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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

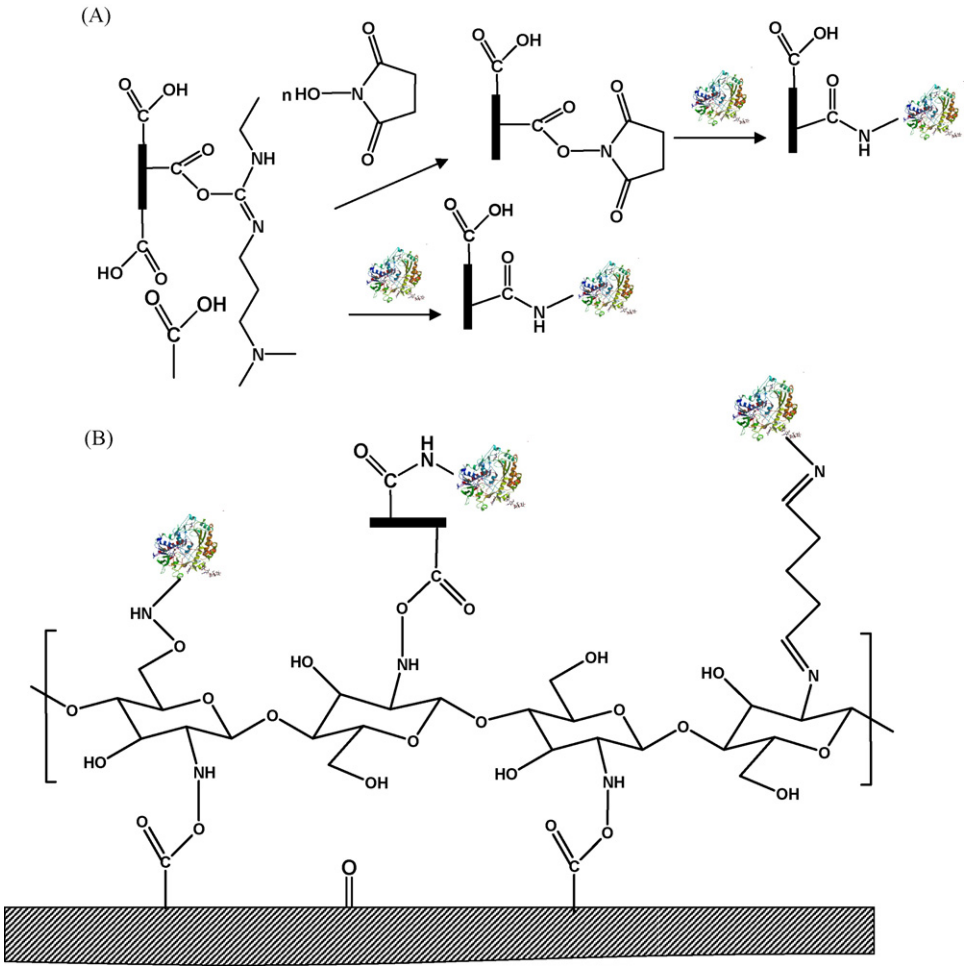

Fig. 1. Scheme of possible ways of enzyme immobilisation at electrodes modified with chitosan and MWCNTs: (A) enzyme attachment directly to CNTs by EDC–NHS; (B) enzyme linked to both chitosan and to CNTs by EDC–NHS and GA.

(1% m/v), 5 μL of BSA (2.5%, m/v) and 5 μL of GA (2.5%, v/v) were mixed carefully with a thin glass rod directly on top of the electrode and left to react for 1 h. Before use, the excess of GA was washed out with 20 μL of phosphate buffer saline (pH 7.0).

The scheme of enzyme immobilisation on the electrode is presented in Fig. 1.

