Concanavalin A Immobilized Magnetic Poly(glycidyl methacrylate) Beads for Antibody Purification

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ABSTRACT: Concanavalin A (Con A) immobilized magnetic poly(glycidyl methacrylate) (mPGMA) beads in monosize and spherical for (1.62 μm in diameter) were used for the purification of human immunoglobulin G (IgG) from human plasma. Con A was immobilized by covalent binding onto the mPGMA beads. The maximum IgG adsorption on the mPGMA-Con A beads was observed at pH 6.0. The nonspecific IgG adsorption onto the plain mPGMA beads was very low (0.22 mg/g). Scatchard analysis of the adsorption isotherm for IgG on mPGMA-Con A beads showed an affinity constant (K_a) of $1.39 \times 10^5\ M^{-1}$ and a theoretical maximum adsorption capacity of 109.1 mg/g. An apparent IgG adsorption capacity of 66.2 mg/g was observed under the experimental conditions. IgG adsorption capacity from human plasma was observed as 48.0 mg/g. The adsorption of human serum albumin from plasma was 2.0 mg/g. The total protein adsorption was determined to be 50.0 mg/g. IgG molecules could be repeatedly adsorbed and eluted with the mPGMA-Con A beads without noticeable loss in the IgG adsorption capacity. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1867–1874, 2012

Key words: magnetic beads; separation techniques; chromatography; lectin affinity chromatography; concanavalin A; IgG

INTRODUCTION

Biomolecular interactions of proteins with carbohydrates are among the nature's specific affinity interactions.[1] They are especially important in cellular growth, communication, recognition, and adhesion between cells etc.[2] The reversible interaction of lectins with glycoconjugates, including glycosylated proteins, is a well-known phenomenon.[3] Owing to these properties, lectins from plants and invertebrates have been used for many years for the detection of carbohydrate moieties of glycoconjugates,[4–6] protein purification,[7,8] fractionation,[9] structural characterization,[10,11] immobilization of a variety of glycosylated proteins,[12,13] detection of biomarkers of certain clinical conditions[14] and cell separation and sorting.[15–17]

As an affinity ligand, lectins have numerous advantages because of their specific and reversible interactions with certain sugars.[18] Lectin affinity chromatography is carried out under mild conditions which highly preserves biological activities of the target molecules.[1,2] Con A is a tetrameric metalloprotein found in the jack bean (*Canavalia ensiformis*) and can bind α-D-mannose and α-D-glucose with high molecular specificity.[19,20]

Antibodies are a group of biologically active glycoproteins 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 affinity ligands to purify other high-value proteins of pharmaceutical importance such as cytokines and blood-clotting factors.[21] Downstream processing of antibodies like other bioproducts, could constitute most of the manufacturing costs. With the growing expansion of the biotechnology industry, and increasing need for huge amounts of antibodies, efficient and well-designed product recovery methods are very essential in developing a downstream process.[22–24] Moreover, IgG removal from human plasma is employed for the treatment of immune disorders including systemic lupus erythematosus, alloimmunization, and cancer.[25–28] Multi-lectin affinity columns have been used for the purification of IgG1 and IgG4 class antibodies through the interaction with chain C region. For these reasons Con A immobilized polymeric beads could be a potentially attractive tool for IgG purification.

In this study, the magnetic poly(glycidyl methacrylate) [mPGMA], beads were prepared in monosize form and Con A molecules were immobilized for the efficient purification of IgG from human plasma.

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In the first part, the mPGMA beads were prepared by dispersion polymerization of glycidyl methacrylate (GMA) in the presence of Fe_3O_4 nanopowder. Then, Con A molecules were immobilized covalently through active epoxy groups of GMA. After that, IgG adsorption properties of the mPGMA-Con A beads from aqueous solutions and human plasma were investigated at different experimental conditions in a magnetically stabilized fluidized bed (MSFB) system. Elution of IgG and reusability of the magnetic beads were also investigated and the results are presented.

EXPERIMENTAL

Materials

Concanavalin A (Con A) from Jack Bean, human IgG (lyophilized) and magnetic nanopowder (Fe_3O_4, 20–50 nm) were supplied from Sigma Chemical Co. (St. Louis, MO). 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) (PVP) (MW: 30.000, BDH Chemicals Ltd., Poole, England) were selected as the initiator and the steric stabilizer, respectively. AIBN was recrystallized from methanol. 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 all the glassware was rinsed with deionized 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.

