Antibody Purification by Concanavalin A Affinity Chromatography

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ABSTRACT: Concanavalin A (Con A) immobilized poly(2-hydroxyethyl methacrylate) (PHEMA) beads in a spherical form (100–150 μm in diameter) were used for the affinity chromatography purification of human immunoglobulin G (IgG) from aqueous solutions and human plasma. PHEMA adsorbents were prepared by suspension polymerization. Bioligand Con A was then immobilized by covalent binding onto PHEMA beads. The maximum IgG adsorption on the PHEMA/Con A beads was observed at pH 6.0. The nonspecific IgG adsorption onto the plain PHEMA adsorbents was very low (ca. 0.17 mg/g). Higher adsorption values (up to 54.3 mg/g) were obtained when the PHEMA/Con A beads were used from aqueous solutions. A higher adsorption capacity was observed for human plasma (up to 69.4 mg/g) with a purity of 82.5%. The adsorption capacities of other blood proteins were 2.0 mg/g for fibrinogen and 4.2 mg/g for albumin. The total protein adsorption was determined to be 76.0 mg/g. IgG molecules could be repeatedly adsorbed and desorbed with the PHEMA/Con A beads without noticeable loss in the IgG adsorption capacity. J Appl Polym Sci 97: 1202–1208, 2005

Key words: adsorption; separation techniques; chromatography

INTRODUCTION

Antibodies are biologically active proteins produced by plasma cells in response to the presence of foreign substances. They offer exciting potential as diagnostic and therapeutic substances and also serve as bioaffinity ligands for purifying other high-value proteins of pharmaceutical importance, such as cytokines and blood-clotting factors.[1] Because of the importance of antibodies in the biotechnology industry, they are purified from various sources with different chromatographic techniques, including high-performance liquid chromatography, size exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, histidine affinity chromatography, and thiophilic chromatography.[2–8] However, antibodies are purified by affinity chromatography because of its high selectivity. Among the affinity techniques, protein A affinity chromatography is a well-known and popular method for purifying antibodies.[9] Protein A binds with different affinities to the Fc region of immunoglobulins from a variety of sources; for example, it binds to human, rabbit, and pig immunoglobulin G (IgG) with high affinity, to horse and cow IgG with lower affinity, and to rat IgG only very weakly. It exhibits a very high specificity and can, therefore, be employed as a one-step procedure for the purification of antibodies. Because of this specificity, protein A chromatography is now commonly used on a pilot scale for the purification of immunoglobulins to be used in clinical tests and therapy.[10] However, despite its high selectivity, protein A chromatography also has some drawbacks that are worth considering: (1) a considerable amount of protein A may be released from the matrix and such contamination cannot, of course, be tolerated in clinical applications, and (2) the cost of these biomolecules tends to be very high.[11] In addition, ligands such as protein A and protein G are difficult to immobilize in the proper orientation.

Bioaffinity chromatography on immobilized lectins has been extensively used to purify glycoproteins.[12–14] This work explores the performance of concanavalin A (Con A) affinity beads for human IgG purification from human plasma. The purification of IgG is generally required for immunodiagnostics, immunoaffinity chromatography, and immunotherapy. Moreover, IgG removal from human plasma is employed for the treatment of immune disorders, alloimmunization, and cancer.[15–17] For this reason, the clinical application of Con A immobilized adsorbents could be a potentially attractive tool. In this study, hydrophilic and blood-compatible poly(2-hydroxyethyl methacrylate) (PHEMA) beads were prepared in a spherical form by suspension polymerization. The hydroxyl groups on these adsorbents were activated by cyanogen bro-

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mide, and then Con A molecules were covalently coupled to the beads through the active sites. IgG adsorption on PHEMA/Con A beads from aqueous solutions containing different amounts of IgG at different pHs, temperatures, and ionic strengths and also from human plasma is reported here.

EXPERIMENTAL

Materials

Con A from jack bean and lyophilized human IgG were supplied by Sigma (St. Louis, MO). 2-Hydroxyethyl methacrylate (HEMA) was purchased from Sigma and was purified by vacuum distillation under a nitrogen atmosphere. Ethylene glycol dimethacrylate (EGDMA; Buchs, Fluka, Switzerland) was used as the crosslinking agent. 2,2′-Azobisisobutyronitrile (AIBN; BDH, Poole, United Kingdom) was used as the initiator. The dispersion medium was a saturated aqueous solution of magnesium oxide (MgO; Sigma). All other chemicals were purchased from available commercial sources and used as obtained. All water used in the experiments was purified with 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.

