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Purification of the specific immunoglobulin G_1 by immobilized metal ion affinity chromatography using nickel complexes of chelating porous and nonporous polymeric sorbents based on poly(methacrylic esters) Effect of polymer structure

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Abstract

Ni^{2+} complexes of the chelating nonporous and porous bead sorbents based on methacrylic esters crosslinked with ethylene dimethacrylate were used in isolation of the horseradish peroxidase-specific immunoglobulin IgG_1 from the crude mouse ascitic fluid by immobilized metal ion affinity chromatography (IMAC). Iminodiacetic and aspartic acids were attached to porous poly(glycidyl methacrylate) beads differing in size, morphology and chemical composition. Ethylenediaminetriacetic acid and quinolin-8-ol chelating groups were attached mainly to the surface hydroxyl groups in nonporous poly(diethylene glycol methacrylate) beads through spacers. The latter sorbents exhibited better kinetic characteristics than the former but a very low IgG_1 sorption capacity. In a single-step IMAC procedure, the best efficiency in the specific IgG_1 purification was obtained with porous sorbents (recovery 92%, purity 73%). Differences in IMAC separations are discussed from the point of view of morphology of polymer beads as well as of the type and concentration of chelating ligands.

Keywords: Immobilized metal affinity chromatography; Affinity sorbents; Stationary phases, LC; Immunoglobulins; Proteins; Metal complexes; Quinolinol; Iminodiacetic acid; Aspartic acid; Ethylenediaminetriacetic acid

1. Introduction

Application of specific immunoglobulins (IgGs) in diagnostics [1], immunotherapy [2,3], immunochromatography [4] and controlled drug delivery [5,6] is based on their extremely high selectivity and sensitivity in recognition of antigens against which they are directed. A unique chance for desired altering IgG specificity promotes widening of their applications, which in turn stimulates the development and improvement of their purification processes.

For diagnostics and especially for medical applications, specific IgGs have been thoroughly purified using a combination of various physicochemical methods, mainly precipitation and chromatography

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[7–10]. The leading purification technique is the bioaffinity chromatography with immobilized proteins A and G [11,12] because of very high binding selectivity of most IgG types. However, leaching of cytotoxic biological ligands may be a serious problem in IgG purification by biochromatography if its medical application is intended [13]. As an alternative to biochromatography, a number of other chromatographic methods such as ion-exchange [14,15], hydroxyapatite [16], hydrophobic [17], thiophilic [18] dye ligand [19], protein A–mimetic [20] and immobilized metal ion affinity chromatography (IMAC) [21] are under development in purification of the specific IgGs. Each of the methods has some advantages and disadvantages but the main merit of IMAC consists in its simplicity, universality, stability and cheapness of the chelating supports. In addition, the IMAC supports ensure the milder elution conditions of the specific IgGs, keep their biological activity and give the wider opportunities for controlling the protein binding affinity. It is not surprising that IMAC has been recently recognized as a nucleus for generation of a high-performance and economic standard procedures for purification of specific IgGs [22]. The purification of IgGs and of other proteins and enzymes by IMAC is based on the affinity of histidine (His) residues for transition metal ions coordinated with chelating groups [21–24]. The specific mammalian IgGs have a highly conserved His cluster at the junctions of the Cγ2 and Cγ3 domains [25,26]. The highest concentration of His residues is in the Cγ3 domain. Besides His, which supports the strongest interactions with the coordinated transition metal ions, several other surface residues of amino acids such as tryptophan> tyrosine > phenylalanine > arginine ~ methionine ~ glycine contribute to the protein binding [22].

It has been already known that the selectivity in IMAC is influenced by a wide range of variables, such as the type of chelating group, the coordinated metal ion, the kind and concentration of salt, and additives to either enhance or selectively elute bound proteins [22,27–38]. In addition to the mentioned factors, structural parameters (porosity, pore size distribution) and chemical composition of polymer support contribute to protein binding and elution indirectly due to their influence on the distribution of chelating ligands in polymer support as well as on spatial accessibility and arrangement of the transition metal complexes. However, this aspect has been insufficiently taken into consideration in IMAC investigations.

The aim of the study is the comparison of hydrophilic chelating sorbents based on the bead copolymers of diethylene glycol methacrylate (DEGMA) or glycidyl methacrylate (GMA) with ethylene glycol dimethacrylate (EDMA). The nonporous DEGMA–EDMA beads were modified with ethylenediaminetriacetic acid (EDTRIA) and quinolin-8-ol (HQ) while aspartic aicd (ASP) and iminodiacetic acid (IDA) were attached to surface of pores in GMA–EDMA beads. Ni^{2+} complexes of new chelating sorbents were used in purification of the horseradish peroxidase (HRP)-specific IgG_1 from the crude mouse ascitic fluid. Influence of chemical composition, morphology and dimensions of polymer beads on modification with the chelating ligands, on formation of Ni^{2+} complexes, and on the efficiency of IMAC separations is discussed.

2. Experimental

2.1. Chelating sorbents

The porous GMA–*co*-EDMA (GMA) polymer beads (7–13, 12–20 and 60–100 μm) and nonporous DEGMA–*co*-EDMA (DEGMA) beads (20–40 μm) were prepared by suspension polymerization by the known procedures [39,40]. The specific surface area and the total pore volume of polymer beads was measured by nitrogen adsorption using a Quantasorb (Quantachrome, CA, USA) and by mercury porosimetry in Porosimeter 225 (Carlo Erba, Milan, Italy), respectively (Table 1 and in Figs. 1–3). The morphology of the unmodified polymer beads were analyzed by transmission electron microscopy (TEM). Image analysis was performed with Lucia 32G-4.24 (Laboratory Imaging). Analyzed micrographs were pre-processed by the program Atlas (Tescan) in order to reduce the noise on the TEM micrographs and to obtain sufficient contrast between pores and polymer matrix. The error of the image analysis procedure was estimated at 5–7%.

