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Effect of IDA and TREN chelating agents and buffer systems on the purification of human IgG with immobilized nickel affinity membranes

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Abstract

The purification of IgG from human plasma was studied by comparing two affinity membranes complexed with Ni(II), prepared by coupling iminodiacetic acid (IDA) and Tris(2-aminoethyl)amine (TREN) to poly(ethylenevinyl alcohol), PEVA, hollow fiber membranes. The Ni(II)-TREN-PEVA hollow fiber membrane had lower capacity for human IgG than the complex Ni(II)-IDA-PEVA, but with similar selectivity. The IgG in peak fractions eluted from the Ni(II)-IDA-PEVA with a stepwise concentration gradient of Tris–HCl pH 7.0 (100–700 mM) reached a purity of 98% (based on IgG, IgM, IgA, albumin, and transferrin nephelometric analysis). Adsorption IgG data at different temperatures (4–37 °C) were analyzed using Langmuir model resulting in a calculated maximum capacity at 25 °C of 204.6 mg of IgG/g of dry membrane. Decrease in K_d with increasing temperature (1.7×10^{-5} to 5.3×10^{-6} M) indicated an increase in affinity with increased temperature. The positive value of enthalpy change (26.2 kJ/mol) indicated that the adsorption of IgG in affinity membrane is endothermic. Therefore, lower temperature induces adsorption as verified experimentally.

Keywords: Affinity membrane; Purification; Human IgG; Adsorption; IMAC

1. Introduction

Human immunoglobulin G (IgG) is an important plasma protein with many applications in therapeutics, immunodiagnosis and immunochromatography. These applications generally require highly pure IgG [1]. Among different purification methods, the membrane affinity chromatography has gained special attention because of its lower mass-transfer limitations than the traditional column techniques, since solutes are transported to binding sites primarily by convection. Affinity membranes also provide high flow rate, low pressure drop, easy scale-up, mechanical stability and high productivity [2–4]. However, if biological compounds like immunoglobulins are purified from complex solutions such as blood plasma or supernatant of hybridoma cell culture, the use of membranes in dead-end filtration mode has the drawback of membrane fouling. To solve this problem, cross-flow filtration operation mode has been used to treat such feeds [5].

Affinity ligands such as protein A and G are the most widely adsorption systems for IgG purification for analytical-scale (diagnostics) applications. Despite their high selectivity towards IgG, these systems are not present in therapeutic IgG purification schemes from human plasma due to high cost of the adsorbents and the possibility of leakage of ligands [6]. In order to overcome these problems pseudobioaffinity chromatography using affinity ligands such as immobilized metal ion, thiophilic, hydrophobic, dye and histidine have been studied [7]. Among these ligands, immobilized metal ion is an interesting alternative [8–11]. The Immobilized Metal Ion Affinity Chromatography (IMAC) method exploits the chemical affinity of a target protein towards the metal ions immobilized onto stationary phase [12–15]. These interactions derive mainly from the coordina-

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tion bonds formed between certain sites side-chain groups on the protein surface, mainly the imidazole group of histidine, and metal ions [13–15].

The ligands (metal chelates) in IMAC are low cost and have high stability, capacity, simplicity, and selectivity. It is a versatile technique since the same ligand can be used for the purification of different proteins and the same chelating resin can be used to chelate different metal ions [13–15]. An important aspect in IMAC method is the occasional leakage of metal ion from the resin, leading to metal ion contamination of the final product. In this case, a column packed with metal-free matrix derivatized with a strong chelating ligand such as TED (Tris(carboxymethyl) ethylenediamine) downstream the IMAC column could be used to trap any metal ions present in the eluate without altering the chromatographic time or the purification effectiveness [14].

Adsorption of immunoglobulins from different sources on IMAC matrices has been reported by many authors [8–11,16–19]. With regard to human IgG purification and interactions studies, the common chelating groups used are imminodiacetic acid (IDA) [8,9,11] and imidazole [10,19].

Proteins retention on IMAC supports is affected by a wide range of variables, such as surrounding chemical environment (depending on salt concentration and pH hydrophobic and electrostatic interactions can take place), nature of chelating groups and the metal ion specific [13–15]. The choice of the chelating group is of paramount relevance. Each chelating ligand has its own selectivity and capacity adsorption towards a specific protein. Normally, the more polydentate is the chelating ligand, the lower is the capacity for protein adsorption but higher is the selectivity [14,20,21]. The empirical order for the adsorption capacity of proteins with accessible histidines and chelating ligands is IDA (tridentate) > NTA (nitrilotriacetic acid, tetradentate) ≥ CM-Asp (carboxymethylated aspartic acid, tetradentate) > TED (pentadentate) [20].

The purpose of this study is to evaluate influence of IDA and TREN (Tris(2-aminoethyl)amine) chelating agents and Mops-acetate (25 mM pH 7.0 with 1.0 M NaCl) and Tris–HCl (25 mM pH 7.0 with or without 1.0 M NaCl) buffer systems on the purification of human IgG with immobilized nickel affinity PEVA (polyethylene vinyl alcohol) hollow fibers membranes chromatography. The IDA (tridentate chelator) was chosen due to reported successful applications for IgG purifications [8,9,11,18]. The TREN agent (quadridentate chelator) was chosen due to its high selectivity towards goat IgG [17]. A binding study was performed at different temperatures and the adsorption isotherm data were analyzed using Langmuir model. The thermodynamic parameters (ΔG°, ΔH°, ΔS°) and the breakthrough curves were estimated since they are the basis for process design, scale-up, and optimization of large-scale IMAC IgG separation process.

2. Experimental

2.1. Materials

The agarose gel (Sepharose® 6B, wet bead diameter of 45–165 μm, specific surface area of 75 m^2/g) and electrophoresis calibration kit for molecular mass determination (myosine, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 113 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) were provided by Amersham Biosciences (Sweden). Epichlorohydrin, Coomassie brilliant blue and Tris(hydroxymethyl amino methane) were purchased from Merck (Germany). Nickel sulphate, disodium ethylenediamine tetra-acetate (EDTA), crystalline bovine serum albumin (BSA), iminodiacetic acid (IDA), and Tris(2-aminoethyl)amine (TREN) were obtained from Sigma (USA). Prepurified human immunoglobulin G (containing 98.3% of IgG according nephelometric analysis of IgG, IgM, IgA, albumin (HSA) and transferrin done in our laboratory) was provided by Aventis Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (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(ethylene vinyl alcohol) (PEVA) hollow fiber cartridges (2 m^2 surface area) were obtained from Kuraray (Japan). The hollow fiber had an internal diameter of 200 μm, a wall thickness of 20 μm, a molecular mass cut-off of 600 kDa and specific surface area of 49.5 m^2/g [22].