3. Results and discussion

3.1. Characterisation of the carbon nanotubes modified graphite-epoxy composite electrodes

The graphite-epoxy resin composite electrode unmodified and modified with chitosan (Chit) and with chitosan and carbon nanotubes (Chit-CNT) immobilised by covalent binding with EDC–NHS were characterised by cyclic voltammetry and impedance spectroscopy in the presence of hexaammineruthenium (III) chloride as electroactive species. The results from the three kinds of electrode were compared in order to evaluate the advantages of immobilisation with incorporation of nanotubes for further applications.

3.1.1. Cyclic voltammetry

Cyclic voltammograms of hexaammineruthenium (III) chloride at bare (GrEC), chitosan (GrEC/Chit) and chitosan-nanotubes (GrEC/Chit-CNT) modified electrodes are shown in Fig. 2A. As can be clearly observed, the response to ruthenium (III) is slightly decreased by the chitosan coating (GrEC/Chit) compared with the bare electrode (GrEC), meaning that chitosan is causing a small decrease in the electroactive surface of the electrode. Chitosan has a relatively poor conductivity [32] and this effect of partially blocking the electrode was previously reported [33]. However, when nanotubes are added to the film coating a large increase in the response occurred. In all cases, at bare or modified electrode, the current peak increases linearly with the square root of scan rate for both oxidation and reduction, consistent with a diffusion-controlled process (see Fig. 2B for GrEC/Chit-CNT modified electrode). The slopes for the reduction process were: 9.7, 6.0 and 24 $\mu A\,mV^{1/2}\,s^{-1/2}$ for bare, chitosan and chitosan-nanotube modified electrodes, respectively, suggesting that carbon nanotube modification leads to a higher electroactive area. In all cases the peaks are well defined and quasi-reversible, and by the addition of carbon nanotubes an improvement in the reversibility of the redox process of $Ru(NH_3)_6Cl_3$ was observed, the peak separation decreasing from 92 mV with GrEC/Chit to 57 mV with GrEC/Chit-CNT at 10 $mV\,s^{-1}$. By applying the Randles–Sevcik equation and using a diffusion coefficient for hexammineruthenium (III) of $9.1 \times 10^{-6}\,cm^2\,s^{-1}$ [34] the calculated electroactive area was 0.126, 0.078 and 0.312 cm^2 for bare (GrEC), Chit (GrEC/Chit) and Chit-CNT (GrEC/Chit-CNT), respectively, a factor of 2.5 greater for the Chit-CNT modified electrode compared with the bare electrode.

The heterogeneous electron transfer standard rate constant, k_0, was estimated as the average among different values calculated at different scan rates, using the difference between anodic and cathodic peak potentials.

