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Human IgG adsorption using dye-ligand epoxy chitosan/alginate as adsorbent: influence of buffer system

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Abstract The aim of this work was to evaluate the ability of Cibacron Blue F-3GA dye-ligand chitosan/alginate epoxide on human IgG adsorption under different buffer systems at 25 mmol L^{-1}: Morpholinoethane sulfonic acid, morpholinopropane sulfonic acid, hydroxyethylpiperazine ethanesulfonic acid and tris-hydroxymethyl aminomethane (Tris–HCl). Batch adsorption studies were performed in order to evaluate the effect of buffer pH and nature on IgG uptake as well the equilibrium isotherms. Chromatographic experiments were performed with both human IgG (high purity) and human serum and each buffer with NaCl 1.0 M was used in the elution step. Best results (maximum equilibrium adsorption capacity of 110.9 mg g^{-1}) were found at pH 7.8 with Tris–HCl buffer. The Langmuir–Freundlich model provided a good fit for the adsorption isotherm. In chromatographic experiments with high purity IgG and Tris/HCl buffer, IgG dynamic adsorption capacity onto E-Ch/Al-Cibacron was 13.4 mg g^{-1}, which accounted for 60 % of IgG injected into the column. Chromatographic experiments with diluted (tenfold) human serum were conducted and it was found by electrophoresis that IgG is preferentially adsorbed with respect to other proteins using all buffers systems, especially with Tris–HCl. The amount of desorbed protein from E-Ch/Al-Cibacron was around 7.0 mg g^{-1} for all buffers. The new stationary phase showed high affinity for IgG and may be a potential adsorbent for human IgG purification.

Keywords Adsorption · Dye-ligand affinity chromatography · Human immunoglobulin G · Human serum · Cibacron blue · Chitosan

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1 Introduction

Human immunoglobulin G (IgG) is an important group of proteins present in human blood with a wide range of diagnosis and therapeutic applications. The therapeutic prescription is indicated in cases of congenital or acquired immunodeficiency, in the treatment of selective antibody deficiency, autoimmune diseases and some kinds of cancer which require large intakes of high purity protein (Burnouf and Radosevich 2001). There are several methods to purify IgG, mainly by precipitation and chromatographic techniques including ion exchange, hydrophobic interaction, gel filtration and affinity chromatography (Özkara et al. 2004; Yavuz et al. 2006).

Affinity chromatography is an adsorption technique that exploits the specific interaction between an immobilized ligand and the molecule which will be separated (Vijayalakshmi 1989). This method is extensively used for separation, identification and purification of macromolecules since it is based on the high specificity of the protein–ligand bonding (Denizli and Piskin 2001).

The pseudobiospecific ligands used in affinity chromatography show the ability to form bonds with proteins by hydrophobic, electrostatic or coordination interactions (Vijayalakshmi 1989). Among these ligands, thiophilic, biomimetic, peptides, amino acids, dyes, and metal ions or

multimodal ones, have been frequently reported in literature aiming at antibody purification due to their low production costs, commercial availability and easy immobilization (Denizli and Piskin 2001; Yavuz et al. 2006; Gondim et al. 2012; Ghose et al. 2013; Rajak et al. 2012; Sadavarte et al. 2014; Wolfe et al. 2014). Dyes are considered as alternative ligands in affinity chromatography since they are able to reversibly bind to most biomolecules and have been successfully used with proteins (Bayramoğlu et al. 2007; Denizli and Piskin 2001; Gondim et al. 2012; Wongchuphan et al. 2009; Yavuz et al. 2006).

The selection of the supporting matrix for the immobilization of the ligand is of paramount relevance in affinity chromatography. The stationary phases are usually commercial polymeric-based resins specially prepared for this purpose, such as agarose, cellulose, dextran and poly(acrylate). They are highly porous so as to allow for a high degree of ligand immobilization (Denizli and Piskin 2001) and they have functional surface groups (hydroxyl, carboxyl, amine), which may be used for further derivatization and immobilization of ligands. Stationary phases based on chitosan are an interesting alternative due to its availability and easy manipulation. Chitosan is a biodegradable and non-toxic hydrophilic polysaccharide, normally obtained by alkaline deacetylation of chitin, a natural biopolymer abundantly found in the exoskeleton of shells of crustaceans, insects, and fungi cell walls (Cetinus et al. 2007; Feng et al. 2009; Hoven et al. 2007). Besides its low cost, the matrix can be easily modified by gelation and such agents as alginate or κ-carrageenan, which are able to improve chemical stability and mechanical strength (Adriano et al. 2008; Kamari et al. 2009; Krajewska 2004; Nadavala et al. 2009;).

Thus, the aim of this work was to study the influence of four different biological buffers in human IgG adsorption onto Cibacron Blue F-3GA dye-ligand epoxy chitosan/alginate (E-Ch/Al-Cibacron), extending the application presented in a previous work (Gondim et al. 2012). The dye-ligand affinity adsorbent was characterized by different techniques (differential scanning calorimetry, thermogravimetric analysis, differential thermogravimetric analysis and scanning electron microscopy) and tested for IgG adsorption in both batch and fixed bed column systems. The effect of the buffer system on IgG adsorption kinetics and equilibrium was evaluated, as well as the selectivity of the adsorbent when used in the purification of IgG from human serum.

