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Adsorption of peptides and small proteins with control access polymer permeation to affinity binding sites. Part II: Polymer permeation-ion exchange separation adsorbents with polyethylene glycol and strong anion exchange groups

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ABSTRACT

In chromatographic separations, the most general problem in small biomolecule isolation and purification is that such biomolecules are usually found in extremely low concentrations together with high concentrations of large molecular weight proteins. In the first part of this work, adsorption and size exclusion chromatography (AdSEC) controlled access media, using polyethylene glycol (PEG) as a semi-permeable barrier on a polysaccharide Immobilized Metal Affinity Chromatography (IMAC) matrix was synthesized and used to develop chromatographic adsorbents that preferentially adsorb and separate low molecular weight biomolecules while rejecting large molecular weight proteins. In this second part, we expand the concept of controlled access polymer permeation adsorption (CAPPA) media by grafting polyethylene glycol (PEG) on a high capacity polysaccharide ion exchange (IEX) chromatographic resin where PEG acts as a semi-permeable barrier that preferentially allows the permeation of small molecules while rejecting large ones. The IEX resin bearing quaternary ammonium groups binds permeated biomolecules according to their ion exchange affinity while excluding large biomolecules by the PEG barrier and thus cannot compete for the binding sites. This new AdSEC media was used to study the retention of peptides and proteins covering a wide range of molecular weights from 1 to 150 kDa. The effect of protein molecular weight towards retention by ion exchange was performed using pure protein solutions. Recovery of insulin from insulin-spiked human serum and insulin-spiked human urine was evaluated under polymer controlled permeation conditions. The CAPPA media consisted of agarose beads modified with amino-PEG-methoxy and with trimethyl ammonium groups, having chloride capacities between 20 and 40 μeq/mL and were effective in rejecting high molecular weight proteins while allowing the preferential adsorption of small proteins and peptides.

1. Introduction

In the first part of this work we describe the problems associated with the purification of peptides and small proteins when in the presence of large molecular weight proteins. In general, 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. Ion exchange chromatography is considered one of the most common separation and purification techniques for proteins, its application however, to the specific isolation of small biomolecules when in the presence of larger proteins on a larger than analytical scale is still underdeveloped.

According to Yamamoto et al. [1] two facts established ion exchange chromatography (IEX) as a separation technique for proteins. The first one was the development of hydrophilic adsorbents (cellulose-based) by Peterson and Sober [2] and the second was the work of Porath and Flodin [3] with the synthesis of cross-linked dextran gels. Ion exchange chromatography of proteins is based on ionic interactions between charged residues in the proteins and immobilized charged ligands of opposite charge. Due to the nature of the interaction, the adsorption process by ion exchange is reversible and easily controlled to separate proteins once they are retained by the adsorbent. This simplicity in operation makes ion exchange chromatography the most common separation and purification technique for proteins [4].

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Proteins and peptides are in general good candidates for ion exchange separation due to the presence of ionizable groups on their structure. Thus, at a given pH, they present a positive or negative charge according to their isoelectric point and are able to interact with a cation or anion exchanger, respectively. Protein desorption from an ion exchanger is usually performed by an increase in the ionic strength by either stepwise or gradient elution procedures [5].

A selectivity problem emerges from IEX since all the proteins with the appropriate charge could be retained by an ion exchanger. Similarly to the problems described in the first part of this work, another significant problem arises when recovering small proteins or peptides at low concentrations from a complex mixture, such as human serum, that contains a high concentration of large proteins that could adsorb on the ion exchange sites generally with higher strength than smaller proteins because of their higher charge density. In the literature, as mentioned in the first part of this work, several methods have been reported to restrict the undesired protein interactions at binding sites while trying to adsorb small biological solutes. Yoshida et al. [6] reported the first efforts to isolate small solutes from larger proteins with the analysis of small proteins and drugs from plasma, introducing with this the method now known as restricted access media (RAM) analysis [7–9]. Additionally, several restricted access adsorbents have also been developed with ion exchange (cationic and anionic) adsorption sites [10–19]. However, as in most cases, these adsorbents have been used as analytical tools for the analysis of small molecules from complex mixtures. RAM systems with ion exchangers as adsorption sites have been also used in the analysis of small analytes from fermentation broths and in the separation and analysis of RNA from plasmid DNA [20–23]. As described previously in the first part of this work, Kanda et al. [24] reported the immobilization of hydrophobic sites and grafting of polyethylene glycol on silica particles for the analysis of drugs in serum and plasma. Katagi et al. [25] explored this concept and extended the RAM media by replacing the hydrophobic sites with a strong cation exchanger for the identification of heroin metabolites in human urine. Considering that all the RAM media found in the literature has been developed for analytical scale applications, our efforts have been directed towards the development of preparative controlled restricted media by using agarose-based adsorbents for incorporation of polyethylene glycol and immobilized metal ions as adsorption sites as described in the first part of this work, and strong ion exchangers as adsorption sites as presented in this second part of the work [26,27].

Polyethylene glycol has been the polymer of choice in the modification of surfaces to prevent or control protein adsorption or develop non-fouling surfaces. Polyethylene glycol surface grafts have gained considerable attention as being able to produce stable films that provide resistance to nonspecific protein adsorption. The use of PEGylated surfaces and biomolecules has increased considerably in diverse areas from biomedical to biotechnological applications [28,29]. Although the protein-repelling property of PEG has been attributed to many molecular mechanisms, a compelling picture has not emerged. Two theories have been considered towards the molecular description of the interaction between grafted PEG and proteins for the creation of non-fouling surfaces. The first theory considers that the PEG layer induces a steric repulsion of proteins associated with an entropic repulsion originated from the compression of the PEG layer [30–32]. The second one considers that the affinity of water molecules towards oxygen atoms in the PEG chains is high enough to avoid protein interaction [33]. 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 [34].

The incorporation of the PEG polymer barrier on the surface of the adsorptive chromatographic matrix adds additional mass transport resistances that need to be elucidated in order to effectively model the dynamic behavior of transport and adsorption of proteins in these polymer restricted media. This article shows the inherent problems in the field and should be considered as an initial investigation of the effect of restricted permeation polymers on the relation of binding between target solutes and immobilized ligands. Wang and Liapis [35] in their work with cross-linked agarose as a matrix and dextran as polysaccharide ligand extender, using microscopic molecular dynamics modeling and simulations and macromolecular continuum models have shown that when adsorption equilibrium is reached, the concentration of the adsorbed solutes exhibits the shape of the density distribution of the immobilized ligands, suggesting that the former could be used to study the density distribution of the latter. In future work we will study the effect of PEG density on the distribution of adsorbed solute and its relation to the distribution of adsorbed ligand on the matrix surface.

The isolation of small biomolecules while rejecting larger proteins with the use of polyethylene glycol surface grafts has been complemented recently by other methodologies described in the literature. Alternative approaches to incorporate adsorption and size exclusion have been also considered and reported by the incorporation of adsorption sites in highly ordered mesoporous silica particles. Tian et al. [36], reported the effective introduction of hydrophobic siloxane bridges by calcination of the silanol groups in the mesoporous silica matrix and very sharp extractions of peptides from human plasma were obtained. In a different study, Tian et al. [37] also reported the incorporation of strong cation-exchange (SCX) and strong anion-exchange (SAX) groups on the porous nanoparticles for selective enrichment of endogenous peptides. In the same research group, Hu et al. [38] reported the incorporation and attachment of chelated titanium phosphonate as binding site inside the mesoporous silica particles and sharp serum phosphopeptides analytical separations were also obtained with this adsorptive, size exclusion system. In this second part of the work, the synthesis and application of control access polymer permeation adsorbent (CAPPA) systems using ion exchange chromatography with quaternary amine anion-exchange groups and PEG as the control access polymer grafted on a high capacity chromatographic polysaccharide matrix is described and used for the isolation, purification and recovery of small proteins and peptides from complex biological systems.

In the first part of the work we introduced the most general schematic concept of adsorption and size exclusion (AdSEC) with control access polymer permeation adsorption (CAPPA) on an agarose matrix. In this second part Fig. 1 illustrates the immobilization on the polysaccharide matrix of the anion exchange groups between PEG derivatives that restrict the access to larger proteins thus preventing its interaction with the surface bound ion exchange groups.

