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Adsorption of peptides and small proteins with control access polymer permeation to affinity binding sites. Part I: Polymer permeation-immobilized metal ion affinity chromatography separation adsorbents with polyethylene glycol and immobilized metal ions

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ABSTRACT

Despite the many efforts to develop efficient protein purification techniques, the isolation of peptides and small proteins on a larger than analytical scale remains a significant challenge. Recovery of small biomolecules from diluted complex biological mixtures, such as human serum, employing porous adsorbents is a difficult task mainly due to the presence of concentrated large biomolecules that can add undesired effects in the system such as blocking of adsorbent pores, impairing diffusion of small molecules, or competition for adsorption sites. Adsorption and size exclusion chromatography (AdSEC) controlled access media, using polyethylene glycol (PEG) as a semi-permeable barrier on a polysaccharide matrix, have been developed and explored in this work to overcome such effects and to preferentially adsorb small molecules while rejecting large ones. In the first part of this work, adsorption studies were performed with small peptides and proteins from synthetic mixtures using controlled access polymer permeation adsorption (CAPPA) media created by effectively grafting PEG on an immobilized metal affinity chromatography (IMAC) agarose resin, where chelating agents and immobilized metal ions were used as the primary affinity binding sites. Synthetic mixtures consisted of bovine serum albumin (BSA) with small proteins, peptides, amino acids (such as histidine or Val[4]-Angiotensin III), and small molecules-spiked human serum. The synthesized hybrid adsorbent consisted of agarose beads modified with iminodiacetic (IDA) groups, loaded with immobilized Cu(II) ions, and PEG. These CAPPA media with grafted PEG on the interior and exterior surfaces of the agarose matrix were effective in rejecting high molecular weight proteins. Different PEG grafting densities and PEG of different molecular weight were tested to determine their effect in rejecting and controlling adsorbent permeation properties. Low grafting density of high molecular weight PEG was found to be as effective as high grafting density of low molecular weight PEG in the rejecting properties of the semi-permeable synthesized media.

1. Introduction

In general, the same methods to isolate large molecular size proteins are used to recover and isolate peptides and other small biomolecules (hormones, nucleotide fractions, for example) however, the methodologies for more efficient preparative purifications targeted to peptides and small proteins are still underdeveloped. Size exclusion chromatography (SEC) has been so far the method of choice to separate small from large biomolecules [1], however, this technique has a limited sample volume capacity. When adsorption is superimposed on SEC media, large volumes of extracts must be processed to purify trace components, but disturbing accumulation of undesired substances occurs at the same time. In addition, more difficulties are encountered when the target molecules are of low molecular weight and present in a biological fluid at very dilute concentrations. For example, the isolation at a preparative scale of small biomolecules and or biomarkers from human serum is in general hindered by the high concentration of human albumin.

In this work, a concept that considers specific adsorption and size exclusion in the same chromatographic media is explored for the development of more effective separations of small proteins and peptides from complex biological systems. In the first part of the work, to introduce this new type of preparative adsorbent system, immobilized metal ion affinity chromatography (IMAC) with chelating agents and immobilized metal ions, as pseudo affinity ligands were used to test the CAPPA concept, using polyethylene

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glycol as the control access polymer and as the solid matrix, Novarose, an agarose derivative, a high capacity chromatographic polysaccharide resin already used very successfully in IMAC preparative isolation of biomolecules.

Immobilized metal affinity chromatography (IMAC) since its introduction by Porath et al. [2], has developed as a powerful technique that permits protein isolation and purification by exploiting the interaction of proteins with immobilized ions, such as Cu(II), via coordination processes through selected amino acid residues, e.g., histidyl residues. Due to the nature of this type of interactions, IMAC is recognized as a pseudo-affinity chromatography since all the proteins that are able to interact with the immobilized ions will be retained. A significant problem, common to practically all adsorption-based bioseparations arises when recovering small proteins or peptides at low concentrations from a complex mixture containing small and large molecules, such as human serum, where the large molecules hinder the specific adsorption of target low molecular weight compounds by adsorbing non-specifically or obstructing the reach of affinity metal sites by the target molecules.

In the literature, several methods have been explored to diminish undesired protein interaction at binding sites. The concept of combining polymer restriction (say, size exclusion) with other modes of interaction (hydrophobic interactions for example) is well established. Restricted access media (RAM) systems are not new; in fact such RAM matrices for analytical applications have been in existence for quite some time. In general RAM materials packings have been developed for the direct analysis and separation of protein solutions containing small analytes such as drugs and are characterized by mixed hydrophilic–hydrophobic properties. In the past years, the use of restricted-access media (RAM) columns has become an important tool for direct high-throughput analysis of biological fluid samples. For instance, to diminish adsorption of large molecules while allowing adsorption of small molecules, Yoshida et al. [3] developed protein-coated porous silica for drug analysis in plasma, introducing with this approach new ways to isolate smaller proteins, method now known as restricted access media (RAM) analysis [4–6]. The same concept was used by Desilets et al. [7] in the development of adsorbents prepared from reverse phase silica supports that contained an outer layer of hydrophilic polymer as a semi-permeable surface. This hydrophilic layer (detergents Tween or Brij) was able to restrict the adsorption of proteins into the alkylsilane phase while allowing penetration of small molecules. The latter concept was further developed by Kanda et al. [8], these researchers prepared an adsorbent, based on silica particles, for analysis of drugs in serum and plasma that contained hydrophobic sites as adsorption points and a semi-permeable layer of polyethylene glycol or oligoglycerol. In this case, not only the external surface but also the internal surface of the adsorbent contained both the adsorption sites and the semi-permeable barrier. All the aforementioned work has been applied mainly as analytical techniques. Later Stone [9] and Guzman and Porath [10] applied the semi-permeable surface concept with IMAC. They developed agarose-based adsorbents that contained immobilized ions as adsorption sites and polyethylene glycol as semi-permeable barrier to recover small molecules from complex biological mixtures.

The use of polyethylene glycol to prevent protein adsorption onto surfaces is widely known after the work of Mori et al. [11], these researchers observed that adsorption of blood components significantly decreased with the presence of grafted polyethylene oxide. Polyethylene glycol (PEG), also known as polyethylene oxide or polyoxyethylene, is a polymer with ethylene oxide repeating units ($HO{-}CH_2{-}[CH_2{-}O{-}CH_2]_n{-}CH_2{-}OH$) with several properties that makes it attractive to biomedical and biotechnological applications including phase partitioning, peptide and protein modification and non-fouling surfaces [12].

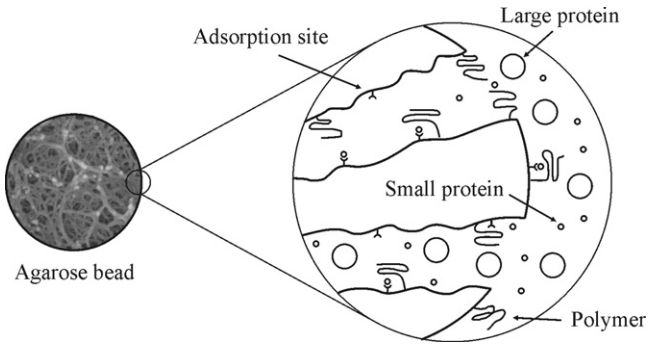

Fig. 1. *Ad*sorption and *Size E*xclusion (AdSEC) schematic concept using *C*ontrolled *A*ccess *P*olymer *P*ermeation *A*dsorbent (CAPPA) media.

The concept described in this work of adsorption and size exclusion (AdSEC) with control access polymer permeation adsorption (CAPPA) on an agarose matrix is illustrated schematically in Fig. 1. Here, the PEG derivatives are grafted outside and inside the polysaccharide structure, restricting the access to the bound affinity sites on both the inside and outside of the particles. Fig. 2 illustrates the immobilization on the matrix of the chelating agent iminodiacetic acid (IDA), loaded with Cu(II) ions, between PEG derivatives that restrict the access to larger proteins, thus preventing its interaction with the surface bound chelating agent.

The main objective of the present methodology is the separation of low-abundance, low molecular weight (LMW) proteins or peptides with elimination of interfering high molecular weight (HMW) proteins.

The first part of this work describes the effect of PEG grafting density and PEG polymer length in agarose particles modified with IDA-Cu(II) sites for the recovery of small peptides and LMW proteins from synthetic and complex biological systems.

