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Bilirubin removal from human plasma in a packed-bed column system with dye-affinity microbeads

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Abstract

A dye-ligand, Cibacron Blue F3GA, was covalently coupled with the poly(EGDMA-HEMA) microbeads. The affinity sorbent carrying 16.5 μmol Cibacron Blue F3GA per gram polymer was then used to remove bilirubin from human plasma in a packed-bed column system. Bilirubin adsorption from human plasma on the unmodified poly(EGDMA-HEMA) microbeads was 0.32 mg/g, while much higher adsorption values, up to 24.2 mg/g, were obtained with the dye-attached microbeads. The bilirubin adsorption capacity of the microbeads decreased with an increase in the recirculation rate of plasma. Bilirubin adsorption increased with increasing temperature, and the maximum adsorption achieved at 37°C (32.5 mg bilirubin/g polymer). Bilirubin molecules interacted directly with the immobilized Cibacron Blue F3GA molecules. Contribution of albumin adsorption on bilirubin adsorption was also significant. © 1998 Elsevier Science B.V.

Keywords: Hyperbilirubinemia; Cibacron Blue F3GA; Affinity microbeads; Bilirubin

1. Introduction

Bilirubin, a bile pigment, is formed as a result of the catabolism of hemoglobin from aged red blood cells in all mammals [1]. Although its physiological functions in the human body are not fully understood, it has been suggested that it probably serves as a chain-breaking antioxidant. It deposits in tissue, especially in the brain and is toxic. Disorders in the metabolism of bilirubin, especially common among newborn infants, may cause jaundice, a yellow discoloration of the skin and other tissues [2].

Many techniques have been used for the removal of bilirubin directly from plasma of patients suffering from hyperbilirubinemia such as hemodialysis, phototherapy and hemoperfusion. Hemoperfusion treatment, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for bilirubin is a most promising technique [3–15]. 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]. It has been shown that uncharged resins can adsorb bilirubin from aqueous media [8]. Idezuki used anion-exchange synthetic fibers, and clinically applied this sorbent system in a selective bilirubin separation [9]. Sideman et al. suggested the application of hemoperfusion to the removal of the bilirubin from jaundiced newborn babies by using albumin-deposited macroreticular resin [10]. Brown prepared oligo-

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peptide functionalized polyacrylamide beads as an affinity sorbent system for bilirubin removal [11]. Chandy and Sharma used polylysine immobilized chitosan beads for selective bilirubin removal [12]. Yamazaki et al. developed poly(styrene-divinyl benzene)-based sorbents, and successfully applied the treatment in more than 200 patients with hyperbilirubinemia [13]. Morimoto et al. used plasma exchange and plasma adsorption with styrene-divinyl benzene resin and removed bilirubin from hepatectomized patients. This plasma adsorption system provided the possibility for an improved supportive therapy for hepatic failure, especially for patients with hepatic coma and hyperbilirubinemia [14]. Plotz et al. conjugated human serum albumin with agarose using the cyanogen bromide and reported high bilirubin binding capacity [15].

In our recent studies, we have produced bioaffinity sorbents in which poly(HEMA) or poly(EGDMA-HEMA) microbeads have been used as the basic carrier, and several bio-ligands (e.g. protein A, DNA, heparin, collagen) and dye-ligands (e.g., Cibacron Blue F3GA, Congo Red, Alkali Blue 6B) were incorporated into these microbeads for removal of several substances (e.g., proteins, pathogenic antibodies, cholesterol) from aqueous media, including plasma [16–20]. In this study, we attempted to utilize Cibacron Blue F3GA-attached poly(EGDMA-HEMA) microbeads as a specific sorbent for removal of bilirubin from human plasma in a packed-bed column system. The performance of these sorbents at different conditions are presented in this communication.

2. Experimental

2.1. Cibacron Blue F3GA-coupled polymeric microbeads

The poly(EGDMA-HEMA) microbeads were used as the carrier 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-hydroxyethylmethacrylate (HEMA, Sigma, St. Louis, MO, USA) in an aqueous medium, as described in our previous papers [21,22]. Benzoyl peroxide (BPO) and poly(vinyl alcohol) (PVAL) (M_r 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 pore former 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, 4, 12 and 50 ml, and 0.06 and 0.2 g, respectively. Polymerizations were carried out at 600 rpm at 65°C for 4 h and then 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 washing.

