Poly(acrylamide-allyl glycidyl ether) Cryogel as a Novel Stationary Phase in Dye-Affinity Chromatography

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ABSTRACT: Poly(acrylamide-allyl glycidyl ether) [poly-(AAm-AGE)] cryogel was prepared by bulk polymerization which proceeds in an aqueous solution of monomers frozen inside a glass column (cryo-polymerization). After thawing, the monolithic cryogel contains a continuous polymeric matrix having interconnected pores of 10–100 μm size. Cibacron Blue F3GA was immobilized by covalent binding onto poly(AAm-AGE) cryogel via epoxy groups. Poly(AAm-AGE) cryogel was characterized by swelling studies, FTIR, scanning electron microscopy, and elemental analysis. The equilibrium swelling degree of the poly-(AAm-AGE) monolithic cryogel was 6.84 g H_2O/g cryogel. Poly(AAm-AGE) cryogel containing 68.9 μmol Cibacron Blue F3GA/g was used in the adsorption/desorption of human serum albumin (HSA) from aqueous solutions and human plasma. The nonspecific adsorption of HSA was very low (0.2 mg/g). The maximum amount of HSA adsorption from aqueous solution in acetate buffer was 27 mg/g at pH 5.0. Higher HSA adsorption value was obtained from human plasma (up to 74.2 mg/g). Desorption of HSA with a purity of 92% from Cibacron Blue F3GA attached poly(AAm-AGE) cryogel was achieved using 0.1*M* Tris/HCl buffer containing 0.5*M* NaCl. It was observed that HSA could be repeatedly adsorbed and desorbed with poly(AAm-AGE) cryogel without significant loss in the adsorption capacity. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 1808–1816, 2007

Key words: dye-affinity chromatography; cryogels; protein purification; albumin

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein present in blood plasma.[1] It consists of a single, nonglycosylated, polypeptide chain containing 585 amino acid residues, and has many physiological functions which contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution, and metabolism of many endogeneous and exogeneous substances including bile acids, bilirubin, long-chain fatty acids, amino acids (notably tryptophan, thyrosine and cysteine), steroids (progesterone, testosterone, aldosterone, cortisol), metal ions such as copper, zinc, calcium and magnesium, and numerous pharmaceuticals.[2] HSA commonly used for therapeutic purposes such as shock, burns, hypoalbuminemia or hypoproteinemia, surgery, trauma, cardiopulmonary bypass, the acute respiratory distress syndrome, hemodialysis, acute nephrosis, hyperbilirubinemia, acute liver failure, ascites and sequestration of protein-rich fluids in acute peritonitis, pancreatitis, mediastinitis, and extensive cellulitis.[3] Albumin therapy has multiple effects including volume expansion, increased serum albumin concentration and colloid osmotic pressure, and hemodilution. HSA is at present commonly isolated from human plasma by Cohn's classical blood fractionation procedure.[4] Albumin is currently used in greater volume than any other biopharmaceutical solution that is available, and worldwide manufacturing is of the order of hundreds of tonnes annually. Cohn's method concerns precipitation of proteins using ethanol with varying pH, ionic strength, and temperature. But this technique, which is the oldest method of industrial fractionation of blood proteins, is not highly specific and can give partially denaturated proteins.[5]

Recently, dye-ligand affinity chromatography has been used extensively in laboratory and large scale protein purification.[6–10] Dye-ligands are commercially available, inexpensive, and can easily be immobilized, especially on matrices bearing hydroxyl groups. Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins mimicking the structure of the substrates, cofactors, or binding agents for those proteins. A number of textile dyes, known as reactive dyes, have been used for protein purification. Most of these reactive dyes consist of a chromophore (either azo dyes, anthraquinone, or phathalocyanine),

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linked to a reactive group (often a mono- or dichloro-triazine ring). The interaction between the dye ligand and proteins can be by complex combination of electrostatic, hydrophobic, and hydrogen bonding. Cibacron Blue F3GA is an anthraquinone textile dye that interacts specifically and reversibly with albumin.[11]

Conventional packed-bed columns possess some inherent limitations such as the slow diffusional mass transfer and the large void volume between the beads.[12] Although some new stationary phases such as the non-porous polymeric beads[13] and perfusion chromatography packings are designed to resolve these problems, these limitations cannot be overcome in essence.[14] Recently, cryogel materials are considered as a novel generation of stationary phases in the separation science.[15–21] Cryogels are a very good alternative to protein purification with many advantages. Several advantages of cryogels are large pores, short diffusion path, low pressure drop, and very short residence time for both adsorption and elution. Cryogels are also cheap materials and they can be used as disposable avoiding cross-contamination between batches.

