Purification of Immunoglobulin G from Human Plasma by Metal-Chelate Affinity Chromatography

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Received 17 May 2002; accepted 24 October 2002

ABSTRACT: A new group of specific-affinity beads with metal chelates as ligands and synthetic polymer beads as support matrices was prepared and tested. This study focused on developing metal-chelated poly(hydroxyethyl methacrylate) (PHEMA) gel beads in a spherical form (100–150 μm in diameter) for the separation of immunoglobulin G (IgG) from human plasma. Crosslinked PHEMA gel beads were prepared by suspension polymerization. The metal-complexing ligand L-histidine was then immobilized by covalent binding onto these gel beads. Different transition-metal ions, including Zn(II), Ni(II), Co(II), and Cu(II), were chelated on these gel beads. An elemental analysis of immobilized L-histidine for nitrogen was estimated to be 401.9 μmol of L-histidine/g of PHEMA. The nonspecific IgG adsorption onto plain PHEMA gel beads was negligible (ca. 0.17 mg/g). Higher adsorption values (up to 3.5 mg/g) were obtained in which L-histidine-immobilized PHEMA gel beads were used from aqueous solutions. A remarkable increase in the IgG adsorption capacities were achieved from human plasma with L-histidine-immobilized gel beads (up to 44.8 mg/g). Further increases in the IgG adsorption capacities of the metal-chelated gel beads were observed when human plasma was used (up to 79.6 mg/g). The metal-chelate affinity gel beads allowed the one-step separation of IgG from human plasma. The recognition range of metal ions for surface histidines from human plasma followed this order: Cu(II) > Ni(II) > Zn(II) > Co(II). IgG molecules could repeatedly be adsorbed and desorbed with these metal-chelated PHEMA gel beads without a noticeable loss in their IgG adsorption capacity. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 1567–1572, 2003

Key words: immunoglobulin G; PHEMA; metal-chelated beads; affinity chromatography

INTRODUCTION

Immunoglobulins are a group of biologically active proteins produced by plasma cells in response to the presence of foreign substances. Immunoglobulins have proven to be useful proteins for therapeutic and diagnostic applications.[1] They also serve as bioaffinity ligands for purifying other high-value proteins of pharmaceutical importance, such as cytokines and blood-clotting factors. Different chromatographic techniques, including high-performance, size exclusion, ion-exchange, hydrophobic interaction, hydroxyapatite, and thiophilic, have been used for the separation of immunoglobulins.[2–5] However, immunoglobulins are often purified by affinity chromatography because of its high selectivity. Among the affinity techniques, protein A affinity chromatography is a well-known method for purifying immunoglobulins.[6,7] Protein A binds with different affinities to the Fc regions of immunoglobulins from a variety of sources; for example, it binds to immunoglobulin G (IgG) from humans, rabbits, and pigs with high affinity, binds to horse and cow IgG with lower affinity, and binds to rat IgG only very weakly.[8] It exhibits a very high specificity and can, therefore, be employed as a one-step procedure for the purification of immunoglobulins. Because of this high specificity, protein A affinity chromatography is now commonly used on a large scale for the purification of immunoglobulins to be used in clinical tests and/or therapy.[9] However, despite its high selectivity, protein A affinity chromatography also has some drawbacks that are worth considering: (1) a considerable amount of protein A may leak from the support and such contamination cannot, of course, be tolerated in clinical applications, and (2) the cost of these supports tends to be very high. In addition, these types of bioligands are difficult to immobilize in the proper orientation. They are also susceptible to degradation.

Immobilized metal-chelate affinity chromatography (IMAC) is widely used for protein purification. Transition-metal ions can form stable complexes with electron-rich compounds and may coordinate molecules containing oxygen, nitrogen, and sulfur by ion–dipole interactions. Metal-complexing ligands are first-row transition-metal ions incorporated by iminodiacetic acid, nitrilotriacetic acid, amino salicylic acid, and carboxymethylated amino acids.[10] IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for metal ions. The separation is based on the differential binding abilities of the

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Journal of Applied Polymer Science, Vol. 89, 1567–1572 (2003)

proteins to interact with chelated metal ions to a support.[11–14] The dominating electron-donating group in a protein is the imidazole side chain of histidine, whereas the N-terminus of the protein contributes to a lesser extent. In addition, the thiol group of cysteine would be a good electron donor, but it is rarely present in the appropriate reduced state.[15] The number of histidine residues in the protein is of primary importance in the overall affinity for immobilized metal ions. In addition, factors such as the accessibility, microenvironment of the binding residue, cooperation between neighboring amino acid side chains, and local conformations play important roles in biomolecular adsorption.[16] Aromatic amino acids and the amino terminus of the peptides also have some contributions.[17] The low cost of metals and the ease of regeneration of the supports are the attractive features of metal affinity separation.

