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The effect of NaCl on the adsorption of human IgG onto CM-Asp–PEVA hollow fiber membrane-immobilized nickel and cobalt metal ions

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Abstract Over the past decade, immobilized metal-affinity adsorbents have attracted increasing interest for purification of natural and recombinant immunoglobulin G (IgG). In this work, nickel and cobalt metal ions complexed with CM-Asp (carboxymethylaspartate) immobilized on poly(ethylenevinyl alcohol) (PEVA) hollow fiber membranes were evaluated for purification of human IgG from serum. The buffer system and NaCl had important effects on human serum protein adsorption in both adsorbents. Efficient purification of IgG was accomplished in sodium phosphate buffer without NaCl at pH 7.0. Under this condition, the electrostatic interactions are important for adsorption. The Ni(II)-CM-Asp–PEVA had a protein adsorption capacity of 17.5 mg of IgG mL^{-1} fiber when human serum diluted was loaded in crossflow filtration mode and the eluted IgG had a purity of 82.6 % (based on total protein and IgG, IgM, HSA, and Trf nephelometric analysis). Fitting the experimental IgG adsorption data to the Langmuir and Langmuir–Freundlich models showed that Ni(II)-CM-Asp and Co(II)-CM-Asp had Langmuirean and non-Langmuirean behavior, respectively, with positive cooperativity for IgG-Co(II)-CM-Asp binding, probably due to multipoint interactions ($n = 2.12 \pm 0.31$). Thus, these membranes can be considered as alternative adsorbents for the purification or depletion of IgG from human serum.

Keywords Human IgG · Purification · Affinity membrane · IMAC · CM-Asp

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1 Introduction

Immobilized metal ion affinity chromatography (IMAC) is a method that has been used for protein purification based on coordinative interactions between the target protein in solution and the metal ions chelated to a multidentate ligand immobilized on a solid phase (adsorbent) (Bresolin et al. 2009; Gutiérrez et al. 2007; Porath 1988; Todorova and Vijayalakshmi 2006; Ueda et al. 2003). These coordination bonds are formed between immobilized metal ions and some amino acid residues such as the imidazole group of histidine, the thiol group of cysteine, the indolyl group of tryptophan, and the terminal amine group exposed on the surface of the proteins. Other interactions in IMAC involving electrostatic and/or hydrophobic interactions, as well as hydrogen bonding can also play a role (Gaberc-Porekar and Menart 2005; Gutiérrez et al. 2007; Porath 1992; Todorova and Vijayalakshmi 2006).

Many authors have used this technique for antibody purification (Boden et al. 1995; Bresolin et al. 2010; Hale and Beidler 1994; Kracalikova and Bleha 2008; Prasanna and Vijayalakshmi 2010; Todorova-Balvay et al. 2004; Vançan et al. 2002; Uygun et al. 2012). Polyclonal and monoclonal antibodies (immunoglobulin G, IgG) are important proteins with applications in immunodiagnosis, therapeutics, and immunochromatography. Most studies report the purification of IgG from different sources by

IMAC using copper or nickel ions chelated with the tridentate [iminodiacetic acid (IDA)] chelating ligand (Altintas et al. 2007; Hale and Beidler 1994; Kracalikova and Bleha 2008; Kracalikova et al. 2006; Prasanna and Vijayalakshmi 2010; Rajak et al. 2012; Ribeiro et al. 2008; Serpa et al. 2005; Vançan et al. 2002).

The nature of the chelating ligand, the metal ion, the composition of the mobile phase, and the support material affect the binding affinity of the proteins in IMAC. Different results for selectivity and capacity of the adsorbent IMAC can be achieved by changing these parameters. For example, the selectivity of the adsorption increases the more polydentate is the chelating ligand whereas the capacity for protein adsorption decreases (Chaga 2001; Sharma and Agarwal 2001). Of the different chromatographic support materials available for IgG purification by IMAC, porous hydrophilic gel beads are the most frequently employed. However, gel beads are not recommended for the antibody purification process on an industrial scale due to the high pressure drop, gel compression, flow rate, and diffusion limitations (slow diffusion of IgG molecules into the pores of beads) (Bueno et al. 1995; Klein 1991; Thömmes and Kula 1995). Over the past two decades microporous membranes have been employed as chromatographic matrices in downstream processing because of their higher productivity, easier scale-up, lower pressure drop, and predominance of convective transport of the biomolecules (Barroso et al. 2010; Boi et al. 2008; Borsoi-Ribeiro et al. 2013; Ribeiro et al. 2008; Serpa et al. 2005).

In previous work, Ribeiro and coworkers described the application of two chelating ligands, IDA and TREN [Tris(2-aminoethyl)amine], covalently linked to poly(ethylene vinyl alcohol) (PEVA) hollow fiber membranes for purification of IgG from human plasma in which both chelating ligands were complexed with nickel ions (Ribeiro et al. 2008). The present work extends this application of Ribeiro and coworkers' studies and describes investigations on the interactions of human IgG with transition metal ions chelated to CM-Asp (carboxymethylaspartate) immobilized on PEVA hollow fiber membranes. The selection of CM-Asp aimed to study the effect of the tetradentate chelating ligand on the selectivity for IgG purification using affinity membrane. Moreover, we compared the binding efficiency, specificity, and protein yield of both Ni(II)- and Co(II)-based IMAC with and without NaCl in the buffer systems. The objective was to contribute to a better evaluation of the potential of this chelating ligand for antibody purification in comparison with that of a conventional chelating ligand, IDA. Equilibrium data on adsorption of human IgG at different initial concentrations were obtained in a series of batch experiments and the experimental data were analyzed in terms of both the Langmuir and the Langmuir–Freundlich adsorption isotherm models.

2 Experimental

2.1 Materials

Epichlorohydrin, nickel sulphate, cobalt sulphate, and coomassie brilliant blue were purchased from Merck (Germany). L-aspartic acid (Asp), Disodium ethylenediaminetetraacetic acid (EDTA), crystalline bovine serum albumin (BSA), 3-(*N*-morpholino)-propanesulfonic acid (Mops), hydroxyethylpiperazine ethanesulfonic acid (Hepes), and human serum from human male AB plasma were obtained from Sigma-Aldrich (USA). Acrylamide, bis-acrylamide, dithiotrietol, and sodium dodecyl sulphate (SDS) for SDS-PAGE analysis were purchased from Bio-Rad (USA). High molecular mass markers kit for SDS-PAGE (myosine, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 113 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was provided by GE Healthcare (USA). Human immunoglobulin G was provided by Aventis Behring (Germany). The nephelometric reagents were obtained from Beckman Coulter (USA). The stirred ultrafiltration cell and YM10 membrane (nominal molecular mass cut-off of 10 kDa) were purchased from Millipore (USA). The poly(ethylenevinyl alcohol) (PEVA) hollow fiber cartridge (Model Eval 5A, 2 m^2 surface area) was obtained from Kuraray (Japan). The hollow fiber had an internal diameter of 200 μm, a wall thickness of 20 μm and a molecular mass cut-off of 600 kDa. 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.

