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Efficient removal of albumin from human serum by monosize dye-affinity beads

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Abstract

Cibacron Blue F3GA was covalently attached onto monosize poly(glycidyl methacrylate) [poly(GMA)] beads for removal of human serum albumin (HSA) from human serum. Monosize poly(GMA) beads, 1.6 μm in diameter, were produced by dispersion polymerization. Cibacron Blue F3GA loading was 1.73 mol/g. HSA adsorption experiments were performed by stirred-batch adsorption. The non-specific adsorption of HSA was low (0.8 mg/g polymer). Dye attachment onto the *monosize beads* significantly increased the HSA adsorption (189.8 mg/g). The maximum HSA adsorption was observed at pH 5.0. With an increase of the aqueous phase concentration of sodium chloride, the adsorption capacity decreased drastically. The equilibrium adsorption of HSA significantly decreased with increasing temperature. The elution *studies* were performed by adding 0.1 M Tris/HCl buffer containing 0.5 M NaSCN to the HSA solutions in which adsorption equilibria had been reached. The elution results demonstrated that the adsorption of HSA to the adsorbent was reversible. The depletion efficiencies for HSA were above *87%* for all studied concentrations. To test the efficiency of HSA removal from human serum, proteins in the serum and eluted portion were analyzed by two-dimensional gel electrophoresis. Eluted proteins include mainly albumin, and a small number of nonalbumin proteins such as apo-lipoprotein A1, sero-transferrin, haptoglobulin and α1-antitrypsin were bound by the dye-affinity beads. *IgA was not identified in eluted fraction*.

Keywords: Cibacron Blue F3GA; Monosize beads; Poly(GMA); Albumin removal; Dye-ligand chromatography; Affinity depletion

1. Introduction

Serum proteins may often serve as indicators of disease and is a rich source for biomarker discovery. However, the large dynamic range of proteins in serum makes the analysis very challenging because high abundance of proteins tend to mask those of lower abundance [1]. The high abundance of albumin and immunoglobulin, which comprise about 80% of total serum protein, is a major problem in proteome studies, which use serum, plasma, cerebrospinal fluid or synovial fluid samples [2]. Depletion of abundant serum proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers [3]. A variety of depletion methods for specific removal of highly abundant proteins from body fluids have been developed. Several major strategies are available concerning the mechanims of removal of HSA and IgG. In the case of HSA, depletion can be achieved by either dye-ligands such as the widely recognized Cibacron Blue F3GA and derivatives thereof [4], or specific antibodies [5]. *The high specificity of antibodies provides excellent selectivity. However, in spite of its high selectivity, antibody carrying adsorbents also has some drawbacks which are worth considering: (i) antibody may leak from the matrix and such contamination cannot, of course, be tolerated in clinical applications; (ii) the cost of these materials tends to be very high. The antibody ligands are difficult to immobilise in the proper orientation. They are also susceptible to degradation during the sterilization and cleaning procedures*. A dye-affinity resin for removal of HSA has the advantage of high loading capacity as compared to an antibody-based system but has been shown to lack specificity [6,7]. *Reversible adsorption could provide the possibility of using such dyes in an immobilized form and, in this way, having the advantages of the use of dye-affinity adsorbents, saves time and cost*. Other methods reported include a proprietary polypeptide affinity matrix that removes albumin together with IgG, but is now apparently unavailable [8], and a method based on the size separation in a

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centrifugal filtration device that was, perhaps predictably, unsuccessful [9]. The next challange in serum protein analysis is the depletion of the high concentration of IgGs. The removal of IgG is commonly achieved by immobilizing protein A of protein G onto the affinity resins, which binds to the Fc region of the IgG [10], but specific antibodies can also be used. In addition, the depletion of IgG in human plasma is employed for the treatment of immune disorders including systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, alloimmunization and cancer [11–14].

Affinity adsorbents with large specific surface areas for adsorption are desirable to attain high binding capacity *with fast binding kinetics* [15]. To increase the surface area to the practically useful level of *100 m^2/g*, either *highly* porous materials with large pore size or nonporous nanosized beads have to be employed. Due to these reasons, *micron-sized affinity adsorbents such as silica and polystyrene-based particles have been gaining* more attention for the rapid high-performance liquid chromatography of biomolecules [16]. A major advantage of the non-porous adsorbents is that significant intraparticle diffusion resistances are absent; this is particularly useful for the rapid analysis of proteins with high efficiency and resolution [17]. The rapid separation makes it very useful for quality control, on-line monitoring, and purity check of biomolecules such as peptide mapping of recombinant products. *However, silica is unstable in extreme pH, and polystyrene-based particles are hydrophobic, which makes them exhibit pronounced nonspecific protein adsorption [18]. Therefore, the development of other polymeric adsorbents with low nonspecific protein adsorption is desirable. Poly(glycidyl methacrylate) has attracted much attention for its hydrophilic characteristics*.