2. Experimental

2.1. Chemicals

Novarose 100 Act^{H} and 300 Act^{H} beads from Inovata (Broma, Sweden) were used. The structure of these two gel derivatives are 9.5% and 9% cross-linked agarose, respectively, with a mean particle size of 40 μm. Glycidyl trimethyl ammonium chloride (GMAC), sodium hydroxide, sodium cyanoborohydride, sodium carbonate, ethyleneglycol (EG), tris(hydroxymethyl) aminomethane (TRIS), sodium chloride, methoxy polyethylene glycols (mPEG-OH) of molecular weights 2 and 5 kDa, thionyl chloride, absolute ethanol, ammonium hydroxide, sodium periodate, hydrochloric acid, sodium chloride, sodium nitrate, silver nitrate, potassium

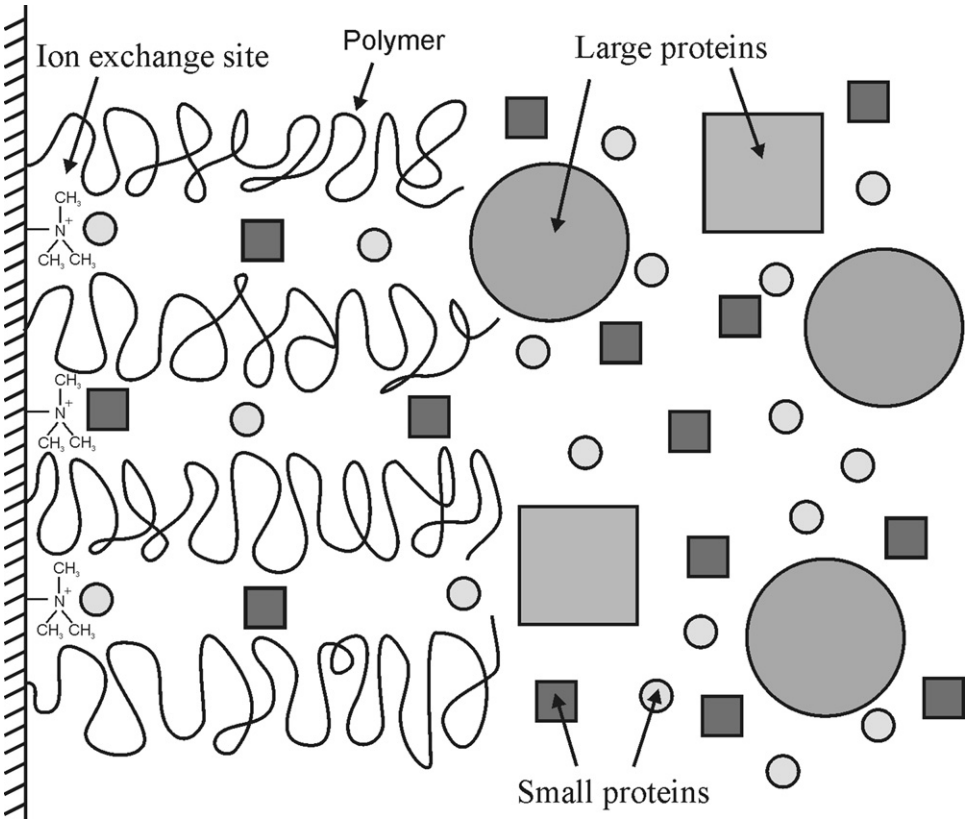

Fig. 1. Adsorption and Size Exclusion (AdSEC) schematic concept using Control Access Polymer Permeation Adsorbent (CAPPA) media with Nov-PEG:QA interactions.

chromate, trinitrobenzene-sulphonic acid (TNBS), bovine insulin (INS), bovine serum albumin (BSA), α-lactalbumin (LAC), ovalbumin (OVA), bovine IgG (BIgG), and human serum were purchased from Sigma–Aldrich (St Louis, MO, USA). Cholecystokynin (CCK-8), a peptide hormone, was obtained from Bachem AG. SDS-PAGE material for electrophoretic analysis was obtained from Bio-Rad (Hercules, CA, USA).

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

2.3. Synthesis of mPEG-NH_2 derivatives

The CAPPA media was synthesized by first coupling trimethyl ammonium groups to a chromatographic agarose matrix followed by the grafting of amino-PEG methoxy derivatives on the surface. The procedure for the production of mPEG-NH_2 from mPEG-OH followed the one reported by Birkenmeier et al. [39] and described in more detail in the first part of this work together with the schematic of the synthesis of the mPEG-NH_2 derivative.

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

Chemical modification of Novarose 100 or 300 with mPEG-NH_2 (2 kDa or 5 kDa) was carried out as follows: 0.5 g of suction dried gel was reacted with 2.5 g of polymer dissolved in 10 mL of 1 M Na_2CO_3 for 24 h at pH 10.3 and 50 °C (except when noted, see Table 1 for temperature and pH conditions and sequence of steps for the preparation of all gels used in this study). The introduction of quaternary ammonium (QA) moieties on the PEG grafted gels, in general, was performed as follows: 0.5 g of suction dried gel was dispersed in 2 mL of 4 M NaOH and 0.01 g of $NaBH_4$ and 1 mL of GMAC were added. Reaction proceeded for 1.5 h at room temperature with agitation. The resulting adsorbents will be denominated henceforth, Nov(100)-PEG:QA or Nov(300)-PEG:QA CAPPA derivatives. In some cases, the need to incorporate more PEG was evident and in these particular cases (last two gels described in Table 1) polyethylene glycol was also grafted on the gels after partial oxidation of Nov(100)-PEG:QA gels with $NaIO_4$. To accomplish the oxidation, 0.5 g of suction dried gel was dispersed in 2 mL of DI water and 0.025 g of $NaIO_4$ were added. Oxidation was allowed to occur for 1.5 h at room temperature with agitation. Chemical modification of partially oxidized Nov(100)-PEG:QA:OX with mPEG-NH_2 (2 kDa or 5 kDa) was carried out as follows: 0.5 g of suction dried gel was reacted with 2.5 g of polymer dissolved in 10 mL of 0.1 M PO_4 (pH 7.0) containing 0.006 g of $NaCNBH_3$ for 24 h at 50 °C with agitation.

Blockage of free activated groups on the Novarose 100 or 300 gels after chemical modification was performed with ethyleneglycol (EG) or TRIS. For EG blockage, 0.5 g of the modified suction dried gel was reacted with 5 mL of an EG solution at 0.5 M containing 1 M NaOH for 24 h at room temperature. For TRIS blockage (when working with oxidized Novarose gels), 0.5 g of modified suction dried gel was reacted with 4 mL of 1 M TRIS (pH 7.4) containing

Table 1
Summary of conditions for the preparation of Nov-PEG:QA CAPPA derivatives.

System	Modification number				
	1st	2nd	3rd	4th	5th
Nov(300)-QA	GMAC	EG			
Nov(300)-PEG:QA[1]	PEG[a] 5k (25 °C)	GMAC	EG		
Nov(300)-PEG:QA[2]	PEG 5k (40 °C)	GMAC	EG		
Nov(100)-QA	GMAC	EG			
Nov(100)-PEG:QA[1]	PEG 5k (40 °C)	GMAC	EG		
Nov(100)-PEG:QA[2]	PEG 5k (pH 11.0)	GMAC	EG		
Nov(100)-PEG_2:QA	PEG 2k	GMAC	EG		
Nov(100)-QA:OX	$NaIO_4$	GMAC	TRIS		
Nov(100)-PEG:QA:OX	$NaIO_4$	PEG 5k	GMAC	TRIS	
Nov(100)-PEG:QA:PEG	PEG 5k	GMAC	$NaIO_4$	PEG 5k	TRIS
Nov(100)-PEG_2:QA:PEG_2	PEG 2k	GMAC	$NaIO_4$	PEG 2k	TRIS

[a] PEG refers to mPEG-NH_2.

