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Evaluation of immobilized metal membrane affinity chromatography for purification of an immunoglobulin G_1 monoclonal antibody

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Abstract

The large scale production of monoclonal antibodies (McAbs) has gaining increased relevance with the development of the hybridoma cell culture in bioreactors creating a need for specific efficient bioseparation techniques. Conventional fixed bead affinity adsorption commonly applied for McAbs purification has the drawback of low flow rates and colmatage. We developed and evaluated a immobilized metal affinity chromatographies (IMAC) affinity membrane for the purification of anti-TNP IgG_1 mouse McAbs. We immobilized metal ions on a poly(ethylene vinyl alcohol) hollow fiber membrane (Me^{2+}-IDA-PEVA) and applied it for the purification of this McAbs from cell culture supernatant after precipitation with 50% saturation of ammonium sulphate. The purity of IgG_1 in the eluate fractions was high when eluted from Zn^{2+} complex. The anti-TNP antibody could be eluted under conditions causing no loss of antigen binding capacity. The purification procedure can be considered as an alternative to the biospecific adsorbent commonly applied for mouse IgG_1 purification, the protein G-Sepharose.

Keywords: Monoclonal antibody; IgG_1; Purification; Affinity membrane; IMAC

1. Introduction

Antibody-based technologies are recognized as key factors in recent advances made by biotechnology industries. Application of monoclonal antibodies (McAbs) in immunodiagnostics, immunotherapy, immunoaffinity chromatography, immunoscintiligraphy, and controlled drug delivery is based on their extremely high selectivity and sensitivity in the recognition of antigens against which they are directed [1]. Traditionally, McAbs have been produced in ascites fluids, but recent advances in hybridoma technology have enabled the large scale production of there antibodies in mammalian bioreactor systems [2].

A variety of approaches exists for purification of McAbs from cell supernatant. Separation is usually initiated with ammonium sulphate precipitation for concentration of the protein material followed by a gel based chromatographic unit operation designed to remove impurities [3]. In particular, due to their high affinity for Fc antibody domain, proteins A and G are the most widely used antibody affinity ligands allowing rapid and highly selective separation of antibodies from different biological fluids [1]. However, these gels require harsh elution conditions and have the disadvantages of low capacity [3], low chemical and proteolytic stability [4]. Thus, these resins cannot withstand cleaning procedures in harsh conditions, and, after long time exposure to cell

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supernatant, eluted monoclonal antibodies could be heavily contaminated by the whole ligand or ligand fragments leaking due to column degradation. As consequence, additional purification steps could be required [4]. As an alternative for affinity chromatography using proteins A and G as ligand, a number of other chromatographic methods such as ion exchange, dye ligand, synthetic peptide ligand, histidine (His) ligand, thiophilic, hydrophobic, and immobilized metal affinity chromatographies (IMAC) are under development [1,4–7]. Although each one of these methods has some advantages and disadvantages compared with other chromatographic methods based on affinity interactions, IMAC is an interesting alternative to biospecific high affinity ligands in terms of ligand stability, capacity, simplicity, recovery of active protein and cost [3,5,8,9]. The purification of antibodies and other proteins and enzymes by IMAC is based mainly on the affinity of histidine residues for transition metal ions coordinated with chelating groups [10].

Adsorption of immunoglobulins from different sources on IMAC matrices has been reported by many authors. Porath and Olin [10] studied the adsorption of human IgG on agarose-IDA-Ni^{2+}; Boden et al. [3] purified goat IgG on Novarose-TREN-Cu^{2+}; Hale and Beidler [11] studied the purification of humanized murine and murine IgG_1 on Tosoh-Haas-TSK-IDA-Ni^{2+}; Vançan et al. [12] purified human IgG on agarose-IDA-Cu^{2+}, and Mészárosová et al. [13] studied the interactions of immunoglobulins with immobilized metal affinity sorbents based on hydrophilic methacrylate polymers. However, there are some drawbacks for large scale applications of the soft gels used by these authors. Flow rates, and thus performances, are limited by the compressibility of the gels and pore diffusion. Recently, the idea of using microporous membranes as support matrices for affinity separation was introduced, since they provide higher flow rates, much lower pressure drops, easier scale-up, mechanical stability and higher productivities [14]. As example, flat-sheet microfiltration membranes functionalized with IDA-Cu^{2+} and IDA-Zn^{2+} showed high effectiveness for immunoglobulin adsorption [15,16].

The work reported here aimed to obtain fundamental data to evaluate the potential use of immobilized metal ion affinity membrane chromatography for the development of large scale purification processes for monoclonal antibody (an IgG_1 monoclonal antibody was used as model protein). We prepared an affinity membrane from polyethylene vinyl alcohol (PEVA) hollow fibers by covalently linking iminodiacetic acid (IDA) to them. The adsorption of IgG_1 monoclonal antibody anti-TNP (an irrelevant antibody used as a negative control of the same isotype in ELISA tests) [17] onto Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} metal ions, immobilized by chelation onto the IDA-PEVA membrane, was then studied. Adsorption isotherm was determined at 25 °C with a static method and analyzed using two models, namely, the Langmuir and Langmuir–Freundlich models. Parameters pertinent to the adsorption processes such as the dissociation constant, maximum capacity, and cooperativity were analyzed and discussed. The effects of variables on IgG_1 breakthrough curves, presence of contaminant proteins and filtrate flow rate were studied.

