Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 795 (2003) 93–103
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Novel metal-chelate affinity adsorbent for purification of immunoglobulin-G from human plasma

Adil Denizli[a,*], Murat Alkan[b], Bora Garipcan[b], Serpil Özkara[b], Erhan Pişkin[b]

[a]*Department of Chemistry, Biochemistry Division, Hacettepe University, Ankara, Turkey*
[b]*Bioengineering Division, Hacettepe University, Ankara, Turkey*

Received 11 March 2003; received in revised form 19 June 2003; accepted 30 June 2003

Abstract

Metal-chelating ligand and/or comonomer 2-methacrylolyamidohistidine (MAH) was synthesized by using methacryloyl chloride and L-histidine methyl ester. MAH was characterized by NMR and FTIR. Spherical beads with an average diameter of 75–125 μm were produced by suspension polymerization of methylmethacrylate (MMA) and MAH carried out in an aqueous dispersion medium. Poly(MMA-MAH) beads had a specific surface area of 37.5 m^2/g. Poly(MMA-MAH) beads were characterized by water uptake studies, FTIR, SEM and elemental analysis. Elemental analysis of MAH for nitrogen was estimated as 34.7 μmol/g of polymer. Then, Cu^{2+} ions were chelated on the beads. Cu^{2+}-chelated beads with a swelling ratio of 38% were used in the adsorption of human-immunoglobulin G (HIgG) from both aqueous solutions and human plasma. The maximum adsorption capacities of the Cu^{2+}-chelated beads were found to be 12.2 mg/g at pH 6.5 in phosphate buffer and 15.7 mg/g at pH 7.0 in MOPS. Higher adsorption value was obtained from human plasma (up to 54.3 mg/g) with a purity of 90.7%. The metal-chelate affinity beads allowed one-step separation of HIgG from human plasma. The adsorption–desorption cycle was repeated 10 times using the same beads without noticeable loss in their HIgG adsorption capacity.

Keywords: Metal-chelate affinity beads; Purification; Immunoglobulin G

1. Introduction

Monoclonol antibodies have been developed as diagnostic tools and therapeutic agents for hematological malignancies and also serve as affinity ligands for purifying other high-value proteins of pharmaceutical importance such as cytokines and blood-clotting factors [1]. For diagnostics and especially for medical applications, specific immunoglobulins (IgGs) have been thoroughly purified using a combination of various physicochemical methods, mainly precipitation and chromatography [2]. Fractionation by ethanol precipitation is the most used process to purify IgG from human plasma on industrial scale (27 ton/year) [3]. Protein A chromatography is a well known and popular method of purifying IgGs [4–7]. Protein A binds with different affinity to the Fc region of IgGs from a variety of sources, e.g., it binds to IgG from human, rabbit and pig with high affinity, binds horse and cow IgG with lower affinity and binds rat IgG only a very weakly [8]. Protein A chromatography exhibits a very high

*Corresponding author. P.K. 51, Samanpazarı, 06242 Ankara, Turkey. Tel./fax: +90-312-299-2163.
E-mail address: denizli@hacettepe.edu.tr (A. Denizli).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00550-6

specificity and can therefore be employed as a one-step procedure for the purification of antibodies. Due to this specificity, protein A chromatography is now commonly used on a pilot scale for the purification of IgGs to be used in clinical tests [9]. However, in spite of its high selectivity, protein A chromatography also has some drawbacks which are worth considering: (i) a considerable amount of protein A may leak from the matrix and such contamination cannot, of course, be tolerated in clinical applications; (ii) the cost of these materials tends to be very high. In addition, it is difficult to immobilise protein A in the proper orientation. It is also susceptible to degradation.

Metal chelate affinity chromatography of proteins, with metal chelate linked to Sepharose, was introduced by Porath et al. in 1975 [10] and since then it has been adopted for the purification of many therapeutic proteins, peptides, nucleic acids, hormones and enzymes [11–17]. Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by metal chelate affinity chromatography [18] as have human growth hormone [19], cytochrome c [20], pectinase [21], carboxypeptidase B [22], histidine tagged glutathione *S*-transferase [23] and peptides containing histidine residues [24] and phosphopeptides [25] poly-histidine tagged glutaryl acylase [26], trypsin inhibitor [27], poly-histidine tagged recombinant proteins [28], apoflavoproteins [29]. Many transition metals can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N and S by ion dipole interactions. Metal ion ligands are first-row transition metal ions (Zn^{2+}, Ni^{2+}, Cu^{2+} and Fe^{3+}) incorporated by iminodiacetic acid, nitrilotriacetic acid, tris(carboxymethyl)ethylenediamine. IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. The separation is based on the interaction of a Lewis acid (electron pair donor), i.e., a chelated metal ion, with an electron acceptor group on the surface of the protein [30–34]. Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Co-operation between neighboring amino acid side chains and local conformations play important roles in biomolecule binding. Aromatic amino acids and the amino terminus of the peptides also have some contributions [35]. The low cost of metals and the reuse of adsorbents for hundred of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.