In this article, we propose histidine as a new ligand for use in IMAC for IgG. The imidazole of histidine has a chelating property with transition-metal ions. The purification of IgG is generally required for the purposes of immunodiagnostics and immunotherapy. Moreover, IgG removal from human plasma is employed for the treatment of immune disorders, alloimmunization, and cancer.[18,19] For this reason, clinical applications of metal-chelated gel beads could be a potentially attractive tool.[20] In this study, poly(hydroxyethyl methacrylate) (PHEMA) gel beads were produced by suspension polymerization. These gel beads were activated, and L-histidine was covalently coupled. Then, Zn(II), Ni(II), Co(II), and Cu(II) were chelated onto the gel beads. IgG adsorption onto PHEMA gel beads from human plasma is reported here.

EXPERIMENTAL

Preparation of the PHEMA gel beads

2-Hydroxyethylmethacrylate (HEMA) was purchased from Sigma (St. Louis, MO) and was purified by vacuum distillation under a nitrogen atmosphere. Ethylene glycol dimethacrylate (EGDMA; Merck, Darmstadt, Germany) was used as the crosslinking agent. The polymerization initiator was 2,2′-azobisisobutyronitrile (AIBN; BDH, Poole, United Kingdom). The dispersion medium was a saturated aqueous solution of magnesium oxide (MgO; Sigma). Crosslinked PHEMA gel beads were prepared by a suspension polymerization method.[21] The polymerization was carried out in an aqueous dispersion medium containing MgO, which was used to reduce 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 polymeric gel beads about 100–150 μm in diameter with a narrow size distribution, we set the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA and AIBN, and the agitation rate to be 1:3 (v/v), 1:10 (v/v), 0.33 (mol of EGDMA/mol of HEMA) and 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 polymeric gel beads were separated from the polymerization medium by filtration, and the residuals (e.g., monomer and MgO) were removed by a cleaning procedure.[22] Briefly, gel beads 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, until we were assured that the gel beads were clean. The purity of the gel beads was followed by observations of the changes in the optical densities of the samples taken from the liquid phase in the recirculation system and also from the DSC thermograms of the gel beads obtained with a differential scanning microcalorimeter (Mettler, Zurich, Switzerland). The optical density of uncleaned gel beads was 2.86. However, after the cleaning operation, this value was reduced to 0.06. In addition, when the thermogram of uncleaned gel beads was recorded, it had a peak around 60°C. This peak might have originated from AIBN. However, after this cleaning procedure, between 30 and 100°C, no peak was observed on this thermogram.

L-Histidine immobilization

The metal-complexing ligand L-histidine was purchased from Sigma. For the preparation of L-histidine-immobilized PHEMA, the following procedure was applied. Dry gel beads (20 g) were weighed and transferred into a L-histidine solution (3.0 g of L-histidine/50 mL of tetrahydrofuran) containing 1.4 g of NaH as a reaction catalyst. This immobilization reaction was carried out under constant gentle magnetic stirring at 40°C for 24 h. At the end of this reaction period, the L-histidine-immobilized gel beads were removed by filtration and washed extensively with methanol and water for the removal of weakly adsorbed L-histidine molecules, and they were then dried in vacuum for 24 h. The amount of L-histidine in the gel beads was determined by elemental analysis (CHNS-932, Leco, Chicago, IL). The amount of L-histidine immobilization on the gel beads was calculated from these data by a consideration of the nitrogen stoichiometry. When not in use, the resulting gel beads were kept under refrigeration in a 0.02% NaN_3 solution.

The leakage of the L-histidine from the gel beads was followed by the incubation of the fully wetted gel beads with 10 mL of a phosphate-buffered saline (PBS; pH 7.4) solution for 24 h at room temperature (25°C). The leakage experiments were carried out at a stirring rate of 50 rpm. L-Histidine released after this incubation was measured in the liquid phase spectrophotometrically.