2. Experimental

2.1. Material

The agarose gel (Sepharose® 6B), the agarose gel immobilized protein G (protein G-Sepharose Fast Flow), and electrophoresis calibration kit for molecular mass determination were provided by Amersham Biosciences (Sweden). Epichlorohydrin and coomassie brilliant blue were purchased from Merck (Germany). Tris(hydroxyethyl amino ethane), copper sulphate, zinc sulphate, nickel sulphate, cobalt sulphate, disodium ethylenediamine tetra-acetate (EDTA), crystalline bovine serum albumin (BSA), iminodiacetic acid, imidazole, Dulbecco's modified Eagle's medium, rabbit IgG-anti-mouse, polyclonal mouse IgG, peroxidase-labelled sheep anti-mouse IgG_1, H_2O_2, *o*-phenylenediamine (OPD), Tween 20, and human immunoglobulin G (prepared from Cohn Fraction II, III, electrophoretic purity approximately 99%, named in this work as "human IgG") were purchased from Sigma (USA). Casein was provided by Calbiochem (USA). The stirred ultrafiltration cell and YM10 membrane (nominal molecular mass cut-off of 10 kDa) were purchased from Amicon (USA). The water used in all experiments was ultrapure water obtained using a Milli-Q System (Millipore, USA). All other chemicals were of analytical reagent grade.

The poly(ethylenevinyl alcohol)—PEVA—hollow fiber cartridges (Model Eval 4A, 1 m^2 surface area) were purchased from Kuraray (Osaka, Japan). The hollow fiber had an internal diameter of 200 μm, a wall thickness of 20 μm and a nominal molecular mass cut-off of 600 kDa.

2.2. Synthesis of the affinity gels

Agarose (Sepharose-6B) activation with epichlorohydrin and coupling to iminodiacetic acid were carried out as described by Porath and Olin [10]. The chelating capacity of the gel for Cu^{2+} ion was about 60 μmol of Cu^{2+} per milliliter of the gel bed, determined according the method described by Belew and Porath [18].

2.3. Immobilization of IDA onto PEVA hollow fiber membrane

2.3.1. Cut membrane derivatization

A commercially available PEVA hollow fiber cartridge was disassembled, the hollow fibers were removed and finely cut in pieces of around 2 mm in length. The cut PEVA membranes were activated with epichlorohydrin as described by Bueno et al. [19] and IDA was coupled to them as described by Porath and Olin [10]. The Cu^{2+} chelating capacity of the membrane was about 38 μmol of Cu^{2+} per milliliter of the

membrane bed, determined by atomic absorption spectrometry using EDTA solution as blank. The derivatized membrane are referred to as IDA-PEVA in this work.

2.3.2. Minicartridge derivatization

A small scale cartridge of PEVA hollow fibers was manufactured using fibers from a commercially available cartridge. The fibers were cut and assembled in a minicartridge of 4.5 cm effective length. The amount of fibers in this cartridge was 0.14 g dry mass, with 71.6 cm^2 surface area and 0.16 cm^3 fiber bed volume. The hollow fiber bed volume (V_b) was calculated as follows:

$$V_b = \pi(r_o^2 - r_i^2)L_e N_f \quad (1)$$

where r_o and r_i are the outer and inner radius of the hollow fibers, respectively, L_e is the effective length and N_f is the number of hollow fibers in the cartridge. The PEVA hollow fiber cartridge was activated with epichlorohydrin and IDA was coupled to it as described previously in this work.

2.4. Preparation of antibodies from cell culture supernatant (IgG_1 precipitate solution)

Anti-TNP IgG_1 mouse monoclonal antibody was produced by culturing the hybridoma cell lines 1B21B6 [17] in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 500 mL spinner flasks, at 37 °C, under 5% CO_2 atmosphere for 48 h. In this hybridoma culture medium there was not the presence of any other antibody or subclass (there was only IgG_1) [17]. A 200 mL aliquot of culture supernatant was centrifuged at 320 × *g* for 10 min to remove cells, then filtered through a 0.22 μm filter (Millipore, EUA). The protein content of supernatant was then precipitated with 50% saturation of ammonium sulphate at 4 °C for 1 h with gentle stirring. The resulting suspension was then centrifuged at 10,000 × *g* for 15 min, and the IgG_1 containing precipitate was collected. This precipitate was dissolved in 25 mM Tris–HCl buffer pH 7.0 and dialysed for 30 h at 4 °C against three 2-L changes of the equilibration buffer described ahead. This anti-TNP IgG_1 containing solution was called "IgG_1 precipitate solution" in this work. It contained 196 μg of IgG_1 per milliliter (5.5 mg of total protein per milliliter).

2.5. IgG_1 determination by ELISA

Determination of monoclonal antibody concentration was performed by solid-phase enzyme-linked immunosorbent assay (ELISA) using microtitre plates coated with rabbit, anti-mouse IgG as described by Leo et al. [17]. Polyclonal mouse IgG was used as standard protein. A 50 μL (2 μg/mL) volume of rabbit IgG-anti-mouse diluted in 50 mM bicarbonate buffer pH 9.2 was added to the wells of a microtitre plate and incubated at 37 °C for 1 h. The plates were incubated at 4 °C for 18 h and then washed with 20 mM PBS buffer pH 7.2 containing 0.05% of Tween 20 and blocked with 20 mM PBS buffer pH 7.2 containing 2% casein and 1% bovine serum at 37 °C for 1 h. After washing, a 50 μL aliquot of samples or standard diluted in 20 mM PBS buffer pH 7.2 containing 1% casein was added to the wells and incubated at 37 °C for 1 h. Plates were once more washed and 50 μL of peroxidase-labelled sheep anti-mouse IgG_1 diluted in 20 mM PBS buffer pH 7.2 containing 1% casein was added to the wells and the plates were further incubated at 37 °C for 1 h. Plates were once more washed with the same buffer describe above; 50 μL of reaction substrate containing 0.03% H_2O_2 and 0.4 mg/mL OPD in 50 mM citrate-phosphate buffer pH 5.0 were added to the reaction mixture. Plates were incubated at 25 °C for 30 min and the reaction was terminated with the addition of 25 μL of 4N sulphuric acid solution. The absorbance was measured at 492 nm (Multiskan MS, Labsystems, Finland).

