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ORIGINAL PAPER

The influence of carbon nanotubes and polyazine redox mediators on the performance of amperometric enzyme biosensors

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Abstract Biosensors with novel modified electrode structures for glucose determination have been developed by using different combinations of multiwalled carbon nanotubes (CNTs) and polyazine redox polymer, poly(neutral red) or poly(brilliant cresyl blue) on glassy carbon electrodes (GCE). CNT films were formed using functionalised CNTs, covalently immobilised by crosslinking in a chitosan matrix, and the azine dyes were electropolymerised directly on GCE or on top of the GCE modified with CNTs. Glucose oxidase (GOx) was immobilised by crosslinking with glutaraldehyde on top of the GCE modified with CNT, poly(azines), or combinations of poly(azines) and CNT. An assembly with enzyme/nanotube mixture immobilised on top of polyazine films was also studied. The mechanism of functioning was investigated in the presence and absence of oxygen and also by using bienzymatic devices containing glucose oxidase and catalase. Of the combinations studied, the best performance was obtained with a PBCB/CNT/GOx biosensor at a potential of −0.3 V vs. SCE with a detection limit of 11 μmol L^{-1}.

Keywords Functionalised carbon nanotubes · Chitosan matrix · Azine dyes · Redox polymer · Glucose oxidase · Catalase

Introduction

Recent developments in nanotechnology have paved the way for a large number of new materials and devices, which have useful functions for electrochemical biosensor applications. Through nanostructures, it is possible to control the fundamental properties of materials without changing their chemical composition. Considerable attention has been devoted to carbon nanotubes (CNTs), which are rolled-up graphene sheets in a nanoscale tube form [1] with high chemical stability and biocompatibility. Due to their high surface area, a large enhancement in electrochemical response is potentially achievable together with possible electrocatalytic effects. Besides CNTs, conducting polymers have also been extensively studied as active nanostructured materials; electrochemical characterisation and applications of conducting polymers for sensors and biosensors have recently attracted a lot of interest [2]. There are different methods to synthesise these active materials, but the most widely-used is electrochemical polymerisation in which a high positive potential is applied to monomers which undergo electrochemical oxidation forming cation radical reactive species, thus initiating polymerisation.

Even though matrices of conjugated polymers [3–5] and CNTs [6–8] individually lead to good results when applied in biosensor assemblies, some properties such as mechanical stability, sensitivity and the possibility of multiple compound detection are poor. To overcome this, films comprising both CNTs and polymers have been prepared. The combination of CNTs with conducting polymers leads to composites which are mechanically stronger, more conductive and less susceptible to thermal degradation [1] when compared to either CNTs or polymer materials alone.

In previous work [9], we have evaluated different crosslinking agents for the immobilisation of functionalised carbon nanotubes into chitosan matrices and used the one with the best properties, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide together with N-hydroxy-succinimide (EDC-NHS), for the development of sensors [10] and biosensors

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[11]. Redox indicator dyes, including neutral red and brilliant cresyl blue, have been used for the modification of electrode surfaces [12, 13], since they possess stable redox-active properties and have been found to be convenient redox mediators in enzyme biosensors [14, 15].

The aim of the present work was to contribute to developing convenient, rapid and sensitive methods for the determination of analytes in natural samples using electrochemical biosensors. Biosensor configurations based on the combination of CNTs with the polymerised azines neutral red and brilliant cresyl blue have been developed and tested. Glucose biosensors were prepared by immobilising glucose oxidase on glassy carbon electrodes modified by CNTs, by poly(azine) or by a mixture of both CNT and poly(azine). For comparison, GOx was immobilised together with CNTs on top of poly(azine) modified electrodes. All biosensors were evaluated for their response to glucose and the mechanism was investigated in the presence and absence of oxygen, as well as by using bienzymatic biosensors with glucose oxidase and catalase (Cat). Among the combinations studied, the best performance was obtained for the PBCB/CNT/GOx biosensor at a potential of −0.3 V vs. SCE with a sensitivity of 36.3 μA mM^{-1} cm^{-2} and a detection limit of 11 μmol L^{-1}.

