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Development of supermacroporous monolithic adsorbents for purifying lectins by affinity with sugars

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ABSTRACT

Affinity techniques are frequently used to purify biocompounds, because of specific interactions observed in many cases. One example are the lectins, proteins connected in a reversible manner and specific to carbohydrates or sugar-containing molecules. Four different methods were investigated (epoxy, Schiff base, glutaraldehyde and ethylenediamine) to immobilize the carbohydrate *N*-acetyl-D-glucosamine (D-GlcNAc) on the surface of supermacroporous cryogels made for lectin purification. The glutaraldehyde method presented the highest immobilization capacity of D-GlcNAc (147.77 mg/g), while the ethylenediamine method presented the lowest capacity (32.47 mg/g). FTIR spectra analysis confirmed the presence of the immobilized carbohydrate. The cryogels containing D-GlcNAc immobilized by the different methods were characterized in terms of swelling capacity, degree of expansion, porosity and constituent fractions. Results showed that the activation methods did not affect the macroporous structure. Images obtained from scanning electron microscopy evidenced the presence of interconnected macropores in the structure of the cryogels produced. The cryogels presented even lower flow resistance in the permeability analysis. Finally, the cryogel modified by the glutaraldehyde method was used in the Concanavalin A lectin adsorption process, presenting an adsorptive capacity of 44.49 mg/g and high stability after five cycles of use.

1. Introduction

Traditional fixed beds present a high saturation capacity because of the high surface area of adsorbent particles. Highly concentrated or particulate solutions however challenge the limits of fixed beds in relation to their capacity and flow resistance. These limitations have led to the development of supermacroporous polymeric monoliths for biomolecule separation purposes [1].

Among the possible monoliths used in biomolecule purification processes are polyacrylamide cryogels, obtained from the polymerization of acrylamide molecules (Aam) formed with the crosslinking agent *N*,*N*′-methylene-bis-acrylamide (BAam) with or without other monomers added, under moderate freezing conditions, using the cryofreezing technique [2]. The use of polyacrylamide cryogel has been reported by many authors [1–7].

Polymeric cryogels present elevated porosity with large pores (greater than 10 mm), which allows the use of more concentrated solutions or solutions containing particles. Moreover, the high chemical and mechanical resistance and low flow resistance gives these monoliths unique characteristics for use in bioseparation. As a consequence of their macroporous structure however, such monoliths present lower adsorptive capacity compared to conventional columns [8].

Thus, the need has arisen for structural modifications of cryogels to increase their adsorptive capacity. To purify molecules that present affinity with carbohydrates, such as lectins [9], a viable alternative is to transform the cryogels into affinity monoliths by means of immobilizating carbohydrates (whether modified or not) on their surfaces. Diverse activation methods on the surface of cryogels followed by biomolecule immobilization have been reported. Many of these methods are based on ligand immobilization via covalent bonds, using epoxy radicals present on the surface of the monoliths to react with specific groups [10,11]. One compound used to provide epoxy radicals in the synthesis process of polyacrilamide cryogels is allyl-glycidyl ether (AGE) [12].

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Existing immobilization methods using covalent bonds include the epoxy, Schiff base and glutaraldehyde methods. The epoxy method involves the nucleophilic attack of amine groups of molecules on the monolithic epoxy groups, leading to the formation of a stable secondary amine bond [13]. The disadvantage of this method is that no spacer arm is inserted between the support and the linker molecule, which may result in a lower immobilization capacity.

The Schiff base method involves the conversion of the epoxy groups in groups diols followed by their oxidation, forming aldehydes that may react with primary amines, forming a Schiff base. This method has a greater immobilization rate than the Epoxy method, but has the disadvantage of using reducing agents to stabilize the formed Schiff bases, which may affect the capacity of the immobilized ligand [13].

In the glutaraldehyde method an adsorbent containing reactive epoxy groups (epoxy-activated) is initially converted to the amine-activated form by means of epoxy group reactions with reagents containing an amine group, such as ethylenediamine [14] or hexanediamine [15]. The amine-activated monolith reacted with the glutaraldehyde, forming an aldehyde-activated monolith capable of reacting with amine radicals of the linker molecule. The support is used in the same way as with the Schiff base method [10]. The principle advantage compared to the Schiff base method is the formation of a long spacer arm between the monolith and the ligand, avoiding possible steric hindrance effects [14,15], which may result in a higher ligand immobilization capacity and purification of the target molecule.

Based on these types of interactions, a possible approach for immobilizing the molecules that present aldehyde groups in their structure would be by means of producing an amine-activated adsorbent capable of interacting with the carbonyl radical present in the linker molecules, as occurs, for example, in the activation using the hydrazide enrichment method [10]. A little explored alternative is to produce amine-activated monoliths with ethylenediamine to immobilize the ligand that contains aldehyde groups.