2 Experimental

2.1 Materials

Human IgG and human serum were supplied by Sigma (USA). Chitosan (Polymar, Brazil), sodium alginate (VETEC, Brazil) and Cibacron Blue F3GA (Polysciences, USA) were used to synthesize the adsorbent. Morpholinoethane sulfonic acid (MES), morpholinopropane sulfonic acid (MOPS), hydroxyethylpiperazine ethanesulfonic acid (HEPES) and Tris-hydroxymethyl aminomethane (Tris–HCl) were supplied by Sigma (USA). The following chemicals used in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis were supplied by Sigma: acrylamide, bis-acrylamide, SDS and dithiothreitol. All chemicals were of analytical grade and were used without further purification. The water used to prepare buffers and other solutions was ultrapure (Milli-Q System, Millipore, USA).

2.2 Chitosan/alginate epoxide preparation

Chitosan/alginate Epoxide stationary phase was prepared according to Gondim et al. (2012) and Rodrigues et al. (2013). Chitosan was dissolved in 400 mL acetic acid 5 % (v/v). After that, 40 mL methanol and 4 mL anhydride acetic were added. Sodium alginate was joined to the mixture, which was kept under mechanical agitation for 1 h, so that chitosan and alginate concentrations were 2.5 % (m/m). The solution was coagulated into NaOH 0.1 mol L^{-1} for 4 h under moderate agitation. The gel was filtered and thoroughly rinsed with distilled water until it reached neutral pH. Chemical modification of chitosan/alginate gel was carried out in a glass reactor. 10.0 g gel and 100 mL dimethylformamide were added into the reactor and maintained at 60 °C for 30 min. Then, 0.8 g KOH dissolved in 3 mL isopropanol and 10 mL epichlorohydrin were added to the reaction medium and maintained under agitation at 60 °C overnight. Then, the thus obtained solids were washed again with distilled water until neutrality.

2.3 Cibacron Blue F3GA immobilization onto epoxide chitosan/alginate

Cibacron Blue F3GA dye was immobilized onto E-Ch/Al according to the procedure described by Ruckenstein and Zeng (1998) and adapted by Gondim et al. (2012). Firstly, 1.0 g E-Ch/Al was added to 100 mL dye solution (1 mg mL^{-1}) and stirred at 60 °C for 1 h. After that, 5 mL NaCl 20 % (m/v) were added to the suspension and the pH was corrected to 10.5 with 2 mL of Na_2CO_3 (25 % m/v), so as to enhance dye adsorption. This suspension was kept at 60 °C for 4 h. The dye-loaded solids were washed with distilled water, methanol, NaCl 2 mol L^{-1}, Urea 6 mol L^{-1}, Tween 80 (1 % m/v) and buffer solutions 25 mmol L^{-1} (Tris–HCl, MOPS, MES and HEPES).

2.4 Characterization

2.4.1 Thermogravimetric analysis (TGA) and differential thermogravimetric analysis

A Shimadzu DTG-60H (Japan) thermal analyzer was used with a N_2 flow rate of 50 mL min^{-1}, in a temperature range from 30 to 900 °C with a heating rate of 10 °C min^{-1}.

2.4.2 Differential scanning calorimetry (DSC)

A Shimadzu DSC-60 (Japan) thermal analyzer was used with a N_2 flow-rate of 100 mL min^{-1}, in a temperature range from 30 to 500 °C with a heating rate of 10 °C min^{-1}.

2.4.3 Scanning electronic microscopy (SEM)

In order to analyze the surface of the adsorbent and to perform elemental analysis, a LEO 440i Oxford scanning electron microscope coupled with energy dispersive X-ray system (SEM/EDS) (Cambridge, England) was used.

2.5 Batch adsorption

In order to determine the effect of the pH in different buffer systems on IgG adsorption kinetics and equilibrium onto E-Ch/Al-Cibacron, several experiments were carried out (in duplicate) at 22 °C (±1 °C) in batch mode. The pH ranges used in the experiments for all buffers were: Tris–HCl (7.2; 7.8; 8.4; 9.0), MES (5.5; 6.1; 6.7) HEPES (6.8; 7.5; 8.2) and MOPS (6.5; 7.2; 7.9). In each experiment, 15 mg adsorbent was placed inside acrylic tubes containing 3.0 mL of the IgG solution (1.0 mg mL^{-1}). The tubes were agitated end-over-end in an orbital shaker (Tecnal TE-165, Brazil) during 2 h. For kinetic experiments, the same procedure for pH investigation using different buffers was used. The samples of supernatant were collected at fixed time intervals in order to obtain the IgG concentration histories. For the measurement of adsorption isotherms, different initial concentrations (1.0–9.0 mg mL^{-1} for IgG) at the same solid/liquid dose were used. Tubes were shaken for enough time to reach the equilibrium (3.0 h). In all these experimental procedures, the supernatant were collected, centrifuged at 10,000 rpm for 10 min (refrigerated microcentrifuge Cientec CT-15000R, USA) and the protein equilibrium concentration in the liquid phase (supernatant) was determined by absorbance at 280 nm (UV–Vis spectrophotometer Biomate 3, ThermoScientific, USA).

The amount of protein adsorbed per unit mass of adsorbent (mg g^{-1}) was calculated from the mass balance described in Eq. (1):

$$q = \frac{V_{sol}(C_0 - C_{eq})}{m_{ads}} \quad (1)$$

where C_0 and C_{eq} (mg mL^{-1}) are the initial and equilibrium protein concentration in liquid phase, q (mg g^{-1}) is the amount of protein adsorbed in solid phase, m_{ads} (g) is the mass of adsorbent and V_{sol} (mL) is the liquid phase volume.

