman *et al.* have suggested the application of hemoperfusion to the removal of the bilirubin from jaundiced newborn babies by using albumin deposited macroreticular resin [12]. Brown has prepared oligo-peptide functionalized polyacrylamide beads as affinity sorbent system for bilirubin removal [13]. Chandy *et al.* have used poly-lysine immobilized chitosan beads for selective bilirubin removal [14]. Yamazaki *et al.* have developed poly(styrene-divinyl benzene) based sorbents, and succesfully applied in the treatment of more than 200 patients with hyperbilirubinemia [15]. Morimoto *et al.* have used plasma exchange and plasma adsorption with styrene-divinyl benzene resin and removed bilirubin from hepatectomized patients. This plasma adsorption system provided a possibility for an improved supportive therapy for hepatic failure, especially for patients with hepatic coma and hyperbilirubinemia [16]. Plotz *et al.* have conjugated human serum albumin with agarose using the cyanogen bromide and reported high bilirubin binding capacity [17].

In the present study, we developed an alternative sorbent for bilirubin removal. Alkali Blue 6B was selected as the affinity ligand, which was shown as a

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good ligand for affinity separation of albumin in our previous studies [18]. In addition, a further increase was expected in the bilirubin removal by direct interaction of bilirubin molecules with the immobilized Alkali Blue 6B molecules. Poly(EGDMA-HEMA) microbeads were used as the carrier matrix, which were produced by a suspension polymerization as also described in our earlier publications [19]. In the present paper, we report our preliminary experiments related to bilirubin adsorption behaviour of this new sorbent, in which plasma samples were obtained from a patient with hyperbilirubinemia.

EXPERIMENTAL

Preparation of Alkali Blue 6B-Derivatized Microbeads

The poly(EGDMA-HEMA) microbeads were selected as the carrier matrix for the synthesis of affinity sorbent for bilirubin removal. The microbeads were produced by a modified suspension polymerization of the respective comonomers, i.e., ethylene-glycol dimethacrylate (EGDMA, Rohm, Germany) and 2-hydroxy-ethylmethacrylate (HEMA, Sigma, St. Louis, MO, USA) in an aqueous media as described before [19]. Benzoyl peroxide (BPO) and polyvinyl alcohol (PVAL) (M_n: 100,000, 98% hydrolyzed, Aldrich, Rockford, IL, USA) were used as the initiator and the stabilizer, respectively. Toluene (Merck AG, Darmstadt, Germany) was utilized as the diluent and used as received. Dispersion medium was distilled water. In order to produce polymeric microbeads of about 150-200 μm in diameter and with a narrow size distribution, the amounts of EGDMA, HEMA, toluene, water, BPO and PVAL were 8 ml, 4 ml, 12 ml, 50 ml, 0.06 g, 0.2 g, respectively. Polymerizations were carried out at an agitation rate of 600 rpm at 65°C for 4 hours and at 90°C for 2 hours. After cooling, the polymeric microbeads were separated from the polymerization medium by filtration, and the residuals (e.g. unconverted monomer, toluene) were removed by a cleaning procedure given in detail elsewhere [20].

Alkali Blue 6B was used as the specific affinity ligand which was obtained from BDH (UK). Three grams of poly(EGDMA-HEMA) microbeads were magnetically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80°C for 4 hours with 100 ml of the Alkali Blue 6B aqueous solution containing 4.0 g NaOH. In order to change the extent of Alkali Blue 6B immobilization, the initial concentration of the Alkali Blue 6B in the medium was varied between 0.1 and 4.0 mg/ml. After incubation, the Alkali Blue 6B-derivatized microbeads were filtered, and washed with distilled water and methanol several times until all the physically

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attached Alkali Blue 6B molecules were removed, which was assured by following the leakage of the dye molecules to the washing media after each treatment. Alkali Blue 6B leakage after this treatment was measured in the liquid phase spectrophotometrically at 630 nm. The dye-derivatized microbeads were stored at 4°C with 0.02% sodium azide to prevent microbial contamination. The leakage of the Alkali Blue 6B from the microbeads was followed by treating the microbeads with fresh human plasma samples for 24 hours at room temperature.

The amount of Alkali Blue 6B immobilized on the microbeads was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen and sulfur stoichiometry.