In this study, we propose metacryloylamidohistidine (MAH) as a new metal-chelating ligand for use in the IMAC for IgG. Imidazole group of MAH has a chelating property with transition metal ions. The purification of IgG is generally required for the purposes of immuno-diagnostics, immunochromatography and immunotherapy. Moreover, IgG removal from human plasma is employed for the treatment of immune disorders, allo-immunization and cancer [36]. For this reason, clinical applications of metal-chelated beads could be a potentially attractive tool [37,38]. MAH was synthesized by methacryloyl chloride and histidine. Poly(methylmethacrylate-methacryloylamidohistidine) [p(MMA-MAH)] beads were produced by suspension polymerization of MAH and MMA. Then, Cu^{2+} ions were chelated on these beads. MAH was characterized by FTIR and NMR. p(MMA-MAH) beads were characterized by FTIR, SEM, elemental analysis and swelling test. Then, HIgG adsorption on the beads from aqueous solutions containing different amounts of HIgG, at different pH values, and also from human plasma were also performed. Desorption of HIgG and reusability of these metal-chelate affinity adsorbents were also tested.

2. Experimental

2.1. Materials

Human immunoglobulin-G (lyophilized), L-histidine methylester dihydrochloride (97%), methacryloyl chloride and morpholinopropanesulfonic acid (MOPS) were supplied by Sigma (St Louis, MO, USA). Methylmethacrylate (MMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4 °C until use. Benzoylperoxide (BPO) was obtained from Fluka. Poly(vinyl alcohol) (PVAL; MW 100 000, 98%

hydrolyzed) was supplied from Aldrich (USA). All other chemicals were of reagent grade and were purchased from Merck (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) 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.

2.2. Synthesis of MAH

For synthesis of MAH, the following experimental procedure was applied: 5.0 g of L-histidine methylester and 0.2 g of hydroquinone were dissolved in 100 ml of dichloromethane. This solution was cooled down to 0 °C; 12.7 g of triethylamine were added to the solution; 5.0 ml of methacryloyl chloride were poured slowly into the solution under nitrogen atmosphere and then this solution was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, unreacted methacryloyl chloride was removed from the medium by extraction with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was dissolved in ethanol.

2.3. Preparation of poly(MMA-MAH) beads

Poly(MMA-MAH) beads were prepared by a suspension polymerization. A typical procedure may be summarized as follows: The stabilizer, PVAL, was dissolved in 50 ml deionized water for the preparation of the continuous phase. The dispersion phase was prepared by mixing MMA (4.0 ml), EGDMA (8.0 ml), MAH (1.0 g) and toluene (12.0 ml) in a test tube. The initiator, BPO (100 mg), was dissolved in this homogeneous solution. The dispersion phase was added to the continuous medium in a glass-sealed polymerization reactor (100 ml) placed in a water bath equipped with a temperature-control system. The polymerization reactor was heated to 65 °C within about 30 min by stirring the polymerization medium at 600 rpm. The polymerization was conducted at 65 °C for 4 h and at 90 °C for 2 h. After completion of polymerization, the reactor content was cooled to room temperature. A washing procedure was applied after polymerization to remove the diluent and any possible unreacted monomers and other ingredients from the beads. The polymer beads were filtered and resuspended in ethyl alcohol. The suspension was stirred for about 1 h at room temperature and the beads were separated by filtration. The beads were washed twice with ethyl alcohol and then four times with deionized water using the same procedure. When not in use, the beads were kept under refrigeration in 0.02% sodium azide solution for preventing of microbial contamination.

2.4. Characterization studies

FTIR spectra of MAH and poly(MMA-MAH) beads were obtained by using a FTIR spectro-photometer (FTIR 8000 Series, Shimadzu, Japan). The dry beads (about 0.1 g) were mixed with KBr (0.1 g, IR Grade, Merck), and pressed into a pellet form and the FTIR spectrum was then recorded.

The proton NMR spectrum of MAH monomer was taken in $CDCl_3$ on a Jeol GX-400 300 MHz instrument. The residual non-deuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to $CHCl_3$.

The specific surface area of the poly(MMA-MAH) beads was measured by nitrogen adsorption with an ASAP2000 instrument (USA, Micromeritics). The average size and size distribution of the polymer beads were determined by screen analysis performed by using Tyler Standard Sieves.

Water uptake ratio of the poly(MMA-MAH) beads were determined in distilled water. The experiment was conducted as follows: initially dry beads were carefully weighed before being placed in a 50-ml vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25 °C) for 2 h. The bead sample was taken out from the water, wiped using a filter paper, and weighed. The weight ratio of dry and wet samples was recorded. The water content of the poly(MMA-MAH) beads were calculated by using the following expression:

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

where m_o and m_s are the weights of beads before and after uptake of water, respectively.

The surface morphology of the beads was examined using scanning electron microscopy (SEM). The beads were initially dried in air at 25 °C for 7 days before being analyzed. A fragment of the dried bead was mounted on a SEM sample mount and was sputter coated with gold for 2 min. The sample was then mounted in a scanning electron microscope (JEM 1200 EX, Jeol, Tokyo, Japan). The surface of the sample was then scanned at the desired magnification to study the morphology of the poly(MMA-MAH) beads.

To evaluate the amount of MAH incorporation the poly(MMA-MAH) beads were subjected to elemental analysis using an Elemental Analyzer (Model CHNS-932, Leco, USA).

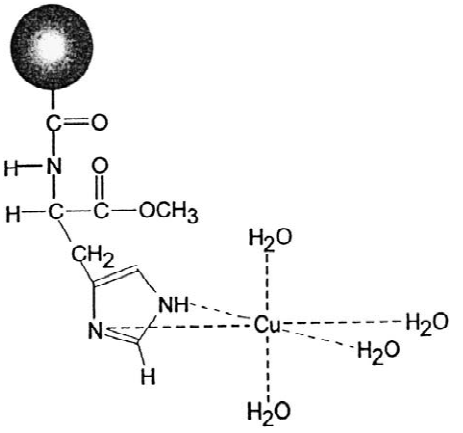

Fig. 1. Schematic diagram for the chelation of Cu^{2+} ions through the beads.