The Langmuir, Freundlich and Langmuir–Freundlich models, as described by Eqs. (2), (3) and (4) respectively, were used to fit the experimental data by using the iterative Levenberg–Marquardt fitting method (Origin® software, Microcal, USA).

$$q = \frac{q_m C_{eq}}{K_D + C_{eq}} \quad (2)$$

$$q = K_f (C_{eq})^{1/a} \quad (3)$$

$$q = \frac{q_m (C_{eq})^n}{K_{DLF} + (C_{eq})^n} \quad (4)$$

where q_m (mg g^{-1}) is the maximum adsorption capacity; C_{eq} (mg mL^{-1}) is the concentration of protein in solution at equilibrium; K_f, K_D and K_{DLF} (mol L^{-1}) are the dissociation and the apparent dissociation constants of Freundlich, Langmuir and Langmuir–Freundlich equations, respectively, which indicate the affinity between protein and adsorbent; a and n are the coefficients for Freundlich and Langmuir–Freundlich, respectively.

2.6 Chromatographic experiments

The chromatographic runs were carried out using a peristaltic pump (Watson-Marlow, USA) and a fraction collector (C-660, Büchi, Switzerland). The dye-affinity adsorbent (0.5 g) was suspended in water, degassed, and packed without compression into a glass column (10.0 cm × 1.0 cm I.D., GE Healthcare, USA) to a bed height of 1.4 cm (corresponding to a stationary phase volume of 1.0 mL).

Chromatographic experiments were carried out with a fixed concentration of IgG (1.0 mg mL^{-1}) and flow rate (0.8 mL min^{-1}—superficial velocity of 61.1 cm h^{-1}). The experimental runs were carried out at 22 °C (± 1 °C). The column was initially equilibrated with a given buffer (25 mmol L^{-1}). After that, a load of protein (high purity IgG or human serum) diluted in buffer was injected into the column. For all experiments, after protein loading, the column was washed with the given buffer until the absorbance at 280 nm in the outlet reached the baseline before protein injection. Elution was performed with the buffer containing 1.0 mol L^{-1} NaCl. After each experiment, the column was regenerated with 25 mmol L^{-1} NaOH, followed by rinsing with Milli-Q water and the buffer to restore the column to its

initial condition for a new experiment. Fractions (1.6 mL) were collected at the exit of the column during all chromatographic steps (A—adsorption/W—washing/E—elution/R—regeneration). Concentration was monitored by UV/Vis detector (Thermo Scientific BioMate 3, USA) at 280 nm to measure protein concentration. For human serum experiments, the same procedure was used and the total protein concentration of each fraction was measured by the Bradford method (Bradford 1976).

2.7 Analytical methods

2.7.1 Protein determination

The total protein content was analyzed by the Coomassie blue method according to the procedure described by Bradford (Bradford 1976) using Bovine Serum Albumin (BSA) as the reference protein. The intensity of the color was measured at 595 nm using a UV–Visible spectrophotometer.

2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of protein samples was performed in 7.5 % polyacrylamide gels under denaturing reducing conditions (Laemmli 1970) using a Mini-Protean III System (Bio-Rad, USA). The runs were carried out at 180 V in 7.5 % separation gels with a 4 % stacking gel. Protein bands were developed by Coomassie Blue.

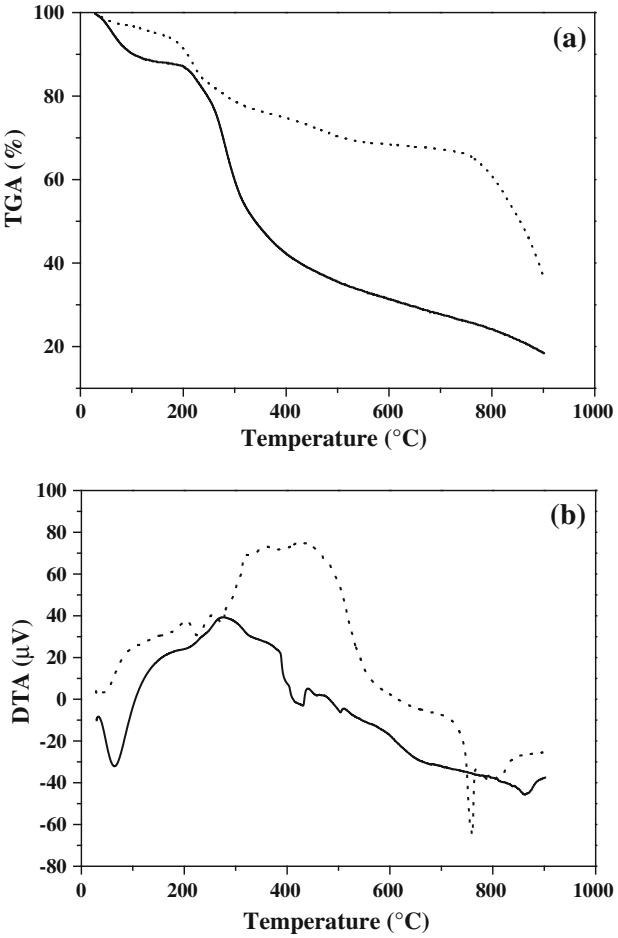

Fig. 1 Thermogravimetry analysis: **a** TGA; **b** DTA. *Dashed line* E-Ch/Al without immobilized dye; *continuous line* E-Ch/Al-Cibacron

3 Results and discussion

In a previous work (Gondim et al. 2012), the synthesis of E-Ch/Al-Cibacron was firstly reported. The adsorbent was characterized in terms of Fourier transform infrared spectroscopy (FTIR) and preliminary results of human IgG adsorption from single solution and human serum using phosphate buffer were presented. Those results indicated that IgG was preferentially adsorbed onto E-Ch/Al-Cibacron and IgG could be purified from human serum. In the present work, the characterization of this material is extended and IgG adsorption in batch and fixed bed experiments were performed. The effect of the buffer system was also evaluated, using Tris–HCl, MES, MOPS and HEPES buffers.