Experimental

Reagents and buffers

All reagents were of analytical grade and were used without further purification. Glucose oxidase (GOx, E.C. 1.1.3.4, from *Aspergillus Niger*, 24 U/mg) was acquired from Fluka (www.sigmaaldrich.com). Catalase (Cat, E.C. 232–577-1, from bovine liver, 2,950 U/mg), α-D(+)-glucose, glutaraldehyde (GA) (25% v/v in water) and bovine serum albumin (BSA) were purchased from Sigma (www.sigmaaldrich.com). Hydrogen peroxide (H_2O_2) 35% was from José M. Vaz Pereira (www.vazpereira.pt). Brilliant cresyl blue (BCB) was obtained from Fluka (www.sigmaaldrich.com) and neutral red (NR) (65% dye content) was from Aldrich (www.sigmaaldrich.com).

Multi-walled carbon nanotubes (MWCNTs) were obtained from NanoLab (www.nano-lab.com). Chitosan (Chit) of low molecular weight with a degree of deacetylation of 80% was obtained from Aldrich,(www.sigmaaldrich.com), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma (www.sigmaaldrich.com) and N-hydroxysuccinimide (NHS) from Fluka (www.sigmaaldrich.com).

All solutions were prepared using Millipore Milli-Q nanopure water (resistivity >18 MΩ cm, www.millipore.com). The supporting electrolyte for sensor evaluation was sodium phosphate buffer saline (NaPBS) (0.1 M NaH_2PO_4/Na_2HPO_4 + 0.05 M NaCl), pH 7.0. For dye electropolymerisation different electrolytes were used, as previously optimised [16, 17]: 0.025 M KH_2PO_4/K_2HPO_4 (KPB) + 0.1 M KNO_3, pH 5.5 for neutral red and 0.1 M NaH_2PO_4/Na_2HPO_4 (NaPB) + 0.1 M KNO_3 pH 7.0 for brilliant cresyl blue.

Stock solutions of 100 mmol L^{-1} glucose and 10 mmol L^{-1} hydrogen peroxide (calibrated by permanganate titration) were prepared in phosphate buffer supporting electrolyte and were kept at 4 °C.

Methods, instruments and cell

Measurements were performed in a 15 mL, one-compartment, cell containing a glassy carbon (GC) modified electrode (electrodes with geometric area 0.24 and 0.28 cm^2 were used) as working electrode, a platinum foil auxiliary electrode and a saturated calomel electrode (SCE) as reference. Voltammetric and amperometric experiments were carried out using a CV-50 W Voltammetric Analyser from Bioanalytical Systems, (West Lafayette, IN, USA, www.bioanalytical.com) controlled by BAS CV-2.1 software.

Electrochemical impedance spectroscopy (EIS) measurements were performed using a Solartron 1,250 Frequency Response Analyser, coupled to a Solartron 1,286 Electrochemical Interface (Solartron Analytical, UK, www.solartronanalytical.com) 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, with an rms perturbation voltage of 10 mV.

Scanning electron microscopy (SEM) images were obtained using a Jeol JSM-5310 (www.jeol.com) scanning electron microscope. Indium tin oxide (ITO) electrodes (Sigma-Aldrich, UK, www.sigmaaldrich.com) were used as substrate and modification was done in the same way as on GC electrodes, see next section.

pH measurements were performed with a CRISON 2001 micro pH-meter (www.crison.es). All experiments were performed at room temperature, 25±1 °C.

Preparation of the modified electrodes

Functionalisation of the carbon nanotubes

Multi-walled carbon nanotubes (MWCNT) were purified and functionalised as described elsewhere [18]. A mass of 120 mg of MWCNT was stirred in 10 mL of a 5 M nitric acid solution for 20 h, in order to cause partial destruction of the CNTs and introduce -COOH groups at the ends and sidewall defects of the CNTs. 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 were then dried in an oven at 80 °C for 24 h.

Immobilisation of the carbon nanotubes

First, 100 mg of Chit powder was dissolved in 10 mL of 1.0% v/v acetic acid solution and stirred for 3 h at room temperature to ensure complete dissolution, obtaining a 1.0% m/m chitosan solution, which was stored at 4 °C.

A 1.0% m/v MWCNT solution was prepared by dispersing 2 mg of functionalised MWCNTs in 200 μL of 1.0% m/m Chit in 1.0% v/v acetic acid solution and sonicating for 2 h. This dispersion was used to modify the electrode and covalently immobilise the CNTs following the procedure described in [11]. Briefly, this consists in placing a 10 μL drop of the 1% m/v MWCNT solution in 1.0% m/m Chit on the electrode and leaving to dry in air at room temperature, repeating this step and then dropping 10 μL of 0.1 M phosphate buffer saline (pH 7.0) twice in order to deprotonate the amino groups of Chit by changing the pH. Finally, 10 μL of 0.5% m/v EDC-0.5% m/v NHS cross-linking agent in the same buffer solution was dropped onto the surface and left to dry for 2 h.