2.5. Incorporation of Cu^{2+} ions

Chelates of Cu^{2+} ions with poly(MMA-MAH) beads were prepared as follows: 1.0 g of the beads was mixed with 50 ml of aqueous solutions containing 5 ppm Cu^{2+} ions, at constant pH of 4.1 (adjusted with HCl and NaOH), which was the optimum pH for Cu^{2+} chelate formation and at room temperature. A 1000 ppm atomic absorption standard solution (containing 10% HNO_3) was used as the source of Cu^{2+} ions. The flask was stirred magnetically at 100 rpm for 1 h (sufficient to attain equilibrium). The concentration of the Cu^{2+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (AA800, Perkin-Elmer, Bodenseewerk, Germany). The Cu^{2+} chelation step is depicted in Fig. 1. The amount of adsorbed Cu^{2+} ions was calculated by using the concentrations of the Cu^{2+} ions in the initial solution and in the equilibrium.

Cu^{2+} leakage from the poly(MMA-MAH) beads was investigated with media pH (6.0–8.0), and also in a medium containing 2.0 *M* NaCl. The bead suspensions were stirred 24 h at room temperature. Cu^{2+} ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer. It should be also noted that immobilized metal containing beads were stored at 4 °C in the 10 m*M* Tris–HCl buffer (pH 7.4) with 0.02% sodium azide to prevent microbial contamination.

2.6. Adsorption of HIgG from aqueous solutions

The capacity of the adsorbent for HIgG was determined in batch mode. In short, 100 mg of the adsorbent prepared as described above were equilibrated, after charging Cu^{2+} ions with 25 m*M* MOPS and phosphate buffers. Then, the beads were incubated with 10 ml of HIgG solution for 2 h, in flasks agitated magnetically at 150 rpm. Effects of initial concentration of HIgG, pH and buffer type on the adsorption capacity were studied. To observe the influence of buffer to the adsorption capacity, the adsorption studies were carried out using 25 m*M* MOPS and phosphate buffers (contained 0.15 *M* NaCl) within their respective buffering ranges. To observe the effects of the initial concentration of HIgG on adsorption, it was changed between 0.1 and 2.0 mg/ml. HIgG concentration determined by measuring the absorbance at 280 nm, with a molar absorptivity of 14.0 for a 1% solution of IgG. The amount of adsorbed HIgG was calculated as:

$$Q = [(C_o - C)V]/m \quad (2)$$

Here, Q is the amount of HIgG adsorbed onto unit mass of beads (mg/g); C_o and C are the concentrations of HIgG in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/ml); V is the volume of the

aqueous phase (ml); and m is the mass of the beads used (g).

2.7. Desorption and repeated use

HIgG desorption experiments were performed in a buffer solution containing 1.0 *M* NaCl. HIgG adsorbed beads were placed in the desorption medium and stirred for 1 h at 25 °C, at a stirring rate of 100 rpm. The final HIgG concentration within the desorption medium was determined by spectrophotometry. In the case of Cu^{2+} carrying adsorbents, desorption of Cu^{2+} ions were also measured in the desorption media by means of an atomic absorption spectrometry. The desorption ratio was calculated from the amount of HIgG adsorbed on the beads and the amount of HIgG desorbed by using the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{amount of HIgG \{or } Cu^{2+}\} \text{ desorbed}}{\text{amount of HIgG \{or } Cu^{2+}\} \text{adsorbed on the beads}} \times 100 \quad (3)$$

To check reusability of the Cu^{2+}-chelated poly-(MMA-MAH) beads, HIgG adsorption–desorption operation was done 10 times by using the same adsorbent. After each HIgG adsorption–desorption experiment, the Cu^{2+} ions was stripped with 25 m*M* EDTA at pH 4.9, and the Cu^{2+} loading procedure was applied again. For sanitization, the beads were washed with 50 m*M* NaOH solution for 30 min after each adsorption–desorption cycle. After this procedure, beads were washed with distilled water for 30 min, then equilibrated with the MOPS buffer for the next adsorption–desorption cycle.

2.8. Adsorption of HIgG from human plasma

Adsorption of HIgG from human plasma were studied batch-wise. The plasma pool used in initial adsorption experiments consisted of platelet-poor plasma, prepared from freshly drawn human blood. The plasma was aliquoted and stored at −20 °C. The total IgG concentration in this pool was 9.45 g/l. The beads were incubated at 20 °C for 2 h with 10 ml plasma. Plasma samples were diluted with MOPS and phosphate buffer solutions, in which the pH of the buffers were 7.0 and 6.5, respectively. Dilution ratios were 1/2 and 1/10. The amounts of HIgG adsorbed on the beads were determined by a solid-phase enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG antibody (Sigma, I-9384) diluted 1/1000 in 50 m*M* $NaHCO_3$, pH 9.6, was adsorbed onto polyvinyl chloride microtitre plates at 4 °C for 12 h. The plates were washed with phosphate buffer containing 0.05% Tween 20 (washing buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% bovine serum albumin, and 0.1% sodium azide (microorganism blocking buffer). Samples (2.5 ml, neutralized with 0.5 ml of 1.0 *M* trisodium citrate) or controls containing known amounts of HIgG were added and incubated at 37 °C for 1 h. Bound HIgG was detected with the anti-HIgG labeled with biotin (Sigma, B-3773) followed by peroxidase-conjugated streptavidin (Sigma) and *o*-phenylenediamine. The absorbance was measured at 492 nm.