This work reports on the purification of HSA from human plasma by dye affinity chromatography with a cryogel column. Poly(acrylamide-allyl glycidyl ether) [poly(AAm-AGE)] cryogel was prepared by bulk polymerization which proceeds in aqueous solution of monomers frozen inside a glass column (cryo-polymerization). Poly(AAm-AGE) cryogel was characterized using FTIR, scanning electron microscope (SEM), elemental analysis, and swelling test. HSA adsorption on the poly(AAm-AGE) cryogel from aqueous solutions containing different amounts of HSA, at different pH's and ionic strengths, and also from human plasma was also performed. In the last part, desorption of HSA and stability of these materials was tested.

EXPERIMENTAL

Materials

Acrylamide (AAm, more than 99.9% pure, electrophoresis reagent), allyl glycidyl ether (AGE, 99%), N,N′-methylene-bis(acrylamide) (MBAAm), and amonium persulfate (APS) were supplied from Sigma (St. Louis, MO). *N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). Cibacron Blue F3GA was obtained from Polyscience (Warrington, USA) and used without further purification. HSA (98% pure by gel electrophoresis, fatty acid free, 67 kDa) was purchased from Aldrich (Munich, Germany). All other chemicals were of the highest purity commercially available and were used without further purification. Coomassie Blue for the Bradford protein assay was from BioRad (Richmond, CA). 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. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before using the glassware was rinsed with deionized water and dried in a dust-free environment.

Production of poly(AAm-AGE) cryogel

Production of poly(AAm-AGE) cryogel was performed using the Arvidsson et al.'s procedure.[22] AGE was selected to insert reactive epoxy groups in the cryogel. Briefly, monomers (10 mL of AAm, 1 mL of AGE) were dissolved in deionized water, and the mixture was degassed under vacuum for about 5 min to eliminate soluble oxygen. Total concentration of monomers was 6% (w/v). The cryogel was produced by free radical polymerization initiated by TEMED (120 μL) and APS (100 mg). After adding APS (1% (w/v) of the total monomers), the solution was cooled in an ice bath for 2–3 min. TEMED (1% (w/v) of the total monomers) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (5 mL, id. 0.8 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at −12°C for 24 h and then thawed at room temperature. Extensive cleaning procedure for removal of unconverted monomers and initiator was performed. Briefly, washing solutions (i.e. a dilute HCl solution and a water-ethanol mixture) were recirculated through the monolithic cryogel column, until to be assured that the cryogel column is clean. Purity of the monolithic cryogel was followed by observing the change of optical densities of the samples taken from the liquid phase in the recirculation system, and also from the DSC thermograms of the cryogel obtained by using a differential scanning microcalorimeter (Mettler, Switzerland). Optical density of the original monolithic cryogel was 1.67. But after the applying of cleaning procedure this value was reduced to 0.02. In addition, when the thermogram of the uncleaned monolithic cryogel was recorded, it has a peak around 50°C. This peak might be originated from TEMED. But after applying of this cleaning procedure, between 30 and 100°C any peak was not observed on this thermogram. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

Cibacron Blue F3GA immobilization

Cibacron Blue F3GA immobilization studies were carried out in a recirculating system equipped with a water jacket for temperature control. The cryogel

was washed with 30 mL of water. Then, 100 mL of Cibacron Blue F3GA solution (5 mg/mL) containing NaOH (5 g) was pumped through the glass column under recirculation at 80°C for 2 h. Under these experimental conditions, a chemical reaction took place between the chloride group of the Cibacron Blue F3GA and the epoxide group of the poly(AAm-AGE) cryogel. The adsorption was followed by monitoring the decrease in UV absorbance at 630 nm. After incubation, the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was washed with distilled water and methanol, until all the physically adsorbed Cibacron Blue F3GA was removed. The modified cryogel was then stored at 4°C with 0.02% sodium azide to prevent microbial contamination.

Characterization of cryogel

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed on porous filter until washing was clear. Then it was sucked dry and then transferred to preweighed vial and weighed ($m_{\text{wet gel}}$). After drying to constant mass in the oven at 60°C, the mass of dried sample was determined ($m_{\text{dry gel}}$). The swelling degree was calculated as:

$$S = (m_{\text{wet gel}} - m_{\text{dry gel}})/m_{\text{dry gel}} \quad (1)$$

The morphology of a cross section of the dried cryogel was investigated by SEM. The sample was fixed in 2.5% gluaraldehyde in 0.15M sodium cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to +10°C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40°C and the pressure to $\sim$ 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of +40°C resulted in dried cryogel sample. Finally, it was coated with gold–palladium (40 : 60) and examined using a JEOL JSM 5600 SEM (Tokyo, Japan).