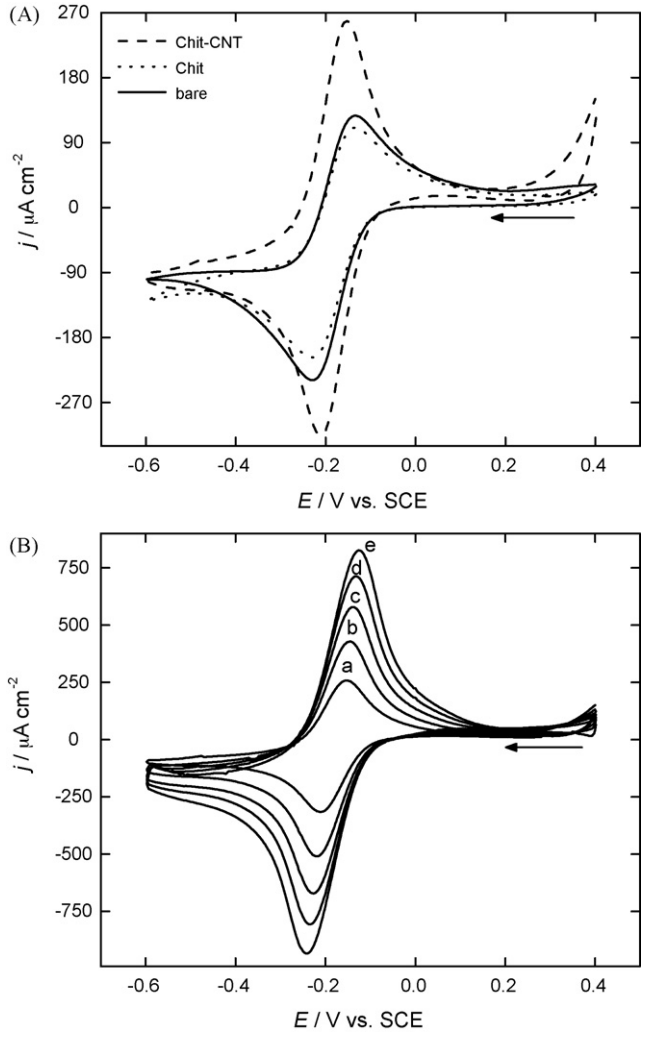

Fig. 2. Cyclic voltammograms, after background subtraction, of 3.0 mM $Ru(NH_3)_6Cl_3$ in 0.1 M KCl at (A) GrEC; GrEC/Chit and GrEC/Chit-CNT electrodes at scan rate 10 mV s^{-1} and (B) GrEC/Chit-CNT electrode, at different scan rates: (a)10, (b) 20, (c) 30, (d) 40 and (e) 50 mV s^{-1}.

The parameter Λ is a quantitative measure of reversibility [35]:

$$\Lambda = \frac{k_0}{(D_O^{\alpha_a} D_R^{\alpha_c} (nF/RT)\nu)^{1/2}} \quad (1)$$

in which D_O and D_R are the diffusion coefficients of oxidised and reduced species, respectively, with α_a and α_c the charge transfer coefficients for oxidation and reduction, and ν is the scan rate.

When $D_R = D_O = D$, and assuming $\alpha_a = \alpha_c = 0.5$:

$$\Lambda = k_0 D^{-1/2} \left(\frac{nF\nu}{RT}\right)^{-1/2} \quad (2)$$

The degree of reversibility, expressed as

$$\Psi = \Lambda \pi^{1/2} \quad (3)$$

has values which depend on the difference between anodic and cathodic peak potentials.

By substituting Eq. (3) in Eq. (2):

$$k_0 = \Psi \left(\frac{D\nu}{\pi} \frac{nF}{RT}\right)^{1/2} \quad (4)$$

The values of k_0 were 0.99×10^{-3}; 1.00×10^{-3} and 1.48×10^{-3} cm s^{-1} for bare, chitosan and chitosan-nanotubes modified electrodes, demonstrating that the electron transfer kinetics are similar at bare and chitosan modified electrodes and that carbon nanotubes significantly improve the electron transfer.

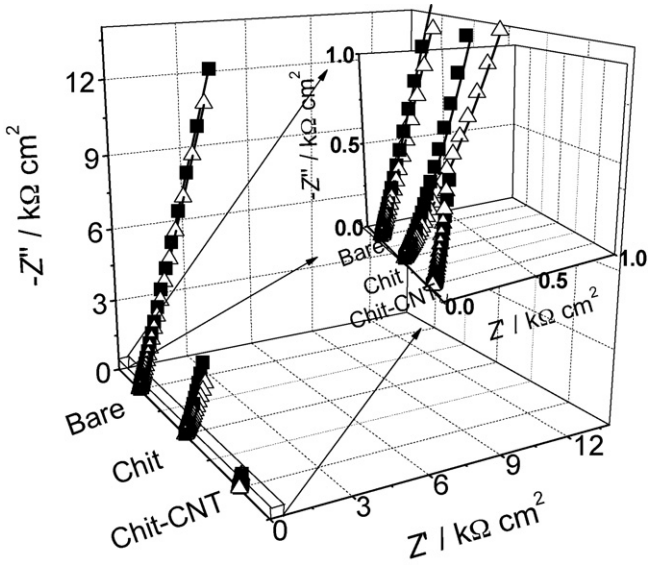

Fig. 3. Complex plane impedance spectra at GrEC, GrEC/Chit, and GrEC/Chit-CNT electrodes at OCP in 0.1 M KCl (black squares) and 0.1 M KCl + 3 mM $Ru(NH_3)_6Cl_3$ (white triangles). Lines indicate equivalent circuit fitting.