In order to show the selectivity, adsorption of other blood proteins (i.e., albumin and fibrinogen) was also studied. The beads (100 mg) were incubated with a human plasma (10 ml) containing albumin (37.3 mg/ml), fibrinogen (2.3 mg/ml) and IgG (9.5 mg/ml) at room temperature for 2 h. Total protein concentration was measured by using the total protein reagent (Ciba Corning Diagnostics, Halstead, Essex, UK; Catalog Ref. No: 712076) at 540 nm which is based on Biuret reaction. Chronometric determination of fibrinogen was performed by using Fibrinogen-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France). Albumin concentration was determined by using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics); Catalog Ref. No: 229241) which is based on bromocresol green dye method. HIgG concentration was determined by ELISA as described above.

The purity of HIgG was assayed by sodium dodecylsulfate–polyacrylamide gel electrophoresis using 10% separating gel 9×7.5 cm) and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brilliant 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 HIgG were used as standards.

3. Results and discussion

3.1. Properties of polymer beads

Poly(methylmethacrylate - methacryloylamidohistidine) [poly(MMA-MAH)] beads were in the spherical form mostly in the size range of 75–125 μm. Specific surface area of the beads was found to be 37.5 m^2/g. The beads are crosslinked matrices. The equilibrium swelling ratio of the poly(MMA-MAH) beads is 38%. Compared with PMMA (5.6%), the water uptake ratio of the poly(MMA-MAH) beads increased. Several explanations can be offered. First, incorporating MAH actually introduces more hydrophilic functional groups into the polymer chain, which can interact with more water molecules within the polymer matrices. Second, reacting MMA with MAH effectively decreased the molecular mass. Therefore, water molecules penetrate into the polymer network more easily, resulting in an improvement of water uptake in aqueous solutions.

2-Methacryloylamidohistidine (MAH) was selected as the metal complexing ligand. In the first step, MAH was synthesized from L-histidine methylester and methacryloyl chloride. Then, MAH was incorporated into the bulk structure of the PMMA beads by suspension polymerization. The molecular formula of poly(MMA-MAH) beads is given in Fig. 2.

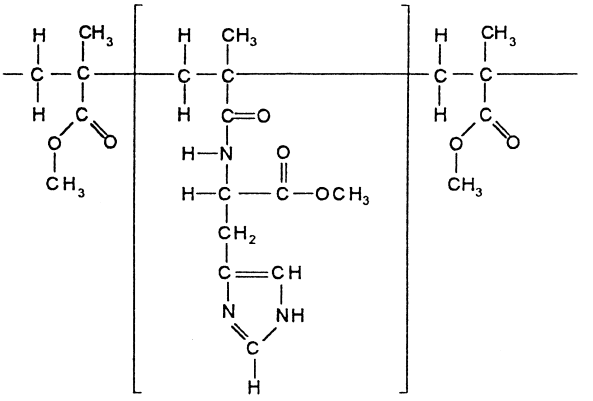

Fig. 2. The molecular formula of poly(MMA-MAH) beads.

^{1}H NMR was used to determine the synthesis of MAH structure. ^{1}H NMR spectrum is shown to indicate the characteristic peaks from the groups in MAH monomer. These characteristic peaks are as follows: ^{1}H NMR ($CDCl_3$): δ 1.99 (t; 3H, $J=7.08$ Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, $-OCH_3$) 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).

FTIR spectrum was undertaken to determine the structure of the copolymer beads (Fig. 3). The FTIR spectrum of poly(MMA-MAH) with characteristic peaks appear at 3586 cm^{-1} (characteristic hydroxyl, OH stretching vibration), at 1516 cm^{-1} amide II vibration. The carbonyl peak appears at 1645 cm^{-1} is associated with the amide and carbonyl vibration of MAH. These data confirmed that the poly(MMA-MAH) copolymer beads was formed with functional groups MAH.

The surface structure of poly(MMA-MAH) beads is exemplified by the electron micrograph in Fig. 4. As seen here, the beads have a rough surface. The presence of pores within the bead surface is clearly seen in this photograph. It can be concluded that the beads have a microporous interior surrounded by a rough surface. The roughness of the bead surface should be considered as a factor providing an increase in the surface area. In addition, these micropores reduce diffusional resistance and facilitate mass transfer because of high internal surface area.

Concentration of the incorporated chelating groups was found from nitrogen analysis using an elemental analyzer. The incorporation of MAH was found to be 34.7 μmol MAH/g polymer. Note that MMA and other ingredients in the polymerization recipe do not contain nitrogen. This nitrogen amount determined by elemental analysis comes from only incorporated MAH groups into the polymeric structure.