IDA (Sigma–Aldrich, Germany) was bound to the

Table 1
Characteristics of nonporous DEGMA–EDMA and porous GMA–EDMA polymer beads

Polymer support	Bead size (μm)	Epoxy groups (mmol/g)	Pore surface (m^2/g)	Pore volume (ml/g)	Porosity (%)	Volume swelling in water	
						(ml/ml dry)	(ml/g)
DEGMA–*co*-EDMA	20–40	–	0.01	–	–	1.53	1.35
GMA–*co*-EDMA							
1	7–13	2.67	68	0.70	52	1.54	3.80
2	12–20	4.22	69	0.55	60	–	–
3	60–100	0.96	30	0.34	27	1.26	3.20

epoxy groups in porous GMA beads by known single-step procedure [41] and will be published elsewhere in detail. The attachment of ASP (Sigma–Aldrich) to epoxy groups in GMA beads have been described [42]. The total concentration of epoxy groups in the beads was determined from the 910 cm^{-1} peak area of the I.R. spectra recorded on a Perkin-Elmer 577 spectrometer using KBr pellets [43]. An original three-step modification procedure for incorporation of HQ (Fluka, Germany) or EDTRIA ligands to nonporous DEGMA beads will be published [44]. Concentration of the bound chelating groups was found from nitrogen analysis using an Elemental Analyzer 240 CHN (Perkin-Elmer, CA, USA) and also by titration (Table 3).

In purification of specific IgGs by IMAC, mainly Ni^{2+} complexes of IDA chelating groups has shown the best separation efficiency [22] and were investigated in the present study. Coordination of Ni^{2+} ions with the chelating groups can be carried out both in the static and dynamic conditions but the latter are preferable from the point of view of chromatographic process automation. Therefore, for IMAC separations, the chelating sorbents were coordinated with Ni^{2+} ions in the dynamic conditions, as described in Section 2.2. Concentration of nickel in solutions was measured by atomic absorption spectrometry using a Spectrometer 3110 (Perkin-Elmer). The formation of Ni^{2+} complexes in static conditions was also studied within the pH range of 4.5–8.

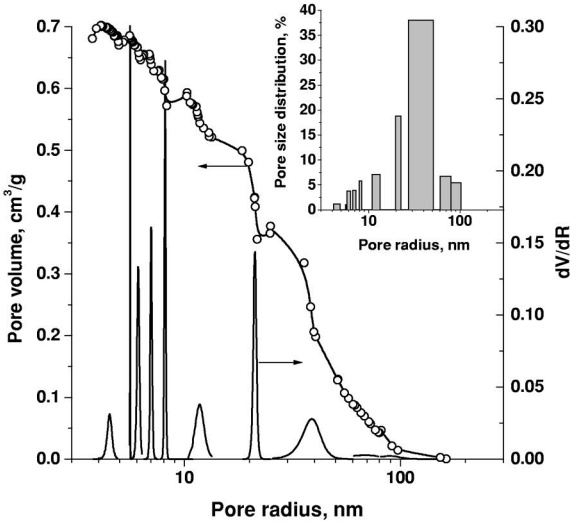

Fig. 1. Structure characteristics of porous GMA-1 polymer beads with diameters 7–13 μm.

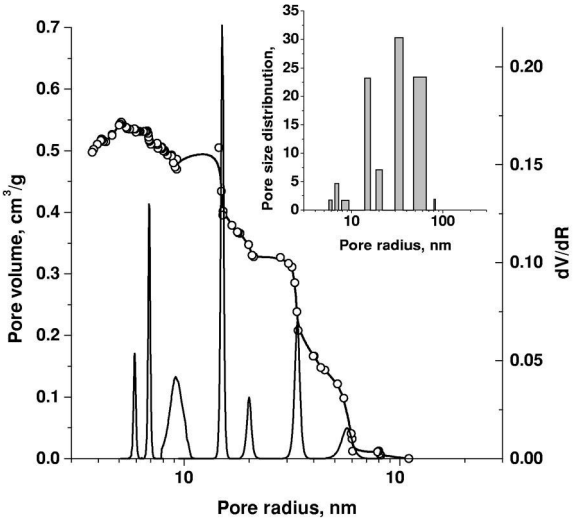

Fig. 2. Structure characteristics of porous GMA-2 polymer beads with diameters 12–20 μm.

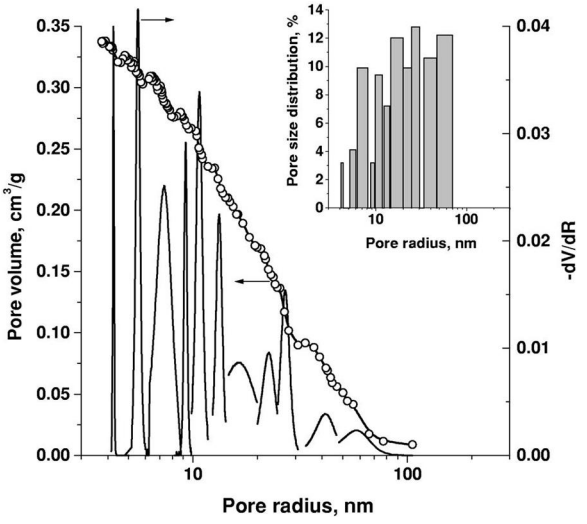

Fig. 3. Structure characteristics of porous GMA-3 polymer beads with diameters 60–100 μm.