FTIR spectra of the Cibacron Blue F3GA, the poly(AAm-AGE) cryogel, and Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

To evaluate Cibacron Blue F3GA immobilization amount, the poly(AAm-AGE) cryogel was subjected to elemental analysis using a Leco Elemental Analyzer (Model CHNS-932, USA).

Chromatographic procedures

HSA-adsorption from aqueous solutions

The HSA adsorption studies were carried out in a column system equipped with a water jacket for temperature control. The cryogel was washed with 30 mL of water and then equilibrated with 25 mM phosphate buffer containing 0.1M NaCl (pH 7.4). Then, the prepared HSA solution (50 mL of the aqueous HSA solution) was pumped through the column for 2 h. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. Effects of HSA concentration, pH of the medium, and ionic strength on the adsorption capacity were studied. The flow rate of the solution was changed in the range of 0.2–2.0 mL/min. To observe the effects of the initial concentration of HSA on adsorption, it was changed between 0.1–3.0 mg/mL. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of ionic strength, NaCl solution was used at ionic strength values of 0.01 and 0.6.

Desorption and repeated use

In all cases, adsorbed HSA molecules were desorbed using 0.1M Tris/HCl buffer containing 0.5M NaCl. In a typical desorption experiment, 50 mL of desorption agent was pumped through the cryogel at a flow rate of 0.2 mL/min for 1 h. The final HSA concentration in the desorption medium was spectroscopically determined. When desorption was achieved, the cryogel was cleaned with 1M NaOH and then re-equilibrated with 25 mM phosphate buffer containing 0.1M NaCl (pH 7.4). The desorption ratio was calculated from the amount of HSA adsorbed on the cryogel and the final HSA concentration in the desorption medium. To test the repeated use of poly(AAm-AGE) cryogel, HSA adsorption-desorption cycle was repeated for 10 times using the same cryogel column. To regenerate and sterilize, after desorption, the cryogel was washed with 1M NaOH solution.

HSA-purification from human plasma

Human blood is collected from thoroughly controlled voluntary blood donors. Each unit separately controlled and found negative for HBS antigen and HIV I, II, and hepatitis C antibodies. No preservatives are added to the samples. Human blood was collected into EDTA-containing vacutainers and red blood cells were separated from plasma by centrifugation at 4000 $\times$ g for 30 min at room temperature,

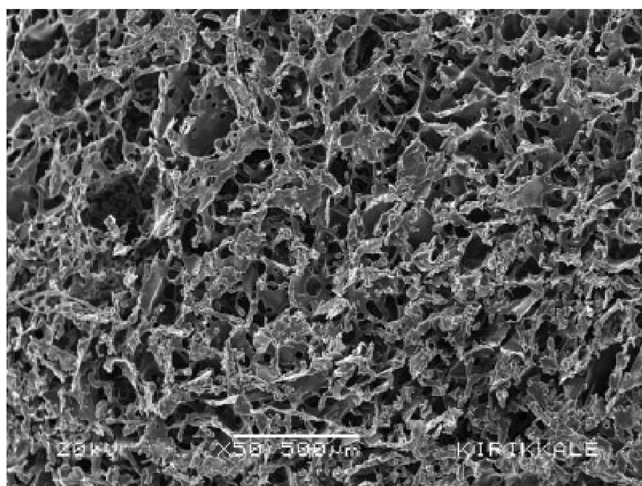

Figure 1 SEM of the inner part of the supermacroporous poly(AAm-AGE) monolithic cryogel matrix.

then filtered (3 μm Sartorius filter) and frozen at −20°C. Before use, the plasma was thawed for 1 h at 37°C. Before application, the viscous sample was diluted with 25 m*M* phosphate buffer containing 0.1*M* NaCl (pH 7.4). Dilution ratios were 1/2 and 1/10. 50 mL of the human plasma with a HSA content of 37.7 mg/mL was pumped through the cryogel column at a flow rate of 0.2 mL/min for 2 h. HSA concentration was determined by using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics, Halstead, Essex, England; Catalog Ref. No: 229241) which is based on bromocresol green (BCG) dye method.[23] To show dye specificity, adsorption of other blood proteins (i.e., fibrinogen and γ-globulins) was also monitored. Total protein concentration was measured by using the total protein reagent (Ciba Corning Diagnostics, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm which based on Biuret reaction.[23] Chronometric determination of fibrinogen according to the Clauss method on plasma was performed by using Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France).[24] γ-Globulin concentration was determined from the difference.

