Polyacrylamide-pectin cryogel: adsorption of human immunoglobulin G in metal chelate

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**Abstract**

Polyacrylamide-pectin (pAAm-pec) and polyacrylamide-pectin-iminodiacetic acid (pAAm-pec-IDA) monoliths synthesized by cryogelation technique were used as stationary phase in chromatography to adsorb human immunoglobulin G (hIgG). The cryogels pAAm-pec-IDA chelated with copper, nickel, and cobalt metal ions exhibit ideal characteristics for use in immobilized metal-ion affinity chromatography (IMAC), such as a porosity of 65-75% and a pore size ranging from 20 to 100 µm. They also supported flow rates of up to 4 mL/min in a column 1.0 cm of internal diameter. The adsorption of hIgG on cryogels was dependent on the buffer system and pH. The highest amount of adsorbed hIgG was observed in MOPS buffer pH 6.5, resulting in 87%, 90%, and 77% hIgG fed on pAAm-pec-IDA-Co(II), pAAm-pec-IDA-Cu(II), and pAAm-pec-IDA-Ni(II), respectively. Elution was carried out under mild conditions, with the addition of 100 mmol/L imidazole to the adsorption buffer. The pAAm-pec-IDA-Me(II) cryogels have the potential for adsorbing and purifying IgG from human serum.

*Keywords*: Cryogel; IMAC; hIgG; Purification; Adsorption

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# 1. Introduction

Adsorption chromatography is a widely used technique for protein purification, in which traditional polysaccharide gels or synthetic polymers are commonly used as stationary phase. These traditional gels, however, may exhibit preferential pathways and slow diffusional mass transfer[1]. To get over these issues, continuous blocks with a porous solid structure (known as monoliths) were proposed as chromatographic stationary phase for the biomolecule purification. Monoliths minimize the aforementioned issues associated with fixed bed chromatography [2]. The highly porous structure of monoliths provides low resistance to fluid flow due to the interconnection of its pores and channels. Moreover, high flow rates can be achieved with moderate pressures due to negligible diffusion within the pores. Depending on the purpose, monoliths can be synthesized using a variety of synthetic and natural polymers, including acrylamide, methacrylate, chitosan, alginate or pectin [2].

When the hydrogel is synthesized at temperatures below the freezing point of water (-20 to -5ºC), they

are called cryogels. Because of their porous structure, cryogels can be repeatedly compressed to 50% of their initial volume without causing pore structure damage[3]. For the cryogel to be utilized in the chromatography, it must be porous, hydrophilic and resistant to flow. Cryogels can therefore be made using only synthetic monomers, like acrylamide and methacrylate, or hybrid cryogels can be created by mixing them with polysaccharides, like chitosan and alginate [4,5,6]. Pectin is a polysaccharide that has not been extensively studied; it is obtained from renewable, sustainable, and low-cost sources, and is widely used in food engineering as a gelling agent.

The present study explores the viability of employing polyacrylamide-pectin cryogel (pAAm-pec) as the stationary phase in immobilized metal-ion affinity chromatography (IMAC) for the adsorption of immunoglobulin G (hIgG). For this, iminodiacetic acid (IDA), the immobilized chelating ligand, is complexed with cobalt, copper, and nickel metal ions. The reversible interactions between chelated metal ions in the stationary phase and accessible amino acid residues on the protein surface are the basis of the IMAC adsorption technique.

# 2. Materials and Methods

*2.1. Materials*

Acrylamide (AAm), bisacrylamide (BAAm), human immunoglobulin G (hIgG), and epichlorohydrin (ECH) were obtained from Sigma-Aldrich (USA). TEMED (N,N,N',N'-tetra-methylenediamine), ammonium persulfate, SDS (sodium dodecyl sulfate) were supplied by Cytiva (USA). Pectin (pec) GENU type LM 102-AS was purchased from CPKelco (Brazil). The remaining reagents were of analytical grade. Milli-Q ultrapure water (Millipore, USA) was used to prepare all solutions.