2.2. Immobilization of IDA and TREN chelating ligands onto PEVA hollow fiber membranes and agarose gel beads

Hollow fibers from a commercially available PEVA hollow fiber cartridge were removed and finely cut in pieces of around 2 mm in length. The cut PEVA membranes and agarose gel beads (Sepharose-6B) were activated with epichlorohydrin as described by Porath and Olin [8]. Briefly, the matrices were mixed in a reaction flask with 50.0 mL of 2 M NaOH, 5.0 mL of epichlorohydrin, and 0.27 g of $NaBH_4$ for 15 min at room temperature. Then, an additional 50.0 mL of NaOH and 25.0 mL of epichlorohydrin were added in portions, and the suspension was allowed to react overnight. The activated matrices were washed with water on a Büchner funnel, sucked dry, and returned to the reaction flask. A 130.0 mL of 2 M Na_2CO_3, 26.0 g of disodium iminodiacetate (IDA) were added to the adsorbents. The suspension was kept at 60 °C overnight with slow stirring. The adsorbents were washed with water until the washings were neutral [8]. The TREN was coupled to the activated matrices as described by Boden et al. [17]. Briefly, the activated matrices were mixed in a reaction flask with 25.0 mL water and 5.0 mL of TREN. The reaction was allowed proceed for 48 h, at room temperature. The excess of TREN was removed by washing the adsorbents with water until the washings were neutral [17]. In this work, the derivatized membranes and gels are referred to as IDA-PEVA, IDA-agarose, TREN-PEVA and TREN-agarose.

A small-scale PEVA hollow fiber cartridge was manufactured using fibers from a commercially available cartridge (Kuraray, Japan). The fibers were cut and assembled in a minicartridge of 12 cm effective length as described by Serpa et al. [18]. The amount of fibers in this cartridge was 0.21 g dry mass, with 113 cm^2 surface area, A_p, ($A_p = 2\pi r_o L_e N_f$, 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 minicartridge (150)) and 0.248 cm^3 fiber bed volume, V_b, ($V_b = \pi(r_o^2 - r_i^2)L_eN_f$). The PEVA hollow fiber minicartridge was activated with epichlorohydrin and IDA and TREN were coupled to it as above-mentioned.

The chelating capacity of the adsorbents for Ni(II) ion was determined according the method described by Belew and Porath [23]. Briefly, the Ni(II)-charged adsorbents were equilibrated with 5–6 column volumes of 25 mM sodium phosphate buffer pH 7.0 in 1.0 M NaCl followed by elution with EDTA 50 mM pH 7.0. The total amount of Ni(II) ions in the eluate was determined by spectrophotometrically at 384 nm using EDTA solution as blank.

2.3. Analysis of plasma proteins and immunoglobulins

Protein concentration was determined by the method of Bradford [24] using bovine serum albumin (BSA) as reference protein. In experiments containing prepurified IgG, the protein concentration was determined by measuring the absorbance at 280 nm. The concentrations of IgG, IgA, IgM, albumin (HSA), and transferrin in the fractions collected at the chromatographic experiments were determined nephelometrically using an Array Protein System (Beckman, USA), according to the method provided by the manufacturer.

2.4. 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) according to Laemmli [25]. The gels were stained with silver nitrate according to Morrissey [26].

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 at flow rate equal 0.5 mL/min. For studies concerning the influence of the buffer on IgG adsorption, the following loading buffers were used: buffer A—25 mM Mops-acetate (MA) in 1.0 M NaCl pH 7.0; buffer B—25 mM Tris–HCl in 1.0 M NaCl pH 7.0 and buffer C – 25 mM Tris–HCl pH 7.0. In elution, pH of buffer A was ranged from 7.0 to 4.0, concentration of Tris in buffers B and C was ranged from 100 to 700 mM.

The IDA- and TREN-PEVA cut fibers (1.25 g dry mass) and IDA- and TREN-agarose gels were separately suspended in the loading buffer described above, degassed and packed into columns (20.0 cm × 1.0 cm I.D., Amersham Biosciences, Sweden) to give bed volumes of approximately 5.0 mL. Nickel ion was loaded into the IDA-PEVA columns by passing 50 mM nickel sulphate solution in water through the column until saturation. The TREN-PEVA adsorbers were charged with nickel by passing 50 mM nickel sulphate in 10 mM acetate solution at pH 7.0 through the columns until saturation, as described by Sharma and Agarwal [21]. Non-specifically bound metal was removed by washing the columns with the loading and elution buffers used in chromatographic experiments described ahead. The columns were equilibrated with loading buffer when no further metal was detected in the out-stream.

Human plasma (1.7 mL) five-times diluted with an appropriate loading buffer was injected into the column previously equilibrated with loading buffer. After protein injection, the column was washed with loading buffer until the absorbance values of eluate became close to zero. Elution was carried out with a stepwise pH gradient (pH 6.0, 5.0, 4.0 for the case of Ni(II)-IDA-PEVA columns and pH 6.0 and 5.0 for the case of Ni(II)-TREN-PEVA columns) for MA solution as the loading buffer or with a stepwise gradient of 100–700 mM for Tris–HCl (with or without salt) as the loading buffer [27]. The absorbance of the eluate was monitored at 280 nm. Column regeneration (removal of remaining adsorbed proteins) was achieved by washing the column with 100 mM EDTA solution at pH 7.0. Fractions of 4.0 mL were collected during the chromatographic experiments and protein content was determined using the method of Bradford [24]. The fractions were then pooled for SDS-PAGE and nephelometric analysis of IgA, IgG, IgM, albumin, and transferrin.

2.6. Batch IgG adsorption on Ni(II)-(IDA/TREN)-PEVA supports

The IgG adsorption experiments (stirred tank batch adsorption) for isotherm determination were carried out in Eppendorfs tubes using human IgG (Aventis Behring) at 25 °C for all adsorbers except for Ni(II)-IDA-PEVA, for which experiments were performed at four different temperatures (5, 14, 25 and 37 °C). The Ni(II)-IDA-PEVA and Ni(II)-TREN-PEVA cut fibers (12.5 mg dry mass) were equilibrated with 25 mM Tris–HCl pH 7.0 buffer and 25 mM MA 1.0 M NaCl buffer at pH 7.0 (loading buffers), respectively. Then, 1.0 mL of human IgG preparation was added to the tubes (diluted in loading buffer for total protein concentration from 1.0 to 30 mg/mL). The tubes were rotated end-over-end at 6 rpm for 16 h to allow equilibrium to be established. After 16 h, the cut fibers and the liquid phase were separated by centrifugation and the unbound IgG concentration in this liquid phase (C) was measured by UV spectrophotometry at 280 nm. The adsorbed IgG mass, Q, was determined as the difference between the amount of IgG added and that present in the liquid phase after equilibrium divided by the dry mass of the adsorbent. Plotting Q^* against C^* yielded the equilibrium isotherm. Data were fitted to the Langmuir isotherm model [28] (Eq. (1)) using non-linear least squares and the Levenberg–Marquardt method:

$$Q^* = \frac{Q_m C^*}{K_d + C^*} \quad (1)$$

in which C^* is the IgG liquid-phase equilibrium concentration, Q^* is the IgG surface concentration, Q_m is the maximum IgG binding capacity and K_d is the apparent dissociation constant.

