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Application of functionalised carbon nanotubes immobilised into chitosan films in amperometric enzyme biosensors

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ABSTRACT

A new approach for building a bio-conductive interface for enzyme immobilisation is described. This strategy permits very simple preparation of the enzyme biosensor and also reveals direct electron transfer features. A graphite-epoxy resin composite (GrEC) electrode modified with functionalised multi-wall carbon nanotubes (MWCNTs) immobilised by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide together with N-hydroxysuccinimide (EDC–NHS) in a chitosan (Chit) matrix was prepared and characterised by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in the presence of hexaammineruthenium (III) chloride. It was then used as a base for glucose oxidase (GOx) immobilisation by the simple method of crosslinking with glutaraldehyde (GA) with bovine serum albumin (BSA) as carrier protein. The resulting mediator-free biosensor was applied to the determination of glucose in amperometric mode at different applied potentials and the mechanism of reaction was also investigated by cyclic voltammetry, with and without dissolved oxygen in solution. Analytical parameters, as well as reproducibility, repeatability and stability were determined. Interferences were assessed using different compounds usually present in natural samples, such as wines, juices or blood, in order to evaluate the selectivity of the developed biosensor. The novel combination of carbon nanotubes immobilised with chitosan crosslinked with EDC–NHS and glucose oxidase immobilised by crosslinking with glutaraldehyde offers an excellent, easy to make biosensor for glucose determination without interferences.

1. Introduction

A new era in the field of nanotechnology has begun with the discovery of carbon nanotubes (CNTs) by Iijima in 1991 [1]. Consisting in single-wall carbon nanotubes (SWCNTs)—a single sheet of graphene rolled seamlessly in a cylinder of 1–2 nm diameter and multi-wall carbon nanotubes (MWCNTs)—several concentric tubes of graphene inside one other with diameters typically ranging from 2 to 100 nm, separated by a distance of 0.3–0.4 nm [2–9], this class of nanomaterials has attracted enormous interest. Due to their unique physical and chemical properties, CNTs have been extensively researched for electrocatalytic and sensing applications including fabrication of electrochemical sensors and biosensors [2–9].

CNTs promote electron transfer reactions of many compounds and their use as electrode modifiers leads to a decrease of the overpotential, a decrease of the electrode response time and/or an increase of the reaction rate of various electroactive substrates [2–10], in comparison with conventional carbon electrodes [3,11]. The electroactivity of CNTs is ascribed to the presence of reactive groups on its surface and/or defect-areas of the nanotubes [4,7,9], and the advantages of using CNTs for electrode surface modification in the development of new designs of electrochemical sensors and biosensors have been recently highlighted by many authors [2–16]. Nevertheless, the low solubility of CNTs in most solvents is the major challenge to their use as modifiers in the fabrication of chemical sensors and/or biosensors. The strategies most employed to disperse CNTs are end and sidewall functionalisation [4,17,18], use of surfactants with sonication [19], and polymer wrapping [20]. In an attempt to develop more sensitive biosensors, different enzymes have been immobilised together with carbon nanotubes [9,21]. The immobilisation of enzymes is a key step in the fabrication of biosensors and the biocompatibility of the matrix, its easy preparation and/or its stability is of extreme importance. Chitosan (Chit), a linear β-1,4-linked polysaccharide (similar to cellulose) that is obtained by the partial deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish fulfils these requirements. Chitosan (Chit) possesses distinct chemical and biological properties [22], because chitosan has reactive amino and hydroxyl groups in its linear polyglucosamine high molar mass chains which are amenable to chemical modification [22–26]. In addition, Chit is biocompatible, biodegradable, is a

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non-toxic, natural and high mechanical strength biopolymer with an excellent film-forming ability and is also a very good matrix for enzyme and/or biomacromolecule immobilisation [23]. Methods for chitosan film preparation described in the literature [27] can be broadly divided into four groups: solvent evaporation, neutralisation, crosslinking and ionotropic gelation methods. Chitosan has also been investigated for the development of electrochemical biosensors together with carbon nanotubes [28].

This work focuses on the development of novel enzyme biosensors, illustrated here using the model enzyme, glucose oxidase. The enzyme was immobilised onto carbon nanotubes entrapped into chitosan matrices by covalent binding with EDC–NHS. The mechanism of EDC–NHS binding with chitosan and MWCNT was previously described [29], in which it was observed that carbon nanotubes present higher loading in the immobilised film, the electrodes modified in this way exhibiting the highest electroactive area and the fastest electron transfer compared with other crosslinkers tested.

The present study concerns the electrochemical characterisation of electrodes modified with carbon nanotubes by means of cyclic voltammetry and impedance spectroscopy and its application in the development of glucose biosensors. The resulting biosensor was tested for its response to glucose by cyclic voltammetry and fixed-potential amperometry. The mechanism of the biosensor, investigated in the presence and absence of oxygen revealed evidence of direct electron transfer between carbon nanotubes and the redox centre of the enzyme. The analytical parameters of the biosensor as well as its reproducibility, stability and selectivity were also evaluated.

2. Experimental

2.1. Reagents and buffers

All reagents were of analytical grade, and the solutions were prepared using water of resistivity not less than 18 MΩ cm from a Millipore Milli-Q nanopure water.