3.1.2. Electrochemical impedance spectroscopy

Electrochemical impedance spectra, see Fig. 3, were recorded at bare (GrEC); chitosan (GrEC/Chit) and chitosan-nanotubes (GrEC/Chit-CNT) electrodes, crosslinked with EDC–NHS, in 0.1 M KCl solution, with and without 3.0 mM $Ru(NH_3)_6Cl_3$, at the open circuit potential (OCP), which was ~+0.15 V *vs.* SCE. The impedance spectra were recorded at three equal electrodes of each type in order to study the reproducibility of the sensing layers.

All bare and Chit-modified electrodes exhibited similar behaviour with the impedance values varying about 20% between equal electrodes (not shown). Bare electrodes exhibited a non-ideal capacitive behaviour over the whole frequency range and the imaginary part of impedance reached a value of ~12 kΩ cm^2 at 0.1 Hz, as seen in Fig. 3. There was no change in the spectra with and without $Ru(NH_3)_6^{3+}$ at OCP at this kind of electrode showing that only charge separation occurs in the presence of a redox couple.

The GrEC/Chit electrodes had values of imaginary impedance which were much lower (Fig. 3), attributed to a decreased resistance of the modified electrodes. This demonstrates that both resistivity and electrode surface layer change with the modification of the graphite-epoxy composite, as expected.

The spectra at the GrEC/Chit-CNT electrodes were different, with a semicircle in the high frequency region and a linear part in the middle and low frequency region. The imaginary impedance value at 0.1 Hz dropped to 0.4 kΩ cm^2, again showing an increase in conductivity of these electrodes after modification with CNTs.

Impedance spectra were analysed by fitting to equivalent electrical circuits. A full analysis of the impedance spectra will be provided elsewhere. Almost all spectra could be fitted to a circuit consisting of a cell resistance in series with two constant phase elements (CPE):–R_Ω–CPE_1–CPE_2–, where R_Ω is the cell resistance. The first CPE describes the high frequency region corresponding to the electrode–electrolyte interface, and the second to the low frequency region, due to the fact that a composite electrode is being used (bare electrode) or to the modifying layer. The film capacitance significantly increases with modification of the electrode and, after modification with CNT, reaches 3 mF cm^{-2} and further increases slightly in the presence of redox species, indicative of incorpora-

tion within the modifying layer. The introduction of CNTs makes the layer less uniform than without nanotubes, suggesting that their dispersion is not perfect, but is still better than at unmodified composite electrodes.

The impedance results show that the modification of graphite-epoxy composite with CNTs attached to the electrode surface by crosslinked chitosan has a much better conductivity and more uniform electrode surface than unmodified electrodes.

3.2. Characterisation of the glucose biosensor

In order to evaluate possible applications of the electrode modified with chitosan and CNT, glucose oxidase was immobilised as described in Section 2. Different immobilisation methods were investigated, but the best was found when enzyme was crosslinked with glutaraldehyde on the top of a Chit-CNT electrode. These biosensors were characterised by cyclic voltammetry, impedance spectroscopy and fixed-potential amperometry in buffer solution in the presence of glucose, and the influence of oxygen was also investigated.

Cyclic voltammograms recorded in 0.1 M NaPBS pH 7.0 electrolyte using the biosensor described above had one quasi-reversible redox couple due to the electrochemical behaviour of CNTs [36,37] and an irreversible reduction peak at −0.2 V, as shown in Fig. 4A, dashed line. This peak disappears after glucose addition (Fig. 4A, solid line) and a decrease in the reduction current as well as an increase in the oxidation current in the potential region from −0.1 to −0.6 V was observed. The redox couple ascribed to CNTs did not change after glucose addition. In order to clarify the reduction processes occurring in the negative potential region, baseline correction was performed and, after this, two clear reduction peaks appeared at −0.2 and at −0.43 V, corresponding to oxygen and FAD reduction, respectively. FAD reduction in the absence of glucose was found to occur at −0.44 V *vs.* Ag/AgCl at CNT-RTIL modified electrodes [38] and at −0.465 V *vs.* SCE at single walled CNT modified Au electrodes [39] in phosphate buffer, pH 7.0. The FAD reduction peak has also been observed at −0.43 and at −0.455 V *vs.* Ag/AgCl at graphene-RTIL modified electrodes [40] and at CNT-chitosan-Au nanoparticle-modified electrodes [41], respectively, in phosphate buffer, pH 7.4. In all these cases a direct electron transfer from GOx to CNT modified electrodes was reported.