Chelation of metal ions

The chelation of metal ions, including Zn(II), Ni(II), Co(II), and Cu(II), from the single-metal-ion solutions was investigated in batch adsorption equilibrium experiments. An aqueous metal-ion solution (20 mL) was treated with the PHEMA gel beads for 2 h (equilibrium adsorption time). The flask was magnetically stirred at an agitation rate of 100 rpm at room temperature (25°C). The initial concentration of the metal ion was 30 mg/L. The medium pH was 5.0. Nitrate salts were used for the preparation of standard metal-ion solutions. The concentration of the metal ions in the aqueous phase, after the desired treatment periods, was measured with a graphite furnace atomic absorption spectrophotometer (AAS 5EA, Carl Zeiss Technology, Jena Zeiss Analytical Systems, Germany). The instrument response was periodically checked with known metal solution standards. The experiments were performed in replicates of three, and the samples were analyzed in replicates of three as well. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples to determine the margin of error.

IgG adsorption from an aqueous solution

The adsorption of IgG (catalog no. 160101, Sigma) on the metal-chelated PHEMA gel beads was studied batchwise. PHEMA gel beads containing metal ions were incubated with 50 mL of an IgG solution at 20°C for 2 h (i.e., equilibrium time). The pH of the solution was 7.4. The initial concentration of IgG was 1.0 mg/mL. After the IgG adsorption, for the removal of the nonspecifically adsorbed IgG molecules, the PHEMA gel 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* $NaHCO_3$ (pH 9.5) and 0.5*M* NaCl. The protein concentration in aqueous solutions was measured with the Folin–Lowry method.[23] The protein sample (200–400 μL) was diluted to 1.0 mL with 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 (SDS) 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 (Uppsala, Sweden) Novaspec II spectrophotometer. A calibration curve was established with bovine serum albumin of known concentrations so that the protein concentration in the solution could be related to the absorbance of the sample. The IgG adsorption capacity of the gel beads was determined by the measurement of the remaining concentration of IgG in the adsorption medium.

IgG adsorption from human plasma

The adsorption of IgG from human plasma on the metal-chelated PHEMA gel beads was studied batchwise. Fresh human blood was used in all experiments. Fresh frozen plasma was obtained from University Hospital (Hacettepe, Ankara, Turkey). Blood samples were centrifuged at 1000 g for 30 min at room temperature for the separation of plasma. PHEMA gel beads containing metal ions were incubated at 20°C for 20 min with 20 mL of human plasma. PBS (pH 7.4, 0.9% NaCl) was used for the dilution of human plasma.

The amount of IgG adsorbed through metal ions on the PHEMA gel beads was determined by a solid-phase-enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG (I-9384, Sigma) diluted to 1/1000 in 50 m*M* $NaHCO_3$ (pH 9.6) was adsorbed onto poly(vinyl chloride) 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% bovine serum albumin, and 0.1% sodium azide (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 anti-human-IgG labeled with biotin (B-3773, Sigma) followed by peroxidase-conjugated streptavidin (Sigma) and *o*-phenylenediamine. The absorbance was measured at 492 nm.

Desorption and repeated use

The desorption of IgG was studied in 50 m*M* ethylenediaminetetraacetic acid (EDTA). IgG-adsorbed PHEMA gel beads were placed in this desorption medium and stirred continuously (at a 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 ELISA. The desorption ratio was calculated from the amount of IgG adsorbed on the gel beads and the final IgG concentration in the desorption medium.

To test the reusability of the metal-chelated PHEMA gel beads, we repeated the IgG adsorption–desorption procedure 10 times with the same polymeric support.

After each IgG adsorption–desorption experiment, the metal ions were stripped with 50 m*M* EDTA at pH 4.9, and the metal-ion chelation procedure was applied again. After the desorption of IgG, L-histidine and metal-ion leakage from the gel beads was also monitored continuously.

RESULTS AND DISCUSSION

Characteristics of the PHEMA gel beads

L-Histidine-immobilized and metal-chelated PHEMA adsorbents were prepared as a metal-chelate affinity adsorbent for the separation of IgG from the human plasma. The main criteria of PHEMA selection were its high mechanical strength, chemical and biological stability, and good blood compatibility. Previous observations led to the conclusion that the PHEMA gel beads used in this study were resistant to the adsorption of blood proteins and the adhesion of blood cells.[24] The PHEMA gel beads prepared in this study were hydrophilic and crosslinked structures. The simple incorporation of water weakened the secondary bonds within the gel beads. This enlarged the distance between the entanglement polymer chains and caused the uptake of water. The equilibrium water uptake ratio of the PHEMA adsorbents was 55% (w/w). The water uptake properties of the PHEMA gel beads did not change after L-histidine immobilization.