RESULTS AND DISCUSSION

A supermacroporous monolithic cryogel was produced by polymerization in the frozen state of monomers, acrylamide (AAm), and allyl glycidyl ether (AGE) with *N*,*N*′-methylene-bis(acrylamide) (MBAAm) as a crosslinker in the presence of APS/*N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED) as initiator/activator pair. The functional epoxy groups on the surface of the pores in monolithic cryogels allowed their modification with the ligand, Cibacron Blue F3GA. The SEM of the internal structure of the monolithic cryogel is shown in Figure 1. Poly(AAm-AGE) cryogel produced in such a way have non-porous and thin polymer walls, large continuous interconnected pores (10–100 μm in diameter) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. The general shape of HSA can be viewed as three tennis balls in a can or cylinder. The Stokes radius is 3.9 nm. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. The equilibrium swelling degree of the poly(AAm-AGE) monolithic cryogel was 6.84 g H_2O/g dry cryogel. Poly(AAm-AGE) monolithic cryogel is opaque, sponge like, and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1–2 s restored its original size and shape.

Biomimetic dye-ligand Cibacron Blue F3GA is covalently attached on poly(AAm-AGE) cryogel. The FTIR bands observed at 1160 cm^{-1} was assigned to symmetric stretching of S=O, as also pointed out on the chemical structure of the Cibacron Blue F3GA (Fig. 2). The split of the band at 3300–3500 cm^{-1} indicates also SO_3H and NH_2 groups. These bands show the attachment of Cibacron Blue F3GA within the poly(AAm-AGE) cryogel. The visual observations (the color of the cryogel) ensured attachment of dye molecules. The dye content was 68.9 μmol/g dry cryogel. Note that AAm, AGE, and other chemicals in the polymerization formula do not contain sulphur. This sulphur amount determined by elemental analysis originated from only immobilized dye into the polymeric structure.

The Cibaron Blue F3GA-immobilized cryogel was extensively washed with methanol until to ensure that there is no dye leakage from any of the dye-immobilized cryogel and in any media used at

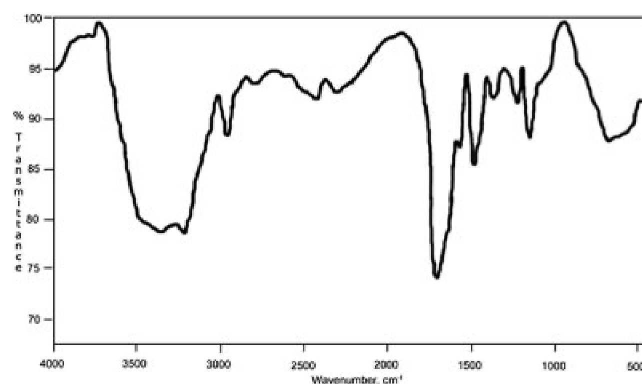

Figure 2 FTIR spectrum of Cibacron Blue F3GA attached poly(AAm-AGE).

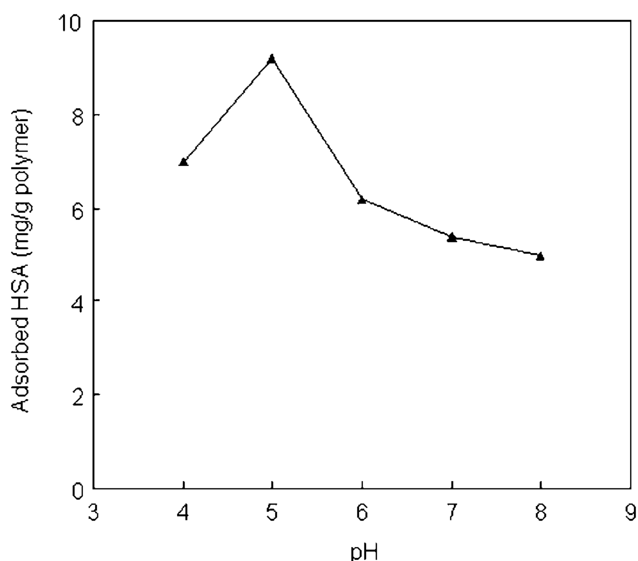

Figure 3 Effect of medium pH on the HSA adsorption. Cibacron Blue F3GA content, 68.9 μmol/g; HSA concentration, 0.5 mg/mL; flow rate, 0.2 mL/min; T, 20°C. Each point is an average of five parallel studies.

adsorption-desorption 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 was released in the alkaline medium too. The release in the strongly alkaline medium indicates the existence of strong ionic interactions. The release in neutral medium might just be the physically occluded dye along with any weakly/physically bonded dye. The studies of Cibacron Blue F3GA leakage from the poly-(AAM-AGE) cryogel showed that there was no dye leakage in any medium used throughout this study, even after long period of time (more than 24 weeks).