Methods

Preparation of the PHEMA adsorbents

The PHEMA adsorbents were prepared by suspension polymerization.[18] The polymerization was carried out in an aqueous dispersion medium containing MgO, which was used to decrease the solubility of the monomer (HEMA) in the medium. The monomer phase, containing HEMA, EGDMA, and AIBN, was added to the dispersion medium within a laboratory-type reactor (i.e., a two-necked flask with a volume of 500 mL) provided with a blade-type stirrer. To produce spherical adsorbents about 100–150 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1:3 (v/v), 1:10 (v/v), 0.33 (mol of EGDMA/mol of HEMA), 0.0015 (mol of AIBN/mol of HEMA), and 600 rpm, respectively. The polymerization was carried out at 70°C for 3 h and then at 90°C for 1 h. After cooling, the adsorbents were separated from the polymerization medium by filtration, and the residuals (e.g., monomer and MgO) were removed by a cleaning procedure.[19] Briefly, adsorbents were transferred into a reservoir, and washing solutions (i.e., a dilute HCl solution and a water–ethanol mixture) were recirculated through the system, which also included an activated carbon column, to make sure that the adsorbents were clean. The purity of the adsorbents was followed by the observation of the changes in the optical densities of the samples taken from the liquid phase in the recirculation system and also with the DSC thermograms of the adsorbents obtained with a differential scanning microcalorimeter (Mettler, Greifensee, Switzerland). The optical density of the uncleaned adsorbent was 2.86. However, after the cleaning operation, this value was reduced to 0.06. In addition, when the thermogram of the uncleaned adsorbent was recorded, it had a peak around 60°C. This peak might have originated from AIBN. However, after the application of this cleaning procedure, between 30 and 100°C, no peak was observed in this thermogram.

CNBr activation

Before the activation process, the beads were kept in distilled water for about 24 h, washed with a glass filter with a 0.5*M* NaCl solution, and resuspended in 18 mL of water. A sodium carbonate buffer (2 mL, pH 10.5) was added, and the mixture was stirred slowly. The mixture was placed in a fume hood, and a glass pH electrode was immersed into this solution. A CNBr aqueous solution (100 mL) with an initial concentration of 50 mg/mL was prepared. The pH of this solution was quickly adjusted to 11.5 with 2*M* NaOH. The suspension was gently agitated at room temperature (25°C), and the activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, unreacted sites were quenched via washing with 0.1*M* $NaHCO_3$, and any remaining active groups (e.g., isourea) on the surfaces were blocked by a treatment with ethanol amine (pH 9.1) and an $FeCl_3$ solution for 1 h. Then, the activated beads were washed four times with a sodium citrate buffer (pH 6.5) and distilled water containing 0.5*M* NaCl.

Con A immobilization

One gram of the freshly activated beads was magnetically stirred (at 50 rpm) at a constant temperature of 25°C for about 2 h (i.e., equilibrium time) with 25 mL of a Con A solution containing 100 μM $CaCl_2$, 100 μM $MnCl_2$, and 2% (w/v) glucose. The immobilization of Con A was carried out in a phosphate buffer (pH 7.4) at 4°C for 24 h. Con A was covalently bound to the CNBr activated beads through a coupling reaction between the free amine groups on Con A and the imidocarbonate groups on the activated beads. The initial concentration of Con A in the medium was 2.0 mg/mL. After incubation, the PHEMA/Con A beads were filtered and washed with distilled water and the phosphate buffer several times until all the physically attached Con A molecules were removed. The amount

of Con A immobilization on the CNBr activated beads was determined by the measurement of the decrease of the Con A concentration and also by the consideration of the Con A molecules adsorbed nonspecifically (amount of Con A adsorbed on the unmodified beads) by the Lowry method.[20] The protein sample (200–400 μL) was diluted to 1.0 mL with phosphate-buffered saline (PBS). Thereafter, 1.0 mL of a freshly prepared Lowry reagent was added. After 30 min of incubation at room temperature, 500 μL of freshly prepared Folin-Ciocalteu's reagent was added, and the solution was mixed with a vortex. The blank solution was prepared analogously to the protein sample with 200–400 μL of a 3.0% sodium dodecyl sulfate solution in PBS instead of the protein solution. After 30 min of incubation at room temperature, the absorbance of the protein sample was measured at 730 nm against the blank solution with a Pharmacia LKB Novaspec II spectrophotometer (Upsala, Sweden). A calibration curve was established with bovine serum albumin with known concentrations to relate the protein concentration in the solution to the absorbance of the sample. The leakage of Con A from the beads was followed by the treatment of the beads with PBS for 24 h at room temperature. Con A released after this treatment was measured in the liquid phase spectrophotometrically at 280 nm.