L-Histidine molecules were immobilized covalently onto the PHEMA gel beads. It is accepted that amide bonds form between the amino groups of L-histidine and the carbonyl groups of PHEMA. L-Histidine leakage was also investigated in PBS solutions. There was no detectable histidine leakage. An elemental analysis of plain PHEMA and the L-histidine-immobilized PHEMA gel beads was performed, and the attachment of the L-histidine was found to be 401.9 μmol/g from nitrogen stoichiometry.

Effect of the metal type

IgG adsorption from aqueous solutions

Different mechanisms of metal affinity interactions with proteins have been proposed,[25] but the macromolecular recognition of proteins with chelated metal ions obviously still remains unclear. In one proposed mechanism, the formation of a coordination complex structure between the protein and chelated metal ion is considered to be the major binding mode. However, more than one type of interaction mechanism is operational.[26] The major functional groups on a protein contributing toward the interaction with a chelated metal ion consist of the histidine residue and the sulfur atom of the sulfidryl group of the free cysteine residue. While a free cysteine residue being maintained in a natural protein is rare, the exposed histidine residue is the dominant binding site in protein adsorption with an chelated metal ion.[27] Factors influencing the interactions include the number of electron-donating groups on the protein surface, the medium pH, the protein concentration, the type of metal ions, the ligand density, and the type and size of chelating ligand.

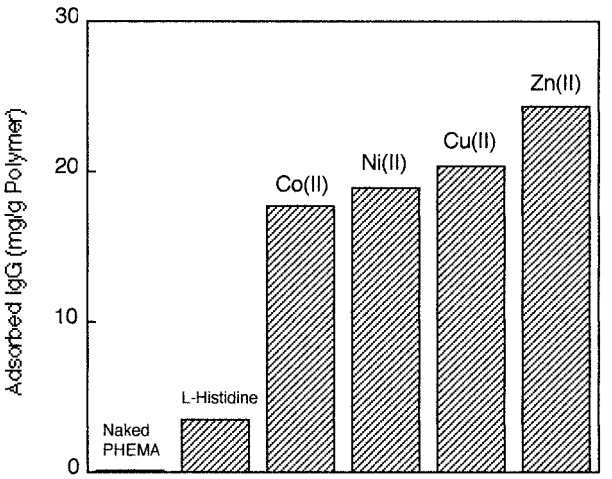

Figure 1 Effect of the metal-ion type on IgG adsorption on PHEMA gel beads (L-histidine loading = 401.9 μmol/g; IgG concentration = 1.5 mg/mL; temperature = 25°C).

Imidazole nitrogen donor atoms in immobilized histidine molecules are the most common binding sites for metal ions. The metal loading values were 71.8 μmol/g for Zn(II), 168.4 μmol/g for Cu(II), 195.1 μmol/g for Co(II), and 219.7 μmol/g for Ni(II) ions. Figure 1 shows the effects of metal-ion incorporation on the IgG adsorption from aqueous solutions. The IgG adsorption capacity on naked PHEMA gel beads was 0.17 mg/g. It should be noted that a negligible amount of protein adsorbed nonselectively on the gel beads was one of the most important requirements. A hydrated water layer on the polymer surface reduced the hydrophobic interaction between the protein and the polymer. The maximum IgG binding capacity of L-histidine-immobilized gel beads was 3.5 mg/g. IgG adsorption capacities on metal-chelated gel beads were 24.3 mg/g for Zn(II), 20.4 mg/g for Cu(II), 18.9 mg/g for Ni(II), and 17.7 mg/g for Co(II) ions under the same conditions. We showed that metal-ion incorporation significantly increased the IgG adsorption capacity of the gel beads. Transition-metal ions have a high affinity for the peptide sequences His-Gly-His, His-Tyr-NH, and His-Trp.[28] This significant IgG adsorption onto metal-chelated affinity gel beads could be due to its greater number of histidine residues, which interacted with the proteins. The IgG adsorption capacity was demonstrated toward the metal ion with the effects in the following order: Zn(II) > Cu(II) > Ni(II) > Co(II).