Given the above, the objective of this work was to evaluate different methods of carbohydrate immobilization in the production of supermacroporous polymeric cryogels, verifying the immobilization capacity of the sugar used, physical characteristics and viability of use.

2. Materials and methods

2.1. Reagents

Acrylamide (AAm), bis-acrylamide (BAAm) and allyl-glycidyl-ether (AGE) were used to produce the cryogels. The carbohydrate used for the assays was *N*-acetyl-D-glucosamine (D-GlcNAc). All of the above were from Sigma-Aldrich (St. Louis, USA). The other reagents used in the experiment are described throughout the methodology. All reagents used had, at a minimum, the PA-ACS degree of purity.

2.2. Synthesis of monolithic cryogels

For cryogel synthesis, methodologies were adapted from those proposed by Kumar et al. [16] and Yao et al. [17]. Four g of AAm, 1.2 g of BAAm and 0.8 g of AGE were dissolved in 100 mL of distilled water, with 1% m/m of ammonium persulfate (APS) and 1% m/m of *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) added relative to total monomer mass. After homogenization, the solution was immediately poured into 5 mL plastic syringes, sealed and immersed in a thermostatic bath at −20.0 °C for 24 h. The syringes were left at 4 °C for 4 h to thaw the existing water. Afterward, the syringes containing the cryogels were placed in a heating oven at 60 °C until the cryogels were completely dry, at which point they had the extremities cut (to remove defective parts) and the masses were measured on an analytical balance (accuracy 0.0001 g). The cryogels were then washed with 150 mL of distilled water, using a peristaltic pump at 1.5 mL min^{-1}, re-dried in the heating oven and weighed to verify the quantity of monomers that had not polymerized. After this, the cryogels were ready to be subjected to the different carbohydrate immobilization methods.

2.3. Carbohydrate immobilization

In this work the modified carbohydrate *N*-acetyl-D-glucosamine (D-GlcNAc) was used to evaluate the different methods of immobilization tested. This molecule has in its structure a carbonyl radical and an amine radical, which may react with the amine-activated and aldehyde-activated cryogels, respectively. Moreover, lectins from different sources show selectivity for this molecule and are suitable for use in affinity chromatography [18].

For D-GlcNAc immobilization, four methods were used, with the objective of determining which method had the highest immobilization capacity. A schematic with the probable sugar immobilization mechanism for each method is presented in Fig. 1. The quantity immobilized in each method was quantified by difference in the D-GlcNAc solutions before and after recirculation, using the dinitrosalicylic acid (DNS) method [19]. All solutions were recirculated through the cryogels in the form of monoliths inside the syringes, using a peristaltic pump at a flow rate 1.5 mL min^{-1}. In each evaluated immobilization method eight base-cryogels were randomly selected from among those produced. The average values obtained for the concentration of D-GlcNAc immobilized from the different methods used were compared using the Tukey test ($p > 0.05$).

The different methodologies used for carbohydrate immobilization are described in detail in Sections 2.3.1 to 2.3.4.

2.3.1. Epoxy method

D-GlcNAc was immobilized with the epoxy method following Babac et al. [20] and Mallik et al. [13] with modifications. Cryogels were initially washed with 30 mL of absolute ethanol, followed by 30 mL of ethanol + distilled water (1:1) and finally 30 mL of distilled water, for a weak nucleophilic attack on the epoxy groups. The wash initially used 15 mL of solution followed by a 10 min rest and the wash being completed with the other 15 mL. Afterward, the cryogels were washed with a 40 mL sodium carbonate buffer 50 mM pH 9.5. Next, 25 mL of 4 mg mL^{-1} of D-GlcNAc-GlcNAc solution in the sodium carbonate buffer was recirculated by the cryogels at room temperature for 16 h to covalently couple the sugar. Afterward, the cryogels were washed with 30 mL of the carbonated buffer, followed by 40 mL of a solution of ethanolamine 0.1 M in the same buffer to block unreacted epoxy groups. The columns were again washed with 30 mL of the buffer, followed by 40 mL of distilled water and 40 mL of phosphate buffer 20 mM pH 7.2. The syringes containing the cryogels filled with the phosphate buffer were closed and stored at 4 °C until use.

2.3.2. Immobilization with the Schiff base method

Methodology was adapted from Luo et al. [15] and Mallik et al. [13]. Cryogels were initially washed with 30 mL of HCl 0.1 M. Next, the syringes containing the cryogels were closed, filled with HCl and placed in a BOD heating oven at 50 °C for 12 h to open the epoxy rings. Afterward, the cryogels were washed with 30 mL of distilled water. Next, 40 mL of period acid 0.1 M was recirculated for 1 h to oxidize the hydroxyl groups (from the epoxy ring opening) to a carbonyl group and then washed with 30 mL of distilled water and 30 mL of sodium citrate buffer 0.1 M pH 6.4. The cryo-