The thermodynamic adsorption parameters (ΔG°, ΔH°, ΔS°) for different temperature were determined as described

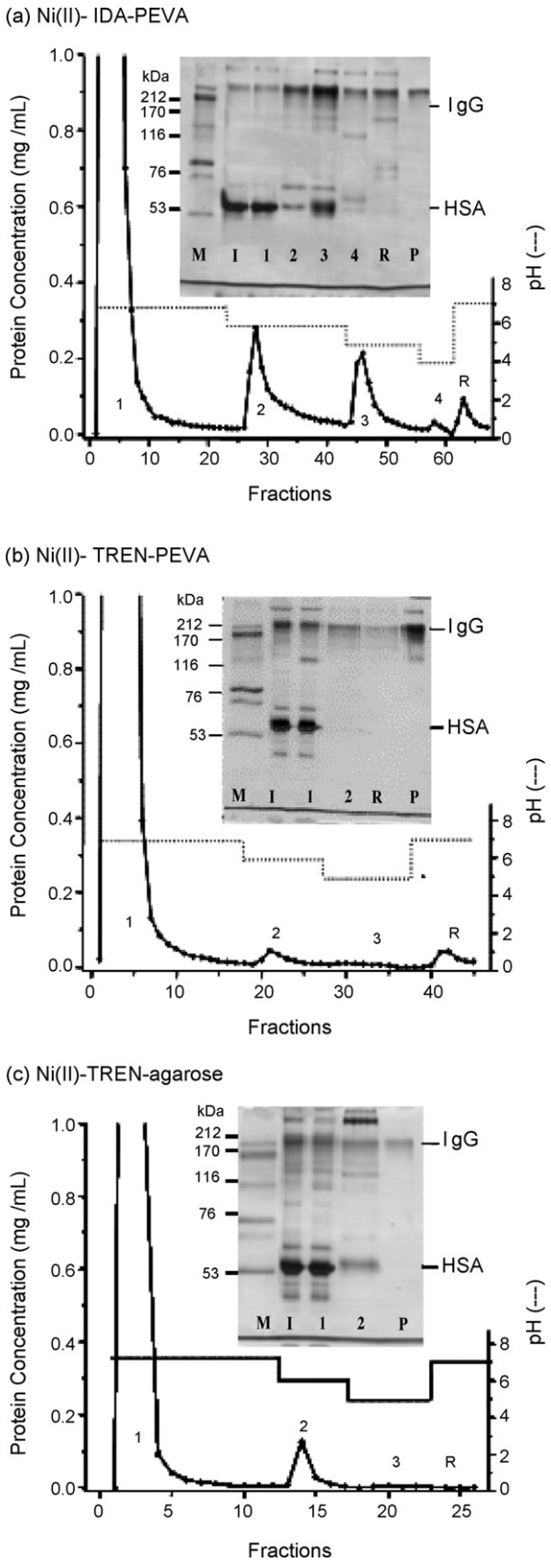

Fig. 1. Effect of the chelating ligand type in column chromatography of human plasma on affinity supports under the stepwise pH gradient. (a) Ni(II)-IDA-PEVA; (b) Ni(II)-TREN-PEVA and (c) Ni(II)-TREN-agarose. Loading buffer: 25 mM MA in 1.0 M NaCl, pH 7.0; elution with 25 mM MA in 1.0 M NaCl under the stepwise pH gradient 7.0–4.0 (a and b) and 7.0–5.0 (c). Bed column 5.0 mL; flow rate 0.5 mL/min; fraction volume 4.0 mL. Plasma solution injected: 8.5 mL. Regeneration with 100 mM EDTA, pH 7.0. Inset: SDS-PAGE under non-reducing conditions. Numbers in chromatograms and SDS-PAGE correspond to the combined peak fractions. M, molecular weight protein marker; I human plasma solution; R combined fractions of the peak eluted at regeneration; P human IgG standard (Aventis, Behring).

by Haupt et al. [29]. From the van't Hoff reaction isotherm:

$$\Delta G = \Delta G^\circ - RT \ln K_d \quad (2)$$

in which ΔG is the change in apparent free Gibbs energy, R is the ideal gas constant, T is the temperature and ΔG° is standard Gibbs energy change. At equilibrium, $\Delta G = 0$ and the Eq. (2) reduces to:

$$\Delta G^\circ = RT \ln K_d \quad (3)$$

This Eq. (3) allows ΔG° to be calculated at a given temperature from the dissociation constant. The temperature dependence of K_d is given by van't Hoff reaction isobar. In its integrated form:

$$\ln K_d = \frac{\Delta H^\circ}{RT} + J \quad (4)$$

where J is an integration constant and ΔH° is a standard enthalpy change. When plotting K_d as a function of $1/T$, ΔH° is given by the slope if a straight line is obtained (in that case, ΔH° is temperature-independent in this temperature range). From the Gibbs–Helmholtz relationship:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

the standard entropy change ΔS° can be obtained.

2.7. Cross-flow filtration of human plasma through Ni(II)-IDA-PEVA hollow fiber minicartridge

These experiments in minicartridge were carried out at 25 °C with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA). After coordination of nickel ions with IDA-PEVA hollow fiber, the adsorbent was equilibrated with the loading buffer (25 mM Tris–HCl pH 7.0 without NaCl) at inlet flow rate equal 1.0 mL/min. Human plasma solution diluted 1:5 in loading buffer was pumped through the minicartridge in a cross-flow mode at inlet flow rates of 1.0 and 1.4 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, with residence time, t_R, of 24 and 17 s, respectively (t_R was calculated by dividing the membrane interstitial volume by the filtrate flow rate [3,30]). The filtrate outlet absorbance at 280 nm was monitored with UV detector. The loading of the protein solution was stopped when the absorbance at 280 nm at filtrate outlet got constant after an initial decay. 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 [18]: 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 was kept 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 of these steps, washing was stopped when 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–HCl buffer pH 7.0 without NaCl (100–700 mM). 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 100 mM EDTA pH 7.0 and with the loading buffer