2.6. Protein determination

Protein concentration was determined by the method of Bradford [20]. BSA was used as reference protein.

2.7. Sodium dodecyl sulfate–polyacrylamide gel elecrophoresis (SDS–PAGE)

SDS–PAGE (10% or 12% acrylamide gels) under non-reducing conditions using a Protean II System (Bio-Rad, USA) was carried out according to Laemmli [21]. The gels were stained with silver nitrate according to Morrissey [22].

2.8. Column preparation

The cut IDA-PEVA fibers (1.6 g dry mass) were suspended in the equilibration buffer described ahead, degassed and packed into a column (10 cm × 1.0 cm i.d.) to give a bed volume of approximately 5.0 mL. Copper, nickel, zinc or cobalt ion was loaded into the IDA-PEVA column by passing 50 mM sulphate solution of the specific metal ion in water through the column until saturation. This matrix saturation was visually detected for all ions except for the case of zinc whose excess in the out-stream was detected by titration with 2 M Na_2CO_3 [23]. Non-specifically bound metal was removed by washing the column with the adsorption and elution buffers used in chromatographic experiments described ahead. When no further metal was detected in the flow stream out of the column, the cut fibers were equilibrated with adsorption buffer. The derivatized membrane with immobilized metal ion is referred to as Me^{2+}-IDA-PEVA in this work.

2.9. Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) at 25 °C at a linear velocity of 38.2 cm/h (30.0 mL/h).

2.9.1. Preliminary IgG_1 purification experiments in Me^{2+}-IDA-PEVA

A column packed with IDA-PEVA cut fibers and saturated with metal ions as described previously here was washed with 200 mM Tris–HCl buffer pH 7.0 and then equilibrated with 25 mM Tris–HCl buffer pH 7.0 (equilibration buffer). The column was loaded with 5.0 mL of the IgG_1 precipitate solution (about 50 mg of protein). After protein injection, the column was washed with equilibration buffer until no protein was detected in the column out-stream (the protein absorbance of the eluate was monitored at 280 nm). Elution was carried out with a Tris discontinuous step gradient (50–200 mM with 50 mM increments in the equilibration buffer at pH 7.0) [24]. Regeneration of the column was done by washing with 50 mM EDTA solution at pH 6.5. Fractions of 5.0 mL were collected during the chromatographic experiments and their protein content was determined by the method of Bradford [20]. The fractions were then pooled and concentrated using a stirred ultrafiltration cell and a YM10 membrane for SDS–PAGE analysis and IgG_1 concentration determination by ELISA.

2.9.2. Protein G-agarose chromatography for the production of purified IgG_1 preparation

A column (10 cm × 1.0 cm i.d.) packed with protein G-agarose (protein G-Sepharose Fast Flow, bead volume of 5.0 mL) was equilibrated with 20 mM phosphate buffer pH 7.4. A volume of 50.0 mL of the IgG_1 precipitate solution (about 500 mg of protein) was loaded into the column which was then washed with the 20 mM phosphate buffer pH 7.4 until the absorbance of the effluent at 280 nm reached zero. Elution was performed with 0.1 M citrate buffer at pH 2.6 and the eluted fractions were immediately neutralized with 1 M Tris–HCl pH 8.0. The absorbance of the eluate was monitored at 280 nm. The eluted fractions of several chromatographics runs were pooled and the buffer was changed to 25 mM Tris–HCl buffer pH 7.0. The eluted fractions pool was concentrated using a stirred ultrafiltration cell and a YM10 membrane. This solution (6.0 mg/mL in terms of total protein) was called purified IgG_1 preparation and it was used for protein adsorption studies.

2.10. Protein adsorption studies

2.10.1. Analysis of equilibrium data

Langmuir model: this model considers that the adsorption process takes place on a surface composed of a fixed number of adsorption sites of equal energy, and that one molecule is adsorbed per adsorption site until a monolayer coverage is achieved [25]. The Langmuir model can be described by the equation:

$$Q = \frac{Q_m C}{K_d + C} \tag{2}$$

in which C is the protein liquid-phase equilibrium concentration; Q, the protein surface concentration; Q_m, the maximum protein binding capacity and K_d is the apparent dissociation constant. However, adsorbent surfaces are rarely homogeneous and there are a number of classical isotherm models for heterogeneous surfaces with continuous energy distribution such as Langmuir–Freundlich model [26].

Langmuir–Freundlich model: the single component Langmuir–Freundlich model is:

$$Q = \frac{Q_{MLF} C^n}{K_d^* + C^n} \tag{3}$$

in which K_d^* is the apparent dissociation constant that includes contributions from ligand binding to monomer, monomer-dimer, and more highly associated forms of the protein; Q_{MLF}, the maximum binding capacity; and n is the Langmuir–Freundlich coefficient. By analogy with protein–multiple ligand interactions it has been suggested that Eq. (3) works well to model adsorption cooperativity [27,28]. For purely independent noninteracting sites, $n = 1$. For positive cooperativity of the protein binding sites, $n > 1$, while negative cooperativity in the binding process is indicated when $0 < n < 1$. The value of n can, thus, be employed as an empirical coefficient, representing the type and the extent of cooperativity present in the binding interaction.

The Langmuir–Freundlich reduces to the Freundlich model ($Q = FC^n$, in which F is the Freundlich constant) at low concentrations, and for the case of a homogeneous surface, it reduces to the Langmuir model [29]. In this work the parameters of Eqs. (2) and (3) were evaluated by fitting the Langmuir and Langmuir–Freundlich models to the experimental data employing the interative fitting method of Levenberg–Marquardt.