The aim of this study was to prepare monosize dye-affinity beads for *efficient* removal of albumin from human serum. So far, only a few dye-affinity adsorbents were reported for the removal of albumin from human serum [1,3,7,19]. Poly(glycidyl methacrylate) [poly(GMA)] monosize beads were obtained by dispersion polymerization of GMA. Dye-affinity beads were characterized by FTIR, SEM and elemental analysis. Then, albumin removal studies from aqueous *protein* solutions and human serum were performed. Elution of albumin and reusability of these dye-affinity adsorbents were also tested.

2. Experimental

2.1. Materials

Human serum albumin (HSA, 98% pure by gel electrophoresis, fatty acid free, 67 kDa) was purchased from Aldrich Chem. Co. (Milwaukee, WI, USA) and used as received. Cibacron Blue F3GA was obtained from Polyscience (Warrington, USA) and used without further purification. Glycidyl methacrylate (GMA, Fluka A.G., Buchs, Switzerland) was purified by vacuum distillation and stored in a refrigerator until use. Azobisisobutyronitrile (AIBN) and poly(vinyl pyrrolidone) (MW: 30.000, BDH Chemicals Ltd., Poole, England) were selected as the initiator and the steric stabilizer, respectively. AIBN was recrystallized from methanol. Ethanol (Merck, Germany) was used as the diluent without further purification. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use, the glassware was rinsed with deionised water and dried in a dust-free environment. All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed bed system.

2.2. Preparation of poly(GMA) beads

The dispersion polymerization was performed in a sealed polymerization reactor (volume: 500 ml) equipped with a temperature control system. A typical procedure applied for the dispersion polymerization of GMA is given below. The monomer phase was comprised of 40 ml GMA. Two hundred and fifty milligrams of AIBN was dissolved into the monomer phase. The resulting medium was sonicated for about 5 min at 200 W within an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. Four grams of poly(vinyl pyrrolidone) were dissolved in a homogeneous solution of ethanol (100 ml) and water (100 ml) placed in a polymerization reactor. The reactor content was stirred at 500 rpm during the monomer addition, completed within about 5 min, and the heating was started. Then reactor was purged with bubbling nitrogen for about 5 min. Then, the sealed reactor was placed in a shaking water bath at room temperature. The initial polymerization time was defined when the reactor temperature was raised to 65 °C. The polymerization was allowed to proceed under nitrogen atmosphere at 65 °C for 4 h (stirring rate: 500 rpm). After completion of the polymerization period, the reactor content was cooled down to room temperature and centrifuged at 5000 rpm for 10 min for the removal of dispersion medium. This *polymerization* reaction led to the formation of white beads. Poly(GMA) beads were redispersed within 10 ml of ethanol and centrifuged again under similar conditions. The ethanol washing was repeated three times for complete removal of unconverted monomers and other components. Finally, poly(GMA) beads were redispersed within 10 ml of water (0.10%, by weight) and stored at room temperature.

2.3. Dye attachment to poly(GMA) beads

Cibacron Blue F3GA was covalently attached to the poly(GMA) monosize beads. First, Cibacron Blue F3GA was dissolved in 100 ml of water (dye concentration: 5 mg/ml). This aqueous dye solution was transferred to poly(GMA) beads (total mass: 1.0 g) in 100 ml distilled water, and then 4.0 g of NaOH were added. The medium was heated at 80 °C in a sealed reactor and was stirred magnetically for 4 h. Under these experimental conditions, a chemical reaction took place between the chlorine-containing group of the Cibacron Blue F3GA and the epoxy groups of the GMA monomer. In order to remove the non-specifically attached dye molecules, an extensive cleaning procedure was applied, which was as follows: The beads were

first washed with deionized water. The monosize beads were dispersed in methanol, and the dispersion was sonicated for 2 h in an ultrasonic bath. At the last stage, beads were washed again with deionized water. Cibacron Blue F3GA-attached poly(GMA) beads were stored at 4 °C with 0.02% sodium azide to prohibit microbial contamination.

The release of the Cibacron Blue F3GA from the dye-attached monosize beads was investigated at different pH values in the range of 4.0–8.0. It should be noted that these media were the same, which were used in the HSA adsorption experiments given below. Cibacron Blue F3GA release was also determined in the medium at 0.05 M Tris/HCl buffer containing 0.5 M NaSCN, which was the medium used the HSA elution experiments. The medium with the Cibacron Blue F3GA-attached beads was incubated for 24 h at room temperature. Then, beads were removed from the medium, and the Cibacron Blue F3GA concentration in the supernatant was measured by spectrophotometry at 630 nm.