2. Experimental

2.1. Chemicals

Novarose 300/40 Act^H spherical beads from Inovata (Bromma, Sweden) were used. The bead structure is 9% cross-linked agarose with a mean particle size of 40 μm and functionalized with a reactive derivative of epibromohydrin. Iminodiacetic acid (IDA), sodium carbonate, methoxy polyethylene glycols (mPEG-OH) of molecular weights 2 kDa and 5 kDa, sodium hydroxide, sodium bicarbonate, 3-(N-morpholino) propanesulfonic acid (MOPS), sodium acetate, sodium chloride, sodium phosphate, copper sulfate, bovine serum albumin (BSA), lysozyme (LYS), insulin (INS), bovine γ-globulin (BIgG), ribonuclease A (RNase A), ethanolamine (ETA), tryptophan, histidine, thionyl chloride, ethanol, ammonium hydroxide, and human serum were purchased from Sigma–Aldrich (St. Louis, MO, USA). SDS-PAGE material for electrophoretic analysis was obtained from Bio-Rad (Hercules, CA, USA). The peptides His-Met, Tyr-Pro-Pro-Trp and Val^4-Angiotensin III were obtained from Bachem AG (Bubendorf, Switzerland).

2.2. Instrumentation

Chromatographic analyses were performed using a Gilson HPLC system (Middleton, WI, USA) equipped with two isocratic pumps, a mixer, a manual injection valve (with a 0.5 mL sample loop), a UV–vis detector, and a fraction collector. The system was controlled by the Unipoint software from Gilson. The chromatographic column was a glass column from Amersham Biosciences (Uppsala, Sweden) having an internal diameter of 0.5 cm and a length of 5 cm. Electrophoretic analyses were performed using a Hoefer miniVE

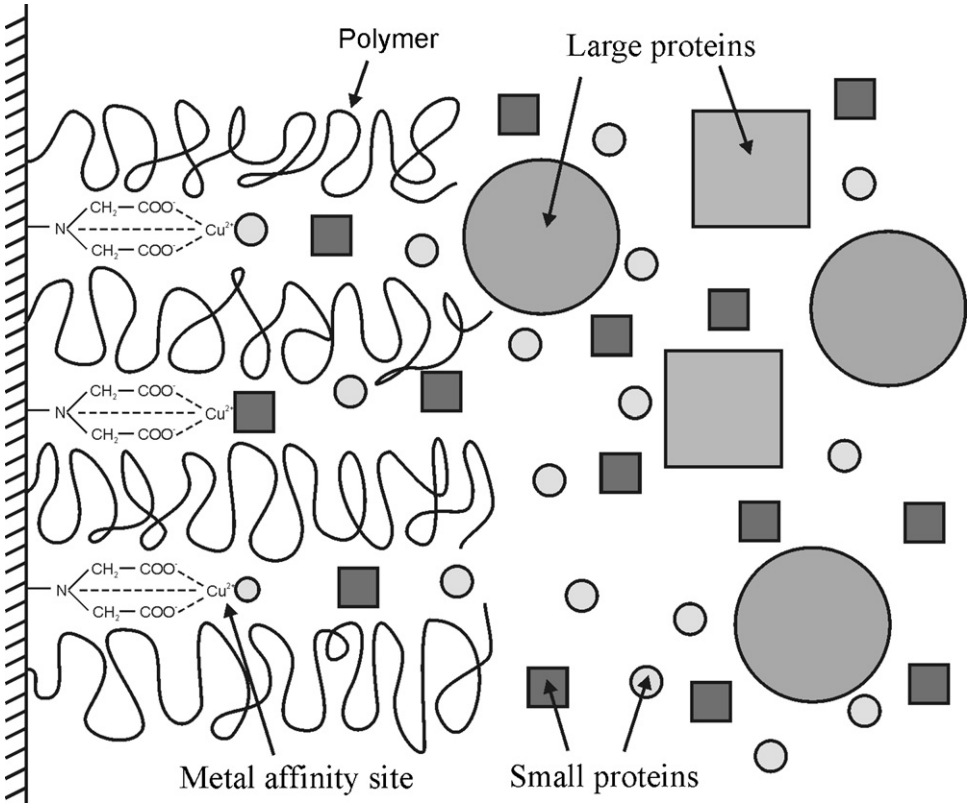

Fig. 2. Illustration of protein adsorption–permeation with Nov-PEG:IDA interaction.

equipment (Hoefer Inc. San Francisco, CA, USA). Synthesis of mPEG-NH_2 derivatives was performed using a Parr (Moline, IL, USA) mini reactor with a 4843 controller.

2.3. Synthesis of mPEG-NH_2 derivative

The procedure for the production of mPEG-NH_2 from mPEG-OH followed the method reported by Birkenmeier et al. [13] where the ammonia–water solution was substituted with an ammonia–ethanol solution as follows. 60 g of mPEG-OH (2 kDa or 5 kDa) were melted at 66 °C removing water with vacuum. 5 mL of freshly distilled thionyl chloride were added and the mixture was rotated at 66 °C under a nitrogen atmosphere for 6 h. Thionyl chloride excess was removed by vacuum and the mixture was added to 2 L of absolute ethanol. Precipitation was allowed to occur at 4 °C overnight. The product was filtered through a #1 Whatman paper to remove excess ethanol and placed in an oven at 50–55 °C to dry. The dried product weighted 56 g and was dissolved in 120 mL of hot absolute ethanol (50 °C). To this solution were added 200 mL of ammonia-saturated ethanol solution. The mixture was placed in a mini reactor where it was heated to 100 °C for 4 h. Afterwards, the mixture was concentrated under vacuum adding absolute ethanol to help remove unreacted ammonia. The concentrated solution was dried in an oven at 50–55 °C for 2 days to give the mPEG-NH_2 derivative. The reaction steps for this synthesis are schematically depicted in Fig. 3.

2.4. Synthesis of hybrid Nov-PEG:IDA CAPPA media

These hybrid adsorbents were prepared by first modifying the matrix with IDA groups followed by incorporation of methoxy-PEG derivatives. Incorporation of IDA groups to the agarose matrix was performed as follows: 1 g of vacuum dried gel was mixed with 10 mL of a solution consisting of 6% (w/v) IDA and 1 M Na_2CO_3. The reaction was allowed to proceed for 1.5 h at room temperature with agitation. Afterwards, the gel was washed with DI water and vacuum dried. 0.5 g of vacuum dried gel was mixed with a solution consisting of 10 mL of 1 M Na_2CO_3 with a certain amount of dissolved PEG polymer (2.5 g for mPEG-NH_2-5k attachment and 1.25 g for mPEG-NH_2-2k attachment). The reaction was allowed to proceed for 24 h at 25 or 40 °C with agitation. After IDA and PEG coupling, the gel was reacted with 10 mL of 0.5 M ethanolamine (ETA) solution for 24 h at 25 °C to block the remaining active groups on

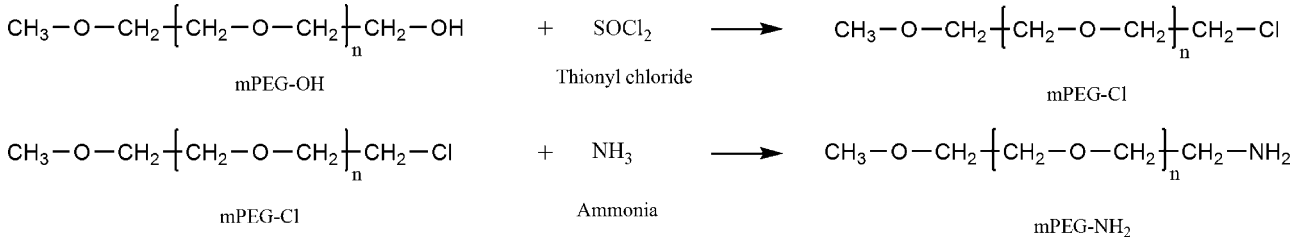

Fig. 3. Schematic representation of synthesis of mPEG-NH_2 derivative.