2.10.2. Equilibrium binding analysis

Stirred tank batch adsorption experiments were used to collect data for equilibrium binding analysis. Purified IgG_1 preparation was the anti-TNP McAbs feed material and finely cut IDA-PEVA fibers were the adsorbent. The cut Zn^{2+}-IDA-PEVA fibers (14.5 mg dry mass) were weighed in 500 μL Eppendorf tubes. The membranes were then equilibrated with degassed 50 mM Tris–HCl buffer at pH 7.0. Then 300 μL of purified IgG_1 preparation were added to the tubes (diluted with Tris–HCl to total protein concentrations from 0.5 to 5.0 mg/mL). The tubes were rotated end-over-end at 6 rpm for 3 h to allow equilibrium to be established. After this, the supernatant was removed and the unbound protein concentration in the liquid phase was determined by the method of Bradford [20]. The adsorbed protein mass, Q, was determined by the difference between the concentration of protein fed and that present in the supernatant C after equilibrium, multiplied by the feed volume (300 μL). Plotting Q against C yielded the equilibrium isotherm.

2.11. IgG_1 precipitate solution cross flow filtration in Zn^{2+}-IDA-PEVA hollow fiber cartridge

These experiments were carried out at 25 °C with an automated chromatography system. Prior to the experiments, the equilibration buffer (50 mM Tris–HCl, pH 7.0) was pumped through the Zn^{2+}-IDA-PEVA minicartridge for 10–15 min in dead-end mode at inlet flow rate equal 1.0 mL/min. Feedstream of IgG_1 precipitate solution at 6.5 mg/mL in terms of total protein was pumped through the minicartridge in a cross flow mode in open loop at inlet flow rates of 0.6, 1.4, and 2.4 mL/min. The inlet flow rate (Q_i) and filtrate flow rate (Q_F) were kept constant using two peristaltic pumps to fix the ratio Q_F/Q_i to 0.50. The corresponding residence times (t_R) of each filtrate flow rate were calculated by dividing the membrane interstitial volume by the filtrate flow rate [14]. The filtrate outlet passed through the UV detector to monitor the absorbance at 280 nm (protein breakthrough determination). After loading the IgG_1 precipitate solution, the unadsorbed protein was washed out with 50 mM Tris–HCl buffer pH 7.0. Four washing steps at different modes were used [19]: a cross-flow filtration, lumen, shell, and backflushing wash. The lumen side of the fibers was washed by pumping buffer into the lumen inlet, with the filtrate exit valves closed. For washing the shell side of the fibers, buffer was pumped into the shell inlet and out of the shell outlet to the waste (retentate exit valve closed). The backflushing wash was carried out by closing the shell outlet and pumping buffer into the shell inlet, through the membrane, and out of the lumen outlet. For each one of these washing steps, the minicartridge was washed with the buffer until the absorbance of effluent at 280 nm reached the baseline. The adsorbed protein was eluted in backflushing mode with a discontinuous step gradient of Tris (100–700 mM of Tris–HCl buffer pH 7.0). The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution was completed, the cartridge was sequentially washed at frontal mode with 50 mM EDTA pH 6.5 and with the loading buffer to restore it to its initial conditions for carrying out the next experiment.

Protein concentrations in the non-retained and retained fractions were determined by the Bradford method [20] and analyzed by SDS–PAGE under non reducing conditions. Breakthrough curves were plotted as the ratio of the total protein concentration in the filtrate (C_f) to that in the feed stream (C_0) as a function of the volume of protein solution throughput.

3. Results and discussion

3.1. Metal selection and comparison of Zn^{2+}-IDA-PEVA and Zn^{2+}-IDA-agarose for IgG_1 purification

The anti-TNP mouse IgG_1 monoclonal antibodies containing culture supernatant contained also a number of proteins of various molecular masses. The fetal bovine serum proteins transferrin and albumin were the main protein impurities. Salt precipitation of the culture supernatant was performed with 50% saturation ammonium sulphate. This technique led to an enrichment of the McAbs (data not shown).

In order to select the metal ion for IgG_1 purification, preliminary adsorption experiments using IDA-PEVA cut fibers were performed with McAbs from IgG_1 precipitate solution on different transition metals ions—Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+}- using Tris–HCl buffer at pH 7.0. The different immobilized metal ions affected differently the adsorption and elution of IgG_1 (Fig. 1 and Table 1).

The IgG_1 was almost completely adsorbed on all metals studied according to the SDS–PAGE assay (Fig. 1) since the flowthrough and washing streams (peaks 1) did not contain virtually any IgG_1. A value of 55% of the loaded culture supernatant protein content was displaced from Cu^{2+}-IDA-PEVA with the loading buffer, while for others metal ions studied, above 92% of proteins was displaced. This suggests a strong interaction between IgG_1 with these metal ions in the presence of Tris buffer since only a weak interaction would be avoided by a weak competitive agent such as the Tris ion. When comparing the Cu^{2+}-IDA-PEVA, Co^{2+}-IDA-PEVA, Ni^{2+}-IDA-PEVA, Zn^{2+}-IDA-PEVA, the eluted IgG_1 co-eluted with decreasing amounts of contaminating protein and thus with increasing purity when changing the metal ion from Cu^{2+} to Co^{2+}, Ni^{2+} and to Zn^{2+}. This corresponds to a weaker interaction between the proteins and the metal charged IDA-PEVA when changing the metal ion from Cu^{2+} to Co^{2+}, Ni^{2+} and to Zn^{2+}. The purity of IgG_1 in the eluated fractions was high when eluted from Zn^{2+} complex. Analysis of Fig. 1c revealed that the majority of albumin eluted in the flowthrough. The fractions eluted with Tris were found to consist primarily of IgG_1 with small amount of other proteins. Although there is the presence of these proteins additional purification is facilitated by the near complete removal of serum albumin. Thus, Zn^{2+} was the most suited metal ion for IMAC purification of this monoclonal IgG_1.

