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Direct electron transfer of glucose oxidase at glassy carbon electrode modified with functionalized carbon nanotubes within a dihexadecylphosphate film

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ABSTRACT

A glassy carbon electrode modified with functionalized multiwalled carbon nanotubes (CNTs) immobilized by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) in a dihexadecylphosphate film was prepared and characterized by cyclic voltammetry and scanning electron microscopy. It was used as a support for FAD or glucose oxidase (GOx) immobilization with EDC/NHS crosslinking agents. Cyclic voltammetry of GOx immobilized onto the surface of CNTs showed a pair of well-defined redox peaks, which correspond to the direct electron transfer of GOx, with a formal potential of −0.418 V *vs.* Ag/AgCl (3 M KCl) in 0.1 M phosphate buffer solution (pH 7.0). An apparent heterogeneous electron transfer rate constant of 1.69 s^{-1} was obtained. The dependence of half wave potential on pH indicated that the direct electron transfer reaction of GOx involves a two-electron, two-proton transfer. The determination of glucose was carried out by square wave voltammetry and the developed biosensor showed good reproducibility and stability. The proposed method could be easily extended to immobilize and evaluate the direct electron transfer of other redox enzymes or proteins.

1. Introduction

Since the discovery of carbon nanotubes (CNTs) by Iijima in 1991 [1] they have attracted considerable attention owing to their unique properties [2–6]. CNTs have been widely used for the development of chemically modified electrodes, promoting electron transfer reactions of many compounds, by decreasing the overpotential and increasing the reaction rate of many electroactive substrates, and/or decreasing the electrode response time, thus enhancing the sensing capabilities of sensors and biosensors [7–14]. The similarity in length scales of CNTs and redox enzymes can benefit interactions between them [15,16]. Additionally, functionalization in acid solution creates binding sites such as carboxylic groups on the surface of the nanotubes, that may be favorable for biosensor applications [5,6,15,17]. The high surface area with these abundant sites also may offer special opportunities for the adsorption and entrapment of chemical/biological molecules.

EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) is widely used for immobilizing biomolecules through covalent binding [18]. It is one of the so-called "zero-length" cross-linkers, since it mediates the formation of amide linkages without leaving a spacer molecule [19]. In addition to reacting with carboxyl groups, EDC alone forms a stable complex with exposed amino groups. The use of N-hydroxysuccinimide (NHS) and EDC can be used to reduce side reactions, increase the stability of the active intermediate and enhance yields [20,21].

Dihexadecylphosphate (DHP) (Fig. 1A) is a type of surfactant with a polar head and two long hydrophobic tails [22]. This material can be dispersed in water by ultrasonic agitation and its dispersion can form a very stable film on electrode surfaces after the evaporation of water, probably via hydrogen bonds (Fig. 1B) and has been used in sensors [23] and biosensors [24].

The direct electron transfer of enzymes at electrodes can be applied to the study of enzyme-catalyzed reactions in biological systems, for the investigation of the structure of enzymes, mechanisms of redox transformation of enzyme molecules and metabolic processes involving redox transformation [25]. Unfortunately, enzymes usually have big and complicated structures, and their redox centers are deep within the structure; hence, it is difficult for the enzymes to exchange electrons with the electrode surface directly. For this reason, nanomaterials are often employed

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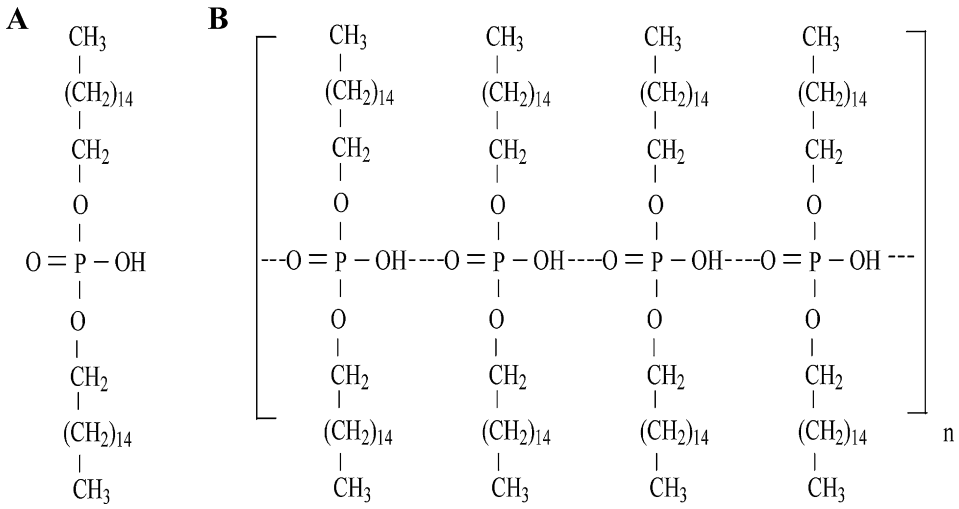