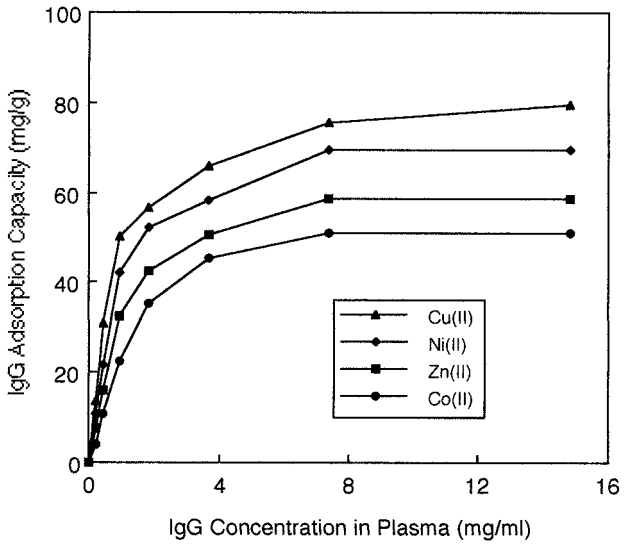

Figure 2 IgG adsorption from human plasma through L-histidine immobilized and metal-chelated onto PHEMA gel beads (L-histidine loading = 401.9 μmol/g; temperature = 25°C).

IgG adsorption from human plasma

Figure 2 gives the adsorption data for the L-histidine-immobilized and metal-chelated PHEMA gel beads. Pseudospecific adsorption (i.e., the adsorption of IgG molecules onto the PHEMA gel beads through L-histidine molecules) was significant (up to 44.8 IgG/g) and increased with an increasing initial concentration of IgG in the plasma. Immobilized L-histidine proved useful also for the direct capture of IgG from healthy human plasma. The IgG molecules adsorbed on immobilized L-histidine via their Fab part.[29] As expected, the amount of IgG adsorbed to PHEMA gel beads via L-histidine molecules reached almost a plateau value around 8.0 mg/mL because of the saturation of active binding sites. There was a further increase in the adsorption capacity of IgG from human plasma with metal-chelated gel beads. The maximum IgG binding capacity of metal-chelated gel beads was 79.6 mg/g for Cu(II) ions, 69.5 mg/g for Ni(II) ions, 58.6 mg/g for Zn(II) ions, and 51.2 mg/g for Co(II) ions under the same conditions. We showed that metal-ion incorporation significantly increased the IgG adsorption capacity of the gel beads. This was due to the specific interactions between the IgG molecules and chelated metal ions. The recognition range of metal ions for surface histidines followed this order: Cu(II) > Ni(II) > Zn(II) > Co(II). This affinity trend agreed well with reported tendencies of chelated metal ions for various surface histidine residue distributions. Each of the four metal ions can coordinate up to six electron donors, and their affinity toward imidazole follows this order: Cu(II) > Ni(II) > Zn(II) > Co(II).[30] A protein needs at least two histidines to be adsorbed on Ni(II), Zn(II) and Co(II) chelated adsorbents, whereas Cu(II)-chelated adsorbents can recognize any histidine distribution, even a single histidine on a protein surface.

Comparison with the related literature

Different affinity supports have been reported in the literature for human-IgG adsorption. Denizli et al.[19] reported an adsorption capacity of 24.0 mg of IgG/g with protein A-immobilized PHEMA adsorbents. Klein et al.[31] used poly(caprolactam) hollow fibers as the carrier matrix and immobilized recombinant protein A as a specific bioligand. They reported human-IgG adsorption capacities of around 12.4–28.3 mg/cm^3. Kim and coworkers[32,33] used hydrophobic amino acid (e.g., phenylalanine and tryptophan)-containing membranes based on polyethylene and obtained 50 mg/g of polymer for bovine γ-globulin. Füglistaller[34] used different commercial protein A affinity chromatography matrices. He presented adsorption capacities of 0.7–20.1 mg of IgG_3/g. Müller-Shulte et al.[35] used several polymeric carriers made of different polymers and histidine as the dye ligand. Their IgG_1 adsorption values were 0.05–0.23 mg of IgG_1/mL of sorbent. Bueno et al.[36] used poly(ethylene vinyl alcohol) hollow-fiber cartridges carrying L-histidine, and they reported dynamic adsorption values up to 77.7 mg of IgG/g of polymer. Teng et al.[37] synthesized a novel biomimetic ligand for IgG and achieved a maximum adsorption capacity of 51.9 mg/g with Sepharose 6B. The IgG adsorption capacity obtained in this study (up to 80 mg of IgG/g) would seem to be sufficient for us to propose these metal-chelated affinity gel beads as IMAC supports.