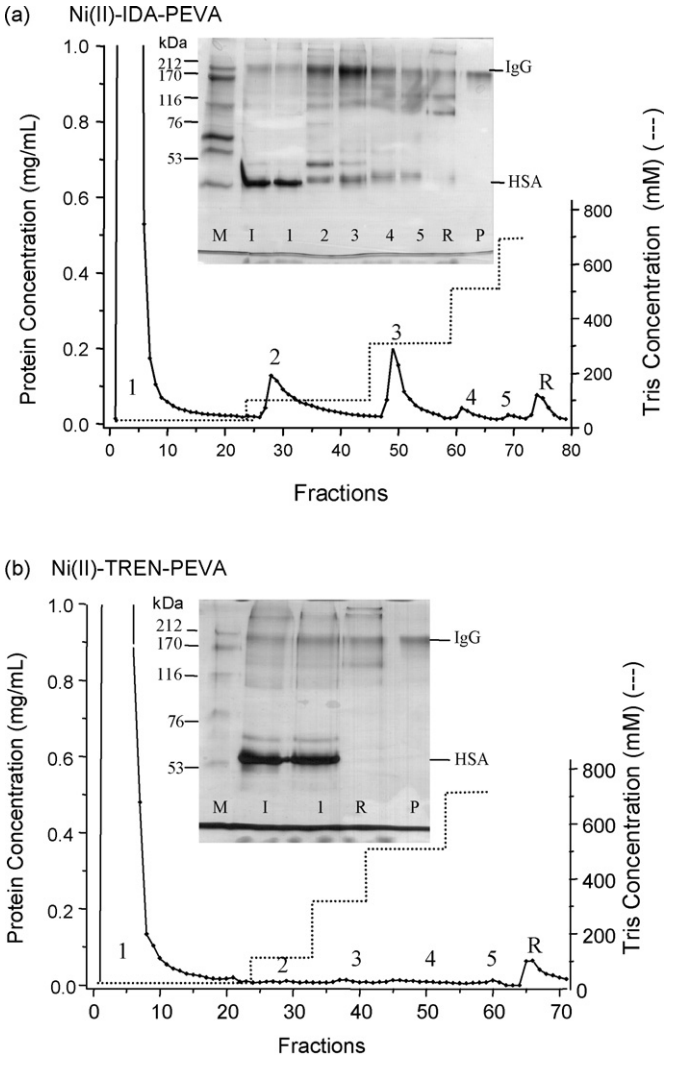

Fig. 2. Effect of the chelating ligand type in column chromatography of human plasma on affinity supports under the stepwise concentration Tris gradient. (a) Ni(II)-IDA-PEVA and (b) Ni(II)-TREN-PEVA. Loading buffer: 25 mM Tris–HCl in 1.0 M NaCl, pH 7.0. Elution under the stepwise concentration gradient of Tris 100–700 mM in the loading buffer. Bed column 5.0 mL; flow rate 0.5 mL/min; fraction volume 4.0 mL. Plasma solution injected: 8.5 mL. Regeneration with 100 mM EDTA, pH 7.0. Inset: SDS-PAGE under non-reducing conditions. Numbers in chromatograms and SDS-PAGE correspond to the combined peak fractions. M, molecular weight protein marker; I human plasma solution; R combined fractions of the peak eluted at regeneration; P human IgG standard (Aventis, Behring).

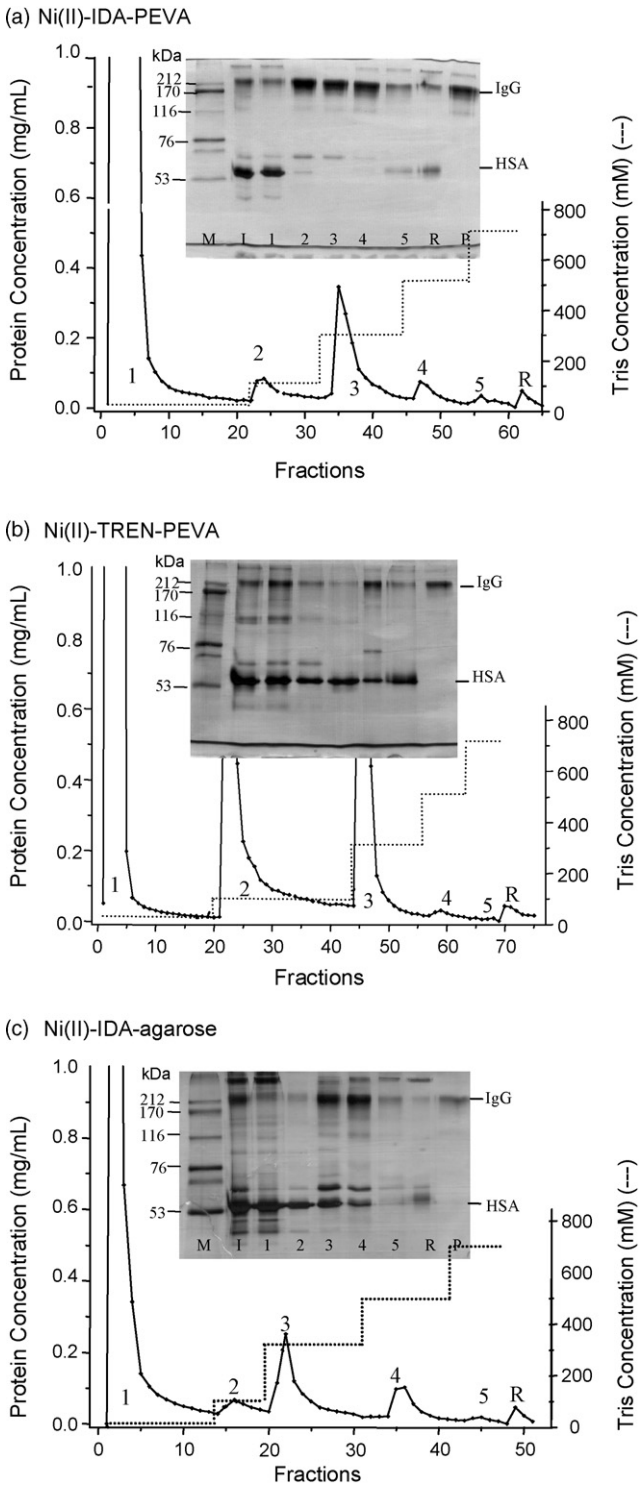

Fig. 3. Effect of the chelating ligand type in column chromatography of human plasma on affinity supports. (a) Ni(II)-IDA-PEVA; (b) Ni(II)-TREN-PEVA and (c) Ni(II)-IDA-agarose. Loading buffer: 25 mM Tris–HCl, pH 7.0; elution under the stepwise concentration gradient of Tris 100–700 mM in Tris–HCl buffer, pH 7.0. Bed column 5.0 mL; flow rate 0.5 mL/min; fraction volume 4.0 mL. Plasma solution injected: 8.5 mL. Regeneration with 100 mM EDTA, pH 7.0. Inset: SDS-PAGE under non-reducing conditions. Numbers in chromatograms and SDS-PAGE correspond to the combined peak fractions. M, molecular weight protein marker; I human plasma solution; R combined fractions of the peak eluted at regeneration; P human IgG standard (Aventis, Behring).