2.2 Immobilization of CM-Asp on cut PEVA hollow fiber membranes

Initially, the hollow fibers were removed from a commercially available PEVA hollow fiber cartridge and cut into fine pieces around 3 mm in length. The cut PEVA membranes were activated with epichlorohydrin as described by Borsoi-Ribeiro et al. 2013, Bresolin et al. (2010), and Ribeiro et al. (2008) and the CM-Asp was coupled to the activated support (Mantovaara et al. 1991). In short, 8 g of aspartic acid were dissolved in 50 mL of sodium carbonate solution at 1.0 mol L^{-1} and the pH was adjusted to 11.5 with anhydrous pellets of NaOH. This solution was added to the epoxy-activated adsorbent and left under stirring for 24 h. Then, the adsorbent was washed with water and 1.0 mol L^{-1} $NaHCO_3/Na_2CO_3$ solution at pH 10.5. After washing, 30 mL of 1.0 mol L^{-1} $NaHCO_3/Na_2CO_3$ solution at pH 10.5 was added to the adsorbent. Then, 12.6 g of

bromoacetic acid was dissolved in 30 mL of 4.0 mol L^{-1} NaOH and the pH was adjusted to 10.5 with 10 mol L^{-1} NaOH. The bromoacetate solution was added to the adsorbent and the suspension was stirred overnight at 25 °C. Next, the adsorbent was washed with deionized water. The chelating capacity of the membrane for Ni(II) and Co(II) metal ions was determined, respectively, in accordance with the method described by Belew and Porath (1990). The derivatized membrane is referred to as CM-Asp–PEVA in this work.

2.3 Minicartridge derivatization

A small-scale PEVA hollow fiber minicartridge was manufactured in our laboratory. The fibers from a commercially available cartridge (Kuraray, Japan) were cut and assembled in a minicartridge having a length of 12 cm as described by Aquino et al. (2006), Bresolin et al. (2010), and Serpa et al. (2005). The amount of fiber in this cartridge was 0.21 g dry mass with a 113 cm^2 surface area and a 0.248 cm^3 fiber bed volume. The hollow fiber surface area (A_p) and bed volume (V_b) were calculated as follows:

$$A_p = 2\pi r_o L_e N_f \quad (1)$$

$$V_b = \pi\left(r_o^2 - ri^2\right) L_e N_f \quad (2)$$

where r_o and ri 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 minicartridge (150). The PEVA minicartridge was activated with epichlorohydrin and CM-Asp was coupled to it as described by Bresolin et al. (2010).

2.4 Column preparation

The CM-Asp–PEVA finely cut fibers (1.25 g dry mass) were suspended in water, degassed, and packed into columns (20.0 × 1.0 cm^2 I.D., GE Healthcare, USA) to give a bed volume of 5.0 mL. The CM-Asp adsorbents were loaded until saturation with nickel and cobalt metal ions by passing 50 mmol L^{-1} nickel and cobalt sulphate solution in water through the columns. Then the columns were washed sequentially with water and elution buffer used in chromatographic experiments (adsorption buffer with 500 mmol L^{-1} imidazole) to remove the weakly immobilized metal ions. After that, the column was equilibrated with loading buffer. The derivatized membrane with immobilized metal ion is referred to as Me(II)-CM-Asp–PEVA in this work.

2.5 Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) at 25 °C and a flow rate of 30.0 mL h^{-1} (38.2 cm h^{-1}). For the studies concerning the effect of the buffer system on human serum protein adsorption on Me(II)-CM-Asp–PEVA, the following loading buffers at 25 mmol L^{-1} at pH 7.0 containing 2.0 mmol L^{-1} imidazole with or without 1.0 mol L^{-1} NaCl were used: sodium phosphate (NaP), Mops and sodium acetate (MA), and Hepes. Then human serum diluted 5 times with the appropriate loading buffer was injected into the column and unadsorbed proteins were removed by washing the adsorbent with the loading buffer until no protein was detected in the column outstream. Adsorbed proteins were subsequently eluted with a stepwise gradient of imidazole (20, 100, and 500 mmol L^{-1}) in the same loading buffer at pH 7.0. After each experiment, the column was regenerated with 100 mmol L^{-1} EDTA solution at pH 7.0. During all the chromatographic experiments, the absorbance of the eluate was monitored at 280 nm, fractions of 2.0 mL were collected and the protein content was determined with the Bradford method (Bradford 1976). The pools of washing, elution, and regeneration fractions were used for SDS-PAGE and nephelometric analysis of IgG, IgM, human serum albumin (HSA), and transferrin (Trf).

2.6 Equilibrium binding analysis

The equilibrium adsorption experiments for determination of human IgG isotherm were carried out in a batch experimental set-up in duplicate at 25 °C. Amounts of 12.5 mg dry mass of finely cut Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA fibers were introduced into the eppendorf tubes and equilibrated with 25 mmol L^{-1} NaP buffer at pH 7.0 containing 2.0 mmol L^{-1} of imidazole (loading buffer). Then 1.0 mL of human IgG solutions were added to the eppendorf tubes (diluted in the loading buffer for total protein concentrations ranging from 0.5 to 40.0 mg mL^{-1}). The tubes were rotated end-over-end at 6 rpm for 16 h to allow equilibrium to be established. At the end of this period, the cut fibers and the liquid phase were separated by centrifugation and the unbound protein concentration in the liquid phase (C) was measured by UV spectrophotometry at 280 nm [extinction coefficient of 1.36, according to the analytical curve performed using high-purity IgG (Aventis Behring, Germany)]. The amount of adsorbed IgG, Q, was determined as the difference between the amount of IgG initially added and that present in the liquid phase after equilibrium was achieved divided by the dry mass of the adsorbent. Plotting Q* against C* yielded the equilibrium isotherm. The parameters of the Langmuir (Eq. 3) and Langmuir–Freundlich (Eq. 4) isotherm models were fitted to the experimental data employing the interative fitting method of Levenberg–Marquardt using Origin® (Microcal, USA):

$$Q = \frac{Q_m C}{K_d + C} \quad (3)$$

$$Q = \frac{Q_m C^n}{K_{dLF} + C^n} \quad (4)$$

in which C is the protein liquid-phase equilibrium concentration; Q is the protein surface concentration; Q_m is the maximum protein binding capacities (mg g^{-1} dry); K_d is the dissociation constant (mol L^{-1}); K_{dLF} is the apparent dissociation constant that includes contributions from ligand binding to monomer, monomer–dimer, and more highly associated forms of the protein (mol L^{-1}); and n can be employed as an empirical coefficient, representing the type and the extent of cooperativity in the binding interaction (dimensionless).