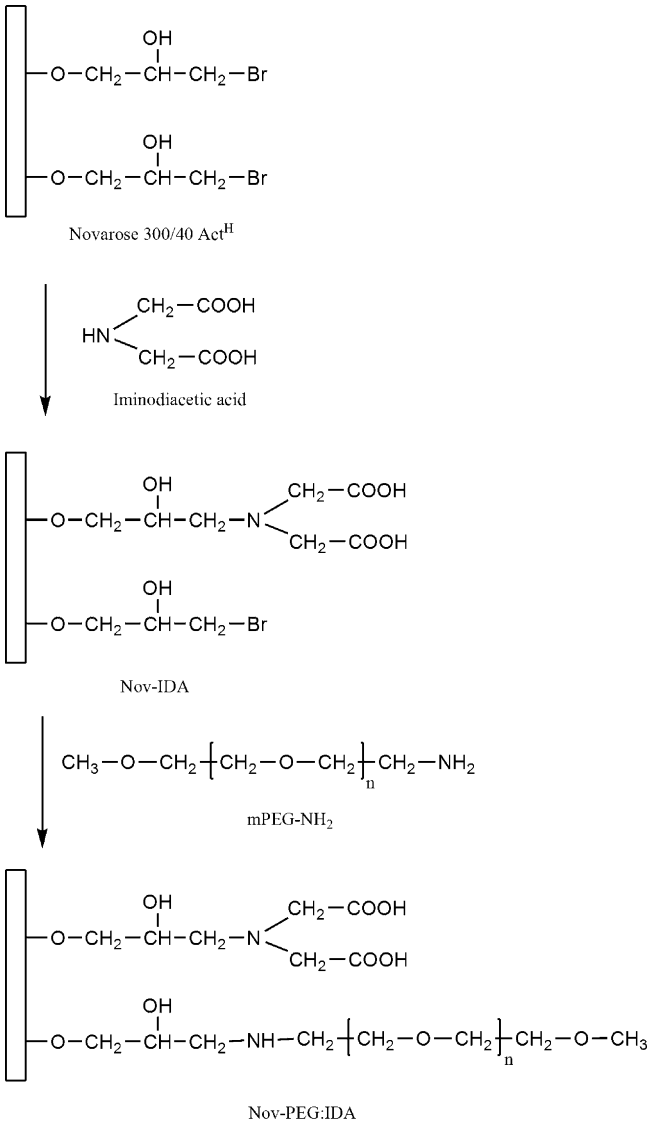

Fig. 4. Schematic representation of the coupling of IDA and grafting of mPEG-NH_2 to activated agarose media.

the surface of the matrix. The reactions for the incorporation of IDA and PEG moieties are depicted schematically in Fig. 4.

2.5. Determination of copper Cu(II) and PEG concentration on hybrid CAPPA media

Copper capacity was measured as follows. A 0.5 cm ID glass column was packed with modified gel to a height of 2.6 cm to give a column volume of 0.5 mL. The gel was washed with 1 M HCl and DI water until reaching neutral pH. Then a 10 mM $CuSO_4$ solution was fed at a flow rate of 0.5 mL/min until saturation. Afterwards, DI water was fed to the column to remove copper ions from the tubing and the void space of the column. The complete process was followed by measuring the absorbance at 825 nm. Once the breakthrough and the washing curves were obtained, copper capacity was measured by the mass balance expressed in Eq. (1). The latter was also the procedure for loading the column with Cu(II) ions for protein or peptide adsorption experiments.

$$\text{Cu(II)capacity}(\mu\text{mol/mL}) = \frac{F}{V}\left(c_{m0}t_f - \int_0^{t_w} c_m\ dt\right) \quad (1)$$

where c_m is the measured copper concentration, mM, c_{m0} is the fed copper concentration, mM, t is the time, min, t_f is the copper feeding time, min, t_w is the final time of experiment, min, F is the flow rate, mL/min, and V is the gel volume, mL.

PEG concentration grafted on the adsorbent was measured indirectly by using the Cu(II) capacity measurement (difference analysis). A gel previously modified with IDA to give an approximate Cu(II) capacity of 20 μmol/mL was then modified with PEG. After PEG attachment, the gel was then further reacted with IDA for a second time, Cu(II) capacity was measured again and the difference between Cu(II) capacities for a gel with and without grafted PEG was considered to be the PEG concentration on the hybrid gel (the maximum copper capacity of a gel reacted with IDA for 24 h was 70–80 μmol/mL).

2.6. Chromatographic adsorption methods

2.6.1. Protein capacity by frontal analysis of model protein systems

For frontal analysis studies, a specified amount of CAPPA gel was packed in a 0.5 cm ID glass column and saturated with copper ions with a 10 mM Cu(II) solution. The system was equilibrated with Buffers B (25 mM sodium acetate, 0.5 M NaCl, pH 4.0) and A (25 mM MOPS, 0.5 M NaCl, pH 7.0) with 30 column volumes. A protein solution was prepared in Buffer A. This solution was fed to the column at a flow rate of 0.5 mL/min for a specified time until the absorbance in the effluent was equal to the absorbance of the inlet solution. Afterwards, the gel was washed with several column volumes of Buffer A to remove non-specifically bound protein, until the absorbance of the effluent returned to the base line. Once the washing step was completed, Buffer B was introduced to remove specifically chelated bound protein. The breakthrough and washing curves were generated by monitoring the absorbance of the effluent at 280 nm. Protein capacity was calculated using Eq. (1) as well. Control adsorption experiments were performed without grafted PEG.

2.6.2. Adsorption by pulse studies of model protein and peptide systems

For pulse studies, a specified amount of CAPPA gel was packed in a 0.5 cm ID glass column and saturated with copper ions with a 10 mM Cu(II) solution as in the previous case. The system was equilibrated with Buffers B (25 mM sodium acetate, 0.5 M NaCl, pH 4.0) and A (25 mM MOPS, 0.5 M NaCl, pH 7.0) with 30 column volumes as well. A protein, peptide or mixture solution was prepared in Buffer A and 0.5 mL of sample was injected to the system at a flow rate of 0.5 mL/min, absorbance of the eluent was followed at 280 or 220 nm. After injection the system was fed with Buffer A for a specified time until the absorbance returned to the base line and Buffer B was then fed to remove adsorbed molecules. When measuring absorbance at 220 nm Buffer A was 25 mM phosphate, 0.5 M NaCl, pH 7.0; while Buffer B was 100 mM phosphate, 0.5 M NaCl, pH 4.0. Control adsorption experiments were performed without grafted PEG.

2.6.3. Protein adsorption from human serum by frontal analysis

A specified amount of CAPPA gel was packed in a 0.5 cm ID glass column and saturated with copper ions with a 10 mM Cu(II) solution. The system was equilibrated with Buffers B (100 mM phosphate, 0.5 M NaCl, pH 4.0) and A (25 mM phosphate, 0.5 M NaCl, pH 7.0) with 30 column volumes. 25 mL of human serum were diluted to 100 mL with Buffer A and were fed to the system at a flow rate of 0.5 mL/min. Absorbance of the eluate was followed at 220 nm. After finishing with sample feeding, the system was fed with Buffer A until the absorbance of the effluent returned to the base line and Buffer B was fed to remove adsorbed molecules. 1-min

fractions were collected and SDS-PAGE analysis was performed on selected fractions. Control adsorption experiments were performed without grafted PEG.

2.7. Analysis of protein chromatographic results

SDS-PAGE analyses of protein containing solutions were performed according to protocols described by Laemmli [14]. The gel was a 10% T and was run at 220 V or 20 mA after sample application (20 μL).

3. Results and discussion

3.1. Synthesis of mPEG-NH_2 derivative

The synthesis of the methoxy amino PEG derivative was carried out using this procedure routinely with a yield in most cases of 62% and assessed by measuring the concentration of amino groups in the PEG derivative using the trinitrobenzene-sulphonic acid (TNBS) test.

3.2. Synthesis of hybrid Nov-PEG:IDA CAPPA media, Cu(II) capacity and PEG concentration

All the agarose derivatives of activated Novarose used in the preparation of hybrid CAPPA media exhibited a copper capacity after exhausted reaction with IDA for 24 h of 70–80 μmol/mL. The gels after functionalization with different grafting densities of PEG derivatives exhibited all uniform properties in terms of copper capacity measurements according to Table 1. For instance, after coupling IDA and measuring copper bound capacity as 18 μmol/mL and with a low PEG grafted density of a 16–17 μmol/mL a further carboxymethylation of the gels provided an increase in copper capacity of 33–35 μmol/mL. Similarly, for gels with high PEG grafted density of around 50 μmol/mL, and copper capacity of 18 μmol/mL, further carboxymethylation resulted in an increase in copper binding of 5–10 μmol/mL. Similar results were obtained for the two size derivatives of m-PEG-NH_2 of 2 and 5 kDa.