HSA adsorption from aqueous solutions

Effects of pH

Figure 3 shows the effect of pH on the adsorption of HSA onto Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel. The maximum adsorption of HSA was observed at pH 5.0, which is the isoelectric point of HSA. With the increase of pH above and below the pH 5.0, the HSA adsorption capacity decreased. The decrease in the HSA adsorption capacity can be attributed to electrostatic repulsion effects between the identically charged groups. At the isoelectric points, proteins have no net charge and therefore, the maximum protein adsorption from aqueous solution is usually observed at this point. In addition, these interactions between the dye and protein molecules may result both from the ionization states of several groups on both the ligands (i.e. Cibacron Blue F3GA) and amino acid side chains in HSA structure, and from the conformational state of protein molecules at this pH. It should be also noted that nonspecific adsorption (i.e. adsorption on poly(AAm-AGe) cryogel) was independent of pH and it was observed at the same at all the pH values studied.

Effects of HSA concentration

Figure 4 shows the HSA adsorption isotherm of the plain and dye-affinity cryogels. Note that one of the main requirements in dye-affinity chromatography is the specificity of the affinity adsorbent for the target molecule. The nonspecific interaction between the support, which is the poly(AAm-AGE) cryogel in the present case, and the molecules to be adsorbed, which are the HSA molecules here should be minimum to consider the interaction as specific. As seen in this figure, negligible amount of HSA was adsorbed nonspecifically on the poly(AAm-AGE) cryogel, which was 0.2 mg/g dry cryogel. While dye-immobilization significantly increased the HSA coupling capacity of the cryogel (up to 27 mg/g). The amount of HSA adsorbed per unit mass of the poly(AAm-AGE) cryogel 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 cryogel. This increase in the HSA binding capacity may have resulted from cooperative effect of different interaction mechanisms such as hydrophobic, electrostatic, and hydrogen bonding

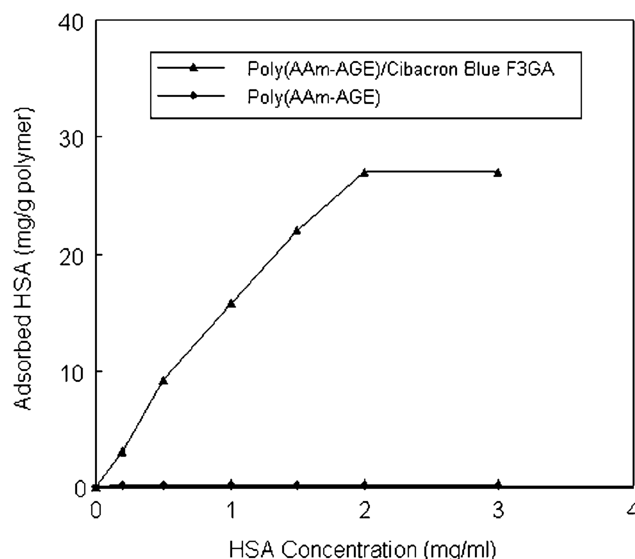

Figure 4 Effect of the concentration of HSA on the HSA adsorption. Cibacron Blue F3GA content, 68.9 μmol/g; pH, 5.0; Flow rate, 0.2 mL/min; T, 20°C. Each point is an average of five parallel studies.

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.

Adsorption isotherms

Two important physicochemical aspects for evaluation of the adsorption process as a unit operation are the kinetics and the equilibria of adsorption. Modeling of the equilibrium data has been done using the Langmuir and Freundlich isotherms.[25] The Langmuir and Freundlich isotherms are represented as follows eqs (2) and (3), respectively.

$$1/q_e = (1/g_{\max}) + [1/(g_{\max} b)](1/C_e) \quad (2)$$

$$\ln q_e = 1/n(\ln C_e) + \ln K_F \quad (3)$$

where b is the Langmuir isotherm constant, K_F is the Freundlich constant, and n is the Freundlich exponent. $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The ratio of q_e gives the theoretical monolayer saturation capacity of magnetic beads.

Some model parameters were determined by nonlinear regression with commercially available software and are shown in Table I. Comparison of all theoretical approaches used in this study shows that the Freundlich equation fits the experimental data best.