Bilirubin Removal from Human Plasma

Bilirubin removal from human plasma with the unmodified and Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads was studied batchwise. The blood samples were obtained from the patients with hyperbilirubinemia. The plasma was separated by centrifugation at 500 g for 30 minutes at room temperature. Since, bilirubin is destroyed by exposure to direct sunlight or any other source of ultraviolet light, including fluorescent lighting, all adsorption experiments were carried out in a dark room. 10 ml of the plasma freshly separated from the patient were incubated with a 100 mg of the unmodified and Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads at different temperatures (i.e., 4°C, 25°C and 37°C) for 2 hours. Poly(EGDMA-HEMA) microbeads containing different amounts of Alkali Blue 6B on their surfaces were utilized. The amounts of bilirubin removed were determined by Malloy/Evelyn modifed colorimetric test by measuring the decrease in the bilirubin concentration in the plasma samples [21]. Total protein and albumin concentrations in the plasma samples both before use and after treatment were determined by Biuret and brom cresol green methods, respectively [22, 23].

RESULTS AND DISCUSSION

In this study, we aimed to prepare a specific sorbent for bilirubin removal from patients with hyperbilirubinemia. Alkali Blue 6B was used as the affinity ligand for specific binding of bilirubin molecules. Poly(EGDMA-HEMA) microbeads were selected as the carrier matrix. Details of preparation and characterization of both the unmodified and Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads were given in our previous papers [24, 25].

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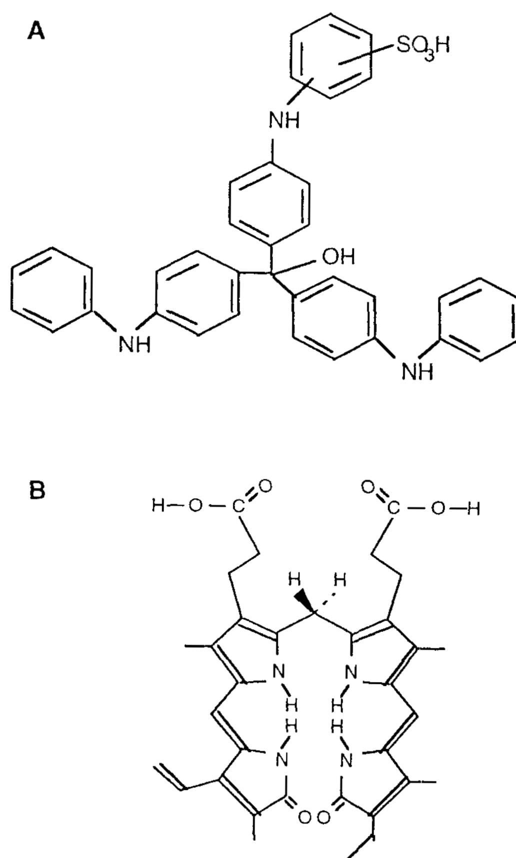

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Figure 1. Chemical structures of (A) Alkali Blue 6B and (B) bilirubin.

Chemical structures of Alkali Blue 6B and bilirubin molecules are shown in Figure 1. Alkali Blue 6B molecules were covalently immobilized into the poly(EGDMA-HEMA) microbeads. Covalent bonds were formed as a result of the condensation reactions between the aromatic amine groups of the dyes and the hydroxyl groups of the HEMA, under alkaline conditions [18]. It should be also mentioned that there is no Alkali Blue 6B release from dye-derivatized microspheres in any adsorption and desorption media. Bilirubin is a hydrophobic molecule due to intramolecular hydrogen bonds. The bilirubin molecule has a nonpolar backbone with two carboxylic groups which are partly ionized in the physiological blood pH [26]. Hence, the adsorption of bilirubin on the Alkali Blue 6B-derivatized poly(EGDMA-HEMA) sorbent can either be based on physical attachment due to

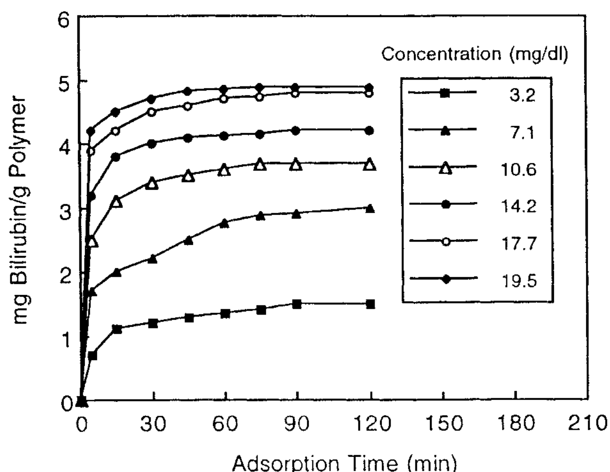

Figure 2. Adsorption rates of bilirubin from human plasma containing different amounts of bilirubin: ligand surface concentration: 17.0 μmol Alkali Blue 6B/g polymer; temperature: 25°C.