2.2. Mouse ascitic fluid

The crude mouse ascitic fluid containing HRP-specific IgG_1 (Seva-Imuno, Prague, Czech Republic) was clarified by filtration through Whatman paper (twice) followed by dialysis through a Cuprophane membrane against distilled water to remove sodium azide. Dialysis proceeded during 16 h at 5 °C. The clarified mouse ascitic fluid contained proteins (19.95 mg/ml) and specific IgG_1 (2.05 mg/ml). Conductivity of the dialyzed ascitic fluid was 2.686 mS/cm, pH 7.77. After dialysis, the ascitic fluid was 1.25 times diluted with a buffer A (0.25 *M* potassium phosphate solution, pH 8.5, containing 2.5 *M* NaCl), filtered through a Millex-GP (0.22 μm) filter (Millipore, Bedford, MA, USA) and stored in a refrigerator.

2.3. Column IMAC

The IMAC procedure was carried out using a HR-5-column (Pharmacia Biotech, Uppsala, Sweden) packed with 1.5–2 ml of a chelating sorbent, which was first equilibrated with 0.1 *M* sodium acetate solution containing 0.5 *M* NaCl, pH 4.5 (10 bed volumes, BVs). Then 5–15 BVs of 0.1 *M* $NiCl_2$ in the equilibration buffer was passed through a sorbent at a flow-rate of 0.25 ml/min. During coordination of Ni^{2+} ions with the chelating groups, absorbance of the filtrate was monitored at the wavelength 405 nm using a Uvicord SD connected to the column. As a rule, the absorbance of the filtrate at the end of the step was the same as in the initial $NiCl_2$ solution. Unsorbed metal ions were removed by washing a sorbent with the equilibration buffer (10 BVs). Then the sorbent was equilibrated with the loading buffer (0.05 *M* potassium phosphate solution containing 0.5 *M* NaCl, pH 8, 10 BVs). The clarified crude ascitic fluid (2 ml) was passed through the sorbent at a flow-rate of 0.25 ml/min. Unsorbed proteins were removed by washing the sorbent with the loading buffer (15–20 BVs). Sorbed proteins were eluted using a stepwise pH gradient (6; 5.5; 5; 4.5) followed by an imidazole concentration gradient (0.1; 0.5 *M*) and finally with 0.05 *M* Tris buffer containing 0.5 *M* NaCl and 0.05 *M* EDTA, pH 8. Concentration of proteins in the eluate fractions was determined by the Warburg and Christian method [45]. Eluate fractions with maximum protein concentration were analyzed by the Bradford [46] method and sodium dodecylsulphate–polyacrylamide electrophoresis (SDS–PAGE). Concentration of HRP-specific IgG_1 was determined by enzyme-linked immunosorbent analysis (ELISA) titration.

2.4. SDS–PAGE

SDS–PAGE was carried out in a vertical Mini-Hoefer SE 200 apparatus by the discontinuous buffer method under the nonreducing conditions described by Laemlli [47]. Gels were stained, fixed and dried in accordance with the manufacture's instructions. A standard protein mixture (Pharmacia Biotech) was used as a high-molecular-mass protein marker.[1]

3. Results and discussion

3.1. Morphology of polymer beads

The specific surface area of the nonporous

[1]The stacking gel (T 4%, C 2.5%) and the separating gradient gel (T 7.5–10%, C 2.5%) were used with a spacer thickness of 1 mm. T=[g amylamide+g *N,N′*-methylenebisacrylamide (Bis)]/100 ml solution, C=g Bis/%T.

DEGMA beads in the dried state was negligible (0.01 m^2/g) compared with that of the porous GMA beads (68–30 m^2/g) (Table 1). The latter differed in porosity, pore volume and pore size distribution (Figs. 1–3). Porous GMA beads have a very developed heterogeneous structure, which is formed owing to porogens (cyclohexanol and dodecan-1-ol) used in their synthesis [39,40]. Analysis of the cross-section of the unmodified GMA beads (Fig. 4a,b) has shown that the fraction of pores was a somewhat higher (0.49±0.02) in GMA-1 beads than in GMA-3 ones (0.39±0.02). The mercury porosimetry analysis of the beads supported these results. The main porosity in GMA-1 beads was formed by pores with diameters of 102–55 nm (38%) and 45–39 nm (19%). Larger pores with diameters of 160–205 nm (5%) and 121–158 nm (7%) were also found in GMA-1 beads. In GMA-2 beads, pores with diameters of 96–130 nm (24%), 60–74 μm (30%) and 28–32 μm (23%) were the main. A small quantity of larger pores with diameter of 159–166 nm (2%) was presented in these beads, too. A set of pores with continuous decrease in diameters formed the porous structure of GMA-3 beads. According to amount of pores with diameters higher than 40 nm, porous GMA beads can be ordered as follows: GMA-1 (69%)>GMA-2 (63%)>GMA-3 (46%). The amount of pores with diameters less than 40 μm changed in the opposite order: GMA-1 (25%)< GMA-2 (31%)<GMA-3 (49%). The amount of the rest of pores of intermediate size, which have not been shown in the distribution diagrams did not exceed 6%.

3.2. Concentration of the chelating groups

For incorporation of various chelating ligands to the DEGMA and GMA beads, the reactivity of hydroxyl and epoxy groups, respectively, were used. It is obvious that distribution of the side chains of DEGMA and GMA moieties, bearing the groups to be modified, is a factor affecting the ligand attachment. Three samples of porous GMA beads differing in chemical composition as well as in their structural parameters and bead sizes were chosen to compare the effect of spatial factor and polymer morphology on attachment of the chelating ligands such as IDA and ASP. The following chemical composition of GMA beads was obtained based on the fact of practically complete conversion (96%) of copolymerized monomers: [GMA]:[HPMA]:[EDMA]= 8:0:4 (GMA-2), 5:3:4 (GMA-1) and 2:6:4 (GMA-3) (where HPMA is 2-hydroxypropyl methacrylate). It was reasonable to assume that the longer distance between two neighboring GMA moieties in GMA-3 beads will create less spatial hindrances for ligand attachment and hence a higher modification degree of epoxy groups could be achieved. This assumption was confirmed to a certain extent. Comparing the ratios of the available epoxy groups with that of the experimentally found IDA groups in modified beads, one can clearly see that the modification degree was the smallest (13%) in GMA-2 beads and approximately twice higher in GMA-3 (24%) and. GMA-1 (21%) beads, i.e., the higher content of epoxy group in the matrix, the more spatial hindrances existed for their binding with ASP and IDA groups.

