Antibody Purification Using Porous Metal–Chelated Monolithic Columns

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Received 22 July 2005; accepted 29 November 2005
DOI 10.1002/app.23894
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A novel monolithic material was developed to obtain efficient and cost-effective purification of IgG from human plasma. The porous monolith was obtained by bulk polymerization in a glass tube of 2-hydroxyethyl methacrylate (HEMA) and *N*-methacryloyl-(L)-histidine methyl ester (MAH). The poly(HEMA-MAH) monolith had a specific surface area of 214.6 m^2/g and was characterized by swelling studies, porosity measurement, FTIR, scanning electron microscopy, and elemental analysis. Then the monolith was loaded with Cu^{2+} ions to form the metal chelate. Poly-(HEMA-MAH) monolith with a swelling ratio of 74% and containing 20.9 μmol MAH/g was used in the adsorption/desorption of IgG from aqueous solutions and human plasma. The maximum adsorption of IgG from an aqueous solution in phosphate buffer was 10.8 mg/g at pH 7.0. Higher adsorption was obtained from human plasma (up to 104.2 mg/g), with a purity of 94.1%. It was observed that IgG could be repeatedly adsorbed and desorbed with the poly(HEMA-MAH) monolith without significant loss of adsorption capacity. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 395–404, 2006

Key words: adsorption; chromatography; protein separation techniques

INTRODUCTION

Packed-bed columns have inherent limitations such as slow diffusional mass transfer and a large void volume between the beads, leading to the efficiency of conventional columns remaining in the range of 10,000–30,000 plates/column in the past two decades.[1] Although some new stationary phases such as monosize nonporous beads[2–4] and perfusion chromatography packings have been designed to resolve these limitations, in essence they have not been overcome.[5] Recently, monolithic columns have been considered a novel generation of stationary phases for the separation of biomolecules because of their easy preparation, excellent flow properties, and high performance compared to those of conventional beads.[6–10]

Antibodies are biologically active proteins produced by plasma cells in response to the presence of foreign substances. The growing role of antibodies in biomedical research and devolopment is widely acknowledged. Antibody-based in vivo diagnostics and therapeutics are gaining wider approval from regulatory agencies around the world.[11] At present, the most widely used technique for antibody purification is affinity chromatography on protein A adsorbents.[12–14] The high specificity of protein A for the Fc antibody domain provides excellent chromatographic selectivity.[15] Despite this, protein A sorbent has drawbacks worth considering: (1) it could leak from the matrix, causing contamination, which is intolerable in clinical applications; and (2) it would tend to be very high in cost. Ligands such as protein A or G are difficult to immobilize in the proper orientation. They also are susceptible to degradation during cleaning procedures. To avoid complications from the use of protein A sorbents, several available alternative purification techniques can be used: ion exchange chromatography, hydrophobic interaction chromatography, dye–ligand chromatography, thiophilic chromatography, histidine affinity chromatography, and molecular sieving.[16–18] However, a comparison of these techniques is of little significance because they lack the selectivity of protein A. Among the techniques, metal-chelate affinity chromatography is a promising alternative in downstream processing for the purification of antibodies.[19–21]

Immobilized metal affinity chromatography (IMAC) is a sensitive technique for protein separation that enables distinguishing between proteins differing by only a single histidine residue on the surface.[22–27] It is assumed that proteins interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Cooperation between neighboring amino acid side chains and local conformations play important roles in protein binding. Aromatic amino acids

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Journal of Applied Polymer Science, Vol. 101, 395–404 (2006)
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and the amino terminal of the peptides also contribute.[28] The low cost of metals and the ability to reuse adsorbents hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.

This article reports on the purification of an IgG antibody from human plasma by metal-chelate affinity chromatography with a monolith column. Poly(HEMA-MAH) monolith is a copolymer of 2-hydroxyethyl methacrylate (HEMA) and *N*-methacryloyl-(L)-histidine-methylester (MAH), which was obtained by bulk polymerization. The poly(HEMA-MAH) monolith was characterized by scanning electron microscopy (SEM), porosity measurement, FTIR, elemental analysis, and swelling tests. Then Cu^{2+} ions were chelated through imidazole groups on the MAH reactive functional groups of the polymeric structure. The ability of the monoliths to adsorb IgG from aqueous solutions containing different IgG concentrations at different pHs and ionic strengths and from human plasma was investigated. Desorption of IgG and material stability also were tested.

EXPERIMENTAL

Materials

Immunoglobulin G (IgG) (Sigma, cat. no. 160101), L-histidine methylester, and methacryloyl chloride were supplied by Sigma (St. Louis, MO). Hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. Potassium persulfate (KPS) was obtained from Fluka (Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) and then a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Synthesis of MAH

The synthesis and characterization of MAH were performed as described previously.[29] In the experimental procedure for synthesis of MAH, 5.0 g of L-histidine hydrochloride and 0.2 g of hydroquinone were dissolved in 100 mL of a dichloromethane solution, which was cooled to 0°C. Then 12.7 g of triethylamine was added to the solution, followed by the addition of 5.0 mL of methacryloyl chloride, which was poured in slowly. Then this solution was stirred magnetically at room temperature for 2 h, after which hydroquinone and unreacted methacryloyl chloride were extracted with a 10% NaOH solution. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether–cyclohexane mixture and then dissolved in ethyl alcohol. ^{1}H-NMR, performed in $CDCl_3$ on a JEOL GX-400 300 MHz instrument, was used to determine if the MAH structure was synthesized. The residual nondeuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in parts per million downfield relative to $CHCl_3$. The ^{1}H-NMR spectrum shows the characteristic peaks of the groups in the MAH monomer as follows—^{1}H-NMR ($CDCl_3$): $\delta = 1.99$ (t; 3H, J = 7.08 Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, O—CH_3) 4.82–4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86 (δ; 1H, J = 7.4 Hz, NH), 7.82 (δ; 1H, J = 8.4 Hz, NH), 6.86–7.52 (m; 5H, aromatic).