3.1 Characterization

3.1.1 Thermal analysis

The thermogravimetric curve shown in Fig. 1a indicates that E-Ch/Al without dye (dashed line) has a greater thermal stability as compared to E-Ch/Al-Cibacron (continuous line). When subjected to a temperature range of 30–900 °C, a mass loss of 63 % was observed for E-Ch/Al whereas the mass loss for E-Ch/Al-Cibacron was 79 %. For better visualization of the stages of mass loss, the differential (DTA) curves are shown in Fig. 1b. The DTA curve of E-Ch/Al under N_2 showed peaks characteristic of four stages of mass loss: dehydration (25–90 °C); deacetylation and depolymerization (250–400 °C); final residues degradation (750 °C), in agreement with Chaves et al. (2009). The products of decomposition around 400 and 750 °C are characterized as carbonaceous material (Soares et al. 2004).

From the DSC curves (Fig. 2), the first order transition and the formation of endothermic and exothermic peaks can be clearly identified. There is an endothermic peak around 70 °C and an exothermic peak at 250 °C, probably due to the decomposition of sodium alginate (Rajendran and Basu 2009). As observed, the two curves are significantly different, since the endothermic peaks in the range of 200–300 °C disappear and an exothermic peak at about

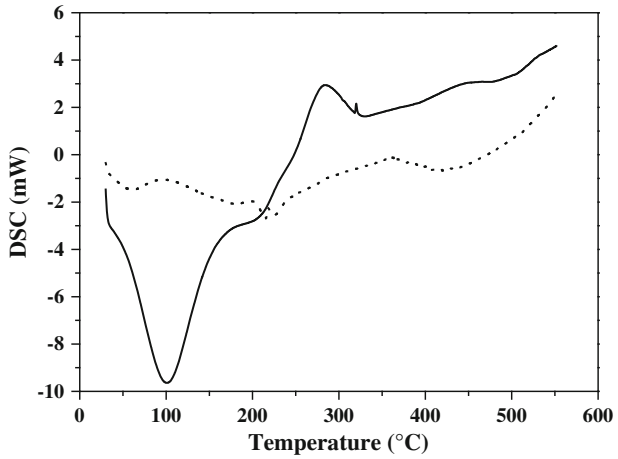

Fig. 2 DSC analysis. *Dashed line* E-Ch/Al without immobilized dye. *Continuous line* E-Ch/Al-Cibacron

280 °C shows up, suggesting that the Cibacron Blue immobilization is effective, leading to the formation of a new product.

3.1.2 Scanning electron microscopy (SEM)

The scanning electron micrographs of E-Ch/Al without dye and E-Ch/Al-Cibacron are shown in Fig. 3a, b, respectively. The solids surface has numerous wrinkles and pores, similar to those observed in the study of Kosaraju et al. (2006). The immobilization of the dye does not seem to have caused significant morphological differences between the samples. The high surface roughness in both micrographs suggests that there were no structural changes associated with immobilization of the dyes, which is in agreement with similar records in the literature (Wu et al. 2006; Yavuz et al. 2006).

The pore formation also occurred when chitosan microparticles were subjected to an epoxy modification (Subramanian et al. 2006). According to SEM presented by these authors, the surface can be considered very similar to the results shown in the present study. Wrinkle formation was also observed in SEM by Chen et al. (2008), who studied chitosan adsorbents modified with different molar ratios of epichlorohydrin.

3.2 IgG batch adsorption

3.2.1 Effect of different buffers on IgG adsorption

The results presented in Fig. 4 demonstrate that IgG adsorption is influenced by the pH and the type of buffer. When HEPES was used as buffer at pH 6.8, the highest IgG amount adsorbed was reached (76.56 mg g^{-1}). In a previous work (Gondim et al. 2012) the IgG adsorption onto