The spatial arrangement of the chelating ligands (Fig. 5) attached to the side GMA and DEGMA chains was estimated to evaluate the accessibility of pores to the ligands. The ligand arrangement was estimated by quantum mechanical calculations performed in the MNDO format using the Gaussian 98 program package. The geometry of the model molecules was fully optimized using the gradient optimization routine in the program. The size of the side GMA and DEGMA groups was characterized by interatomic distances between the carboxyl carbon and farthest atoms in the chelating group (Table 2). The size of the side GMA moiety with attached IDA or ASP does not exceed 0.8–1.0 nm. Hence, theoretically, all pores in porous GMA beads might be accessible for the ligand attachment. However, it is very likely that the smallest pores (8–10 nm) are less accessible for the ligand attachment due to high pore tortuosity and kinetic reasons.

The same theoretical estimation was carried out for the nonporous DEGMA beads modified with EDTRIA and HQ groups. The reaction mixture contained DEGMA (0.799 mmol/g), 2-hydroxyethyl methacrylate, HEMA (0.106 mmol/g) and EDMA (0.117 mmol/g). The chemical composition of the prepared beads was as follows: [DEGMA]: [HEMA]:[EDMA]=37:7:5. It was found that only 12 and 10% of hydroxy groups were spatially accessible for attachment of EDTRIA and HQ groups, respec-

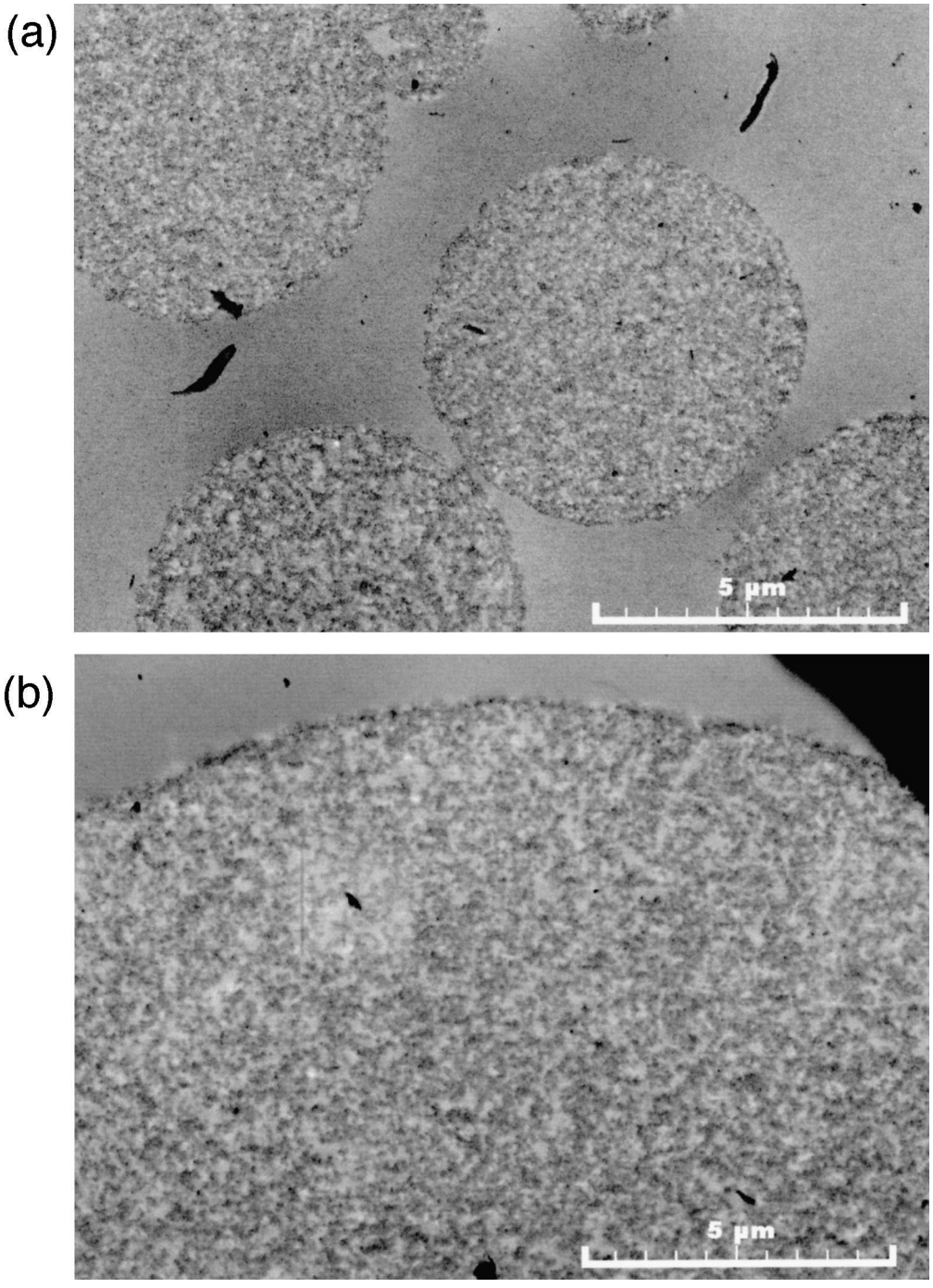

Fig. 4. TEM micrographs of porous GMA-1 (a) and GMA-3 (b) beads.