Preparation of poly(HEMA-MAH) monolithic column

The poly[hydroxyethyl methacrylate-*N*-methacryloyl-(L)-histidinemethylester] [poly(HEMA-MAH)] monolithic column was prepared by in situ polymerization in a glass tube, using potassium persulfate as the initiator. Toluene and EGDMA were the pore former and the crosslinker, respectively. Potassium persulfate (15 mg) was dissolved in a mixture of monomers (HEMA: 1.0 mL; EGDMA: 250 μL) and porogenic diluent (toluene: 750 μL). MAH (10 mg) was dissolved in HEPES (500 μL). These monomer solutions were mixed, and then the final solution was purged with nitrogen for 15 min. The glass tube (100 × 10 mm inside diameter) was filled with the above mixture and then sealed with pieces of silicone rubber tubing plugged with silicone stoppers. Polymerization was allowed to proceed at 75°C for 45 min. The tube was then attached to a chromatographic system. Ethyl alcohol (50 mL) and water (50 mL) were pumped through the column at a flow rate of 1.0 mL/min in order to remove the unreacted monomers and porogenic diluents in the monolith after completion of polymerization. The monolith was stored in buffer containing 0.02% sodium azide at 4°C until use.

Incorporation of Cu^{2+} ions

The investigation of Cu^{2+} chelation was carried out in a recirculating system equipped with a water jacket for temperature control. The monolith was washed with 30 mL of water. Then 40 mL of a Cu^{2+} solution [50 mg/L (pH 4.1), adjusted with HCl and NaOH] was pumped through the column under recirculation at room temperature for 2 h. A 1000-ppm atomic absorption standard solution (containing 10% HNO_3) was the source of the Cu^{2+} ions. The concentration of the Cu^{2+} ions in the resulting solution was determined

with a graphite furnace atomic absorption spectrometer (GFAAS, Analyst 800/Perkin Elmer, USA). The instrument response was periodically checked with known metal solution standards. The experiments were performed in triplicate, as were analyses of the samples. For each set of data, standard statistical methods were used to determine the mean and standard deviation. A 95% confidence interval was calculated for each set of samples in order to determine the margin of error. The Cu^{2+} concentrations in the initial and final solutions were used to calculate the amount of Cu^{2+} ions adsorbed.

Cu^{2+} leakage from the poly(HEMA-MAH) monolith was investigated in media whose pH varied between 5.0 and 8.0 and also in a medium containing 1.0*M* NaCl. The monolith was stirred for 24 h at room temperature. Then the concentration of Cu^{2+} ions in the supernatants was determined using an atomic absorption spectrophotometer. Note that the metal-chelated monolith was stored at 4°C in a 10 m*M* Tris buffer (pH 7.4).

Characterization of monoliths

Surface area and porosity measurements

The surface area of the monolith sample was determined in a BET isotherm of nitrogen using an ASAP2000 instrument (Micromeritics, Norcross, GA). Pore volume and average pore diameter greater than 20 Å were determined up to 2000 kg/cm^2 with a mercury porosimeter (Carlo Erba, model 200).

Swelling test

Monolith water uptake ratios were determined in distilled water as follows: A dry monolith was carefully weighed and then placed in a 50-mL vial containing distilled water. The vial was put into an isothermal water bath at 25°C for 24 h. The monolith was removed from the water, wiped with a filter paper, and weighed. The water content of the monolith (ratio of the dry sample mass to the wet sample mass) was calculated using the following expression:

$$\text{Water uptake ratio } \% = [(W_s - W_o)/W_o] \times 100 \quad (1)$$

where W_o and W_s are the mass of the monolith before and after uptake of water, respectively.

Surface morphology

Scanning electron microscopy was used to analyze the morphology of cross sections of dried monolith. The samples were initially dried in air at 25°C for 7 days before use in the SEM analysis. A fragment of dried monolith was mounted on a SEM sample mount and sputter-coated for 2 min. The sample was then mounted in a scanning electron microscope (Raster Electronen Microscopy, Leitz-AMR-1000, Germany), and the surface was scanned at the desired magnification.

Elemental analysis

Elemental analysis (Leco elemental analyzer, Model CHNS-932, USA) was performed to evaluate how much MAH was incorporated into the poly(HEMA-MAH) monolith.

FTIR

The FTIR spectrum of the poly(HEMA-MAH) monolith was obtained with an FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). Dry monolith (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany) and pressed into pellet form, after which the FTIR spectrum was recorded.

Chromatographic procedures

IgG adsorption from aqueous solutions

Investigation of IgG adsorption was carried out in a recirculating system equipped with a water jacket for temperature control. The monolith was washed with 30 mL of water and then equilibrated with 25 m*M* phosphate buffer containing 0.1*M* NaCl (pH 7.4). Then the prepared IgG solution was pumped through the column under recirculation for 2 h. The adsorption was followed by monitoring of the decrease in UV absorbance at 280 nm. The effects of flow rate, IgG concentration, pH of the medium, and ionic strength on adsorption capacity were studied. The flow rate of the solution (i.e., 50 mL of the aqueous IgG solution) was varied in the range of 0.5–4.0 mL/min. To observe the effects of the initial concentration of IgG on adsorption, it was varied between 0.05 and 2.0 mg/mL. To determine the effects of pH and temperature on adsorption, they were varied between 4.0 and 8.5 and 4°C and 37°C, respectively. To observe the effects of ionic strength, NaCl was used at ionic strengths of 0.01 and 0.1.