Table 1
Effect of loading and elution buffers in column chromatography of human plasma on Ni(II)-IDA-PEVA and Ni(II)-TREN-PEVA adsorbents

Ligand	Buffer system	Washing (%)[a]			Elution (%)[a]			Regeneration (%)[a]			Total recovery (%)[a]		
		IgG	HSA	Protein	IgG	HSA	Protein	IgG	HSA	Protein	IgG	HSA	Protein
IDA	A	53.4	94.6	80.4	34.2	0.0	7.8	2.6	0.0	0.9	90.2	94.5	89.1
	B	54.6	93.9	85.1	31.4	0.0	6.9	0.0	0.0	0.9	86.1	93.9	92.9
	C	60.4	111.6	100.1	48.9	0.0	9.4	0.5	0.0	0.4	109.9	111.6	110.0
TREN	A	97.2	106.2	111.1	1.7	0.0	0.8	0.6	0.0	0.5	99.4	106.2	112.4
	B	93.9	97.1	92.4	0.0	0.0	0.8	1.1	0.0	0.7	94.9	97.1	93.8
	C	84.6	88.2	83.5	14.6	37.7	26.4	0.0	0.0	0.6	98.7	103.8	110.5

A: 25 mM MA in 1.0 M NaCl, pH 7.0 (stepwise pH gradient); protein mass loaded: 112.5 and 105.4 mg for IDA and TREN, respectively.
B: 25 mM Tris–HCl in 1.0 M NaCl, pH 7.0 (discontinuous steps gradient of Tris); protein mass loaded: 119.7 and 105.1 mg for IDA and TREN, respectively.
C: 25 mM Tris–HCl, pH 7.0 (discontinuous steps gradient of Tris); protein mass loaded: 110.2 and 107.9 mg for IDA and TREN, respectively.
[a] Percentage relative to injected protein mass.

to restore it to its initial conditions for carrying out the next experiment.

Protein concentrations in the retained and non-retained fractions were determined by the Bradford [24] method and nephelometric analysis 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_i) as a function of the volume of protein solution throughput.

3. Results and discussion

3.1. Effect of chelating ligand type and buffer nature on IgG separation from human plasma by column chromatography

Untreated human plasma solution was loaded onto Ni(II)-IDA-PEVA or Ni(II)-TREN-PEVA cut fibers columns. Adsorption of human IgG on both affinity membranes was studied using two different buffers systems containing 1.0 M NaCl according to two desorption strategies: lowering the pH (using MA buffer) and increasing the competitive agent (Tris–HCl) concentration. In order to increase the selectivity for IgG NaCl was removed from the loading buffer for the plasma solution chromatography on Ni(II)-IDA-PEVA and Ni(II)-TREN-PEVA. This procedure represents a departure from traditional IMAC by exploiting electrostatic interaction and it has been shown to be effective in the purification of monoclonal antibodies purification on Zn(II)-IDA-PEVA [18]. The elution was performed with discontinuous steps gradient of Tris (100–700 mM in Tris–HCl buffer at pH 7.0) as before. The results were compared with data obtained by performing similar experiments onto Ni(II)-TREN-agarose and Ni(II)-IDA-agarose. The buffer systems and chelating ligands affected differently the adsorption and elution of IgG (Figs. 1–3 and Table 1).

According to the SDS-PAGE and nephelometric analysis, the IgG was not completely adsorbed onto adsorbents studied for all experiments done, since the saturation amount of IgG was injected into the column. For the two buffer systems containing NaCl tested (Figs. 1 and 2), the total amounts of adsorbed protein (eluted and regeneration fractions) were similar (9.8 and 9.4 mg for Ni(II)-IDA-PEVA and 1.4 and 1.5 mg for Ni(II)-TREN-PEVA, for MA and Tris–HCl buffers, respectively). The Ni(II)-TREN adsorber had lower capacity for IgG than the complex Ni(II)-IDA. This was expected since IDA is a tridentate chelator, disposing three sites for coordination with the protein while TREN, theoretically a quadridentate chelating ligand, disposes only two sites to interact with the protein (whether it is a tri- or tetradentate is still an open question in the literature) [13,31]. Furthermore, for IDA-PEVA, the nickel binding capacity (1.57 μmol of Ni(II)/m^2 of membrane) is higher than that of TREN-PEVA (0.51 μmol of Ni(II)/m^2 of membrane).

According to the SDS-PAGE (Figs. 1 and 2) and nephelometric analysis, the best results of IgG purification in buffer containing NaCl were obtained with Ni(II)-TREN-PEVA using

Table 2
Separation of human plasma proteins by column chromatography on Ni(II)-IDA-PEVA adsorbent

Step	Tris (mM)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	TRF (mg)	TP[a] (mg)	Purity %	Purification factor
Loaded	25	18.2	4.7	1.7	53.6	3.8	110.2	16.5	
Washing	25	11.0	5.6	1.5	59.8	3.2	110.3	10.0	0.60
Elution	100	1.9	0.0	0.0	0.0	0.3	2.2	86.4	5.24
	300	5.7	0.0	0.0	0.0	0.6	6.3	90.5	5.48
	500	1.1	0.0	0.0	0.0	0.0	1.4	78.6	4.76
	700	0.2	0.0	0.0	0.0	0.0	0.5	40.0	2.42
Regeneration	EDTA	0.1	0.0	0.0	0.0	0.0	0.5	20.0	1.21
Total		20.1	5.6	1.5	59.8	4.1	121.3	–	–

[a] TP: total protein.

MA buffer, although with low capacities (Table 1). The IgG could be eluted at mild condition with few impurities at pH 6.0 (0.3 mg of IgG recovered) and with EDTA 100 mM (0.1 mg of IgG recovered). Albumin, transferrin, IgA and IgM concentrations were below detection limit in all eluted fraction. At elution fraction at pH 6.0, IgG purification factor was 2.5 and IgG purity was 43%. This result was compared with data obtained with Ni(II)-TREN-agarose gel in the adsorption of IgG from human plasma solution (Fig. 1c). The nickel binding and protein adsorption capacities of TREN-agarose gel were higher than those for TREN-PEVA membrane (0.95 μmol of Ni(II)/m^2 and 2.04 mg of protein/g adsorbent, and 0.51 μmol of Ni(II)/m^2 and 1.12 mg of protein/g adsorbent, respectively, for the gel and the membrane). However, the lower protein adsorption capacity of Ni(II)-TREN-PEVA could be associated with the higher adsorption selectivity for IgG than for contaminants proteins: the fractions eluted from Ni(II)-TREN-PEVA (Fig. 1b) contained less contaminants than the fractions eluted from Ni(II)-TREN-agarose (Fig. 1c).