Fig. 1. Molecular structure of DHP (A) and polymerized form (B).

in biosensors, which improve the analytical signal and promote electrocatalysis [26].

Glucose oxidase (GOx), a flavin enzyme, has been extensively used in the monitoring of blood glucose levels in diabetics. However, the active site of GOx, flavin adenine dinucleotide (FAD), is deeply embedded within a protective protein shell, and so direct electron transfer for GOx is extremely difficult [16]. Some work has been carried out to understand the communication between this active site and electrodes [16,24]. Wu and Hu developed a glucose biosensor using a Au–DHP composite [24]. Zhao et al. showed direct electron transfer of glucose oxidase molecules on carbon nanotube powder microlectrodes [26]. Cai and Cheng reported the direct electron transfer of glucose oxidase promoted by carbon nanotubes dispersed in cetyltrimethylammonium bromide (CTAB) [25]. Liu et al. reported the direct electron transfer of glucose oxidase and a glucose biosensor based on a carbon nanotubes/chitosan matrix [16].

In this work, a biosensor based on the direct electron transfer (DET) of glucose oxidase is presented in which the enzyme is immobilized in a new film containing functionalized carbon nanotube (CNTs) and dihexadecylphosphate on the surface of a glassy carbon electrode (GCE).

2. Experimental

2.1. Chemicals

Glucose oxidase (from *Aspergillus niger*, type II) D(+) glucose, flavin adenine dinucleotide (disodium salt, 96%) and dihexadecylphosphate (DHP) were obtained from Sigma. Multiwalled carbon nanotubes (20–30 nm in diameter, 1–2 nm wall thickness and 0.5–2 μm in length and 95% purity), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (98%) and N-hydroxysuccinimide (98%) were purchased from Aldrich. All other chemicals were of analytical grade. All the solutions were prepared with Millipore Milli-Q nanopure water (resistivity >18 MΩ cm). The 0.1 M phosphate buffer solutions, which were made from Na_2HPO_4 and NaH_2PO_4, were always employed as supporting electrolyte.

2.2. Apparatus

The voltammetric measurements were performed with a three electrode system, including the biosensor GOx–CNTs–DHP/GCE as working electrode, a platinum foil as counter electrode, and Ag/AgCl (3.0 M KCl) as reference electrode at 25 °C. Voltammetric measurements were carried out using an Autolab Ecochemie model PGSTAT12 (Utrecht, The Netherlands) potentiostat/galvanostat controlled by GPES 4.9 software. The morphologies were verified using a FEG-SEM (Supra 35-VP, Carl Zeiss, Germany) equipment with electron beam energy of 25 keV.

2.3. Functionalization of the multiwalled carbon nanotubes

The carbon nanotubes were initially submitted to a chemical pretreatment using a mixture of concentrated sulfuric and nitric acids 3:1 (v/v) for 12 h at room temperature. After this, the suspension was filtered, the solid was washed with ultrapure water until pH 6.5–7.0 and then it was dried at 120 °C for 5 h.

2.4. Preparation of GOx–CNTs–DHP/GCE biosensor

The GC electrode (diameter 3 mm) was polished sequentially with metallographic abrasive paper (No. 6) and slurries of 0.3 and 0.05 μm alumina microparticles to a mirror finish. After being rinsed with Milli-Q water, it was sonicated in absolute ethanol and then with Milli-Q water for about 1 min, respectively.