IgG adsorption from an aqueous solution

IgG adsorption of the PHEMA/Con A beads was studied at various pH values, either in an acetate buffer (0.1*M*, pH 5.5–6.0) or in a phosphate buffer (0.1*M*, pH 6.5–8.0). The initial IgG concentration was 0.5 mg/mL in the corresponding buffer. To determine the adsorption capacities of the PHEMA/Con A beads, the concentration of IgG in the medium was varied in the range 0.05–1.0 mg/mL. The ionic strength was changed in the range of 0.01–0.1. The adsorption experiments were conducted for 2 h at 25°C with continuous stirring. After the IgG adsorption, to remove the nonspecifically adsorbed IgG molecules, the PHEMA beads were washed with a 0.1*M* borate buffer and 0.15*M* NaCl (pH 8.8), with 2*M* urea and 0.15*M* NaCl, and finally with 0.1*M* NaHCO3 (pH 9.5) and 0.5*M* NaCl. The protein concentrations in the aqueous solutions were measured with the Lowry method.[20] The adsorbed amount of IgG was calculated as follows:

$$q = [(C_i - C_t)V]/m \quad (1)$$

where q is the amount of IgG adsorbed onto the unit of mass of the adsorbent (mg/g); C_i and C_t are the concentrations of IgG in the initial solution and in the supernatant after adsorption, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the beads (g).

Human IgG adsorption from human plasma

The adsorption of IgG from human plasma on the PHEMA/Con A beads was studied batchwise. Fresh human blood was used for all the experiments. The blood samples (500 mL) were supplied by a healthy donor at the University Hospital (Hacettepe, Ankara). The blood samples were centrifuged at 1000 g for 30 min at room temperature to separate plasma. PHEMA beads containing Con A were incubated at 20°C for 20 min with 2 mL of human plasma. PBS (pH 7.4, 0.9% NaCl) was used for the dilution of the human plasma. The amount of IgG adsorbed through Con A on the PHEMA beads was determined by a solid-phase enzyme-linked immunosorbent assay (ELISA).[21] Human anti-IgG (I-9384, Sigma) diluted to 1/1000 in 50 m*M* $NaHCO_3$ (pH 9.6) was adsorbed to poly(vinyl chloride) microtiter plates at 4°C for 12 h. The plates were washed with PBS containing 0.05% Tween 20 (a wash buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% bovine serum albumin, and 0.1% sodium azide (a blocking buffer). Samples (2.5 mL, neutralized with 0.5 mL of 1.0*M* trisodium citrate) or controls containing known amounts of IgG were added and incubated at 37°C for 1 h. Bound IgG was detected with the anti-IgG labeled with biotin (B-3773, Sigma) followed by peroxidase-conjugated streptavidin (Sigma) and *o*-phenylenediamine. The absorbance was measured at 492 nm.

To determine the Con A specificity, the adsorptions of blood proteins (i.e., albumin and fibrinogen) were also studied. The Con A immobilized beads were incubated with a human plasma containing albumin (38.4 mg/mL), fibrinogen (2.5 mg/mL), and g-globulin (12.5 mg/mL) at room temperature for 2 h. The total protein concentration was measured with the total protein reagent (catalog ref. no. 712076, Ciba Corning Diagnostics, Ltd., Halstead, England) at 540 nm, which was based on the Biuret reaction. The chronometric determination of fibrinogen according to the Clauss method on plasma was performed with Fibrinogene-Kit (ref. nos. 68452 and 68582, Biomerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France). The human serum albumin concentration was determined with Ciba Corning albumin reagent (catalog ref. no, 229241, Ciba Corning Diagnostics), which was based on the bromocresol green dye method. The IgG concentration was determined by ELISA.