Desorption and repeated use

In all cases, the adsorbed IgG molecules were desorbed using a 1.0*M* NaCl solution. In a typical desorption experiment, 50 mL of the desorption agent was pumped through the monolith column at a flow rate of 1.0 mL/min for 1 h. The final IgG concentration in the desorption medium was spectroscopically determined by a solid-phase enzyme-linked immunosorbent assay (ELISA) method. When desorption was

achieved, the monolith was cleaned with 50 mM sodium hydroxide and then reequilibrated with 25 mM phosphate buffer containing 0.1M NaCl (pH 7.4). The desorption ratio was calculated as the ratio of the amount of IgG adsorbed on the monolith to the final IgG concentration in the desorption medium.

To test the repeated use of monoliths, the IgG adsorption–desorption cycle was repeated 10 times using the same monolith. To regenerate and sterilize, after desorption, the monolith was washed with 1M sodium hydroxide solution.

IgG adsorption from human plasma

Human blood was collected in EDTA-containing vacutainers. Centrifugation at 4000 g for 30 min at room temperature was used to separate the red blood cells from the plasma, which was then filtered (3-μm Sartorius filter) and frozen at -20°C. Prior to use, the plasma was thawed for 1 h at 37°C. Before application, the viscous sample was diluted with 25 mM phosphate buffer containing 0.1M NaCl (pH 7.4) to ratios of 1:2 and 1:10. Then 50 mL of the human plasma with an IgG content of 14.6 mg/mL was pumped through the monolith column at a flow rate of 1.0 mL/min for 1 h. The amount of IgG adsorbed on the monoliths was determined using the solid-phase ELISA method. Human anti-IgG (Sigma, I-9384) diluted 1:1000 in 50 mM $NaHCO_3$ (pH 9.6) was adsorbed to PVC microtiter 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% BSA, and 0.1% sodium azide (blocking buffer). Samples (2.5 mL, neutralized with 0.5 mL of 1.0M trisodium citrate) or controls containing known amounts of IgG were added and incubated at 37°C for 1 h. Bound IgG was detected with biotin-labeled anti-IgG followed by peroxidase-conjugated streptavidin and *o*-phenylenediamine. Absorbance was measured at 492 nm.

Adsorption of albumin and fibrinogen also was monitored. The monolith had contact with human plasma containing albumin (37.2 mg/mL), fibrinogen (2.2 mg/mL), and γ-globulin (14.6 mg/mL) at room temperature for 2 h in a continuous system described previously. The flow rate was kept constant at 1.0 mL/min. Total protein concentration was measured using a total protein reagent (Ciba Corning Diagnostics Ltd., Halstead, Essex, UK; catalog ref. no. 712076) at 540 nm, which is based on the Biuret reaction. Chronometric determination of fibrinogen on plasma was performed according to the Clauss method using Fibrinogene-Kit (Ref. Nos. 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France). Human serum albumin concentration was determined according to the bromocresol green (BCG) dye method using Ciba Corning albumin reagent (catalog ref. no. 229241). IgG concentration was determined by ELISA as described above.

The purity of IgG was assayed by sodium dodecylsulfate–polyacrylamide gel electrophoresis using 10% separating gels (9 $\times$ 7.5 cm) and 6% stacking gels 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.

RESULTS AND DISCUSSION

In this study, we prepared a monolith for specific metal-chelating affinity separation of IgG from human plasma. MAH was used as the metal-chelating affinity ligand to chelate Cu^{2+} ions for specific binding of IgG molecules. According to mercury porosimetry data, the average pore size of the monolith was 820 nm. Total pore volume was 3.85 mL/g, and porosity was more than 83%. These results indicated that the pore volume and pore size were sufficiently large to ensure modest resistance to the mobile phase. The equilibrium swelling ratio of the poly(HEMA-MAH) monolith was 74%. Compared to the equilibrium swelling ratio of poly(HEMA) (35%), the water uptake ratio of the poly(HEMA-MAH) monolith was increased. Increasing of surface area may affect the swelling ratio of the matrix. The specific surface area of the monolith was found to be 214.6 m^2/g by the BET method. The poly(HEMA-MAH) monolith had a larger surface area than did the poly(HEMA) monolith (65.8 m^2/g). The MAH content of the polymerization mixture was responsible for this large surface area. Therefore, more water molecules penetrated the entanglement polymer chains, resulting in increased polymer water uptake in aqueous solutions.

The monolithic columns prepared in glass tubes were observed by scanning electron microscopy (SEM), which showed (Fig. 1) a structure typical of monolithic materials. The monoliths were composed of small and interconnected globules that formed a monolithic porous structure. The globules were 2 μm in size and irregular. There also were many 2-μm-diameter pores on the bulk structure of the globules. The large pores between the clusters were 1 μm in size. Using flow rates suitable for chromatographic purposes, this open structure allowed liquid to be forced through the polymer without compressing it. The back pressure of the monolith was only about 5.8 MPa when the flow rate reached 5.0 mL/min.