1.0 mg of multiwalled carbon nanotubes (CNTs) and 1.0 mg of DHP was added to 1.0 mL of 1.0 mM phosphate buffer solution (pH 7.0) and subjected to ultrasonication for 2 h to give a 1.0 mg/mL stable black CNTs suspension. An aliquot of 200 μL of solution containing EDC (1.0 mM) and NHS (20 mM) was added in suspension for the carboxyl coupling reaction, carried out for 2 h with magnetic stirring. The CNTs suspension was then mixed with 3 mg of GOx thoroughly and was stirred for 2 h; GOx molecules were linked by coupling onto the surface of CNTs during mixing. Finally, 8 μL of the mixture was cast onto the surface of a GC electrode and the solvent allowed to evaporate at ambient temperature for 12 h. This electrode is designated GOx–CNTs–DHP/GCE. If not used immediately, the electrode was stored at 4 °C in a refrigerator in 0.1 M phosphate buffer solution (pH 7.0).

The same procedure was employed to fabricate FAD–CNTs–DHP/GCE in which FAD was used instead of GOx. An aliquot of 8 μL of a mixture containing 1.0 mg of CNTs, 1.0 mg of DHP, 1.0 mL of 1.0 mM phosphate buffer solution and 200 μL of 1.0 mM EDC and 20 mM NHS solution and 3 mg of FAD was cast onto the surface of a CG electrode and the solvent was evaporated for 12 h.

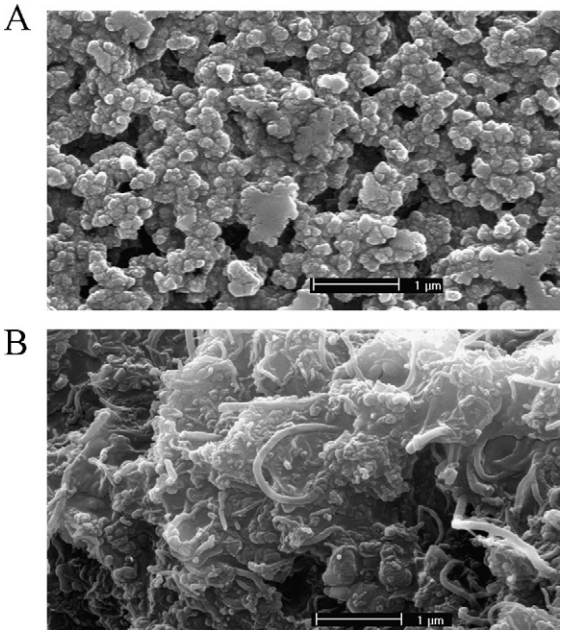

Fig. 2. SEM images of DHP and CNTs–DHP on the surface of GC electrode.

3. Results and discussion

3.1. Characterization of CNTs

CNTs are insoluble in most solvents [27,28], and especially in water. It has been reported, however, that its dispersity can be improved by wrapping the CNTs using some molecules such as poly(*p*-phenylenevinylene) [28], CTAB [25], or Nafion [29]. When CNTs were sonicated for 2 h with DHP causes the formation of a stable black suspension in water. The changes visible by naked eye and the dispersity of CNTs in surfactant solution were clearly observed. A homogeneous, well-distributed suspension of CNTs is observed in 1% mg/mL DHP whereas no such dispersity is observed in water.

FTIR measurements of functionalized and non-functionalized CNTs (data not shown) were made, exhibited strong absorption at 1645 cm^{-1}, corresponding to carboxylic acid [30]. A significant increase in the absorption due to the carbon nanotubes pretreated with a mixture of nitric and sulfuric acid occurred, compared with untreated carbon nanotubes, which can be related to the increase in the concentration of carboxylic groups resulting from CNT functionalization. These results are in agreement with previous reports [25,31].

The electroactive area of GCE and CNTs–DHP/GCE was estimated in 0.1 M KCl in the presence of 1.0 mM $[Fe(CN)_6]^{4-}$ (data not shown) according to the Randles–Sevcik equation [32]:

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} \nu^{1/2} C \quad (1)$$

where I_p is the cathodic peak current (A), A is the electroactive area (cm^2), D is the diffusion coefficient of $[Fe(CN)_6]^{4-}$ in solution (6.2×10^{-6} cm^2 s^{-1}), n is the number of electrons transferred in the redox reaction, ν is the potential scan rate (V s^{-1}), and C is the $[Fe(CN)_6]^{4-}$ concentration in bulk solution (mol cm^{-3}). The electroactive areas of the CNTs–DHP/GCE and the bare GCE were calculated to be 0.100 ± 0.008 cm^2 and 0.050 ± 0.004 cm^2 ($n = 5$), respectively. The CNTs increased the electroactive surface area by a factor of 2 compared with the bare electrode. Fig. 2 shows SEM images of DHP and CNTs–DHP on the surface of the GCE electrode. It can be seen that the DHP film has a uniform nanostructured form with holes and DHP–CNTs presented CNTs distributed homogenously in the film.