Glucose oxidase, in the presence of glucose, consumes oxygen when catalysing the oxidation of glucose to gluconolactone. Thus, the oxygen reduction peak decreased in magnitude after addition of glucose to the buffer solution and the anodic current increased in the potential region from −0.6 to −0.1 V due to $FADH_2$ oxidation or a FAD regeneration reaction (Fig. 4A).

Fig. 4B presents cyclic voltammograms at the biosensor in the absence and the presence of glucose in the absence of dissolved oxygen. The oxygen reduction peak decreased significantly but was still visible; it might be that some oxygen was trapped in the modifying layer, which is much more difficult to remove by nitrogen bubbling. Moreover, after glucose addition no significant changes were observed, especially in the region of $FADH_2$ oxidation (Fig. 4B). This fact leads us to surmise that the glucose oxidation process requires oxygen:

$$C_6H_{12}O_6 + FAD \xrightarrow{O_2} C_6H_{10}O_6 + FADH_2 \quad (8)$$

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (9)$$

Thus glucose can be determined directly either by the redox process of the co-enzyme FAD (Eq. (8)), or by oxygen consumption (Eq. (9)). There was no clear evidence of electrocatalytic hydrogen peroxide reduction in the potential region studied; probably it overlaps with the other electrochemical process at the electrode surface. A similar glucose biosensor behaviour was found at electrodes composed of polyvinylpyrrolidone-protected graphene/polyethylenimine-functionalised ionic liquid/GOx [40]. Such a biosensor was sensitive to both O_2 and H_2O_2 and in the presence of glucose the oxygen reduction peak decreased so that the oxidation current in the same potential region was increasing. The oxygen reduction peak occurred at −0.29 V and FAD reduction at −0.49 V *vs.* Ag/AgCl. Moreover, after solution deoxygenation FAD reduction significantly decreased [40].