2.4. Characterization of monosize beads

The amounts of attached Cibacron Blue F3GA on the monosize beads were obtained by using an elemental analysis instrument (Leco, CHNS-932, USA). The amount of Cibacron Blue F3GA attachment on the monosize beads was calculated by considering the sulfur stoichiometry.

The morphology and size of the monosize beads were observed by scanning electron microscopy (JEOL, JEM 1200 EX, Tokyo, Japan). Polymer samples were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed in the electron microscope.

Fourier transform infrared (FTIR) measurements were performed on a Shimadzu FTIR 8000 Series spectrometer in normal transmission mode using a KBr detector over the range of 400–4000 cm^{-1} at a 2 cm^{-1} resolution averaged over 64 scans. All spectra were baseline-corrected and normalized to a thickness of 1 μm. The polymer beads were degassed overnight in a vacuum oven maintained at 60 °C before FTIR measurements.

The epoxy group content in the synthesized poly(GMA) samples was determined by the HCl–pyridine titration method.

2.5. HSA removal from aqueous solutions

The effects of initial HSA concentration, pH, ionic strength and temperature on the adsorption capacity of Cibacron Blue F3GA-attached poly(GMA) beads were studied. The adsorption experiments were carried out batchwise in the media at different pH values. The pH of the adsorption medium was varied between 4.0 and 8.0 using different buffer systems (0.1 M CH_3COONa–CH_3COOH for pH 4.0–6.0, 0.1 M K_2HPO_4–KH_2PO_4, for pH 7.0 and 0.1 M Tris/HCl for pH 8.0). Ionic strength of the adsorption medium was changed in the range of 0.05–0.5 by adding NaCl. HSA concentration was varied between 0.5 and 8.0 mg/ml. In a typical adsorption experiment, HSA was dissolved in 100 ml of buffer solution, and 250 mg of monosize beads were added. Then the adsorption experiments were performed for 2 h at 25 °C at a stirring rate of 100 rpm. At the end of this equilibrium period, HSA adsorption was determined by measuring the initial and final concentration of HSA within the adsorption medium using Coomassie Brilliant Blue as described by Bradford [20]. The protein adsorption capacity was calculated by mass balance.

2.6. Elution studies

The elution of HSA was carried out using 0.05 Tris/HCl buffer containing 0.5 M NaSCN at room temperature. The HSA adsorbed beads (250 mg) were placed in the elution medium and stirred for 1 h, at 25 °C, at a stirring rate of 100 rpm. The final HSA concentration within the elution medium was determined by using Coomassie Brilliant Blue as described by Bradford [20]. The elution ratio was calculated from the amount of HSA adsorbed on the monosize beads and the amount of HSA eluted into the medium.

In order to test the reusability of the dye-attached poly(GMA) beads, HSA adsorption–elution procedure was repeated 20 times by using the same polymeric adsorbent. It should be also noted that, after elution of HSA, possible dye release was also monitored.

2.7. HSA removal from human serum

HSA adsorption from human serum with Cibacron Blue F3GA-attached poly(GMA) beads was studied batch-wise. The blood is collected from thoroughly controlled voluntary blood donors. Each unit separately controlled and found negative for *hepatitis B-specific* antigen and HIV I, II and hepatitis C antibodies. No preservatives are added to the samples. Blood samples were centrifuged at 500 × *g* for 3 min at room temperature to separate the serum. The serum samples were filtered using 0.45 μm cellulose acetate microspin filters (Alltech, Deerfield IL, USA). The original serum of the healty donor contained 38.3 mg HSA/ml as determined by bromocresol green (BCG) dye method at 628 nm [21]. *Total protein content of crude and depleted serum samples were determined using the DC Protein Assay (Bio-Rad) according to the manufacturers instructions with bovine serum albumin (BSA) as standards (Pierce, Rockford, IL, USA). Total protein concentration in crude serum was 62 mg/ml. In order to remove IgG*, the freshly separated human serum (100 ml) was pumped into the Protein A-sepharose column (10 cm × 0.9 cm inside diameter) equipped with a water jacket for temperature control. Equilibration of the *Protein A-Sepharose 4B* column (Sigma) was performed by passing four column volumes of sodium acetate buffer (pH 5.2) before injection of the serum. *When serum passes through the column, the IgG molecules adsorbed on the Protein A-Sepharose 4B adsorbent. The treated serum, which passed from the column, consists mainly of albumin and other serum proteins*. After that the serum is ready for dye-affinity removal of albumin. Analysis of IgG was performed by a nephelometer assay (Beckman Array 360, USA). *The concentration of IgG in crude serum was determined to 8.5 mg/ml*. The concentration of remaning IgG in serum sample was very low. This stated a depletion ratio of 99% IgG in serum sample. Then, 25 ml of the freshly separated human serum was incubated with 250 mg of polymer beads pre-equilibrated

with acetate buffer (pH 5.0) for 2 h. These experiments were conducted at 20 °C and a stirring rate of 100 rpm. The amount of HSA adsorbed by Cibacron Blue F3GA-beads was determined by measuring the initial and final concentration of HSA in serum. Phosphate buffered saline (PBS, pH 7.4, NaCl: 0.9%) was used for dilution of human serum. In order to test the dye performance, two-dimensional gel electrophoresis (2DE) was carried out as described in details previously [22].