Preparation of Con A-immobilized mPGMA beads

mPGMA monosize beads were synthesized and characterized as previously described in detail elsewhere [29–31]. Con A was immobilized covalently through epoxy groups of GMA. Epoxy groups of GMA were opened in an alkaline medium (pH 10.0). One gram of the magnetic beads was added to each reaction vessel. Totally, 100 μ*M* of $CaCl_2$, 100 μ*M* $MnCl_2$ and 2% glucose (w/v) were also added to 25 mL of reaction medium (pH 7.5, 0.1*M* phosphate buffer) to protect carbohydrate binding sites of Con A during covalent immobilization procedure.[18] The reaction was continued for 24 h at 4°C while stirring mechanically at 150 rpm. At the end of this period, the magnetic beads were separated by centrifugation at 10,000 rpm for 5 min, and the supernatant was collected to determine the amount of immobilized Con A. The magnetic beads were washed with distilled water and buffer solutions several times and then dried under vacuum. The amount of immobilized Con A onto the mPGMA beads was determined spectrophotometrically by measuring the absorbance of the initial Con A solution and the supernatant after the immobilization procedure at 280 nm, by also considering the noncovalently adsorbed Con A molecules in the washing solutions. Con A leakage from the mPGMA beads was controlled periodically throughout the study. The Con A immobilized mPGMA beads (mPGMA-ConA) were kept at 4°C in the presence of bacteriostatic agent (0.01% NaN_3) when not in use.

Adsorption studies from aqueous solutions

IgG adsorption experiments from aqueous solutions were carried out in a magnetically stabilized fluidized bed (MSFB) system using BioRad economic column (diameter: 1.0 cm, length: 10.0 cm) surrounded by a magnetic field generator. Effects of medium pH (5.5–8.0), IgG concentration (0.1–3.0 mg/mL), flow rate (1.0–4.0 mL/min) and magnetic field (6.4–19.1 mT) on the IgG adsorption capacity were investigated. IgG adsorption was periodically checked by taking samples from the adsorption media. Protein concentration was determined spectrophotometrically at 280 nm and the adsorption amount was calculated from the difference between the initial and the final protein concentration by using the following equation:

$$Q = (C_i - C_{f)}\ V/m \quad (1)$$

Where C_i and C_f are the initial and final IgG concentrations (mg/mL), respectively, V is the volume of the protein solution (mL) and m is the amount of magnetic beads used (g).

Adsorption studies from human plasma

IgG adsorption from human plasma was also performed. The blood was supplied from a healthy donor. Blood samples were centrifuged at 3000 rpm for 5 min at room temperature to separate the plasma. Totally, 25 mL of the freshly separated human plasma was incubated with mPGMA-Con A beads pre-equilibrated with phosphate buffered saline, PBS (pH 7.4). These experiments were continued at 20°C for 2 h. The original plasma of the donor contained 16.0 mg/mL IgG as determined by ELISA. Human anti-IgG (Sigma, I-9384) diluted 1/1000 in 50 m*M* $NaHCO_3$, pH 9.6, was adsorbed to poly(vinyl chloride) microtitre plates at 4°C for 12 h. The plates were washed with PBS containing 0.05% Tween 20 (wash buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% bovine serum albumin, and 0.1% sodium azide (blocking buffer). Samples (2.5

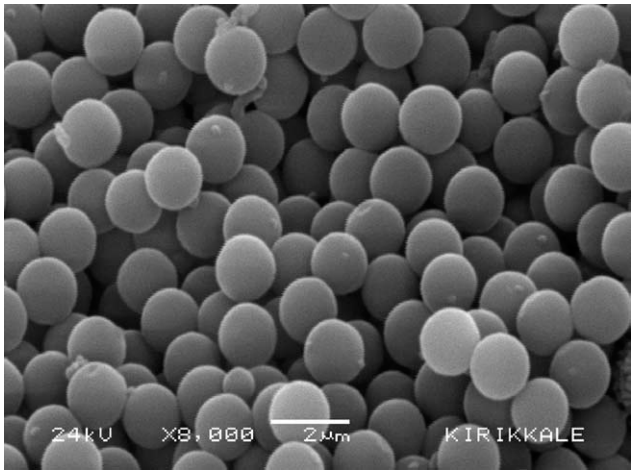

Figure 1 Scanning electron micrograph of mPGMA beads.