For Ni(II)-IDA-PEVA, the use of Tris–HCl pH 7.0 without NaCl (Fig. 3a) seemed to decrease the contamination for albumin compared to the experiment done with Tris–HCl pH 7.0 with 1.0 M of NaCl (Fig. 2a). An explanation for that fact could be that albumin (charged negatively at pH 7.0) might be interacting electrostatically with Tris (a positively charged molecule at pH 7.0) rather than forming coordination with the metal nickel. When salt is added, electrostatic interactions are reduced and as a result the coordination bond with the metal is facilitated. Moreover, the capacity for IgG on Ni(II)-IDA-PEVA increased in the absence of NaCl in the Tris–HCl buffer (6.1 and 9.0 mg of total IgG eluted in Tris–HCl buffers with and without salt, respectively).

For Ni(II)-IDA-PEVA, the elution by increasing Tris concentration (without NaCl) provided the purification of IgG with very few impurities. Only transferrin was detected by nephelometric analysis at the elution fractions using 100 and 300 mM (Table 2). Albumin, IgA, and IgM were not detected by nephelometric analysis, although electrophoresis analysis showed little contamination by albumin at the elution using Tris–HCl 700 mM and in regeneration fractions. The combination of these three fractions eluted with 100, 300, and 500 mM of Tris–HCl provided the purification of 8.70 mg of IgG (6.96 mg/g dry membrane) with very few contaminants, corresponding to a yield of 43.3%, a purity of 88%, and a purification factor of 5.3. This system also showed better results in terms of capacity than that of Ni(II)-TREN-PEVA in presence of MA 25 mM, 1.0 M NaCl pH 7.0.

In order to compare the performance of Ni(II)-IDA-PEVA and Ni(II)-IDA-agarose for IgG adsorption, experiments were carried out with human plasma diluted in Tris–HCl buffer without salt. The Ni(II)-IDA-agarose showed higher adsorption capacity (8.72 and 19.2 mg of protein/g adsorbent, respectively, for the membrane and the gel), but lower selectivity (Fig. 3c). The higher capacity observed for IDA-agarose probably is not due to the nickel binding capacity of the IDA-agarose (1.52 μmol of Ni(II)/m^2) which is similar to that of the IDA-PEVA (1.58 μmol of Ni(II)/m^2).

For Ni(II)-TREN-PEVA, the use of Tris–HCl buffer without NaCl as loading buffer (Fig. 3b) increased the amount of total protein adsorbed, mostly albumin, compared to MA and Tris–HCl buffer containing NaCl, probably due to the nature of the ligand. This suggests the possibility of strong electrostatics interaction between proteins and the ligand Ni(II)-TREN when using the buffer Tris–HCl pH 7.0 without salt. The complex Ni(II)-TREN has a positive net charge, while the albumin (p*I* equal to 4.9) has a negative net charge at pH 7.0. As a result, the use of a buffer with low ionic strength, favors the electrostatic interaction between proteins and ligand thus favoring the adsorption of albumin. The ligand could be working as an ion-exchanger in the presence of low ionic strength. The presence of 1.0 M of NaCl on the buffer Tris–HCl drastically decreased the adsorption of proteins.

3.2. Thermodynamic parameters of IgG adsorption onto Ni(II)-IDA-PEVA

The adsorption isotherms for the binding of IgG onto immobilized Ni(II)-TREN-PEVA (25 mM MA buffer pH 7.0 with 1.0 M NaCl) and Ni(II)-IDA-PEVA (25 mM Tris–HCl buffer pH 7.0) at 25 °C and different temperatures, respectively, are shown in Fig. 4a. The Langmuir model satisfactorily described the adsorption data (correlation coefficient of 0.95–0.99) (Table 3). The dependency of the equilibrium dissociation constant (K_d) versus 1/*T* for the binding of IgG on the Ni(II)-IDA-PEVA membrane was analyzed in terms of van't Hoff plots (Fig. 4b and Table 3).

The results obtained by fitting the data at 25 °C showed that, depending on the chelating ligand used (IDA or TREN), the

Table 3
Langmuir constants and thermodynamic parameters in adsorption of human IgG on Ni(II)-IDA-PEVA and Ni(II)-TREN-PEVA supports

Affinity ligand	T^a (°C)	Q_m (mg/g)	K_d (mol/L)	R^b	Variance	Standard deviation	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (kJ/mol)
Ni(II)-IDA[c]	4	262.7 ± 8.6	$(1.7 \pm 0.2) \times 10^{-5}$	0.98	206.30	14.36	−24.7		183.8
	15	228.0 ± 7.2	$(9.9 \pm 1.3) \times 10^{-6}$	0.98	153.71	12.40	−27.0		184.6
	25	204.6 ± 4.6	$(6.1 \pm 0.7) \times 10^{-6}$	0.98	95.85	9.79	−29.1	+26.2	185.6
	37	159.4 ± 4.6	$(5.3 \pm 0.8) \times 10^{-6}$	0.95	121.67	11.03	−30.6		183.2
Ni(II)-TREN[d]	25	93.9 ± 2.9	$(2.8 \pm 0.3) \times 10^{-5}$	0.99	3.62	1.90	–	–	–

[a] *T*: temperature.
[b] *R*: correlation coefficient.
[c] Tris–HCl buffer pH 7.0 without NaCl.
[d] MA buffer pH 7.0 containing 1.0 M NaCl.

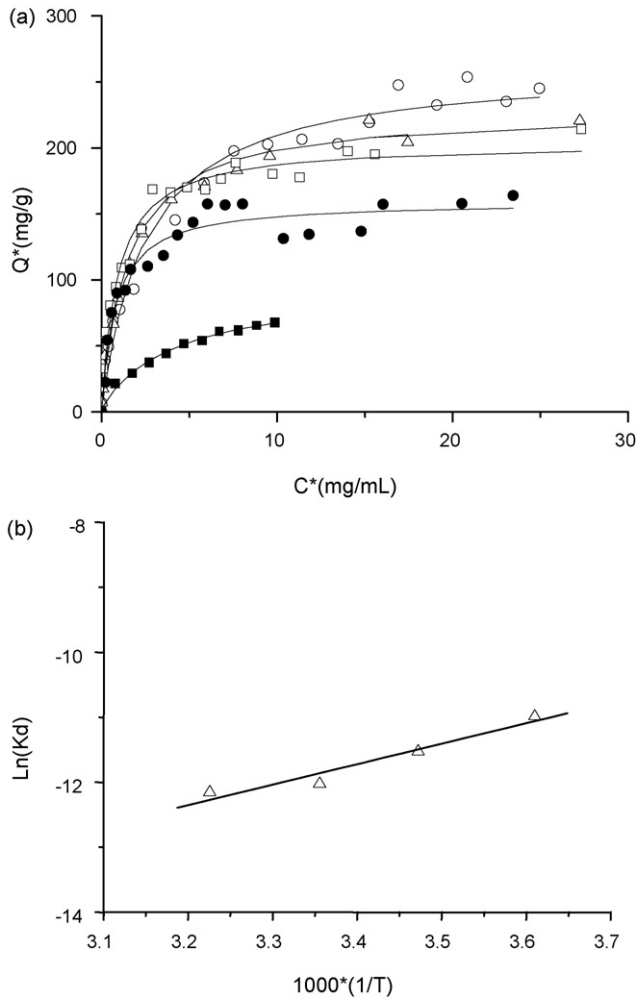

Fig. 4. (a) Isotherm of human IgG adsorption on Ni(II)-IDA-PEVA in 25 mM Tris–HCl pH 7.0 at (◯) 4 °C, (△) 15 °C (□) 25 °C and (●) 37 °C; and Ni(II)-TREN-PEVA in 25 mM MA containing 1.0 M NaCl, pH 7.0 at (■) 25 °C. The solid lines correspond to fitting (non-linear regression) the experimental data in accordance with Langmuir model. (b) van't Hoff plots ($\ln K_d$ vs. $1/T$) for Ni(II)-IDA-PEVA.