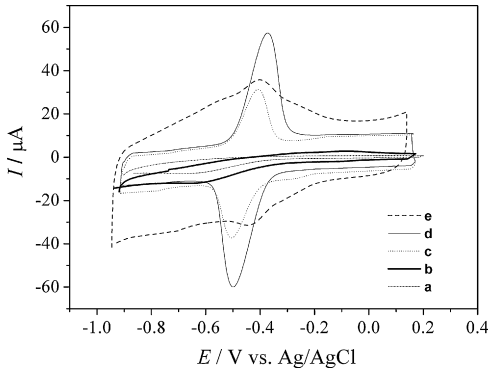

Fig. 3. CVs of a DHP/GCE (a); CNTs–DHP/GCE (b); CNTs–DHP/GCE in the presence of FAD solution (1.0 mM) (c); FAD–CNTs–DHP/GCE (d); and GOx–CNTs–DHP/GCE (e) in N_2-saturated 0.1 M phosphate buffer solution (pH 7.0) at scan rate 50 mV s^{-1}.

3.2. Characterization of GOx–CNT–DHP/GCE biosensor

Fig. 3 shows cyclic voltammograms (CVs) obtained for GOx–CNTs–DHP/GCE, FAD–CNTs–DHP/GCE, DHP/GCE and GCE electrodes in N_2-saturated 0.1 M phosphate buffer solution and for CNT–DHP/GCE in N_2-saturated 0.1 M phosphate buffer solution (pH 7) containing 1.0 mM FAD in solution.

Before enzyme immobilization, CVs of GCE and CNTs–DHP/GCE in 0.1 M phosphate buffer solution (pH 7) were also recorded. The shapes of the CVs indicate that there is no influence of the DHP in DHP/GCE (Fig. 3a, short dashed dotted line) or in CNTs–DHP/GCE (Fig. 3b, bold solid line), meaning that DHP did not interfere in the electron transfer between the CNTs and the GCE support.

FAD linked onto the CNTs (FAD–CNTs–DHP/GCE) in phosphate buffer solution and CNTs–DHP/GCE with FAD in solution displayed a well-defined CV (Fig. 3c, dotted line and 3d, solid line, respectively) with a formal potential of −0.450 V *vs.* Ag/AgCl (3.0 M KCl). Similarly, GOx linked onto CNTs by EDC/NHS coupling (GOx–CNTs–DHP/GCE) presented two well-defined peaks with a formal potential of −0.418 V (Fig. 3e, dashed line).

The presence of both peaks (reduction and oxidation of FAD and GOx(FAD)) of FAD–CNTs–DHP/GCE and GOx–CNTs–DHP/GCE electrodes indicates that EDC/NHS coupling can be an important contributor to the electrochemical behavior of the biosensor and the process of enzymatic immobilization on CNTs in DHP films. EDC and NHS promote the reaction of carboxylic groups of CNTs with amino groups of the enzyme as observed in Fig. 4.

The biosensor presented well-defined anodic and cathodic peaks at −0.405 and −0.432 V (scan rate 50 mV s^{-1}), respectively. The cathodic and anodic peak currents are of similar magnitude and nearly symmetric, with an I_{pa}/I_{pc} ratio equal to one, a formal potential of −0.418 V and peak separation (ΔE_p) 27 mV, a value suggesting a quasi-reversible 2-electron redox reaction of a surface-confined species.