Concentration of the incorporated MAH was found to be 20.9 μmol/g polymer using nitrogen stoichiometry. Note that HEMA and other chemicals in the polymerization formula do not contain nitrogen. Elemental analysis determined that this nitrogen deter-

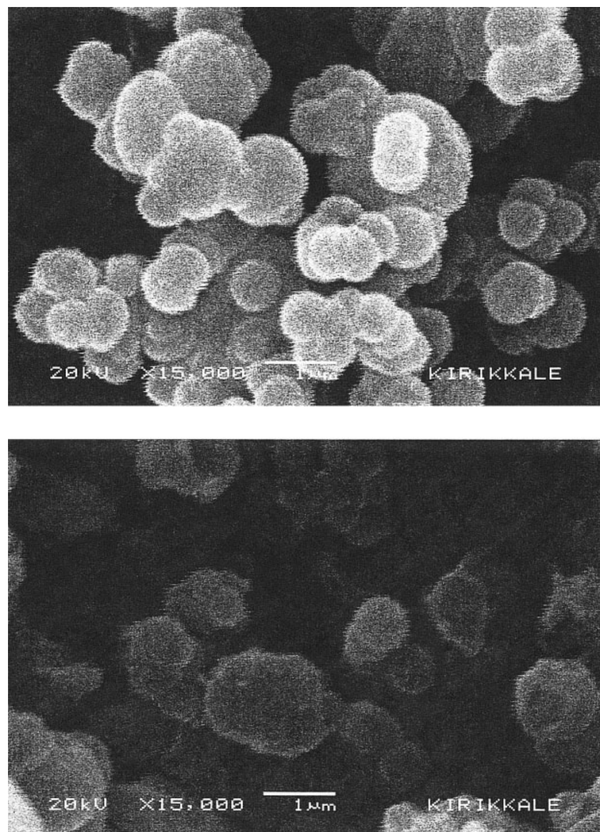

Figure 1 SEM micrographs of poly(HEMA-MAH) monolith.

mined only came from the MAH groups incorporated into the polymeric structure.

The FTIR spectrum of the poly(HEMA-MAH) monolith had the characteristic stretching vibration bands of hydrogen-bonded alcohol, O—H, around 3586 cm^{-1} and carbonyl, at 1645 cm^{-1}, and the absorption bands of amide II, at 1516 cm^{-1}.

The Cu^{2+} chelation step is shown in Figure 2. The imidazole nitrogen donor atom incorporated into the MAH group was the most common binding site for metal ions. The amount of chelated Cu^{2+} on poly-(HEMA-MAH) monolith was measured as 16.3 μmol/g polymer. Mass stoichiometric analysis

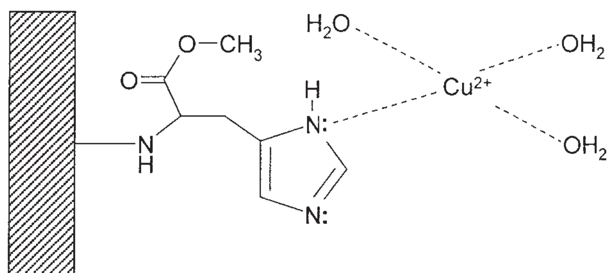

Figure 2 Schematic diagram for the chelation of Cu^{2+} ions through the monolith.

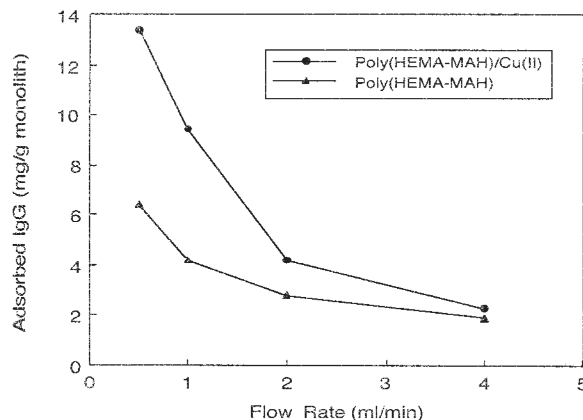

Figure 3 Effect of flow rate on IgG adsorption: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; IgG concentration, 0.8 mg/mL; adsorbing buffer PBS, pH 7; temperature, 25°C.

showed that 1 incorporated MAH molecule interacted around 1 Cu^{2+} ion (20.9 μmol MAH/g:16.3 μmol Cu^{2+}/g). Because MAH has two coordinating sites of nitrogen atoms, it could form a ternary complex that was coordinated water molecules at vacant coordination sites of the Cu^{2+}–MAH complexes. Investigation of leakage of Cu^{2+} from the poly(HEMA-MAH) monolith detected no leakage in any of the adsorption and desorption media, suggesting that the washing procedure was satisfactory for the removal of the nonspecific adsorbed Cu^{2+} ions from the monolith.

Adsorption of IgG from aqueous solutions

Effect of flow rate

Adsorption capacity at different flow rates is shown in Figure 3. Adsorption capacity decreased significantly, from 13.4 to 2.3 mg/g for the Cu^{2+}-chelated poly-(HEMA-MAH) monolith and from 6.4 to 1.9 mg/g for the poly(HEMA-MAH) monolith, with flow rate increasing from 0.5 to 4.0 mL/min. One explanation for such a phenomenon would be a faster ligand–protein (i.e., MAH-Cu^{2+}–IgG) dissociation rate compared to the association rate. Hence, at a high flow rate the adsorbate (i.e., protein molecules) would pass through the metal-chelated monolithic column without adsorption.