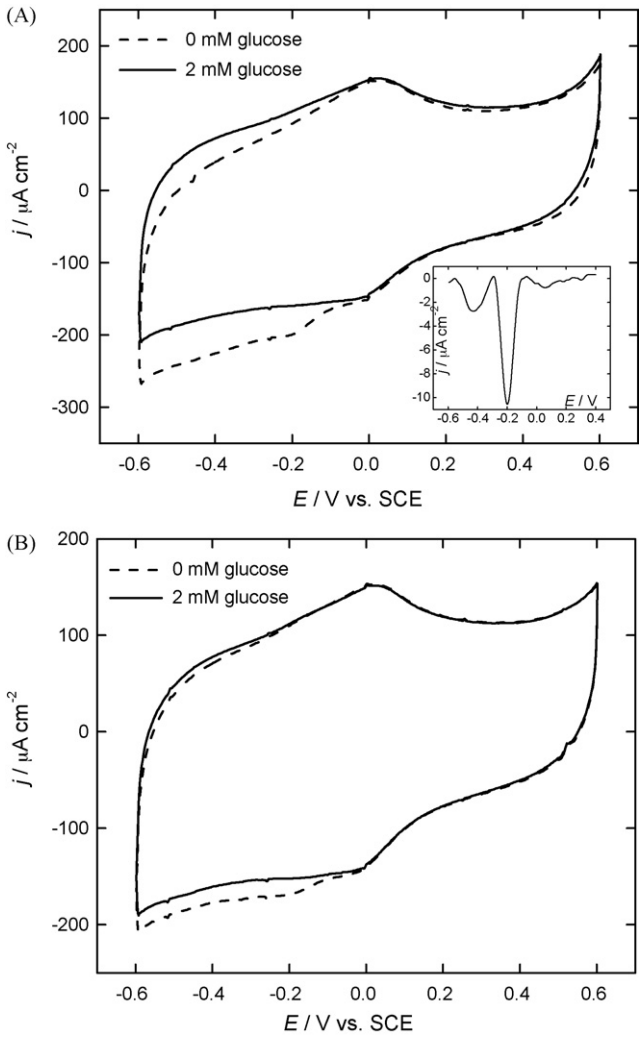

Fig. 4. Cyclic voltammograms at GrEC/Chit-CNT/GOx electrode before (dashed line) and after (solid line) 2 mM glucose addition in the presence (A) and absence (B) of O_2. Supporting electrolyte 0.1 M NaPBS, pH 7; scan rate 10 mV s^{-1}. Inset in (A) is the reduction process without glucose, after baseline correction.

Impedance spectra were recorded at the GrEC/Chit-CNT/GOx biosensor in the presence and absence of dissolved oxygen in the buffer solution. There is no significant difference in spectra with or without dissolved oxygen in solution which are similar to those shown in Fig. 3 without GOx. Addition of glucose to the buffer solution slightly decreased the values of the imaginary impedance and the real part of impedance became very slightly higher. In the literature, impedance spectra have a similar profile to those recorded here [33,36,37,42].

3.3. Amperometric glucose biosensor

A variety of immobilisation procedures have been used the in biosensor construction. Immobilisation by crosslinking and covalent binding has some important advantages such as good sta-

bilisation of the enzyme and fast and easy procedures. For this reason, crosslinking with glutaraldehyde and covalent binding with EDC–NHS were used.

Biosensors were prepared by first immobilising the nanotubes and afterwards the enzyme using either glutaraldehyde or EDC–NHS. On testing under the same conditions, using glutaraldehyde gave a good response but it was observed that in the case of immobilisation with EDC–NHS the biosensor exhibited very little response to glucose—it might be that EDC–NHS is somehow deactivating the enzyme. Some explanation for this behaviour can be found in Ref. [43] where criticisms concerning covalent immobilisation of proteins on nanotubes using EDC–NHS are made: the authors suggest that by crosslinking proteins with EDC–NHS supramolecules or aggregates of protein might be formed. On the other hand, carbodiimide is able to exert a strong bond between enzyme and electrode, but it promotes this fixation by covalent bonding with carboxylic groups. Since the enzyme active centre could contain this kind of group, the immobilised enzyme could have a lower activity. It seems that using GA both the enzyme and the nanotubes in chitosan are bonded, resulting in a more robust biosensor. This is in agreement with previous work [44], where, by studying the conditions for GOx immobilisation using EDC–NHS and GA, it was found that the best response was using GA.

Regarding glutaraldehyde crosslinking, two different strategies were evaluated to optimise the glucose response. In the first, the biosensor was prepared by first immobilising two layers of Chit-CNT on the graphite-epoxy resin composite and afterwards immobilising glucose oxidase, i.e. Chit-CNT/Chit-CNT/GOx-GA. In the second, a Chit-CNT layer was first cast on the electrode, then glucose oxidase was immobilised with GA and finally another, second layer of Chit-CNT was spread in a sandwich-like configuration, i.e. Chit-CNT/GOx-GA/Chit-CNT. These two types of biosensor assembly were tested at −0.2 V *vs.* SCE in phosphate buffer saline pH 7.0, adding known amounts of glucose standard solution. The results (data not shown) revealed that by placing glucose oxidase on top of Chit-CNT a better response to glucose is obtained, meaning that diffusion of the enzyme substrate to the enzyme active centre is slower than that of peroxide to the electrode substrate surface. The sensitivity of this biosensor was a factor of 3.3 greater than with the sandwich configuration and the detection limit was lower at 22 μM compared to 48 μM. This can be explained by the fact that when enzyme is placed on top of the nanotubes it is easier for the glucose substrate to diffuse to the active centre of the enzyme and react. For comparison, crosslinking of MWCNTs was also performed without chitosan but the electrode stability was not sufficiently good. Thus, chitosan appears to be an important aid for casting carbon nanotubes on substrate electrode surfaces.