The effect of scan rate was studied on the voltammetric response of GOx–CNTs–DHP/GCE (Fig. 5), which shows an increase of both cathodic and anodic peak currents and of peak-to-peak separation with increase in scan rate. The anodic and cathodic peak currents show a linear relation with scan rate in the range from 20 to 400 mV s^{-1}, indicating that the process is controlled by the redox monolayer species adsorbed on the electrode surface (GOx) [24,32], described by the Laviron equation [33,34]:

$$E_{pc} = E^0 - \frac{(2.3RT)}{\alpha nF}\left\{\log\frac{\alpha nF}{RT} + \log[\upsilon] - \log[k]\right\} \quad (2)$$

$$E_{pa} = E^0 - \frac{(2.3RT)}{(1-\alpha)nF}\left\{\log\frac{(1-\alpha)nF}{RT}(\log[\upsilon] - \log[k])\right\} \quad (3)$$

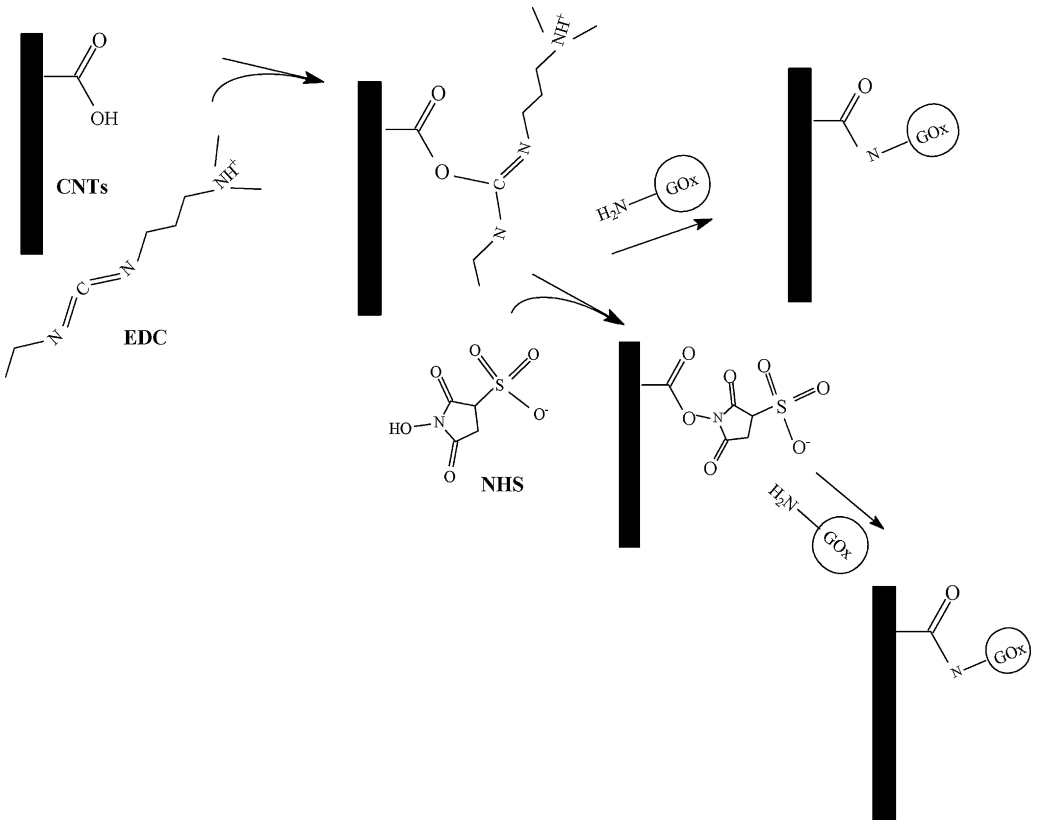

Fig. 4. Scheme of the reaction of EDC and NHS with the CNTs and the enzyme GOx.

$$\Delta E_p = \frac{(2.3RT)}{(1-\alpha)nF}\left\{\alpha\log(1-\alpha)+(1-\alpha)\log\alpha-(1-2\alpha)\log\frac{RT}{nF}\right.$$
$$\left.-(1-2\alpha)\log[k]\right\}+\frac{2.3RT(1-2\alpha)}{(1-\alpha)nF}\log[\upsilon] \quad (4)$$

where E_{pa} and E_{pc} are the anodic, and cathodic peak potentials, respectively, $\Delta E_p = E_{pa} - E_{pc}$, α is the cathodic transfer coefficient, υ is the potential scan rate ($V\,s^{-1}$), and k is the heterogeneous electron transfer rate constant (s^{-1}).