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London Forces between the nonpolar bilirubin molecules and the hydrophobic sites on the Alkali Blue 6B, or by electrical attraction between the carboxyl groups on the bilirubin molecules and the amino groups on the Alkali Blue 6B molecules.

Bilirubin Adsorption

Adsorption Rate

In this group of experiments, we used human plasma samples obtained from a patient with hyperbilirubinemia, in which the total bilirubin concentration was 19.5 mg/100 ml. Underivatized and Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads were incubated with the plasma samples for 2 hours at room temperature in the dark. Figure 2 gives the adsorption rate curves which were obtained by following the decrease of the concentration of bilirubin within the plasma samples with time. These curves indicate once again that the adsorption process completed within about 1 hour, and this value can be considered as the equilibrium time for bilirubin adsorption. It can be seen that the adsorption rate increased with increasing bilirubin concentration, which may be due to high driving force, which is the bilirubin concentration difference between the liquid (i.e., the plasma) and the solid (i.e., the microbeads) phases, in the case of high bilirubin concentration.

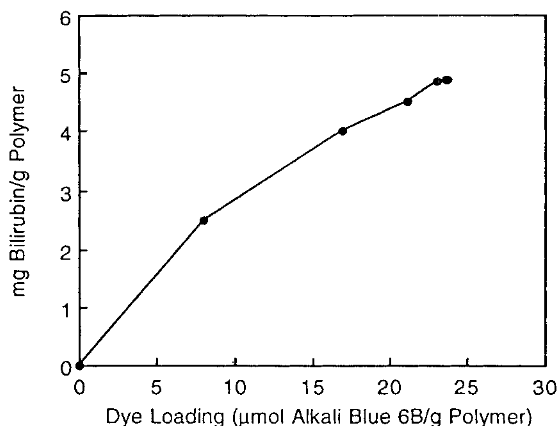

Figure 3. Effect of Alkali Blue 6B surface concentration on bilirubin adsorption: bilirubin initial concentration: 19.5 mg/100 ml; temperature: 25°C.

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Adsorption Capacity

Effect of Ligand Surface Concentration

Figure 3 indicates the adsorption capacities of the sorbent microbeads containing different amounts of Alkali Blue 6B. Note that the adsorption capacities were evaluated by using the initial and equilibrium concentrations of bilirubin in the adsorption media. As seen here, when the number of Alkali Blue 6B molecules on the microbeads increased the amount of adsorbed bilirubin also increased in the studied region, as expected, due to the number of available active functional groups on the Alkali Blue 6B ligands for interaction with bilirubin molecules.

Effects Of Bilirubin Initial Concentration

In this group of studies, the underivatized poly(EGDMA-HEMA) and Alkali Blue 6B-derivatized microbeads were incubated with the human plasma samples containing different amounts of bilirubin (1.6-19.5 mg/100 ml). Bilirubin solutions were obtained by dilution of plasma.

Figure 4 shows the bilirubin adsorption isotherms for unmodified and Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads. Note that albumin adsorption capacities of Alkali Blue 6B-derivatized microbeads are also given on an enlarged graph on the figure. Notice that there was a very low non-specific bilirubin adsorption (i.e., the adsorption onto the underivatized poly(EGDMA-HEMA) microbeads) which was about 0.32 mg bilirubin/g polymer. There is no functional