Electropolymerisation of the azines

Poly(neutral red) or poly(brilliant cresyl blue) films were obtained by electropolymerisation using potential cycling [16, 17]. Prior to use, the GC electrode was polished with diamond spray (Kemet International Ltd., UK, www.kemet.co.uk) of decreasing particle size down to 1 μm, followed by polishing with 0.3 μm alumina particles and washing with ultrapure water. The state of the surface was monitored by cyclic voltammetry in supporting electrolyte.

The polymer was formed directly on the GC or on the CNT-modified GC electrode. In the optimised procedures, electropolymerisation of NR was carried out by cycling 20 times from -1.0 to + 1.0 V *vs*. SCE at 50 mV s^{-1} in 1 mM NR + 0.025 M KPB + 0.1 M KNO_3 (pH 5.5) [16] and of BCB by cycling 30 times between −0.6 V and +1.0 V *vs.* SCE at 50 mV s^{-1} in 0.1 mM BCB + 0.1 M NaPB + 0.1 M KNO_3 (pH 7.0) [17].

Enzyme immobilisation

Glucose oxidase was immobilised by cross-linking with glutaraldehyde and bovine serum albumin. The enzyme solution was prepared by dissolving 40 mg BSA and 10 mg GOx in 1 mL of 0.1 M NaPBS (pH 7.0); each 10 μL of this solution was mixed with 5 μL of GA (2.5% v/v in water). For drop-coating, 10 μL of the mixture were placed onto the electrode previously modified with CNT, with polymer, or both. For immobilisation of enzyme/CNT mixtures, the same concentrations of GOx, GA, BSA and CNTs were used.

To prepare bienzymatic biosensors, 3.3 mg/mL of Cat were added to the enzyme mixture, a drop of 10 μL being placed onto the modified electrode. Thus, the GOx concentration was maintained, and the concentration of Cat was sufficiently high to ensure decomposition of all hydrogen peroxide generated in the GOx enzymatic reaction.

Electrodes with enzymes were left to dry at room temperature for at least 1 h, after which the biosensors could be used immediately. When not in use, enzyme electrodes were kept at 4 °C in phosphate buffer electrolyte, pH 7.0.

Results and discussion

Electropolymerisation at CNT-modified and unmodified glassy carbon electrodes

The effect of modification of the electrode by CNTs on the electropolymerisation of the azines by potential cycling was investigated. Figure 1 shows poly(neutral red) (PNR) and poly(brilliant cresyl blue) (PBCB) film growth at bare (insets) and CNT-modified glassy carbon electrodes during electropolymerisation. In the case of neutral red, polymer growth, as shown by the increase in the cyclic voltammetric current peaks at −0.5 V, was similar on CNT-modified and bare GC surfaces, with a larger amount of polymer formed in the presence of CNT. The second pair of peaks at ~0.0 V is much more evident at CNT-modified electrodes, in agreement with previous observations [19]. For PBCB formation, the differences in voltammetric profile are greater than for PNR but the ratio of peak current of the main polymer redox couple (~0.0 V) at CNT-modified to that at bare glassy carbon is approximately 3 whereas it is around 5 for PNR (couple at ~ −0.5 V). A contributing factor may be the different pH values of the electrolyte used for polymerisation: pH 5.5 for NR and pH 7.0 for BCB. It might be that at pH 5.5 there are COO^- groups from CNTs that are able to bind with NH_3^+ from NR and lead to a more stable structure polymer than at pH 7.0 for BCB.

To our knowledge, no previous study of electropolymerisation of BCB on top of CNT has been published. The literature only describes a sensor for epinephrine using PBCB and CNT obtained by in situ electropolymerisation from a solution containing BCB and CNT dispersed in dihexadecyl phosphate [20] and a PBCB/CNT composite for the determination of ethanol prepared by fixed potential polymerisation [21].

