Magnetic particles as affinity matrix for purification of antithrombin

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Abstract: Immobilization of biomolecules onto insoluble supports is an important tool for the fabrication of a diverse range of functional materials. It provides advantages: enhanced stability and easy separation. In this work two different magnetic composites were synthesized (MAG-PANI-HS and mDAC-HS) to human antithrombin purification. The magnetic particles (MAG) were obtained by co-precipitation method of iron salts II and III and subsequently coated with polyaniline (MAG-PANI particles). Dacron (polyethylene terephthalate) suffered a hydrazinolysis reaction to obtain a powder (Dacron hydrazide) which was subsequently magnetized (mDAC particles) also by co-precipitation method. Heparan sulfate (HS) was immobilized to MAG-PANI and mDAC retained respectively 35µg and 38.6µg per of support. The magnetic composite containing HS immobilized (MAG-PANI-HS and mDAC-HS) was incubated with human blood plasma (1mL) and then washed with NaCl gradients. Electrophoresis of proteins present in eluates showed bands of antithrombin (58kDa). A reduction in the antithrombin activity was detected in plasma that were incubated in the composites magnetic with HS immobilized, suggesting that the antithrombin was removed of the human blood plasma and then purified. Therefore, the above results suggest that both preparations: MAG-PANI-HS and mDAC-HS are able to affinity purify antithrombin, an important component of blood coagulation.

Keywords: affinity, heparan sulfate, immobilization, magnetic particles, purification.

1. Introduction

Different types of materials have been used as supports for the immobilization of biomolecules, including the supports that exhibit magnetic properties. In addition to being used as adsorbents in magnetic separation, these particles within the micrometer to the nanometer scale, are being used in a growing number of medical applications due to non-toxicity [1,2]. In general, the most suitable inorganic supports have high mechanical strength, good thermal stability, resistance to organic solvents and to attack by microorganisms [3]. Biomolecules immobilized on magnetic supports may be used in the pre analyte concentration, magnetic separation adsorbents, molecular identification of organic and inorganic molecules [4]. Magnetic particles as Dacron ferromagnetic [5] and magnetite coated with polyaniline [6] has been used as supports for immobilization of enzymes. Biomolecules is immobilized to a solid support by adsorption physical, hydrogen bonding, electrostatic interactions or covalently attached. Covalent immobilization is considered to...
result in higher biomolecule activity, reduce nonspecific adsorption, and greater stability of immobilized biomolecules compared to that by physical adsorption, furthermore the biomolecule immobilized covalently cannot be removed from the support by washing with buffers and/or detergents [7].

Affinity separation is a good protein purification method which is based on the formation of reversible and specific complexes between an immobilized molecule and the ligands to be purified. When using a magnetic affinity chromatography has the great advantage of to get a rapid process of separation [8].

Heparan sulfate (HS) is a type of proteoglycan and specific glycoproteins that containing long and sulfated sugar chains that consist of repeating disaccharide units composed of D-glucosamine and D-glucuronic acid or L-iduronic acid. HS have a variety of functions, affecting cell growth, cell adhesion, cell migration, angiogenesis and anticoagulation [9]. So, the HS is able to interact with these and a wide variety of proteins. This work intends to immobilize the heparan sulfate in magnetic particles to affinity purify human blood plasma proteins.

2. Experimental Procedure

2.1. Synthesis of the magnetic particles

The synthesis of Dacron ferromagnetic according to Amaral et al. [5]. First, Dacron was converted to Dacron-hydrazide and then magnetized by co-precipitation method of iron salts II and III. The magnetic particles (MAG) were obtained also by co-precipitation method of iron salts II and III and subsequently coated with polyaniline (MAG-PANI) according to Neri et al [6].

2.2. Immobilization of heparan sulfate on magnetic particles

First, a solution of heparan sulfate 3 mg/mL was functionalized carbodiimide (EDAC) and N-hydroxysuccinimide (NHS). The carboxylic groups of heparan sulfate were activated with EDAC and formed an intermediate compound (O-acylurea) which contains an ester grouping quite reactive and easily undergo hydrolysis. So, was added the NHS that reacted with the ester group of the intermediate compound, leaving it more stable. Thus, the amino group present on the support reacted with the carbonyl of the ester and formed a covalent amide bond [10]. Aliquot 1 ml of heparan sulfate functionalized solution was incubated in each 30 mg of MAG, MAG-PANI and mDAC under light stirring for 72 hours at 25 °C. The amount of heparan sulfate immobilized to the supports was determined according to the methodology used by Oliveira et al. [11].

2.3. Purification of antithrombin

The test of human antithrombin purification by affinity was conducted as follows: for each 30 mg of MAG-PANI-HS and mDAC-HS (magnetic particles with covalently immobilized heparan sulfate) was added 1 mL of human plasma. This mixture was stirred for 60 minutes at 4 °C. Then, with the aid of a magnetic field, the supernatants were collected. Subsequently successive washes with phosphate buffer pH 7.2 in increasing concentrations of NaCl (0.5 M, 1.0 M and 1.5 M) to remove non-adsorbed proteins. A solution of 2.0 M NaCl was added to elute proteins complexed to HS. Electrophoresis of proteins SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) 12,5% was performed to the corresponding fractions collected eluates 2M. These human blood plasmas that were incubated in the
magnetic derivatives: MAG-PANI-HS and mDAC-HS, were subjected to the antithrombin amidolytic activity kit (TriniCHROM™ Antithrombin IIa - Trinity Biotech SA - New Jersey, USA).