dissociation constant K_d can differ by one order of magnitude (6.1×10^{-6} and 2.8×10^{-5} mol/L for IDA and TREN, respectively) and the maximum IgG binding capacity (Q_m) can double (204.6 and 93.9 mg/g for IDA and TREN, respectively).

Chelating affinity matrices should have K_a values above 10^5 (K_d smaller than 10^{-5} M) to assure efficient adsorption, without risking ligate elution during washing [32]. The dissociation constants (K_d) measured (10^{-6} M for Ni(II)-IDA-PEVA and 10^{-5} M for Ni(II)-TREN-PEVA) were typical for pseudobiospecific affinity ligands. The IDA which is weakly acid forms a double five-membered ring chelate with tetra- and hexa-coordinate metal ions. Theoretically, TREN is a tetradentate chelator with four nitrogens atoms, three of which are primary in nature and the fourth is ternary. Thus, TREN can occupy four of the six coordination sites of the Ni(II) ion [33]. Consequently, Ni(II)-TREN-PEVA has a higher dissociation constant (K_d) and a lower maximum IgG binding capacity (Q_m) than the corresponding IDA-membrane.

An increase in temperature from 4 to 37 °C caused a decrease in equilibrium dissociation constant from 1.7×10^{-5} to 5.3×10^{-6} M for Ni(II)-IDA-PEVA, indicating an increase in affinity with increased temperature. If the interaction between the protein and the immobilized ligand involved a significant hydrophobic contribution, the contact surface between the protein and the immobilized ligand should increase the affinity of protein for the adsorbent at higher temperature [34]. Although, experimentally, affinity increases at higher temperature, the opposite is observed for the binding capacity. The higher IgG binding capacity onto immobilized Ni(II)-IDA-PEVA at lower temperature indicates that coulombic forces are the main forces prevailing during the adsorption process [34].

The ΔG° values for IgG adsorption onto Ni(II)-IDA-PEVA were calculated for each temperature. In accordance with adsorption being a favorable process, the ΔG° values were all negative, ranging from −24.7 to −30.6 kJ/mol (Table 3). These values are of similar magnitude to those reported by Hutchens and Yip [35] in their study of the binding of several proteins onto immobilized IDA-Cu(II)-agarose and also similar to the results of Finette et al. [34] in their study of binding of hen egg white lysozyme (ΔG° ranging from −23.2 to −24.3 kJ/mol) and human serum albumin (ΔG° ranging from −28.3 to −29.4 kJ/mol) onto immobilized IDA-Cu(II)-Fractosil 1000.

The values of ΔG° decrease with increased temperature, indicating that the adsorption reaction is spontaneous and more favorable at higher temperature. The positive value of enthalpy change (ΔH°), indicates that the adsorption of IgG onto Ni(II)-IDA-PEVA is endothermic. Positive values for the ΔS° were obtained, and according to Ross and Subramanian [36], from the point of view of water structure, positive values for ΔS° and ΔH° are frequently taken as typical evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by positive value of ΔS° and a negative value of ΔH°, while negative entropy and enthalpy changes arise from van der Waals forces and hydrogen bonds. Even though, the binding of IgG to Ni(II)-IDA might involve hydrophobic interaction, strongly supported by positive values of ΔS° and ΔH°, electrostatic interaction and coordination bonds should not be excluded. Accordingly, it is not possible to account for the thermodynamic parameters of human IgG-Ni(II)-IDA-PEVA on the basis of a single intermolecular force model.

3.3. Chromatography of human plasma on Ni(II)-IDA-PEVA hollow fiber membrane minicartridge

Fig. 5a displays the human plasma solution breakthrough curves for a Ni(II)-IDA-PEVA minicartridge at filtrate flow rate of 0.5 and 0.7 mL/min, corresponding to t_R of 24 and 17 s, respectively. The non-retained fractions (filtrate fractions) were analyzed with SDS-PAGE (Fig. 5b). Initially, the protein band with molecular mass of approximately 150 kDa (corresponding to IgG) was not detected in SDS-PAGE, leading to the conclusion that the IgG concentration in the filtrate was zero, reflecting complete adsorption of the IgG molecules onto the immobilized