The amount of immobilized Cu^{2+} on poly(MMA-MAH) beads was measured and it is about 39.5 μmol/g polymer. Unit mass of the poly(MMA-MAH) beads carries 34.7 μmol MAH which was found by elemental analysis. From the mass stoichiometry, it seems that one incorporated MAH

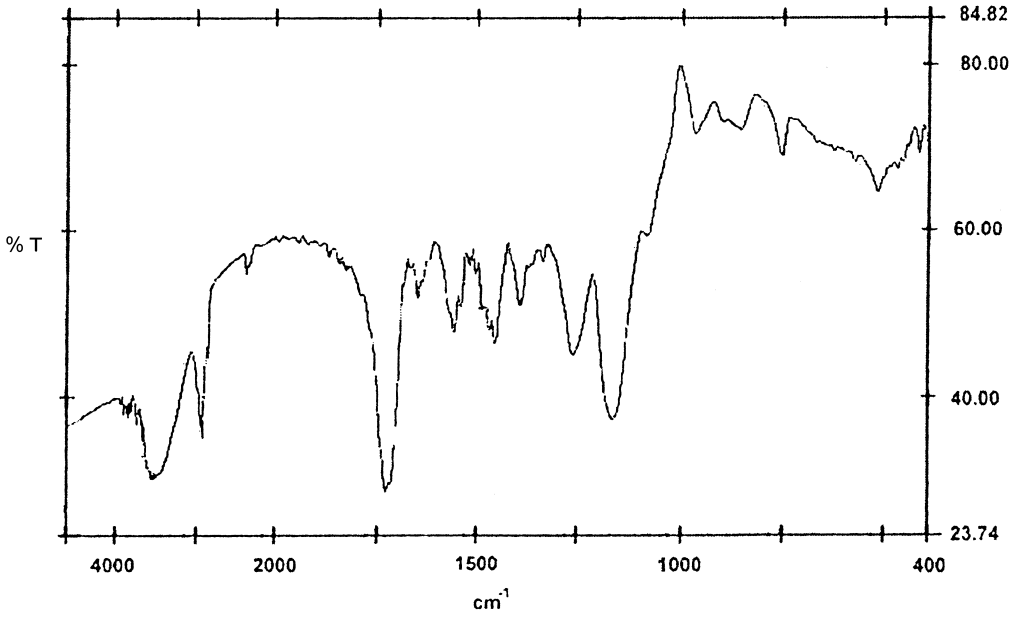

Fig. 3. FTIR spectrum of poly(MMA-MAH) beads.

molecule interacts around one Cu^{2+} ion. Since MAH has two coordinating sites of nitrogen atoms, it can form a ternary complex which is coordinated water molecules at vacant coordination sites of Cu^{2+}-MAH complexes (Fig. 1). Studies aimed at detecting leakage of Cu^{2+} from the poly(MMA-MAH) beads revealed no leakage in any of the adsorption and desorption media, and implied that the washing procedure was satisfactory for the removal of the non-specific adsorbed Cu^{2+} ions from the beads.

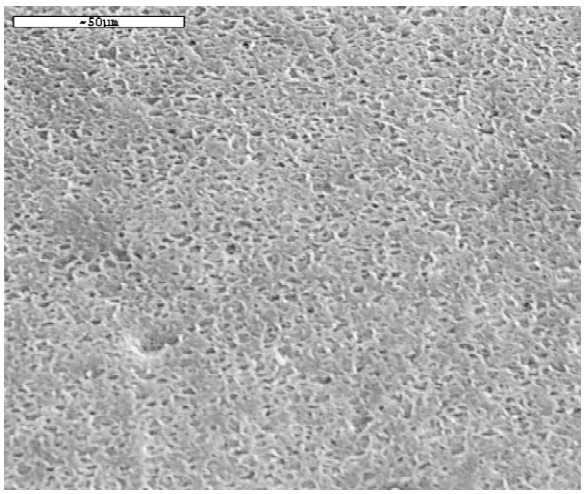

Fig. 4. SEM micrograph of poly(MMA-MAH) bead.

3.2. Adsorption of human-IgG from aqueous solutions

3.2.1. Effect of pH

HIgG adsorption onto chelating affinity beads seemed to depend on the buffer system used [39,40]. To clarify this point, equilibrium adsorption studies were carried out using MOPS and phosphate buffers within their respective buffering ranges. Fig. 5 shows IgG adsorption capacity in these buffer systems at different pH values. Buffer ranges are 6.5–8.0 for MOPS and 6.0–8.0 for phosphate. In MOPS buffer, adsorption capacity is higher than phosphate buffer. Maximum adsorption capacities are observed at pH 7.0 for MOPS (14.5 mg/g) and at pH 6.5 for phosphate buffer (11.7 mg/g). Below and over the maximum adsorption pH values, adsorption capacities decreased significantly. It should be also noted that the adsorption onto PMMA beads were about 0.5 mg/g for MOPS at pH 7.0 and 0.4 mg/g for phosphate at pH 6.5. From the structure of the buffer ion used, it is obvious that phosphate carries one or

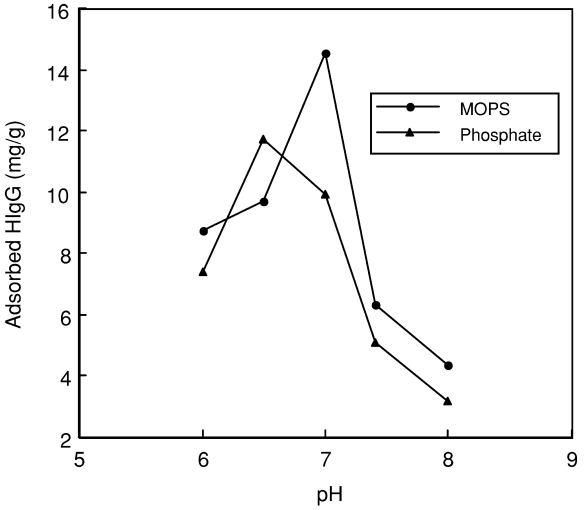

Fig. 5. Effect of buffer type on HIgG adsorption. HIgG concentration, 1.0 mg/ml; MAH content, 34.7 μmol/g; Cu^{2+} loading, 39.5 μmol/g; time, 2 h; $T = 25$ °C.

