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Nanoconfinement of glucose oxidase on mesoporous carbon electrodes with tunable pore sizes

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ABSTRACT

By using a series of nanoporous carbons with a controlled distribution of pore sizes we have demonstrated the effect of commensurate confinement in the nanopores of the carbon electrodes in the electrochemical and enzymatic activity of immobilized glucose oxidase. The nanoconfinement resulted in more efficient oxidation of glucose through direct electron tunneling of the flavin adenine dinucleotide (FAD) site and the electrode surface. The electrochemical and enzymatic activity was boosted in carbon materials with pores which size matched the dimensions of the enzyme. This is attributed to the conformational changes of the biomolecule in the nanoconfined state, and the proximity of the FAD active site and the carbon electrode pores/walls boosts the electron transfer even in the absence of a mediator. The thermal profiles of the immobilized enzyme provided direct evidence of the conformational changes in the nanoconfined state, and their correlation with the average mesopore size of the carbon material. For the material showing the most adequate porosity, the nanoconfined enzyme retained the electrocatalytic activity towards glucose oxidation -even in the absence of mediator-, and at a broad range of concentrations. This approach is essential to make further clear some critical issues about the immobilization of enzymes on nanoporous carbon electrodes for bioelectrochemical applications.

1. Introduction

Besides the extensive use of graphite and carbon black as electrodes, many different types of carbon forms (i.e., graphene and derivatives, carbon nanotubes, diamond related compounds, nanofibres or nanoporous carbons) exhibiting varied bulk, structural and surface properties have been extensively explored as main components in the formulation of carbon-based inks for electrochemical applications [1–3]. Particularly in the field of electrochemical biosensing, nanoporous carbon-based electrodes offer interesting opportunities due to their physicochemical features that combine mechanical and chemical stability, biocompatibility, relatively high conductivity and mostly importantly, reproducible up-scale synthesis for mass production of electrochemical devices [4–7].

Nanoporous carbons (NPC) as electrodes allow a quick and accurate monitoring of a pool of metabolites due to the enhanced stability of the confined molecules inside the pores. Thus, the choice of a carbon material as electrode may not only be dictated by parameters such as electron transfer rates, stability, mass transfer limitations and/or redox potentials, but also by its textural characteristics (in terms of surface area, pore volume and distribution of pore sizes) that control the host-guest interactions, and thus the stability of the nanoconfined molecule. NPC are thus ideal electrode materials for the design of third generation electrochemical sensors and biosensors based on achieving direct electron transfer (DET) processes between the electrode surface and the immobilized molecules [8–12]. The key aspect of such biosensors is to achieve improved selectivity and lower redox potentials, while preserving the stability of the immobilized molecules, since DET allows the measurement of the substrate of the enzyme without the need of mediators or complications associated to solution system [13–14].

However, the redox/active sites of most enzymes are often deeply buried within the protein, hindering direct electron transfer communication between the enzyme and the electrode surface. This is the case, for instance of glucose oxidase (GOX), where the flavin adenine dinucleotide (FAD) moiety, is deeply embedded in the protein shell of GOX. Immobilization on porous supports is perhaps the simplest alternative to overcome these drawbacks; however, this scenario may become quite complex, since the immobilization can induce conformation changes on the biomolecule that would reduce its enzymatic activity, even if direct electrical transfer at the electrode surface is achieved [15–17]. Indeed,

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understanding the confinement of immobilized molecules on nanopore spaces is still challenging.

The objective of this work was to explore the utilization of NPC with a varied distribution of pore sizes in the mesoporous range, to investigate the impact of nanoconfinement of glucose oxidase on such electrodes on the long term stability and enzymatic response for glucose biosensing. To attain this goal, nanoporous carbon gels prepared by sol-gel polycondensation of resorcinol-formaldehyde mixtures were used as electrode materials for the immobilization of the enzyme. These materials can be easily synthesized with tunable pore architectures by adjusting the synthesis conditions [18–21], thus allowing to obtain carbons combining large specific surface areas with a proper network of transport pores to favor the diffusion and adsorption of bulky molecules [22,23]. The novelty of the work resides on providing a deep insight on the role of the tight nanoconfinement of an immobilized enzyme inside the porosity of carbon electrodes, aiming at correlating the electrochemical and enzymatic response with the pore dimensions of the carbon-based electrode materials. This approach is essential to make further clear some critical issues about the immobilization of enzymes on nanoporous carbon electrodes in bioelectrochemical applications.

2. Experimental

2.1. Materials and reagents

All chemicals were of analytical reagent grade and were used without further purification. Glucose oxidase (E.C. 1.1.3.4, from Aspergillus niger), glucose d-(+)-glucose (97%) and ferrocene monocarboxylic acid were obtained from Sigma-Aldrich. Nafion perfluorinated ion-exchange (5 wt% solution in 90% alcohol) was obtained from Fluka. GOX was dissolved in phosphate buffer saline (PBS) solution (0.1 M, pH = 7.6) and stored at 4 °C until use (concentrations ranging from 10–200 μM).