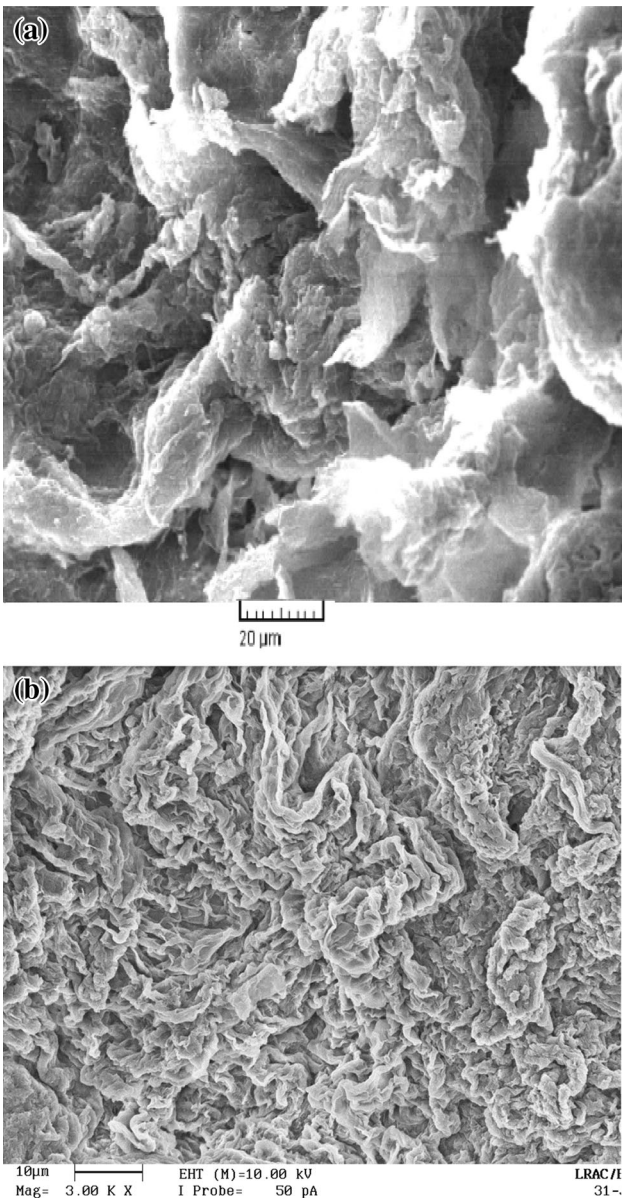

Fig. 3 SEM micrograph at 3000 zoom of E-Ch/Al without immobilized dye (**a**) and E-Ch/Al-Cibacron (**b**)

E-Ch/Al-Cibacron was carried out using only sodium phosphate 25 mmol L^{-1} as buffer at different pH values. At pH 6.0, the highest amount adsorbed was 43.34 mg g^{-1}.

Among all buffers, MES presented the lowest amount of adsorbed IgG as compared to MOPS, HEPES and Tris–HCl. However, the amount adsorbed was higher to that measured for sodium phosphate, as reported by Gondim et al. (2012). In this study, IgG adsorption capacity was superior to 43.34 mg g^{-1} for all buffers. For the sake of comparison, Wongchuphan et al. (2009) studied rabbit IgG (high purity) adsorption in dye ligand immobilized *Streamline* resin. These authors investigated a large range of pH (between 4.0 and 9.0, with three different buffers)

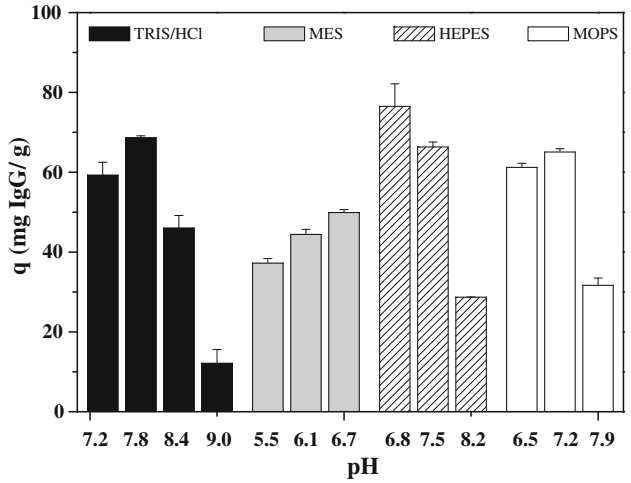

Fig. 4 Effect of buffer solutions at different pHs on IgG adsorption onto E-Ch/Al-Cibacron

and found rabbit IgG uptake of 32.0 mg mL^{-1} at pH 6.0. Hence, according to the present results, E-Ch/Al-Cibacron has shown higher adsorption capacity than those so far reported in the literature.

At this point, the kinetic and equilibrium adsorption experiments were conducted with Tris–HCl (pH 7.8), HEPES (pH 6.8) and MOPS (pH 7.2).

3.2.2 Adsorption isotherms

Kinetic tests were performed before the isotherm measurements. Equilibrium was reached after 3 h of contact between IgG and E-Ch/Al-Cibacron (results not shown). The adsorption isotherms of IgG are shown in Fig. 5 (A, B and C). All isotherms show non-linear behavior, compatible with a description by Langmuir, Freundlich and Langmuir–Freundlich equations. The amount of IgG adsorbed onto E-Ch/Al-Cibacron in full saturation, i.e. maximum adsorption capacity, reaches high concentrations, well beyond those measured in the studies of pH and type of buffer (Fig. 4).

The three adsorption isotherm models (Langmuir, Langmuir–Freundlich and Freundlich) were applied to fit the experimental data for IgG adsorption onto E-Ch/Al-Cibacron. The adsorption models parameters are shown in Table 1. The Freundlich model shows a poor fit, when compared to the other two models. The maximum IgG adsorption capacities were very similar for all buffers (around 100.0 mg g^{-1}), reaching 117.5 mg g^{-1} when Tris–HCl at pH 7.8 was used.

Yavuz et al. (2006) studied the adsorption of IgG and its subclasses (IgG_1, IgG_2, IgG_3 and IgG_4) using poly (hydroxypropyl methacrylate) gel beads with immobilized Reactive Green 5 as adsorbent. The presence of