Comparison with other supports

Different support systems have been used for albumin adsorption/purification from various sources including human serum. Nigel et al. used dye-incorporated Sepharose CL-6B-200 and they reported bovine serum albumin (BSA) adsorption capacities around 1–3 mg/g moist gel.[26] Denizli et al. used dye affinity supports including monosize poly(glycidyl methacrylate) and poly(methyl methacrylate-hydroxyethyl methacrylate) beads, polyamide hollow fibers, poly(hydroxyethyl methacrylate), and magnetic poly(hydroxyethyl methacrylate) macrobeads, poly(ethylene glycol dimethacrylate-glycidyl methacrylate) monolith, and they obtained 35–220 mg/g polymer for HSA.[27–32] Nash and Chase used poly(vinyl alcohol) modified poly(styrene-divinyl benzene) beads carring different dye ligands.[33] They presented adsorption capacities of 11.7–27 mg HSA/g. Boyer and Hsu used Sepharose beads carrying different amounts of Cibacron Blue F3GA (2–25 μmol/mL) and reported adsorption values up to 55.9 mg/g (BSA) polymer.[34] Zeng and Ruckenstein reported 10.2 mg HSA/g adsorption capacity with Cibacron Blue F3GA-attached-polyethersulfone supported chitosan supports.[35] Li and Spencer used Cibacron Blue F3GA-attached polyethylene imine coated titania and achieved 4.4 mg HSA/g.[36] Chase reached 14 mg BSA/g with Cibacron Blue F3GA-attached Sepharose CL-6B.[37] Tuncel et al. reported 60 mg BSA/g adsorption capacity with Cibacron Blue F3GA-attached poly(vinyl alcohol)-coated monosize polystyrene beads.[38] Muller-Shulte et al. used several polymeric carriers made of different polymers, and Cibacron Blue F3GA as the dye-ligand.[39] Their albumin adsorption values were in the range of 0.19–0.81 mg HSA/mL support. McCreath et al. developed liquid pefluorocarbon chromatographic supports carrying C.I. Reactive Blue 4 and the maximum capacity of the flocculated emulsion for HSA was found to be 1.81 mg/mL.[40] Gu et al. employed Cibacron Blue F3GA-attached microporous poly(tetrafluoroethylene) capillary membrane and they achieved 198.5 mg/g albumin adsorption capacity.[41] Zhang et al. used a series of aminated chitosan microspheres carrying Cibacron Blue F3GA and they obtained maximum adsorption capacity as 108.7 mg albumin per gram support.[42] Ma et al. prepared nonporous micrometer sized magnetic poly(styrene-divinyl benzene-glycidyl methacrylate) having CIbacron Blue F3GA and they shoed that the magnetic microspheres had high adsorption capacity of albumin (80.2 mg/g).[43] The affinity support described here was comparable with the conventional and commercially available carriers.

TABLE I
Adsorption Constants of Langmuir and Freundlich Isotherms

Langmuir adsorption isotherm	Freundlich adsorption isotherm
$Q_{\max} = 43.9$ mg/g	$K_F = 15.0$
$B = 0.65$	$n = 0.79$
$R^2 = 0.953$	$R^2 = 0.969$

Adsorption dynamics

To quantify the extent of uptake in adsorption kinetics, the kinetic models (Pseudofirst- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations.[44] The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$\log(q_e - q_t) = \log(q_{1\,\mathrm{cal}}) - (k_1 t)/2.303 \quad (4)$$

where q_e is the experimental amount of HSA adsorbed at equilibrium (mg/g); q_t is the amount of HSA adsorbed at time t (mg/g); k_1 is the equilibrium

TABLE II
The First Order and Second Order Kinetic Constants for Poly(Am-AGE) Cryogel

HSA concentration (mg/mL)	q_e (mg/g)	First order kinetics			Second order kinetics		
		k_1 (min^{-1})	q_e	R^2	k_2 (g/mg/min)	q_e (mg/g)	R^2
0.1	2.4	0.081	2.84	0.957	0.0010	10.28	0.966
0.2	3.0	0.078	3.52	0.957	0.0006	14.18	0.962
0.5	9.2	0.081	9.93	0.978	0.0022	16.92	0.978
1.0	15.8	0.092	17.37	0.983	0.0016	27.55	0.985
1.5	22.0	0.100	24.57	0.951	0.0018	33.56	0.953
2.0	26.8	0.093	24.56	0.946	0.0036	32.90	0.967
3.0	27.0	0.120	25.23	0.937	0.0053	31.75	0.988

rate constant of first order adsorption (1/min); and $q_{1\,cal}$ is the adsorption capacity calculated by the pseudofirst-order model (mg/g).

The rate constant for the second-order adsorption could be obtained from the following equation:

$$(t/q_t) = (1/k_2 q_{2\,cal^2}) + (1/q_{2\,cal})t \quad (5)$$

where k_2 is the equilibrium rate constant of pseudo-second-order adsorption [g/(mg min)]; $q_{2\,cal}$ is the adsorption capacity calculated by the pseudosecond-order kinetic model (mg/g).