Cibacron Blue F3GA was supplied by Sigma (USA) and used as received. It 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 molecules under alkaline conditions. Briefly, 3 g 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. Under these conditions, a chemical reaction takes place between the group of the Cibacron Blue F3GA having chloride and hydroxyl groups of the HEMA, with the elimination of HCl, resulting in the coupling of Cibacron Blue F3GA to the poly(EGDMA-HEMA) microbeads. The initial concentration of the Cibacron Blue F3GA in the medium was 3.0 mg/ml. After incubation, the solution cooled down to room temperature and the Cibacron Blue F3GA-coupled microbeads were then filtered and washed with distilled water and methanol several times until all the physically attached Cibacron Blue F3GA molecules were removed.

The amount of Cibacron Blue F3GA attached on the microbeads was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen and sulfur stoichiometry. 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 modified microbeads were stored at 4°C with 0.02% sodium azide to prevent microbial contamination.

2.2. Bilirubin removal from human plasma

The adsorption studies were carried out in a packed-bed column system, in which an adsorption column (with an internal diameter of 0.9 cm and height of 10 cm) equipped with a water jacket for temperature control was used. The column was filled with the unmodified or dye-attached poly(EGDMA-HEMA) microbeads containing 16.5 μmol Cibacron Blue F3GA per gram. The blood samples having different bilirubin initial concentrations were obtained from different patients with hyperbilirubinemia. The plasma was immediately separated by centrifugation at 500 *g* for 30 min at room temperature and then it was collected in a samplepool and stored in the dark, by covering the plasma tubes with aluminum foil, in a refrigerator before use. Since bilirubin is 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. In a typical continuous column system, 50 ml of the plasma freshly separated from the patient was recirculated through the column containing polymeric microbeads for 2 h. The bilirubin concentrations in the plasma samples were determined by the Malloy/Evelyn modifed colorimetric test method [23]. Total protein and albumin concentrations in the plasma samples both before use and after treatment were determined by Biuret and bromocresol green dye methods, respectively [24,25].

In the first group of experiments, the volumetric recirculation rate of the aqueous phase (i.e., 50 ml of the human plasma with a bilirubin content of 18.6 mg/100 ml) was varied between 0.5 and 2.5 ml/min. The temperature was kept constant at 25°C.

In the second group of experiments, bilirubin adsorption from human plasma containing 18.6 mg/100 ml was studied at three different temperatures, i.e., 4, 25 and 37°C. The volumetric recirculation rate was 1 ml/min.

Adsorption rates were obtained both in the continuous recirculation packed-bed column system and in batch-wise experiments. Human plasma samples containing different amounts of bilirubin were used in these experiments. The changes in the bilirubin concentration with time was followed to obtain the adsorption rate curves. The volumetric recirculation rate in the continuous column studies was 1 ml/min, while the agitation rate in the batch experiments was 100 rpm. These studies were performed at a constant temperature of 25°C.

In the last group, bilirubin adsorption from human plasma containing different amounts of bilirubin was studied at 25°C. The volumetric recirculation rate was 1 ml/min.

3. Results and discussion

In the present study, we aimed to prepare a specific sorbent for bilirubin removal from human plasma obtained from patients with hyperbilirubinemia in a packed-bed column system. Poly(EGDMA-HEMA) microbeads were selected as the sorbent. Cibacron Blue F3GA was used as the affinity dye-ligand for specific binding of bilirubin molecules. It is reported that Cibacron Blue F3GA has no adverse effect on biochemical systems [26]. However, all commercial reactive dyes (including Cibacron Blue F3GA) contain various impurities which may affect their biochemical and related use [26–28]. Reactive dyes have been purified by a number of chromatographic procedures such as thin-layer chromatography, high-performance liquid chromatography and column chromatography on Silica gel or Sephadex [29]. However, it is suggested that purification of reactive dyes is necessary only when free dyes are used [27]. In cases where immobilized dyes are used, purification of the dye before immobilization is not likely to be necessary, because few of the contaminants will be immobilized on the support matrix, and proper washing of the matrix should remove adsorbed contaminants [27].

