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Carbon nanotubes non-covalently functionalized with cytochrome c: A new bioanalytical platform for building bienzymatic biosensors

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ABSTRACT

We report for the first time the use of glassy carbon electrodes (GCE) modified by drop-coating with multi-walled carbon nanotubes (MWCNTs) dispersed in cytochrome c as platform for the construction of bienzymatic biosensors, using the glycoenzyme glucose oxidase (GOx) as model. The sensitivity of the resulting biosensor at −0.050 V towards glucose using hydroquinone (H_2Q) as redox mediator was (96 ± 2) μA M^{-1}, with a linear range between 1.00×10^{-4} M and 1.00×10^{-3} M and a detection limit of 8 μM. The biosensor demonstrated to be highly reproducible with RSD. values of 6.1% for five different bioelectrodes. The combination of the excellent dispersing properties of Cyt c, the stability of MWCNTs-Cyt c dispersion and the intimate interaction between Cyt c, MWCNTs and GOx, allowed the construction of an efficient glucose biosensor successfully used to determine glucose in commercial beverages.

1. Introduction

The combination of nanomaterials and biomolecules to build bioanalytical platforms has demonstrated to be an advantageous alternative for developing electrochemical biosensors [1,2]. The excellent electronic and structural properties of carbon nanotubes (CNTs) have converted them in a very attractive material to develop electrochemical sensors [3–5]. One of the most widely used strategies for the incorporation of CNTs in electrochemical transducers is the non-covalent functionalization with polymers, biomolecules, and surfactants [6–8]. These functionalization agents produce a change in the surface energy of the CNTs that avoids their aggregation and allow to obtain stable CNTs dispersions in polar solvents [9]. Recently, we have reported the efficient non-covalent functionalization (dispersion) of multi-walled carbon nanotubes (MWCNTs) with the protein cytochrome c (Cyt c), the robustness of the dispersion once deposited at glassy carbon electrodes (GCE) (GCE/MWCNTs-Cyt c), the direct electron transfer of Cyt c that supports the CNTs, and the efficient electrochemical sensing of hydrogen peroxide at GCE/MWCNTs-Cyt c [10].

Usually, enzymatic glucose biosensors are based on the use of the enzyme glucose oxidase (GOx) [11]. An alternative approach to improve the analytical performance of the biosensor is the construction of bienzymatic peroxidase/GOx biosensors [11–14]. In these systems, the H_2O_2 generated by GOx is subsequently reduced by horseradish peroxidase (HRP) which is electrically connected to the electrode surface at low potentials [14]. Compared to monoenzyme biosensors, this cascade scheme amplifies the electrochemical responses producing an enhancement of the biosensor sensitivity because H_2O_2 can easily diffuse to the nearby HRP and immediately react with HRP. Moreover, the removal of H_2O_2 by HRP could also reduce peroxide-induced degradation of GOx [15]. Only few works based on GOx/Cyt c bienzymatic biosensor have been proposed. Song et al. co-entrapped GOx and Cyt c on both gold nanoparticles-chitosan nanocomposites [15] and poly(diallyldimethylammonium chloride)-graphene nanosheets-gold nanoparticles hybrid nanocomposites modified glassy carbon electrodes [16]. A GOx/Cyt c biosensor based on glassy carbon electrode modified with gold nanoparticles-polyaniline nanospheres has also been developed [17].

We are reporting here a new concept of a bienzymatic biosensor based on the use of GCE/MWCNTs-Cyt c as a bioanalytical platform to build supramolecular architectures through the immobilization of an additional enzyme able to generate hydrogen peroxide. To evaluate the capability of this new electrochemical biointerface, we selected the glycoprotein glucose oxidase (GOx) as a model enzyme to develop a glucose biosensor. In the following sections we discuss the influence

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of the concentration of Cyt c that supports the MWCNTs, the concentration of GOx used as biorecognition layer, the advantages of using hydroquinone as redox mediator, and the effect of adding successive enzyme layers on the effectiveness of the biosensor for glucose quantification.