3. Results and discussion

3.1. Characteristics of poly(GMA) beads

Fig. 1 shows a representative SEM picture of the monosize (R.S.D. <1%) poly(GMA) beads with a diameter of 1.6 μm. As seen here, micropheres were obtained in the highly monosize form. Polydispersity index (PDI) value of poly(GMA) beads was calculated to be around 1.006. FTIR spectroscopy was used to show attachment of Cibacron Blue F3GA within poly(GMA) beads (Fig. 2). These spectra were thickness-normalized and base-line-corrected and no other processing was performed. There are five main vibrational modes: oxirane groups (910 cm^{-1}), aromatic C=C vibration (1075 cm^{-1}), symmetric stretching of S=O (1155 cm^{-1}), asymmetric stretching of S=O (1280 cm^{-1}) and aromatic C–N vibration (3400 cm^{-1}) as also pointed out on the chemical structure of the dye (Fig. 3). These absorption bands may be considered as an indication of the presence of Cibacron Blue F3GA within the poly(GMA) beads.

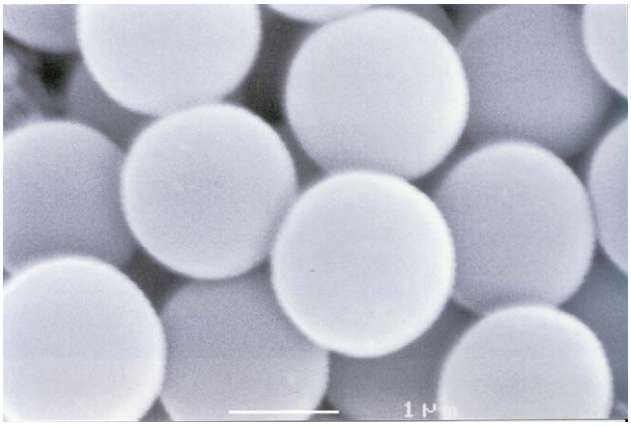

Fig. 1. SEM photograph of monosize poly(GMA) beads.

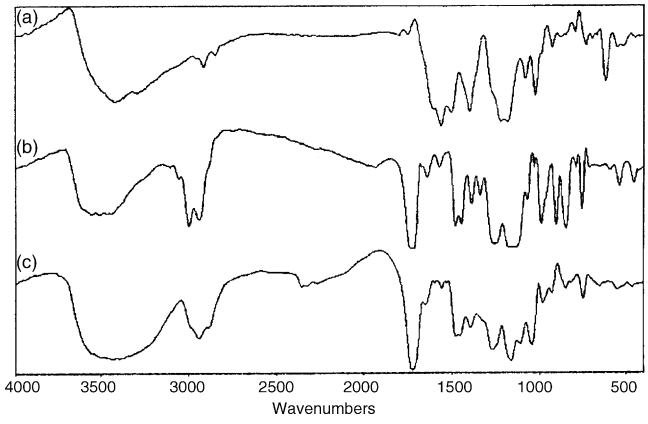

Fig. 2. FTIR absorbance spectra of (a) poly(GMA) beads and (b) Cibacron Blue F3GA-attached poly(GMA) beads. Baseline correction and thickness normalization were only processes that were done on these spectra.

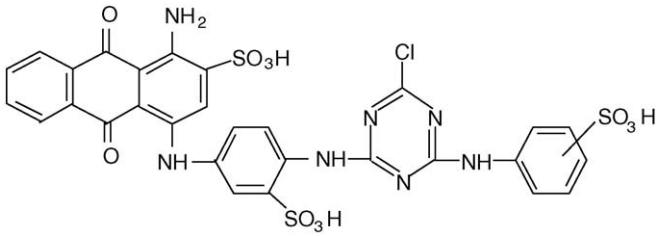

Fig. 3. Chemical structure of Cibacron Blue F3GA.