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. Immobilized IgG was detected with the anti IgG labeled with biotin followed by peroxidase-conjugated streptavidin and *o*-phenylenediamine. The absorbance was measured at 492 nm.

To show the ligand specificity, competitive protein adsorption (i.e., human serum albumin (HSA and fibrinogen) was also measured. The mPGMA-Con A beads were incubated with a human plasma containing HSA (37.6 mg/mL), fibrinogen (2.0 mg/mL), and IgG (16.0 mg/mL). Total protein concentration was measured by using the total protein reagent (Ciba Corning Diagnostics, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm based on Biuret reaction. 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). HSA concentration was determined by using Ciba Corning HSA Reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 229241) based on bromocresol green (BCG) dye method.

Purity of the IgG was assessed using SDS-PAGE with 8% separating and 5% stacking gels stained with 0.25% Coomassie Brilliant R 250 (w/v) in an acetic acid/methanol/water mixture (4 : 1 : 5 v/v) and destained in an ethanol/acetic acid/water mixture (1 : 4 : 6 v/v). Electrophoresis was run for 3 h at 120 V. Sigma grade human IgG was used as a standard and myosin, β-galactosidase, ovalbumin, and lysozyme were used as a marker.

Elution and repeated use

Elution of IgG was carried out using 50% ethylene glycol solution and 2.0*M* NaCl solution. 25 mL elution solution was passed through the IgG adsorbed MSFB column for 2 h at 25°C. The final IgG concentration within the elution medium was determined spectrophotometrically. Elution ratio was calculated from the amount of IgG adsorbed on the mPGMA-Con A beads and the amount of IgG eluted into the medium.

To show the reusability of the mPGMA-Con A beads, IgG adsorption-elution procedure was repeated ten times by using the same group of the polymeric beads. It should be also noted that, after elution of IgG, Con A leakage was also monitored.

RESULTS AND DISCUSSION

Properties of the beads

mPGMA beads were produced by dispersion polymerization of GMA. The surface morphology of mPGMA beads was shown by the scanning electron micrograph in Figure 1. As can be seen from the figure, the mPGMA beads are highly uniform in size and nonporous. The physicochemical properties of the mPGMA beads were given in Table I.

Con A was used as a bioligand and Con A immobilized mPGMA beads were used as an adsorbent for IgG purification. Concanavalin A (Con A) recognize the carbohydrade moiety of the IgG molecule.[24] Con A immobilization onto mPGMA beads was 12.5 mg Con A onto per gram of mPGMA beads.

Adsorption of IgG from aqueous solutions

Effect of pH

The effect of pH on the adsorption capacity of IgG on the mPGMA-Con A beads was investigated and the obtained results are shown in Figure 2. It can be seen that the adsorption capacity of IgG increased with increasing pH from 5.0 to 6.0 and further decrease of 8.0. The maximum adsorption amount of IgG was 37.7 mg/g which was observed at pH 6.0. Specific interactions between the carbohydrate moieties of the glycoprotein IgG and the saccharide binding sites of the Con A at pH 6.0 may result from the more folded conformational state of IgG at

TABLE I
Some Physicochemical Properties of the mPGMA Beads

Particle diameter	1.62 ± 0.01 μm
Polydispersity index	1.008
Specific surface area	3.2 m^2/g
Epoxy group content (theoretical)	3.46 mmol/g
Epoxy group content (experimental)	3.00 mmol/g
Swelling ratio	52%
Wet density	1.09 g/mL
Fe_3O_4 incorporation	4.5%
Resonance of magnetic field	2055 Gauss
g factor	2.28