2.7 Crossflow filtration of human serum solution in the Ni(II)-CM-Asp–PEVA hollow fiber minicartridge

The cross flow filtration experiments in the minicartridge to determine dynamic capacity were carried out at 25 °C with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) as described in Ribeiro et al. (2008). After loading the CM-Asp–PEVA membranes with metal ions, the hollow fiber minicartridge was washed with 10 column volume of water. To prevent the leakage of metal ions on elution, the column was washed with more drastic elution condition (adsorption buffer with 500 mmol L^{-1} imidazole). The adsorbent was equilibrated with the loading buffer (25 mmol L^{-1} NaP buffer containing 2.0 mmol L^{-1} imidazole at pH 7.0) at an inlet flow rate of 1.0 mL min^{-1}. Human serum (4.0 mL) diluted 2.5 and 5 times in loading buffer was pumped through the minicartridge in a crossflow mode at an inlet flow rate of 1.0 mL min^{-1}. The inlet flow rate (Qi) and the filtrate flow rate (Q_F) were kept constant using two peristaltic pumps to fix the Q_F/Qi ratio at 0.50, with a residence time, t_R, of 24 s (t_R was calculated by dividing the membrane interstitial volume by the filtrate flow rate (Aquino et al. 2006; Thömmes and Kula 1995). The filtrate outlet absorbance at 280 nm was monitored with a UV detector. The unabsorbed protein was washed out of the cartridge with loading buffer. Four washing steps were performed until the absorbance of effluent at 280 nm reached the baseline (Serpa et al. 2005; Ribeiro et al. 2008; Bresolin et al. 2010; Borsoi-Ribeiro et al. 2013): crossflow filtration, lumen wash, shell wash, and backflushing. The adsorbed protein was eluted in the backflushing mode with a discontinuous step gradient of imidazole at pH 7.0 (20, 100, and 500 mmol L^{-1}). The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution was completed, the cartridge was washed in frontal mode with 100 mmol L^{-1} EDTA at pH 7.0 and then with the loading buffer to restore it to its initial conditions for the next experiment.

Protein concentrations in the retained and unretained fractions were determined by the Bradford method (Bradford 1976) and nephelometric analysis. Breakthrough curves were plotted as the ratio of the total protein concentration in the filtrate (C_f) to that in the feed stream (C_o) as a function of the volume of protein solution throughput.

2.8 Analytical methods

2.8.1 Protein determination

Protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as reference protein. In experiments containing purified human IgG, the protein concentration was determined by measuring the absorbance at 280 nm [extinction coefficient of 1.36, according to the analytical curve performed using high-purity IgG (Aventis Behring, Germany)].

2.8.2 SDS-PAGE electrophoresis

The chromatographic fractions were analyzed by SDS-PAGE electrophoresis (7.5 % acrylamide gels) under non-reducing conditions using a Mini-Protean III System (Bio-Rad, USA) in accordance with Laemmli (1970). The gels were stained with silver nitrate in accordance with Morrissey (1981).

2.8.3 Nephelometric quantification of IgG, IgM, HSA, and Trf

The concentrations of IgG, IgM, HSA, and Trf in the fractions collected in the chromatographic and crossflow filtration experiments were determined nephelometrically using an Array Protein System (Beckman, USA). The lowest concentrations detected were 0.0093 mg mL^{-1} for IgG, 0.0062 mg mL^{-1} for HSA, 0.0069 mg mL^{-1} for IgM, and 0.0035 mg mL^{-1} for Trf.

2.8.4 Determination of the amount of immobilized metal

The 1.0 mL nickel- and cobalt-loaded hollow fiber membranes in the columns were washed with ten column volumes of Milli-Q water, ten column volumes of sodium 25 mmol L^{-1} acetate buffer, at pH 4.0, and ten column volumes of Milli-Q water, followed by elution with 50 mmol L^{-1} EDTA at pH 6.5. The total amounts of Ni(II) and Co(II) in the eluate were determined by reading the absorbance in triplicate on a UV–Vis spectrophotometer (DU 650 Beckman Coulter, USA) at 384 and 466 nm,

respectively, using EDTA solution as blank, and comparing them to analytical curves performed with Ni(II) and Co(II) sulphate solutions in 50 mmol L^{-1} EDTA at pH 6.5 (Serpa 2002).

2.8.5 *Zeta potential for Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA*

The CM-Asp–PEVA finely cut fibers was prepared according to the procedure explained on Sect. 2.4 for both metal ions. The zeta potential was measured in quadruplicate by a SurPASS electrokinetic analyzer (Anton-Paar GmbH, Austria). A cylindrical cell was used and the measurements were done with Hepes 25 mmol L^{-1} at pH 7.0 as electrolyte.

3 Results and discussion

3.1 Effect of buffer system and NaCl on the capacity for serum protein adsorption onto Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA

The metal-protein interaction in IMAC depends on the operational conditions, especially the composition and the NaCl content of the chromatography buffers. The conditions usually employed in IMAC for adsorption are buffers with a pH between 7.0 and 8.0, and with NaCl to eliminate non-specific electrostatic interactions (Porath 1988; Ueda et al. 2003; Winzerling et al. 1992). The adsorption capacity and selectivity of IMAC adsorbent can be handled favorably by regulating the NaCl concentration in adsorption and elution buffers.

Preliminary adsorption experiments for studying the effect of NaCl on the adsorption of human IgG using CM-Asp–PEVA cut fibers were performed with human serum on Cu(II), Ni(II), and Co(II) immobilized metal ions. SDS-PAGE results for eluates from chromatographic experiments suggest that the Cu(II)-CM-Asp–PEVA had a low selectivity for human IgG (IgG was recovered with the main impurities HSA and Trf, data not shown). Sulkowski showed that Cu(II) has the ability to adsorb larger amounts of peptides and protein that contain at least one or two accessible histidine residues (Sulkowski 1989). Aromatic amino acids such as tryptophan are also important in the retention of proteins in IMAC. However, a tryptophan residue can be considered to have less effect than histidine on the retention of proteins in IMAC. Cysteine also affects the retention of proteins in IMAC (but only when in reduced form) (Sulkowski 1989). Vançan et al. (2002) also observed that both HSA and IgG were adsorbed onto Cu(II)-IDA-agarose when a solution of human plasma (in a Mops-imidazole buffer system containing 1.0 mol L^{-1} NaCl) was chromatographed. Therefore, Ni(II) and Co(II) were the metal ions most suited for IMAC purification of human IgG human on CM-Asp–PEVA.