The purity of IgG was assayed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) with 10% separating gel (9 × 7.5 cm), and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in an acetic acid/methanol/water

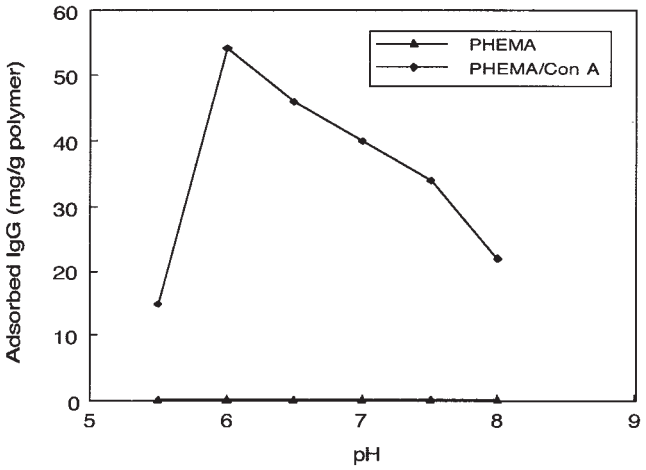

Figure 1 Effect of the pH on the IgG adsorption (Con A loading = 56.8 mg/g, IgG concentration = 0.5 mg/mL, temperature = 25°C).

mixture (1:5:5 v/v/v) and destained in an ethanol/acetic acid/water mixture (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.

Desorption and repeated use

The desorption of IgG was studied in a 2*M* NaCl aqueous solution. IgG-adsorbed PHEMA/Con A beads were placed in this desorption medium and stirred continuously (at a magnetic stirring rate of 600 rpm) for 1 h at room temperature. The total volume of the desorption medium was 50 mL. The final IgG concentration in the desorption medium was determined by solid-phase ELISA. The desorption ratio was calculated from the amount of IgG adsorbed on the adsorbents and the final IgG concentration in the desorption medium. To test the reusability of the PHEMA/Con A beads, the IgG adsorption–desorption cycle was repeated 10 times with the same polymeric adsorbent. Moreover, after the desorption of IgG, Con A leakage from the polymeric structure was also monitored continuously.

RESULTS AND DISCUSSION

PHEMA/Con A beads were prepared for the purification of IgG from human plasma. The main selection criteria for PHEMA were its physiological acceptability, mechanical strength, chemical and biological stability, low nonspecific adsorption of proteins, and good blood compatibility. The observations showed that the PHEMA adsorbents used in this study were resistant to the adhesion of blood proteins and blood cells.[22] The PHEMA adsorbents prepared in this study were rather hydrophilic and crosslinked structures, that is, hydrogels. The simple incorporation of water weakened the secondary bonds within the hydrogels. This enlarged the distance between the polymer chains and caused the uptake of water. The equilibrium water uptake ratio of the PHEMA beads was 55% (w/w). The aqueous swelling properties of the PHEMA beads were not observed to change after derivatization with Con A. Con A molecules were immobilized covalently to the PHEMA beads. Con A leakage was investigated in a PBS solution. Ligand leakage was not observed from the PHEMA/Con A beads.

Human IgG adsorption from aqueous solutions

Effect of the pH

Figure 1 shows the effect of the pH on the IgG adsorption, which was very significant. The maximum IgG binding capacity of the affinity beads was found to lie at 54.3 mg of IgG/g; this was observed at pH 6.0. Significantly lower adsorption capacities were obtained below and above pH 6.0. The amount of IgG coupled onto the PHEMA/Con A beads as a function of pH exhibited two adsorption domains, as shown in Figure 1. The amount of IgG coupled onto Con A containing beads showed a maximum at pH 6.0 (isoelectric point of IgG = 6.2), with a significant decrease at lower and higher pH values. Pseudospecific interactions between IgG and Con A molecules at pH 6.0 may have resulted from the conformational state of the IgG molecules (more folded structure) at this pH. Moreover, nonspecific IgG adsorption on plain PHEMA beads was independent of pH and was observed to be the same at all the pH values studied.