0.006 g $NaCNBH_3$ for 1 h at room temperature. Table 1 describes a summary and characteristic steps in the preparation of the adsorbents synthesized and studied in the present work. As an example the system Nov(100)-PEG:QA:PEG represents Novarose 100 that was chemically modified five times: the first one with PEG (5 kDa), the second one with GMAC to attach trimethyl ammonium groups, the third one with $NaIO_4$ to oxidize the gel, the fourth one with PEG (5 kDa) to increase PEG grafting density and the last one with TRIS to block remaining active groups generated from the oxidation step. Fig. 2 illustrates the reactions involved in the preparation of the derivatives of Nov(100)-PEG:QA:PEG.

2.5. Determination of ion exchange capacity of QA-CAPPA derivatives

Chloride capacity of strong base groups in the modified gels was quantified according to a protocol described by Harland [40]. The method consisted in packing specified amounts of modified gel in a 0.5 cm ID glass column to a height of 2.9 cm, approximately, followed by a washing step with 1 M HCl (5 CV) and DI water until reaching neutral pH. A solution of 2.5 N ammonium hydroxide was then fed (10 CV) followed by a rinse with DI water until it was ammonia free. A 2.5% NaCl solution was then fed (10 CV) and the gel was washed once more with DI water until it was chloride free. Finally, adsorbed chloride ions were removed by feeding a solution of 1% sodium nitrate (6 CV) collecting the eluate. The eluate was titrated with 0.01 N $AgNO_3$ using potassium chromate (5%) as indicator. Chloride capacity was determined using Eq. (1). The flow rate during the experiments was set to 0.5 mL/min.

$$\text{Capacity } (\mu\text{eq/mL}) = \frac{TN}{V} \times 1000 \quad (1)$$

where T is $AgNO_3$ titre (mL), N is $AgNO_3$ normality, and V is gel volume (mL).

2.6. Protein adsorption by pulse studies

For pulse studies of peptides and proteins and mixtures, a specified amount gel (that includes all the derivatives synthesized in the study) was packed in a 0.5 cm ID glass column and washed with 1 M HCl and DI water until neutral pH. The system was equilibrated with Buffers B (50 mM TRIS, 1.0 M NaCl, pH 8.3) and A (50 mM TRIS, pH 8.3) with 20 column volumes. A protein, peptide or mixture solution was prepared in Buffer A and 0.5 mL of sample was injected to the system following the absorbance of the eluent at 280 nm. After sample injection, the system was fed with Buffer A until the absorbance returned to the base line and Buffer B was introduced to remove adsorbed molecules. The flow rate during the experiments was set to 0.5 mL/min.

2.7. Determination of protein capacity of QA-CAPPA adsorbents

Protein capacity for QA-CAPPA adsorbents was measured by batch equilibrium studies. In this case, 50 mg of modified adsorbent was contacted, after equilibration with Buffer A (50 mM TRIS, pH 8.3), with 10 mL of protein solution of specified concentration. Protein adsorption was allowed to occur at 25 °C with agitation until equilibrium conditions were reached. To determine the protein concentration adsorbed at the surface of the adsorbent, a mass balance given by Eq. (2) was used.

$$q = \frac{V\rho(c_0 - c_e)}{m} \quad (2)$$

where q is the solid protein concentration (protein capacity, mg/mL), c_0 is the initial liquid protein concentration (mg/mL), c_e is the equilibrium liquid protein concentration (mg/mL), V is the liquid volume (mL), ρ is the apparent density (g of suction dried gel per mL of settled volume), m is the wet mass of modified gel (mg).

2.8. SDS-PAGE analysis

SDS-PAGE analyses of protein containing solutions was performed according to protocols described by Schagger [41]. The gel was a 10% T and it was run at 200 V or 30 mA after sample application (10 μL). Proteins were visualized by silver staining.

3. Results and discussion

3.1. Synthesis of mPEG-NH_2 derivatives

The synthesis of the methoxy amino PEG derivatives 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:QA CAPPA media and determination of ion exchange capacity and PEG concentration

All the agarose derivatives of Nov-PEG:QA CAPPA media exhibited an ion exchange capacity gauged by the chloride capacity of trimethyl amine groups covalently attached to Novarose 300 or 100 after modification. Average chloride capacities of 38 and 26 μeq/mL were obtained for Nov 300 and Nov 100, respectively. The gels after functionalization with different grafting densities of PEG derivatives exhibited all uniform properties in terms of chloride capacity measurements as shown in Table 2. According to the literature, immobilized trimethyl amine groups are the strongest basic functional groups for anion exchange applications [42]. These groups are essentially always charged and retain the same known charge

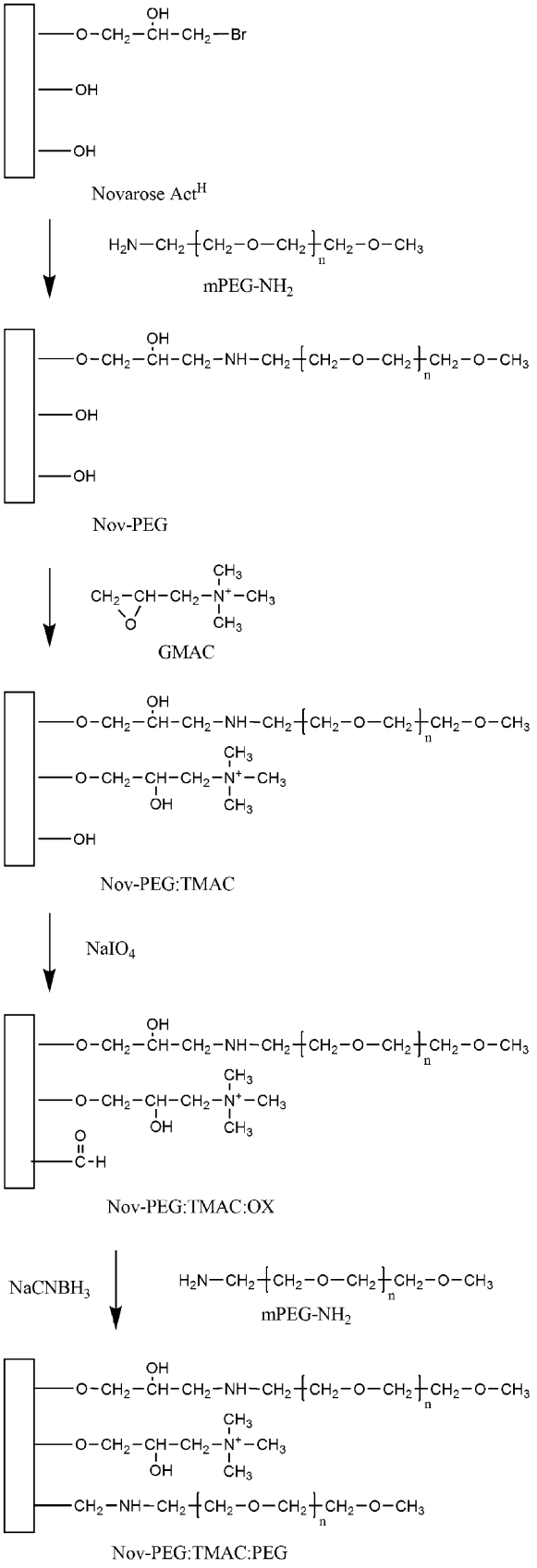

Fig. 2. Schematic representation of the coupling of QA groups and grafting of mPEG-NH_2 to activated agarose gels.

Table 2
Chloride capacity determination for all Nov-PEG:QA derivatives.

System	Chloride capacity, μeq/mL
Nov(300)-QA	40
Nov(300)-PEG:QA[1]	35
Nov(300)-PEG:QA[2]	38
Nov(100)-QA	29
Nov(100)-PEG:QA[1]	25
Nov(100)-PEG:QA[2]	26
Nov(100)-PEG_2:QA	27
Nov(100)-QA:OX	25
Nov(100)-PEG:QA:OX	23
Nov(100)-PEG:QA:PEG	25
Nov(100)-PEG_2:QA:PEG_2	24

Table 3
Physical properties of studied proteins and peptides.