2. Experimental

2.1. Chemicals and solutions

Carbon nanotubes (MWCNTs, (30 ± 15) nm diameter, (1–5) μm length and purity higher than 95%), were supplied from Nanolab (USA). Cytochrome c (Cyt c) from horse heart, glucose oxidase (GOx) (Type X-S, Aspergillus niger, EC 1.1.3.4, 157,500 units/g of solid), Nafion (Naf) and hydroquinone (H_2Q) were supplied by Sigma. Glucose, lactose and galactose were obtained from Merck. Fructose and maltose were supplied by Mallinckrodt. NaH_2PO_4 and Na_2HPO_4 were purchased from Baker. Other chemicals were of analytical grade and used without further purification. Pepsi® and Gatorade® were obtained in a local market.

A 0.050 M phosphate buffer solution pH 7.40 was employed as supporting electrolyte. Ultrapure water ($\rho = 18.2$ MΩ cm) from a Millipore-MilliQ system was used for preparing all aqueous solutions.

2.2. Apparatus

Sonication treatments were carried out with an ultrasonic processor VCX 130 W (Sonics and Materials, Inc.) of 20 kHz frequency with a titanium alloy microtip (3 mm diameter).

Electrochemical experiments were carried out with Autolab (PGSTAT 128N Eco-Chemie) and TEQ_04 potentiostats. Bare glassy carbon electrodes (GCE, CH Instruments, 3 mm diameter) and modified GCE were used as working electrodes. A platinum wire and Ag/AgCl, 3 M NaCl (BAS, Model RE-5B) were used as auxiliary and reference electrodes, respectively. All potentials are referred to this reference electrode. A magnetic stirrer under controlled speed provided the convective transport during the amperometric measurements.

2.3. Preparation of MWCNTs-Cyt c dispersion

MWCNTs were oxidized using a mixture of sulfuric and nitric acids (3:1 v/v) for 5 h under ultrasonic stirring. The oxidized MWCNTs were repeatedly washed with distiller water up to reach pH ~7. The dispersion was prepared by mixing 1.0 mg of oxidized MWCNTs with 1.0 mL of 0.12 mg mL^{-1} Cyt c solution (prepared in water) followed by sonication with a sonicator probe for 5.0 min. The amplitude was 50% and the sample was kept in ice-bath during the procedure.

2.4. Preparation of the bienzymatic biosensor (GCE/MWCNTs-Cyt c/GOx/Naf)

Before modification, the GCEs were polished with alumina slurries of 1.0, 0.3 and 0.05 μm for 1 min each, rinsed thoroughly with deionized water, sonicated for 30 s in water, and finally dried under a N_2 stream. After that, GCE/MWCNTs-Cyt c was prepared by casting 20 μL of MWCNTs-Cyt c dispersion onto the glassy carbon surface, followed by the evaporation of the solvent at room temperature for 60 min. The bioanalytical bienzymatic platform was prepared by drop-coating the GCE/MWCNTs-Cyt c with 10 μL of 0.75 mg mL^{-1} GOx. After drying, the resulting electrode was modified by deposition of 5 μL 0.5% v/v Naf solution.

The multilayered system was obtained by repeating the previous scheme (successive deposition of MWCNTs-Cyt c and GOx) and the resulting electrodes were named as GCE/$(MWCNTs\text{-}Cyt\ c/GOx)_n$/Naf, being "n" the number of MWCNTs-Cyt c/GOx bilayers.

2.5. Procedure

Amperometric experiments were performed in stirred 0.050 M phosphate buffer solution pH 7.40 containing 0.050 mM hydroquinone by applying −0.050 V as working potential and allowing the transient current to reach a steady-state value prior to the addition of the analyte and the subsequent current monitoring. All experiments were conducted at room temperature.