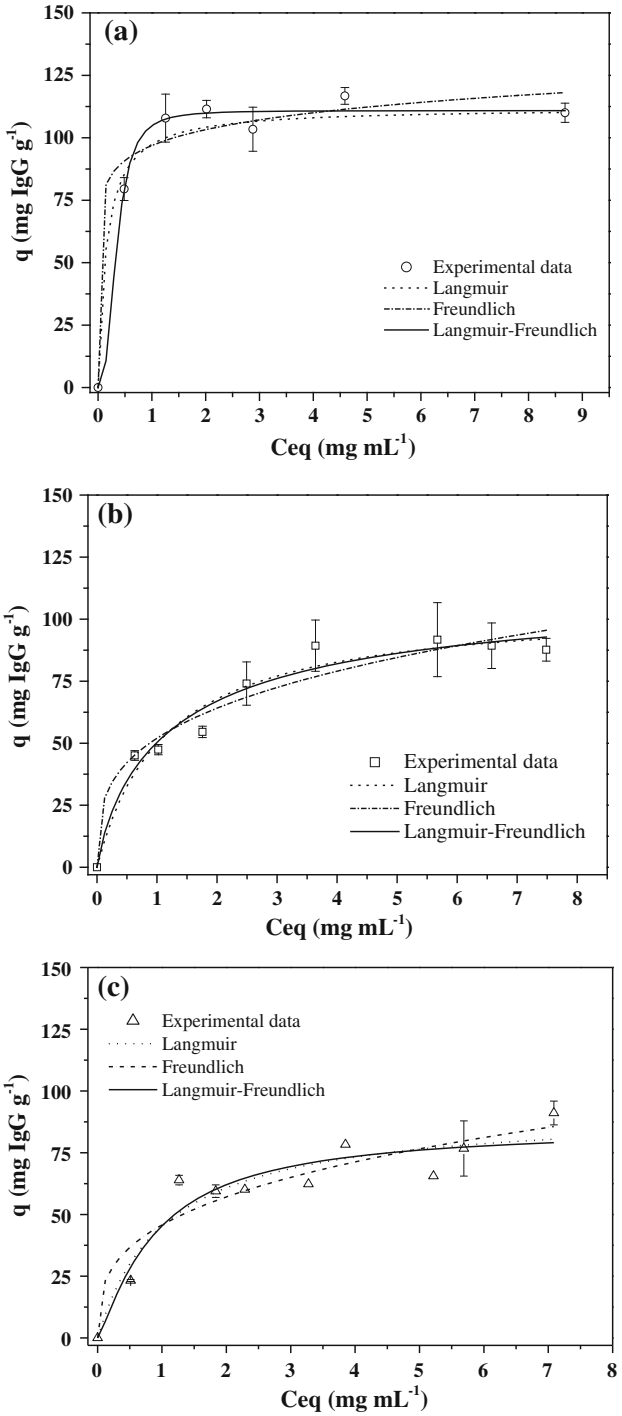

Fig. 5 Adsorption isotherm of IgG onto E-Ch/Al-Cibacron at different buffers solutions: **a** Tris–HCl, **b** MOPS and **c** HEPES. Theoretical profile: The *lines* correspond to fitting (nonlinear regression) of experimental data according to Langmuir, Langmuir–Freundlich and Freundlich models

immobilized dye led to an increase in IgG adsorption from 0.5 mg g^{-1} (without dye) to 71.0 mg g^{-1} (with dye) at pH 7.0. The authors argued that the binding mechanism between IgG and the immobilized dye were hydrophobic

Table 1 Adjusted parameters of Langmuir (L), Freunlich (F) and Langmuir–Freundlich (LF) models fitted to experimental data of IgG adsorption onto E-Ch/Al-Cibacron at buffers MOPS (pH 6.5), HEPES (6.8) and Tris–HCl (pH 7.8) 25 mmol L^{-1}

Parameters	E-Ch/Al-Cibacron								
	Tris–HCl pH 7.8			MOPS pH 6.5			HEPES pH 6.8		
	L	LF	F	L	LF	F	L	LF	F
q_m (mg g^{-1})	117.5	110.9	–	105.9	114.3	–	92.3	85.4	–
K_D ($\times 10^6$ mol L^{-1})	3.4	–	–	5.9	–	–	6.4	–	–
K_F ($\times 10^4$ mol L^{-1})	–	–	6.5	–	–	3.5	–	–	3.0
K_{DLF} ($\times 10^6$ mol L^{-1})	–	0.38	–	–	8.5	–	–	5.9	–
R^2	0.98	0.99	0.92	0.97	0.97	0.95	0.91	0.91	0.89
χ^2	31.9	22.2	75.4	37.5	42.6	49.2	72.2	81.6	85.7
a			10.9			3.3			3.1
n	–	2.7	–	–	0.84	–	–	1.2	–

L Langmuir, *LF* Langmuir–Freundlich, *F* Freundlich

and electrostatic interactions. After 10 adsorption–desorption cycles, these authors reported only 11 % reduction in adsorption capacity. Again E-Ch/Al-Cibacron (reported in the present work) shows superior adsorption capacity as compared to results found in the literature.

An important parameter that qualifies the affinity between the protein and the adsorbent is the dissociation constant (K_D, K_f or K_{DLF}). When the values of these constants are between 10^{-4} and 10^{-8} mol L^{-1}, the ligand may be considered as pseudobioespecific (Vijayalakshmi 1989). According to the values presented n Table 1, all dissociation constants values were between 10^{-4} and 10^{-6} M, indicating that this adsorbent has a moderate affinity for IgG. These K_D results were similar to others found in the literature (Bayramoğlu et al. 2005, 2007; Wongchuphan et al. 2009) for IgG adsorption by dye-ligand affinity chromatography. Bayramoğlu et al. (2007) studied IgG adsorption onto poly (2-hydroxylethylmethacrylate) membrane with immobilized Reactive Green 5 and obtained K_D of 1.6×10^{-6} M. Bayramoğlu et al. (2005) used Reactive Red 120 immobilized onto a polymer and obtained K_D of 2.8×10^{-6} M. Wongchuphan et al. (2009) used dye-ligands immobilized onto *streamline* resin and they obtained k_D of 2.9×10^{-6} M for rabbit IgG adsorption. All afore-mentioned adsorbents were considered to be average affinity chromatographic stationary phases and thus were thought to be suitable for IgG purification.