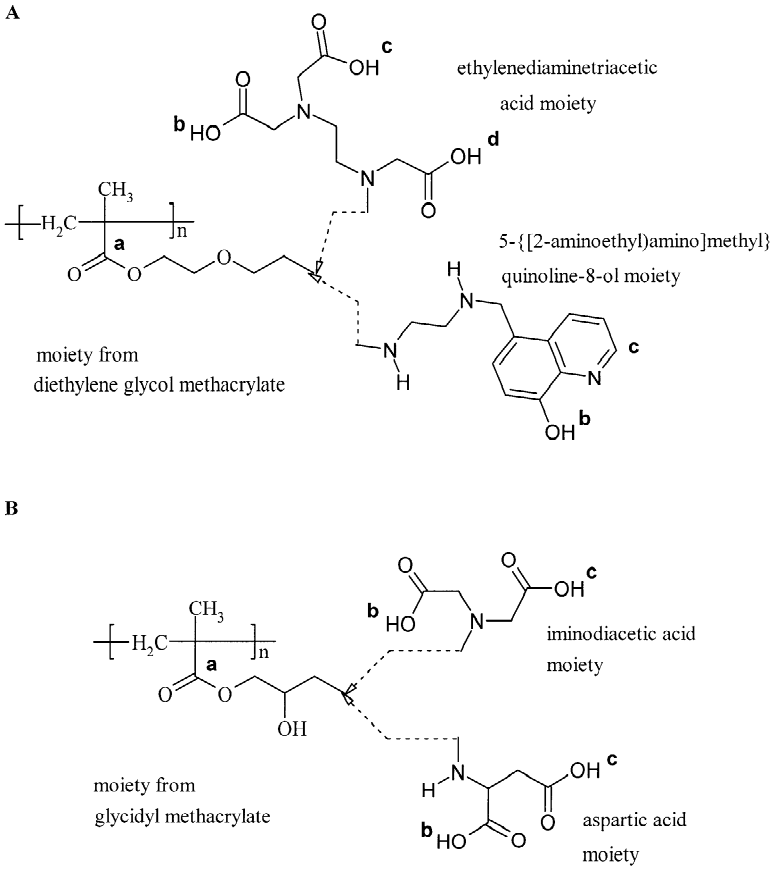

Fig. 5. Chemical structure of the chelating ligands. (A) EDTRIA, HQ chelating groups attached to the DEGMA moiety, (B) IDA, ASP chelating groups attached to the GMA moiety.

tively, having larger dimensions than IDA. Obviously, these chelating groups were attached mainly on the surface of nonporous beads.

Table 2
Distance between the most distant atom in the chelating ligands and carboxyl carbon atom in GMA and DEGMA moieties (see Fig. 5)

Chelating ligand	Distance (Å)		
	a–b	a–c	a–d
EDTRIA	9.001	9.805	12.159
HQ	14.618	15.551	–
IDA	7.861	9.878	–
ASP	8.843	9.790	–

3.3. Coordination of Ni^{2+} ions with the chelating ligands

The volume concentration of the chelating groups, their accessibility to coordination with transition metal ions, spatial arrangement and pH stability of the formed complexes affect the efficiency of protein separations by IMAC to a great extent [31,35,48–50]. The studied chelating sorbents were coordinated with Ni^{2+} ions both in static and dynamic conditions.

The results of static experiments can be summarized as follows: practically all EDTRIA and HQ groups in nonporous DEGMA sorbents as well as IDA and ASP groups in porous GMA sorbents were coordinated with Ni^{2+} ions. In dynamic conditions, a

Table 3
Characteristics of chelating sorbents

Chelating sorbent	Volume swelling in water		Ligand content (μmol/ml swollen sorbent)		Ni^{2+} content (μmol/ml swollen sorbent)			Relative Ni^{2+} content, $Ni_8^{2+}/Ni_{4.5}^{2+}$
	(ml/ml dry)	(ml/g)	Analysis	Titration	pH 4.5[a]	pH 4.5	pH 8	
EDTRIA–DEGMA	1.25	1.70	–	75	73	71	69	97.2
HQ–DEGMA	1.08	1.50	–	57	56	53	52	98.1
IDA–GMA-1	1.63	4.00	115	120	113	103	101	98.1
ASP–GMA-2	1.23	3.00	134	151	151	36	28	77.8
IDA–GMA-3	1.40	3.60	70	86	67	17	16	94.1
IDA–SFF	–	–	–	45	43	32	31	96.9

[a] Static sorption.

decrease in the coordination degree of Ni^{2+} ions was observed in porous GMA sorbents with increasing bead sizes (Table 3). This phenomenon can be explained mainly by kinetic reasons. Concentrations of Ni^{2+} complexes were 94–98%, when change of pH from 4.5 to 8 except for Ni^{2+}–ASP complex, which was less stable (78%).

3.4. IMAC separations

The specific IgG_1 was completely sorbed with the porous GMA-1 sorbent having the highest concentration of Ni^{2+} complexes and pore volume (Fig. 6). Similar results were obtained using chelating IDA–SFF in the Ni^{2+} form. Approximately 90% of the specific IgG_1 in the loaded ascitic fluid did not sorb on IDA–GMA-3 sorbent having the smallest concentration of Ni^{2+} complexes and pore volume (Table 4) and (Fig. 7).