Protein	Molecular weight, Da	Dimensions (nm^3)	Isoelectric point
BSA[a]	67,000	11.6 × 2.7 × 2.7	4.7–4.8
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
LAC[a]	14,200	3.7 × 3.2 × 2.5	4.3
OVA[c]	43,500	7.0 × 4.5 × 5.0	4.6
CCK-8	948		3.8

[a] [44].
[b] [45].
[c] [46].

density regardless of the operating pH [43]. Agarose is a polymer with agarobiose as repeating unit with four hydroxyl residues susceptible of modification, and from this work the chloride capacity reported in Table 2 shows that between 1 and 2% of these hydroxyl groups were modified with the anion exchange group. For the case of modified Novarose 300, considering a maximum chloride capacity of 40 μeq/mL and a uniform distribution of trimethyl amine groups, there would be an ion exchange group for every 12.5 agarobiose residues while for the case of modified Novarose 100, considering a chloride capacity of 25 μeq/mL, there would be an ion exchange group for every 20 agarobiose residues. Despite this apparent low substitution, trimethyl amine groups could be found as close as 1.9 nm considering hydroxyl residues lying in a double helix on a straight line (according to the agarose structure that is later described). Thus, multipoint interactions between acidic residues in the protein and ion exchange groups in the adsorbent could occur.

3.3. Protein adsorption analysis on Nov(300)-QA adsorbents

Novarose 300 modified with QA was tested with BSA (2 mg/mL), BIgG (1 mg/mL), LAC (0.5 mg/mL), INS (0.5 mg/mL) and OVA (1 mg/mL). The chromatograms obtained in the adsorption experiments are shown in Fig. 3. Two of the large molecular weight proteins studied, BSA and OVA were completely retained by the system Nov(300)-QA as shown in Fig. 3A and B, respectively. Fig. 3C presents the chromatogram obtained for the adsorption and desorption of BIgG where some of the protein (60%) was not adsorbed most likely due to local size exclusion effects from the agarose matrix that has an exclusion limit of 300 kDa. The low molecular weight proteins LAC (data not shown) and INS were likewise completely retained by the adsorbent as shown in Fig. 3D for the case of INS.

Table 3 shows physical properties such as the isoelectric points and molecular weights of the proteins and peptides used in this work [44–46]. Adsorbed proteins were desorbed simply by an increase in buffer ionic strength corroborating that the proteins were retained by an ion exchange process. From these results it was clearly seen that the system Nov(300) modified quaternary

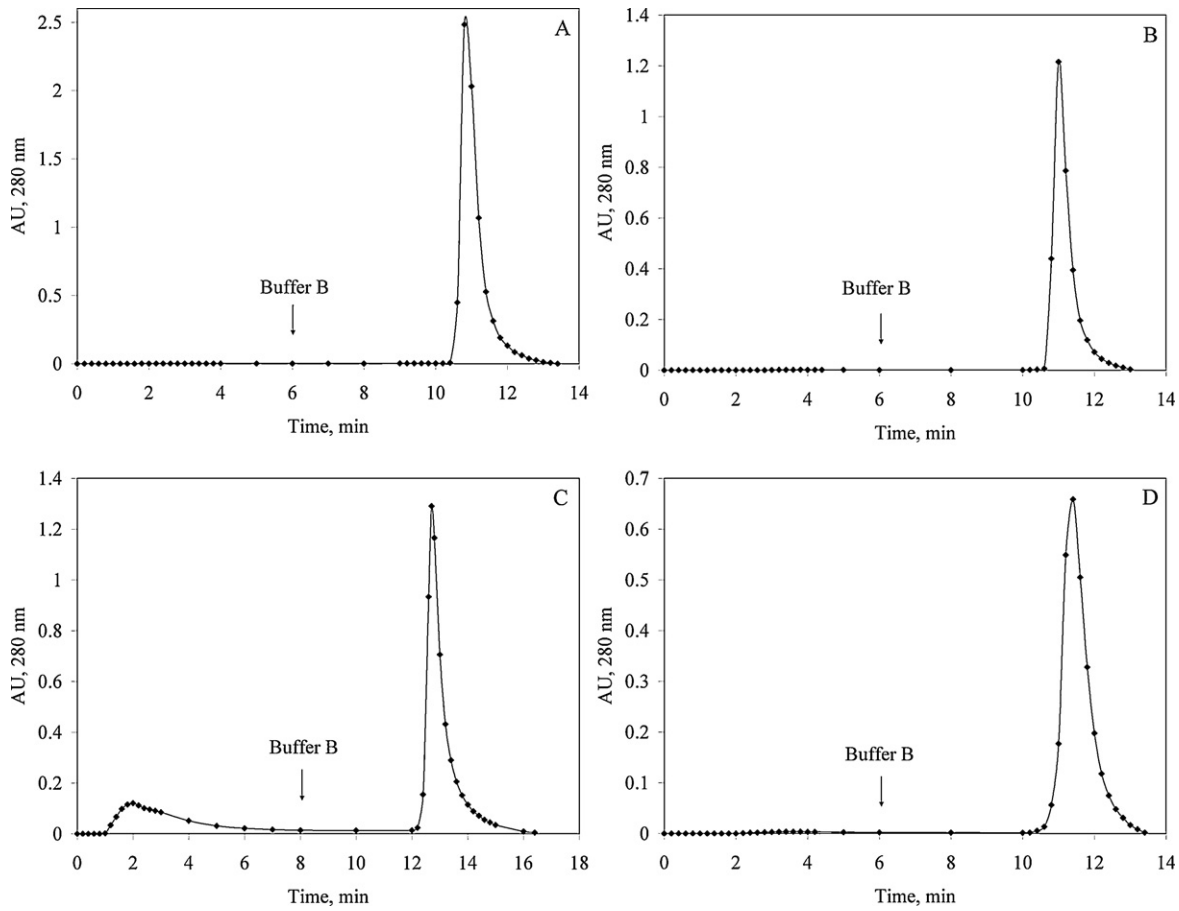

Fig. 3. Pulse studies chromatograms for Nov(300)-QA. (A) BSA, (B) OVA, (C) BIgG and (D) INS. The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. After sample injection, Buffer A was fed until the absorbance returned to the base line and Buffer B was introduced to remove adsorbed molecules. The arrow indicates the point where buffer B was fed to the column for the particular sample.

ammonium groups was not able to discriminate between molecular weights of proteins with isoelectric points lower than the adsorption buffer pH (8.3). At pH 8.3, glutamic and aspartic acid are fully deprotonated and thus, contribute to the retention of most proteins by ion exchange. The proteins here were retained by the adsorbent due to the recognized fact that hydrophilic amino acids such as glutamic and aspartic acid are most likely located on the surface of the proteins and that, according to the amino acid sequence of the selected proteins, the number of these residues is abundant (revealed by the isoelectric points of the proteins).

According to Arnott et al. [47], agarobiose units dimerize to form a double helix that has a pitch of 1.9 nm and each strand has three-fold helical symmetry. This double helix structure was also confirmed by Schafer and Stevens [48]. After the double helices are formed, it is presumed that they aggregate and form fibers containing 10–10^4 helices [47,49]. Later, these fibers are cross-linked to obtain a rigid gel. Thus, multipoint interactions could occur if acidic residues in the studied proteins lie on straight lines with a separation distance of at least 1.9 nm. Furthermore, multipoint interactions could also result from simultaneous interaction between acidic residues in the protein and ion exchange groups from different helixes. For instance, consider the case of LAC; Asp-64 is separated by 2 nm from residues Asp-83 and Asp-84. Moreover, Glu-113 and Glu-121 are separated by 2.4 nm. All these acidic residues are located on the surface of the protein and are water accessible [50]. For the case of OVA, Glu-270 is separated by 3.6 nm from Asp-70 and by 2.3 nm from Asp-83 while residues Asp-98 and Glu-281 are separated by 2.9 nm. Nevertheless, proteins are not rigid bodies and they can suffer conformational changes when they recognize a charged surface, which could also favor multipoint interactions. INS only contains four glutamic acid residues on its structure and due to the size of the molecule, multipoint interactions would be difficult to occur. Even low exposed charged residues in the structure of a protein are a sufficient condition for proteins to interact with charge surfaces.

3.4. Protein adsorption analysis on hybrid Nov(300)-PEG:QA adsorbents

The hybrid systems Novarose 300 modified with QA groups and PEG with a molecular weight of 5 kDa (mPEG-NH_2-5k) were tested with BSA, BIgG, LAC, INS and OVA. Fig. 4 shows the results for BSA pulse studies. For attachment of PEG at 25 °C, the amount of adsorbed BSA in this derivative (Nov(300)-PEG:QA^1) was reduced 62% (compared to the system Nov(300)-QA) as shown in Fig. 4A while in system where PEG was attached at 40 °C (Nov(300)-PEG:QA^1) the adsorption of BSA was reduced 69% as shown in Fig. 4B.

