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Pore size effect in the amount of immobilized enzyme for manufacturing carbon ceramic biosensor

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ABSTRACT

Understanding the mechanism of enzyme immobilization in porous designed matrices is important issue to develop biosensors with high performance. Mesoporous carbon ceramic materials with conductivity and appropriated textural characteristics are promising candidates in this area. In this work, carbon ceramic materials were synthesized using the sol-gel method by planning the experimental conditions to obtain materials with different pore size, from 7 to 21 nm of diameter. The study of the influence of pore size in the biomacromolecules immobilization capacity was performed using glucose oxidase enzyme as probe. The influence of textural characteristics of material in the amount of enzyme immobilized, as well as, its performance as biosensor, was studied. On the surface of highest pore size matrix, it was possible to immobilize the highest amount of enzyme, resulting in better electrochemical response. With this simple material, composed only by silica, graphite and enzyme, which was improved by the amount of immobilized enzyme through the enlargement of matrix pore size, it was possible to prepare an electrode to be applied as biosensor for glucose determination. This electrode presents good reproducibility, sensitivities of 0.33 and 4.44 μA mM^{-1} cm^{-2} and detection limits of 0.93 and 0.26 mmol L^{-1}, in argon and oxygen atmosphere, respectively. Additionally, it can be easily reused by simple polishing its surface.

1. Introduction

The interest in developing materials containing enzymes has been rising in the last decades because of the numerous applications in enzymatic catalysis and in the preparation of biosensors [1–5]. These materials present high selectivity and specificity, minimized impurities, easier product separation and environmental acceptance, when compared with non-enzymatic systems [6]. An important aspect when enzymatic materials are being developed is the immobilization and stabilization of the biomolecules on adequate substrates. Although the adsorption of enzymes and proteins in solid matrices has been widely studied, the ability to control the amount adsorbed, the interaction between enzyme and matrix surface, and the location of the enzyme in the pore structure are still an important field to be investigated [7]. There are several reports dealing with the enzyme immobilization on porous materials, however the immobilization is commonly accompanied by drastic reduction in the textural characteristics such as surface area and pore volume. This behavior can be consequence of fully entered enzyme inside of pores or also due to immobilization on external surface leading to pore blocking [7]. For immobilization inside of pores, it should be taking into account also the remained free space into the pore to provide sufficient enzyme mobility and retain its catalytic activity. Additionally, the free space eases the substrate diffusion to the catalytic sites [8]. Therefore, to synthesize porous materials with adequate pore structure allowing the immobilization of enzyme, without the loss of textural properties, is still a challenge [7,9].

Concerning electrochemical sensors, there is a recent interest in the preparation of devices based on carbon ceramic materials [10–12]. These materials are obtained by sol-gel synthesis method, based on hydrolysis and polycondensation of silicon precursor

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reactants, in the presence of carbon source. The main feature of this approach is that the sol-gel synthesis method allows designing characteristics such as texture, morphology, composition and chemical reactivity of the synthesized materials [13,14]. In this way, it is possible to obtain carbon ceramic materials with essential characteristics to be used as matrices for enzyme immobilization, as mechanical rigidity and the possibility of chemical functionalization provided by silica. On the other hand, the conductivity provided by the carbon moiety allows their use as electrochemical devices. The surface of electrodes can be renewed by polishing, which affords the creation of a reproducible and reusable system with easy handling, improving its technological importance.

Even presenting such advantages, the carbon ceramic electrodes have been not often applied as enzymatic biosensors [15–20]. Biosensors based on glucose oxidase enzyme are a significant share of publications in the field of biosensors [21–23] and even so, there are only a few reports of glucose oxidase immobilized on carbon ceramic materials [18–20]. Glucose plays essential role in diabetes disease and in food industry, justifying the need for quantification devices [24]. The papers that deal with carbon ceramic electrodes and glucose oxidase enzyme present studies of the electrochemical behavior and the biosensor performance. Nevertheless, a physicochemical characterization of the matrix is also necessary aiming to elucidate the influence of the textural characteristics of the matrix in the amount of enzyme immobilized and how these aspects are related to the electrochemical response.

In this work, carbon ceramic materials were synthesized with planned textural characteristics by changing the experimental conditions in order to obtain materials with different pore size and surface area. These materials were used as matrices for glucose oxidase immobilization aiming to prepare carbon ceramic electrodes. The influence of textural characteristics in the amount of enzyme immobilized, as well as, in the performance as biosensor, was studied.