According to the agarose composition, each disaccharide unit (agarobiose) presents four hydroxyl groups that could be potentially modified or activated. An approximate analysis of the agarose gel reveals a concentration of hydroxyl groups equal to 2000 μmol/mL (considering hydroxyl groups that were used for cross-linking of the agarose gel) which indicates that only 1% of these hydroxyl groups were modified with IDA. That is, considering a homogeneous distribution of IDA groups and that each IDA group chelates a single Cu(II) ion, there would be an IDA moiety for every 25 agarobiose residues. PEG concentration, obtained by a copper binding difference analysis, revealed values in the range 15–50 μmol/mL. This range of PEG attachment was differentiated by low and high grafting PEG density gels. IDA groups could be considered more homogeneously distributed in the gel than the PEG moieties due to the generally accepted agarose structure as will be discussed later. High PEG grafting density was achieved by increasing reaction temperature during grafting reaction. Increasing temperature does not only affect the reaction rate but the conformation of polymer chains in solution. It has been determined that increasing temperature increases polymer–polymer interactions favoring a high grafting density [15,16].

Table 1
Cu(II) capacity and PEG concentration of synthesized adsorbents.

System	Cu(II) capacity, μmol/mL[a]	PEG concentration, μmol/mL[a]
Nov-IDA	20	0
Nov-PEG:IDA^L	18	17
Nov-PEG_2:IDA^L	18	16
Nov-PEG:IDA^H	18	48
Nov-PEG_2:IDA^H	18	49

[a] mL refers to volume of packed gel, superscript L or H refers to low or high PEG grafting density, respectively. Subscript 2 refers to PEG with a molecular weight of 2 kDa.

3.3. Protein capacity by frontal analysis

Protein capacity by frontal analysis with the systems Nov-IDA-Cu(II) (as control adsorbent) and Nov-PEG:IDA-Cu(II)^L (low polymer grafting density) was measured with BSA, BIgG, LYS, INS, and ConA as model proteins since they are known to present a high affinity toward immobilized copper ions with IDA as chelating agent. BSA has 2–3 histidyl residues on its surface that could interact with immobilized Cu(II) ions, through coordination bonds, its residue His 3 located at the N-terminus has been recognized as the site for Cu(II) binding [17,18]. Similarly, BIgG has 4 histidyl residues on its Fc domain and according to Hale and Beidler [19] and Todorova-Balvay et al. [20], the sequence His 433-Asp 434-His 435 is the most likely sequence that contributes toward metal coordination. LYS, despite having a single histidyl residue on position 15, presents a strong retention on IDA-Cu(II) [17]. INS contains 2 histidyl residues (His 5 and His 10) that could interact with immobilized copper ions [21]. ConA is composed of identical subunits with 6 histidyl residues per subunit which can account for the strong binding with the system IDA-Cu(II) [22].

The results of frontal analyses are shown in Fig. 5, as a control, the system Nov-IDA without Cu(II) ions was also analyzed to discard non-specific interactions. In Fig. 5a, for BSA analysis, the capacity of the system Nov-IDA-Cu(II) (with no grafted PEG) was 15 mg/mL of packed gel. The breakthrough curve obtained for the system without Cu(II) ions and without grafted PEG represents the dead volume of the chromatographic system used for the frontal analysis. No BSA was adsorbed in the absence of immobilized Cu(II) ions. For the system with grafted PEG (Nov-PEG:IDA-Cu(II)^L), BSA capacity was greatly reduced to 0.3 mg/mL. This implies that the PEG moieties are in fact restricting the access of the BSA molecule to the matrix surface. Similar results were obtained in the analysis of BIgG and ConA (data not shown) with 87 and 95% in protein capacity reduction, respectively, in the presence of grafted PEG. Fig. 5b shows the results of frontal analysis for the lower molecular weight protein lysozyme (LYS). In this case, for the system loaded with Cu(II) ions but with no grafted PEG (Nov-IDA-Cu(II)) a LYS capacity equal to 1.7 mg/mL was obtained, a result comparable to values reported in the literature in IMAC separations. A 28% reduction in LYS capacity was obtained for the system containing grafted PEG. In this case as well, as with other proteins, no LYS was adsorbed for the system without Cu(II) ions (Nov-IDA). In the case of insulin (INS), a lower molecular weight protein (data not shown), a 33% adsorption capacity reduction was obtained with grafted PEG. From these results it is clear that the presence of grafted PEG affects protein adsorption with the immobilized metal ions. Low molecular weight proteins (LYS and INS) are rejected by grafted PEG to a lesser extent than high molecular weight proteins such as BSA or BIgG.

As previously described, immobilized copper ions could be considered as being homogeneously distributed in the agarose gel with one immobilized metal ion (for a capacity of 20 μmol/mL) for every 25 agarobiose residues. Even when it can be considered that there is one grafted PEG for every 25 agarobiose units, the substitution cannot be considered homogeneously distributed throughout the gel, considering the general accepted agarose structure. According to Arnott et al. [23], agarobiose units dimerize to form a double helix that has a pitch of 1.9 nm and each strand has a three-fold helical symmetry. This double helix structure was also confirmed by Schafer and Stevens [24]. After the double helices are formed, it

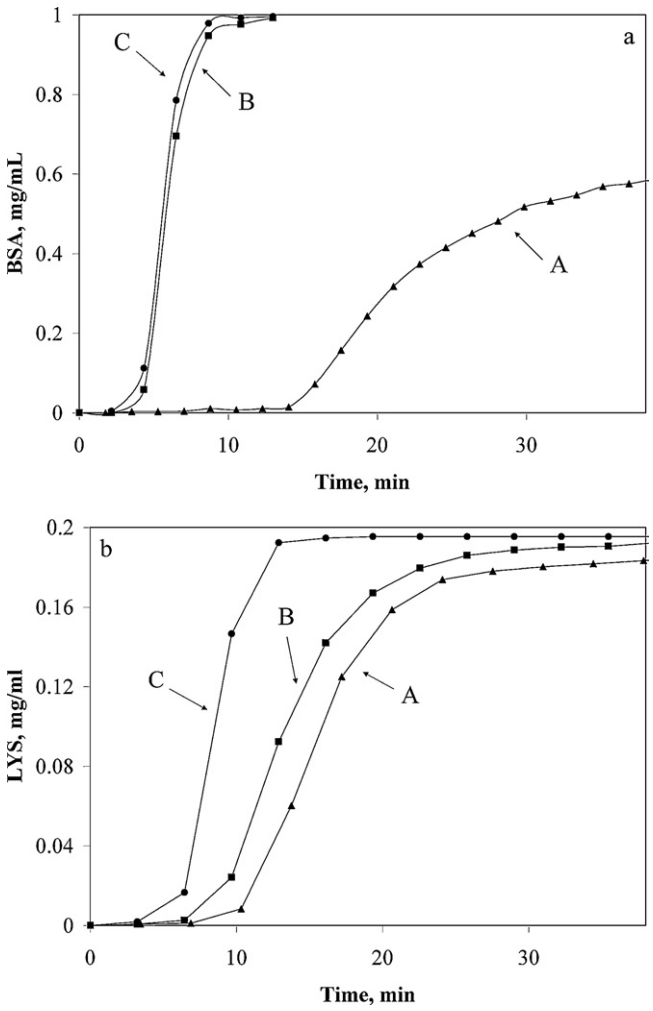

Fig. 5. Frontal analysis of BSA (a) and LYS (b). (A) Nov-IDA-Cu(II), (B) Nov-PEG:IDA-Cu(II)^{L}, (C) Nov-IDA. BSA feeding concentration was 1 mL/mL while LYS was 0.2 mg/mL. The flow rate was 0.5 mL/min.

is presumed that they aggregate and form fibers containing 10–10^4 helices [23,25], that when cross-linked the fibers form a rigid gel. Presumably, PEG groups could be incorporated into the agarose matrix on the surfaces of these cross-linked fibers, however in a very non homogeneous substitution and distribution in the gel since many interior double helices in the matrix will not be able to interact with the PEG molecules in a way to favor uniform grafting of the polymer. Nonetheless, immobilized copper ions that are located on the surface of the fibers are the ones responsible for metal coordination with a protein. Thus, the protein will experience the attraction of the metal ions but the rejection of the grafted PEG as well. The latter is one of two potential configurations that could be considered to occur in the process of protein rejection (Fig. 1). A second possible conformation is also depicted in Fig. 1 and considers that a grafted PEG molecule could be partially blocking the entrance of a pore in the agarose matrix preventing large molecules to enter while allowing diffusion of small molecular weight compounds.