The theoretical content of epoxy groups, calculated on the basis of the feed composition, i.e. GMA content in the monomer mixture was 4.2 mmol/g. The content of epoxy groups on the surface of the poly(GMA) sample determined by the HCl–pyridine method differs from the theoretical value (3.8 mmol/g). Some of the epoxy groups usually remain inside a poly(GMA) beads and are not accessible for subsequent reactions or for analytical determinations.

Dye loading was 1.73 mmol Cibacron Blue F3GA/g polymer. The visual observations (the colour of the monosize beads) ensured attachment of dye molecules. The Cibacron Blue F3GA-attached beads were extensively washed with methanol, until it was ensured that there was no dye leakage from any of the dye-attached beads and in any media used at adsorption–elution steps. The release of dye-molecules was also measured in three different kinds of media. There was no measurable release of dye into the acidic medium (pH 2.0). Dye was released in the neutral medium while some were released in the alkaline medium too. The release in the strongly alkaline medium indicates the existance of strong ionic interactons. The release in neutral medium might just be the physically occluded dye along with any weakly/physically bonded dye. It can be said that there was not a significant increase in the amount of dye released (more than 20 weeks).

3.2. HSA removal from aqueous solutions

Effect of albumin concentration: Fig. 4 shows the HSA adsorption isotherm of the poly(GMA) and dye-attached poly(GMA) beads. Note that one of the main requirements in affinity chromatography is the specificity of the adsorbent. The non-specific interaction between the support, which is the poly(GMA) beads in the present case, and the molecules to be adsorbed, which are the HSA molecules here, should be minimum in order to consider the interaction as specific. As seen in this figure, non-specific HSA adsorption was 0.8 mg/g. This non-specific adsorption may be contributed to either non-specific interactions of the poly(GMA) matrix or the reaction of HSA with epoxide groups. While dye-attachment significantly increased the HSA binding capacity of the beads (up to

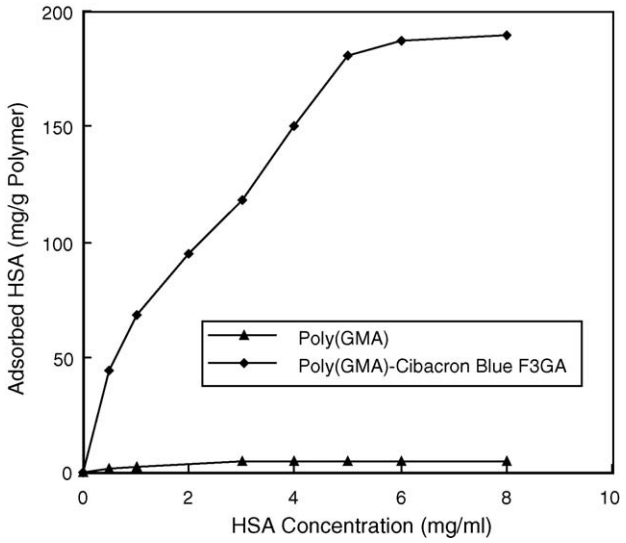

Fig. 4. Effect of HSA concentration on adsorption capacity. Dye loading: 1.73 mmol/g; pH 5.0; *T*: 25 °C.

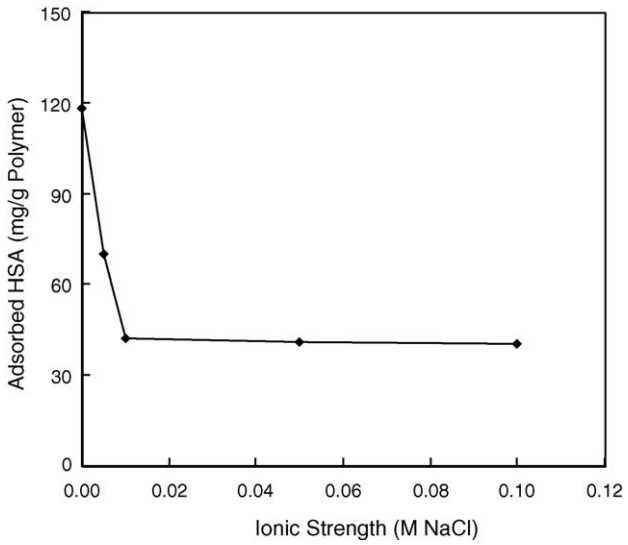

Fig. 6. Effect of ionic strength on adsorption capacity. Dye loading: 1.73 mmol/g; HSA concentration: 3.0 mg/ml; pH 5.0, and *T*: 20 °C.