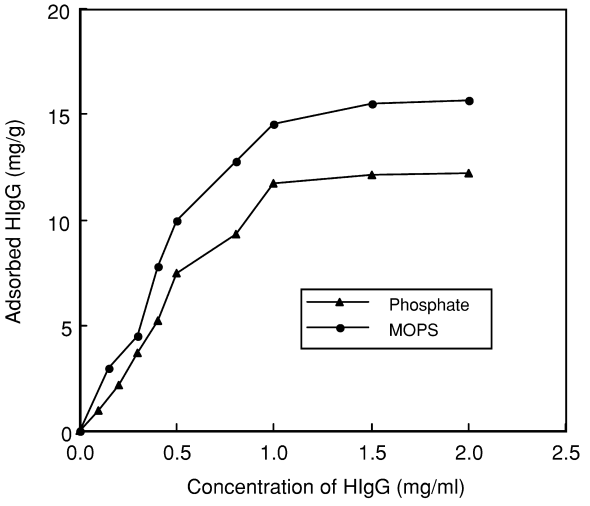

Fig. 6. Effect of initial HIgG concentration on adsorption capacity; MAH content, 34.7 μmol/g; Cu^{2+} loading, 39.5 μmol/g; pH 6.5 for MOPS; pH 7.0 for phosphate; time 2 h; $T = 25$ °C.

more charge of the same sign (negative), whereas zwitterionic buffer MOPS carries two charges of opposite sign below its pK_a (6.2). In MOPS buffer at pH 7.0, HIgG conformational structure enhance the interaction of the biomolecule with the matrix [Cu^{2+}-chelated poly(MMA-MAH beads] against in phosphate buffer.

3.2.2. Effect of HIgG concentration

Fig. 6 shows the effect of initial HIgG concentration on HIgG adsorption. As presented in this figure, with increasing HIgG concentration, the amount of HIgG adsorbed per unit mass increases almost linearly at low concentrations, below about 1.0 mg/ml, then increases less rapidly and approaches saturation. The steep slope of the initial part of the adsorption isotherm represents a high affinity between HIgG and Cu^{2+}-chelated groups. It becomes constant when the protein concentration is greater than 1.0 mg/ml. Negligible amount of HIgG molecules adsorbed on the plain PMMA beads which was about 0.4 mg/g for phosphate buffer and 0.5 mg/g for MOPS buffer at pH 7.0. Cu^{2+}-chelation significantly increased the HIgG adsorption capacity of the beads up to 12.2 mg/g at pH 6.5 in phosphate buffer and 15.7 mg/g at pH 7.0 in MOPS. It is clear that this increase is due to specific interaction between chelated Cu^{2+} ions and HIgG molecules.

3.3. Regeneration of the beads

Regeneration is a crucial step in all affinity chromatography techniques. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. In this study, more than 90% of the adsorbed HIgG molecules was removed easily from the chelating beads in all cases when NaCl was used as desorption agent. Note that there was no Cu^{2+} release in this case which shows that Cu^{2+} ions are attached to MAH groups on the beads surface by strong chelate formation. With the desorption data given above, we concluded that NaCl is a suitable desorption agent for the Cu^{2+}-chelated beads. In order to show the reusability of the Cu^{2+}-chelated poly(MMA-MAH) beads, the adsorption–desorption cycle was repeated 10 times using the same modified beads from aqueous HIgG solution. As seen in Fig. 7, there was no significant loss in the adsorption capacity of the beads. Moreover, no obvious changes of the morphology of the beads were found in the recycling process when examined

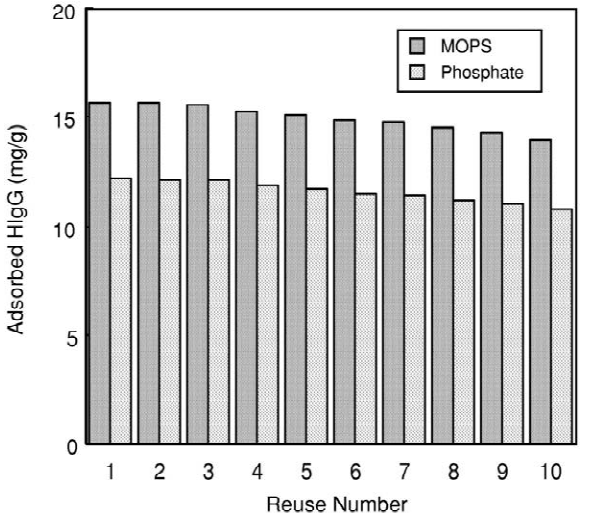

Fig. 7. Repeated use of poly(MMA-MAH) beads: MAH content, 34.7 μmol/g; Cu^{2+} loading, 39.5 μmol/g; initial concentration of HIgG, 2.0 mg/ml; pH 6.5 for MOPS; pH 7.0 for phosphate; time 2 h; $T = 25$ °C.

visually. These results demonstrated the stability of the present solid support as a metal-chelate affinity adsorbent.