Ni^{2+}–EDTRIA and Ni^{2+}–HQ complexes of nonporous DEGMA beads sorbed only 18–17% of the specific IgG_1 and 34–36% of accompanying proteins from the loaded ascitic fluid, respectively. The amounts of the sorbed specific IgG_1 (and proteins) increased with increasing volume concentration of Ni^{2+} complexes and volume of pores in IDA–GMA-3, ASP–GMA-2 and IDA–GMA-1 sorbents as follows: 18% (and 27%), 42% (and 32%) and 100% (and 72%), respectively. However, there is no proportionality between the concentration of Ni^{2+} complex in the studied sorbents and their IgG_1 sorption capacity. Obviously, a partial inaccessibility of Ni^{2+} complexes for binding large protein molecules exists in porous GMA sorbents. Only a somewhat lower sorption capacity of the chelating IDA–SFF to the specific IgG_1 and proteins in comparison with that of the IDA–GMA-1 sorbent having three times higher concentration of Ni^{2+} complexes supports this assumption. Some notion about selectivity of the chelating sorbents can be obtained from comparison of their sorption capacities to the IgG_1 and proteins. These ratios decrease in the order: 18 (HQ)>16 (EDTRIA)>15 (IDA–GMA-3)>6.7 (ASP–GMA-2) >6.4 (IDA–GMA-1)>5 (IDA–SFF). The stronger the specific IgG_1 binding with Ni^{2+} complexes was the harsher elution conditions were required for its elution. The specific IgG_1 eluted from Ni^{2+}–IDA–

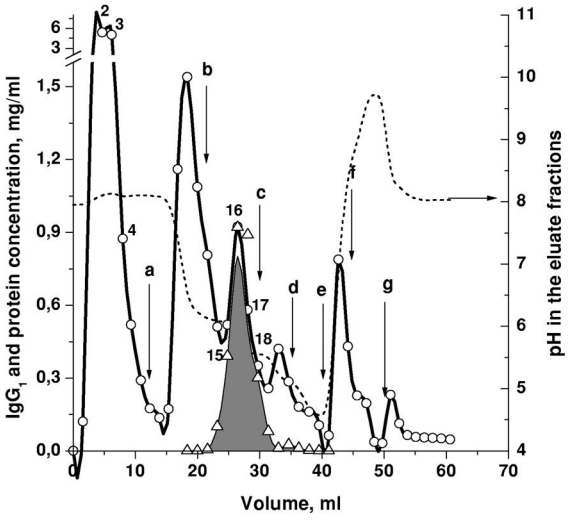

Fig. 6. IMA chromatography of the crude mouse ascitic fluid on Ni^{2+}–IDA–GMA-1 sorbent. Volume of the sorbent, 1.5 ml; loaded ascitic fluid, 2 ml. Flow rate, 0.25 ml/min. Elution was carried out using 0.1 *M* acetate buffer containing 0.5 *M* NaCl, pH 6 (a); pH 5.5 (b); pH 5 (c); pH 4.5 (d); 0.1 *M* (e) and 0.5 *M* (f) imidazole; 0.05 *M* Tris buffer containing 0.05 *M* EDTA and 0.5 *M* NaCl (g).

Table 4
IMA chromatography of the crude mouse ascitic fluid containing the HRP-specific IgG_1 using Ni^{2+} complexes of the chelating nonporous DEGMA and porous GMA sorbents

Chelating sorbent	Unsorbed (mg/ml swollen sorbent)		Sorbed (mg/ml swollen sorbent)		Recovery (%)		IgG_1 in the main fraction	
					IgG_1	Proteins		
	IgG_1	Proteins	IgG_1	Proteins			Amount (mg/ml)	Purity (%)
EDTRIA–DEGMA	1.26	8.9	0.28	4.53	90	70	0.123	89.8
HQ–DEGMA	1.70	11.49	0.35	6.41	83	70	0.338	72.2
IDA–GMA-1	0	4.78	2.05	13.12	92	89	1.886	73.3
ASP–GMA-2	1.18	12.12	0.87	5.79	67	70	0.185	88.5
IDA–GMA-3	1.72	12.98	0.33	4.92	95	81	0.316	80.8
IDA–SFF	0	7.51	2.05	10.39	90	82	1.238	65.3

Amount of the loaded specific IgG_1 (accompanying proteins): 2.05 (17.91) mg/ml for IDA–SFF, HQ–DEGMA, IDA–GMA-1, ASP–GMA-2, IDA–GMA-3 sorbents and 1.54 (13.43) mg/ml for EDTRIA–DEGMA sorbent.

GMA-1 and GMA-3 complexes with 0.1 *M* acetate buffer, pH 6–5.5 containing 0.5 *M* NaCl. A decrease in pH of the elution buffer to 5.5–5 was needed to elute the specific IgG_1 from Ni^{2+}–SFF, Ni^{2+}–ASP–GMA-2 and Ni^{2+}–EDTRIA–DEGMA complexes (Fig. 8a). From the former two complexes, the specific IgG_1 was completely eluted with 0.1 *M* imidazole. The strongest binding of the specific IgG_1 was observed with the Ni^{2+}–HQ–DEGMA complex. The IgG_1 was desorbed from this complex with EDTA-containing buffer (Fig. 8b).

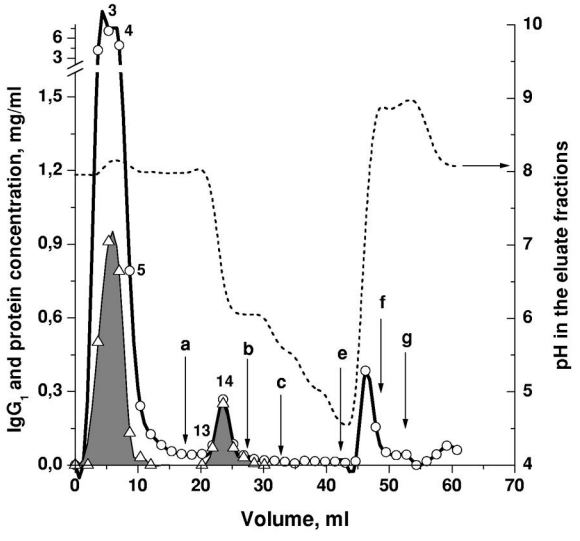

Fig. 7. IMA chromatography of the mouse ascitic fluid on Ni^{2+}–IDA–GMA-3 sorbent. For details, see Fig. 6.