Cibacron Blue F3GA molecules were covalently attached to the polymeric microbeads. It is accepted that ether linkages were formed between the reactive triazine ring of the dye and the hydroxyl groups of the sorbent. Elemental analysis of the unmodified and Cibacron Blue F3GA-attached poly(EGDMA-HEMA) microbeads was performed and, from the

nitrogen stoichiometry, the attachment of the dye was found to be 16.5 μmol/g. Cibacron Blue F3GA leakage was also investigated in human plasma. Cibacron Blue F3GA leakage was not observed from any of the dye-attached microbeads, even after a long period of time (more than 2 months). Preparation and characterization details of both the unmodified and Cibacron Blue F3GA-attached poly-(EGDMA-HEMA) microbeads were given elsewhere [21,22].

3.1. Column performance

3.1.1. Effects of flow-rate on adsorption

In this group of experiments, the volumetric recirculation rate of the aqueous phase was varied between 0.5 and 2.5 ml/min; other parameters were kept constant, as given in the figure legends. The adsorption capacity (the amount of bilirubin adsorbed per gram of the microbeads in 2 h) at different flow-rates are given in Fig. 1. The adsorption capacity decreased significantly from 28.8 to 9.7 mg bilirubin/g polymer with the increase of the flow-rate from 0.5 to 2.5 ml/min. This decrease may be explained as follows: the residence time in the column decreases with increasing flow-rate, which does not give enough time for the bilirubin molecules to interact with the sorbent. In addition, the increase in the flow-rate may cause channeling in the sorbent bed, which results a decrease in the effective use of the sorbent microbeads in the column. Therefore, low adsorption capacities are observed at high flow-rates. Note that at flow-rates lower than 1.0 ml/min we faced some technical problems in our experimental set-up, such as difficulties in sampling, therefore we performed all other adsorption tests at a flow-rate of 1.0 ml/min.

3.1.2. Effects of temperature on bilirubin adsorption

In this group of experiments, bilirubin adsorption studies were performed at 4, 25 or 37°C. Other parameters were kept constant as given in the figure legend. The effect of temperature on the adsorption of bilirubin by the Cibacron Blue F3GA-attached microbeads is shown in Fig. 2. As seen here, the amount of adsorbed bilirubin per unit amount of the sorbent increased with increasing temperature. The maximum bilirubin adsorption was 32.5 mg bilirubin/g polymer, which was obtained at 37°C. Since this is body temperature, we did not attempt to work at higher temperatures.

This result is in contrast to that observed in most adsorption studies. In general, it is known that adsorption decreases with increasing temperature, because adsorption is an exothermic process. Similar to our results, Takase and Baba found increased bilirubin adsorption with increasing temperature

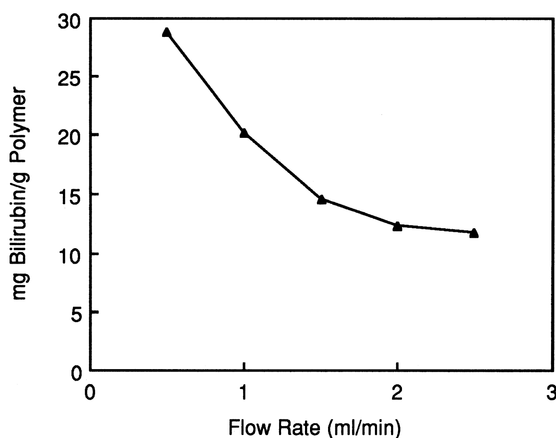

Fig. 1. Bilirubin adsorption at different flow-rates. Ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; bilirubin initial concentration, 18.6 mg/100 ml; temperature, 25°C; and total plasma volume, 50 ml.