3.4. Adsorption of HIgG from plasma

L-Histidine proved useful for the capture of HIgG directly from human plasma [41–43]. The IgG molecules were found to be adsorb on L-histidine via their Fab part. The adsorption of IgG from normal plasma diluted to varying extents with MOPS and phosphate buffers was measured after 2 h equilibrium adsorption time. Table 1 gives the adsorption data. As seen here, lower adsorption of HIgG was obtained for human plasma diluted with phosphate buffer. But, there was a pronounced adsorption of HIgG (up to 54.3 mg/g) onto the Cu(II)-chelated poly(MMA-MAH) adsorbent for plasma diluted with MOPS. The purity of HIgG was assayed by SDS–PAGE. The purity of HIgG obtained was found to be 90.7%. It is worth to point that the adsorption of HIgG onto the Cu^{2+}-chelated adsorbents was higher than those obtained in the studies in which aqueous solutions were used. This is due to the high initial concentration of HIgG in the plasma. HIgG, has a

Table 1
HIgG adsorption from human plasma: HIgG concentration before dilution: 9.45 mg/ml

Dilution agent	Adsorption capacity (mg/g)
Plasma (undiluted)	54.3±2.12
1/2 diluted plasma (MOPS, pH 7.0)	41.3±1.42
1/2 diluted plasma (phosphate, pH 6.5)	35.8±1.24
1/10 diluted plasma (MOPS, pH 7.0)	34.2±1.12
1/10 diluted plasma (phosphate, pH 6.5)	26.9±0.97

molecular mass of 150 000 and consists of four peptide chains; two identical light chains. These chains are linked by strong disulphide bonds into a Y- or T-shaped structure with hinge-like flexible arms. Thus an IgG molecule would expand and contract significantly with the variation of the ionizable groups in the molecule. This high HIgG adsorption may also be due to suitable conformation of HIgG molecules within their native medium (i.e., human plasma pH 7.4) for interaction with Cu(II)-chelated groups onto the poly(MMA-MAH) adsorbents.

In order to show the chelated-Cu^{2+} specificity, adsorption of other blood proteins (i.e., albumin and fibrinogen) was also studied. There was a pronounced adsorption of HIgG onto the Cu^{2+}-chelated poly(MMA-MAH) adsorbent for undiluted plasma (54.3 mg/g). Adsorption capacities were found to be 2.7 mg/g for fibrinogen and 18.5 mg/g for albumin. The total protein adsorption was determined as 76.4 mg/g. HIgG adsorption ratio is around 71% (54.3 mg HIgG per gram adsorbent: 76.4 mg total proteins per gram adsorbent). Fibrinogen and albumin adsorption ratios are 4.5% (3.5 mg fibrinogen per gram sorbent:76.4 mg total proteins per gram sorbent) and 24.2% (18.5 mg albumin per gram sorbent:76.4 mg total proteins per gram sorbent). HIgG adsorbed more than HSA under physiological condition, although the initial concentration of HIgG to HSA was in the ratio (9.5:37.3 mg/ml). This is probably due to the specific interactions between IgG molecules and chelated Cu^{2+} ions. Transition metal ions have a high affinity to the peptide sequences His–Gly–His, His–Tyr–NH and His–Trp [44]. This significant IgG adsorption onto metal-chelated affinity gel beads

could be due to its greater number of histidine residues, which are interacting with the proteins.

4. Conclusions

Immunoglobulins are purified from various sources by using conventional affinity adsorbents carrying the bio-ligands as protein A and G [45–48]. However, it is great challenge to obtain regulatory approval of an adsorbent containing biological ligands on an industrial scale. Bio-ligands are expensive and sensitive to the process operating conditions. They can lose activity or leach into the products by the harsh elution and cleaning conditions commonly used in the separation processes. The time consuming and high cost of chelating procedure has inspired a search for suitable low-cost adsorbents. The main advantage of IMAC consists in its simplicity, universality, stability and cheapness of the chelating supports [37]. In addition, the IMAC supports ensure the milder elution conditions of the specific IgGs, keep their biological activity. In this study, a novel methacryloylamidohistidine containing metal-chelate affinity adsorbent for the separation of IgG from human plasma was prepared. This new approach for the preparation of metal-chelating affinity adsorbent has many advantages over conventional techniques. An expensive and critical step in the preparation process of metal-chelating adsorbent is coupling of a chelating ligand to the adsorption matrix. In this procedure, comonomer MAH acted as the metal-chelating ligand, and no need to activate the matrix for the chelating-ligand immobilization. Another major issue is that of slow release of this covalently bonded chelators off the matrix. Ligand release is a general problem encountered in any affinity adsorption technique which caused a decrease in adsorption capacity. It is well known that ligand leakage from the adsorbent causes contaminations that will interfere with analysis of the purified biomolecule. Ligand immobilization step was also eliminated in this approach. MAH was polymerized with MMA and there was no ligand leakage. Poly(MMA-MAH) affinity adsorbent was also cheap, resistance to harsh chemicals and high temperatures. These results are encouraging and suggest that further studies should be done to develop poly(MMA-MAH)/Cu^{2+} metal-chelated adsorbents.