Araldit epoxy resin and Araldit hardener were purchased from Ceys S.A. (Spain). Graphite powder (grade #38) was obtained from Fisher Scientific Corporation (USA). Multi-walled carbon nanotubes (MWCNTs) were obtained from NanoLab (USA). Chitosan of low molecular weight with a degree of deacetylation of 80%, glucose oxidase (GOx; E.C. 1.1.3.4) from *Aspergillus niger*, type II, lyophilized powder, 15,000–25,000 U/g solid, α-D(+)glucose, glutaraldehyde (GA) (25%, v/v solution), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma–Aldrich (Germany). N-hydroxysuccinimide (NHS) and potassium chloride were from Fluka (Germany) and hexaamminеruthenium (III) chloride was acquired from Merck (Germany).

For electrochemical experiments the supporting electrolyte was sodium phosphate buffer saline (NaPBS) (0.1 M Na_2HPO_4/NaH_2PO_4 + 0.05 M NaCl) pH 7.0.

2.2. Instruments and cell

Cyclic voltammetry and amperometry experiments were performed using a PalmSens potentiostat from Palm Instruments BV (The Netherlands) running with PS Lite 1.7.3 software. All measurements were carried out using an electrochemical cell with three electrodes: the bare graphite-epoxy resin composite (GrEC) electrode, GrEC/Chit, GrEC/Chit-CNT and GrEC/Chit-CNT/GOx as working electrode, a platinum wire as the auxiliary and a saturated calomel electrode (SCE) as reference. Electrochemical impedance measurements were carried out using a Solartron 1250 Frequency Response Analyser, coupled to a Solartron 1286 Electrochemical Interface (UK) controlled by ZPlot software. The frequency range used was 65 kHz to 0.1 Hz with 10 frequencies per decade, and integration time 60 s. The pH measurements were done with a CRISON 2001 micro pH-meter (Spain). All experiments were performed at room temperature, 25 ± 1 °C.

2.3. Preparation of the electrode

2.3.1. Preparation of the epoxy resin composite

Graphite-epoxy composite electrodes were used as base electrodes, prepared from graphite powder and Araldit epoxy resin plus Araldit hardener. Graphite powder and epoxy resin, mixed with hardener, were hand-mixed in a ratio of 60:40 (m/m) as described previously [30]. The resulting paste was placed into the tip of a 1 mL insulin plastic syringe, and a copper rod with diameter equal to the inner size of the syringe was inserted to give the external electrical contact [31]. Before each use, the surface of the electrode was wetted with Milli Q water and then thoroughly smoothed, first with abrasive paper and then with polishing paper Kemet (UK).

2.3.2. Functionalisation of the carbon nanotubes

Multi-walled carbon nanotubes (MWCNTs) were purified and functionalised as described elsewhere [9]. A mass of 120 mg of MWCNTs was stirred in 10 mL of a 5 M nitric acid solution for 20 h. The solid product was collected on a filter paper and washed several times with nanopure water until the filtrate solution became neutral (pH ≅ 7). The functionalised MWCNTs obtained were then dried in an oven at 80 °C for 24 h. Nitric acid usually causes the significant destruction of carbon nanotubes and introduces –COOH groups at the ends or at the sidewall defects of the nanotube structure.

2.3.3. Immobilisation of the carbon nanotubes

A 1.0% (m/m) chitosan solution was initially prepared by dissolving 100 mg of Chit powder in 10 mL of 1.0% (v/v) acetic acid solution and stirred for 3 h at room temperature until complete dissolution occurred. The Chit solution was stored under refrigeration at 4 °C when not in use.

A 1.0% (m/v) functionalised MWCNTs in 1.0% (m/m) chitosan dispersion was prepared by sonication of 2 mg of functionalised MWCNTs in 200 μL of 1.0% (m/m) Chit in 1.0% (v/v) acetic acid solution for 2 h.

All film electrodes, obtained using the 1.0% (m/m) Chit solution or 1.0% (m/v) functionalised MWCNTs in 1.0% (m/m) chitosan were prepared following the procedure:

(1) dropping 10 μL of 1% (m/m) Chit or 10 μL of 1% (m/v) MWCNTs in 1.0% (m/m) Chit on the GrEC and left to dry. After solvent evaporation, another aliquot of 10 μL of Chit or 10 μL of MWCNT dispersion was added and the electrode was again left for solvent evaporation at room temperature in air for approximately 1 h;
(2) 10 μL of 0.1 M phosphate buffer saline (pH 7.0) solution was dropped on the surface and left to dry for 40 min and this step was then repeated, in order to deprotonate the amino groups of Chit by changing the pH at the electrode surface;
(3) finally, 10 μL of 0.5% (m/v) EDC–0.5% (m/v) NHS in the same buffer solution was dropped on the surface and left to dry for 2 h.

Three equal electrodes of each type, with and without MWCNTs, were prepared in order to evaluate the reproducibility of the sensing layers.

2.3.4. Enzyme immobilisation

Glucose oxidase was immobilised on top of the electrode previously modified with carbon nanotubes as follows: 10 μL of GOx