2. Materials and methods

2.1. Chemicals

Tetraethylorthosilicate (TEOS) (Aldrich), ethanol (Merck), graphite powder (Aldrich), hydrofluoric acid (HF) (Merck, 40%), hydrochloric acid (HCl) (Merck, 37%), 3-aminopropyltrimethoxysilane (APTMS) (Aldrich), glutaraldehyde (GA) (Sigma-Aldrich, 25%), glucose oxidase enzyme (GOD) (Aldrich, E.C. 1.1.3.4 50 KU), glucose (Vetec), *L*-ascorbic acid (Synth), dopamine (Sigma), uric acid (Aldrich), all analytical grade, were used without previous purification. Phosphate buffer solution (PBS) (0.1 mol L^{-1}, pH 7.0) was prepared from NaH_2PO_4 (FMaia) and Na_2HPO_4 (FMaia). Mineral oil was used for the electrode manufacture. All the solutions were prepared in distilled water.

2.2. Synthesis of silica graphite matrices by sol-gel method (SG matrices)

Three carbon ceramic matrices were synthesized in two steps, using TEOS as silica precursor, graphite powder and different quantities of a solution HF/HCl (6.0 mol L^{-1}) as catalyst. Initially, 5.0 mL of TEOS were pre-hydrolyzed in 5.0 mL of ethanol, in the presence of 0.22 mL of distilled water and 0.03 mL of catalyst. The solution was maintained under magnetic stirring, in reflux, at 80 °C by 1 h and then, it was cooled to room temperature. Graphite powder (50 wt%, calculated from the expected SiO_2 weight) was added to this solution and the system was sonicated for 2.5 h at 40 °C. In the second step, 0.73 mL of distilled water were homogenized with 0.68 mL of catalyst and added to the mixture, under constant stirring. The resultant matrix was named SG1. Two more matrices were synthesized changing the amount of catalyst and the water added in the second step. For SG2 matrix, 0.41 mL of distilled water and 1.04 mL of catalyst were added, while for SG3, just 1.59 mL of catalyst. The materials were covered without sealing and stored for solvent evaporation at room temperature. After 15 days, the monoliths were powdered, washed with distilled water and ethanol, and then they were vacuum-dried for 2 h at 80 °C.

2.3. Modifications of SG matrices with APTMS by grafting

The matrices were previously activated by heat treatment at 120 °C, under vacuum for 2 h before modification with APTMS. Then, for each matrix, 1 g was added in a three-neck round bottom flask containing 1 mL of APTMS solubilized in 20 mL of ethanol, the reaction was performed under argon atmosphere, mechanical stirring, at 65 °C. After 24 h, the supernatant was removed and the matrices were vacuum-dried for 2 h at 80 °C. Finally, the modified matrices were washed thoroughly with water and ethanol, vacuum-dried at 80 °C and denominated SG1-AP, SG2-AP and SG3-AP, where AP specifies the aminopropyl group.

2.4. Glutaraldehyde activation

Activation with glutaraldehyde was performed using 1 g of each SG-AP immersed in 10 mL of 5% glutaraldehyde solution (PBS) and kept in shaking for 3 h. After this, the materials were washed with PBS, vacuum-dried at 60 °C for 2 h, and hereafter called SG1-AP-GA, SG2-AP-GA, SG3-AP-GA, where GA specifies glutaraldehyde.

2.5. GOD enzyme immobilization

GOD was immobilized in the modified matrices using a PBS GOD solution containing 20.3 mg mL^{-1} of protein. For this, 1 g of the materials was kept immersed in 8 mL of GOD solution, at 4 °C, under shaking and rest overnight. Protein content in solution was determined by the Bradford assay [25]. The immobilized protein was estimated as the difference between the amount of protein offered to the material and the amount recovered in the supernatant and washing fractions. For SG3 matrix, there was no residual protein in the supernatant. Because of this, a more concentrated solution of glucose oxidase (33.8 mg mL^{-1}) was offered. All materials were washed with PBS, vacuum-dried in ice bath for 2 h and are hereafter called SG1-AP-GA-GOD, SG2-AP-GA-GOD, SG3-AP-GA-GOD.

2.6. Characterization

The N_2 adsorption-desorption isotherms were determined at liquid nitrogen boiling point, using a Tristar 3020 Kr Micromeritics equipment. The samples were previously degassed at 120 °C (except for samples with enzymes, where degassed temperature was 60 °C), under vacuum, for 12 h. The specific surface areas were determined by the BET (Brunauer, Emmett and Teller) multipoint technique and the pore size distribution was obtained by using the BJH (Barret, Joyner and Halenda) method [26]. The thermogravimetric analysis of materials were performed under argon flow on a Shimadzu Instrument model TGA-50 2, with a heating rate of 10 °C min^{-1}, from room temperature up to 600 °C. The temperature range used to estimate the organic contente was 150 to 600 °C.

2.7. Electrochemical measurements

Carbon ceramic electrodes were prepared by pressing (3 tons) 20 mg of the materials with 3 mg of mineral oil. The obtained disks