Electrochemical impedance spectroscopy (EIS) and scanning electron microscopy (SEM) surface characterisation

Unmodified and modified glassy carbon electrodes were characterised by EIS in the presence of the model electroactive species potassium hexacyanoferrate (III) at 0.15 V, the

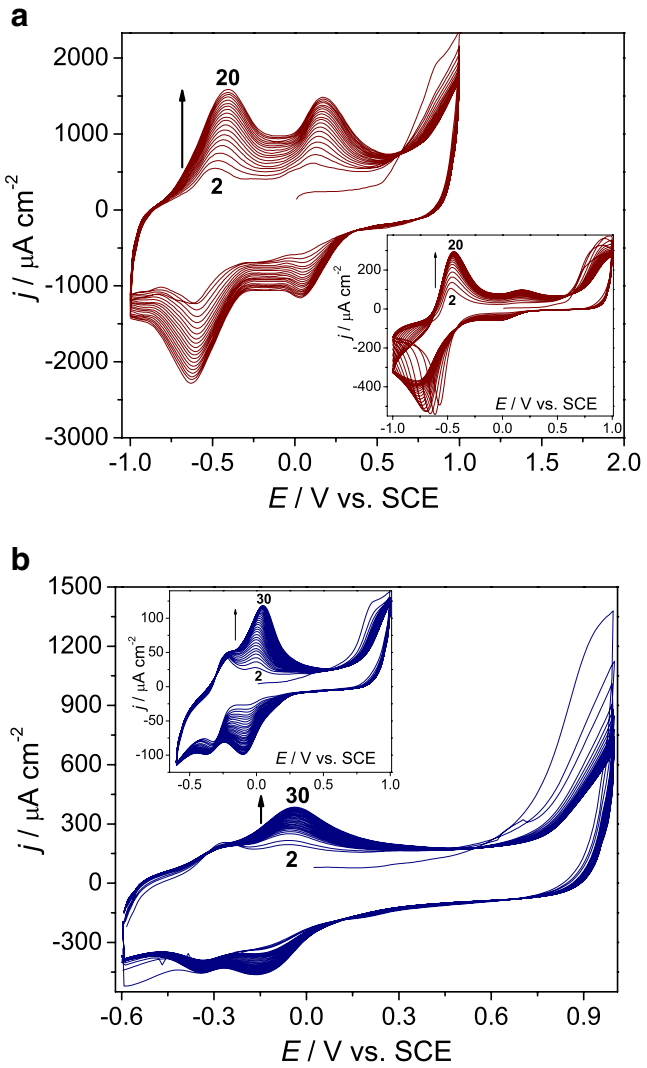

Fig. 1 Electrochemical polymerisation by potential cycling at 50 mV s^{-1} between: (**a**) −1.0 and +1.0 V for neutral red in 1 mM NR, 0.025 M KPB, pH 5.5 + 0.1 M KNO_3; (**b**) −0.6 and +1.0 V for brilliant cresyl blue in 0.1 mM BCB, 0.1 M NaPB, pH 7.0 + 0.1 M KNO_3 at CNT modified GCE. Insets show polymerisation at bare glassy carbon electrodes

formal potential of $[Fe(CN)]^{3-}/[Fe(CN)]^{4-}$ at GC electrodes, in order to examine the ease of charge transfer. Spectra obtained are shown in Fig. 2 and compare different electrode modifications by PNR (Fig. 2a) and PBCB (Fig. 2b). Nearly all spectra had a similar shape, with a semicircle in the high frequency region due to the charge transfer process and a linear part in the low frequency region, due to diffusion control. The only spectra with different shapes were GC modified with just PNR and PBCB polymer films.

Impedance spectra were fitted to equivalent electrical circuits. The circuit used for the bare GC electrode, was a typical Randles circuit consisting of a cell resistance, R_Ω, in series with a parallel combination of a constant phase element, CPE_{dl}, as a non-ideal double layer capacitance ($CPE_{dl} = -(i\omega C_{dl})^{-\alpha}$, where ω is the angular frequency and α the CPE exponent reflecting a non-uniform surface) and a charge transfer resistance, $R_{ct,}$ in series with an open Warburg element W_o. For PNR- and PBCB- modified electrodes, W_0 was replaced by a finite-diffusion Warburg element, reflecting diffusion and charge separation into and through the polymer film as rate-limiting step. For all other spectra, i.e. at CNT, PNR/CNT, PBCB/CNT, CNT/PNR and CNT/PBCB modified electrodes, the Warburg element was replaced with a second CPE, CPE_2, in series with the parallel combination, reflecting the fact that CNT and polymer form distinct layers. The values obtained from fitting are presented in Table 1; the values of cell resistance were in all cases between 11–14 Ω cm^2.