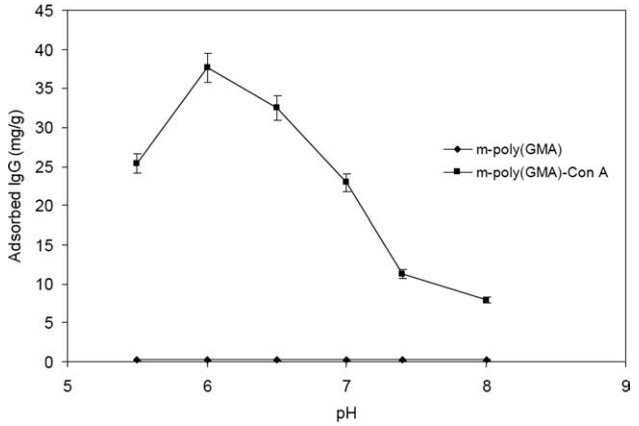

Figure 2 Effect of pH on IgG adsorption. IgG concentration: 0.5 mg/mL; Con A loading: 12.5 mg/g; magnetic field: 6.4 mT; flow rate: 1.0 mL/min; T: 20°C.

this pH. This observation is consistent the results obtained by Guerrier et al. on a porous ceramic material carrying the protein A-mimetic ligand.[32] They reported that optimal pH value for antibody adsorption was in the range of 6.0–6.5. It should be also noted that nonspecific IgG adsorption on plain mPGMA beads is independent of pH and it is observed same at all pH values studied.

Adsorption isotherms

Figure 3 gives the adsorption data on the plain mPGMA beads and mPGMA-Con A beads. The non specific adsorption onto mPGMA beads was very low (about 0.22 mg/g) while specific adsorption (i.e., adsorption of IgG molecules through Con A molecules immobilized onto the mPGMA beads) was significant (up to 66.2 mg/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.[33] As expected, the amount of IgG coupled to mPGMA beads via Con A molecules reached almost a plateau value around 1.5 mg/mL because of the saturation of active binding sites.

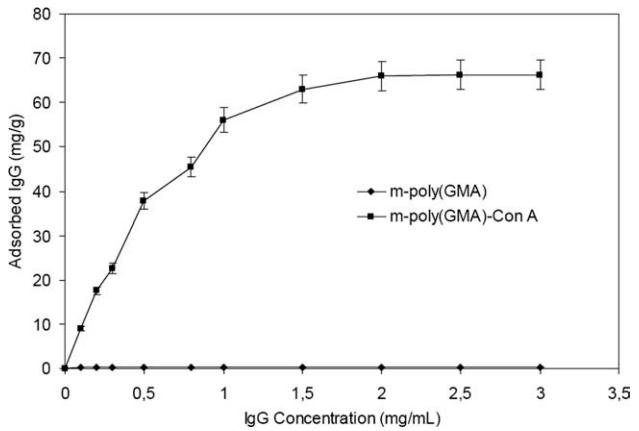

Figure 3 Effect of IgG concentration on IgG adsorption. Con A loading: 12.5 mg/g; pH: 6.0; magnetic field: 6.4 mT; flow rate: 1.0 mL/min; T: 20°C.

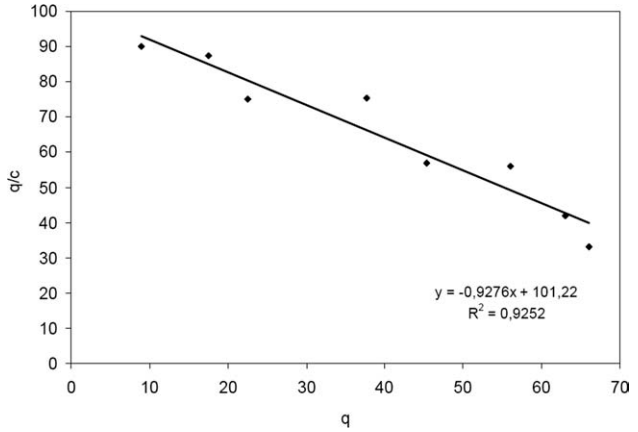

Figure 4 The Scatchard plot of IgG binding to mPGMA-Con A beads.