From the slopes of anodic and the cathodic processes the value of α was 0.48. From the corresponding ΔE_p *vs.* $\log\upsilon$ plot (Fig. 6 inset), a value of $k = 1.69 \pm 0.05\,s^{-1}$ was calculated ($n = 5$), very close to other results in the literature for biosensors using carbon nanotubes ($k = 1.78\,s^{-1}$; $k = 1.61\,s^{-1}$) [26,35] or gold nanoparticles ($k = 1.69\,s^{-1}$) [24] and GOx.

FAD is responsible for the electrochemical response of GOx immobilized onto the heterogeneous surface, which involves two electrons coupled with two protons in the redox reaction [24]. The pH dependence of the biosensor regarding the anodic and cathodic peak potentials of immobilized GOx was studied in N_2-saturated medium. An increase of the pH of the solution (from 5.0 to 8.5) leads to a negative shift in potential for both cathodic and anodic peaks. Fig. 7 (inset) shows a plot of E_{pc} *vs.* pH, which was linear in the pH range studied. The value of the slope obtained was 61.0 mV/pH unit, close to the theoretical value (59.2 mV/pH) at 25 °C for a reversible process involving equal numbers of protons and electrons [24,25].

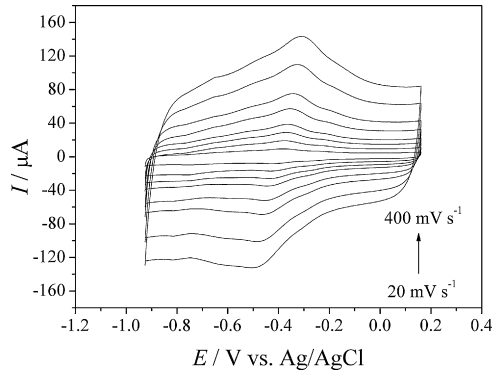

Fig. 5. CVs of GOx–CNTs–DHP/GCE at various scan rates in N_2-saturated 0.1 M phosphate buffer solution (pH 7.0).

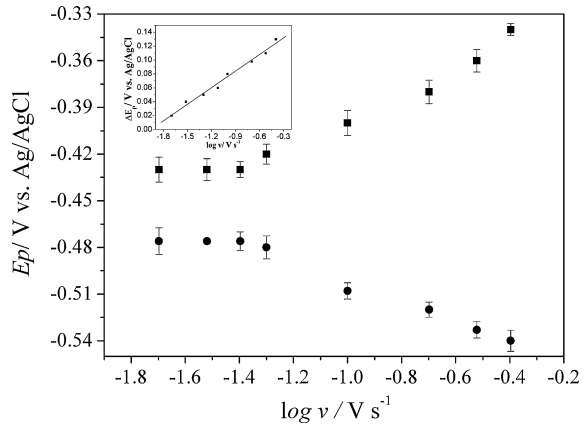

Fig. 6. Plots of the anodic peak and cathodic peak potentials (E_{pc} *vs.* $\log\upsilon$) and ΔE_p *vs.* $\log\upsilon$ (inset).

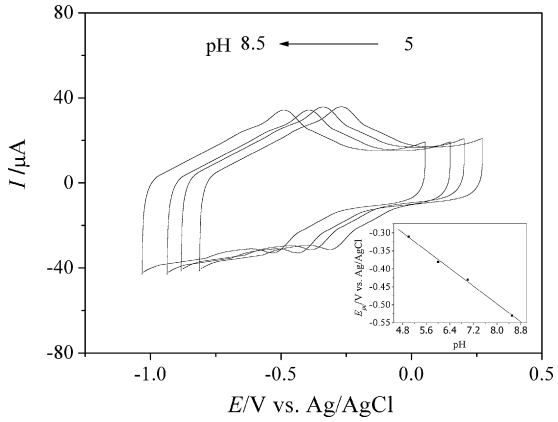

Fig. 7. CVs of the GOx–CNTs–DHP/GCE in N_2-saturated 0.1 M phosphate buffer solution, pH from 5.0 to 8.5. Scan rate 50 mV s^{-1}.

