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New sorbent for bilirubin removal from human plasma: Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads

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Abstract

Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads were investigated as a specific sorbent for bilirubin removal from human plasma. The poly(EGDMA–HEMA) microbeads were prepared by a modified suspension copolymerization technique. Cibacron Blue F3GA was covalently coupled to the poly(EGDMA–HEMA) microbeads via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecule, under alkaline conditions. Bilirubin adsorption was investigated from hyperbilirubinemic human plasma on the poly(EGDMA–HEMA) microbeads containing different amounts of immobilized Cibacron Blue F3GA, (between 5.0–16.5 μmol/g). The non-specific bilirubin adsorption on the unmodified poly(EGDMA–HEMA) microbeads were 0.32 mg/g from human plasma. Higher bilirubin adsorption values, up to 14.8 mg/g, were obtained with the Cibacron Blue F3GA-immobilized microbeads. Bilirubin molecules interacted with these sorbents directly. Contribution of albumin adsorption on the bilirubin adsorption was pronounced. Bilirubin adsorption increased with increasing temperature.

Keywords: Poly(EGDMA–HEMA) microbeads; Bilirubin

1. Introduction

Bilirubin, a yellow–orange bile pigment, is formed as a result of the catabolism of hemoglobin from aged red blood cells. The free bilirubin is toxic, and hence it is transported to the liver as a complex with albumin where it is normally conjugated and excreted into the bile [1]. At high bilirubin concentration may cause hepatic or biliary tract dysfunction, and also permanent brain damage or death in more severe cases [2].

There have been many attempts to remove bilirubin directly from the 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]. Immobilized serum albumin [4], activated charcoal [5] and agar [6] have been used as sorbents in hemoperfusion 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.

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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 and Sharma have used polylysine immobilized chitosan beads for selective bilirubin removal [14]. Yamazaki et al. have developed poly(styrene–divinyl benzene) based sorbents, and successfully 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 this study we proposed an alternative sorbent for bilirubin removal. We selected Cibacron Blue F3GA as the affinity ligand, which was shown to be a good ligand for affinity separation of albumin in our previous studies [18–21]. In addition we were expecting a further increase in the bilirubin removal by direct interaction of bilirubin molecules with the immobilized Cibacron Blue F3GA molecules. Poly(EGDMA–HEMA) microbeads were selected as the carrier matrix, which were produced by a suspension polymerization as also described in our earlier publications [22–25]. In this communication, we report our preliminary experiments related to bilirubin adsorption behaviour of this new sorbent, in which plasma samples obtained from a patient with hyperbilirubinemia.

2. Experimental

2.1. Preparation of Cibacron Blue F3GA-immobilized polymeric 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., ethyleneglycol dimethacrylate (EGDMA, Rohm, Germany) and 2-hydroxyethylmethacrylate (HEMA, Sigma, St. Louis, MO, USA) in an aqueous media as described in our previous papers [22–25]. Benzoyl peroxide (BPO) and poly(vinyl alcohol) (PVAL) (M_w 100 000, 98% hydrolyzed, Aldrich, Rockford, IL, USA) were used as the initiator and the stabilizer, respectively. Toluene (Merck, 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 h and at 90°C for 2 h. 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 [26].

Cibacron Blue F3GA was used as the specific affinity ligand which was obtained from Sigma (USA). A 3-g amount of poly(EGDMA–HEMA) microbeads was magnetically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80°C for 4 h with 100 ml of the Cibacron Blue F3GA aqueous solution containing 4.0 g NaOH. In order to change the extend of Cibacron Blue F3GA immobilization, the initial concentration of the Cibacron Blue F3GA in the medium was varied between 0.1 and 4.0 mg/ml. After incubation, the Cibacron Blue F3GA-immobilized microbeads were filtered, and washed with distilled water and methanol several times until all the physically attached Cibacron Blue F3GA molecules were removed. The modified microbeads were stored at 4°C with 0.02% sodium azide to prevent microbial contamination. The leakage of the Cibacron Blue F3GA from the microbeads was followed by treating the microbeads with fresh human plasma samples for 24 h at room temperature. Cibacron Blue F3GA released after this treatment was measured in the liquid phase spectrophotometrically at 630 nm.

The amount of Cibacron Blue F3GA immobilized on the microbeads was evaluated by using an

elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen and sulfur stoichiometry.