Charge transfer resistance values decreased at the CNT-modified electrodes compared with the bare electrode, but at PNR and PBCB modified electrodes the values are very high, meaning that both polymers hinder electron transfer compared to the GC substrate, whilst CNT greatly improve the reaction rate. At all other modified electrodes, with both CNTs and polymers, the values of R_{ct} are much lower, less so for PNR/CNT, consistent with more difficult charge transfer. The lowest value was obtained for CNT/PBCB, and for CNT/PNR and PBCB/CNT the values are very similar. These results are useful for development of sensors and biosensors, since they provide important information about electron transfer at each modified electrode, and suggest that CNT with PBCB should provide advantages.

SEM was used to examine the surface morphology of films of carbon nanotubes in combinations with poly (neutral red) and poly(brilliant cresyl blue) on ITO electrodes, prepared by the same method as for glassy carbon. Figure 3a shows micrographs of CNT immobilised in chitosan, although it is difficult to distinguish the CNT within the chitosan polymer; higher magnification (Fig. 3b) shows the well known small bundle structure of the nanotubes [22]. Regarding the combination of CNT and poly(azine) in all cases similar morphologies are observed (see CNT/PBCB in Fig. 3c), independent of the CNTs being on top of or under the polymer, indicating a composite structure as previously reported for both PNR and PBCB [19, 20, 22]. The structure is network-like with pore sizes around 10 µm for CNT/PBCB and PNR/CNT composite and more compact with pore sizes less than 5 µm for CNT/PNR and PBCB/CNT.

Glucose determination with different biosensor assemblies

The effect of combining phenazine polymers and CNT on biosensor functioning was studied using the model enzyme glucose oxidase (GOx). Biosensors were prepared by immo-

