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Cholesterol biosensing with a polydopamine-modified nanostructured platinum electrode prepared by oblique angle physical vacuum deposition

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ABSTRACT

This paper reports a novel cholesterol biosensor based on nanostructured platinum (Pt) thin films prepared by Magnetron Sputtering (MS) in an oblique angle (OAD) configuration. Pt thin films were deposited onto a gold screen-printed electrode and characterized using Rutherford Back Scattering (RBS), Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), Cyclic Voltammetry (CV), X-ray Photo-electron Spectroscopy (XPS), Atomic Force Microscopy (AFM) and wetting analysis. Our results confirmed that the film is highly porous and formed by tilted nanocolumns, with an inclination of around 40° and a total thickness of 280 nm. XRD and CV analysis confirmed the polycrystalline nature of the Pt thin film. Cholesterol oxidase (ChOx) was covalently immobilized using a bioinspired polymer, polydopamine (PDA), via Schiff base formation and Michael-type addition. After being immobilized, ChOx displayed apparent activation energy of 34.09 kJ mol^{-1} and Michaelis constant (K_M) values of 34.09 kJ mol^{-1} and 3.65 mM, respectively, confirming the high affinity between ChOx and cholesterol and the excellent ability of the PDA film for immobilizing biological material without degradation. Under optimized working conditions the developed biosensor presented a sensitivity of 14.3 mA $M^{-1}cm^{-2}$ (R^2:0.999) with a linear range up to 0.5 mM and a limit of detection of 10.5 μM (S/N = 3). Furthermore, the biosensor exhibited a fast response (<8 s), good anti-interference properties and high stability after relatively long-term storage (2 months).

1. Introduction

Present days are witnessing a growing demand of new sensor devices for analytical, health, agro-alimentary, pharmaceutical, defense and environmental applications. According to the International Union of Pure and Applied Chemistry (IUPAC) a biosensor is "a self-contained integrated device, which is capable of providing specific semi-quantitative or quantitative analytical information using a biological recognition element which is retained in direct spatial contact with a transduction element" [1]. Biosensors may replace or complement classical analytical methods (e.g. chromatography, capillary electrophoresis and mass spectrometry) simplifying or eliminating sample preparation steps and making field-testing easier and faster with a significant decrease in cost per analysis [2]. In particular, electrochemical biosensors are very attractive because of their low dimensions, high sensitivity and selectivity, low cost, real-time output, simplicity of starting materials or the possibility to develop user-friendly and ready-to-use devices [3,4].

First-generation biosensors based on the detection of enzymatically generated H_2O_2, are still preferred due to its easy and practical implementation [5]. Platinum (Pt), thanks to its outstanding electrocatalytic properties and commercial availability in different configurations, dimensions and alloy compositions, is the most common material for electrochemical detection of H_2O_2 [3–8]. However, classical Pt electrodes present polished and flat surfaces with a low surface to volume ratio with limited sensitivity and capacity for oriented immobilization of anchored biomolecules. Random distribution and poor orientation of biomolecules may induce conformational changes and biomolecule denaturation, as well as constrain the accessibility of the substrate to the active/binding sites [9,10]. These deleterious effects can be avoided

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using nanostructured porous layers which, presenting higher surface/volume ratios and abundant surface sites for an oriented and conformal immobilization of biomolecules, may render a higher biological activity and an enhanced sensing capacity [10–13].

In the present work we combine both a new nanostructured Pt electrode and the bioinspired material polydopamine (PDA) to develop a new kind of cholesterol biosensor. This combination ensures the correct immobilization of the biological recognition element (cholesterol oxidase) and preserves its electrocatalytic activity. Magnetron Sputtering (MS) has been used to fabricate the Pt electrode [14]. This physical vapour deposition technique is typically applied for the fabrication of compact films, a feature that restricts the expected functionality with regard to biomolecules. An alternative to conventional MS consists of using an oblique angle deposition configuration (MS-OAD) with the substrate forming a glancing angle with respect to the target [14–22]. This deposition geometry leads to the formation columnar and highly porous microstructures with mesopores extending from the surface of the film up to the interface with the substrate [18,21].

PDA coatings have attracted considerable interests in the recent years because they can be very effectively applied to modify surfaces. This bioinspired polymer has been usefully applied for biomedical applications, remediation, biomineralization, drug delivery and hyperthermia, as well as for sensor and biosensor manufacturing [23,24]. From a biosensor perspective an interesting feature of PDA is the possibility to covalently graft onto its residual quinone groups other nitrogen and thiol containing molecules through either Schiff base or Michael-type additions [23–27]. This possibility together with a high reactivity and the possibility to cover an almost unlimited number of materials offer many unexplored opportunities for biosensor assemblies [23–32]. In particular, PDA provides a suitable microenvironment for immobilizing a high number of oriented biomolecules with intact catalytic and bio-recognition activities [23,25,33–37].

In the present work we prove that these concepts can be used for the development of a cholesterol biosensor consisting of a Pt porous electrode fabricated by MS-OAD, a PDA layer and a specific enzyme. The high performance of the developed biosensor, characterized by a good analytical response, anti-interference properties and the retention of the enzymatic activity for a long time, proves the feasibility of using these complex electrodes as robust and reliable biosensors.