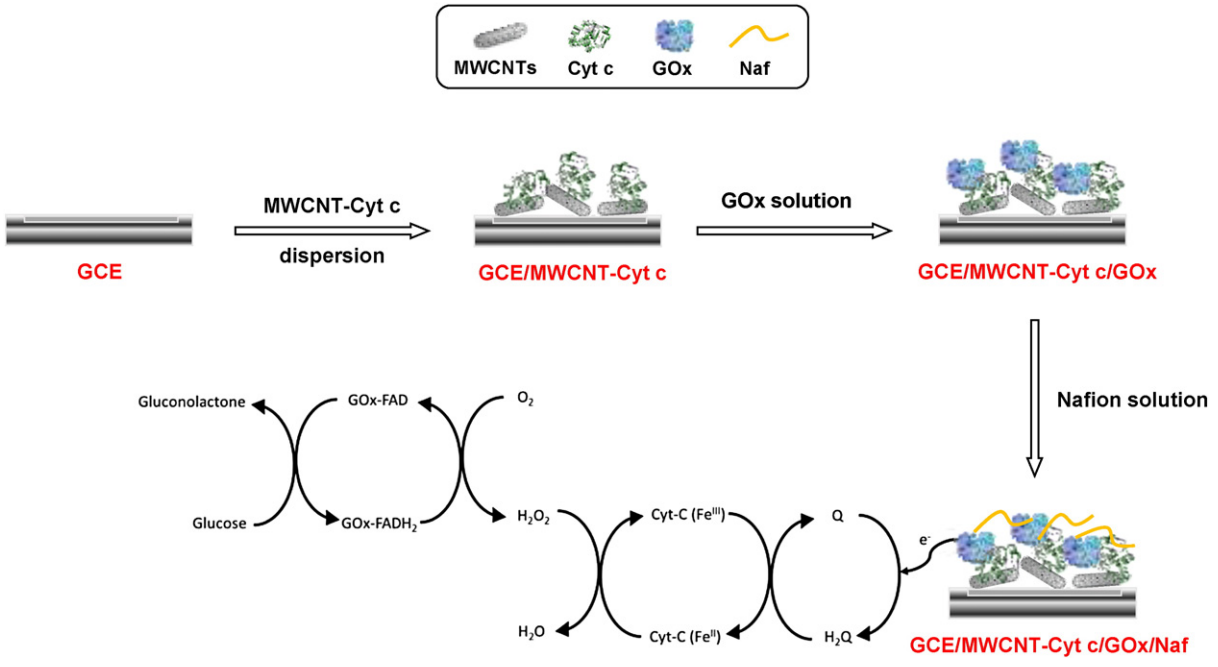

Fig. 1. Schematic display of the steps involved in the construction of GCE/MWCNTs-Cyt c/GOx/Naf biosensor and the reactions that take place at the resulting bioanalytical platform.

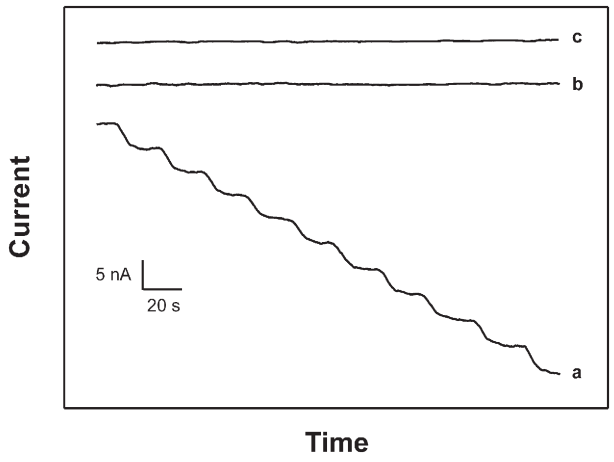

Fig. 2. Amperometric recording for successive additions of 0.10 mM glucose at GCE/MWCNTs-Cyt c/GOx/Naf (a), GCE/MWCNTs/GOx/Naf (b) and GCE/MWCNTs-Cyt c (c) obtained at −0.050 V in the presence of 0.050 mM hydroquinone. Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40.