2.2. Bilirubin removal from human plasma

Bilirubin removal from human plasma with the unmodified and Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads was studied batch wise. The blood samples were obtained from the patients with hyperbilirubinemia. The plasma was separated by centrifugation at 500 *g* for 30 min 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. A 10-ml volume of the plasma freshly separated from the patient was incubated with 100 mg of the unmodified and Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads at different temperatures (i.e., 4°C, 25°C and 37°C) for 2 h. Poly(EGDMA–HEMA) microbeads containing different amounts of Cibacron Blue F3GA on their surfaces were utilized. The amounts of bilirubin removed were determined by Malloy–Evelyn modified colorimetric test by measuring the decrease in the bilirubin concentration in the plasma samples [27]. 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 [28,29].

3. Results and discussion

In this study, we attempted to prepare a specific sorbent for bilirubin removal from patients with hyperbilirubinemia. Cibacron Blue F3GA 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 Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads were given in our previous papers [18–21].

Cibacron Blue F3GA is covalently coupled to the poly(EGDMA–HEMA) microbeads via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA, under alkaline conditions [30]. Note that although the amino acid sequence distribution of HSA is well documented, the precise location of the primary binding site for bilirubin has not yet been established. In our case, we also do not have any clear-cut evidence about the exact interaction points of bilirubin and Cibacron Blue F3GA molecules. However, from the chemical formula of both molecules, we may postulate that these molecules may interact through the amino and carboxyl groups, which are the most probable reactions.

3.1. Effect of ligand surface concentration on bilirubin adsorption

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. The unmodified and Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads carrying different amounts of ligand (i.e., Cibacron Blue F3GA) were incubated with the plasma samples for 2 h at room temperature in the dark. Fig. 1 gives the adsorption rate curves which were obtained by following the decrease of the concentration of bilirubin within the plasma samples with time. As seen here, there were relatively faster adsorption rates were observed at the beginning, and then adsorption equilibria were achieved gradually in about 1 h. Notice that, there

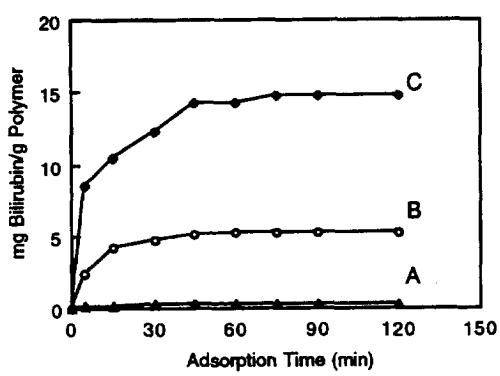

Fig. 1. Adsorption rates of bilirubin from human plasma: bilirubin initial concentration: 19.5 mg/100 ml; temperature: 25°C. Curve A: adsorption onto unmodified poly(EGDMA–HEMA); curve B: adsorption onto poly(EGDMA–HEMA) with 5.0 μmol Cibacron Blue F3GA/g; curve C: adsorption onto poly(EGDMA–HEMA) with 16.5 μmol Cibacron Blue F3GA/g.

was a very low non-specific bilirubin adsorption (i.e., the adsorption onto the unmodified poly(EGDMA–HEMA) microbeads) which was about 0.32 mg bilirubin/g polymer (Curve A, Fig. 1). There are no functional groups on the unmodified 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 rates were observed when the Cibacron Blue F3GA-immobilized poly-(EGDMA–HEMA) microbeads were used (Curve B and C, Fig. 1).

Fig. 2 gives the bilirubin adsorption capacities of the sorbent microbeads carrying different amounts of Cibacron Blue F3GA. Note that the adsorption capacities were evaluated by using the initial and equilibrium concentrations of bilirubin in the adsorption media. As expected, when the number of Cibacron Blue F3GA molecules on the microbeads increased the amount of adsorbed bilirubin also increased in the studied region.

3.2. Effect of bilirubin initial concentration on adsorption

Fig. 3 shows the non-specific and specific adsorption of bilirubin onto poly(EGDMA–HEMA) microbeads. The amount of bilirubin adsorption on the unmodified poly(EGDMA–HEMA) microbeads

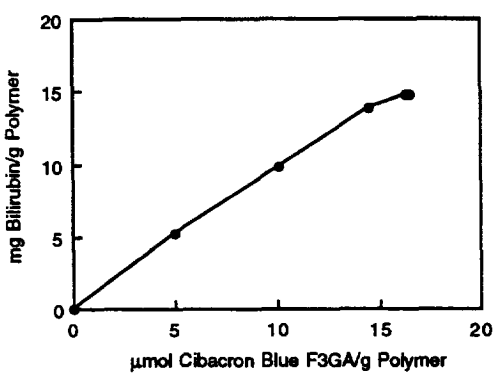

Fig. 2. Effect of ligand surface concentration on bilirubin adsorption: bilirubin initial concentration: 19.5 mg/100 ml; temperature: 25°C.