3.3 Chromatographic experiments with high purity IgG and human serum

Chromatographic experiments were performed by injecting 10 mL of high pure IgG solutions (1.0 mg mL^{-1}) using the same buffers as for adsorption isotherm determinations. The chromatograms are shown in Fig. 6. For all buffers, very similar elution peaks are observed, which indicates IgG has been successfully adsorbed and released,

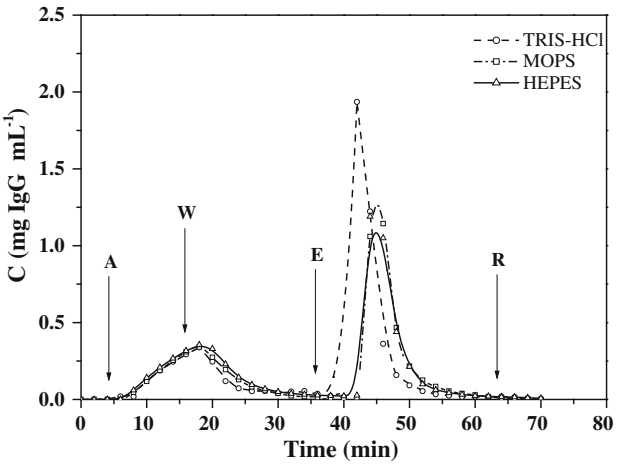

Fig. 6 Effect of the buffer system on the adsorption and elution of high pure IgG onto E-Ch/Al-Cibacron. Adsorption buffer at 25 mmol L^{-1}: Tris–HCl pH 7.8 (*circle*), MOPS pH 6.5 (*square*) and HEPES pH 6.8 (*triangle*). Other conditions: bed volume, 1.0 mL; flow rate, 0.8 mL/min. Protein injected: 10 mL of IgG diluted in adsorption buffer (about 10 mg). Standard deviation of Bradford Method: 1.5 %. Steps: A (adsorption); W (washing); E (elution) and R (regeneration)

corresponding to around 60 % of the injected protein according to mass balances (Table 2). The elution peak of IgG in the experiment performed with Tris–HCl buffer had a larger area than those obtained for the other buffers. The highest amount of IgG could be recovered in this step with this buffer, as can be seen in Table 2 (13.4 mg g^{-1}). For Tris–HCl buffer, not only the highest uptakes in equilibrium were obtained (see Fig. 5a), but also the highest recovered concentration in dynamic mode. Using sodium phosphate as buffer at pH 6.0, a recovered adsorption capacity of 10.6 mg g^{-1} was reported by Gondim et al. (2012).

Figure 7 presents the chromatograms of the experiments performed with Tris–HCl (Fig. 7a), MOPS (Fig. 7b) and

Table 2 Mass balance (MB) of protein adsorption onto E-Ch/Al-Cibacron (0.50 g) with high purity IgG in different buffers for each step: injection (I), adsorption (A), wash (W), elution (E) and regeneration (R)

Steps	Tris–HCl		MOPS		HEPES	
	(mg)	%	(mg)	%	(mg)	%
I	10.7	100	9.2	100	8,9	100
A	1.5	13.9	1.0	11.0	1.1	12.3
W	1.8	17.21	2.5	26.6	2.7	30.8
E	6.5	60.8	5.1	54.8	5.3	59.4
R	0.2	2.1	0.4	4.5	0.1	0.4
MB	10.0	94.0	8.9	96.9	9.2	102.9

IgG concentration determined at 280 nm

HEPES (Fig. 7c) using injected human serum, so that the selectivity of the E-Ch/Al-Cibacron for IgG adsorption may be assessed. The injection volume was 15 mL of human serum diluted 10 times to give a final protein concentration around 4.5–5.0 mg mL^{-1}.

From the results presented in Table 3 (mass balances of chromatographic runs), total adsorbed protein onto E-Ch/Al-Cibacron was 6.62, 6.58 and 7.04 mg g^{-1}, respectively, for Tris–HCl, MOPS and HEPES buffers. When sodium phosphate was used as adsorption buffer (Gondim et al. 2012), the total amount of human serum protein adsorbed onto E-Ch/Al-Cibacron was 4.38 mg g^{-1}.

According to the SDS-PAGE shown in the insets of Fig. 7, high purity IgG is detected in the elution for all buffers used. However, in the run performed with HEPES buffer, the presence of a small amount of human serum albumin (HSA) can also be found. Therefore, this adsorbent showed to be a potential candidate to be used for IgG purification since most protein released upon elution corresponds to IgG for all buffers, especially with Tris–HCl (SDS-PAGE in Fig. 7a).

Despite the immobilized dyes being potential candidates for antibody purification, studies with human serum are still scarce. Bayramoğlu et al. (2007) studied the IgG purification from human serum in batch systems. These authors used HPLC to determine the purity and amount of IgG adsorbed on the pHEMA-RG-5 [poly(2-hydroxyethylmethacrylate) with immobilized Reactive Green 5]. The purity of the IgG eluted from the adsorbent membrane was found to be 81 % with a recovery of 67 %. Wongchuphan et al. (2009) studied the *Streamline* resin with immobilized Reactive Green 5 using a binary synthetic solution containing BSA and rabbit-IgG. From qualitative (electrophoresis) and quantitative (using the Quantity One software) analyses, the authors observed that rabbit-IgG was preferentially adsorbed, accounting for about 64 % of the total protein content adsorbed.