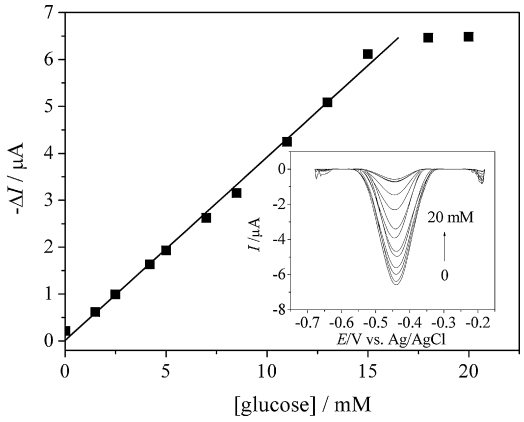

Fig. 9. SWV of GOx–CNTs–DHP/GCE in a pH 7.0 N_2-saturated 0.1 M phosphate buffer solution for different glucose concentrations; inset shows analytical curve.

The variation with pH and the well-defined and stable peaks observed at the GOx–CNTs–DHP/GCE electrode, corresponding to the reaction of GOx(FAD) with $2e^-$ and 2 protons, Eq. (5),

$$GOx(FAD) + 2H^+ + 2e^- \rightleftharpoons GOx(FADH_2) \quad \text{(at the biosensor)} \qquad (5)$$

suggest that immobilizing GOx in CNTs–DHP facilitates its electronic communication between the deeply buried active site of the enzyme and CNTs network by EDC/NHS coupling. Studies with air-saturated and N_2-saturated solution (Fig. 8), demonstrated that the redox peaks are due to GOx–CNTs–DHP/GCE; however, N_2-saturated solution was selected for further studies because it presented a better voltammetric profile.

In air-saturated solution, the reduction of O_2 with formation of hydrogen peroxide is catalyzed by GOx($FADH_2$) in the biosensor as shown in Eq. (6):

$$GOx(FADH_2) + O_2 \rightleftharpoons GOx(FAD) + H_2O_2 \qquad (6)$$

3.3. Determination of glucose by square wave voltammetry (SWV)

The effect of SWV parameters was investigated using 1.0 mM glucose in 0.1 M phosphate buffer solution. The square-wave frequency (f) influences the intensity of the analytical signal and, in turn, the sensitivity of the technique. It was observed that ΔI increases up to 40 Hz, which was selected for further studies. The pulse amplitude (a), that also strongly influences the SWV signal increased linearly up to 50 mV and reached a plateau at a = 60 mV; thus, the amplitude was set at 50 mV for the subsequent analytical application. The scan increment (ΔE_s) influences the effective potential scan rate (the product of the frequency and ΔE_s). Here, ΔI_p increased significantly for ΔE_s values up to 4 mV, then leveling off, so 4 mV was chosen.

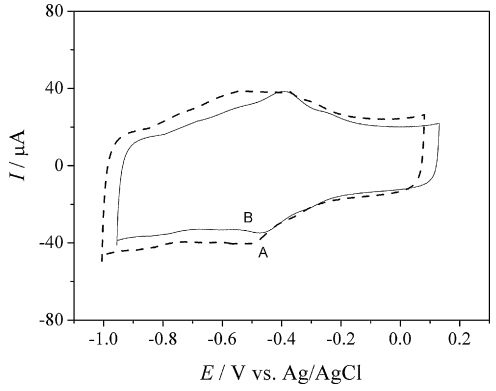

Fig. 8. CVs of GOx–CNTs–DHP/GCE in 0.1 M phosphate buffer solution (pH 7.0): air-saturated (A) and N_2-saturated (B). Scan rate 50 mV s^{-1}.

The SWV response of the GOx–CNTs–DHP/GCE biosensor towards glucose was studied, with the selected parameters, in N_2-saturated 0.1 M phosphate buffer solution (pH 7.0), based on the reaction described in Eq. (5), where FAD is reduced to $FADH_2$ (at the biosensor). In the absence of oxygen, when glucose is added, the formation of $FADH_2$ occurs in the vicinity of the biosensor surface (Eq. (7)), leading to a decrease of the cathodic peak current.

$$\text{Glucose} + GOx(FAD) \rightleftharpoons \text{gluconolactone} + GOx(FADH_2)$$
$$\text{(in the vicinity of the electrode surface)} \qquad (7)$$