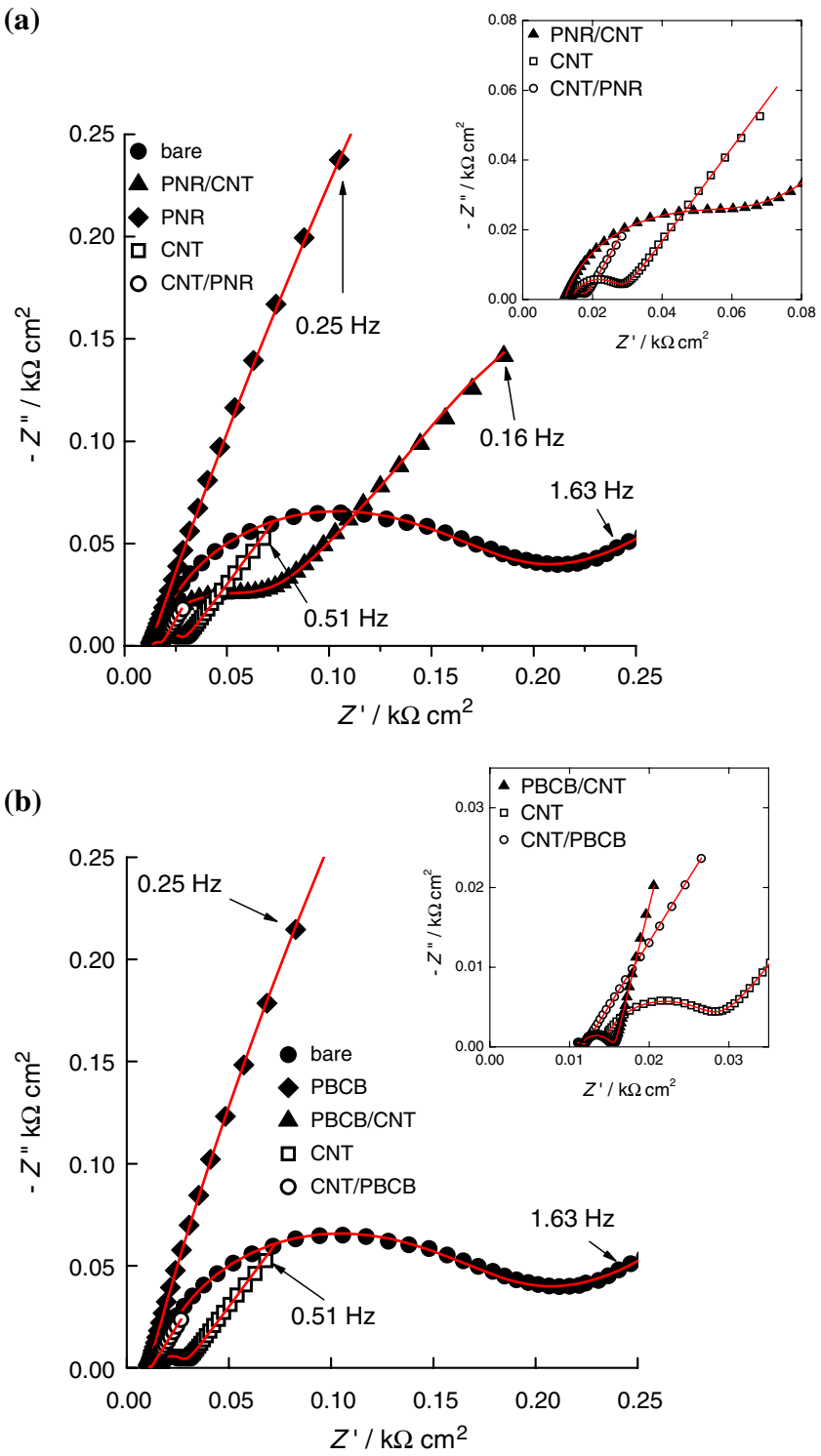

Fig. 2 Complex plane impedance spectra at different stages of electrode assembly for (**a**) PNR and CNT and (**b**) PBCB and CNT modified electrodes in 3 mM $K_3Fe(CN)_6$ / 0.1 M KCl at 0.15 V *vs.* SCE. The magnified insets show the spectra of only CNT and their combination with polymers. *Lines* indicate equivalent circuit fitting, see text for circuits

bilising GOx on top of electrodes covered with PNR, PBCB, CNT, PNR/CNT, PBCB/CNT, CNT/PNR and CNT/PBCB. Amperometric determination of glucose at fixed potentials between −0.3 V and +0.3 V was evaluated for each type of electrode. The mechanism of action was investigated in the absence of oxygen and also using bienzymatic systems with glucose oxidase and catalase (Cat).

Table 1 Data from the fitting to equivalent circuits of spectra from Fig. 2

Electrode modification	C_{dl} / μF cm^{-2} s$^{\alpha-1}$	α_1	R_{ct} / Ω cm^2	C_2 / mF cm^{-2} s$^{\alpha-1}$	α_2
–	12.9	0.85	129	–	–
PNR	168	0.79	4,200	–	–
PBCB	134	0.83	3,100	–	–
CNT	69.1	0.81	13.6	7.5	0.59
CNT/PNR	345	0.76	4.3	10.1	0.67
CNT/PBCB	185	0.81	1.3	30.3	0.64
PNR/CNT	200	0.84	43.4	5.1	0.52
PBCB/CNT	387	0.84	4.5	38.6	0.69

Polymer-modified-electrode biosensors

Biosensors with PNR and PBCB as redox mediators exhibited a similar behaviour: an anodic change in current, associated with an oxidation process between −0.3 and −0.1 V, at 0.0 V a small cathodic change and at positive potentials no response. Comparing the two types of biosensor at −0.3 V (Table 2) the response is three times higher using the PNR-based biosensor compared to PBCB. The linear range was up to 1.3 mM of glucose in both cases and detection limits (three times signal to noise ratio) were similar (19 μM for PNR/GOx and 21 μM for PBCB/GOx). In the absence of oxygen, both biosensors saturate at 0.6 mM but the sensitivity is higher than in the presence of oxygen.

In air-saturated electrolytes, oxidation of glucose might follow two competitive pathways as suggested in [23], involving FAD regeneration and H_2O_2 reduction [15, 17]. In the absence of oxygen, the response saturates at low glucose concentrations because only a small amount of FAD is available compared to oxygen, as also reported in [24] for a biosensor based on glucose oxidase and ferrocene derivative as mediator.

CNT-modified-electrode biosensors

In the case of the CNT/GOx biosensor, between −0.3 V and 0.0 V an oxidation process is observed, at +0.1 and +0.2 V reduction occurs and at +0.3 V there is again oxidation. Analytical parameters calculated from the calibration curves (not shown) at different potentials are presented in Table 3. The oxidation at +0.3 V is attributed to hydrogen peroxide oxidation, also reported in [25] at a basal plane pyrolytic graphite electrode modified with nanotubes and glucose oxidase. Despite the shorter linear range (up to 2.4 mM compared with 4.0 mM) our biosensor had a lower detection limit (18 μM compared to 50 μM).