189.8 mg/g). The amount of HSA adsorbed per unit mass of the dye-affinity beads increased first with the initial concentration of HSA then reached a plateau value which represents saturation of the active adsorption sites (which are available and accessible for HSA) on the beads. This increase in the HSA binding capacity may have resulted from cooperative effect of different interaction mechanisms such as hydrophobic, electrostatic and hydrogen bonding caused by the acidic groups and aromatic structures on the Cibacron Blue F3GA and by groups on the side chains of amino acids on the HSA molecules. It should be mentioned that Cibacron Blue F3GA is not very hydrophobic overall, but it has planar aromatic surfaces that prefer to interact with hydrophobic groups in HSA structure [23].

Effect of pH: The pH was changed from 4.0 (below the isoelectric point of HSA) to 8.0 (above the isoelectric point of HSA). Fig. 5 shows the effects of pH on the adsorption of HSA

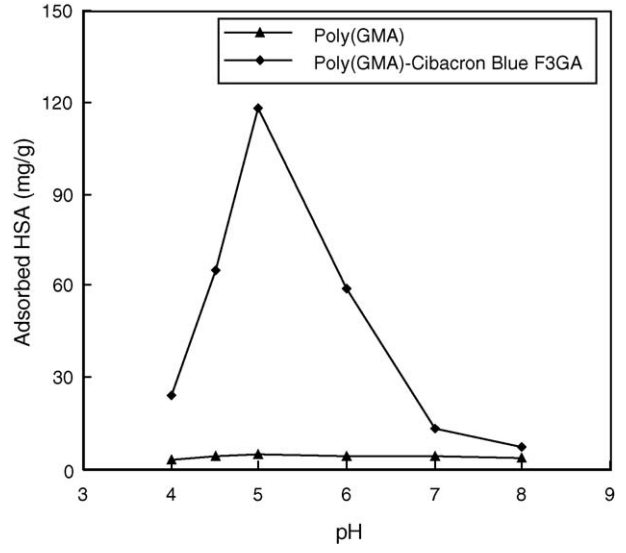

Fig. 5. Effect of pH on adsorption capacity. Dye loading: 1.73 mmol/g; HSA concentration: 3.0 mg/ml; *T*: 25 °C.

to Cibacron Blue F3GA-attached poly(GMA) beads. No significant effect of pH was observed on the physical adsorption of HSA onto the poly(GMA) beads. In all the cases investigated, the maximum adsorption of HSA was observed at pH 5.0. Significantly lower adsorption capacities were obtained in more acidic and in more alkaline pH regions. The decrease in the HSA adsorption capacity in more acidic and more alkaline pH regions can be attributed to electrostatic repulsion effects between the identical charged protein molecules on the surface of the poly(GMA) beads. As it has been shown that proteins have no net charge at their isoelectric points and so the protein solubility in auqeous media decreases. Thus, the maximum adsorption from aqueous solutions is usually observed at their isolectric points [24]. The isoelectric pH of HSA is 4.9 [25]. However, acidic and basic medium caused the protein to bepositively or negatively charged, increasing the solubility of protein in media.

Effect of NaCl concentration: The effect of salt concentration on HSA adsorption is shown in Fig. 6. With an increase of the aqueous phase concentration of sodium chloride, the adsorption capacity decreased drastically. Increasing the ionic strength could promote the adsorption of the dye molecules to the poly(GMA) surface by hydrophobic interaction [26,27]. Moreover, the hydrophobic interactions between the attached dye molecules themselves would also become strong, because it has been observed that the salt addition to a dye solution caused the stacking of the free dye molecules [28]. Thus, the numbers of the attached dye molecules accessible to HSA would decrease as the ionic strength increased, and the adsorption of the HSA to attached dye became difficult. In addition, the ionic interactions decrease with increasing ionic strength due to the Debye screening effect [29]. The adsorption capacity decreased significantly from 118.3 to 40.5 mg/g polymer with the increase of the NaCl concentration from 0 to 0.1 M. The adsorption amount of HSA decreased by about 65.7%.

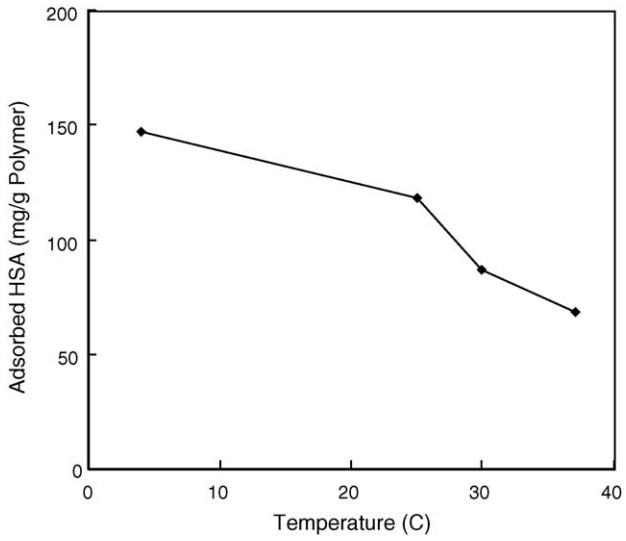

Fig. 7. Effect of temperature on adsorption capacity. Dye Loading: 1.73 mmol/g; HSA concentration: 3.0 mg/ml; pH 5.0.