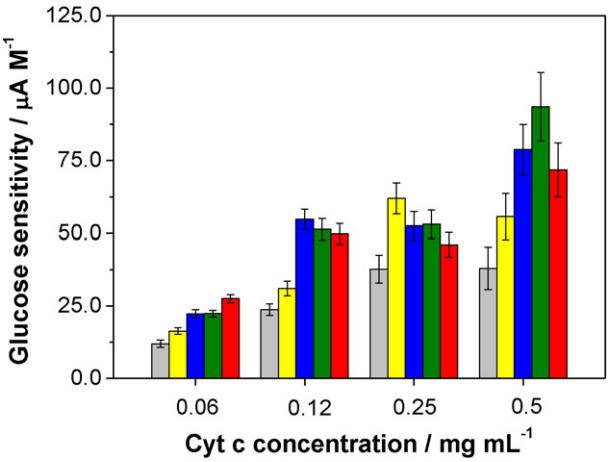

Fig. 4. Sensitivity towards glucose obtained from amperometric experiments at GCE/MWCNTs-Cyt c/GOx/Naf obtained at −0.050 V in the presence of 0.050 mM hydroquinone as a function of the concentration of Cyt c used to disperse the MWCNTs (0.06, 0.12, 0.25 and 0.5 mg mL^{-1}) and the concentration of GOx deposited at GCE/MWCNTs-Cyt c: 0.25 mg mL^{-1} (gray), 0.50 mg mL^{-1} (yellow), 0.75 mg mL^{-1} (blue), 1.00 mg mL^{-1} (green) and 2.00 mg mL^{-1} (red). Supporting electrolyte 0.050 M phosphate buffer solution pH 7.40. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Determination of glucose in real samples

Pepsi® and Gatorade® were used to evaluate the analytical performance of the proposed biosensor by transferring a given aliquot of the beverages stock solutions (diluted with the phosphate buffer) to the cell containing 5.0 mL of 0.050 mM hydroquinone prepared in 0.050 M phosphate buffer solution pH 7.40. In all cases the determination of glucose was performed by amperometry at −0.050 V.

3. Results and discussion

Fig. 1 shows a schematic representation of the different steps involved in the preparation of the bienzymatic biosensor GCE/MWCNTs-Cyt c/GOx/Naf and the reactions that take place at the resulting biosensor. GOx catalyzes the oxidation of glucose to gluconolactone producing hydrogen peroxide in the presence oxygen during the enzymatic regeneration. The hydrogen peroxide is, in turn, catalytically reduced by Cyt c that oxidizes H_2Q, a typical redox mediator of hemeproteins [18], to the corresponding quinone (Q). The electrochemical reduction of the generated Q at the working potential (−0.050 V) was used for monitoring the overall reaction. The working potential was selected as a compromise to obtain a good sensitivity, to minimize potential interference from other electroactive substances, and to obtain a stable baseline.

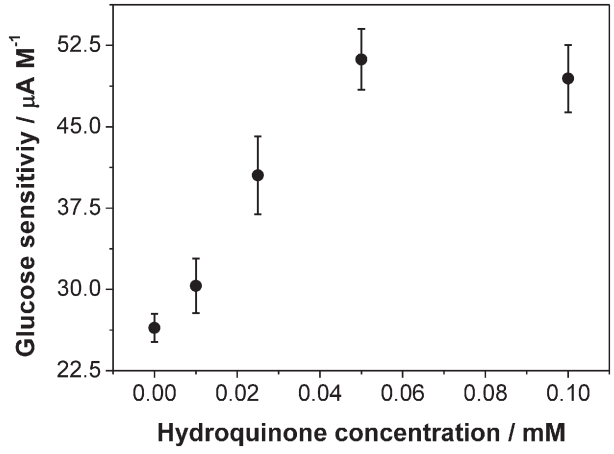

Fig. 3. Sensitivity towards glucose obtained from amperometric experiments at GCE/MWCNTs-Cyt c/GOx/Naf obtained at −0.050 V as a function of the concentration of hydroquinone (0, 0.010, 0.025, 0.050, 0.100 mM). Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40.