Since the system Nov(300)-PEG:QA^2 prevented BSA adsorption by 69%, the proteins BIgG, LAC, INS, and OVA were studied by pulse analysis on this modified system and Fig. 5 shows the chromatograms of these experiments. Fig. 5A presents the results for BIgG analysis where it is clear that this protein was clearly affected by the presence of PEG reducing its adsorption by 44% (considering only the protein that is being retained in the system Nov(300)-QA). OVA adsorption decreased slightly (9%) as shown in Fig. 5B while

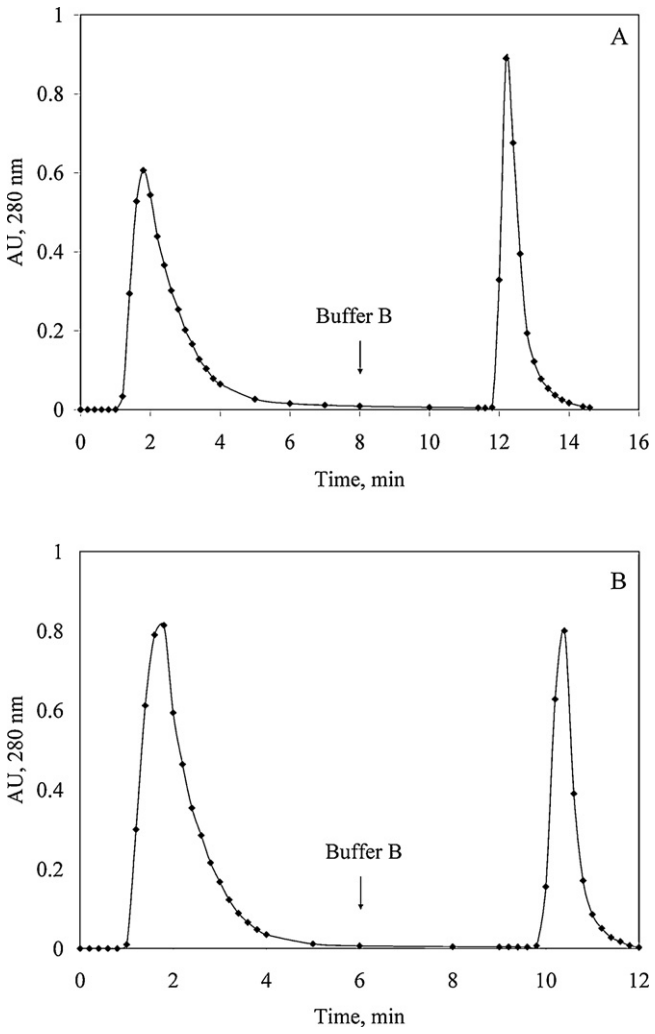

Fig. 4. BSA pulse studies chromatograms for Novarose 300 modified gels. (A) Nov(300)-PEG:QA[1] and (B) Nov(300)-PEG:QA[2]. The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where buffer B was fed to the column to remove adsorbed molecules.

the low molecular weight proteins LAC and INS were unaffected by the presence of grafted PEG and completely adsorbed (see Fig. 5C and D, respectively). From these results it is very clear to see that the small proteins, LAC and INS, are able to interact freely with trimethyl amine groups on the modified systems.

Since the studied proteins continued to adsorb to a different extent, we could foresee to have obtained a low PEG grafting density in the system Nov(33)-PEG:QA[2]. High PEG grafting densities are associated with better rejection properties when analyzing adsorption on flat surfaces [51,52]. High PEG grafting densities are difficult to generate on agarose beads due to the previously described conformation of agarose that tends to form aggregated fibers. Moreover, as the molecular weight of a protein diminishes, the protein is more capable to penetrate the PEG layer with less restriction and reach the adsorption sites more effectively.

An important factor worth mentioning is that the large number of acidic residues present on the studied proteins can contribute towards their retention. If a protein is approaching the agarose surface facing acidic residues towards an adsorption site and faces PEG groups, the protein could be apparently rejected from reaching the surface but then could be adsorbed to a different free adsorption site through different acidic residues. This situation could be more likely to occur for a small protein. If the protein had only one acidic residue on its surface, the rejection would be more efficient even

Table 4
Summary of protein rejection analysis by Nov-PEG:QA derivatives.

System	Protein adsorption reduction[a], %				
	BSA	BIgG	OVA	LAC	INS
Nov(300)-PEG:QA[1]	62				
Nov(300)-PEG:QA[2]	69	44	9	0	0
Nov(100)-PEG:QA[1]	83	58	23	0	0
Nov(100)-PEG:QA[2]	95		87		
Nov(100)-PEG_2:QA	96	83	77	27	40
Nov(100)-PEG:QA:OX	83				
Nov(100)-PEG:QA:PEG	98	94	93	82	46

[a] Compared to the respective system containing only GMAC groups.

at low grafting densities. Protein rejection due to grafted PEG could be possibly associated with the two processes of protein rejection. The first one where an adsorption site on the surface is protected by a close grafted PEG group impairing the adsorption process, as depicted in Fig. 1. The second 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 molecules, as shown in the general principle described in Fig. 1 in the first part of this work.

3.5. Protein adsorption analysis on Nov(100)-QA adsorbents

The system Nov(100)-QA (used as a control) was analyzed by pulse studies of the proteins BSA, BIgG, OVA, LAC and INS. The chromatograms showed that the five studied proteins were retained by the QA moieties and desorbed very effectively by an increase in salt concentration. The chromatograms were similar to the results obtained with the system Nov(300)-QA (data not shown). BIgG was not completely adsorbed by the anion exchange groups (only 66% was retained) most likely due to the size exclusion limit of this Novarose gel (100 kDa). Despite the fact that the concentration of QA groups in the 100 system is lower than the concentration of the correspondent 300 system (see Table 2 for comparison), proteins with molecular weight below 70 kDa were completely retained. The results were tabulated and summarized in Table 4, together with results from derivatives of Nov(300).

3.6. Protein adsorption analysis on hybrid Nov(100)-PEG:QA

Table 4 shows the results of protein pulse analysis on the hybrid Nov-PEG:QA adsorbents. For the system Nov(100)-PEG:QA[1], the rejection of large proteins was only slightly better than with the corresponding high exclusion system of Nov(300) while small proteins (LAC and INS) continued adsorbing with no restriction at all. Since the final objective of this work was to be able to recover small proteins (<15 kDa) the rejection of BIgG and OVA in particular, needed to improve, in order to accomplish this we integrated strategies to increase the PEG grafting density on the gels in order to maximize the rejection of these proteins.

Towards this goal, PEG grafting density was increased by performing the grafting reactions at higher temperature and at a more basic pH. While performing PEG grafting at 40 of 50 °C, the reaction conditions were such that two phases were formed. Phase separation (clouding) is a consequence of the high salt concentration (1 M Na_2CO_3), the basic pH (10.3) and the high temperature. Polyethylene glycols possess clouding points of approximately 100 °C in water for high molecular weight polymers and above 100 °C for low molecular weight polymers [53]. Nonetheless, clouding points can vary due to the presence of inorganic salts. Sodium carbonate can significantly decrease the clouding point with an effect comparable to sulfate salts [54,55]. Similarly, hydroxide ions are also effective in reducing clouding point of polyethylene glycol