Recovery of the specific IgG_1 and accompanying proteins with Ni^{2+}–HQ– and Ni^{2+}–IDA–GMA-3 under the used pH and imidazole concentration gradient achieved only 67–83%. For the rest of chelating sorbents, the recovery of IgG_1 was 90–96% (compared with the sorbed amount). The highest recovery of the specific IgG_1 was obtained using Ni^{2+} complexes of IDA–GMA-1 (92%) and IDA–SFF (90%). The purity of the specific IgG_1 in combined eluate fractions with maximum protein concentration was 73 and 65%, respectively. The purity of a small amount of IgG_1, which was eluted from Ni^{2+}–ASP–GMA-2 and Ni^{2+}–EDTRIA complexes, reached. 88–72%. Transferrin was found as main protein accompanying the eluted HRP-specific IgG_1 (Fig. 9a,b).

The spatial factors play an important role in modification of DEGMA and GMA beads with the studied chelating ligands as well as in their coordination with Ni^{2+} ions and in protein binding. Actually, the amount of the attached chelating groups has increased with decreasing size of polymer beads and increasing the large pore proportion. The amount of modified epoxy groups has also increased from 13 to 24–21% with increasing distance between neighbouring epoxy groups in GMA beads due to increase of HPMA content from 0 to 3–6 mmol/g.

The kinetic factor has manifested itself in coordination of Ni^{2+} ions with the chelating groups in dynamic conditions. The difference between static and dynamic sorption capacities of porous chelating GMA beads for Ni^{2+} ions increased with the bead

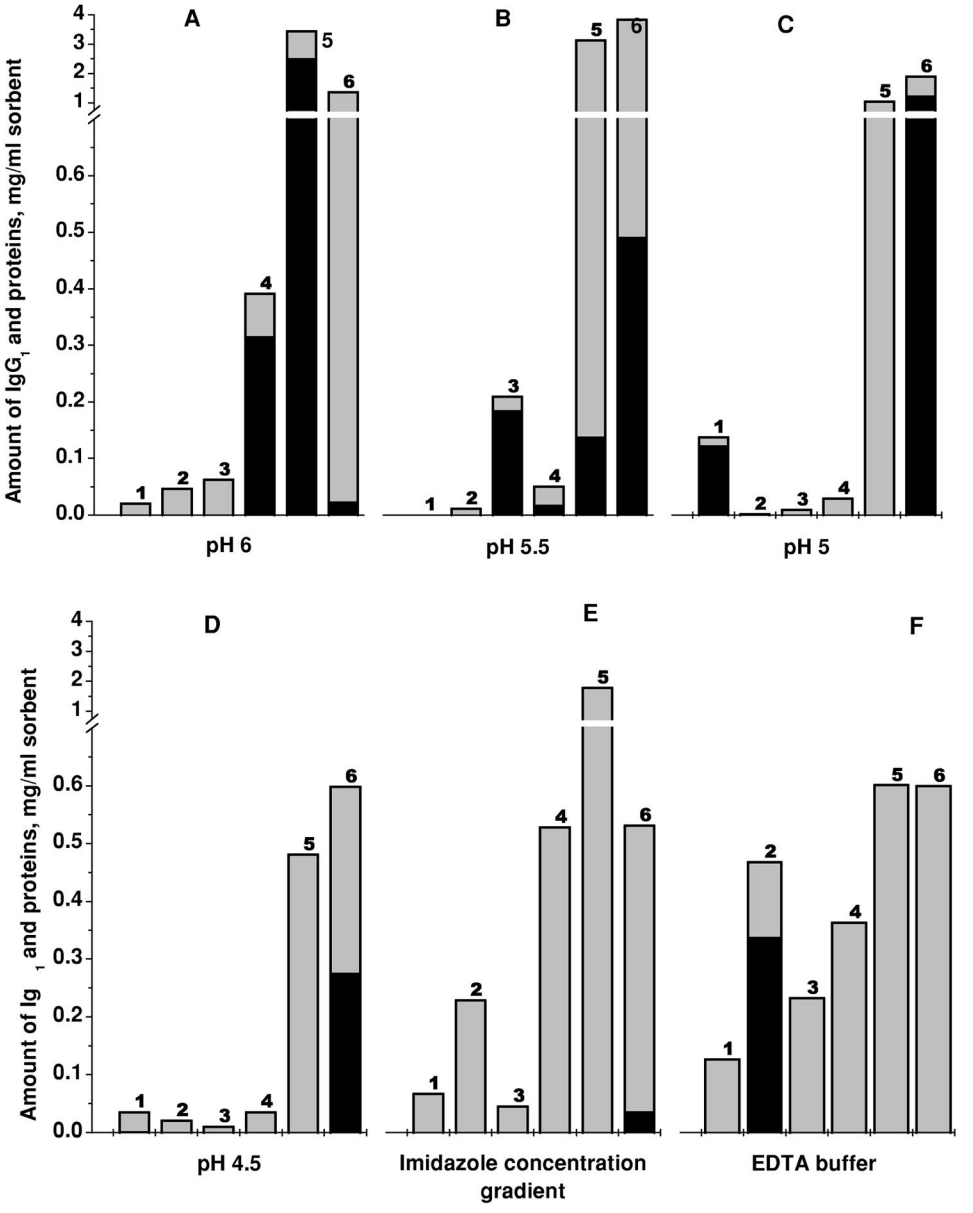

Fig. 8. Elution of the HRP-specific IgG_1 (■) and accompanying proteins (□) from Ni^{2+} complexes of the chelating sorbents under: stepwise pH gradient with 0.1 *M* acetate buffer containing 0.5 *M* NaCl (A–D), 0.1–0.5 *M* imidazole concentration gradient (E) and 0.05 *M* Tris buffer in 0.05 *M* EDTA containing 0.5 *M* NaCl (F).

size and a proportion of small pores. This can be expected bearing in mind that complexation is more complete reaction than simple ion-exchange. High tortuousity and nonuniformity of pores in size, as follows from analysis of porosimetric curves of porous GMA beads, contribute to the kinetics of Ni^{2+} complex formation. In contrast, EDTRIA and HQ chelating groups incorporated on the surface of nonporous DEGMA beads have more opportunities for the best spatial arrangement of Ni^{2+} complexes.