Effect of buffer type

Figure 4 shows the IgG adsorption capacity in different buffer systems at different pHs. IgG adsorption onto the monolith seemed to depend on the pH. The buffer ranges were 5.5–6.5 for MES, 5.5–7.4 for phosphate, 7.0–8.0 for HEPES and Tris-HCl, and 6.5–8.0 for MOPS. In the phosphate buffer, adsorption capacity was higher than in other buffers. Maximum ad-

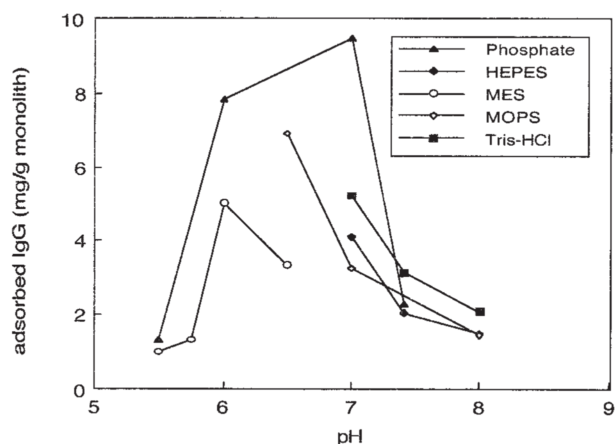

Figure 4 Effect of buffer type on IgG adsorption: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; IgG concentration, 0.8 mg/mL; flow rate, 1.0 mL/min; temperature, 25°C.

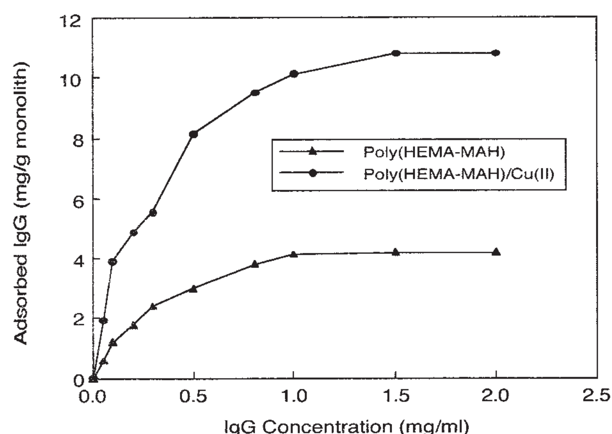

Figure 5 Effect of IgG concentration on adsorption capacity: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; adsorbing buffer PBS, pH 7; flow rate, 1.0 mL/min; temperature, 25°C.

sorption capacity were observed at a pH of 6.5 for MOPS (6.9 mg/g), 7.0 for HEPES (4.2 mg/g), 7.0 for Tris-HCl (5.3 mg/g), 7.0 for phosphate (9.5 mg/g), and 6.0 for MES (5.0 mg/g). Below and above these maximum adsorption pHs, the adsorption capacity decreased significantly. The pKa values for MOPS, HEPES, and MES were pHs of 6.5, 7.0, and 5.4, respectively. From the structure of the buffer ions used, it was obvious that Tris-HCl and phosphate carried one or more charges of the same sign —only positive or only negative, whereas the zwitter ionic buffers MES, MOPS, and HEPES carried two charges of opposite sign below their pKa. It could be clearly observed that maximum adsorption was obtained with phosphate.

Effect of concentration of IgG

Figure 5 shows the effect of IgG concentration on adsorption. IgG adsorption on the poly(HEMA-MAH) monolith was low (about 4.2 mg/g), although adsorption of IgG molecules onto the Cu^{2+}-chelated poly-(HEMA-MAH) monolith through Cu^{2+} ions was significant (up to 10.8 mg/g). As expected, the amount of IgG coupled to monoliths almost reached a plateau of around 1.0 mg/mL because of saturation of the active binding sites.

Effect of ionic strength

Adsorption of IgG by the Cu^{2+} chelated-poly(HEMA-MAH) and poly(HEMA-MAH) monoliths was performed at different NaCl concentrations. The effect of ionic strength on IgG adsorption is shown in Figure 6. As seen here, IgG adsorption capacity decreased with increasing salt concentration. The decrease in adsorption capacity as ionic strength increased can be attributed to the repulsive electrostatic forces between the Cu^{2+}-chelated poly(HEMA-MAH) monolith and protein molecules. When the salt concentration increased in the adsorption medium, this could have led to coordination of the deprotonated amino groups of the histidine with the cations of the salts, resulting in low protein adsorption. The distortion of existing salt bridges between protein molecules and the pseudospecific metal-complexing affinity ligand in the presence of salt also contributed to the low protein adsorption at high ionic strength.

Effect of temperature

The effect of temperature on IgG adsorption was studied in the range of 4°C–37°C. At all temperatures, adsorption of IgG by the poly(HEMA-MAH) monolith was lower than that by the Cu^{2+}-chelated-poly-

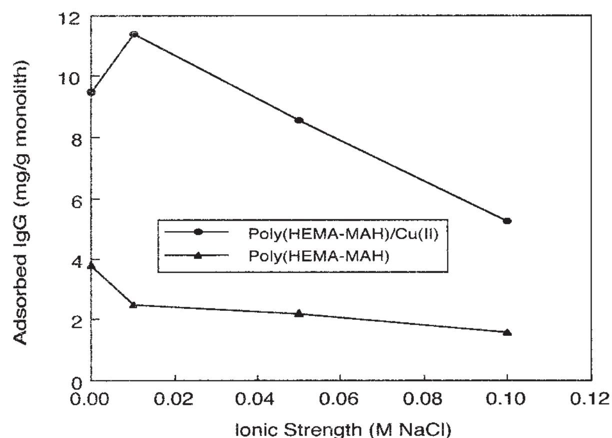

Figure 6 Effect of ionic strength on IgG adsorption: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; IgG concentration, 0.8 mg/mL; flow rate, 1.0 mL/min; adsorbing buffer PBS, pH 7; temperature, 25°C.