The optimised construction methodology for the biosensor in the present work was therefore first to immobilise CNT in a chitosan matrix with EDC–NHS and afterwards the enzyme, glucose oxidase, using the bi-functional crosslinker glutaraldehyde. Enzyme can be crosslinked via EDC–NHS to carbon nanotubes or directly crosslinked with glutaraldehyde to chitosan via its amino groups, as illustrated in Fig. 1. This biosensor is used in the experiments below. Between tests, the biosensors were stored in phosphate buffer saline pH 7.0 at 4 °C.

The biosensor response at different potentials, namely −0.45, −0.3, −0.2, −0.1, 0.0 and +0.45 V *vs.* SCE was evaluated. A current–time curve obtained at −0.3 V and the calibration curves at all potentials are shown in Fig. 5. In all cases, an anodic change in current was observed with the difference that at negative potentials the currents have negative values and at 0.0 and +0.45 V the values are positive. Based on this response and also on cyclic voltammetry experiments carried out with the biosensor in buffer and with addition of glucose with and without oxygen (see Section 3.2) a mechanism for biosensor functioning can be proposed (see also

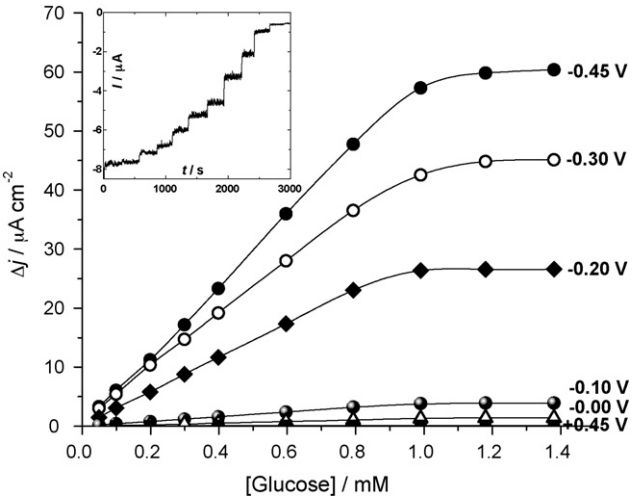

Fig. 5. Calibration curves at GrEC/Chit-CNT/GOx biosensor for glucose in 0.1 M NaPBS pH 7.0 at different applied potentials *vs.* SCE. In the inset is the current *vs.* time response at −0.3 V.

Eqs. (8) and (9)). Thus, at positive potentials, oxidation of hydrogen peroxide occurs and at negative potentials competition between $FADH_2$ oxidation and H_2O_2 and/or O_2 reduction occurs. The analytical parameters obtained from the calibration curves plotted at different potentials are presented in Table 1. As can be seen, the highest response was obtained at −0.45 V *vs.* SCE and is mainly dictated by FAD regeneration; at less negative potentials the sensitivity decreased due to the contribution of two processes leading to a total current which is the sum of both an oxidation and a reduction response.

The performance of the biosensor is highly satisfactory, considering that the sensitivity obtained at −0.45 V is a factor of two thousand higher (60.2 $\mu A\,mM^{-1}\,cm^{-2}$) than in Ref. [21] (31.1 $nA\,mM^{-1}\,cm^{-2}$) which used a biosensor prepared with chitosan and carbon nanotubes at −0.4 V. The sensitivity achieved at +0.45 V (1.38 $\mu A\,mM^{-1}\,cm^{-2}$) is comparable with but less than that of a similar biosensor operating at +0.4 V but which uses a mediator [28] (7.4 $\mu A\,mM^{-1}\,cm^{-2}$), but details of concentration and activity of the enzyme used in the sensor are not specified.