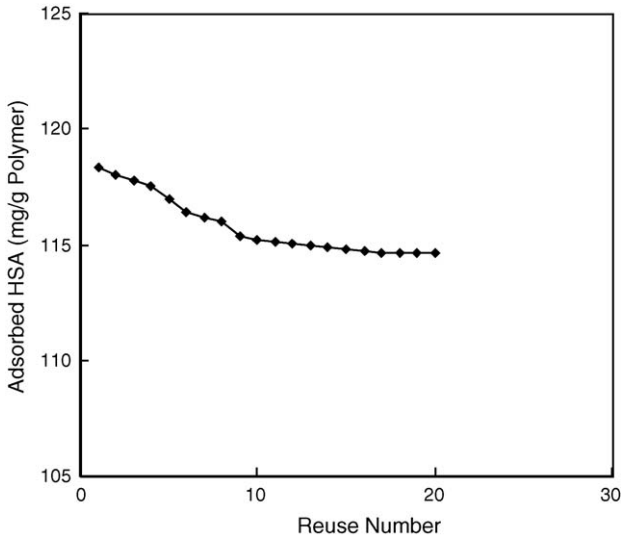

Fig. 8. Repeated use of poly(GMA)-Cibacron Blue F3GA beads. Dye loading: 1.73 mmol/g; HSA concentration: 3.0 mg/ml; pH 5.0 and *T*: 25 °C.

Effect of temperature: The effect of temperature on the equilibrium HSA adsorption capacity of the dye-affinity beads was given in Fig. 7. At all temperatures, non-specific adsorption of HSA due to van der Waals force was very low (0.8 mg/g). No significant effect of temperature was observed on the physical adsorption of HSA onto the poly(GMA) beads. However, the equilibrium adsorption of HSA onto the Cibacron Blue F3GA-attached poly(GMA) beads significantly decreased with increasing temperature and the maximum HSA adsorption was achieved at 4 °C (118.3 mg/g). *It is well known that adsorption is an exothermic process, so adsorption capacity usually decreases with increasing temperature*.

Elution: The elution of HSA from Cibacron Blue F3GA-attached poly(GMA) beads was studied in a batch system. Dye-affinity beads loaded with different amounts of HSA were placed in an elution medium containing 0.05 M Tris/HCl buffer containing 0.5 M NaSCN and the amount of HSA released in 60 min was determined. It should be noted that the pH value was adjusted in the NaSCN containing buffer. Table 1 gives the elution data. More than 95% of the adsorbed HSA was eluted in all cases when NaSCN was used for elution. SCN^-, being a chaotropic ion, disorganized the structure of water, thus stimulating the elution of protein [30]. Note that there was no dye release in this case which shows that Cibacron Blue F3GA molecules are attached to the beads surface by strong chemical bond. With the elution data given above we concluded that 50 mM Tris/HCl buffer (*pH 8.0*) containing 0.5 M NaSCN is a suitable elution agent especially for the Cibacron Blue F3GA carrying beads, and allows repeated use of the affinity adsorbents developed in this study.

Table 1
Elution of HSA

Concentration (mg/ml)	HSA adsorbed (mg/g)	HSA eluted (%)
1.00	68.04	94.6
2.00	95.02	96.4
3.00	118.32	95.5
4.00	150.04	96.0
6.00	187.51	97.2
8.00	189.80	94.3

In order to show the reusability of the Cibacron Blue F3GA-attached beads, adsorption–elution cycles of HSA were repeated 20 times using the same poly(GMA) beads. *After the 20th run a slight decrease of ca. 3% in albumin adsorption capacity has been observed with re-use of the beads (Fig. 8)*. The possibility to regenerate the micron size dye-affinity beads was also considered as a major advantage. Moreover, no obvious changes of the monosize beads were found in the recycling process. These results demonstrated that these fine beads had high stability during the removal process.