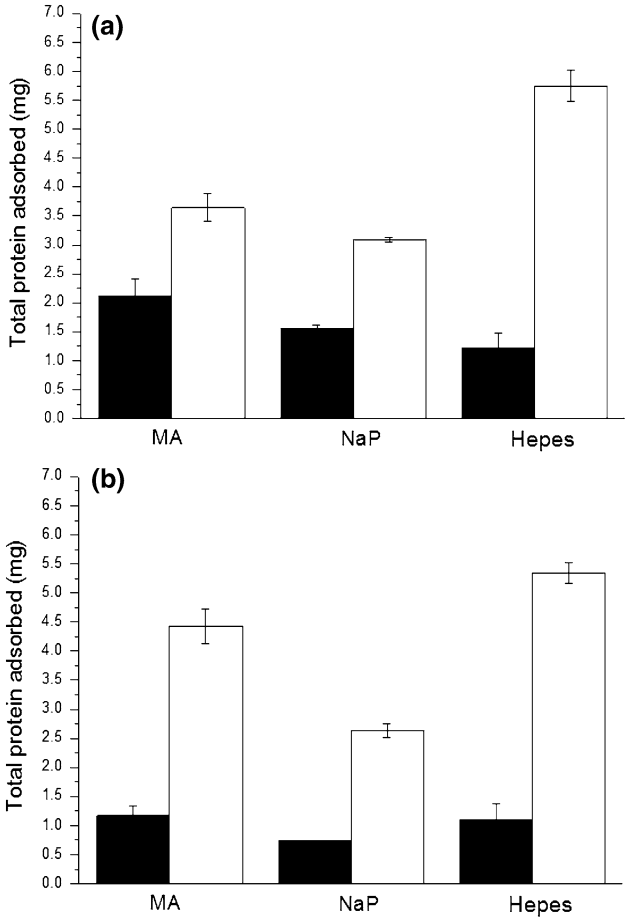

Fig. 1 Effect of NaCl on the adsorption capacity of total human serum protein on the buffer systems studied. **a** Ni(II)-CM-Asp–PEVA and **b** Co(II)-CM-Asp–PEVA. *Black rectangle* with and *white rectangle* without 1.0 mol L^{-1} NaCl Ionic strength: 1.003 for Hepes with NaCl; 0.005 for Hepes without NaCl; 1.100 for NaP with NaCl; 0.100 for Hepes without NaCl; 1.032 for MA with NaCl, 0.035 for Hepes without NaCl

In terms of the capacity for serum protein adsorption, the effect of adding NaCl to the loading buffer was pronounced for all buffer systems and better results were obtained without the addition of NaCl (Fig. 1a, b). The percentage of total serum protein adsorbed (eluted and regeneration fractions) relative to the injected protein mass was similar for both IMAC adsorbents studied (7.4, 7.0, and 11.5 % for Ni(II)-CM-Asp–PEVA and 9.1, 5.0, and 10.1 % for Co(II)-CM-Asp–PEVA for the MA, NaP, and Hepes buffers, respectively) when buffer systems without NaCl were used. Classical IMAC is characterized for high ionic strength, in which electrostatic interactions between the protein and the complex metal-chelating ligand are suppressed and the paired electron type of coordination bonding is promoted (Gutiérrez et al. 2007; Ueda et al.

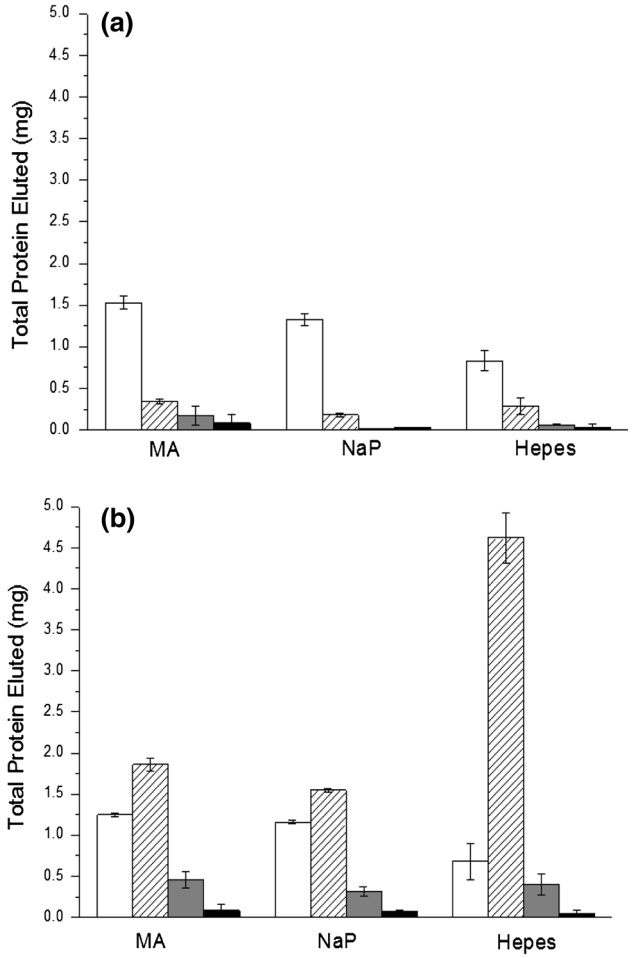

Fig. 2 Human serum protein eluted by increasing the imidazole concentration of the chromatography on the adsorbent Ni(II)-CM-Asp–PEVA **a** with and **b** without of NaCl (*white rectangle*) E1 elution with 20 mmol L^{-1} (*crossed rectangle*) E2 elution with 100 mmol L^{-1} (*gray rectangle*) E3 elution with 500 mmol L^{-1} (*black rectangle*) regeneration 100 mmol L^{-1} EDTA

