Influence of macroporous gold support and its functionalization on lactate oxidase-based biosensors response

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ABSTRACT

A general bioanalytical platform for biosensor applications was developed based on three-dimensional ordered macroporous (3DOM) gold film modified electrodes using lactate oxidase (LOx) as a case study, within the framework of developing approaches of broad applicability. The electrode was electrochemically fabricated with an inverted opal template, making the surface area of the 3DOM gold electrode up to 18 times higher than that of bare flat gold electrodes. These new electrochemical transducers were characterized by using Field Emission Scanning Electron Microscopy (FE-SEM), Atomic Force Microscopy (AFM) and the X-ray diffraction (XRD). The biosensor was developed by immobilization of lactate oxidase (LOx), on a 3DOM gold electrode modified with a self-assembled monolayer of dithiobis-N-succinimidyl propionate (DTSP). The resulting lactate oxidase biosensor was characterized by electrochemical impedance spectroscopy (EIS). The 3DOM gold electrode not only provides a good biocompatible microenvironment but also promotes the increase of conductivity and stability. Thus, the developed lactate oxidase bioanalytical platforms showed higher mediated bioelectrocatalytic activity compared to others previously described based on polycrystalline gold transducers. The response to varying lactate concentrations has been obtained in the presence of hydroxymethylferrocene as redox mediator in solution. Under these conditions, the bioanalytical platform response for DTSP covalently bound enzyme was improved with respect to that obtained in absence of DTSP.

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1. Introduction

Biosensors based on flat gold electrode surfaces modified with self-assembled monolayers are widely used, including fundamental studies of electrochemical processes on modified electrodes. For some applications, such as in vivo measurements, it may be necessary to use miniaturized electrodes and smaller active electrode surface will lead to lower analytical response. Therefore, to achieve a sufficiently high rate of electrochemical conversion, the electrode surface area must be increased artificially by using nanostructured [1] or porous materials [2]. An ideal way to develop metallic macroporous materials with controlled size and the spatial arrangement of the pores is to use colloidal crystal films as templates for subsequent electrodeposition [3–5]. Afterwards, the template is removed by an appropriate solvent and highly ordered structures of macroporous metal are obtained [6,7]. Due to its large surface area to

volume ratio, this kind of structure can be considered as a good candidate for biosensors. Thus, Xia and co-workers developed a 3DOM gold film modified electrode to study the direct electron transfer of hemoglobin [8,9]. Macroporous ultramicroelectrodes [7], 3DOM Prussian blue film and 3DOM gold film modified electrodes have also been prepared for glucose detection [10,11].

Recently, 3DOM gold film modified electrodes have also been used for immunosensor development [12,13]. In these systems, the sensing enhancement was significantly higher than that of respective nonporous electrodes.

The development of methods for the determination of L-lactic acid is of great importance in several areas such as medicine and food industry [14–20].

Our efforts over the past years in this field have been addressed to design L-lactic acid biosensors based on lactate oxidase (LOx). In the present work, we describe the preparation and characterization of a 3D macroporous gold inverse opal films, using polystyrene colloidal crystals as templates, in order to obtain a nanostructured electrodes that can be used in the design of significantly improved lactate biosensors. To achieve the enzyme immobilization we have employed two strategies: (i) direct adsorption onto the 3D macroporous gold surface and (ii) covalent bonding to 3D gold surfaces

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previously modified with a bifunctional reagent containing succinimide functionalities, in particular, 3,3′-dithiodipropionic acid di(N-succinimidyl ester) (DTSP) [19]. The biosensor response was obtained by replacing the natural electron acceptor (O_2) by an artificial mediator (hydroxymethylferrocene, HMF), whose electrochemical behaviour (anodic peak current in cyclic voltammetric response) is followed at −0.16 V, instead of detecting the hydrogen peroxide generated in the lactate oxidation.

This strategy allows minimizing the contribution of interfering substances that could be oxidized at the high potential required for H_2O_2 detection [19,21].

To characterize the morphology and physical properties of the resulting biosensors, microscopic, impedimetric and voltammetric techniques have been used. Their electrocatalytic response has been compared with that obtained under the same conditions using a polycrystalline gold electrode as well as roughened gold electrodes [1].

2. Materials and methods

2.1. Reagents and substrates

The monodisperse polystyrene colloidal spheres solution (500 nm diameter) was obtained from Thermo Scientific as a 1.0 wt% suspension in water. The commercial gold plating solution (ECF 60) containing 10 g/l gold was obtained from Metalor. The gold electrodes used as substrates were prepared by thermal vapor deposition of 10 nm of a chromium adhesion layer, followed by 200 nm of gold, onto standard 1 mm thick glass microscope slides. The gold electrodes were cleaned by sonication in isopropyl alcohol (IPA) for 1 h followed by rinsing with deionized water. Lactate oxidase (EC 232-841-6 from *Pediococcus* species) lyophilized powder containing 41 units/mg solid was obtained from the Sigma Chemical Co. (St. Louis, MO). Stock solution was prepared by dissolving 1.3 mg of the LOx lyophilized powder in 250 μl of 0.1 M, pH 7 phosphate buffer, aliquoted (10 μl) and stored at −30 °C. Under these conditions the enzymatic activities remain stable for several weeks. 0.1 M stock solution of L-(+)lactic acid lithium salt 97% (Sigma) containing 1 mM hydroxymethylferrocene was prepared. 3,3′-dithiodipropionic acid di(N-succinimidyl ester) (DTSP), hydroxymethylferrocene (HMF) and dimethyl-sulfoxide (DMSO) were obtained from Aldrich and used as received. Other chemicals used in this work, such as sulphuric acid and sodium phosphate, were reagent grade quality and used as received without additional purification steps. Sodium phosphate (Merck) was employed for the preparation of buffer solutions (0.1 M, pH 7.0). All the solutions were prepared with ultra pure water by means of a Millipore Milli-Q system (18.2 MΩ cm).