Desorption of IgG

The desorption of IgG from metal-chelated PHEMA gel beads was performed in a batch experimental setup. The adsorbents loaded with IgG were placed within the desorption medium, and the amount of IgG that desorbed in 1 h was determined. Human plasma was used for repeated IgG adsorption cycles. Up to 98% of the adsorbed IgG was desorbed with 50 m*M* EDTA as an elution agent. It must be pointed out that in metal-chelated affinity systems, adsorption (i.e., the binding of protein molecules with chelated ions) is completely reversible. Note that there was no L-histidine release in this case, and this showed that L-histidine molecules were immobilized covalently to the PHEMA gel beads.

To show the reusability of the metal-chelated PHEMA gel beads, we repeated the adsorption–desorption cycle 10 times with the same polymeric gel beads. There was no remarkable reduction in the adsorption capacity of the adsorbents (Fig. 3). The IgG adsorption capacity decreased only 1.0% after three cycles. With the aforementioned desorption data, we concluded that EDTA was a suitable desorption agent

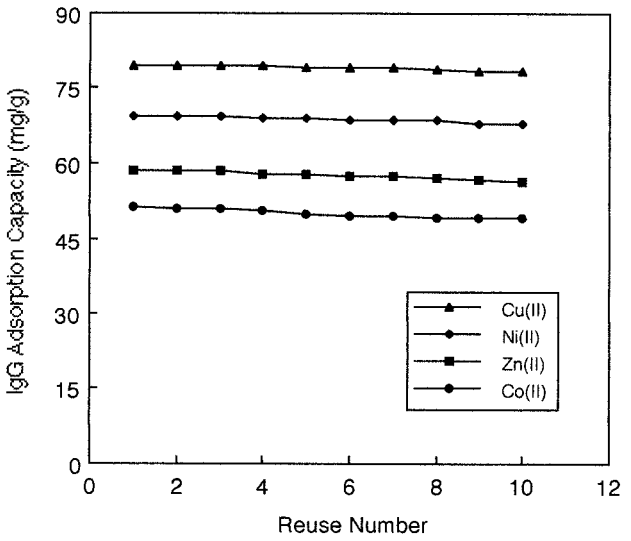

Figure 3 Repeated use of metal-chelated PHEMA gel beads (L-histidine loading = 401.9 μmol/g; temperature = 25°C).

that allowed the repeated use of the metal-chelate affinity adsorbents used in this study.

CONCLUSIONS

Immunoglobulins are widely used for *in vitro* diagnostic purposes. Immunoglobulin preparations have also been used for many decades to induce passive immunity.[38] The medical and commercial relevance of immunoglobulins has stimulated the development of cost- and time-effective purification techniques, including polymeric carriers. Protein A columns cannot be used for large-scale purification because they are very expensive. Pseudospecific adsorbents may hold certain advantages as ligands for industrial affinity separations because they are not likely to cause an immune response in case of leakage into the product.[39] PHEMA gel beads were produced by the suspension polymerization of HEMA. A metal-complexing ligand, L-histidine was then immobilized to these adsorbents to have a loading of up to 401.9 μmol/g, which resulted in an IgG adsorption of 3.5 mg/g from aqueous solutions. A remarkable increase in the IgG adsorption capacities were achieved from human plasma (up to 44.8 mg/g). Further increases were achieved from human plasma (up to 79.6 mg/g). The recognition range of metal ions for surface histidines from human plasma followed this order: Cu(II) > Ni(II) > Zn(II) > Co(II). This affinity trend agreed well with reported tendencies of chelated metal ions for various surface histidine residue distributions. High desorption ratios (>98% of the adsorbed IgG) were achieved with 50 m*M* EDTA. It was possible to reuse these metal-chelated affinity gel beads without a remarkable reduction in the adsorption capacities.

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