Two theories have been considered toward the molecular description of the interaction between grafted PEG with proteins for the creation of non-fouling surfaces. The first theory considers that the PEG layer induces steric repulsion of proteins associated with an entropic repulsion originated from the compression of the PEG layer [26–28]. The second one considers that the affinity of water molecules toward oxygen atoms in the PEG chains is high

Table 2
Specific properties of peptides and proteins used in this work.

Protein or peptide	Molecular weight, Da	Dimensions (nm^3)	Isoelectric point
BSA[a]	67,000	11.6 × 2.7 × 2.7	4.7–4.8
LYS[a]	14,600	4.5 × 3.0 × 3.0	11.1
RNase A[a]	13,700	3.8 × 2.8 × 2.2	9.4
BIgG[b]	150,000	14.2 × 8.5 × 3.8	4.6–7.2
INS[b]	5800	2.0 × 2.5 × 2.0	5.3
ConA[c]	106,000	6.0 × 7.0 × 7.0	4.5–5.5
Trp	204		5.89
His	155		7.6
Tyr-Pro-Pro-Trp	562		6.1
His-Met	286		7.6
Val^4-Angiotensin III	917		9.3

[a] [33].
[b] [34].
[c] [35].

enough to avoid protein interaction [29]. The PEG water affinity relies on the conformation of the polymers that offers two hydrogen bond acceptors in ideal distance for hydrogen bonding with water [30].

In these results however, the introduction of PEG derivatives onto a polysaccharide matrix and its effect on the behavior of protein rejection is not quite clear. Due to the arbitrary formation of fibers that could contain different number of double helices, it is difficult to establish the conformation of grafted PEG in the agarose matrix, nonetheless, the three-dimensional nature of the fibers and the position of reactive hydroxyl groups indicate that the most likely conformation will be of a "mushroom" type. Considering a single double helix and that the grafted PEG is in a "mushroom" conformation, the Flory radius will be given by Eq. (2) [31], where N is the number of monomers (ethylene oxide) and a is the size of the monomers equal to 0.278 nm [29].

$$R_f = aN^{3/5} \quad (2)$$

Thus, for PEG derivatives with a molecular weight of 5 kDa the Flory radius is equal to 5 nm. This will imply that the area of influence of a grafted PEG molecule will be a circle of diameter equal to approximately 10 nm. Considering a single double helix with a single grafted PEG moiety, the polymer will be able to "cover" 30 agarobiose units and thus would influence the access to a copper ion in that double helix. Nonetheless, the area of influence of a single grafted PEG could potentially include several double helices. This coverage could explain the reason underlying the rejecting properties of even a low PEG grafting density in an agarose gel. Even though it is generally recognized that high grafting densities are required for protein rejection, it has been experimentally found, and observed in this work, that a low coverage with a high molecular weight PEG can lead to effective protein resistance since the gaps between anchored chains are covered by this high molecular weight PEG [15,32].

According to the dimensions of some proteins presented in Table 2, proteins that have a dimension smaller than the Flory radius of a grafted PEG 5 kDa molecule (5 nm) are able to penetrate the PEG barrier and coordinate with immobilized Cu(II) ions for a low grafting density, even though protein capacity for LYS and INS decreased by 30% approximately. Large molecular weight proteins are being excluded to a much larger extent (90% approximately). Despite these observations, a high PEG grafting density is expected to influence the adsorption of small proteins like INS to a much higher extent of rejection.

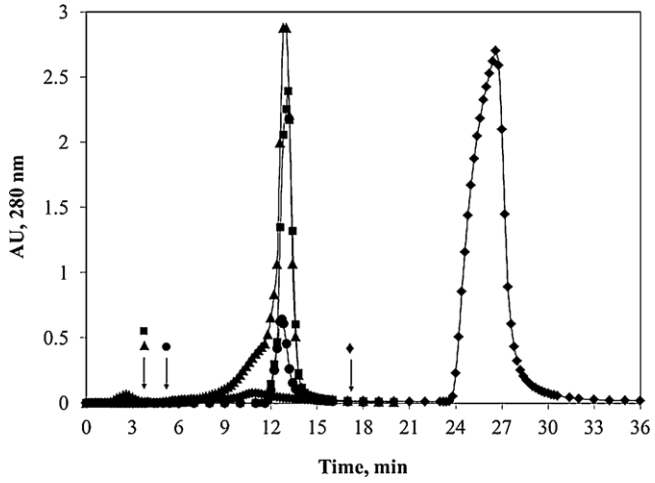

Fig. 6. Pulse studies chromatograms for Nov-IDA-Cu(II). (●) Pure BSA (1.5 mg/mL), (♦) mixture of BSA (1.5 mg/mL) and INS (0.11 mg/mL), (■) mixture of BSA (1.5 mg/mL) and Trp (0.05 mg/mL), (▲) mixture of BSA (1.5 mg/mL) and Tyr-Pro-Pro-Trp (0.05 mg/mL). The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where Buffer B was fed to the column for the particular sample.

3.4. Protein and peptide analysis with pulse studies

Several proteins, peptides and mixtures (composed of BSA plus another protein or peptide) samples were prepared and analyzed by pulse studies on the systems Nov-IDA-Cu(II), Nov-PEG:IDA-Cu(II)^{L} and Nov-PEG:IDA-Cu(II)^{H}. Here also, general results indicate that small proteins and peptides are able to penetrate the hybrid gels. From the pulse studies of binary mixtures it is clear that the hybrid system is able to discriminate between molecules of different sizes allowing the low molecular weight entities to adsorb with the immobilized metal ion while rejecting most of the BSA.

Adsorption. Fig. 6 shows chromatograms for pulse studies using the system Nov-IDA-Cu(II). In the figure, the results show that a pulse of pure BSA (1.5 mg/mL), indicated by the solid dots (●) was almost completely retained by the immobilized Cu(II) ions and later recovered using Buffer B. Similar results (data not shown) were obtained when performing pulse studies with pure samples of INS, Trp and Tyr-Pro-Pro-Trp. The figure also shows the chromatogram obtained when a mixture of BSA (1.5 mg/mL) and INS (0.11 mg/mL), indicated by the solid diamonds (♦) was analyzed with the system Nov-IDA-Cu(II). It is clear that both proteins were adsorbed and recovered without being separated. Similar results were obtained for the case of mixtures of BSA (1.5 mg/mL) with Trp (0.05 mg/mL) and BSA (1.5 mg/mL), solid squares (■) results and with Tyr-Pro-Pro-Trp (0.05 mg/mL), results indicated by the solid triangles (▲) in the figure. Thus, all the test molecules were able to interact with the immobilized Cu(II) ions irrespective of the size of the molecule and separation between different species was not possible.

Desorption. Adsorbed proteins and peptides desorbed with Buffer B (pH 4.0) confirming that all molecules, containing histidyl surface residues, were in fact retained by coordination interaction between the biomolecules and the immobilized Cu(II) ions since the pK_a value of histidine side chain presents values around 6.0. Ionic interactions were suppressed by creating a high ionic strength environment adding a high concentration of NaCl in the adsorption (pH 7.0) and desorption (pH 4.0) buffers. It is generally recognized that histidyl residues are the most likely amino acids responsible to form coordination bonds, through imidazole group in the side chain of histidines, with immobilized metal ions [2]. Tryptophan is also believed to participate to a lesser extent in the retention process [36] through its indole side group when being part of a polypeptide. On the other hand, in the case of pure tryptophan, the carboxylic and amino end groups could participate in the coordination process [37] with immobilized copper ions presenting a stability constant ($\log \beta$) for Cu(II) equal to 15.32 [38] In most cases, this can be lowered with the use of a low pH desorption buffer, thus allowing the removal of tryptophan containing species from the system Nov-IDA-Cu(II).

Fig. 7 shows chromatograms obtained from pulse studies for the system Nov-PEG:IDA-Cu(II)^{L}. The chromatogram for BSA (1.5 mg/mL) pulse study is presented in Fig. 7a where it is evident that most of the BSA molecules (more than 90%) were prevented from adsorbing to the matrix due to the presence of grafted PEG. On the other hand, pure samples of Trp (0.05 mg/mL), Tyr-Pro-Pro-Trp (0.5 mg/mL), and Val^4-Angiotensin III (0.2 mg/mL) were almost entirely retained and also recovered with Buffer B (data not shown). Fig. 7b shows the result of a pulse study of a mixture consisting of BSA (1.5 mg/mL) and the amino acid Trp (0.05 mg/mL). In this case, it can be seen very clearly that nearly all the BSA was rejected while most of the amino acid was retained, and later also recovered by lowering the pH of the running buffer.