In order to maximize IgG_1 elution and to facilitate separation of the albumin from the IgG_1 in the loading step, the IgG_1 precipitate solution was loaded in Zn^{2+}-IDA-PEVA cut fibers column with Tris–HCl buffer pH 7.0 at a higher concentration −50 mM instead of the 25 mM used before—and elution was performed with discontinuous steps gradient of Tris (100–700 mM in Tris–HCl buffer at pH 7.0). The results were compared with data obtained by performing similar experiments onto 2 mL Zn^{2+}-IDA-agarose (0.67 g of dry weight) (Fig. 2 and Table 2).

The 50 mM Tris–HCl buffer (pH 7.0) allowed the albumin to pass through the Zn^{2+}-IDA-PEVA without adsorption (peak 1, 94.9% of total protein). The difference in adsorption capacity and selectivity of Zn^{2+}-IDA-PEVA and Zn^{2+}-IDA-agarose was observed. The adsorption capacity of the Zn^{2+}-IDA-PEVA for the total protein (1.53 mg of protein/g adsorbent) was about twice as low as that of the Zn^{2+}-IDA-agarose (3.24 mg of protein/g adsorbent). However, the considerably lower adsorption capacity of Zn^{2+}-IDA-PEVA can be asso-

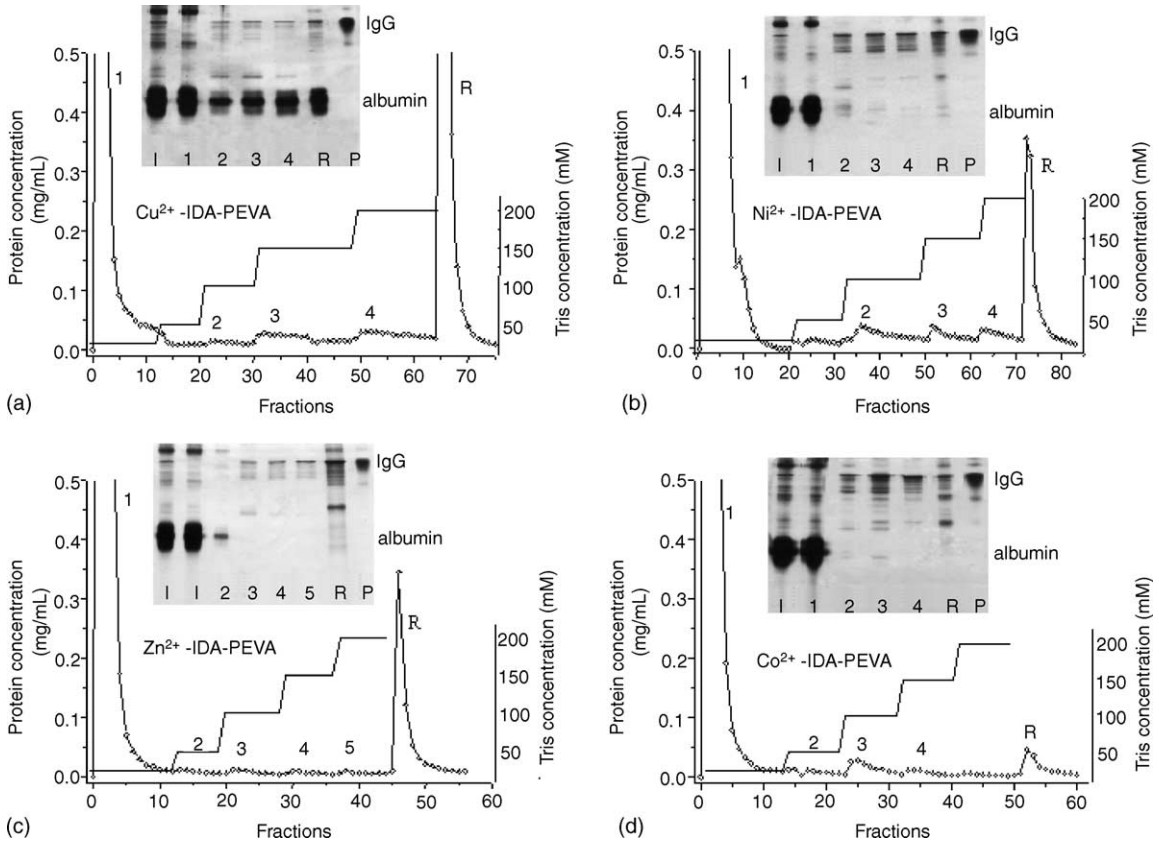

Fig. 1. Effect of the different metal ions on the adsorption and elution by Tris gradient of IgG_1 precipitated chromatography on (a) Cu^{2+}-IDA-PEVA, (b) Ni^{2+}-IDA-PEVA, (c) Zn^{2+}-IDA-PEVA and (d) Co^{2+}-IDA-PEVA. Buffer composition: 25 and 50–200 mM with increments of 50 mM Tris at pH 7.0. Column regeneration: 50 mM EDTA at pH 6.5. Other conditions: bed volume, 5.0 mL; linear velocity, 38.2 cm/h; fraction volume, 5.0 mL. Protein injected: 5.0 mL of IgG_1 precipitate solution (about 50 mg of protein). Insert: SDS–PAGE analysis of fractions from the chromatography on Me^{2+}-IDA-PEVA: I, IgG_1 precipitate solution; numbered lanes represent aliquots of the corresponding pooled fractions of the protein peaks obtained; R, pool of regeneration fractions; P, human IgG (Sigma).

ciated with its higher adsorption selectivity for IgG_1 than for contaminants proteins: the fractions eluted from Zn^{2+}-IDA-PEVA (Fig. 2b, lanes 3–5) contains less contaminants than the fractions eluted from Zn^{2+}-IDA-agarose (Fig. 2a, lanes 3–5).