3. Results and Discussion

The particles: Dacron-hydrazide ferromagnetic (mDAC) and magnetite coated with polyaniline (MAG-PANI) had an excellent magnetic activity by their conduct through a magnetic field of 6000 Oe using a magnetic separation system (Abraxis™ LLC), see Fig. 1.

![Figure 1: Magnetic separation system with a magnetic field of 6000 Oe (Abraxis™ LLC). The magnetic particles have been attracted by the magnet located on the lateral of the plate (indicated by arrows). A) MAG-PANI; B) mDAC.](image)

Approximately 35μg of heparan sulfate offered was immobilized covalently on MAG-PANI and 38.6 μg in mDAC-HS. An amount of 18 ug of HS was adsorbed in each mg of the MAG particles, this value was lower (compared to MAG-PANI) because MAG particles were not coated with polyaniline. After functionalization with EDAC/NHS [10] the covalent immobilization of heparan sulfate happened by linking its carboxyl groups with the amino groups present in MAG-PANI and mDAC. The electrophoresis of the purified proteins with 2 M NaCl (Fig. 2) in both magnetic supports showed bands corresponding to antithrombin (58kDa) and other bands may be plasma proteins that interact with HS and are identified in subsequent studies. The bands corresponding to the antithrombin were more pronounced in supports containing the covalently immobilized HS: MAG-PANI-HS (Fig. 2A) and mDAC-HS (Fig. 2B). Therefore the other supports did not interact with the antithrombin due to absence or small amount of HS. Heparin and heparan sulfate have structures qualitatively similar [12] and the use of heparin affinity chromatography has been used to purify antithrombin for the past four decades [13]. Here, we study the HS immobilized in magnetic particles to use in the purification process of the AT because was already shown that heparan sulfate also can interact with antithrombin [14].
After the purification process, was performed the antithrombin activity of the human blood plasmas that were incubated with MAG-PANI-HS and mDAC-HS. Fig. 3 showed a reduced antithrombin activity of 88% to MAG and 82% to MAG-PANI-HS (Fig. 3A), and 79% in mDAC-HS (Fig. 3B). These reductions suggest that the antithrombin was removed from the human blood plasma and then fixed at HS. Comparing MAG-HS and MAG-PANI-HS, it can be seen that the particle coated with polyaniline showed a more significant AT removal performance, it was probably due to the HS is immobilized covalently on MAG-PANI and only adsorbed (smaller amount) in MAG. So, the magnetic composites with HS have proven useful in the purification of antithrombin process, but according to the results mDAC-HS was a little better because has more HS covalently immobilized and then can removed a large amount of AT when compared to MAG-PANI-HS and MAG-HS.

Figure 2: Electrophoresis of proteins obtained in 2M eluates, SDS/PAGE 12.5%. A) MAG-HS (pure magnetite with heparan sulfate adsorbed), MAG-PANI-HS and MAG (pure magnetite). B) mDAC-HS and mDAC (pure ferromagnetic Dacron).

Figure 3: Antithrombin Activity plasma after direct contact with the supports. A) pure magnetite (MAG), pure magnetite with heparan sulfate (MAG-HS) and MAG-PANI-HS. B) pure ferromagnetic Dacron (mDAC) and mDAC-HS.
Angeli et al. [15] demonstrated the use of affinity matrix of ferromagnetic levan composite to purify lectin, this composite used as matrix affinity makes a fast and easy procedure. We obtained similar results, but use magnetic composites for purification of antithrombin human blood plasma. Recently, Lan et al. [8] conducted a study on angiotensin converting enzyme (ACE) purified through magnetic affinity separation, using a magnetic agarose microsphere. In our study, the affinity matrix is represented by magnetic composite MAG-PANI-HS and mDAC-HS which were capable of interacting with plasma proteins, especially antithrombin. The specificity of these elements led to the removal and identification of specific proteins. Moreover, these magnetic composites described in this work can be applied as affinity matrix to be used in the area of materials science, blood products industry, biochemistry and pharmacology. This is a single and innovative tool and can be exploited in the biotechnology of biomaterials.

4. Conclusions
From the above displayed results one can conclude that the amount of HS immobilized on the particles and the coating of magnetic particles can influence the performance of composites in AT purification process. Thus when comparing the three preparations synthesized in this work, we can see that mDAC-HS proved slightly more efficient because it contained more HS covalently immobilized and so could remove more AT of human blood plasma. Another important fact is that coating of MAG with polyaniline promoted a larger amount of HS immobilized in MAG-PANI particles and thus also more useful in the AT purification process. So, according to the results presented these composites can be applied as magnetic affinity matrices for purification of antithrombin, this human blood plasma protein has an important biological value and the great advantage this method are: easy synthesis and low cost.

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