*2.2. Synthesis of the cryogel, activation with ECH and IDA immobilization*

AAm, BAAm, and pec were dissolved in water (4.74% m/v, 1.26% m/v, and 0.1% m/v, respectively). After degassing, the mixture was cooled to 4ºC, ammonium persulfate (2% v/v) and TEMED (2% v/v) were added. A final volume of 3.0 mL of the final mixture was deposited in previously sealed plastic syringes and placed in a freezer at -20ºC for 16 hours. After polymerization, the cryogels contained in the molds were thawed at room temperature and washed with water [7]. After, the cryogel was activated with ECH and IDA was coupling on the cryogel as described by Fioravante and Bueno5.

*2.3. Adsorption experiments*

All chromatographic experiments were carried out using an Econo Liquid Chromatography System (Bio-Rad, USA) at 25 °C at a flow rate of 1.0 mL/min. The column containing 3 mL of pAAm-pec-IDA cryogel was saturated with metal ions of cobalt, copper, or nickel (Me2+), followed by washing with 25 mmol/L acetate buffer pH 4.0 and then with water. In the case of experiments with pAAm-pec (control), the column was not saturated with metal ions. The columns were equilibrated with adsorption buffer 25 mmol/L MOPS, Tris-HCl or NaP buffer at pH from 6.5 to 8.0 (control experiments) and for IMAC experiments, the buffer contained 2 mmol/L of imidazole to avoid the non-specific interactions between proteins and the chelated metal ions. Two milliliters of hIgG (approximately 2.0 mg of total protein) in adsorption buffer were loaded onto the column, and subsequently washed with adsorption buffer until no protein was detected in the column outlet stream by absorbance at 280 nm. The adsorbed proteins were eluted with buffer containing 100 mmol/L imidazole at pH 7.0 (column loaded with metal ions) or adsorption buffer containing 1.0 mol/L NaCl (control experiments). In IMAC, imidazole, (chemical structure similar to histidine), competes with the target protein for binding to the metal ion, displacing the bound protein from the column.

*2.4. Total protein determination*

Total protein concentrations were determined by the method of Bradford [8], with BSA as a reference protein.

# 3. Results and discussion

Both the pAAm-pec and pAAm-pec-IDA cryogels have an elastic and spongy appearance, with a pore size ranging from 20 to 100 µm, and a porosity of 65-75%, which are desirable physical properties for a chromatographic column.

The IDA was attached to the cryogels (Figure 1) by covalent bonds between the IDA amino group and the epoxy group introduced in the cryogels. Its attachment was quantified by the complexation of metal ions, measured as the difference in the moles of metal ions chelated on the cryogels with and without the chelating agent.



Fig. 1. pAAm-pec-IDA cryogel (a) without metal ion and with metal ions (b) copper, (c) cobalt, and (d) nickel.

It is possible that the control cryogel did not chelate metal ions due to the protonation of the pectin's carboxyl groups. On the other hand, cryogels with immobilized IDA complexed the three studied metal ions. The results in Table 1 show the dependence of the quantity of metal ions chelated on the type of metal.

Table 1. Density of metal ions (µmol/mL) chelated on the pAAm-pec and pAAm-pec-IDA cryogels (3.0 mL of cryogel)

|  |  |  |
| --- | --- | --- |
|  | pAAm-pec | pAAm-pec-IDA |
| Cu(II) | 1.04 | 171.03 ± 3.20 |
| Ni(II) | 0.00 | 92.77 ± 2.07 |
| Co(II) | 0.00 | 132.86 ± 0.83 |

The effect of the buffer system and pH on IgG adsorption was first investigated for the pAAm-pec cryogel (control). The three MOPS, Tris-HCl, and NaP buffers were tested in their respective pH ranges, from pH 6.5 to 8.0. The adsorption of IgG was negatively affected by increasing the pH in all cases (Figure 2).