Amperometric measurements carried out with different biosensors showed that there exists good reproducibility; the relative standard deviation for 4 electrodes was 2.7%, lower than obtained in Ref. [45] with 5 electrodes (4.1%). The stability of the biosensors was evaluated by constructing two calibration curves per week and after 3 weeks 69% of the initial response was maintained, even though this biosensor was also used for cyclic voltammetry and impedance spectroscopy studies during this period. The results are comparable with those in Ref. [45] where the biosensor kept 80% of the initial response after 1 month. When used for continuous measurements at least 7 calibration curves could be recorded successively consisting of 10 successive glucose additions each, without any loss of response, an improvement compared to Ref. [28], where the biosensor remains stable after up to 50 continuous potential

Table 1
Performance of GrEC/Chit-CNT/GOx biosensor at different applied potentials.

E/V *vs.* SCE	Linear range/mM	Sensitivity/$\mu A\,mM^{-1}\,cm^{-2}$	LOD/μM
+0.45	1.0	1.38 ± 0.01	16
0.00	0.8	0.109 ± 0.001	21
−0.10	0.8	4.06 ± 0.03	14
−0.20	0.8	28.8 ± 0.3	22
−0.30	0.8	44.9 ± 0.3	20
−0.45	0.8	60.2 ± 0.8	26

Table 2
Relative response to glucose in the presence of some potential interfering compounds at GrEC/Chit-CNT/GOx—ratio glucose to interferent 1:1.

Compound	Relative response to glucose/%		
	−0.2 V	−0.3 V	−0.4 V
Fructose	100	100	100
Catechol	100	97.1	93.6
Ascorbic acid	56.6	65.8	92.3
Citric acid	96.1	100	100
Tartaric acid	100	100	100
Uric acid	93.4	95.8	100

cycles in cyclic voltammetry or after 15 days of "on-shelf" stability. The performance under continuous use is good without loss of response and offers good stability due to the strong immobilisation of the enzyme to both carbon nanotubes and chitosan, an improvement compared to the biosensor described in Ref. [21] in which the electrode loses 10% of response after 1 week.

The selectivity of the biosensor was evaluated by testing the response of different compounds that exists in natural samples such as juice, wine and blood that could interfere with glucose. These interferents were: fructose, catechol, ascorbic acid, citric acid, tartaric acid and uric acid. The response to the interferents in a ratio 1:1 to glucose at different applied potentials: −0.4, −0.3 and −0.2 V is shown in Table 2. The only one which exhibited a significant effect was ascorbic acid but, as can be seen, its influence is less important (the response decreased from 43% at −0.2 V to 7.7% at −0.4 V) when applying more negative potentials where the response to glucose is, in fact, increasing. However, normally all these compounds are present in concentrations that are smaller than that of glucose so there would be no problem for the determination of glucose in the presence of these substances. This biosensor clearly offers a great advantage compared with [28] which, since it operates at +0.4 V, can easily detect both ascorbate and acetaminophenol which can interfere with the glucose response.

4. Conclusions

The immobilisation of multi-walled carbon nanotubes on graphite-epoxy resin composite electrodes in a matrix of chitosan crosslinked with EDC–NHS leads to an improvement in electrode kinetics as illustrated by hexaammineruthenium (III) chloride using cyclic voltammetry and impedance spectroscopy. This nanotube-modified electrode provided a novel electrode platform for the immobilisation of glucose oxidase with glutaraldehyde. It was found that glucose was detected at this biosensor at different applied potentials, the highest sensitivity being achieved at −0.45 V *vs*. SCE, although less negative potentials, from −0.2 V, can be profitably used. The biosensor showed good reproducibility and good stability under continuous use and storage conditions. Selectivity against a number of interferents was also good and the ascorbic acid response could be reduced using more negative potentials where the response to glucose increases. Thus, the novel combination of carbon nanotubes immobilised with chitosan crosslinked with EDC–NHS and glucose oxidase immobilised by crosslinking with glutaraldehyde offers an excellent and convenient biosensor for glucose determination without interferences.

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