Effect of the IgG concentration

Figure 2 presents adsorption data for the plain and PHEMA/Con A beads. The nonspecific IgG adsorp-

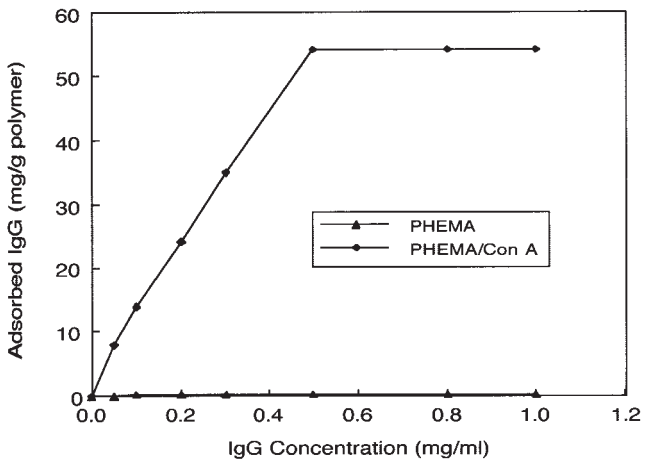

Figure 2 Effect of the IgG concentration on the IgG adsorption (Con A loading = 56.8 mg/g, pH = 6.0, temperature = 25°C).

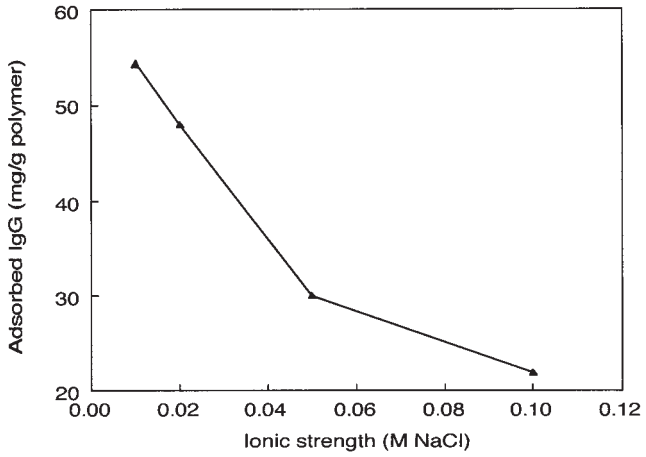

Figure 3 Effect of the ionic strength on the IgG adsorption (Con A loading = 56.8 mg/g, IgG concentration = 0.5 mg/mL, pH = 6.0, temperature = 25°C).

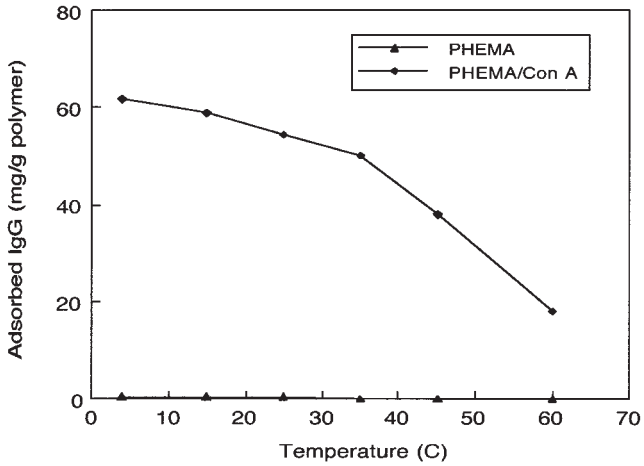

Figure 4 Effect of the temperature on the IgG adsorption (Con A loading = 56.8 mg/g, IgG concentration = 0.5 mg/mL, pH = 6.0.

tion onto the plain PHEMA adsorbents was very low (ca. 0.17 mg/g). However, the specific adsorption (i.e., the adsorption of IgG molecules onto the PHEMA adsorbents through Con A molecules) was significant (up to 54.3 IgG/g) and increased with an increasing initial concentration of IgG in the incubation medium. A specific interaction took place between the carbohydrate moieties of the glycoprotein IgG and one (or more) of the saccharide binding sites of Con A.[13] As expected, the amount of IgG coupled to PHEMA beads via Con A molecules reached almost a plateau value around 0.5 mg/mL because of the saturation of active binding sites.