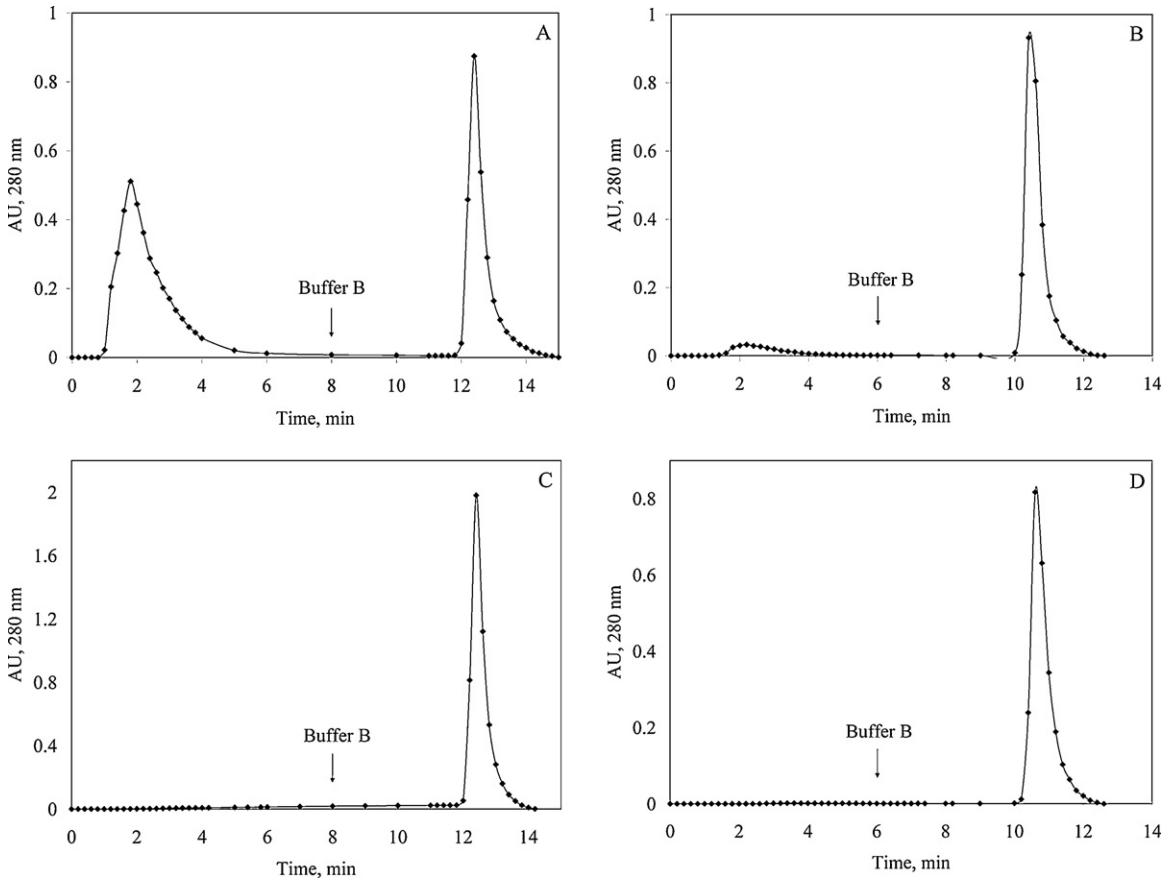

Fig. 5. Pulse studies chromatograms for Nov(300)-PEG:QA^2. (A) BIgG, (B) OVA, (C) LAC and (D) INS. The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where buffer B was fed to the column to remove adsorbed molecules.

aqueous solutions [53]. Formation of two phases favors grafting density since polymer–polymer interactions are increased [52], to our favor, we expected to obtain a higher PEG grafting density by increasing the reaction temperature to 50 °C and the pH to 11.0. Table 4 shows the results from pulse studies of BSA and OVA on the new system with higher PEG grafting densities, Nov(100)-PEG:QA^2 where in fact, an improvement in protein rejection was observed.

To determine the effect of PEG molecular weight on protein rejection, PEG with a molecular weight of 2 kDa was also attached to the agarose matrix. Table 4 shows the results of pulse studies for the system Nov(100)-PEG$_2$:QA. Rejection of BSA and OVA was comparable to that obtained with the system Nov(100)-PEG:QA^2. This again might be due to the arbitrary formation of fibers that could contain different number of double helices, however, it is difficult to establish with certainty the conformation of grafted PEG on the agarose matrix, nonetheless, the three-dimensional nature of the fibers and the position of reactive hydroxyl groups might suggest that the most likely the conformation will be of the "mushroom" type. Considering a single double helix and that the grafted PEG is in a "mushroom" conformation, the Flory radius of the polymer will be given by Eq. (3) [56]; where N is the number of monomers (ethylene oxide) and a is the size of the monomers equal to 0.278 nm [37].

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

For PEG groups with a molecular weight of 5 kDa the Flory radius is equal to 4.7 nm while for PEG 2 kDa would be 2.7 nm. Thus, PEG-2k would be expected to cover less area than its PEG-5k counterpart. Despite this, PEG-2k proved to be as effective as PEG-5k for protein rejection. Furthermore, with PEG-2k grafting BIgG rejection markedly increased. Moreover, LAC and INS were also partially rejected corroborating with this that a higher grafting density was obtained in the new prepared hybrid gels consisting of lower molecular weight PEG, however with more effective grafting.

In order to increase even further the PEG grafting density, the Novarose 100 gel was subjected to a partial oxidation step with iodate [57,58]. This oxidation opens agarobiose unit forming aldehyde groups than can be subjected to reductive amination with mPEG-NH$_2$. Table 4 shows the results from BSA pulse studies on the system Nov(100)-PEG:QA:OX. As a control, the system Nov(100)-QA-OX completely retained BSA.

Finally, in this work, a hybrid IEX-PEG derivative of Novarose 100 was prepared by a double incorporation of PEG moieties that dramatically increased the grafting density of gel derivatives, a gel denominated Nov(100)-PEG:QA:PEG, also shown in the description given in Table 1. Table 4 shows the results from pulse studies with this new double PEG derivative and the chromatographic results obtained with different proteins are given in Fig. 6. The large proteins BSA, BIgG and OVA were clearly prevented from adsorbing (rejection by more than 90%) on the ion exchange sites as shown in Fig. 6A, B and C, respectively; by the presence of the grafted PEG moieties. Fig. 6D and E presents the chromatograms obtained for the adsorption and desorption of LAC and INS, respectively, where it was clear that the increase in PEG grafting density affected its adsorption. Nevertheless, compared with the system Nov(100)-PEG$_2$:QA, LAC rejection increased while INS continued to adsorb to the same extent. From these results it was evident that small proteins were able to penetrate the semi-permeable surface formed by highly grafted PEG.