The performance at −0.3 V with the proposed biosensor is better than in the literature at comparable biosensor assemblies. The linear range up to 1.6 mM, sensitivity 10.1 μA mM^{-1} cm^{-2}, and detection limit 17 μM is better than that achieved in [26] with a MWCNT/APTES/GOx electrode at −0.45 V vs. Ag/AgCl (sensitivity 8.85 μA mM^{-1} cm^{-2}, detection limit 25 μM, but a longer linear range, up to 5 mM). At a CNT-GOx

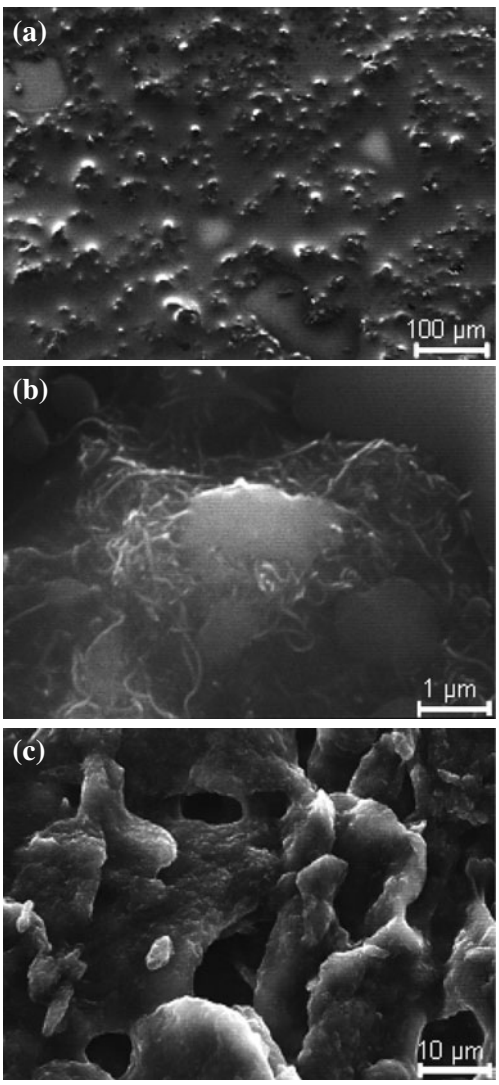

Fig. 3 SEM images on ITO-modified electrodes of (**a**) CNT in chitosan, (**b**) detail of (**a**) at higher magnification; (**c**) CNT/PBCB modified electrode

Table 2 Analytical parameters of different biosensors with CNT, PNR, PBCB and combinations of CNT with PNR or PBCB at −0.3 V *vs*. SCE. Data are average values from three different biosensors

Biosensor	Upper limit of linear range / mM	Sensitivity / $\mu AmM^{-1}cm^{-2}$	LOD / μM
PNR/GOx	1.3	1.68±0.15	19
PBCB/GOx	1.3	1.08±0.09	24
CNT/GOx	1.6	10.1±1.2	17
CNT/PNR/GOx	1.6	30.4±3.2	17
CNT/PBCB/GOx	1.6	10.1±0.9	14
PNR/CNT/GOx	1.3	2.73±0.12	20
PBCB/CNT/GOx	1.6	36.3±3.4	11

biosensor [27], although the sensitivity was higher (34.2 μA mM^{-1} cm^{-2}, at −0.48 V vs. Ag/AgCl) and almost the same detection limit (20 μM), the linear range was only up to 1.0 mM.

The reduction process at +0.1 and +0.2 V is due to hydrogen peroxide (confirmed by independent measurements on the CNT-modified electrode without enzyme following addition of H_2O_2 and also on the CNT/GOx biosensor in the absence of O_2, data not shown).

In order to shed light on the process occurring at negative potentials, measurements without oxygen were performed. There is still an anodic change in current, saturation occurs at much lower concentrations than with oxygen, but not as low as with the polymer biosensors, so CNT improve the GOx response in the absence of O_2. In the presence of oxygen the linear range is up to 1.6 mM and in its absence up to 1.0 mM. In other work with a GOx biosensor and adsorbed (rather than covalently immobilised) CNT no response to glucose was obtained in the absence of oxygen [28], so here the access to the CNT surface within the Chit matrix may be important since sensitivity to glucose in the presence and absence of oxygen was similar.