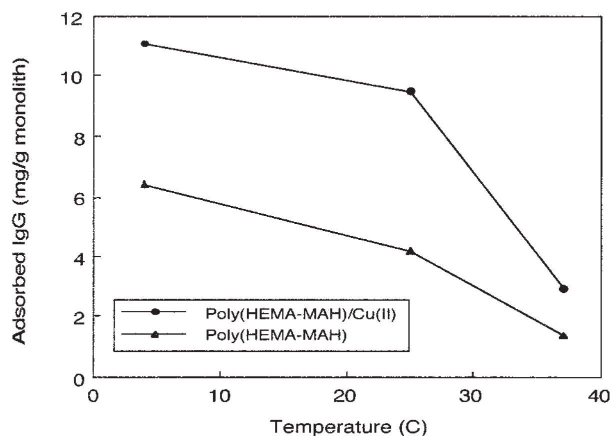

Figure 7 Effect of temperature on IgG adsorption: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; IgG concentration, 0.8 mg/mL; flow rate, 1.0 mL/min; adsorbing buffer PBS, pH 7.

(HEMA-MAH) monolith. No significant effect of temperature was observed on the physical adsorption of IgG by poly(HEMA). However, the equilibrium adsorption of IgG onto the poly(HEMA-MAH) and Cu^{2+}-chelated poly(HEMA-MAH) monoliths decreased significantly with increasing temperature, with maximum adsorption achieved at 4°C (Fig. 7). From 4°C to 37°C, the adsorption capacity of the monoliths decreased about 73% for the Cu^{2+}-chelated poly(HEMA-MAH) and 78% for the poly(HEMA-MAH). A possible explanation for this behavior is the exothermic nature of the adsorption process.

Adsorption Isotherms

An adsorption isotherm was used to characterize the interactions of each protein molecule with the adsorbent. This showed a relationship between the concentration of protein in the solution and the amount of protein adsorbed on the solid phase when the two phases were at equilibrium. The Langmuir adsorption model assumes that molecules are adsorbed at a fixed number of well-defined sites, each of which can hold only one molecule. These sites are also assumed to be energetically equivalent and distant from each other so that there is no interaction between molecules adsorbed on adjacent sites.

Adsorption isotherms were used to evaluate the adsorption properties. The Langmuir adsorption isotherm is expressed by eq. (2). The corresponding transformations of the equilibrium data for IgG resulted in a linear plot, indicating that the Langmuir model could be applied in these systems, described by the equation:

$$Q = Q_{max} \cdot b \cdot C_{eq}/(1 + bC_{eq}) \quad (2)$$

where Q is the amount of IgG adsorbed (mg/g), C_{eq} is the equilibrium IgG concentration (mg/mL), b is the Langmuir constant (mL/mg), and Q_{max} is the maximum adsorption capacity (mg/g). This equation can be linearized so that

$$C_{eq}/Q = 1/(Q_{max} \cdot b) + C_{eq}/Q_{max}. \quad (3)$$

The plot of C_{eq} versus C_{eq}/Q was employed to generate the intercept of $1/Q_{max}b$ and the slope of $1/Q_{max}$.

The maximum adsorption capacity (Q_{max}) data for the adsorption of IgG were obtained from the experimental results (Table I). The correlation coefficients (R^2) were high for Cu^{2+}-chelated poly(HEMA-MAH) monolith. The Langmuir adsorption model can be applied in this affinity adsorbent system.

The other well-known isotherm that is frequently used to describe adsorption behavior is the Freundlich isotherm. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed protein is the sum total of the adsorption on all binding sites. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. This empirical equation takes the form

$$Q_{eq} = K_F(C_{eq})^n \quad (4)$$

where K_F and n are the Freundlich constants.

The adsorption isotherms of IgG were found to be linear over the whole range of concentrations studied, and the correlation coefficients were high. Table I shows the Freundlich adsorption isotherm constants, n and K_F, and the correlation coefficients. The magnitude of K_F and n of the Freundlich model showed that easy uptake of IgG occurred from aqueous medium,

TABLE I
Langmuir and Freundlich Adsorption Constants and Correlation Coefficients for IgG

	Langmuir constants				Freundlich constants		
Type of polymer	q_{ex} (mg/g)	q_m (mg/g)	K_d	R^2	K_F	n	R^2
Polymer	4.2	4.88	3.96	0.995	4.46	0.50	0.994
Polymer/Cu(II)	10.8	11.3	10.7	0.993	10.2	0.27	0.935

TABLE II
First- and Second-order Kinetic Constants for Cu^{2+}-Chelated Poly(HEMA-MAH) Monolith

Initial concentration (mg/mL)	Experimental	First-order kinetic			Second-order kinetic		
	q_{eq} (mg/g)	k_1 (L/min)	q_{eq} (mg/g)	R^2	k_2 (g mg^{-1} min^{-1})	q_{eq} (mg/g)	R^2
0.05	2.61	0.050	2.34	0.959	0.035	2.18	0.946
0.1	3.92	0.053	3.28	0.978	0.027	4.17	0.994
0.2	4.89	0.037	5.29	0.988	0.003	7.36	0.939
0.3	5.55	0.041	5.25	0.921	0.007	6.86	0.974
0.5	8.16	0.023	8.24	0.970	0.008	15.10	0.831
0.8	9.47	0.038	10.60	0.965	0.014	14.50	0.937
1.0	10.12	0.040	11.30	0.976	0.012	15.80	0.902
1.5	10.77	0.046	12.40	0.966	0.009	17.70	0.814
2.0	10.80	0.062	11.80	0.995	0.004	12.80	0.979

with the metal-chelated monolith having a high adsorption capacity.