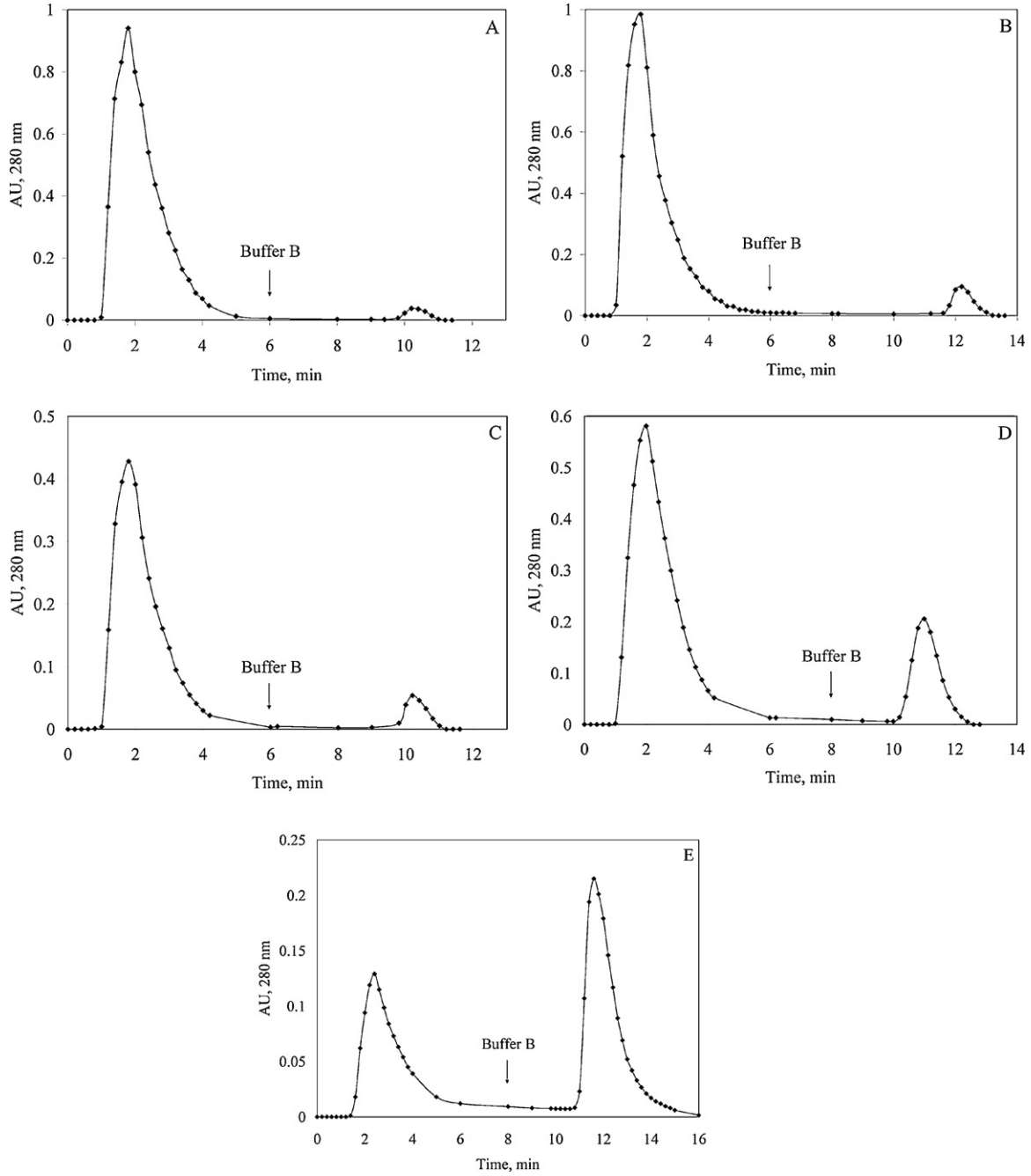

Fig. 6. Pulse studies chromatograms for Nov(100)-PEG:QA:PEG. (A) BSA, (B) BIgG, (C) OVA, (D) LAC and (E) INS. The injection volume was 0.5 mL and the flow rate was 0.5 mL/min. The arrow indicates the point where buffer B was fed to the column to remove adsorbed molecules.

3.7. Protein capacity of QA-CAPPA adsorbents

The results of protein capacities determined for the Novarose 100 matrix derivatives, Nov(100)-QA, Nov(100)-PEG:QA[1], Nov(100)-PEG_2:QA, and Nov(100)-PEG:QA:PEG and with the proteins BSA, LAC and INS are given in Table 5. The results in this table show clearly that the effect of PEG densities on the matrix can in fact decrease the adsorption even of small molecular weight proteins such as insulin. That is why the incorporation of affinity groups, in this case QA groups on the same site as the attached PEG derivative provided a very effective rejection surface, even for small proteins.

3.8. CCK-8 analysis and recovery from a BSA-CCK-8 synthetic mixtures

Recovery of the peptide hormone CCK-8 from a mixture with BSA was evaluated on the hybrid Nov(100)-PEG:QA CAPPA systems. Table 6 shows the results from pulse studies on several adsorbents from a mixture of BSA (1 mg/mL) and CCK-8 (0.05 mg/mL). The results show clearly that very effective rejection of BSA is practically accomplished in all cases, except in the case of the low grafted PEG at 25 °C, where the recovery of BSA was 17%. The recovery of the peptide was, in all cases of single PEG grafted, higher that 90% and only when the PEG grafted density was increased (systems

Table 5
Protein capacity of QA-CAPPA adsorbents.

System	Protein capacity (mg/mL)		
	BSA	LAC	INS
Nov(100)-QA	18.26	30.58	40.97
Nov(100)-PEG:QA[1]		3.17	12.99
Nov(100)-PEG_2:QA			8.94
Nov(100)-PEG:QA:PEG			7.23

Table 6
CCK-8 recovery under the presence of BSA on Nov-PEG:QA derivatives.

System	BSA recovery, %	CCK-8 recovery, %
Nov(100)-QA	100	100
Nov(100)-PEG:QA[1]	17	94
Nov(100)-PEG:QA[2]	5	100
Nov(100)-PEG_2:QA	4	99
Nov(100)-PEG:QA:PEG	2	80
Nov(100)-PEG_2:QA:PEG_2	–	83

with highest PEG grafting density), the recovery of this small peptide was partially affected by the gels, however the CCK-8 rejection was less between 17 and 20%. Nevertheless, at these PEG densities, practically all BSA was rejected. For the system Nov(100)-QA both molecules (BSA and CCK-8) are completely retained by the ion exchange groups, as expected.

3.9. Human serum pulse studies

To test the hybrid adsorbents with real mixtures having a high concentration of proteins, human serum pulse studies were performed where human serum was diluted with Buffer A (1:1). The results of these isolation studies were tabulated and presented in Table 7. The results of the proteins adsorbed in the different gels are represented by the area below the curve from the peak generated from the desorption step by the particular modified system. The amount of protein retained in the systems having grafted PEG, as can be seen clearly, was greatly reduced by the incorporation of grafted PEG, even at a low grafting density (as observed from the SDS-PAGE analysis shown in Fig. 7). It can be observed that even small amounts of HSA and IgG continue to adsorb even with double incorporation of PEG. Again, from these results, it

Table 7
Human serum adsorption on Nov-PEG:QA derivatives.

System	Area below the curve, AU min
Nov(300)-PEG:QA[2]	1.68
Nov(100)-PEG:QA[1]	0.39
Nov(100)-PEG:QA[2]	0.20
Nov(100)-PEG_2:QA	0.24
Nov(100)-PEG:QA:OX	0.66
Nov(100)-PEG:QA:PEG	0.10
Nov(100)-PEG_2:QA:PEG_2	0.17

appears that there is no appreciable difference between the systems Nov(100)-PEG:QA[2] and Nov(100)-PEG_2:QA, indicating with this the effectiveness of permeation of the small molecular weight PEG at high grafting density. These results clearly demonstrates that high molecular weight proteins from human serum can be very effectively prevented from adsorbing to the anion exchange groups grafted on the agarose gels in the presence of grafted PEG.

3.10. Insulin-spiked human serum adsorption analysis

To determine if a small protein could be retained by the hybrid systems in the presence of human serum, insulin was spiked on human serum and pulse studies were performed on specific hybrid Nov-PEG:QA systems. Fig. 8 shows a SDS-PAGE gel analysis of the retained fractions after elution. The system Nov(100)-QA, as expected, effectively retained insulin along with a high concentration of high molecular weight proteins from serum. However, single PEG modified systems are able to retain insulin to different extents. On the other hand, under these extreme grafting conditions, the systems having a double incorporation of grafted PEG were unable to retain insulin, that is, the systems of hybrid derivatives could be designed, with the approach developed here, to reject specific molecular weight proteins with precise molecular weight cut offs. For insulin, in this analysis the best modified system appears to be the gel Nov(100)-PEG_2:QA since not only retained insulin but rejected very effectively a large proportion of high molecular weight proteins from human serum.

3.11. Insulin-spiked human urine adsorption analysis

Insulin analysis studies conducted with mixtures with human urine confirmed the effectiveness of the specific adsorption of small

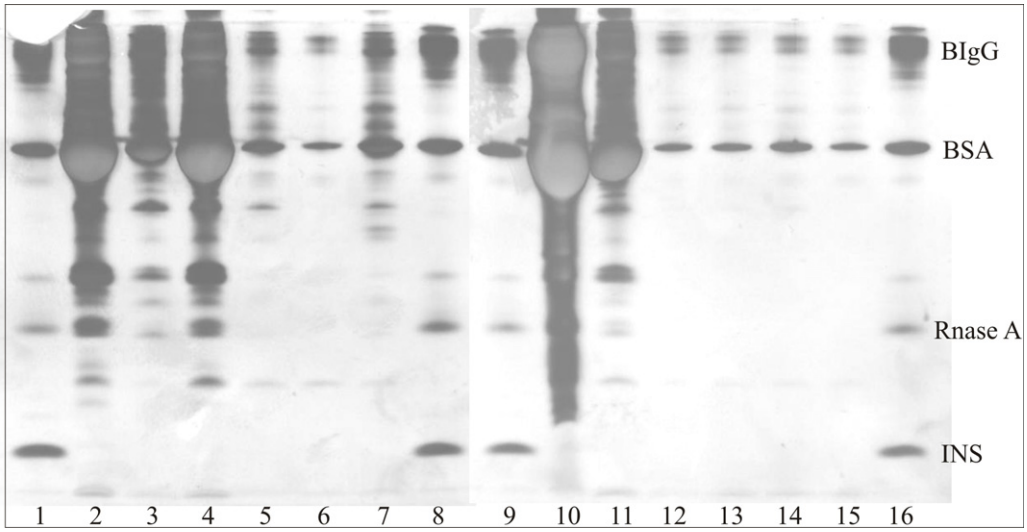

Fig. 7. SDS-PAGE of retained fractions from human serum adsorption studies. (1) Molecular weight standards, (2) Nov(300)-QA, (3) Nov(300)-PEG:QA[2], (4) Nov(100)-QA, (5) Nov(100)-PEG:QA[1], (6) Nov(100)-PEG:QA:PEG, (7) Nov(100)-PEG:QA:OX, (8) and (9) molecular weight standards, (10) human serum, (11) Nov(100)-QA:OX, (12) Nov(100)-PEG:QA[2], (13) Nov(100)-PEG_2:QA, (14) and (15) Nov(100)-PEG_2:QA:PEG_2, (16) molecular weight standards.