Fig. 2 compares the amperometric response of glucose at different electrodes in the presence of 0.050 mM H_2Q. Fig. 2a depicts the amperometric response obtained at GCE/MWCNTs-Cyt c/GOx/Naf biosensor (1.0 mg mL^{-1} MWCNTs in 0.12 mg mL^{-1} Cyt c; 0.75 mg mL^{-1} GOx) at −0.050 V after successive additions of 0.10 mM glucose. A well-defined response is obtained after the different additions of glucose, demonstrating that the cascade system of reactions previously discussed efficiently occurs. At variance with this clear response, no signal was obtained after successive additions of 0.10 mM of glucose neither at GCE modified with MWCNTs dispersed in a Cyt c-free solution and GOx (GCE/MWCNTs/GOx) (Fig. 2b) nor at GCE modified just with MWCNTs-Cyt c (GCE/MWCNTs-Cyt c) (Fig. 2c), clearly indicating the need of having both enzymes immobilized at the electrode surface to obtain the amperometric signal due to the biocatalytic oxidation of glucose.

The effect of the concentration of redox mediator on the response of the bienzymatic electrode to glucose was also evaluated (Fig. 3). There is a sharp increase in the sensitivity with the increment of H_2Q concentration up to 0.050 mM (two-fold higher than that obtained in the absence of the redox mediator). Higher concentrations of H_2Q do not produced any additional improvement in the sensitivity and the steady-state currents become more difficult to reach. Therefore, the selected value was 0.050 mM.

Cyt c/GOx ratio demonstrated to be a critical aspect for the analytical performance of the biosensor. Fig. 4 displays the variation of the sensitivity to glucose obtained from amperometric experiments at −0.050 V as a function of the concentration of GOx deposited at GCE/MWCNTs-Cyt c (between 0.25 and 2.0 mg mL^{-1}) and the concentration of Cyt c used to disperse the MWCNTs (between 0.06 and 0.50 mg mL^{-1}). The sensitivity increases with the increment of Cyt c concentration although the quality of the MWCNTs-Cyt c dispersion becomes worse, the robustness once deposited at the electrode surface is poor, the reproducibility decreases, and the linear range becomes narrow. The best performance in sensitivity without compromising the reproducibility is obtained using 0.12 mg mL^{-1} Cyt c for dispersing 1.0 mg MWCNTs. Regarding the concentration of GOx, the sensitivity towards glucose is enhanced as GOx increases up to 0.75 mg mL^{-1} to remain almost constant thereafter. Therefore, the selected conditions to prepare the biosensor were 0.12 mg mL^{-1} Cyt c and 0.75 mg mL^{-1} GOx.