Effect of the ionic strength

The effect of the ionic strength (adjusted by the addition of NaCl) on the IgG adsorption is presented in Figure 3, which shows that the adsorption capacity decreased with the increasing ionic strength of the binding buffer (phosphate buffer, pH 6.0). The adsorption of IgG decreased by about 59% as the NaCl concentration changed from 0.01 to 0.1*M*. The decrease in the adsorption capacity as the ionic strength increased could be attributed to the repulsive electrostatic interactions between the PHEMA/Con A beads and IgG molecules. When the salt concentration increased in the adsorption medium, the hydrophobic interactions between the immobilized Con A molecules themselves also became strong because the salt addition to the Con A solution caused the stacking of the free Con A molecules. Thus, the numbers of the immobilized Con A molecules accessible to IgG decreased as the ionic strength increased, and the adsorption of IgG to immobilized Con A became difficult.

Effect of the temperature

The effect of temperature on the equilibrium IgG adsorption capacity of the Con A immobilized PHEMA beads is presented in Figure 4. At all temperatures, the nonspecific adsorption of IgG due to the van der Waals force was very low (0.1 mg/g). No significant effect of the temperature was observed on the physical adsorption of IgG onto the PHEMA beads. However, the equilibrium adsorption of IgG onto the PHEMA/Con A beads significantly decreased with increasing temperature, and the maximum IgG adsorption was achieved at 4°C (61.6 mg/g).

IgG purification from human plasma

The adsorption of IgG from human plasma was performed batchwise. Figure 5 shows the adsorption for human serum obtained from a healthy donor. There was a very low nonspecific adsorption of IgG (1.4 mg/g) on the plain PHEMA beads, whereas much

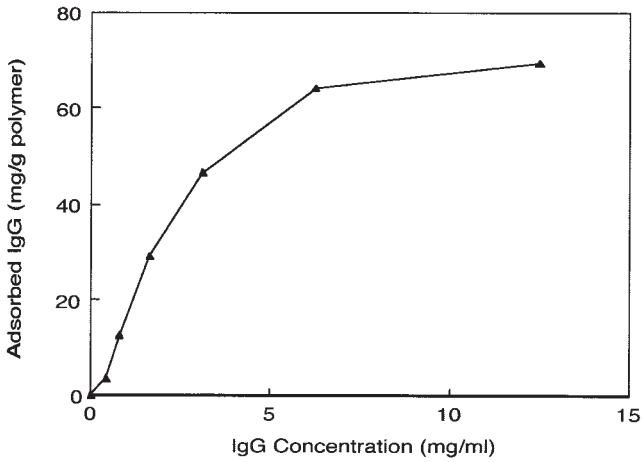

Figure 5 IgG adsorption from the plasma of a healthy donor (Con A loading = 56.8 mg/g, temperature = 4°C, plasma volume = 10 mL).

TABLE I
Comparison of the Adsorption Capacities for IgG of Various Adsorbents

Adsorbent	Ligand	q_{max}	Reference
PHEMA	L-Histidine	44.8	6
PHEMA	Methacryloylamidohistidine	73.8	7
Eupergit, Affigel	Protein A	20.1	10
PHEMA	Protein A	24.0	11
Poly(methyl methacrylate)	Cu^{2+}	54.3	15
Polycaprolactam	Protein A	28.3	23
Polyethylene	Phenylalanine	50.0	24
Sepharose 4B	L-Histidine	0.23	25
Poly(ethylene vinyl alcohol)	L-Histidine	77.7	26
Polysulfone	Protein A	8.8	27
Sartobind	Protein A	0.51	28
Poly(methyl methacrylate)	Protein A/G	6.6	29
Poly(vinyl alcohol)	Protein A	13.2	30
	3-Aminophenol	52.0	31
	4-Amino-*l*-naphthol		
Sepharose CL 6B	Biomimetic ligand	7.0	32
Sepharose 4B	Biomimetic ligand	25.0	33
PHEMA	Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+}	79.6	34
PHEMA	Con A	69.4	This study

higher adsorption values (69.4 mg/g) were obtained when the PHEMA/Con A beads were used. The purity of IgG was assayed by SDS–PAGE, and it was found to be 82.5%. The adsorption of IgG onto the PHEMA/Con A beads was approximately higher than those values obtained in studies in which aqueous solutions were used. This may be explained as follows: the conformational structure of the IgG molecules within their native environment (i.e., human plasma) is much more suitable for specific interactions with attached Con A molecules. The high IgG concentration may also contribute to this adsorption capacity because of the high driving force between the aqueous and solid phases.