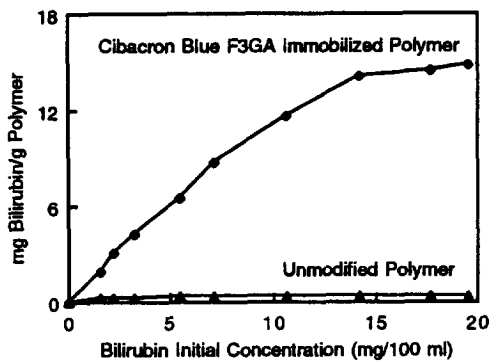

Fig. 3. Effect of bilirubin initial concentration on adsorption: ligand surface concentration: 16.5 μmol Cibacron Blue F3GA/g polymer; temperature: 25°C.

was about 0.32 mg/g polymer, while much higher adsorption values up to 14.8 mg bilirubin/g were achieved in the case of the Cibacron Blue F3GA-immobilized microbeads. The specific bilirubin adsorption increased with the bilirubin initial concentration and reached a plateau (at 14.2 mg bilirubin/100 ml), at which we may assumed 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 literature for bilirubin removal. Davies et al. presented adsorption capacities of 4.0–80 mg bilirubin/g with their anion-exchange resins [31]. 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 [32]. 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 (IONEX) and they obtained the maximum amount of bilirubin in was 7.7 mg/g [33]. The maximum bilirubin adsorption that we achieved with the sorbent system developed in this study was 14.8–18.9 mg bilirubin/g polymer which was quite comparable with the related literature.

3.3. Bilirubin versus albumin adsorption

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 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 [34]. The ideas of removing of bilirubin by using oligopeptide pentads as ligands 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 Cibacron Blue F3GA as the affinity ligand, which was shown as a good ligand for affinity separation of albumin in our previous studies [18–21]. In addition we were expecting a further increase in the bilirubin removal by direct interaction of bilirubin molecules with the immobilized Cibacron Blue F3GA 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 14.2–60.5 mg BSA/g polymer. The total protein adsorption was parallel to the albumin adsorption. Almost in all cases the ratio of the numbers (as μmol) bilirubin molecules to albumin molecules adsorbed on the sorbent microspheres were in the range of 25–30. Note that according to the related literature, each albumin molecule can bind two bilirubin molecules [35]. This is very 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., Cibacron Blue F3GA, in direct interaction. Note that there is an equilibrium between the free and albumin–conjugated bilirubin, therefore when one removes the free form 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.

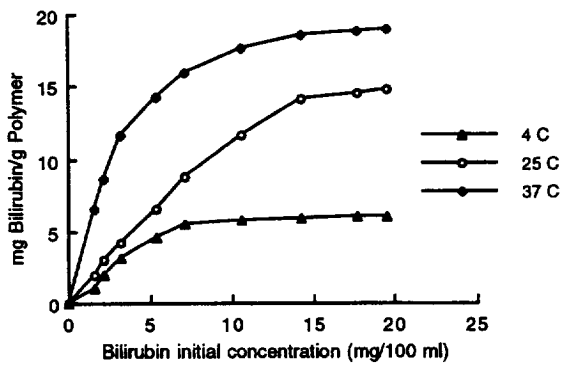

Fig. 4. Effect of temperature on bilirubin adsorption: ligand surface concentration: 16.5 μmol Cibacron Blue F3GA/g polymer; bilirubin initial concentration: 19.5 mg/100 ml;

3.4. Effect of temperature on bilirubin adsorption

We have also studied effect of temperature on bilirubin adsorption. In these experiments, we used the plasma with a total initial bilirubin concentration of 19.5 mg/100 ml. The Cibacron Blue F3GA-immobilized poly(EGDMA–HEMA) microbeads containing 16.5 μmol Cibacron Blue F3GA/g were incubated with this plasma. The bilirubin adsorption curves obtained at three different temperatures, i.e., 4°C, 25°C and 37°C are shown in Fig. 4. The amount of adsorbed bilirubin per unit amount of the sorbent increases with increasing temperature. Note that the maximum bilirubin adsorption was 18.9 mg bilirubin/g polymer. In general, adsorption decreases as temperature increase [36], but in bilirubin case it was different as also reported by others [31,37].

4. Conclusion

In this study, we developed a new sorbent system which composed of Cibacron Blue F3GA as the specific ligand and poly(EGDMA–HEMA) microbeads as the carrier matrix. The results presented in this communication showed that up to 18.9 mg bilirubin per unit mass of the sorbent can be adsorbed at relatively high adsorption rates. Bilirubin

molecules interact directly with the immobilized ligand molecules, and the albumin adsorption does not decrease bilirubin adsorption significantly. 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 fixed-bed columns filled with the sorbent microbeads in extracorporeal recirculation units are under investigation.

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