Table 3 summarizes the IgG_1 yield in the eluted fractions from the Zn^{2+}-IDA-PEVA column. Little or no detectable McAbs were observed in washing and eluted fractions with 100 mM Tris–HCl. The two highest IgG_1 yield and purifica-

Table 1
Mass balance of total protein for chromatographies of IgG_1 precipitate solution with elution by Tris–HCl step concentration gradient

Protein recovery		Metal							
		Cu^{2+}		Ni^{2+}		Zn^{2+}		Co^{2+}	
		(mg)[a]	(%)[b]	(mg)[a]	(%)[b]	(mg)[a]	(%)[b]	(mg)[a]	(%)[b]
Injection	25 mM	48.42	100.0	85.64	100.0	43.05	100.0	38.64	100.0
Washing	25 mM	26.42	54.6	78.76	92.0	43.17	100.3	39.90	103.3
Elution	50 mM	0.46	1.0	0.28	0.3	0.29	0.7	0.35	0.9
	100 mM	0.58	1.2	1.11	1.3	0.34	0.8	0.64	1.7
	150 mM	1.80	3.7	1.13	1.0	0.25	0.1	0.30	0.1
	200 mM	2.02	4.1	1.02	1.2	0.26	0.1	0.16	0.0
Regeneration[c]		17.23	35.6	4.30	5.0	3.27	7.6	0.70	1.8
Total		48.51	100.1	86.60	101.1	47.58	110.5	42.05	108.8

[a] Mass calculated from protein concentration determined by Bradford [16].
[b] Percentage relative to injected protein mass.
[c] EDTA concentration: 50 mM.

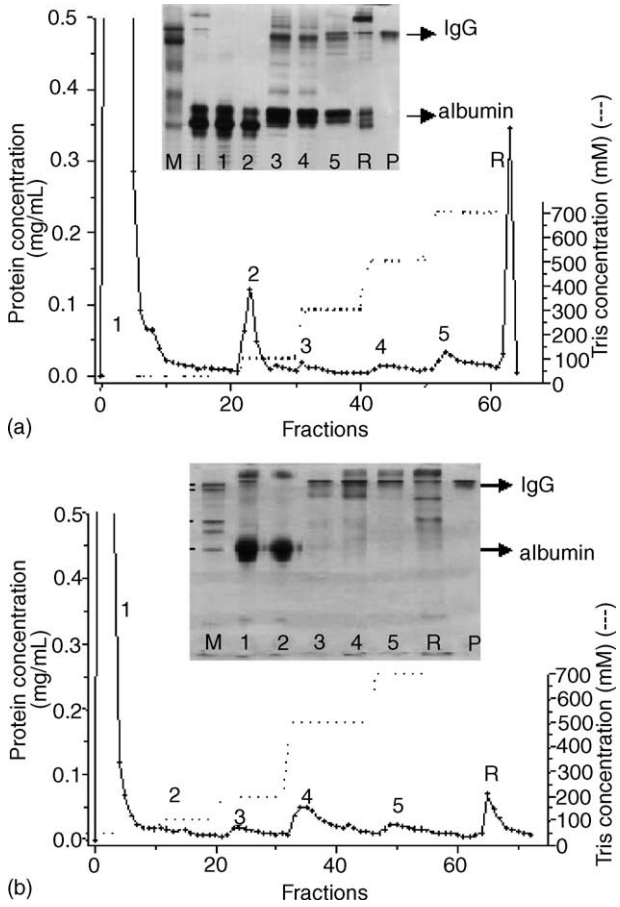

Fig. 2. Adsorption and elution by Tris gradient of IgG_1 precipitate solution chromatography on (a) Zn^{2+}-IDA-agarose and (b) Zn^{2+}-IDA-PEVA. Insert: SDS–PAGE analysis under non-reducing conditions of fractions from the chromatography: M, molecular mass markers (Amersham Biosciences); I, IgG_1 precipitate solution; numbered lanes represent aliquots of the corresponding pooled fractions of the protein peaks obtained; R, pool of regeneration fractions; P, human IgG (Sigma).

tion factor were obtained in the fractions eluted with 500 mM Tris (61.8% of total IgG_1 eluted and purification factor of 18.7) and 700 mM Tris (28.5% of total IgG_1 eluted and purification factor of 15.0). Considering the combination of these two fractions as a product the total yield and purification factor were 90.3% and 33.7%, respectively.

In order to evaluate the effect of experimental purification conditions on the maintenance of the antibody-antigen recognition, 5 mL of IgG_1 precipitate solution (980 μg of starting anti-TNP IgG_1), were purified onto 5 mL Zn^{2+}-IDA-PEVA cut fibers column equilibrated with 50 mM Tris–HCl, pH 7.0. After the antibody elution and neutralization, unbound and bound materials were analyzed for immunoreactivity content by an antigen-specific ELISA. The results indicated that the antibodies were eluted with their antigenic properties conserved, considering that the most of the immunoreactivity (>90%) was recovered in the bound fraction, and only traces in the unbound and washing material (data not shown).

3.2. Thermodynamics of McAbs adsorption

In order to evaluate thermodynamic parameters such as IgG_1 binding capacity (Q_m) and dissociation constant (K_d), adsorption isotherms were determined from the experimental data obtained from the batch adsorption experiments at 25 °C using Zn^{2+}-IDA-PEVA in Tris–HCl buffer 50 mM at pH 7.0. The isotherm was analyzed using Langmuir and Langmuir–Freundlich models (Table 4). The comparison between experimental and theoretical profiles for adsorption of IgG_1 on the Zn^{2+}-IDA-PEVA can be made analysing Fig. 3.