To show the Con A specificity, the adsorption of other blood proteins was also studied. The protein adsorption capacities were 69.4 mg/g for IgG, 2.0 mg/g for fibrinogen, and 4.2 mg/g for albumin. The total protein adsorption was determined to be 76.0 mg/g. The adsorption of other plasma proteins (i.e., fibrinogen and albumin) on the PHEMA/Con A beads was negligible. This low adsorption of fibrinogen and albumin was due to the high affinity of Con A toward IgG molecules.

A comparison of the maximum adsorption capacity, q_{max}, of the PHEMA beads with those of some other bioaffinity adsorbents reported in the literature is given in Table I. The adsorption capacity of PHEMA was relatively high in comparison with that of the other adsorbents. The differences in IgG adsorption were due to the properties of each adsorbent, such as the structure, functional groups, ligand loading, and surface area.

Desorption of IgG

The desorption of IgG from the PHEMA/Con A beads was performed in a batch experimental setup. The adsorbents loaded with IgG were placed within the desorption medium, and the amount of IgG desorbed in 1 h was determined. Human plasma was used for repeated IgG adsorption cycles. Up to 92% of the adsorbed IgG was desorbed with 2.0*M* NaCl as the elution agent. The addition of NaCl could change the charge of the peptide side groups because of their isoelectric points, resulting in the detachment of the IgG molecules from Con A. There was no Con A release in this case, and this shows that Con A molecules were immobilized covalently to PHEMA beads.

To show the reusability of the Con A immobilized PHEMA beads, the adsorption–desorption cycle was repeated 10 times with the same polymeric beads. There was no remarkable reduction in the adsorption capacity of the adsorbents (Fig. 6). The IgG adsorption capacity decreased only 1.0% after 10 cycles. Considering the aforementioned desorption data, we have concluded that NaCl is a suitable desorption agent that allows repeated use of the affinity sorbents used in this study.

CONCLUSIONS

Antibodies are becoming an important class of biomolecules for both the diagnosis and treatment of a large variety of human diseases. Antibodies of the G class are also finding applications for the diagnosis and cure of certain important diseases, such as cancer.[35] The medical and commercial relevance of anti-

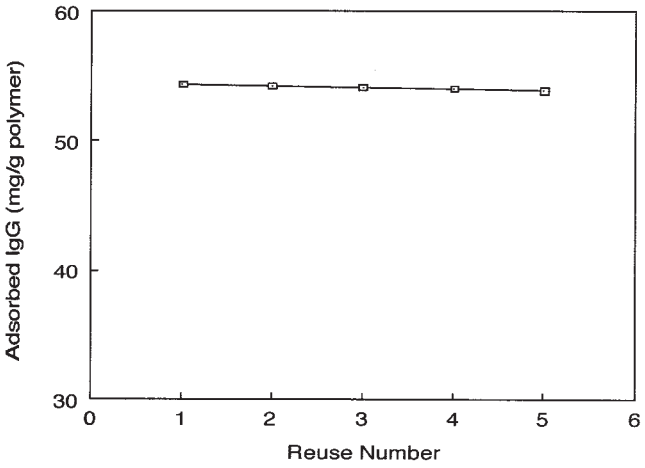

Figure 6 Repeated use of Con A immobilized PHEMA beads (Con A loading = 56.8 mg/g, IgG concentration = 0.5 mg/mL, pH = 6.0, temperature = 25°C).

bodies has stimulated the development of cost- and time-effective purification techniques, including polymeric carriers.[8,36] Protein A affinity chromatography cannot be used for large-scale purification because it is very expensive. Affinity chromatography on immobilized lectins has been extensively used to purify glycoproteins. Here, PHEMA adsorbents were produced by the suspension polymerization of HEMA. A bioligand, Con A, was then immobilized to these beads to have a loading up to 56.8 mg/g, which resulted in an IgG adsorption of 54.3 mg/g from aqueous solutions. The resulting Con A affinity beads displayed a high capacity for IgG (69.4 mg/g) from human plasma and were able to purify the protein to a high purity (>82.5%). Successful desorption ratios (>92% of adsorbed IgG) were achieved with 2.0*M* NaCl. It was possible to reuse the PHEMA/Con A beads without a remarkable reduction in the adsorption capacities.

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