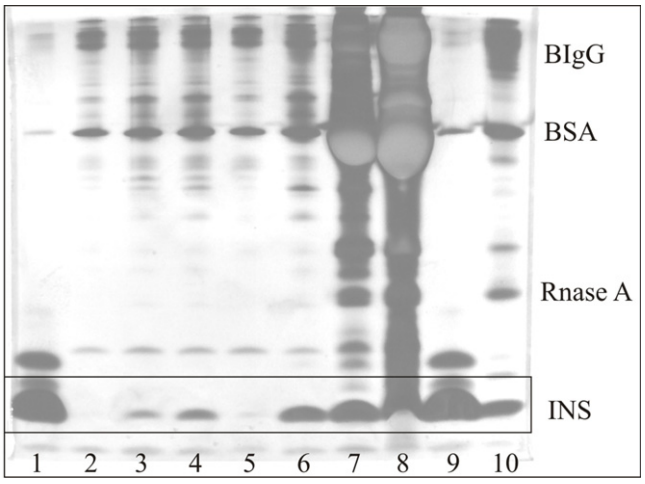

Fig. 8. SDS-PAGE of retained fractions from insulin-spiked human serum experiments. (1) Insulin, (2) Nov(100)-PEG$_2$:QA:PEG$_2$, (3) Nov(100)-PEG:QA^2, (4) Nov(100)-PEG$_2$:QA, (5) Nov(100)-PEG:QA:PEG, (6) Nov(100)-PEG:QA^1, (7) Nov(100)-QA, (8) insulin-spiked human serum, (9) insulin and (10) molecular weight standards.

molecular weight proteins in the presence of a high concentration of proteins from complex systems. In these studies, concentrated human urine (first concentrated 10 times by centrifuge filtration with a membrane of a MWCO of 3 kDa) was spiked with insulin and pulse studies with this sample were performed on selected hybrid Nov-PEG:QA systems. Fig. 9 shows a SDS-PAGE gel analysis of the retained fractions after elution. This analysis clearly showed that the system Nov(100)-PEG:QA^2 was able to retain and recover insulin from the urine samples, however, under these conditions,

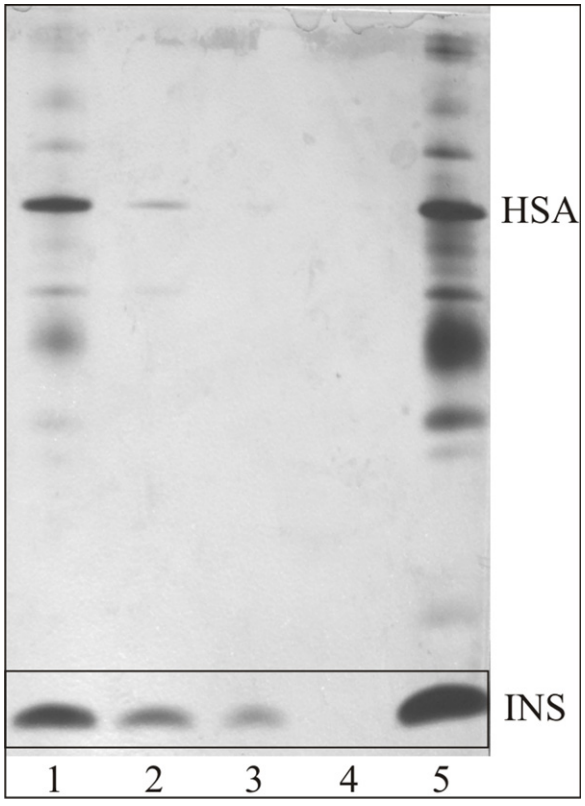

Fig. 9. SDS-PAGE of retained fractions from insulin-spiked human urine experiments. (1) Nov(100)-PEG:QA^2, (2) Nov(100)-PEG$_2$:QA:PEG$_2$, (3) Nov(100)-PEG:QA:PEG, (4) blank and (5) insulin-spiked human urine.

for this gel preparation, even though, the gel rejected a large portion of the proteins in urine, still a considerable amount of albumin and other high molecular weight proteins were able to penetrate the PEG barrier and adsorb on the QA sites on the surface. The systems having a double incorporation of grafted 2k or 5k PEG (the system Nov(100)-PEG:QA:PEG) were able to reject almost all the protein from urine and selectively allow the effective permeation and consequent specific adsorption of insulin from the complex system of the concentrated human urine sample.

From the results presented in Figs. 7–9, it can be observed that the Nov(100)-PEG:QA:PEG can very effectively restrict the adsorption of high molecular weight proteins and that an important factor to obtain useful resolution of target peptides or small proteins is the size and concentration of total protein samples. In the case of human serum for instance, the sample contained high concentration of total protein and even the small protein, insulin, could not reach easily the polymer barrier because of the interaction with the high concentration of protein in the serum sample. On the other hand, in the case of human urine, the concentration of total protein was not high enough as to restrict the insulin from reaching the polymer PEG interface, penetrate the polymer barrier and further bind to the anion exchange groups on the protected surface, restricting however, the adsorption of most high molecular weight proteins from the urine. In order to elucidate the effects and limits of total protein concentration for the gels described here, further studies will be considered at different concentrations of total protein.

4. Conclusions

In this work, new chromatographic polysaccharide adsorbents for potential use in preparative separations were synthesized and used to selectively separate small molecular weight peptides and proteins. The adsorbents combine very effectively two well established biomolecule preparative separation techniques, (1) protein specific adsorption with (2) sharp size exclusion permeation. In the first part of the work, Control Access Polymer Permeation (CAPPA) adsorptive media with IMAC binding sites and PEG permeation was introduced. In this second part of the work, the use of ion exchange binding sites corroborates the effective application of these new preparative adsorbents for low-abundance, low molecular weight (LMW) proteins or peptides with elimination of interfering high molecular weight (HMW) proteins. The high capacity, hybrid polysaccharide agarose adsorbents, Nov-PEG:QA presented here and prepared by incorporating PEG derivatives as control access polymer permeation adsorption (CAPPA) media and quaternary ammonium (QA) adsorption groups as affinity ligands, were synthesized and used to resolve and separate very effectively peptides and small molecular weight proteins from model and complex real protein mixtures of human serum and human urine. The peptide CCK-8 in mixtures with BSA were separated with several of the Nov-PEG:QA adsorbents derivatives while no effective separation was obtained in the absence of PEG on the adsorbents. The hybrid systems were able to isolate very effectively low molecular weight biomolecules from crude human serum compared with the systems without grafted PEG. Similarly, INS was effectively separated from complex mixtures of insulin-spiked human serum and from human urine. The results show that these Nov-PEG:QA adsorbent hybrid systems can practically eliminate in chromatographic experiments all albumin and high molecular weight compounds from model and real protein mixtures with very effective permeation, adsorption and separation of small molecular weight compounds. 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 ion exchange moieties and similar systems is very promising and more research should be conducted to further study these systems. Grafting PEG density and molecular weight are relevant factors and definitely more research work on its effects will help elucidate protein rejection and permeation in these polysaccharide matrices.

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