Table II shows the results for both first order and the second order kinetic models. The results show that the second order mechanism is applicape (R^2 values are the highest). These results suggest that the pseudosecond order mechanisms are predominant and that chemisorption might be the rate-limiting step that controls the adsorption process. The rate-controlling mechanism may vary during the course of the biosorption process with three possible mechanisms rate-limiting occurring.[45] There is an external surface mass transfer or film diffusion process that controls the early stages of the adsorption process. This may be followed by a reaction or constant rate stage and finally by a diffusion stage where the adsorption process slows down considerably.[46]

Effect of flow-rate

The adsorption amounts at different flow-rates are given in Figure 5. Results show that the HSA adsorption capacity onto the poly(AAm-AGE)/Cibacron Blue F3GA cryogel decreases when the flow-rate through the column increases. The adsorption capacity decreased significantly from 9.2 to 0.6 mg/g polymer with the increase of the flow-rate from 0.2 to 2.0 mL/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the HSA molecules and the poly(AAm-AGE)/Cibacron Blue F3GA cryogel at higher flow rates. These results are also in agreement with those referred to the literature.[47] When the flow-rate decreases the contact time in the column is longer. Thus, HSA molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is obtained. In addition, for column operation the cryogel is continuously in contact with a fresh protein solution. Consequently the concentration in the solution in contact with a given layer of cryogel in a column is relatively constant.

Effect of NaCl concentration

The effect of NaCl concentration on HSA adsorption is presented in Figure 6, which shows that the adsorption capacity decreases with increasing ionic strength of the binding acetate buffer. The adsorption amount of HSA decreased by about 93.4% as the NaCl concentration changes from 0.2 to 0.6 *M*. Increasing the NaCl concentration could promote the adsorption of the dye molecules to the polymer surface by hydrophobic interaction. Moreover, the hydrophobic

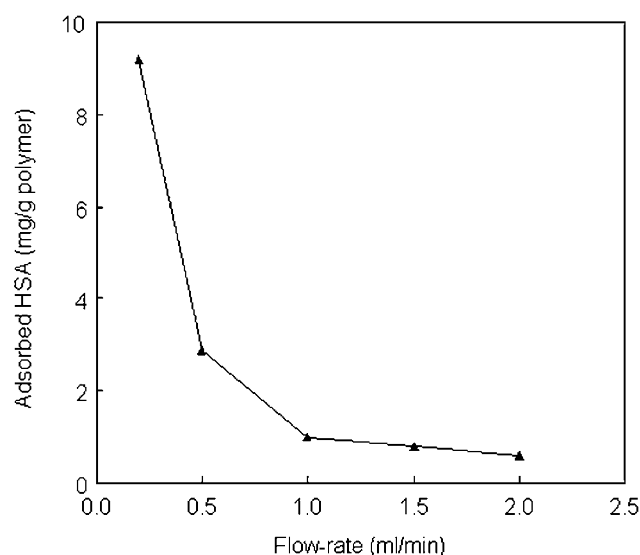

Figure 5 Effect of flow-rate on HSA adsorption. Cibacron Blue F3GA content, 68.9 µmol/g; pH, 5.0; HSA concentration, 0.5 mg/mL; T, 20°C. Each point is an average of five parallel studies.

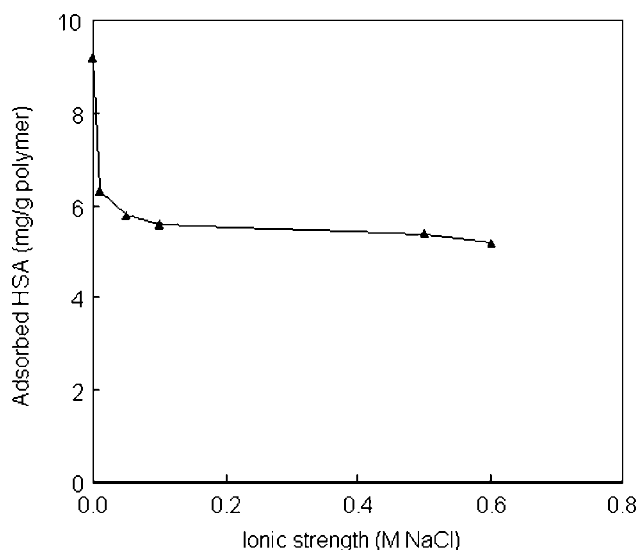

Figure 6 Effect of the NaCl concentration on HSA adsorption. Cibacron Blue F3GA content, 68.9 μmol/g; HSA concentration, 0.5 mg/mL; pH, 5.0; flow rate, 0.2 mL/min; T, 20°C. Each point is an average of five parallel studies.

interactions between the immobilized 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. Thus, the numbers of the immobilized dye molecules accessible to HSA would decrease as the ionic strength increased, and the adsorption of the HSA to immobilized dye became difficult. It is also suggested that an increase in NaCl concentration result in the reduction of electrostatic interactions.[48]

Desorption studies

Desorption of HSA from the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was also carried out in column system. The desorption of HSA is expressed in percentage of totally adsorbed HSA. Up to 98.0% of the adsorbed HSA was desorbed by using 0.1*M* Tris/HCl buffer containing 0.5*M* NaCl as elution agent. The addition of elution agent reduced electrostatic interactions, resulting in the release of the HSA molecules from the attached dye-molecules. Note that there was no Cibacron Blue F3GA release in this case which shows that dye-molecules are bonded strongly to poly(AAm-AGE) cryogel. With the desorption data given earlier, we concluded that 0.1*M* Tris/HCl buffer containing 0.5*M* NaCl is a suitable desorption agent, and allows repeated use of the affinity cryogel used in this study.