Adsorption kinetics modeling

To determine the controlling mechanism of the adsorption process such as mass transfer or chemical reaction, kinetic models were used to test the experimental data. The kinetic models (pseudo-first- and -second-order equations) can be used in this case assuming the measured concentrations equal the adsorbent surface concentrations. 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

$$dq_t/dt = k_1(q_{eq} - q_t) \qquad (5)$$

where k_1 is the rate constant of pseudo-first-order adsorption (1/min) and q_{eq} and q_t are the amounts of protein adsorbed at equilibrium and at time t (mg/g), respectively. After integration by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, gives

$$\log[q_{eq}/(q_{eq} - q_t)] = (k_1 t)/2.303 \qquad (6)$$

Equation (6) can be rearranged to obtain the linear form

$$\log(q_{eq} - q_t) = \log(q_{eq}) - (k_1 t)/2.303 \qquad (7)$$

where a plot of log ($q_{eq} - q_t$) versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process, log q_{eq} should equal the interception point of a plot of log($q_{eq} - q_t$) via t.

In addition, a pseudo-second-order equation based on equilibrium adsorption capacity may be expressed in the form

$$dq_t/dt = k_2(q_{eq} - q_t)^2 \qquad (8)$$

where k_2 (g mg^{-1} min^{-1}) is the rate constant of the pseudo-first-order adsorption process. Integrating eq. (8) and applying the boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, leads to

$$[1/(q_{eq} - q_t)] = (1/q_{eq}) + k_2 t \qquad (9)$$

or, equivalently, the linear form

$$(t/q_t) = (1/k_2 q_{eq}^2) + (1/q_{eq})t \qquad (10)$$

where a plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively. A comparison of the experimental and theoretical adsorption capacity values is presented in Table II. The theoretical q_e values estimated from the pseudo-first- and -second-order kinetic models were very close to the experimental values, and the correlation coefficients were high. The results indicate this metal-chelated monolith was described by both the first-order and the second-order kinetic models.

Adsorption from human plasma

Table III gives the adsorption data. As seen here, there was a pronounced adsorption of IgG (up to 104.2 mg/g) onto the Cu^{2+}-chelated poly(HEMA-MAH)

TABLE III
IgG Adsorption from Human Plasma IgG Concentration Before Dilution: 14.6 mg/mL; MAH Loading: 20.9 μmol/g; Cu^{2+} Loading: 16.3 μmol/g; Flow Rate: 1.0 mL/min; *T*: 25°C

Dilution agent	Adsorption capacity (mg/g)
Plasma (undiluted)	104.2 ± 2.41
1:2 diluted plasma (phosphate pH: 7.4)	62.3 ± 2.80
1:10 diluted plasma (phosphate pH: 7.4)	41.8 ± 2.63

monolith for plasma diluted with PBS. The purity of the IgG, assayed by SDS-PAGE, was found to be 94.1%. It is worth noting that because of the high initial concentration of IgG in the plasma, the adsorption of IgG by the Cu^{2+}-chelated poly(HEMA-MAH) monolith was higher than that obtained with aqueous solutions. IgG has a molecular mass of 150,000 and consists of four peptide chains; two identical light chains are linked by strong disulfide bonds to make a Y- or T-shaped structure with hingelike flexible arms. Thus, an IgG molecule would expand and contract significantly with variation in the ionizable groups in the molecule. This high IgG adsorption may also have been a result of suitable conformation of IgG molecules in their native medium [i.e., human plasma (pH 7.4)] for interaction with the histidine groups of the poly(HEMA-MAH) monolith.

Adsorptions of albumin and fibrinogen also were determined. There was a pronounced adsorption of IgG by the Cu^{2+}-chelated poly(HEMA-MAH) monolith for undiluted plasma (104.2 mg/g). Adsorption capacity was found to be 6.2 mg/g for fibrinogen and 8.5 mg/g for albumin. The total protein adsorption was determined as 119.1 mg/g. The IgG adsorption ratio was around 87.5% (104.2 mg IgG/g adsorbent; 119.1 mg total protein/g adsorbent). The fibrinogen and albumin adsorption ratios were 5.2% (6.2 mg fibrinogen/g sorbent; 119.1 mg total protein/g sorbent) and 7.1% (8.5 mg albumin/g sorbent; 119.1 mg total protein/g sorbent). IgG adsorbed more than HSA under physiological conditions, although the initial concentration ratio of IgG to HSA (mg/mL) was 11.9:37.2. Most of the described methods for IgG capture resulted in coadsorption of several other proteins, among which adsorption of albumin was significant. Adsorption of IgG was significant compared to adsorption of albumin and fibrinogen. Transition metal ions have a high affinity to the peptide sequences His-Gly-His, His-Tyr-NH_2, and His-Trp. The significant IgG adsorption by the Cu^{2+}-chelated monolith could have been a result of its having a greater number of histidine residues, which could interact with the metal ions.