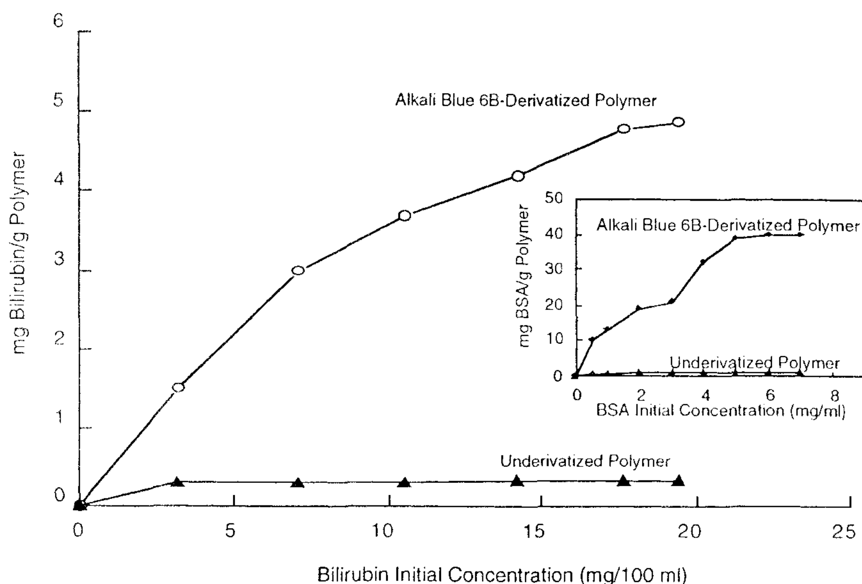

Figure 4. Effect of bilirubin initial concentration on adsorption: ligand surface concentration: 17.0 μmol Alkali Blue 6B/g polymer; temperature: 25°C.

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group on the underivatized poly(EGDMA-HEMA) microbeads which interact with bilirubin molecules, hence, this adsorption may be due to diffusion of bilirubin into the swollen matrix and weak interactions between bilirubin and hydroxyl groups on the surface of microbeads. On the other hand, much higher adsorption values, up to 4.9 mg bilirubin/g, were achieved in the case of the Alkali Blue 6B-derivatized microbeads. The specific bilirubin adsorption increased with the bilirubin initial concentration and reached a plateau (at around 15 mg bilirubin/100 ml), at which we may assume that all the active points available for bilirubin adsorption were occupied with bilirubin molecules.

Note that a wide variety of sorbents with a wide range of adsorption capacities were reported in the literature for bilirubin removal. Dunlop reported bilirubin adsorption capacity between 0.3-5 mg/g with charcoal [27]. The hydrophobic and ionic properties of bilirubin makes it natural to try uncharged resins and anion- exchangers as bilirubin sorbent. Davies *et al.* presented adsorption capacities of 4.0-80 mg bilirubin/g with their anion exchange resins [28]. Chandy and Charma reached adsorption capacities of 0.66-1.13 mg bilirubin/g with the polylysine-immobilized chitosan beads [14]. Zhu *et al.* have reported 0.2-75 mg

bilirubin/g with the polypeptide (i.e., poly-L-lysine, poly-D-lysine and poly-L-ornithine) coated polyamide resin [3]. Henning *et al.* have showed 5-80 mg bilirubin/g with the polyamide resins containing various basic amino acids [29]. Sideman *et al.* reported bilirubin adsorption capacities between 2-24 mg/g with a macroreticular resin [12]. Kanai *et al.* have developed an improved model of anion exchange resin and they obtained the maximum amount of bilirubin in was 7.7 mg/g [30]. Hughes showed 0.3 mg bilirubin adsorption capacity per g albuminated agarose from plasma [31]. The maximum bilirubin adsorption that we achieved with the sorbent system developed in this study was 4.9-6.8 mg bilirubin/g polymer which was quite comparable with the related literature.

Bilirubin Versus Albumin Adsorption

It is generally accepted that bilirubin exists in the serum in two form: direct and indirect. The direct reacting type is thought to be bilirubin conjugated with glucuronic acid, rendering it water soluble, while the indirect is bound to blood protein, albumin [13, 14]. It is reported that some sorbents like activated carbon can remove bilirubin only from the free or soluble phase, and the removal efficiency is limited by the tight binding of bilirubin to albumin [32]. The ideas of removing of bilirubin by using oligopeptide pentands as ligand in preparation of affinity sorbents [13], or alternatively adsorption of albumin-bilirubin conjugates have also been utilized [4]. Starting from the same point, we selected Alkali Blue 6B as the affinity ligand, which was shown as a good ligand for affinity separation of albumin in our previous studies [18]. In addition we were expecting a further increase in the bilirubin removal by direct interaction of bilirubin molecules with the immobilized Alkali Blue 6B molecules.