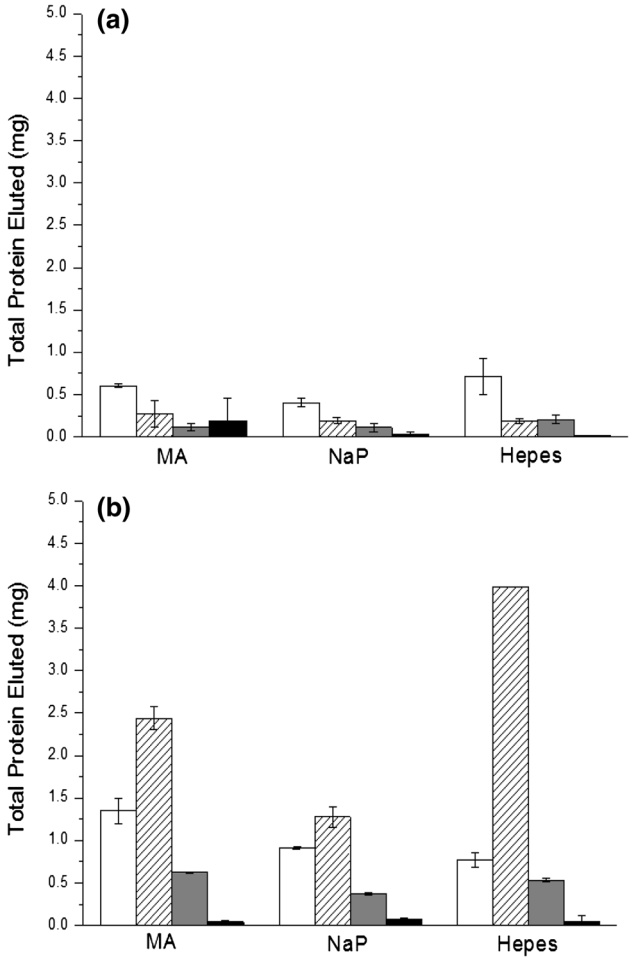

Fig. 3 Human serum protein eluted by increasing the imidazole concentration of the chromatography on the adsorbent Co(II)-CM-Asp–PEVA. **a** With and **b** without of NaCl (*white rectangle*) E1 elution with 20 mmol L^{-1} (*crossed rectangle*) E2 elution with 100 mmol L^{-1} (*gray rectangle*) E3 elution with 500 mmol L^{-1} (*black rectangle*) regeneration 100 mmol L^{-1} EDTA

2003; Zachariou and Hearn 2000). However, for both adsorbents, the condition that favored a higher adsorption capacity was obtained at low ionic strength. In this case, the electrostatic interactions between the protein and the Me(II)-CM-Asp–PEVA (negatively charged at pH 7.0) take place. Probably the coordination bonding between the chelate and protein also play a role.

When human serum solution was applied to Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA with NaCl, the serum protein adsorption was slightly affected by the buffer system. On the other hand, in a salt-independent chromatography, protein adsorption was greatly affected by the buffer system. High adsorption capacities were achieved with the zwitterionic Hepes buffer than with the phosphate and MA buffers. The negatively charged phosphate and acetate groups seemed to inhibit serum protein adsorption, whereas Hepes, which carries two charges of opposite signs below its p*K*a, favored protein adsorption. The electrostatic interactions are probably the main forces involved in serum protein adsorption onto Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA under low ionic strength buffer conditions.

The retention of serum proteins on Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA was stronger at low ionic strength than with NaCl in the elution buffer (Figs. 2a, b, 3a, b). For instance, with NaCl, the most of the serum proteins was eluted with 20 mmol L^{-1} imidazole, whereas at low ionic strength condition most of the protein was eluted with 100 mmol L^{-1} imidazole.

According to the SDS-PAGE analysis (Figs. 4, 5), the increase in selectivity for IgG (decrease in contamination by Trf and HSA) was observed when NaCl was removed from the loading buffer for the human serum solution chromatography on both adsorbents. The addition of NaCl to the loading buffer probably changed the p*K*a of

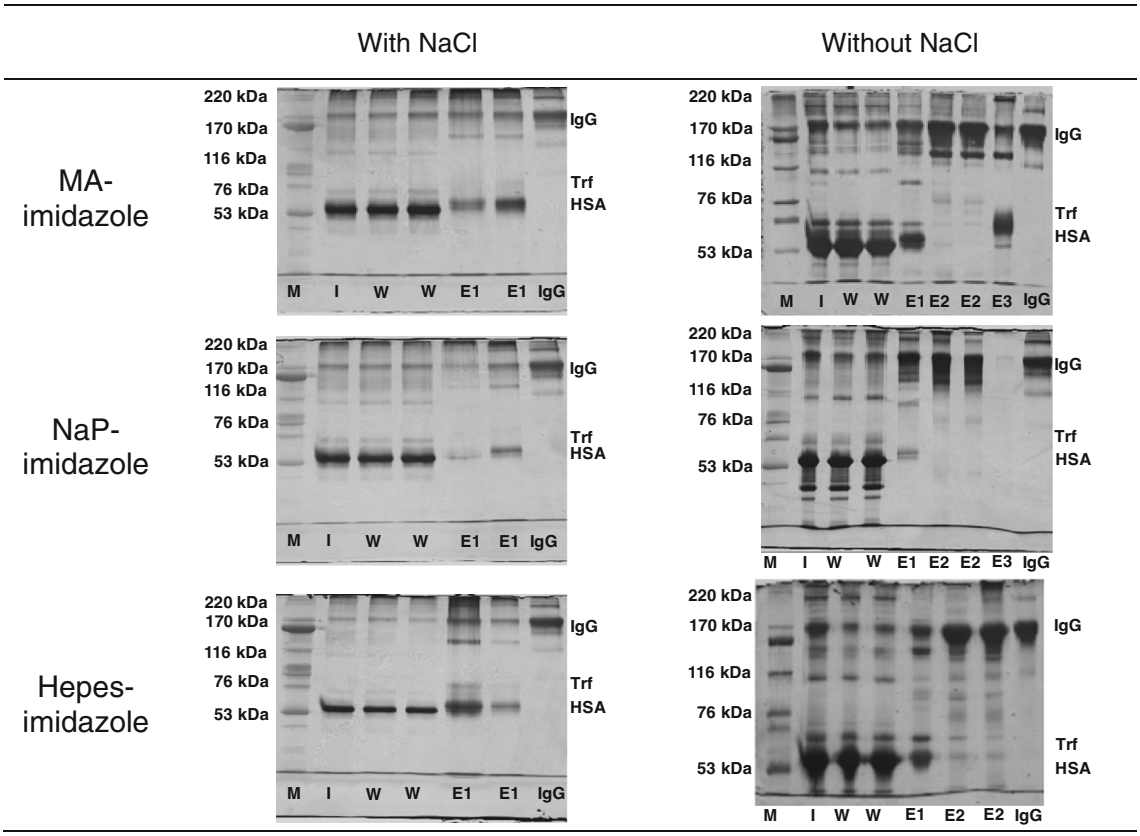

Fig. 4 Effect of the buffer system and NaCl on the selectivity of the Ni(II)-CM-Asp–PEVA. SDS-PAGE analysis under nonreducing conditions of fractions from the chromatography. *M* molecular mass protein marker. *I* injected human serum solution. *W* washing. *E1* elution fractions at 20 mmol L^{-1} imidazole, *E2* elution fractions at 100 mmol L^{-1} imidazole. *E3* elution fractions at 500 mmol L^{-1} imidazole, *R* regeneration. *IgG* human IgG standard (Aventis Behring)