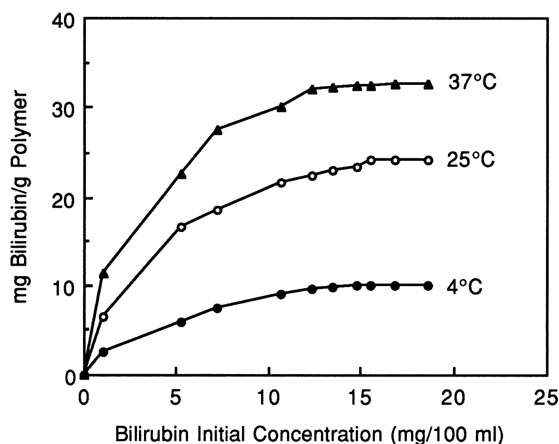

Fig. 2. Effect of temperature on bilirubin adsorption. Ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; bilirubin initial concentration, 18.6 mg/100 ml; flow-rate, 1.0 ml/min; and total plasma volume, 50 ml.

[30]. Davies et al. also examined the effects of temperature on bilirubin removal from solution by anion exchange, and they also reported an increase in the adsorption of bilirubin with temperature [31]. One hypothesis to explain this unexpected behaviour is that conformational changes takes place in the bilirubin molecule with the increase in temperature, which in turn causes an increase in the adsorption capacity [32].

3.2. Adsorption rate

Fig. 3 gives the adsorption rate curves which were obtained by following the changes of the concentration of bilirubin within the plasma with time in the continuous recirculation packed-bed column systems. For comparison, batch-wise experiments were also carried out as mentioned before. Experimental conditions are given in the figure legends.

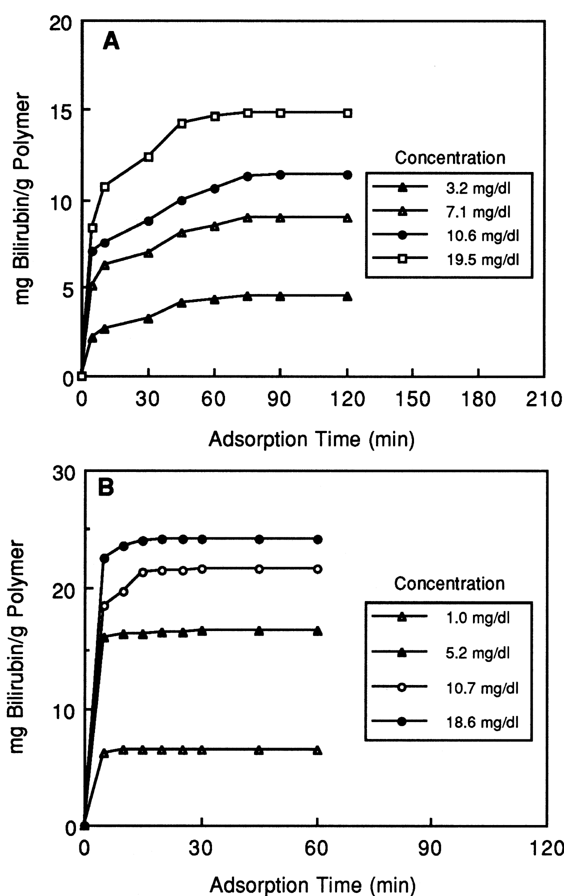

Fig. 3. Adsorption rates of bilirubin from human plasma containing different amounts of bilirubin. (A) Batch system; (B) column system. Ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; flow-rate, 1.0 ml/min for column system; temperature, 25°C; and total plasma volume, 50 ml.

Fig. 3A gives the adsorption rate curves obtained in a batch system. As seen here, adsorption equilibrium is achieved in about 1 h. While the bilirubin adsorption in the continuous column studies was much higher than that observed in the batch system, the equilibrium times were around 15 min (Fig. 3B). Note that, in both cases, adsorption rates increased with increasing bilirubin concentration. This may be due to a high driving force, which is the bilirubin concentration difference between the liquid (i.e., the human plasma) and the solid (i.e., the microbeads) phases, in the case of high bilirubin concentration.