In order to observe the interrelation between albumin and bilirubin adsorptions, we also followed the changes of albumin concentration in the plasma samples before and after each adsorption cycle. Albumin adsorption was in the range of 5-40 mg albumin/g polymer (Figure 4). The total protein adsorption was parallel to the albumin adsorption. In almost all the cases, the ratio of the numbers (as μmols) bilirubin molecules to albumin molecules adsorbed on the sorbent microbeads were in the range of 10-15. Note that according to the related literature, each albumin molecule can bind two bilirubin molecules [33]. This is significantly higher in our case, which means that, there may be adsorption of albumin-bilirubin conjugates, but, bilirubin molecules are preferentially adsorbed by our ligand, i.e. Alkali Blue 6B, in direct interaction. Note that there is an equilibrium between the free and albumin-conjugated bilirubin, therefore when one removes the free form

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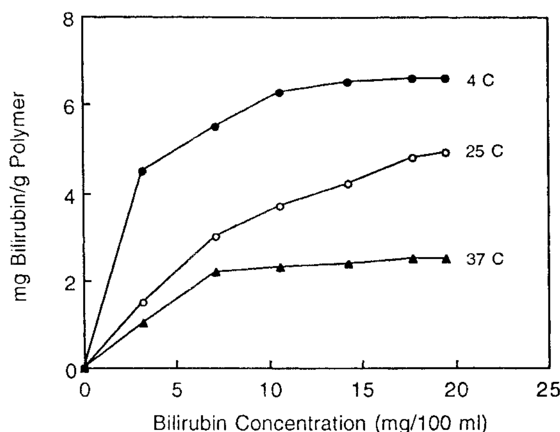

Figure 5. Effect of temperature on bilirubin adsorption: ligand surface concentration: 17.0 μmol Alkali Blue 6B/g polymer; bilirubin initial concentration: 19.5 mg/100 ml.

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by using sorbents, more bilirubin molecules will be released from the albumin-conjugates in order to attain this equilibrium, which, we believe, was also the case in our system. This process will continuously strip bilirubin molecules from the protein conjugate until to reach the adsorption equilibrium between the free bilirubin, the albumin-conjugated bilirubin and the sorbent.

Effects of Temperature on Bilirubin Adsorption

Effects of temperature on bilirubin adsorption was also studied. In these experiments, we used the plasma with a total initial bilirubin concentration of 19.5 mg/100 ml. Alkali Blue 6B-derivatized poly(EGDMA-HEMA) microbeads were incubated with this plasma. The bilirubin adsorption curves at 4 °C, 25 °C and 37 °C, representing the relative amount of bilirubin adsorbed with respect to the bilirubin initial concentrations, are shown in Figure 5. The amount of adsorbed bilirubin per unit amount of the sorbent increases with increasing temperature. Note that the maximum bilirubin adsorption was 6.8 mg bilirubin/g polymer.

In general, adsorption decreases as temperature increase [34], but in the bilirubin case it was different. Takase and Baba has found increased bilirubin adsorption with increasing temperature [35]. Davies *et al.* examined the effects of temperature on bilirubin removal from solution by an anion exchange [28]. They also showed increased adsorption with temperature. As the temperature is increased, the adsorption curve changes to the single species adsorption curve characteristic of

Langmuir adsorption. This reflects a change in the mechanism of adsorption. One hypothesis is that a conformational change takes place in the bilirubin molecule [36]. The bilirubin molecule became from a *cis* to a *trans* configuration with increasing temperature. This would allow for lessened streric hindrance in the binding of bilirubin to the Alkali Blue 6B molecules.

CONCLUSION

In this study, we developed a new sorbent system which composed of Alkali Blue 6B as the specific ligand and poly(EGDMA-HEMA) microbeads as the carrier matrix. These microbeads exhibit high mechanical strength, and therefore, they are suitable even large size column applications. The dye ligand that we use is relatively much less expensive than the ligands utilized in other sorbent systems. The results showed that up to 6.8 mg bilirubin per unit mass of the sorbent can be adsorbed at relatively high adsorption rates. The numbers (as μmols) bilirubin molecules to albumin molecules adsorbed on the sorbent microbeads were in the range of 10-15, which means that, there may be adsorption of albumin-bilirubin conjugates to the sorbent microbeads, but, bilirubin molecules were preferentially adsorbed by our ligand, i.e. Alkali Blue 6B, in direct interaction. It was possible to adsorb more bilirubin at higher temperatures. In the preliminary batch wise experiments allowed us to conclude that this inexpensive sorbent system may be an important alternative to the existing sorbents in the therapy of hyperbilirubinemia. Further studies using packed-bed columns filled with the sorbent microbeads in extracorporeal recirculation units are underinvestigation.

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