coordinate groups on the proteins and also the enhancement of hydrophobic interaction, thus affecting the binding of IgG to the Co((II)- and Ni(II)-CM-Asp–PEVA. This condition (buffer system without NaCl) has also been shown to be effective in the purification of monoclonal antibodies on Zn(II)-IDA-PEVA and human IgG on Ni(II)-IDA-PEVA (Bresolin et al. 2010; Serpa et al. 2005; Ribeiro et al. 2008). For all chromatographic experiments, nephelometric analysis of IgG, IgM, HSA, and Trf in the washing and elution fractions was carried out at low ionic strength (Table 1) except for Co(II)-CM-Asp–PEVA in Hepes buffer with which low selectivity was observed (Fig. 5). The higher purity and purification factor of the eluted IgG were obtained with NaP buffer for both adsorbents (106 and 107 % purities and purification factors of 7.7 and 5.6 for Ni(II)-IDA-PEVA and Co(II)-IDA-PEVA, respectively). Although serum proteins other than IgG were detected by SDS-PAGE for both adsorbents (Figs. 4 and 5), the IgG purity was higher than 100 % (based on the Bradford method and IgG, IgM, HSA, and Trf nephelometric analysis). This was observed because the Bradford method (Bradford 1976) shows a variation in response to different proteins (for instance, high sensitivity to albumin and low sensitivity to IgG) (Lucarini and Kilikian 1999).

The affinity of IgG for Co(II)-CM-Asp was similar to that for Ni(II)-CM-Asp (Figs. 4, 5; Table 1). This result seems to contradict the order of retention strength of proteins on immobilized metal ions established by Sulkowski (1989) (Cu(II) > Ni(II) > Zn(II) ≥ Co(II) (experiments performed in Me(II)-IDA-agarose gel). The metal ion Co(II) requires at least two adjacent histidine residues for binding with protein, while the metal ion Ni(II) requires any two accessible histidine residues. However, the chelating ligand used in this work was different (CM-Asp). Similar results were reported by Chaga et al. (1999) in their study on the purification of lactate dehydrogenase from chicken breast muscle by IMAC, in which Ni(II)-CM-Asp had a lower affinity than Co(II)-CM-Asp for this protein.

The Ni(II)-CM-Asp–PEVA membranes are more selective and have a higher adsorption capacity than the PEVA adsorptive membranes with Ni(II) chelated to the tetradentate TREN (Ribeiro et al. 2008). The higher selectivity of the Ni(II)-CM-Asp–PEVA could be associated with the structure of the chetating ligand. The force

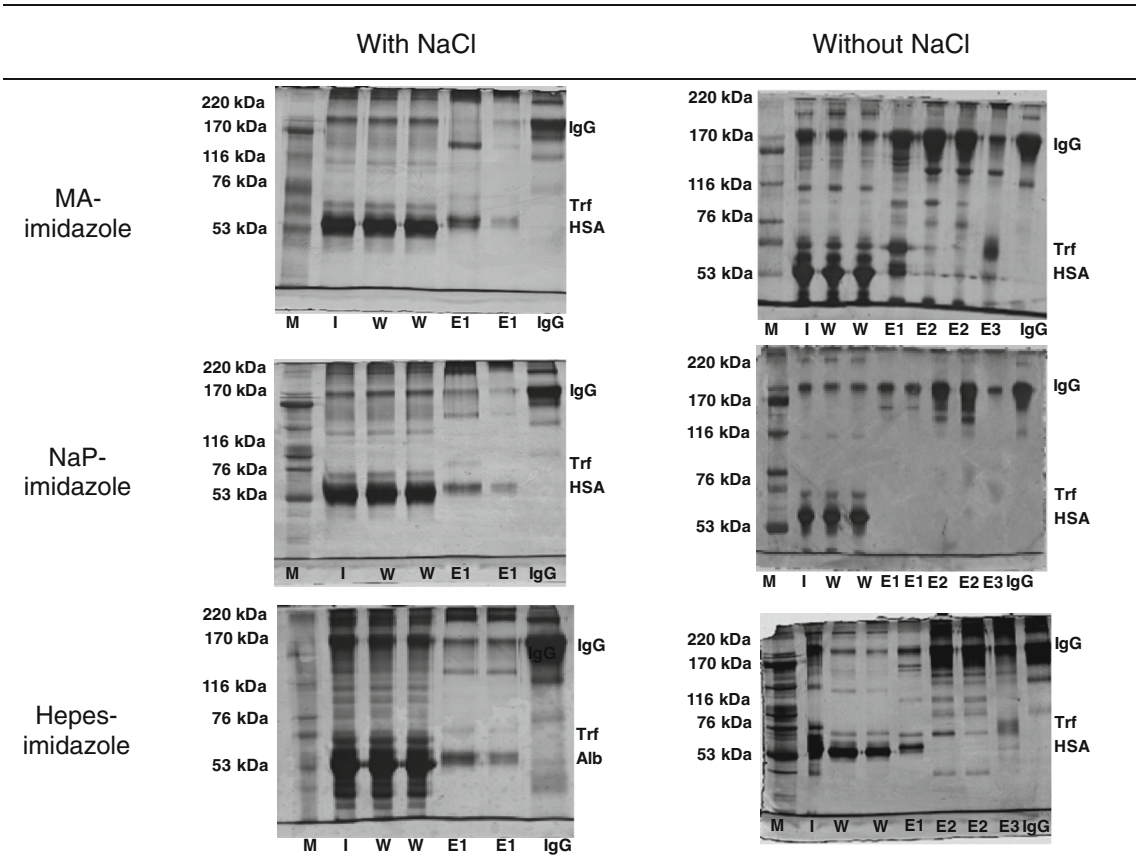

Fig. 5 Effect of the buffer system and NaCl on the selectivity of the Co(II)-CM-Asp–PEVA. SDS-PAGE analysis under nonreducing conditions of fractions from the chromatography on a Co(II)-CM-Asp–PEVA. *M* molecular mass protein marker. *I* injected human serum solution. *W* washing. *E1* elution fractions at 20 mmol L^{-1} imidazole, *E2* Elution fractions at 100 mmol L^{-1} imidazole. *E3* elution fractions at 500 mmol L^{-1} imidazole, *R* regeneration. *IgG* human IgG standard (Aventis Behring)