To show the reusability of the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel, the adsorption-desorption cycle was repeated 10 times using the same dye-affinity poly(AAM-AGE) cryogel. There was no remarkable reduce in the adsorption capacity of the cryogel. The HSA adsorption capacity decreased only 6.4% after 10-cycle. By taking into account the different experimental parameters studied above, it should be possible to scale up the process of HSA separation by increasing the cryogel size by dye-affinity chromatography on Cibacron Blue F3GA-attached poly(AAM-AGE) cryogel.

TABLE III
HSA Adsorption from the Plasma of a Healthy Donor

Dilution agent	HSA concentration (mg/mL)	Adsorption capacity (mg/g)
Plasma (undiluted)	37.7	74.2 ± 3
1/2 diluted plasma	18.8	56.8 ± 2
1/10 diluted plasma	3.8	48.6 ± 3

Cibacron Blue F3GA content, 68.9 μmol/g; flow rate; 0.2 mL/min; T, 20°C. Each point is an average of five parallel studies.

HSA adsorption from human plasma

Table III shows the adsorption for human serum obtained from a healthy donor. There was a low adsorption of HSA (1.43 mg/g) on the poly(AAm-AGE) cryogel, while much higher adsorption values (74.2 mg/g) were obtained when the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel were used. The purity of HSA was assayed by SDS-PAGE (Fig. 7). The purity of HSA

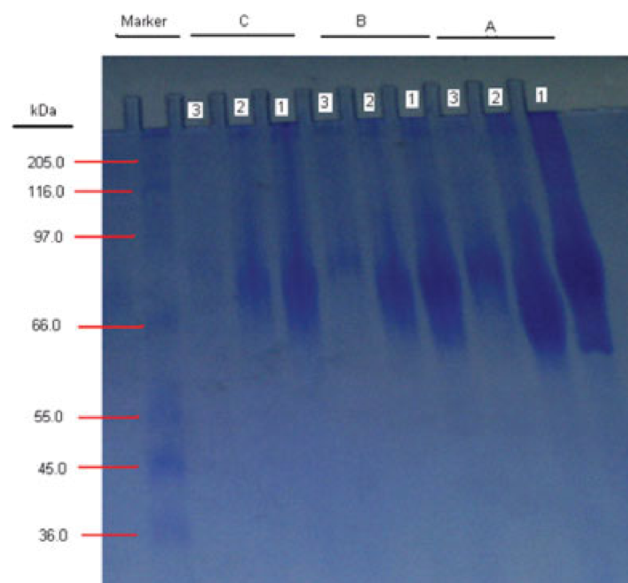

Figure 7 SDS/PAGE of HSA: The purity of HSA was assayed by SDS/PAGE using 10% separating gel (9 × 7.5 cm^2) and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol-water (1 : 5 : 5, v/v/v) and destained in ethanol-acetic acid-water (1 : 4 : 6, v/v/v). Lane A, 1/2 diluted plasma; Lane B, 1/5 diluted plasma; and Lane C, 1/10 diluted plasma. Lane 1, initial; Lane 2, final; Lane 3 after desorption. Equals amounts of sample = were applied to each line.

obtained was found to be 92% after purification. It is worth to note that adsorption of HSA onto the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was approximately 3.0-fold higher than those obtained in the studies in which aqueous solutions were used. This may be explained as follows: the conformational structure of HSA molecule within their native environment (i.e. human plasma) much more suitable for specific interaction with the Cibacron Blue F3GA-attached poly-(AAm-AGE) cryogel.

Competitive protein adsorption was also carried out and interesting results were obtained in these studies. Adsorption capacities were achieved as 1.7 mg/g for fibrinogen and 4.5 mg/g for γ-globulin. The total protein adsorption was determined as 80.8 mg/g. It is worth noting that adsorption of other plasma proteins (i.e., fibrinogen and γ-globulin) on the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel are negligible. It should be noted that HSA is the most abundant protein in plasma. It generally makes up more than half of the total plasma proteins. It may be resulted that this low adsorption of fibrinogen and γ-globulin is due to the high concentration of HSA.

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