Comparison with related literature

Different IgG adsorption capacities have been reported in the literature. Füglistaller was interested in determining the dynamic binding capacities of different commercial protein A affinity chromatography matrices, including Affi-Gel, Eupergit, Ultragel, the Sepharose series, and Prosep A.[15] He found that adsorption capacity varied between 0.7 and 20 mg IgG_3/g. Bueno et al. used poly(ethylene vinyl alcohol) hollow-fiber cartridges carrying L-histidine to determine adsorption; they reported dynamic adsorption of up to 77.7 mg IgG/g polymer.[30] Klein et al. used microporous poly(caprolactam) hollow fibers and flat-sheet membranes as the carrier matrices and immobilized recombinant protein A as the specific bioligand.[31] They reported IgG adsorption capacities of around 12.4–28.3 mg/cm^3. With the use of hydrophobic amino acids (e.g., phenylalanine and tryptophan) containing membranes based on polyethylene, Kim et al. obtained an adsorption capacity of 50 mg/g polymer for bovine gamma globulin.[32] Muller-Shulte et al. used several polymeric carriers made of different polymers, including Biograft, Sepharose 4B, Superose, and Spherosil, with histidine as the pseudospecific ligand.[33] They found maximum IgG_1 adsorption in the range of 0.05–0.23 mg IgG_1/mL sorbent. Denizli et al. reported an adsorption capacity of 24 mg IgG/g with protein A–immobilized PHEMA beads.[34] Using microporous immunoaffinity hollow fibers composed of polysulfone/protein A, Charcosset et al. obtained an adsorption capacity of 8.8 mg/g.[35] Langotz and Kroner reported an adsorption capacity of 0.5 mg/mL rabbit IgG with commercially available Sartobind Epoxy sorbents.[36] Dancette et al. studied the performance of recombinant protein A/G affinity membranes based on poly(methyl methacrylate), and polyacrylonitrile for human and mouse IgG purification, and they obtained a static binding capacity of 6.6 mg IgG/mL membrane.[37] Teng et al. described a fully characterized IgG-binding ligand comprising a triazine scaffold substituted with 3-aminophenol and 4-amino-1-naphthol.[38] They showed that this synthetic ligand mimicking protein A interacted with HIgG and was able to selectively purify IgG from diluted human plasma. They achieved an adsorption capacity of 52 mg/g moist weight gel. Özkara et al. immobilized L-histidine covalently on PHEMA beads, obtaining adsorption of 3.5 mg IgG/g polymer in a batch system.[39] Protein A mimetic synthetic ligands carrying Sepharose 4B and Sepharose CL-6B sorbents were used in the purification of IgG from diluted human plasma, with the maximum adsorption capacity found to be in the range of 7–25 mg/g.[40,41] The IgG adsorption capacity obtained in the present study (up to 104 mg IgG/g) would seem to be sufficient for proposing that these metal-chelated affinity monoliths be used as IMAC supports. Differences in IgG adsorption are a result of the specific properties of each adsorbent, such as structure, functional groups, ligand loading, and surface area.

Desorption and repeated use

In the last step of the affinity separation, the main concern was to desorb the adsorbed protein in the shortest time and the highest amount possible. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. In this study, more than 95% of the adsorbed IgG molecules

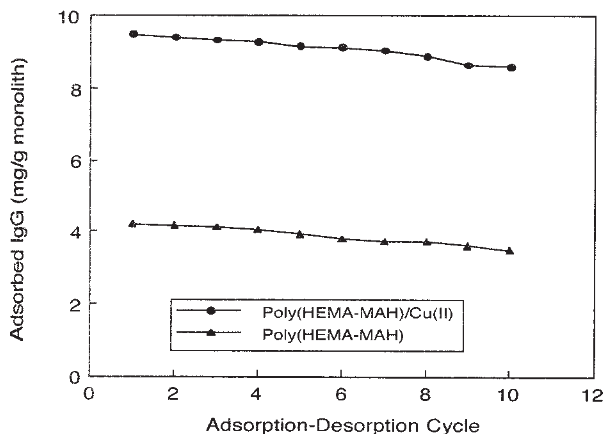

Figure 8 Repeated used of the Cu^{+2} chelated-poly(HEMA-MAH) monolith: MAH loading, 20.9 μmol/g; Cu^{2+} loading, 16.3 μmol/g; IgG concentration, 0.8 mg/mL; flow rate, 1.0 mL/min; pH, 7.0; temperature, 25°C.

were removed easily from the monolith in all cases when 1*M* NaCl was used as the desorption agent. Note that elution of IgG was achieved under the relatively mild conditions employed during affinity chromatography on protein A sorbents.

To show reusability of the monoliths, the adsorption–desorption cycle was repeated 10 times using the same monolith (Fig. 8). For sterilization, after one adsorption–desorption cycle, the monolith was washed with 50 m*M* NaOH solution for 30 min. After this, the monolith was washed with distilled water for 30 min and then equilibrated with phosphate buffer for the next adsorption–desorption cycle. It was observed that the adsorption behavior of IgG to the Cu^{2+}-chelated poly(HEMA-MAH) and poly(HEMA-MAH) monoliths was little changed over 10 cycles. These results demonstrated the stability of the present metal-chelated monolith as an affinity adsorbent.

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