Similar pulse studies results were obtained with the systems consisting of mixtures of BSA (1.5 mg/mL) and the small molecular weight tetrapeptide Tyr-Pro-Pro-Trp (0.05 mg/mL) and with the system BSA (0.5 mg/mL) and the polypeptide Val^4-Angiotensin III (0.1 mg/mL), where despite the fact that all species had affinity toward immobilized Cu(II) ions, the effective separation is quite evident as seen for both systems in Fig. 7c and d, respectively. In this particular case, the small molecule Val^4-Angiotensin III was the only one with a positive charge during adsorption experiments (Table 2). All other molecules (including BSA) had a negative charge during pulse studies. Considering also that LYS had a positive charge during frontal analysis and that the enzyme was also retained in the system Nov-PEG:IDA-Cu(II)^{L}, it can be stated with confidence that the charge of the molecule was not a determining factor for biomolecules to be excluded or to penetrate the barrier formed by the grafted PEG on the hybrid CAPPA gels (more evidence is presented later). A similar result was found by Archambault and Brash [26] when working with grafted PEG on polyurethane–urea surfaces where they determined that there was not any clear effect of the isoelectric point of proteins on the ability of the modified surfaces to inhibit protein adsorption.

A feature observed in these mixture separation systems with Nov-PEG:IDA-Cu(II)^{L} is that the presence of BSA appears not to affect at all, under the experimental conditions, the adsorption of the small molecules, for instance, in the case of BSA and tryptophan, the area below the curve for tryptophan adsorption only was equal to 1.46 AU·CV, while the area below the curve for the adsorbed fraction of the mixture of BSA and tryptophan gave a value of 1.49 AU·CV for the tryptophan area. A similar result was obtained for the mixture of BSA and Val^4-Angiotensin III. This implies that, despite the possible interaction between the BSA and the peptides, the effect of the polymer permeation of PEG prevailed at one point in "filtering" the small analytes through the PEG barrier and rejecting entirely BSA while the analytes reach the immobilized Cu(II) ions on the fibers. This possible situation has been accepted even for a "brush" configuration of grafted PEG [28].

Fig. 8 shows chromatograms obtained from pulse studies for the system Nov-PEG:IDA-Cu(II)^{H}. Fig. 8a, for the pulse study of pure BSA (2 mg/mL) demonstrates that almost all the BSA molecules where prevented from adsorption in the Cu(II) ions. Similar to the results obtained with pure samples of low molecular weight solutes using a system with low PEG grafting density, the system Nov-PEG:IDA-Cu(II)^{H} successfully adsorbed (data not shown) the amino acid His (0.02 mg/mL), the dipeptide His-Met (0.04 mg/mL) and the peptide Val^4-Angiotensin III (0.05 mg/mL). Fig. 8b shows the chromatogram obtained when analyzing the mixture of BSA (1 mg/mL) and His

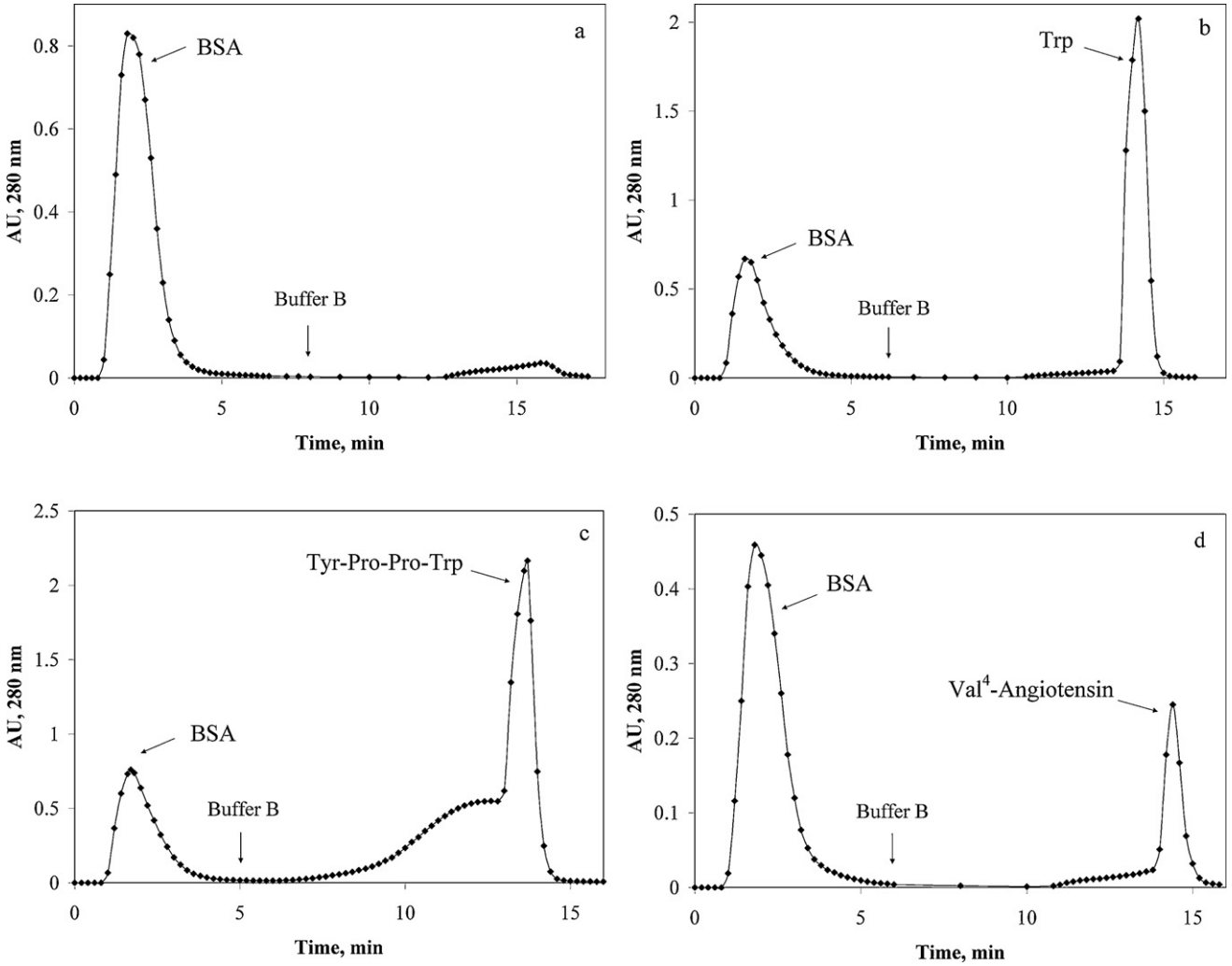

Fig. 7. Pulse studies chromatograms for Nov-PEG:IDA-Cu(II)^{L}. (a) Pure BSA (1.5 mg/mL), (b) mixture of BSA (1.5 mg/mL) and Trp (0.05 mg/mL), (c) mixture of BSA (1.5 mg/mL) and Tyr-Pro-Pro-Trp (0.05 mg/mL) and (d) mixture of BSA (0.5 mg/mL) and Val^4-Angiotensin III (0.1 mg/mL). The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where Buffer B was fed to the column for the particular sample.

(0.01 mg/mL) where, as before, BSA was rejected while His was adsorbed and recovered using Buffer B. The separation of His-Met (0.02 mg/mL) from BSA (1 mg/mL) is presented in Fig. 8c. A similar separation was obtained for the mixture of BSA (1 mg/mL) and Val^4-Angiotensin III (0.025 mg/mL) as shown in Fig. 8d.

For the high PEG grafting density system, His and His-Met, with practically half of the molecules with positive charge during pulse studies were considered to corroborate that charge in fact was not a determining factor for a molecule to be excluded by the grafted PEG chains. Similar results were found with the low PEG grafting density systems and with the high PEG grafting density, when dealing with mixtures of small molecules and BSA. Such results allow us to ascertain that not only the adsorbents in these preparations can help discriminate between molecular weights biomolecules, but allows practically complete recovery of small molecular weight molecules.