References

[1] M.L. Linenberger, D.G. Maloney, I.D. Bernstein, Trends Mol. Med. 8 (2002) 69.
[2] G. Tishchenko, J. Dybal, K. Meszarosova, Z. Sedlakova, M. Bleha, J. Chromatogr. A. 954 (2002) 115.
[3] T. Burnouf, M. Radosevich, J. Biochem. Biophys. Methods 49 (2001) 575.
[4] J.J. Langone, J. Immunol. Methods 55 (1982) 277.
[5] A. Tejeda-Mansir, R. Espinoza, R.M. Montesinos, R. Guzman, Bioproc. Eng. 17 (1997) 39.
[6] A. Denizli, A.Y. Rad, E. Pişkin, J. Chromatogr. B. 668 (1995) 13.
[7] A. Denizli, Y. Arıca, J. Biomater. Sci. Polym. Ed. 11 (2000) 367.
[8] U. Ljunberg, B. Jansson, U. Niss, R. Nilsson, B. Sandberg, B. Nilsson, Mol. Immunol. 14 (1993) 1279.
[9] P. Füglistaller, J. Immunol. Methods 124 (1989) 171.
[10] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[11] M.N. Gupta, S. Jain, I. Roy, Biotechnol. Prog. 18 (2002) 18.
[12] A.E. Ivanov, I.Y. Galaev, S.V. Kazakov, B. Mattiasson, J. Chromatogr. A. 907 (2001) 115.
[13] T. Abudiab, R.R. Beitle, J. Chromatogr. A. 795 (1998) 211.
[14] Y.J. Kim, Biotechnol. Tech. 13 (1999) 837.
[15] A. Denizli, E. Pişkin, J. Chromatogr. A. 731 (1996) 57.
[16] M.Y. Arıca, H.N. Testereci, A. Denizli, J. Chromatogr. A. 799 (1998) 83.
[17] F. Denizli, A. Denizli, M.Y. Arıca, Polym. Int. 48 (1999) 360.
[18] D.C. Rijken, G. Wijngaards, M. Zaal-De Jong, Biochim. Biophys. Acta 580 (1979) 140.
[19] J. Liesiene, K. Racaityte, M. Morkeviciene, P. Valancius, V. Bumelis, J. Chromatogr. A. 764 (1997) 27.
[20] R.D. Johnson, R.J. Todd, F.A. Arnold, J. Chromatogr. A. 725 (1996) 225.
[21] S.A. Camperi, M. Grasselli, O. Cascone, Bioseparation 9 (2000) 173.
[22] M. Marquez-Mendez, J. Biochim. Biophys. Methods 24 (1992) 51.
[23] R.H. Clemmitt, L.J. Bruce, H.A. Chase, Bioseparation 8 (1999) 53.
[24] V.V. Kronina, H.J. Wirth, M.T.W. Hearn, J. Chromatogr. A 852 (1999) 261.
[25] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[26] P. Armisen, C. Mateo, E. Cortes, J.L. Barredo, F. Salto, B. Diez, L. Rodes, J.L. Garcia, R.F. Lafuente, J.M. Guisan, J. Chromatogr. A. 848 (1999) 61.
[27] H.Y. Reina, A.R. Manriquez, B.J. Wong, A.T. Mansir, Biotechnol. Prog. 17 (2001) 729.
[28] S. Noronha, J. Kaufman, J. Shiloach, Bioseparation 8 (1999) 145.

[29] M.H. Hefti, F.J. Milder, B. Boeren, J. Vervoort, W.J.H. van Berkel, Biochim. Biophys. Acta 1619 (2003) 139.
[30] Y.H. Tsai, M.Y. Wang, S.Y. Suen, J. Chromatogr. B. 766 (2001) 133.
[31] A. Denizli, H. Yavuz, B. Garipcan, M.Y. Arıca, J. Appl. Polym. Sci. 76 (2000) 115.
[32] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[33] Q. Luo, H. Zou, X. Xiao, Z. Guo, L. Kong, X. Mao, J. Chromatogr. A. 926 (2001) 255.
[34] A. Denizli, F. Denizli, E. Pişkin, J. Biomater. Sci. Polym. Ed. 10 (1999) 305.
[35] T.T. Yip, Y. Nakagawa, J. Porath, Anal. Biochem. 183 (1989) 159.
[36] S.C. Bansal, B.R. Bansal, H.I. Thomas, J.E. Siegel, J.E. Rhodas, R.M. Terman, Cancer 42 (1978) 1.
[37] E.K.M. Ueda, P.G. Gout, L. Morganti, J. Chromatogr. A 988 (2003) 1.
[38] O. Pitiot, C. Legallais, L. Darnige, M.A. Vijayalakshmi, J. Membr. Sci. 166 (2000) 221.
[39] K. Haupt, S.M.A. Bueno, M.A. Vijayalakshmi, J. Chromatogr. B 674 (1995) 13.
[40] B. Garipcan, A. Denizli, Macromol. Biosci. 2 (2002) 135.
[41] S.M.A. Bueno, K. Haupt, M.A. Vijayalakshmi, J. Chromatogr. B 667 (1995) 57.
[42] A. El-Kak, M.A. Vijayalakshmi, J. Chromatogr. B 604 (1992) 29.
[43] S. Özkara, H. Yavuz, S. Patir, Y. Arica, A. Denizli, Sep. Sci. Technol. 37 (2002) 1.
[44] M.C. Smith, T.C. Ferman, C. Pidgeon, Inorg. Chem. 26 (1987) 1695.
[45] S. Brandt, R.A. Goffe, S.B. Kessler, J.L. Connor, S.E. Zale, Bio/Technology 6 (1988) 779.
[46] K. Kugel, A. Moseley, G.B. Harling, E. Klein, J. Membrane Sci. 74 (1992) 115.
[47] M. Nachman, A.R.M. Azad, P. Bailon, Biotechnol. Bioeng. 40 (1992) 564.
[48] R.C.A. Ventura, R. Zollner, C. Legallais, M.A. Vijayalakshmi, S. Bueno, Biomol. Eng. 17 (2001) 71.