The relationship between the decrease of the reduction peak current and the concentration of glucose was examined. The current decrease (at −0.432 V) is linearly proportional to the concentration of glucose from 0.020 to 15 mM (Fig. 9), following the equation $-\Delta I_p$ (μA) = 0.011 + 30 C (mM) with a correlation coefficient of 0.994. The limit of detection obtained was 9 μM based on a signal-to-noise ratio of three. The GOx–CNTs–DHP/GCE showed good precision with a relative standard deviation of 3.2% for 10 successive determinations of 1.0 mM glucose using the same biosensor. In addition, a relative standard deviation of 4.1% was obtained for measurements of 1.0 mM glucose using ten different biosensors prepared in the same way. The GOx–CNTs–DHP/GCE was kept at 4 °C and its stability from day-to-day was also evaluated by measuring the response at 25 °C, which maintained 89% of the initial activity after 30 days.

The interference of species such as L-ascorbate and cysteine on glucose determination was evaluated by SWV in 1.0 mM glucose solution spiked with a 100-fold excess of interfering species. All the species evaluated presented less than 5% change in the electrode response; hence, at the concentration evaluated they do not affect the determination of glucose using the proposed biosensor.

The biosensor was applied to the determination of the glucose content of human serum samples by the standard addition method and the recovery value for glucose ranged from 96 to 104% (Table 1).

Table 1
Determination of glucose in serum samples.

Sample	Added (mM)	Biosensor (mM)	Recovery (%)
A	5.0	5.2	104
B	5.0	4.9	98
C	5.0	4.8	96

Table 2
Comparison of the analytical data between present work and some recently reported biosensors for glucose determination based on direct electron transfer.

Biosensor	Linear range/mM	Limit of detection/μM	Reference
CNTs@SnO_2–Au_{nano}/GOx	4.0–12.0	5	Li et al. [36]
GOx–Au_{nano}–DHP/GCE	5.0–9.3	100	Wu and Hu [24]
GOx/CNTs–PCD/GCE	0.5–8.0	Not presented	Xue et al. [37]
GOx/Tm_2O_3/Nafion/GCE	1.0–7.0	Not presented	Li et al. [38]
GOx–CNT–DHP/GCE	0.020–15.0	9	This work

CNTs, multiwalled carbon nanotubes; Au_{nano}, gold nanoparticles; PCD, citric acid and D-sorbitol; SnO_2, tin dioxide; Tm_2O_3, thullium oxide; GCE, glassy carbon electrode; GOx, glucose oxidase.

The response characteristics of the proposed method were compared with those reported in the literature with different glucose biosensors based on direct electron transfer (Table 2). It can be seen that the proposed GOx–CNTs–DHP/GCE biosensor has better analytical characteristics than those described in the other reports. It presents a limit of detection (LOD) of 9 μM similar to that of 5 μM [35] and significantly better than the other biosensors. In relation to the linear range, the lower limit is significantly less at 20 μM than all the others (the closest is 5.0 mM) [24] and the upper limit is also higher at 15 mM. The lower LOD and the larger glucose concentration range of the analytical curve obtained in this work can be attributed to using the SWV technique [39]. Moreover, the proposed biosensor presented a stable film, easy to prepare with a precise and accurate response for glucose determination.

4. Conclusions

The construction of an electrochemical biosensor by modification of a glassy carbon electrode with a film containing CNTs and DHP is reported. The GOx–CNT–DHP/GCE biosensor was prepared to detect glucose, using functionalized carbon nanotubes to immobilize the GOx as a model enzyme and EDC/NHS as coupling agent. The evaluation of the GOx–CNT–DHP/GCE electrode demonstrated that DHP has a good ability to retain the bioactivity of GOx due to the formation of a stable and uniform film. Cyclic voltammetry showed a pair of well-defined redox peaks, corresponding to the direct electron transfer of GOx (FAD/$FADH_2$). The presence of the redox peaks indicates that the functionalized CNTs facilitate the direct electron transfer of GOx. The dependence of E_{pc} on pH indicated that the direct electron transfer of GOx involved a two-electron-transfer coupled with two-proton transfer. Possible interfering species in blood such as L-ascorbate and cysteine do not influence glucose determination. The GOx-CNT–DHP/GCE sensor was applied to the determination of glucose in human serum samples with good results. The method presented can be used for the immobilization and evaluation of the direct electron transfer of other enzymes or proteins.

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