The system with high PEG grafting density provided results similar to reports found in the literature that indicate that the higher the grafting density of grafted PEG, the better the protein adsorption resistance [15,16]. As it is shown later (Table 4), high PEG grafting density gel decreased adsorption of BSA by more than 95% compared with the regular gel with no grafted PEG. To determine the effect of PEG grafting density on low molecular weight protein adsorption pulse studies were conducted using RNase A with the systems Nov-IDA-Cu(II), Nov-PEG:IDA-Cu(II)^{L} and Nov-PEG:IDA-Cu(II)^{H}. The results are shown in Fig. 9. In this case, the low PEG grafting density reduced RNAse A adsorption by 22%, while high PEG grafting density reduced adsorption by 91%. From these results it is quite clear and as expected, PEG grafting density dictates the size of the molecules that can reach the adsorption sites and controls effectively the permeation of most solutes.

3.5. Human serum adsorption

Both high and low PEG grafting density adsorbents were tested by pulse studies with samples of human serum. Table 3 summarizes the results of protein adsorption from human serum where the area under the curve corresponds to the desorption peak. SDS-PAGE results for the system Nov-IDA-Cu(II) showed adsorption of albumin, immunoglobulins and transferrin (all high molecular weight proteins). The presence of low PEG grafting density clearly reduced the amount of proteins that could interact with the immobilized Cu(II) ions. 97% of reduction in protein adsorption was obtained using a high grafted PEG density. SDS-PAGE analysis for the PEG-containing systems blocked with ETA is shown in Fig. 10a for selected fractions from the desorption peak. This analysis reveals that several proteins are retained by the system Nov-PEG:IDA-Cu(II)^{L} including albumin and transferrin, along with

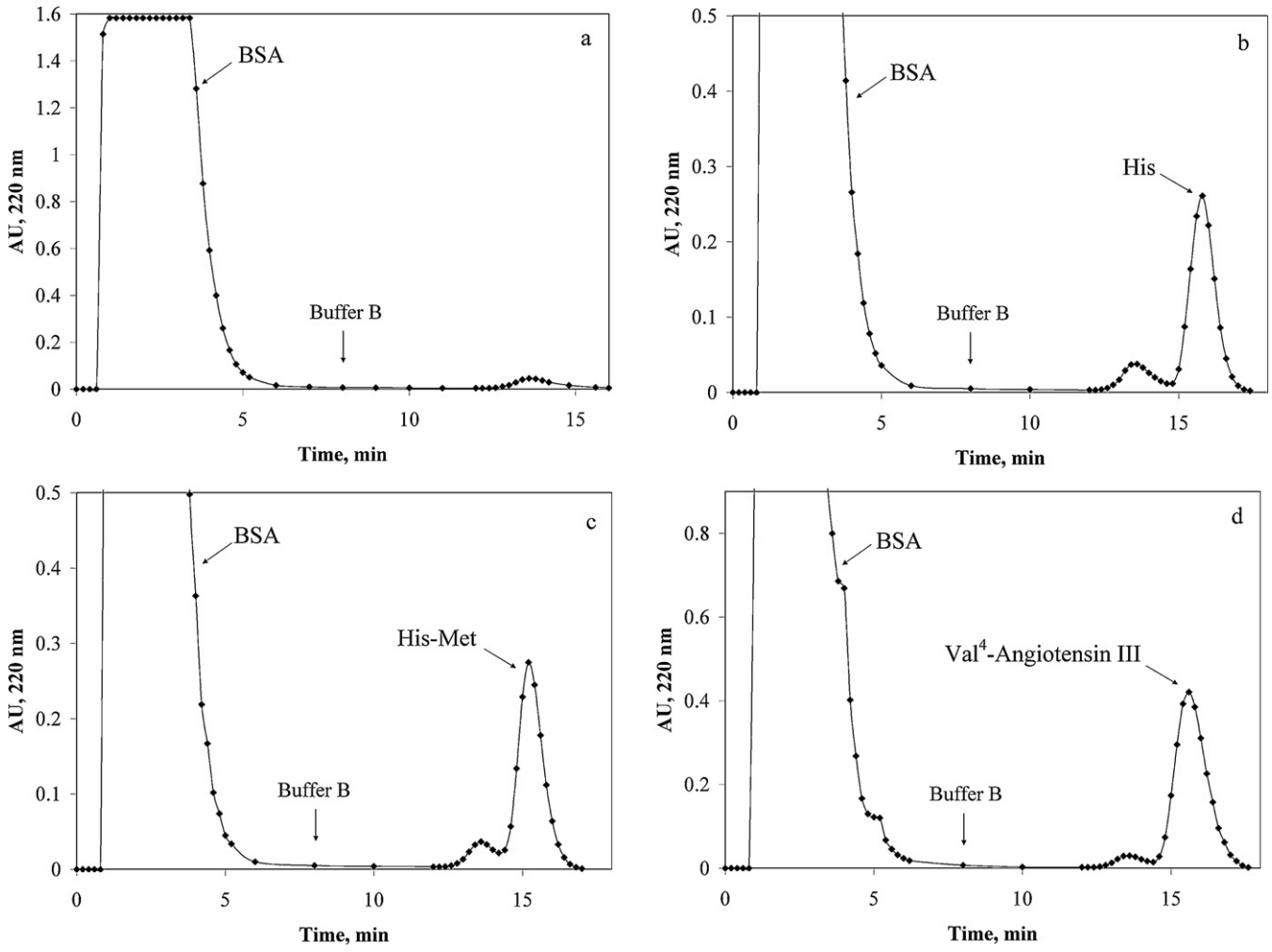

Fig. 8. Pulse studies chromatograms for Nov-PEG:IDA-Cu(II)^{H}. (a) Pure BSA (2 mg/mL), (b) mixture of BSA (1 mg/mL) and His (0.01 mg/mL), (c) mixture of BSA (1 mg/mL) and His-Met (0.02 mg/mL) and (d) mixture of BSA (1 mg/mL) and Val^{4}-Angiotensin III (0.025 mg/mL). The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where Buffer B was fed to the column for the particular sample.

a protein with a molecular weight close to 15 kDa. The system with a high PEG grafting density shows that practically no retention of high molecular weight proteins occurred, while three small molecular weight proteins with molecular weights between 6 and 15 kDa were effectively retained with the CAPPA media. The desorption chromatograms for human serum adsorption studies are shown in Fig. 10b for the systems Nov-PEG:IDA-Cu(II)^{L} and Nov-PEG:IDA-Cu(II)^{H}.

From the latter results, it seems quite clear that grafted PEG, whether at high or low grafting densities, was capable of reducing very effectively, protein adsorption even when working with a highly concentrated protein solution, such as human serum.

Table 3
Human serum adsorption.

System	Cu(II) capacity, μmol/mL	PEG concentration, μmol/mL	Area below the curve, AU min	Adsorption reduction[a], %
Nov-IDA-Cu(II)	20	0	9.6	0
Nov-PEG:IDA-Cu(II)^{L}	18	17	1.2	87
Nov-PEG:IDA-Cu(II)^{H}	18	48	0.3	97

[a] Compared to the correspondent system Nov-IDA-Cu(II).

Table 4
Comparison of BSA adsorption by pulse analysis.

System	Cu(II) capacity, μmol/mL	PEG concentration, μmol/mL	Area below the curve, AU min	Adsorption reduction[a], %
Nov-IDA-Cu(II)	20	0	0.80	0
Nov-PEG:IDA-Cu(II)^{L}	18	17	0.08	90
Nov-PEG$_2$:IDA-Cu(II)^{L}	18	16	0.68	15
Nov-PEG:IDA-Cu(II)^{H}	18	48	0.01	99
Nov-PEG$_2$:IDA-Cu(II)^{H}	18	49	0.03	96

[a] Compared to the system Nov-IDA-Cu(II).

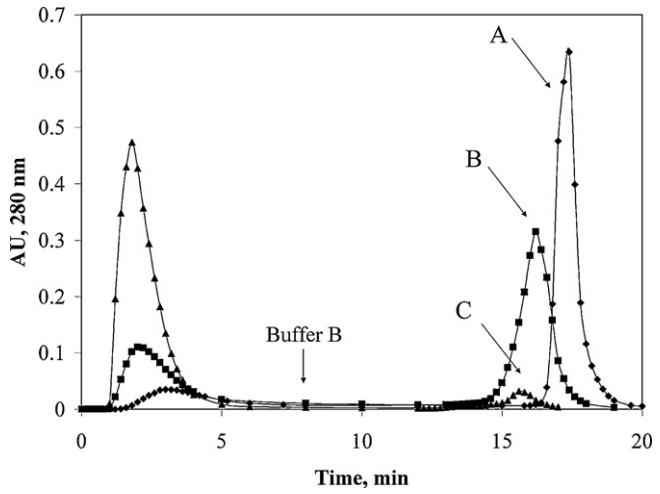

Fig. 9. RNase A pulse studies on studied adsorbents. (A) Nov-IDA-Cu(II), (B) Nov-PEG:IDA-Cu(II)^{L} and (C) Nov-PEG:IDA-Cu(II)^{H}. Sample concentration was 1 mg/mL, sample volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where Buffer B was fed to the column for desorption.