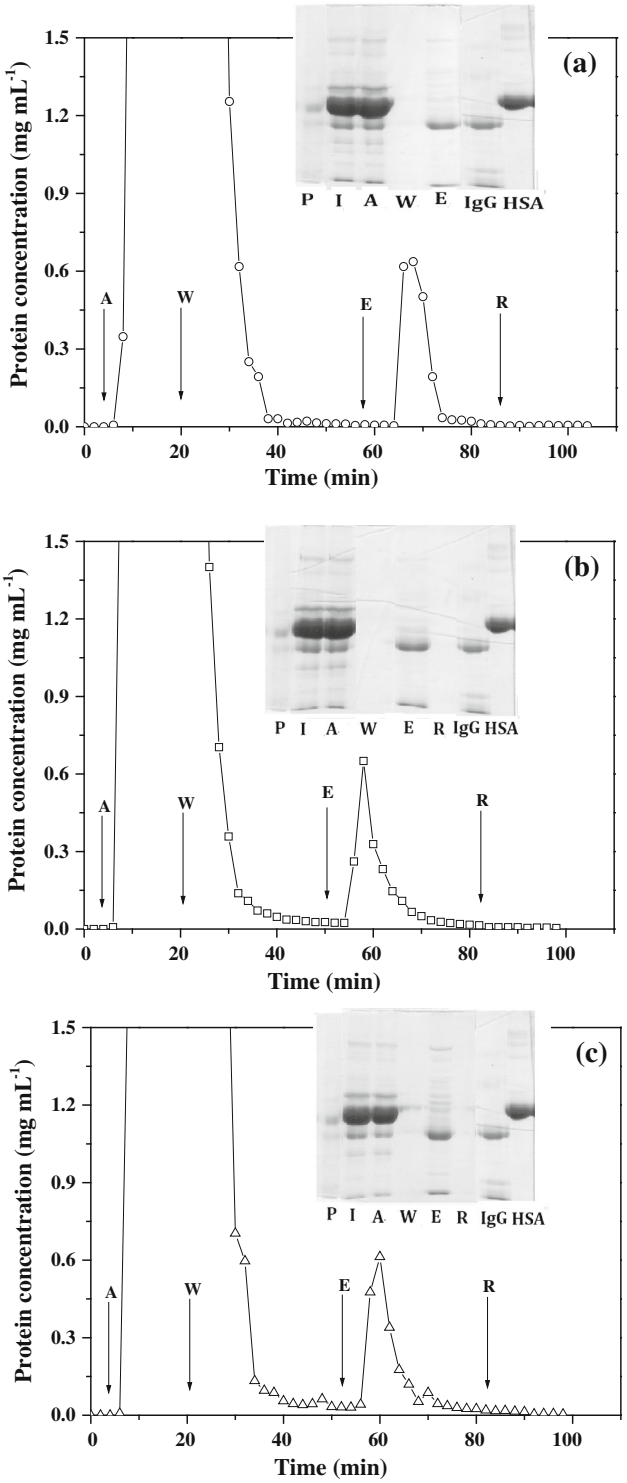

Fig. 7 Effect of the buffer system on the adsorption and elution of IgG from human serum onto E-Ch/Al-Cibacron. Adsorption buffer at 25 mmol L^{-1}: Tris–HCl pH 7.8 (**a**), MOPS pH 6.5 (**b**) and HEPES pH 6.8 (**c**). Other conditions: bed volume, 1.0 mL; flow rate, 0.8 mL/min. Protein injected: 1.5 mL of human serum diluted 10 times in adsorption buffer (final volume of 15 mL, about 70 mg). Standard deviation of Bradford method: 1.5 %. Insert: SDS–PAGE analysis of fractions from the chromatography: P (molecular weight marker), I (injection), A (adsorption); W (washing); E (elution), R (regeneration), IgG (IgG standard), HSA (HSA standard)

Table 3 Mass balance (MB) of protein adsorption onto E-Ch/Al-Cibacron (0.50 g) with human serum diluted in different buffers for each step: injection (I), adsorption (A), wash (W), elution (E) and regeneration (R)

Steps	Tris/HCl		MOPS		HEPES	
	(mg)	%	(mg)	%	(mg)	%
I	67.2	100	68.4	100	77.7	100
A	47.6	70.8	47.9	70.1	46.5	59.9
W	15.0	22.4	16.2	23.7	28.5	36.6
E	3.3	4.8	3.2	4.6	3.3	4.3
R	0.1	0.1	0.1	0.2	0.2	0.3
MB	66.0	98.1	67.4	98.6	78.5	101.1

Total protein determined according to Bradford method (absorbance at 595 nm) (Bradford 1976)

4 Conclusions

Cibacron Blue F3GA immobilized onto chitosan/alginate epoxide showed affinity for human IgG and the adsorption capacity was influenced by the pH and the different buffers used. In batch experiments with E-Ch/Al-Cibacron and in presence of Tris–HCl buffer at pH 7.8, a maximum IgG adsorption capacity of 117.5 mg g^{-1} was reached. Langmuir and Langmuir–Freundlich models were well adjusted to experimental data of batch adsorption and the dissociation constants showed that the E-Ch/Al-Cibacron can be classified as pseudobiespecific adsorbent for affinity chromatography. Chromatographic runs with both pure IgG and human serum also indicated that Tris–HCl buffer at pH 7.8 is a suitable buffer system because it allowed for the highest recovered IgG capacity in the elution step. SDS-PAGE analysis of the eluted fraction in experiments with human serum confirmed that this adsorbent may be used to purify IgG from human serum due to its high selectivity with respect to other serum proteins.

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