The equation for the Langmuir adsorption isotherm, $q = (Q_{max}K_aC)/(1+K_aC)$, can be rearranged to form the Scatchard plot, $q/C = K_aQ_{max} - K_aq$, where q/C is the ratio of bound IgG, K is the association constant and Q_{max} the adsorption capacity (Figure 4). Table II shows the calculated association and dissociation constants and the equilibrium adsorption capacities for the binding of IgG to immobilized Con A and compares these values with those calculated for protein A and histidine ligands.[34,35] The association constant of immobilized Con A for IgG is comparable with that of protein A and histidine carrying adsorbents. The association constant values for the peptide ligands and protein A were in the order of 10^5 M which are within the normal range for affinity chromatography carriers.[36]

Effect of flow-rate

The adsorption amount at different flow-rates is given in Figure 5. The adsorption amount decreased significantly from 37.7 to 10.9 mg/g with the increase of flow-rate from 1.0 to 4.0 mL/min. One explanation for such a phenomenon would be a faster ligand-protein (i.e., Con A-IgG) interaction rate. This is due to decrease in contact time between

TABLE II
Comparison of Binding Isotherms of IgG to Immobilized Protein A, Histidine, and Con A

	Protein A[34]	Histidin[35]	Con A
K_a (M^{-1})	1.33×10^5	6.60×10^5	1.39×10^5
K_d (μM)	7.52	1.52	7.2
Q_{max} (mg/g)	139.42	358.2	109.12

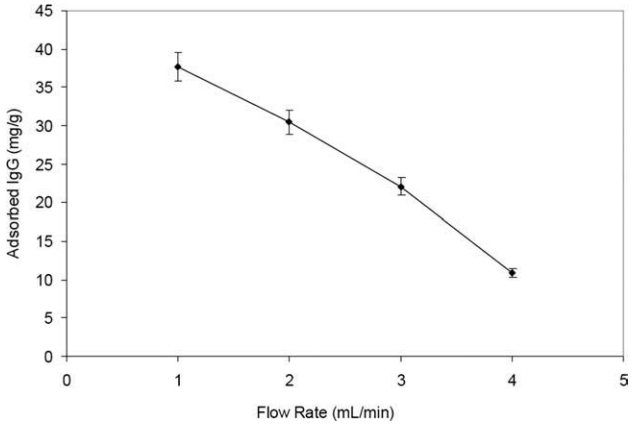

Figure 5 Effect of flow rate on IgG adsorption. IgG concentration: 0.5 mg/mL; Con A loading: 12.5 mg/g; magnetic field: 6.4 mT; pH: 6.0; T: 20°C.

the IgG molecules and the mPGMA-Con A beads at higher flow rates. An increase in flow-rate decreases the retention time. Hence, IgG molecules would pass through the magnetically stabilized fluidized bed column without adsorption at high flow-rates. A second explanation could be increased the nonideal flow hydrodynamics of liquid phase (i.e., IgG solution) and the solid phase (i.e., mPGMA beads) for magnetically stabilized fluidized bed with increasing flow-rate. These phenomena can be summarized by the increase of the axial dispersion model.[37]

Effect of magnetic field

Magnetic properties of polymeric structure was shown using electron mass unit (EMU), showing the behavior of magnetic beads in a magnetic field using a vibrating magnetometer. In EMU spectrum and from Hr value, 2055 Gauss magnetic field was found sufficient to excite all of the dipole moments present in 1.0 g of sample. This value is an important design parameter for a magnetically stabilized fluidized bed or magnetic filtration using the beads. The value of this magnetic field is a function of the flow velocity, bead size, and magnetic susceptibility of solute to be removed. In the literature, this value was found to change from 8 kG to 20 kG for various applications, thus magnetic beads presented in this study will need less magnetic intensity in a magnetically stabilized fluidized bed or for a magnetic filter system.[38] To show the effect of magnetic field on the adsorption capacity of the column, the experiments were carried out in three different magnetic fields. As shown in Figure 6, adsorption amount is decreased

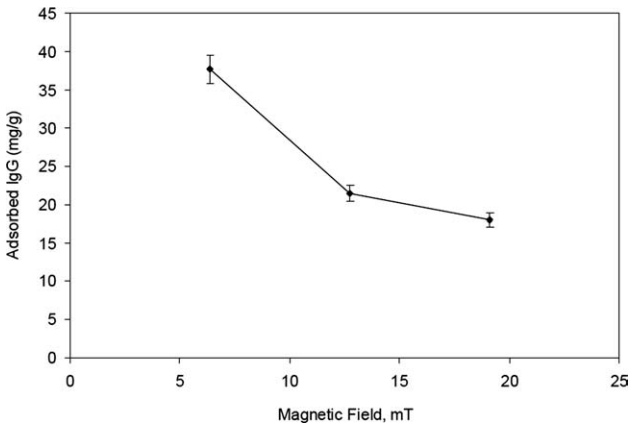

Figure 6 Effect of magnetic field on IgG adsorption. Con A loading: 12.5 mg/g; IgG concentration: 0.5 mg/mL; T: 20°C; pH:6.0.