Table 1 Effect of loading buffers in column chromatography of human serum on Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA adsorbents

Metal	Buffer system	Loading				Washing				Elution				Purity[a] (%)	PF[b]
		IgG (mg)	HSA (mg)	Trf (mg)	TP (mg)	IgG (mg)	HSA (mg)	Trf (mg)	TP (mg)	IgG (mg)	HSA (mg)	Trf (mg)	TP (mg)		
Ni(II)	A	7.20	31.98	2.39	51.27	4.10	31.85	2.24	45.66	2.62	0.63	0.05	3.31	79	5.6
	B	7.14	30.68	2.39	51.88	4.20	30.19	2.19	45.31	2.07	n.d.	n.d.	1.95	106	7.7
	C	7.37	33.00	2.51	51.33	0.79	32.06	0.84	49.53	4.4	0.12	0.21	6.2	71	4.9
Co(II)	A	7.15	31.28	3.00	51.26	4.64	31.69	2.23	50.20	2.61	n.d.	0.31	3.54	74	5.3
	B	9.72	40.96	3.09	51.22	5.83	39.73	2.89	45.31	1.74	n.d	0.03	1.62	107	5.6

Elution discontinuous steps gradient of imidazole

A 25 mmol L^{-1} MA, 2 mmol L^{-1} imidazole, pH 7.0, *B* 25 mmol L^{-1} NaP, 2 mmol L^{-1} imidazole, pH 7.0, *C* 25 mmol L^{-1} Hepes, 2 mmol L^{-1} imidazole, pH 7.0

[a] *Purity* mass ratio of IgG to total protein × 100

[b] *PF* purification factor

and stability of the chelate are affected by number and type of donor atoms (O, S, and N) in the chelating agent (Bresolin et al. 2010; Todorova and Vijayalakshmi 2006). Both chelating ligands are tetradentates with two available sites able to interact with the protein, but TREN contains four nitrogen atoms, three of which are primary in nature and the fourth is ternary, while CM-Asp has three oxygen atoms and one nitrogen atom.

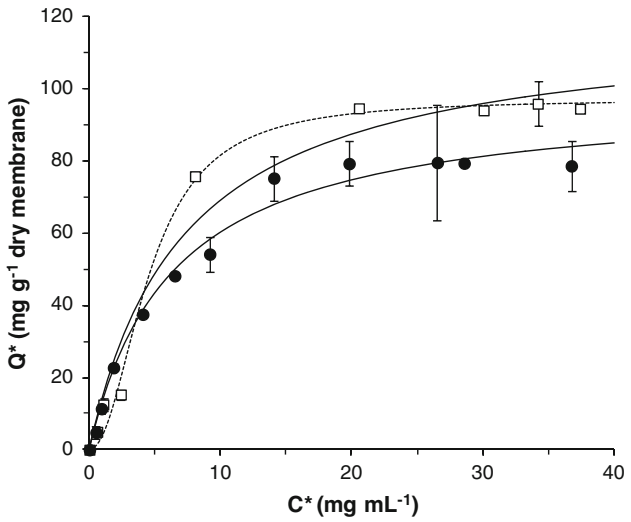

Fig. 6 Experimental adsorption isotherms (*symbol*) for human IgG on *white square* Co(II)-CM-Asp–PEVA and *black circle* Ni(II)-CM-Asp–PEVA with 25 mM NaP, 2 mmol L^{-1} imidazole, pH 7.0 at 25 °C. The *solid* and *dashed lines* correspond to nonlinear regression of experimental data in accordance with the Langmuir and Langmuir–Freundlich models, respectively

3.2 Static and dynamic capacity of affinity hollow fiber membranes

The adsorption isotherms were determined in batch (static) experiment mode with high purity IgG solutions in NaP buffer without NaCl at 25 °C for both IMAC affinity membranes [Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA]. The equilibrium adsorption data were used to fit the parameters of the classical Langmuir and Langmuir–Freundlich adsorption models (Fig. 6). The Langmuir model satisfactorily described the data on human IgG adsorption onto Ni(II)-CM-Asp–PEVA only (correlation coefficient of 0.99) whereas for Co(II)-CM-Asp–PEVA, a correlation coefficient of 0.96 was achieved (Table 2). The values of maximum static binding capacity and dissociation constant were similar for both adsorbents studied (Table 2).

The maximum static binding capacity values obtained for Ni(II)- and Co(II)-CM-Asp–PEVA (98.48 and 119.01 mg g^{-1} of dry membrane or 83.39 and 100.77 mg IgG mL^{-1} hollow fiber, respectively) are in accordance with that previously reported by Ribeiro and co-workers when performing Langmuir analysis of the adsorption isotherm for human IgG on Ni(II) immobilized on tetradentate TREN coupled to PEVA hollow fiber membranes (79.51 mg of IgG mL^{-1} fiber) (Ribeiro et al. 2008). The range of dissociation constant for pseudo affinity ligands in order of magnitude is between 10^{-3} and 10^{-6} mol L^{-1} and the dissociation constant obtained on this work is 10^{-4} mol L^{-1}, which is characterized as pseudoaffinity ligand (Vijayalakshmi 1989). Although both chelating ligands are tetradentates, the dissociation constant (K_d) differed by one order of magnitude (10^{-5} and 10^{-4} mol L^{-1} for Ni(II)-TREN (Ribeiro et al. 2008) and Ni(II)-CM-Asp, respectively). The lower K_d obtained for Ni(II)-TREN-PEVA could be associated with the 1.0 mol L^{-1} NaCl in the adsorption buffer (Mops-acetate, MA) (Ribeiro et al. 2008). With NaCl, the coordination bond with the metal ion is facilitated, whereas the electrostatic interactions are reduced, consequently increasing the affinity of IgG for the metal chelate. The dissociation constant (K_d) measured in this work (10^{-4} mol L^{-1} for Ni(II)- and Co(II)-CM-Asp–PEVA) indicates that human IgG has a low binding affinity for both adsorbents (Vijayalakshmi 1989).