2.2. Instrumentation

2.2.1. Electrochemical cell

The electrochemical measurements were performed in a conventional three-electrode set-up. In the case of catalytic measurements the volume of solution used was 10.0 ml. For 3D macroporous gold biosensor the working electrode was template-coated gold. A large area gold electrode was used as counter electrode. All the potentials are quoted with respect to mercury/mercurous sulphate reference electrode (SMSE). The electroactive surface area of the working electrodes was determined by cyclic voltammetry in 0.50 M H_2SO_4 from the charge under the reduction peak of gold oxide.

A conversion factor of 400 μC cm^{-2} was used [22]. The experiments were performed in solutions thermostated at 25 ± 0.5 °C.

2.2.2. Electrochemical Impedance spectroscopy measurements

EIS experiments were performed with an Autolab PGSTAT 30 potentiostat from Eco-Chemie. The solution was 0.1 M PBS (pH 7), 0.1 M KCl containing 10 mM $K_3Fe(CN)_6$ + 10 mM $K_4Fe(CN)_6$. A sinusoidal potential modulation of ±10 mV amplitude in 10^5–10^{-3} Hz frequency range, spaced logarithmically (120 per 8 decades), was superimposed on the formal potential of the redox couple $[Fe(CN)_6]^{3-/4-}$ (−0.21 V vs. MSE). To obtain the capacitance from the experimental data, the frequency f_i was selected to obtain the maximum value of Z''. The capacitance was calculated as $C = 1/(2\pi f_i R_{CT})$, where R_{CT} is the charge transfer resistance.

EIS results were analysed by fitting the experimental impedance data to Randles equivalent circuit. The electrical equivalent circuit parameters were calculated by fitting the impedance function to the measured spectra with a non-linear least-squares (NLLS) program using Z-plot/Z-view for all the frequencies measured. The criteria used to estimate the fitting quality were evaluated firstly with the low chi-square value and secondly with the low estimative errors (%) for all the components.

2.2.3. SEM, XRD and AFM characterization

The equipment used for the characterization of template-coated gold and macroporous gold samples was a Philips XL30 Scanning Electron Microscope equipped with a Field Emission Gun (FEG) and coupled with an Energy Dispersive X-ray analyzer (EDX DE4i). The samples were examined at 15 keV acceleration voltages.

For the atomic force microscopy (AFM) studies, a JPK NanoWizard II system was used. Measurements were carried out using silicon nitride cantilevers (Model OCML-RC800PSA; 71 kHz; k 0.73 N/m) from Olympus. Tapping mode was used for topographical characterization of the inverse opal gold electrodes electrodeposited on Au/Cr/glass slides as well as for gold electrodeposited on Au/Cr/glass slides prepared under the same conditions of potential and temperature as 3D macroporous gold samples in air. The images were only linearly flattened, in order to account for sample tilt.

The phases present in the coating were determined by X-ray diffractometry (X'Pert PRO XRD, Panalytical) at a glancing angle of 0.5° and 1° using Cu Kα line generated at 40 mA, 45 kV. Start Position [2θ (°)] 30.0200; End Position [2θ (°)] 99.9800; Step Size [2θ (°)] 0.0200; Scan Step Time [s] 2.0000. The measurement of width at half height ($W_{1/2}$) of each of the diffractogram peaks, for the determination of crystallite size, was performed with the X'Pert HighScore Plus program. The diffractometer works with the wavelength of copper, which is 1.5406 nm.

2.3. Procedures

2.3.1. Preparation of macroporous Au electrodes

The method for the self-assembly of the polystyrene (PS) spheres consists basically of the slow evaporation of a small volume of dilute bead suspension (0.05 wt% in water) at the gold substrate surface [23]. To define the geometric area for the electrodeposition step, the electrodes together with the template were coated with a thin varnish film leaving a square window with a desired dimension. The template samples were then put into the commercial gold plating bath (at 25 °C) 5 min prior to the reduction, in order to allow the solution to diffuse all the way throughout the template. Then a potential of −1.05 V vs. SMSE was applied until the required charge had been passed. During the electrodeposition of gold through the template we observed temporal oscillations in the reduction current [6]. These oscillations can be explained by the periodic variation of the active electrode surface area during the growth of gold in the template and they give us an easy and reliable way to control the thickness of the deposit with respect to the number of sphere layers (Fig. 1a).