TABLE III
IgG Adsorption from Human Plasma

Sample	Adsorption capacity (mg/g)
Non-diluted plasma	48 ± 2.45
Diluted plasma (1 : 2, PBS pH 7.4)	23 ± 2.60
Diluted plasma (1 : 5, PBS pH 7.4)	14 ± 2.0
Diluted plasma (1 : 10, PBS pH 7.4)	7 ± 2.55

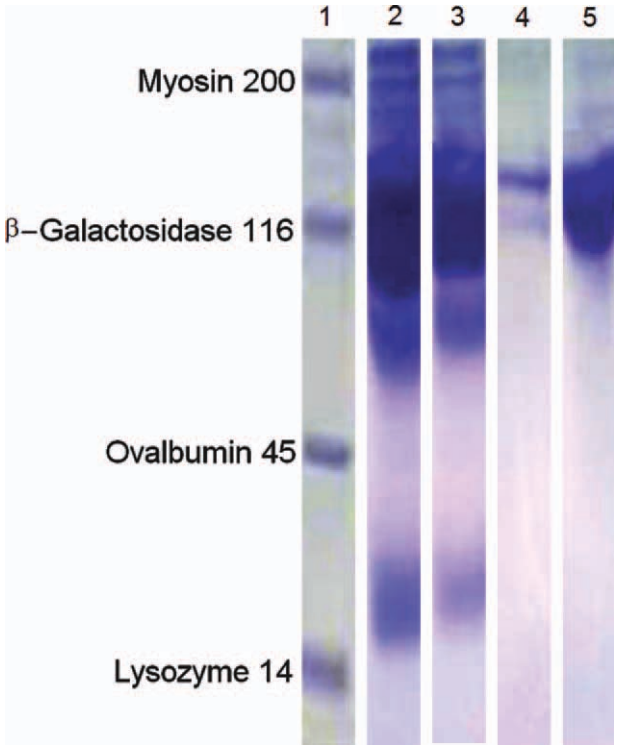

Figure 7 SDS-PAGE of human serum fractions: The fractions were assayed by SDS-PAGE using 8% separating gel (9 × 7.5 cm^2). Stacking gels (5%) were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol-water (1 : 5 : 5, v/v/v) and destained in ethanol-acetic acid-water (1 : 4 : 6, v/v/v). Lane 1: Marker; Lane 2: Initial serum sample; Lane 3: Final serum sample; Lane 4: Standard human IgG sample; Lane 5: Eluted sample. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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

Adsorbent	Ligand	q_{max} (mg/g)	Reference
PHEMA	Con A	69.4	8
Poly(methyl methacrylate)	Cu^{2+}	54.3	22
P(AAm-AGE) Cryogel	Con A	6.7	24
PHEMA cryogel	Protein A	88.1	34
m-poly(EGDMA)	Histidine	320	35
PHEMA beads	L-Histidine	44.8	39
PHEMA beads	Methacryloylamidohistidine	73.8	40
PHEMA beads	Protein A	24.0	41
Poly(methyl methacrylate)	Protein A/G	6.6	42
PHEMA beads	Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}	79.6	43
Toyopearl AF beads	Fc-binding hexapeptide ligands	158	44
P(HEMA-VPBA)	Boronic acid	38.8	45
PHEMA nanoparticles	IMEO	843	46
PHEMA nanoparticles	Phenylalanine	780	47
Bentonite-histidine	L-histidine	89.6	48
UNOsphere SUPrA	Protein A	40	49
Polyacrylamide/ azolactone-activated gel	D-PAM peptide	53-60	50
Poly(GMA-EDMA) beads	L-Histidine	12.5	51
Poly(butadiene-HEMA)	IDA/Cu^{2+}	39.3	52
Poly(GMA) beads	IDA/Cu2+	171.2	53
PGMA/PHEMA composite cryogel	IDA/Cu2+	257	54
PHEMA monolith	Histidine	12.5	55
Poly(LA-Ch)	Lioleic acid	390	56
mPGMA	Con A	66.2	This study

(i.e., from 37.7 to 18 mg IgG per g of mPGMA beads) with the increasing magnetic field from 6.4 to 19.1 mT. This behavior could be attributed to strong agglomeration of magnetic particles in higher magnetic fields, which makes the fluidization of particles more difficult.