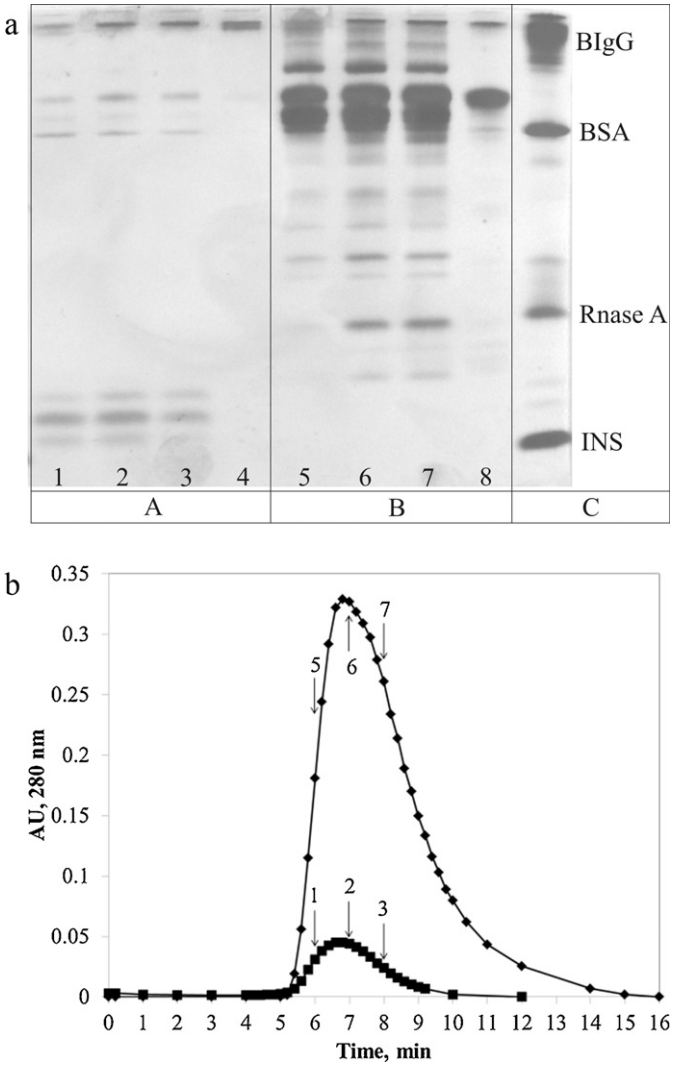

Fig. 10. (a) SDS-PAGE of fractions recovered from human serum adsorption experiments. (A) Nov-PEG:IDA-Cu(II)^{H}, (B) Nov-PEG:IDA-Cu(II)^{L} and (C) molecular weight standards. (1–3) and (5–7) recovered protein from pH change, (4 and 8) recovered protein with EDTA washing. (b) Desorption chromatograms for human serum adsorption experiments for the systems (■) Nov-PEG:IDA-Cu(II)^{H} and (♦) Nov-PEG:IDA-Cu(II)^{L}.

Average typical concentrations of albumin, immunoglobulins and transferrin in the human serum sample are 21.3, 6.5 and 1.3 mg/mL, respectively.

3.6. Spiked human serum pulse studies

The system Nov-PEG:IDA-Cu(II)^{H} was used to test permeation and adsorption with high protein concentration mixture toward its ability to discriminate between small and large molecules. Human serum was spiked with small analytes and its retention characteristics were determined with the hybrid gel. Area below the curve for protein adsorbed from non-spiked human serum sample revealed a value of 1.18 AU min. Areas below the curve for adsorbed fractions of pure samples of His, INS and Val^{4}-Angiotensin III presented values of 1.28, 1.51 and 0.97 AU min, respectively, while the values corresponding to the adsorbed fraction after these molecules were spiked in human serum were (after subtraction of the value corresponding to the non-spiked human serum) 1.25, 1.65 and 0.95 AU min, respectively. These results corroborate the potential of the synthesized polysaccharide CAPPA media for the recovery of small molecular weight molecules present in a mixture containing a high protein concentration of large molecular weight proteins.

3.7. Effect of grafted PEG chain length on the adsorption of BSA

To test the effect of grafted PEG chain length on the permeation and rejection properties, adsorbents containing mPEG-NH_2 with a molecular weight of 2 kDa were prepared at low and high grafting density. In these experiments, the remaining active sites of the adsorbents containing mPEG-NH_2-2k were blocked with ETA and their performance was compared with adsorbents containing mPEG-NH_2-5k for pulse studies with BSA. The results are shown in Table 4. According to theory, the Flory radius of a grafted PEG chain of 2 kDa, considering a "mushroom" configuration, can be obtained with a value of 3 nm. This would imply that the area of influence of a grafted PEG molecule would be a circle of diameter equal to approximately, 6 nm. At low PEG grafting density, it was observed that the low molecular weight grafted PEG rejects protein less efficiently than what it was obtained with the 5 kDa grafted PEG. This result could be explained by the fact that in this situation the immobilized Cu(II) ions are more accessible to binding by BSA since the contact is now less restricted by PEG moieties. On the other hand, an increase in grafting density for the 2 kDA and 5 kDA grafted PEG chains eventually approaches a similar behavior between these two grafted chains, with only a slight enhanced rejection by the high molecular weight PEG. Archambault and Brash [26] found that rejecting properties of grafted PEG 2 kDa or PEG 5 kDa were in fact, similar. Gombotz et al. [39] found that the adsorption of albumin and fibrinogen decreased with an increase in PEG molecular weight with an apparent limit of 3.5 kDa. The results obtained in the present work are comparable with the observations and results of these researchers for high PEG grafting density for the two molecular weights of PEG used in the study. However, it is worth noting that the reported studies in the literature were performed with PEG grafted on flat surfaces while in this work the grafting of PEG chains was performed on a polysaccharide matrix, a much more complex architectural surface.

4. Conclusions

In this work, new chromatographic polysaccharide adsorbents for specific low molecular weight biomolecule preparative separations were synthesized and used to effectively separate low molecular weight peptides and low molecular weight proteins from large molecular weight proteins. In the first part of this work, IMAC was used as the adsorption mode with IDA as the chelating agent.

The high capacity, hybrid polysaccharide adsorbent, Nov-PEG:IDA, prepared by incorporating PEG derivatives as controlled access polymer permeation adsorption (CAPPA) media and IDA loaded with copper metal ions as the affinity ligand, were effectively used to resolve and separate amino acids, peptides and low molecular weight proteins from model and complex real protein mixtures. This high capacity media when modified with IDA and PEG at appropriate grafting densities, excluded BSA almost entirely, restricting its access to the chelating binding sites. The small biomolecules studied in the first part of this work such as the amino acids His and Trp, and the peptides His-Met and Val^4-Angiotensin III, were effectively separated from mixtures containing large amounts of high molecular weight proteins such as BSA or human serum. These adsorbents were also able to very effectively resolve and separate relatively low molecular weight proteins from human serum by rejecting almost entirely all high molecular weight proteins, results that we believe will have an immense impact in the preparative isolation analysis and development of small molecular weight biomarkers. Grafting PEG density and molecular weight are relevant factors and definitely more research work on their effects will help elucidate protein rejection and permeation in these polysaccharide matrices. The results show that these PEG–IMAC hybrid adsorbent systems can practically eliminate in chromatographic experiments all albumin with very effective separation of small compounds, compared with the use of IMAC chromatography alone, or gel permeation alone or by using a sequential separation consisting of IMAC followed by gel permeation or with gel permeation followed by IMAC separation. The potential of the proposed control access polymer permeation (CAPPA) media to develop preparative isolation and purification methods for low molecular weight peptides and proteins based on adsorption and size exclusion chromatographic (AdSEC) media with IMAC adsorbents, as shown in the first part of this work and with other similar systems is very promising. This work represents an initial investigation and shows the inherent problems involved in the development of this dual preparative separation concept. Other systems coupling ion exchange, hydrophobic and affinity adsorption media with polymer permeation that are under investigation will help elucidate theoretical and experimental problems associated in the effective implementation of this methodology.

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