The Langmuir and Langmuir–Freundlich isotherm equations could describe the adsorption data satisfactorily (relatively high coefficients of correlation equal to 0.98 and 0.99, respectively). Nevertheless, the Langmuir–Freundlich isotherm model gave $n > 1.0$. Value of $n = 1.66 \pm 0.47$ for this system indicate positive cooperativity in binding (attractive force due to lateral interactions) and the heterogeneous nature of the adsorption [28]. Similar results ($n > 1$) were reported by Sharma and Agarwal [28] for adsorption of the lysozyme, conalbumin, ovalbumin, wheat germ agglutinin, and bovine serum albumin onto IDA-Cu^{2+} and IDA-Ni^{2+} agarose.

The K_d values measured here for the Langmuir and Langmuir–Freundlich models were of the order of magnitude of 10^{-6} M. This K_d value of the complex IgG_1-Zn^{2+}-IDA in-

Table 2
Mass balance of total protein for chromatographies from IgG_1 precipitate solution with elution by Tris–HCl step concentration gradient on Zn^{2+}-IDA-PEVA and Zn^{2+}-IDA-agarose

Fractions		Zn^{2+}-IDA-PEVA[a]		Zn^{2+}-IDA-agarose[b]	
		(mg)	(%)	(mg)	(%)
IgG_1 precipitate solution		27.5	100	17.5	100
Washing	50 mM Tris	26.1	94.9	15.2	86.8
Elution	100 mM Tris	0.16	0.6	0.61	3.48
	300 mM Tris	0.32	1.2	0.09	0.5
	500 mM Tris	0.91	3.3	0.35	2.0
	700 mM Tris	0.52	1.9	0.45	2.6
Regeneration EDTA		0.54	1.9	0.67	3.8
Total		28.5	103.8	17.3	99.2

[a] Column volume: 5.0 mL (1.6 g of dry mass).
[b] Column volume: 2.0 mL (0.67 g of dry mass).

Table 3
Purification of mouse McAbs from IgG_1 precipitate solution on Zn^{2+}-IDA-PEVA

Fractions		Total Protein[a] (μg)	Antibody[b] (μg)	Antibody to protein mass ratio (μgMcAb/mg)	Yield of McAb[c] (%)	Purification factor
IgG_1 precipitate solution		27500	980	35.7	100	1
Washing	50 mM Tris	26100	26	1.0	2.7	0.03
Elution	100 mM Tris	160	0	0	0	0
	300 mM Tris	320	100	312.5	10.2	8.8
	500 mM Tris	910	606	665.9	61.8	18.7
	700 mM Tris	520	279	536.5	28.5	15.0
Regeneration EDTA		540	86	159.3	8.8	4.5

[a] Dosage of protein by Bradford method.
[b] The amount of McAb in each step was determined by ELISA.
[c] The yield of the McAb was determined as a ratio of the McAb in the eluate fractions to the total McAb present in the feed.

Table 4
Adsorption parameters determined for IgG_1 adsorption onto Zn^{2+}-IDA-PEVA at 25 °C

Isotherm models	Langmuir	Langmuir–Freundlich
Q_m (mg/mL)	54.6 ± 7.2	39.7 ± 4.6
K_d (M)	$(8.1 \pm 2.4) \times 10^{-6}$	$(3.2 \pm 1.7) \times 10^{-6}$
n	–	1.66 ± 0.47
Correlation coefficient	0.98	0.99

dicated medium affinity, which is typical for a pseudobiospecific affinity ligand [30].

3.3. Adsorption breakthrough curves using Zn^{2+}-IDA-PEVA cartridge

Breakthrough curves up to ligand saturation were determined to study the effects of filtrate flow rates on selectivity and capacity of Zn^{2+}-IDA-PEVA cartridge. Fig. 4a shows the total protein breakthrough curves at filtrate flow rates (Q_F) of 0.3, 0.7, and 1.2 mL/min for IgG_1 precipitate solution chromatography on Zn^{2+}-IDA-PEVA cartridge. All curves qualitatively shared the characteristic "S" shape. The non-retained fractions (filtrate fractions) were analyzed by SDS–PAGE (Fig. 4b–d). Initially, the protein band with molecular mass of approximately 150 kDa (corresponding of IgG_1) was not detected in SDS–PAGE, concluding that the IgG_1 concentration in the filtrate was zero, reflecting complete adsorption of the IgG_1 molecules by the immobilized Zn^{2+}. As the loading step proceeded and binding sites became occupied, IgG_1 was detected in the filtrate (breakthrough point). Following breakthrough, filtrate IgG_1 concentration increased and C_f/C_0 asymptotically approached values from 0.5 to 0.65 (depending on the Q_F), at which point steady state was achieved in the membrane and no further adsorption of protein occurred. The C_f/C_0 value is lower than unity because when operating with the tangential mode of filtration a fraction of the protein mass fed to the system is contained in the retentate and does not end up in the filtrate.

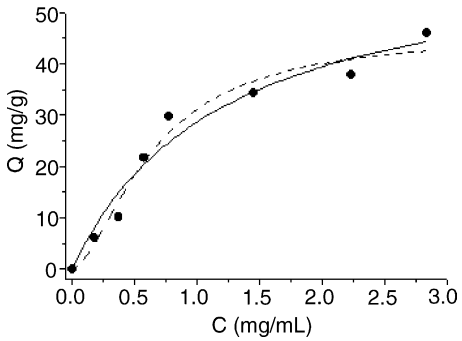

Fig. 3. Experimental adsorption isotherm (symbol) for anti-TNP McAb IgG_1 on Zn^{2+}-IDA-PEVA in 50 mM Tris–HCl, pH 7.0 at 25 °C. The lines correspond to fitting (nonlinear regression) of experimental values on the basis of Langmuir model (solid line), and Langmuir–Freundlich model (dotted line).