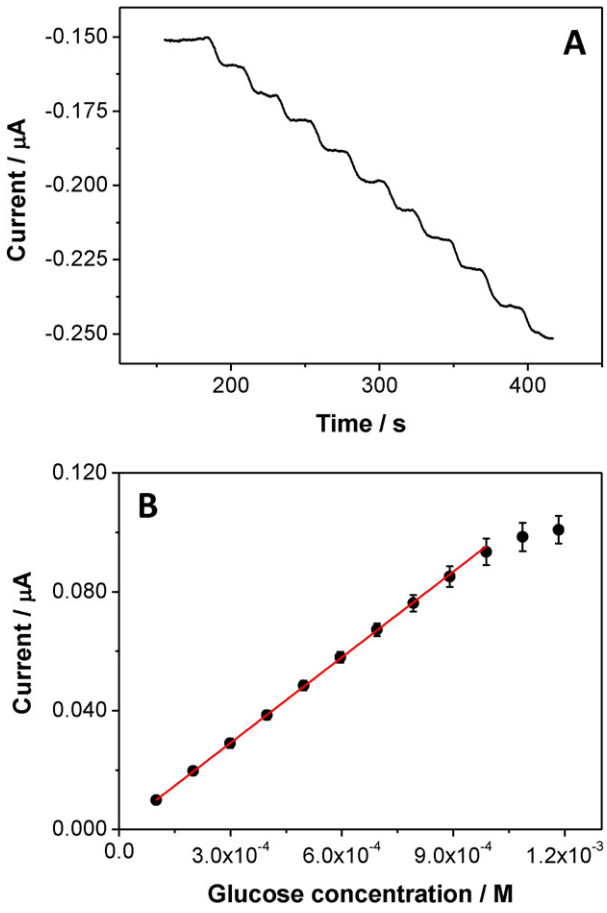

Fig. 5. (A) Amperometric recording for successive additions of 0.10 mM glucose at GCE/(MWCNTs-Cyt c/GOx)$_2$/Naf obtained at −0.050 V in the presence of 0.050 mM hydroquinone. Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40. (B) Calibration plot obtained from the amperometric recording shown in Panel A.

The effect of adding successive enzyme bilayers of MWCNTs-Cyt c and GOx at the glassy carbon surface, GCE/(MWCNTs-Cyt c/GOx)$_n$/Naf, was also investigated. Amperometric experiments showed an increase almost in a factor of 2 when n = 2 ((47 ± 6) µAM^{-1} and (91 ± 5) µAM^{-1} for 1 and 2 bilayers, respectively). A third bilayer did not improve the sensitivity ((62 ± 15) µAM^{-1}) and produced a large decrease in the reproducibility of glucose biosensing, phenomenon attributed to the swelling of the multilayers system that made it unstable.

Fig. 5A displays the amperometric response of GCE/(MWCNTs-Cyt c/GOx)$_2$/Nafion after successive additions of 0.10 mM glucose. A fast (7.5 s to reach the steady-state signal) and well-defined response is obtained after the different additions of glucose. The calibration plot (Fig. 5B) shows a linear range between 0.10 and 1.0 mM glucose, with a sensitivity of (96 ± 2) µAM^{-1} (r^2 = 0.9998) and a detection limit of 8 µM (taken as 3.3 σ/S, where σ is the standard deviation of the blank signal and S the sensitivity). This detection limit is lower than that obtained with GOx/Cyt bienzymatic biosensors based on chitosan modified gold nanoparticles (0.15 mM) [15] and polyaniline modified gold nanoparticles (0.1 mM) [17], and similar to the one obtained with a GOx/Cyt c biosensor based on poly(diallyldimethylammonium chloride)-graphene nanosheets-gold nanoparticles hybrid nanocomposite (11 µM) [16]. Moreover, the proposed biosensor offers a detection limit lower than that obtained with a GOx/HRP biosensor based on carbon nanotubes (0.4 mM) [11]. The reproducibility obtained with 5 different biosensors was 6.1%. The selectivity of the biosensor was evaluated in the presence of 0.30 mM fructose, galactose, maltose and lactose. No interference was observed in any case.

To evaluate the analytical usefulness of the proposed biosensor, we determine the glucose concentration in two commercial beverages, Pepsi® and Gatorade® samples. The results for the glucose concentration in the different beverages, Pepsi® (4.1 ± 0.3) g/100 mL and Gatorade® (2.3 ± 0.2) g/100 mL, exhibit an excellent agreement with the values reported by the suppliers (3.9 and 2.4 g/100 mL, respectively), demonstrating the analytical usefulness of the proposed electrochemical biosensor to determine glucose content in real samples.

4. Conclusions

The work described here reports a new strategy for designing a bienzymatic biosensor based on the deposition of MWCNTs noncovalently functionalized with Cyt c at GCE and further incorporation of the model enzyme GOx. This novel approach associates the efficiency of the functionalization of MWCNTs with Cyt c and the advantages of the intimate contact between MWCNTs, Cyt c and GOx for the biocatalytic activity and electron transfer. The biosensor demonstrated to be sensitive, selective and useful for practical applications, more precisely, for the quantification of glucose in beverages, with excellent correlation with the reported values.

The platform described here represents the starting point to prepare other biosensors just by selecting a hydrogen peroxide-producer enzyme, opening the way to the development of new biosensors.

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