Fig. 2. Effect of the buffering system and pH in the IgG adsorption on pAAm-pec-cryogel. Blue, green, and red correspond at MOPS, Tris-HCl, and NaP buffers, respectively.

The effectiveness of IgG adsorption depended not only on pH but also on the type of buffer used; different quantities of IgG were adsorbed with different buffers at the same pH. This variability in this case can be attributed to the conductivity of the medium (MOPS < 1/kΩc m and NaP and Tris-HCl in the range of 1-3.5 1/kΩcm) [9]. The presence of free ions in the mobile phase may hinder electrostatic interactions between the pAAm-pec cryogel and IgG.

The adsorption of IgG was possible between pH 6.5 and 8.0, but the pH and buffer system optimum of adsorption was MOPS at pH 6.5 (when the buffer is in the zwitterionic form). Since the polyclonal nature of IgG results in a range of isoelectric points (pI from 5.5 to 9.3), they are neutral, negatively charged, and positively charged at the pH values studied [10]. The adsorption of IgG appeared to be governed by electrostatic forces on the pAAm-pec cryogel since elution was possible with salt (NaCl) in the adsorption buffer.

Since the MOPS buffer at pH 6.5 was the optimal condition for the adsorption of hIgG on the pAAm-pec cryogel, this buffer was maintained when using the pAAm-pec-IDA-Me(II) cryogel. Thus, the Cu(II), Ni(II), and Co(II) were chelated on the pAAm-pec-IDA, and the quantification of chelated metals was 57.01 ± 1.05, 30.92 ± 0.69, and 44.29 ± 0.27 µmol/mL, respectively. The molarity of chelated Cu(II) was similar to that reported in the literature; Kumar et al11 reported chelation of 25 to 62 µmol Cu(II)/mL pAAm-IDA cryogel and Sepharose®-IDA, respectively.

In IMAC, salts such as NaCl are typically used in the adsorption buffer to prevent electrostatic interactions between the protein and the metal chelate. In this study, the adsorption buffer was devoid of salt to promote electrostatic interactions between the protein and the metal chelate. This approach enhances the adsorption capacity for the IgG [4,5,6].

As shown in Figure 3, the IDA-Co(II), IDA-Cu(II), and IDA-Ni(II) were able to adsorb hIgG, retaining about 87%, 90% and 77% of the fed protein, respectively. This can be explained by the combination of electrostatics and coordination interactions between the hIgG and the metal ions, allowed by the zwitterionic character of the MOPS buffer, which does not compete for the binding sites on the cryogel. On the other hand, the difference in the amount of hIgG adsorbed could be related to the type of metal ion chelated. According to Sulkowski12, the chelated Co(II) and Ni (II) require several exposed histidine residues on the protein surface for interaction, whereas chelated Cu(II) requires only one.

Fig. 3. Effect of the immobilized metal ion on the hIgG adsorption onto pAAm-pec-IDA cryogel. Red, blue, and green are Co(II), Cu(II), and Ni(II), respectively.

Furthermore, the adsorbed hIgG was eluted using the adsorption buffer containing imidazole as a competitive agent. The imidazole elution method showed an incomplete recovery of the total adsorbed hIgG, resulting in contamination of the eluted fractions with metal ions complexed with EDTA during the subsequent regeneration step. The implementation of a gel filtration column downstream of the pAAm-pec-IDA-Me(II) cryogel columns could facilitate the removal of EDTA-complexed metal ions from the eluate without compromising the overall efficiency of the purification process.

# 4. Conclusion

The acrylamide and pectin based cryogels synthesized in this work showed high porosity, spongy aspect and ideal properties for a stationary phase in chromatography. Both pAAm-pec and pAAm-pec-IDA-Me(II) cryogels were able to adsorb hIgG, demonstrating their potential for use as chromatographic columns, especially in IMAC. Among the buffer systems studied, MOPS at pH 6.5 promoted the adsorption of hIgG in both cryogels. These results highlight the potential of the polyacrylamide-pectin-based cryogels for use as a chromatographic column for protein separation.

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