2.2. Synthesis of the carbon materials

Nanoporous carbon gels were synthesized by the sol-gel polymerization of resorcinol (R) and formaldehyde (F) in water (W), using sodium carbonate (C) as catalyst, as reported elsewhere [20,21]. Briefly, the precursors were mixed at fixed molar ratios (R/W of 0.06, R/F of 0.5 and R/C of 50, 75, 100 and 200) under magnetic stirring and immediately heated in airtight sealed glass vessels for gelation/aging at 95 °C for 4 h in an oven. Afterwards, the wet gels were dried at subcritical conditions at 150 °C for 12 h, and further carbonized at 800 °C under inert atmosphere (i.e., 100 mL/min N_2) for 1 h. Different R/C molar ratios (50, 75, 100 and 200) were used to obtain carbon gels with varied porous features in the mesoporous range. The carbon gels were labeled as Gx, where x refers to the R/C molar ratio (i.e., G50, G75, G100 and G200). The carbon gels were dried at 80 °C overnight and kept in desiccator until use. A commercial carbon black (Super P, TIMCAL) was also used as electrode material for comparison purposes. The nomenclature of this sample was CB.

2.3. Preparation of the inks and electrodes

About 10 mg of solids were dispersed in 5 mL of isopropyl alcohol by sonication; about 20 μL of this ink were casted onto a 3 mm glassy carbon (GC) electrode (previously polished with 0.05 μm alumina slurry) and allowed to dry at room temperature. A Nafion binder film (ca. 5 μL of 5 wt% Nafion aqueous solution) was subsequently deposited on top of the electrode to facilitate the cohesion between the glassy carbon support and the casted ink. The electrodes were then immersed in ca. 20 mL of 0.1 M PBS containing 10 mg/mL of GOX at 4 °C, for 24 h. To remove the weakly bounded enzyme, the electrodes were rinsed several times with distilled water and PBS solution. The casted electrodes were labeled as GC/Z, where Z stands for the NPC material (Gx or CB), and GC/Z/GOX when the enzyme is immobilized on the carbon materials. Free GOX was also casted directly onto the glassy carbon electrode to evaluate the activity of the enzyme on the bare non-porous support. The electrodes were stored at 4 °C in a refrigerator when not used.

2.4. Electrochemical measurements

All the electrochemical measurements were carried out at room temperature in a three electrode configuration cell, using the prepared working electrodes casted on the GC support, a Pt wire as counter electrode and a calomel SCE reference electrode -via Luggin probe-. Deoxygenated 0.1 M PBS solutions were used in all the cases (ca. bubbling N_2 for 30 min and additionally maintaining a N_2-rich atmosphere over the solution). Due to the nanoporous nature of the carbon materials, the electrodes were immersed in 0.1 M PBS buffer solution for at least 6 h prior to the measurements so as to guarantee the access of the electrolyte to the pores (wettability), and then stabilized a few cycles. The electrochemical response of the electrodes was evaluated by cyclic voltammetry at 20 mV/s in a multichannel potentiostat/galvanostat. For evaluating the electrocatalytic response of the immobilized GOX, cyclic voltammograms were recorded in the absence and presence of ferrocene monocarboxylic acid as mediator, in a potential range between −1000 and 1200 mV. The linearity of the electrocatalytic response of the best performing electrode after subsequent injections of glucose was recorded in the presence (ca. +350 mV) and absence (ca. +900 mV) of mediator.

2.5. GOX adsorption

Glucose oxidase adsorption capacity of the studied carbon materials was determined by UV–Vis spectrophotometry from aqueous solution. Briefly, about 50 mg of the carbons were dispersed in a PBS solution of glucose oxidase and allowed to equilibrate for 24 h under continuous stirring. The carbon is then filtered out and the concentration of the enzyme remaining in the solution was determined by UV–Vis spectrophotometry. The amount of GOX adsorbed was calculated from the mass balance of the amount of enzyme remaining in the solution.

2.6. Characterization techniques

The porous features of the carbon materials were evaluated by N_2 adsorption/desorption isotherms at −196 °C using a volumetric analyzer (ASAP 2010, Micromeritics). The samples were degassed at 120 °C for 17 h under vacuum before the analysis. Each isotherm was performed in duplicate (error was below 2%). Ultrahigh purity nitrogen (i.e., 99.995%) was supplied by Air Products. The gas adsorption data was used to determine the specific surface area, S_{BET}, total pore volume, V_{PORES} and full micro-/mesopore size distribution of the samples, the latter using the 2D-NLDFT-HS model assuming pore surface heterogeneity [24].

The interactions of the immobilized enzyme on the carbon gels were investigated using Temperature Programmed Desorption coupled to mass spectrometry (TPD-MS) in a custom-made device. Briefly, the samples were put in a fused-silica reactor and heated up to 873 K (heating rate 2 °C/min) under vacuum. The gases evolved were detected online by the mass spectrometer, recording several *m/z* simultaneously. Before the analysis, the carbon materials were loaded with the enzyme and washed several times to eliminate the weakly adsorbed biomolecule, as indicated above. The blanks corresponding to the carbon gels before the immobilization of the protein were also recorded.

Samples were chemically characterized by elemental analysis; the materials were dried under vacuum at 393 K for 17 h before the analysis. The contents of carbon, hydrogen, and nitrogen were measured in a LECO CHNS-932 microanalyzer (ASTM D-5373). The oxygen content

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