The nature of the metal ion plays an important role in protein adsorption in IMAC. In contrast to that observed with Ni(II)-CM-Asp–PEVA and Ni(II)-TREN-PEVA, the IgG adsorption onto Co(II)-CM-Asp–PEVA deviated from Langmuirean behavior. The Langmuir model showed a poor correlation between the experimental data and the theoretical profiles (correlation coefficient of 0.96). The Langmuir–Freundlich model satisfactorily described the data on human IgG adsorption onto Co(II)-CM-Asp–PEVA (correlaction coefficient of 0.99). The Langmuir model is formulated for homogeneous adsorption, whereas the Langmuir–Freundlich isotherm model is usually adopted for heterogeneous adsorption (Sharma and Agarwal 2001; Yang et al. 2011; Zhou et al. 2012). The n value of 2.12 for Co(II)-CM-Asp–PEVA indicated positive cooperativity in binding and the heterogeneous nature of the adsorption, corroborating the reports describing the cooperative mechanism for the binding of proteins to several IMAC

Table 2 Parameters of the Langmuir and Langmuir–Freundlich isotherm models adjusted to experimental human IgG adsorption data

Metal	Ni(II)	Co(II)	
Isotherm models	Langmuir	Langmuir	Langmuir–Freundlich
Q_m (mg mL^{-1})	83.39 ± 3.37	100.77 ± 8.55	82.14 ± 2.35
K_d (mol L^{-1})	(1.0 ± 0.13) × 10^{-4}	(1.10 ± 1.77) × 10^{-4}	
K_{dLF} (mol L^{-1})			(3.85 ± 1.77) × 10^{-4}
n	–	–	2.12 ± 0.31
Correlation coefficient	0.99	0.96	0.99

Table 3 Total protein and nephelometric analysis of fractions from dynamic binding capacity experiment on a PEVA-CM-Asp-Ni(II)

Fractions	Protein (mg)					IgG purification	
	IgG	IgM	HSA	Trf	PT[a]	Purity[b] (%)	PF[c]
Feed	26.20	2.34	107.04	8.57	190.46	14.1	1.0
Pool of elution	2.70	n.d.	0.15	n.d.	3.27	82.6	5.9

n.d. Values lower than the detectable range of Array Protein System

[a] *Total protein* determined using the Bradford method (Bradford 1976). Average standard deviation of the Bradford method: 1.5 %

[b] *Purity* mass ratio of IgG to total protein × 100

[c] *PF* purification factor

absorbents due to protein-multiple ligand interaction (Johnson et al. 1996; Kracalikova and Bleha 2008). The phenomenon observed in this work is probably due to multisite interactions and/or multipoint attachment since the cobalt metal ion requires two vicinal histidine residues for protein adsorption (Sulkowski 1989).

As similar IgG adsorption capacities were obtained for both chelates, Ni(II)-CM-Asp–PEVA hollow fiber membranes were chosen to evaluate the dynamic binding capacity of human serum proteins. The experiments were performed in crossflow filtration mode using human serum diluted 5 times in 25 mmol L^{-1} NaP buffer without NaCl at pH 7.0 containing 2.0 mmol L^{-1} imidazole in the inlet feedstream (injection of 10 mL of solution containing 190 mg of total protein). The filtrate flow rate was 0.5 mL min^{-1}, corresponding to a t_R of 24 s (corresponding to 176 mL min^{-1} for the 2 m^2 cartridge). Protein concentrations in the feed and eluted fractions were determined by the Bradford method (Bradford 1976) and nephelometry (Table 3).

The dynamic capacity of human IgG adsorption onto Ni(II)-CM-Asp–PEVA was in the range of 17.5 mg of IgG mL^{-1} fiber (20.7 mg of IgG g^{-1} of dry membrane) in the minicartridge. The combination of fractions eluted with 20, 100, and 500 mmol L^{-1} imidazole gave an IgG purity of 82.6 %.

The dynamic capacity of the affinity membrane obtained with the human serum protein was lower than the capacity obtained in static mode (isotherm data), determined with the high-purity IgG. This behavior was expected due to competitive effects, particularly in the weak affinity adsorbents (K_d of the order of magnitude of 10^{-4} mol L^{-1}).

In the minicartridge experiments with Ni(II)-CM-Asp–PEVA, the amount and purity of the eluted IgG (20.7 mg of IgG g^{-1} of dry membrane or 17.5 mg of IgG mL^{-1} fiber and 82.6 % purity, respectively) were lower than those obtained in the minicartridge experiments with Ni(II)-IDA-PEVA (40.5 mg of IgG g^{-1} of dry membrane or 34.29 mg of IgG mL^{-1} fiber and 98 % purity). This probably occurred due to the type of chelating ligand and buffer systems used (NaP-imidazole for Ni(II)-CM-Asp–PEVA and Tris–HCl for Ni(II)-IDA-PEVA). Furthermore, IDA is a tridentate chelator, having three sites for coordination with the protein, while CM-Asp is a tetratentate one in which only two sites are able to interact with the protein (Ribeiro et al. 2008).

4 Conclusions

This work showed that Ni(II)-CM-Asp–PEVA and Co(II)-CM-Asp–PEVA hollow fiber membranes can be used for the purification of IgG from human serum. It was found that the selectivity and the adsorption capacity were remarkably lower for both adsorbents when NaCl (1.0 mol L^{-1}) was added to the adsorption buffer systems. The screening of buffer without NaCl showed that NaP (sodium phosphate) had a higher selectivity for both adsorbents than MA (Mops and sodium acetate) and Hepes buffers. The IgG adsorption isotherms in NaP buffer for chelates Ni(II)-CM-Asp and Co(II)-CM-Asp can be described by the Langmuir and Langmuir–Freundlich models, respectively, with dissociation constant values of the order of magnitude of 10^{-4} mol L^{-1} for both adsorbents, indicating that human IgG has a low binding affinity for both adsorbents. The cooperativity parameter (n) was 2.12 ± 0.31 for IgG-Co(II)-CM-Asp binding, indicating positive cooperativity. The Ni(II)-CM-Asp–PEVA minicartridge experiments showed an adsorption capacity and a purity of eluted IgG of 17.5 mg of IgG mL^{-1} fiber and 82.6 %, respectively, and a purification factor of 5.9 (based on total protein and IgG, IgM, HSA, and Trf nephelometric analysis). A comparison with the performance of immobilized protein-A membranes [11.67 mg of IgG g^{-1} of support (Boi et al., 2008)] showed similar capacity (20.7 mg of IgG g^{-1} Ni(II)-CM-Asp–PEVA).

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