2. Materials and methods

2.1. Reagents, materials and solutions

Cholesterol oxidase (ChOx) (C8868, from microorganism), dopamine (DA), cholesterol, ascorbic acid (AA), Triton X-100 and all other chemicals were obtained from Sigma. The target for MS was supplied by SMP and consisted of a 1 inch diameter disk of high purity (99.99%) platinum. Gold screen-printed electrodes (Au-SPE) (AT220, Dropsens) consisting of a 4-mm diameter gold working electrode, a gold counter electrode and a Ag pseudo-reference electrode were employed as electrochemical transducers. Phosphate buffer solutions (0.1 M, pH 7.4) were prepared in doubly distilled (DI) water (18.2 MΩ cm, Millipore-Q). ChOx was dissolved (1 mg mL^{-1}) in 50 mM PBS with 0.1 M NaCl. PDA monomer solutions (10 mM) were prepared in a PBS solution (pH 7.4) before use. Since cholesterol does not dissolve well in standard buffer solutions, a non-ionic surfactant (Triton X-100) was used [38–40]. Cholesterol stock solutions (5 and 10 mM) were prepared in PBS incorporating 10% (v/v) Triton X-100 and slowly heated until 60 °C during 30 min. The solutions were stored in a refrigerator where they remained stable for 1 month until the appearance of a slight turbidity. Electrochemical tests were carried out in 0.1 M PBS with 1% (v/v/) Triton X-100 (PBST) [38]. The spectrophotometric kit for cholesterol determination was supplied by Linear Chemical SL.

2.2. Preparation and characterization of the nanostructured platinum film electrodes

Nanostructured platinum thin films were deposited onto gold screen-printed (Au-SPE) substrates using the MS-OAD technique in a cylindrical vacuum reactor equipped with a magnetron sputtering target device (A310-XP, by AJA International) that was pumped down to a base pressure lower than 5×10^{-4} Pa. This base pressure value was achieved by a combination of turbomolecular and rotary pumps. The gas used for the deposition was Ar (purity 99.995%) and the working pressure was adjusted at 0.4 Pa with a throttling valve placed between the chamber and the turbo pump. The electrodes were placed 70 mm apart from the target. Preparations were carried out using a unipolar power DC supply at a constant electromagnetic power of 40 W. Deposition of platinum for a deposition time of 1 h was performed at oblique angle by tilting the surface of the electrodes 85° relative to the target [21]. A mask was placed over the electrodes to cover only the 4-mm diameter gold working area (Au-SPE/Pt).

Field emission scanning electron micrographs were collected in a HITACHI S 4800 microscope for doped silicon supported films conveniently diced for cross section observation. Raman and FTIR spectra were taken with a HORIBA HR-800-UV microscope and a JASCO FT/IR-6200 spectrophotometer set in the "Attenuated Total Reflection" (ATR) mode, respectively. Wetting measurements were carried out according to the Young method measuring the contact angle of small water droplets (3 μL) with a Contact Angle System OCA20 (DataPhysics). Topographic images of the surface were taken at the scale of 1 μm × 1 μm with a Nanotec AFM microscope and Dulcinea electronics, working in tapping mode and using high frequency levers. Data were processed with the WSxM software [41]. X-ray photo-electron spectroscopy (XPS) was carried out in a Phoibos-100 spectrometer, working in the pass energy constant mode, with Mg Kα as excitation source. The binding energy scale was referenced at 284.5 eV for the C 1 s peak of some slight contamination of carbon present on the electrode surface. X-ray diffraction spectra, obtained in a grazing angle configuration, were acquired using a Panalytical X'PERT PRO diffractometer. Rutherford back scattering, RBS, spectra were obtained in a tandem accelerator (CNA, Sevilla, Spain) with a ca. 1 mm diameter beam of alpha particles with energy and intensity of ca. 2.1 MeV and 1.7 nA respectively. RBS spectra were analyzed with the SIMRA6.0 program [42].

To evaluate the quality and the effective electrochemical area of the Pt thin film, cyclic voltammetry (CV) experiments were carried out with a DRP-μSTAT400 BiPotentiostat/Galvanostat, while data were acquired with the Dropview 8400 software (DropSens) in a 10 mL glass cell at room temperature. Therefore, a Pt-modified electrode was used as working electrode and a commercial Ag/AgCl (3 M KCl) and a Platinum (Pt) wire as reference and counter electrodes, respectively.

2.3. Biosensor fabrication and characterization

PDA was directly electrodeposited using CV. To this end, a drop (80 μL) of 10 mM DA was deposited onto the full electrochemical cell of the Au-SPE/Pt electrode, and cycled between −0.2 and 0.6 V at a scan rate of 0.1 V s^{-1} (default 20 cycles). Then, electrodes were washed several times with DI water to remove non-polymerized DA and subsequently dried under a N_2 atmosphere. Enzymatic immobilization was done by casting the working electrode with 20 μL of ChOx solution (1 mg mL^{-1}) followed by overnight storage in a wet atmosphere at 4 °C. These biosensors were then washed sev-