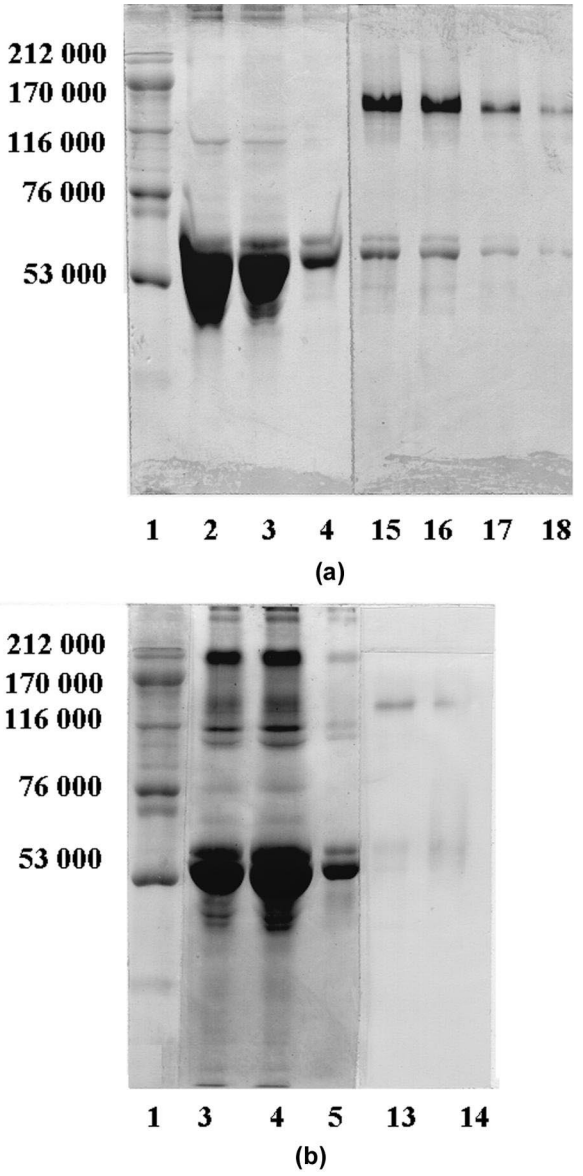

Fig. 9. SDS–PAGE of the eluate fractions in separation of the HRP-specific IgG_1 from the crude mouse ascitic fluid by IMAC on Ni^{2+}–IDA complexes of porous GMA-1 (a) and GMA-3 (b) sorbents. Band 1 high-molecular-mass protein standard (Pharmacia Biotech), other band numbers correspond to those of eluate fractions indicated in Figs. 6 and 7.

But, new spatial hindrances appeared in sorption of the bulky protein molecules with Ni^{2+} complexes both on the surface and in pores.

Analyzing the results of the study we came to the conclusion that preference should be given to highly porous IDA–GMA-1 beads with sizes of 7–13 μm if purification of a preparative quantity of specific IgG_1 is intended. Chelating sorbents with the surface EDTRIA and HQ may be useful in analytical IMAC procedure [41]. The difference in elution of the specific IgG_1 and accompanying proteins was caused by the strength of protein binding with the studied Ni^{2+} complexes, which increased in the order: Ni^{2+}–IDA < Ni^{2+}–ASP < Ni^{2+}–EDTRIA < Ni^{2+}–HQ. However, compared with the Ni^{2+}–IDA–GMA-1 complex, a peak of the specific IgG_1 eluted from the Ni^{2+}–IDA–SFF under somewhat lower pH values. Its main amount was desorbed with a buffer, of pH 5. Obviously, the more mobile matrix of the chelating Sepharose Fast Flow gives more opportunities for intermolecular protein–protein and protein–polymer interactions than those of the rigid GMA–EDMA sorbents. A relatively high sorption of accompanying proteins having lower dimensions than the specific IgG_1 took place in all the chelating sorbents. Promising results were obtained in separation of the HRP-specific IgG_1 from the crude mouse ascitic fluid using a porous GMA-1 sorbent.

4. Conclusion

An attempt was made to find correlation between the morphology of chelating sorbents based on poly(methacrylic) esters and their separation efficiency in purification of the HRP-specific IgG_1 from crude mouse ascitic fluid by IMAC. The nonporous EDTRIA–EGMA–*co*-EDMA or HQ–DEGMA–*co*-EDMA sorbents with surface chelating groups showed only small spatial and kinetic limitations both in coordination with Ni^{2+} ions and in protein binding. In the porous IDA– or ASP–GMA–*co*-EDMA beads, an interrelation between their structural parameters (chemical composition, bead dimensions, pore volume, pore size distribution) and their sorption properties was found. Compared with the chelating IDA–Sepharose Fast Flow, porous IDA–GMA–*co*-EDMA beads (7–13 μm) with developed specific surface area (68 m^2/g) and 67%-amount of pores with diameters higher than 40 nm exhibited a somewhat better efficiency (92% recovery, 73% purity) in purification of the HRP-

specific IgG_1 from the crude mouse ascitic fluid in a single-step IMAC procedure. Finally, it should be mentioned that controlling the spatial arrangement of chelating groups, their distribution in the support as well as the nature and morphology of the latter might be a way for improving the selectivity of a target protein binding and IMAC efficiency.

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