3.3. Albumin removal from serum

Removal of additional abundant proteins can be beneficial in the analysis of serum proteins, and therefore we attempted to remove the IgG class immunoglobulins from human serum. *In the first step of this study, the removal of IgG class proteins was achieved by using Protein A-sepharose 4B column. Protein A binds to the Fc region of the IgG [31]. The depletion efficiency for IgG class proteins was 99% in serum sample. Then, in the second step*, the removal of HSA from human serum was performed batch-wise with dye-affinity beads. *The depletion efficiencies for HSA were above 87% for all studied concentrations*. Results, shown in Table 2, indicate that a large portion of the HSA was bound by the monosize dye-affinity beads. To test the efficiency of HSA removal from human serum, proteins in the serum and eluted portion were analyzed by *two-dimensional gel electrophoresis*. Proteins that were eluted from the dye-affinity beads include albumin, many albumin fragments, and a small number of nonalbumin proteins. A small number of relatively abundant proteins such as apo-lipoprotein A1, serotransferrin, haptoglobulin and α1-antitrypsin were bound by the dye-affinity beads. IgA was not identified in eluted fraction. *The*

Table 2
HSA depletion from the serum of a healthy donor

HSA concentration (mg/ml)	HSA adsorbed[a] (mg/g)	Achieved depletion (%)
0.08	2.81 ± 0.2	94.7
0.15	5.94 ± 0.5	97.5
0.30	11.93 ± 1.0	99.3
0.60	21.80 ± 1.2	90.8
1.20	42.62 ± 2.6	88.8
2.40	84.22 ± 2.9	87.7

Dye loading: 1.73 mmol/g; *T*: 25 °C.
[a] Each data is average of five parallel studies.

presence of these additional proteins may be a consequence of non-specific retention on the dye-affinity beads during adsorption. However, specific binding of proteins to albumin may also be a contributing factor, as albumin is well known as a carrier and transport protein in serum. These results are consistent with published studies [31,32]. Björhal et al. [1] used five different commercially available depletion columns including Aurum Serum Protein Minikit (Bio-Rad, USA), ProteoExtract Albumin/IgG Removal kit (Merck, Germany), Multiple Affinity Removal Column (Agilent Technologies, USA), POROS Affinity Depletion Cartridges (Applied Biosystems, USA) and Albumin-IgG Removal Kit (Amersham Biosciences, Sweden). Aurum Serum Protein Minikit (Bio-Rad, USA) contained Cibacron Blue F3GA as a ligand and they showed minimum achieved albumin depletion was 96.3%. We reached up to 99.3% albumin depletion amount and it may be concluded that dye-affinity monosize poly(GMA) beads are sufficient in terms of efficiency of albumin removal.

4. Conclusions

The serum proteome has been shown to contain information that directly reflects pathophysiological states and represents an invaluable source of diagnostic information for a variety of diseases [33]. Unfortunately, the dynamic range of protein abundance renders complete characterization of this proteome nearly impossible with current analytical methods [34]. To study low abundance proteins, which have potential value for clinical diagnosis, the high abundant species, such as albumin and immunoglobulins, are generally eliminated as the first step in many analytical protocols. But, removal of albumin from serum is problematic because of its extremely high concentration [35]. Monoclonal antibodies to human serum albumin are available from many commercial suppliers. These antibody ligands are extremely specific. But, they are very expensive, due to high cost of production and/or extensive purification steps. In the process of the preparation of specific adsorbents, it is difficult to immobilize antibodies on the supporting matrix with retention of their original biological activity and proper orientation. Precautions are also required in their use (at adsorption and elution steps) and storage. However, albumin is present in human serum at concentrations in the range of 35–45 mg/ml and very large quantities of antibody are required for its quantitative removal. In addition, high capacity adsorbent is required. Synthetic *dye*-ligands should display not only reduced process costs, but also increased resistance to chemical and biological actions, reduction in the amount of contaminants of biological nature and high capacity. Our goal was to find a cost effective and reusable dye-affinity adsorbent having high adsorption capacity for removal of albumin from human serum. *The amount of HSA adsorbed per unit mass of the dye-affinity beads increased then reached a plateau value. This increase in the HSA binding capacity may have resulted from cooperative effect of different interaction mechanisms such as hydrophobic, electrostatic and hydrogen bonding. With an increase of the aqueous phase concentration of sodium chloride, the adsorption capacity decreased drastically due to the Debye screening effect. More than 95% of the adsorbed HSA was eluted in all cases when NaSCN was used for elution. After the 20th run a slight decrease of ca. 3% in albumin adsorption capacity has been observed with reuse of the beads. Up to 99.3% albumin depletion amount was achieved and it may be concluded that dye-affinity beads are sufficient in terms of efficiency of albumin removal*. Based on our evaluations of depletion efficiency, reproducibility and binding specificity of the dye-affinity beads offered the promising depletion approach.

The eluted proteins were assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis using 10% separating gel (9 cm × 7.5 cm) and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid–methanol–water (1:5:5, v/v/v) and destained in ethanol–acetic acid–water (1:4:6, v/v/v). Electrophoresis was run for 2 h with a voltage of 110 V. Human serum albumin, lysozyme and IgG were used as standards.

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