After washing steps, adsorbed proteins were eluted with a Tris discontinuous step gradient (100–700 mM at pH 7.0) and protein concentration in eluted fractions was determined by Bradford method and analyzed by SDS–PAGE. The results were similar to those obtained with the Zn^{2+}-IDA-PEVA cut fibers (IgG_1 adsorption capacity of Zn^{2+}-IDA-PEVA of 3.20 mg/g and 2.75 mg/g for cartridge and cut fibers, respectively).

The effect of varying filtrate flow rate on breakthrough point and reduction of the transmembrane flow was illustrate in Fig. 4a and e. Increasing the filtrate flow rate decreased the residence time (t_R) in the membrane according to

$$t_R = \frac{V_m}{Q_F} \quad (4)$$

where V_m is the membrane interstitial volume; Q_F was varied four-fold from 0.3 to 1.2 mL/min, corresponding to t_R equal to 31.4–7.8 s.

Increasing flow rate did not decrease the amount of total protein bound to the membranes (the mass of total protein eluted was similar for all flow rates studied, around 3.25 mg/g of membrane). Nevertheless, the filtrate flow rate had an effect on the position and shape of the breakthrough curve. With

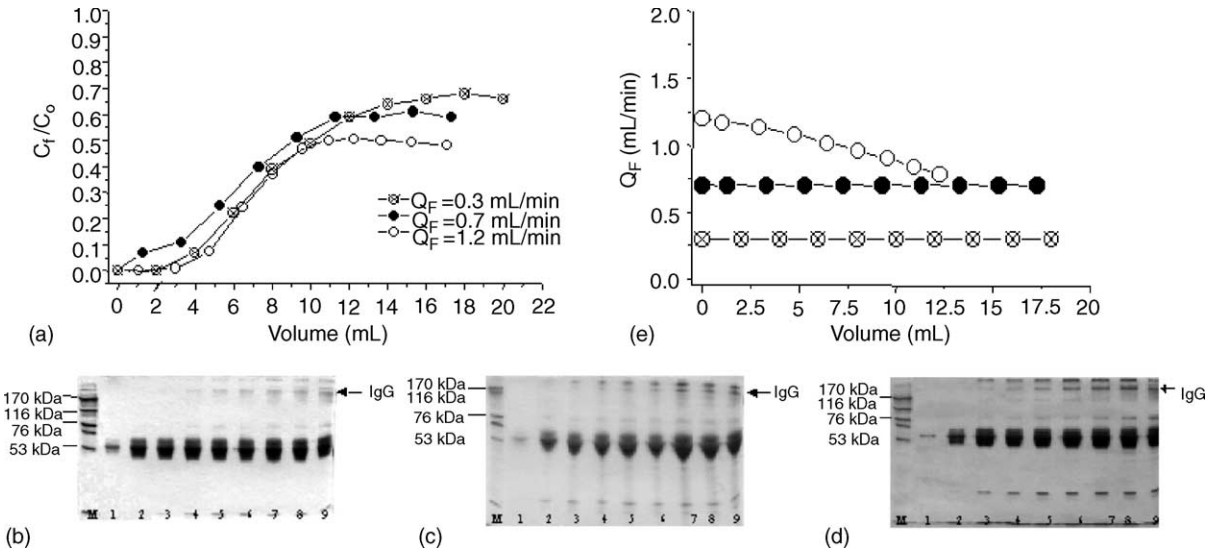

Fig. 4. Analysis of the breakthrough curves of IgG_1 precipitate solution for the Zn^{2+}-IDA-PEVA cartridge. (a) Effect of filtrate flow rate on breakthrough curve. SDS–PAGE analysis under non-reducing conditions of fractions of breakthrough curves. Numbered lanes represent aliquots of the corresponding fractions of the protein obtained at (b) Q_F = 0.3 mL/min; (c) Q_F = 0.7 mL/min; (d) Q_F = 1.2 mL/min, (e) decline in filtrate flow rate during feed of IgG_1 precipitate solution.

decreasing Q_F, the breakthrough point (effluent volume at which IgG_1 was first detected) shifted toward larger IgG_1 precipitate solution volumes and IgG_1 mass correspondingly throughput, ranging from 1.1 to 1.6 mg.

During feeding of the IgG_1 precipitate solution at filtrate flow rate of 1.2 mL/min, a reduction of the transmembrane flow from 1.2 to 0.75 mL/min was experienced, probably due to formation of polarization layer (Fig. 4e). However, decreased initial filtrate flow rates (0.7 and 0.3 mL/min) were constant over the time of the experiment. The most efficient breakthrough curve was attained at the lowest flow rate corresponding to the longest residence time of 31.4 s.

4. Conclusion

We have successfully demonstrated that the Zn^{2+}-IDA-PEVA hollow fiber membrane system is a potential alternative for the purification of monoclonal IgG_1 since the anti-TNP IgG_1 monoclonal antibodies could be adsorbed under mild conditions, near to physiological pH, and at room temperature. Also, this antibody could be eluted under conditions not causing IgG_1 denaturation or loss of antigen binding capacity, as detected by ELISA. Performance comparison with the conventional agarose bead system—Zn^{2+}-IDA-agarose—showed a similar selectivity and a lower capacity for the membrane configuration. However, membrane systems have the advantage of operating at high productivities. In contrast to the biospecific ligand commonly applied for mouse IgG_1 purification, the protein G, the metal chelate ligand is not, or to a much lesser extent, subjected to physical, chemical or microbiological degradation. This facilitates handling and storage of the cartridge considerably.

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