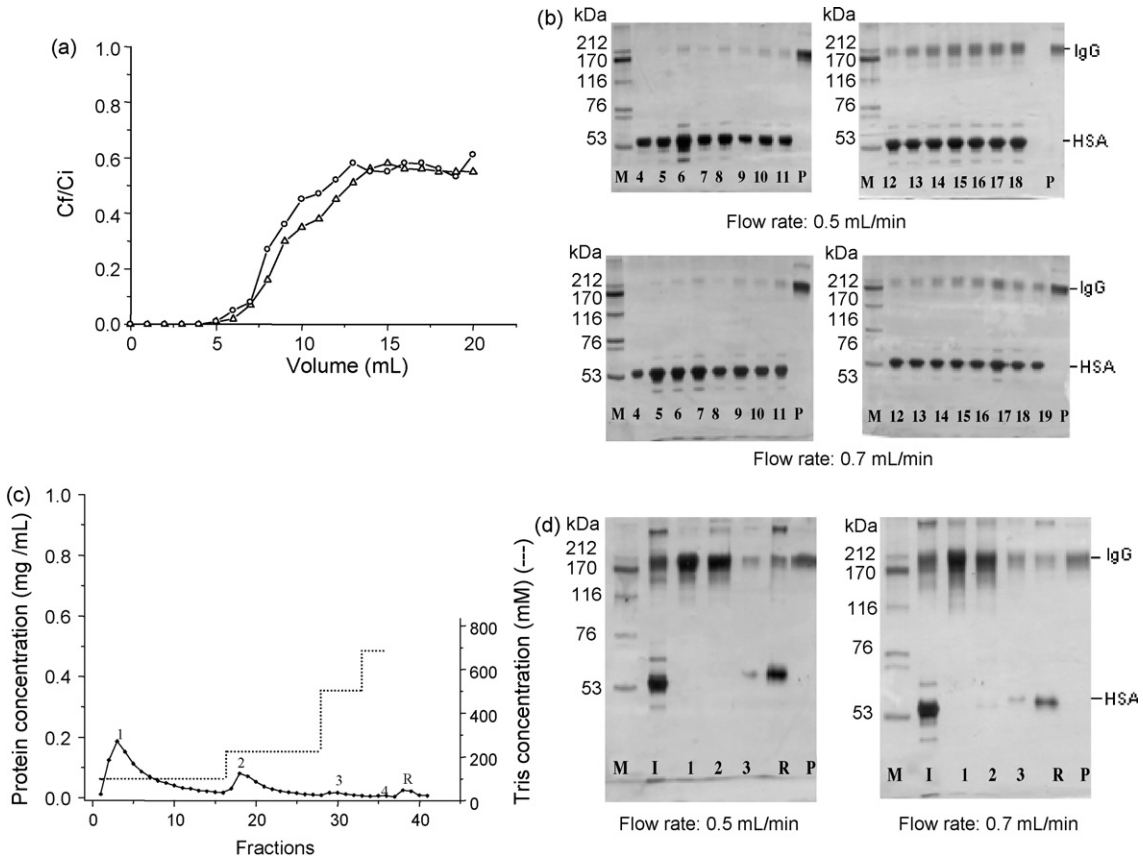

Fig. 5. (a) Separation of IgG from human plasma by cross-flow filtration through Ni(II)-IDA-PEVA hollow fiber membrane minicartridge. Buffer composition: 25 mM Tris–HCl pH 7.0. Flow rate, mL/min: (Δ) 0.5 and (◯) 0.7. (b) SDS-PAGE under non-reducing conditions. Numbers 1–19 in SDS-PAGE correspond to the outlet column fractions; (c) typical elution pattern under the stepwise Tris gradient of the human plasma solution in backflushing mode on the Ni(II)-IDA-PEVA minicartridge. Buffer composition: 100–700 mM with increments of 200 mM Tris at pH 7.0. (d) SDS-PAGE under non-reducing conditions. Numbers in chromatograms correspond to the combined peak fractions.

Ni(II). As the loading step proceeded and binding sites became occupied, IgG was detected in the filtrate (breakthrough point). The breakthrough point (in the range of 6.0–7.0 mL) was the same for both flow rates.

Following breakthrough, filtrate total protein concentration increased and C_f/C_0 asymptotically approached 0.6, at which point steady-state was achieved (no further adsorption of protein occurred). The C_f/C_0 value is lower than unity because a fraction of the protein mass fed to the system is contained in the retentate and does not end up in the filtrate when operating with the tangential mode of filtration.

After washing of the cartridge, elution was performed with discontinuous steps gradient of Tris–HCl buffer at pH 7.0 (100–700 mM) (Fig. 5 c and d). Similar results were obtained for the experiments at filtrate flow rate of 0.5 and 0.7 mL/min (Table 4). High IgG yields were obtained in the fractions eluted with 100 mM (6.6 and 7.2 mg of IgG for filtrate flow rate of 0.5 and 0.7 mL/min, respectively, representing 78% of total IgG eluted). Albumin could be detected with SDS-PAGE at the elution fraction using 500 mM Tris–HCl, but could not be detected with nephelometric essays. Transferrin, IgA, and IgM could not be detected neither with nephelometric assays nor with SDS-PAGE on all eluted fraction. The IgG adsorption capacity on Ni(II)-IDA-PEVA were in the range of 40.5–43.8 mg/g of dry membrane or 76.9 and 83.2 μg/cm^2 of membrane in the minicartridge. The combined peak fractions eluted with 100 and 300 mM of Tris gave an IgG purity of 98 and 99% for filtrate flow rates of 0.5 and 0.7 mL/min, respectively.

In the chromatographic experiments with cut fibers, the amount of IgG eluted (7.2 mg of IgG/g adsorbent) was from 82 to 84% lower than the value obtained in the minicartridge experiments (40.5–43.8 mg of IgG/g adsorbent) (Table 4). Moreover, the selectivity of Ni(II)-IDA-PEVA minicartridge for IgG (Fig. 5d) was different of that on Ni(II)-IDA-PEVA cut fiber column (Fig. 3a). The minicartridge seemed to show better results of IgG selectivity, since with Ni(II)-IDA-PEVA cut fibers column, transferrin was the major impurity. This phenomenon could be due to differences in adsorption affinities for IgG and for transferrin at Ni(II)-IDA-PEVA (transferrin could be adsorbed at Ni(II)-IDA-PEVA with a weaker affinity than IgG) and to differences in operational mode. At cut fiber chromatographic experiments, transferrin, which has a smaller size (76 kDa) than IgG (150 kDa), has its adsorption favored by diffusion into the interior of the pores, forming a protein deposit which might prevent the IgG from entering there. In presence of cross-flow membrane process, the plasma solution is forced from the sur-

Table 4
Effect of flow rate on the separation of IgG and proteins from human plasma on Ni(II)-IDA-PEVA hollow fibers in minicartridge and in column

Step	Tris-HCl mM	Q_F					
		0.5 (mL/min)		0.7 (mL/min)		0.5 (mL/min)	0.7 (mL/min)
		IgG (mg)	TP[a] (mg)	IgG (mg)	TP[a] (mg)	Purity (%)	Purity (%)
Elution	100	6.6	6.7	7.2	7.2	98.5	100.0
	300	1.9	2.0	2.0	2.1	95.0	95.2
	500	0.0	0.3	0.1	0.3	0.0	33.3
	700	0.0	0.2	0.0	0.1	0.0	0.0
Regeneration	EDTA	0.0	0.3	0.0	0.3	0.0	0.0
Total protein eluted (mg)		8.5	8.7	9.2	9.3	97.7	98.9
Capacity (mg/g dry membrane)		40.5	41.4	43.8	44.3	97.8	98.9

[a] TP: total protein.

face into the pores, displacing the transferrin molecules thus eliminating this effect. Convective transport of protein into the pores may favor IgG adsorption.

4. Conclusion

We have demonstrated that the Ni(II)-IDA-PEVA hollow fiber membrane system is a potential alternative for the purification of human IgG. The IgG adsorption was influenced by the buffer nature. The performance of the Ni(II)-IDA-PEVA minicartridge membrane compared to the conventional Ni(II)-IDA-agarose beads showed a higher capacity and higher selectivity for the membrane configuration. The equilibrium adsorption data was analyzed using Langmuir model. At different temperatures, K_d values were in the range of 10^{-5} to 10^{-6} M for Ni(II)-(IDA/TREN)-PEVA hollow fiber membrane. The negative values of ΔG° confirm a favorable adsorption of human IgG onto Ni(II)-IDA-PEVA and the positive value of ΔH° (26.2 kJ/mol) suggests an endothermic nature of adsorption. IgG adsorption could be achieved under mild conditions, near to physiological pH, low ionic strength, and at room temperature. The adsorbed IgG could be eluted under non-denaturing conditions.

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