3.3. Adsorption capacity

Fig. 4 shows the non-specific and specific adsorption of bilirubin onto the unmodified and dye-attached poly(EGDMA-HEMA) microbeads, respectively. Note that one of the main requirements in the affinity system is the specificity of the sorbent. The non-specific interaction between the carrier matrix [here the poly(EGDMA-HEMA) microbeads] and the molecules to be removed (here bilirubin) should

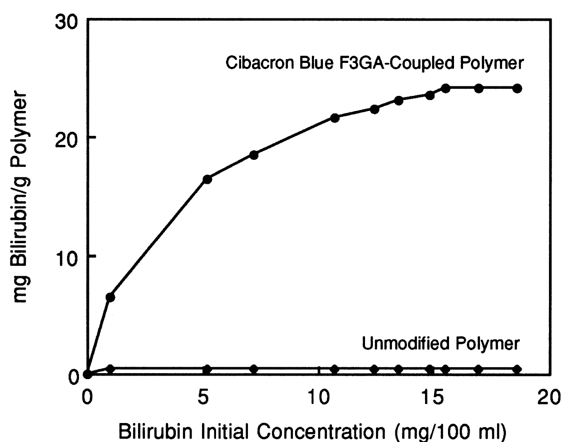

Fig. 4. Effect of bilirubin initial concentration on adsorption. Ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; flow-rate, 1.0 ml/min; temperature, 25°C; and total plasma volume, 50 ml.

be minimum in order to have a high specificity. The amount of bilirubin adsorption on the unmodified poly(EGDMA-HEMA) microbeads was quite low (about 0.32 mg/g polymer), while much higher adsorption values (up to 24.2 mg bilirubin/g) were achieved in the case of the Cibacron Blue F3GA-attached microbeads.

Note that a wide variety of sorbents with a wide range of adsorption capacities were reported in the 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 obtained adsorption capacities of 0.66–1.13 mg bilirubin/g with the polylysine-attached chitosan beads [12]. Zhu et al. reported 0.2–75 mg bilirubin/g with a polypeptide (i.e., poly-L-lysine, poly-D-lysine and poly-L-ornithine)-coated polyamide resin [3]. Henning et al. showed 5–80 mg bilirubin/g with polyamide resins containing various basic amino acids [33]. Sideman et al. reported bilirubin adsorption capacities between 2 and 24 mg/g with a macroreticular resin [10]. Kanai et al. developed an improved anion-exchange resin (IONEX) model and they obtained the maximum amount of bilirubin: 7.7 mg/g [34]. Comparison of these results, shows that the poly(EGDMA-HEMA) column has a high coupling capacity, and that the Cibacron Blue F3GA-attached poly(EGDMA-HEMA) microbeads exhibit a high-affinity adsorption towards bilirubin molecules.

As can be seen in Fig. 4, bilirubin adsorption first increased significantly with the initial bilirubin concentration and then reached plateau values at around 15 mg bilirubin/100 ml, at which point we may assume that all the active binding sites available for bilirubin adsorption were occupied with bilirubin molecules. This is a typical Langmuir type monolayer adsorption behaviour, which can be described by the following equation:

$$q = q_m c/(K_d + c)$$

where q is the amount of bilirubin adsorbed on the sorbent microbeads 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/100 ml); 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 3.9 mg/dl and 29.3 mg/g, respectively.

3.4. Effects of albumin adsorption

It is generally accepted that bilirubin exists in serum in two different 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 blood protein, albumin [11,12]. It is reported that some sorbents, such as 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 [30]. The idea of removing bilirubin by using oligopeptide pentands as ligands in a preparation of affinity sorbents [11], 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 dye-ligand for affinity separation of albumin in our previous studies [35,36]. In addition, we expected a further increase in bilirubin removal by direct interaction of bilirubin molecules with the attached 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. In almost all cases, the ratio of the numbers (μmol) of 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 [37]. 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 adsorption equilibrium is reached between the free bilirubin, the albumin-conjugated bilirubin and the polymeric sorbent.

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