IgG adsorption from human plasma

Table III shows the IgG adsorption values from human plasma. As can be seen from the table, lower IgG adsorption capacity was obtained for diluted human plasma. But there was a significant adsorption of IgG (up to 48 mg/g) on the mPGMA-Con A beads from nondiluted blood. The composition of adsorbed and eluted protein was assayed by SDS-PAGE (Fig. 7). About 90% of the protein was IgG indicating high specificity of the mPGMA-Con A beads even in the presence of large amount of other proteins like albumin.

As clearly seen in Figure 6, IgG in serum (Lane 2) was decreased in Lane 3 after adsorption onto mPGMA-Con A beads. Furthermore, the presence of single band at Lane 5 indicates the high purity of IgG after elution from the mPGMA-Con A beads.

A comparison of the maximum adsorption amount of the mPGMA-Con A beads with those of some other affinity adsorbents reported in the recent literature is listed in Table IV. The adsorption capacity of mPGMA-Con A was comparable with 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.

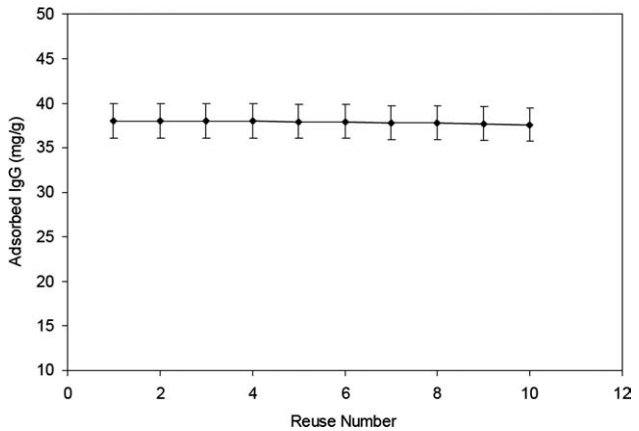

Figure 8 Reusability of the mPGMA-Con A column. Con A loading: 12.5 mg/g; IgG concentration: 0.5 mg/mL; magnetic field: 6.4 mT; flow rate: 1.0 mL/min; T: 20°C; pH:6.0.

Reusability of mPGMA-Con A beads

Reusability is an important criterion for affinity adsorbent. This means that in designing a system, attention must be paid to the chemical stability of the adsorbent.[57] Elution of IgG from the mPGMA-Con A beads was performed using 50% ethylene glycol solution and 2.0*M* NaCl solution. After circulating for 2 h, the released IgG molecules to the

elution medium were measured spectrophotometrically. Elution of IgG is achieved under relatively mild conditions compared to traditional affinity ligands for antibodies and high elution ratio was obtained (more than 98%).

To determine stability and reusability, the mPGMA-Con A column has been used up to ten cycles. For sterilization, after each adsorption-elution cycle, the column was washed with 50 m*M* NaOH solution for 30 min. After this procedure, column was washed with distilled water for 30 min, and then equilibrated with the phosphate buffer for the next adsorption-elution cycle. At the end of 10 adsorption-elution cycles, IgG antibody adsorption capacity decreased only about 2% with ethylene glycol (50%) and NaCl (2.0*M*) as elution solution (Fig. 8).

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. The medical and commercial relevance of proteins/antibodies has stimulated the development of cost- and time-effective purification techniques.[58,59] Bioaffinity chromatography on immobilized lectins has been extensively used to purify glycoproteins. Here, monosize mPGMA beads were produced by dispersion polymerization. A bioligand, Con A, was then immobilized to these beads to have a loading up to 12.5 mg/g, which resulted in an 66.2 mg/g IgG adsorption from aqueous solutions and 48.0 mg/g IgG adsorption from human plasma. Successful elution ratios (98% of adsorbed IgG) were achieved with 50% ethylene glycol and 2.0*M* NaCl. It was possible to reuse the mPGMA-Con A beads without a remarkable reduction in the adsorption capacities.

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