These results can be explained by competition between FAD regeneration (oxidation) and hydrogen peroxide reduction. The proposed mechanism is sustained by results obtained with the bienzymatic biosensor with glucose oxidase and catalase in the absence of oxygen. Catalase catalyses the decomposition of hydrogen peroxide into water and molecular oxygen, the overall reaction being [29]:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

so that peroxide resulting from the reaction of the GOx catalysed reaction of glucose with any remaining dissolved oxygen (it is not possible to completely remove oxygen from the system by bubbling nitrogen) will be removed. The bienzymatic biosensor exhibited a higher anodic response both at −0.3 V and at 0.0 V compared with the monoenzymatic one, supporting this explanation (see Table 3).

The biosensors developed exhibited good reproducibility, the relative standard deviation for three different electrodes was around 8%. The operational stability of the biosensors was evaluated through performing consecutive

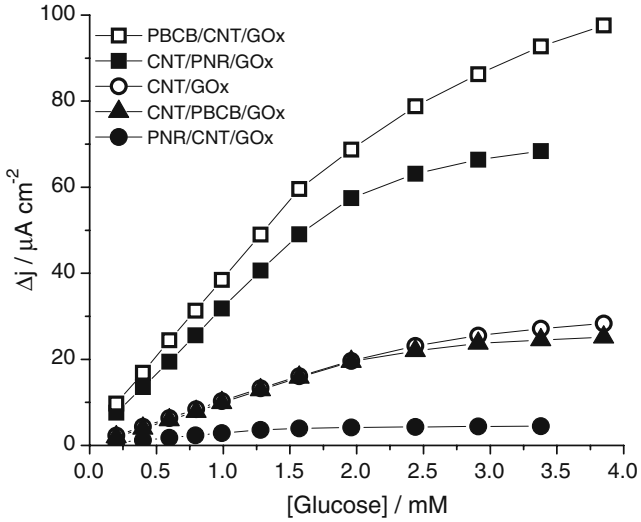

Fig. 4 Calibration curves for glucose at different biosensors combining PNR or PBCB with CNTs in 0.1 M NaPBS pH 7.0 at −0.3 V *vs*. SCE

Table 3 Performance of the CNT/GOx and CNT/GOx + Cat biosensor at different applied potentials. Data are averages from three different biosensors

E / V vs SCE	Process observed	Upper limit of linear range / mM		Sensitivity / $\mu AmM^{-1}cm^{-2}$		LOD / μM	
		GOx	GOx+Cat	GOx	GOx+Cat	GOx	GOx+Cat
−0.3	oxidation	1.6	1.0	10.1±1.2	36.7±3.5	17	13
0.0	oxidation	1.6	1.0	1.36±0.08	6.11±0.7	16	19
+0.1	reduction	1.6	1.0	0.26±0.02	0.38±0.04	16	20
+0.2	reduction	1.6	1.0	0.17±0.01	0.74±0.07	22	19
+0.3	oxidation	2.4	1.0	0.55±0.04	2.61±0.2	18	22

measurements with glucose additions and recording calibration curves at different applied potentials during a whole day. After 100 injections of substrate under these different conditions, the loss of response was always less than 5%.

CNT/polymer-modified-electrode biosensors

Three different biosensor assemblies were analysed: i) polymer/CNT/Gox; ii) CNT/polymer/GOx iii) polymer/CNT-GOx, in order to investigate the role of CNT in the polymer mediation process.

In all cases the biosensor response is fast, 95% of the steady state current being achieved about 10 s after addition of the substrate, similar to with polymer or CNT alone. Experiments carried out in the same range of potentials as before showed that all biosensors prepared by combinations of poly(azine) and CNT exhibited the same dependence on potential as those with only CNT. The highest response was achieved for the PBCB/CNT/GOx biosensor (Table 2 and Fig. 4), about 3.6 times higher than the CNT/GOx biosensor, whilst the PNR/CNT/GOx biosensor exhibited the lowest response, about 3.7 times lower than the CNT/GOx biosensor. For polymers deposited on top of the CNT the behaviour was in the opposite sense: the highest response was exhibited by the CNT/PNR/GOx biosensor (Fig. 4), a factor of 3 higher than CNT/GOx, whilst the response of the CNT/PBCB/GOx biosensor was the same as CNT/GOx. The analytical parameters of all combinations are shown in Table 2, the linear range being almost the same, and detection limits are between 11 and 19 μM.

When CNT were immobilised together with GOx (with BSA and glutaraldehyde, see "Experimental" section) on top of PBCB-modified GC electrodes, the response was smaller than that of CNT/PBCB/GOx; a similar result was obtained in [18]. Both Chit-CNT and GOx possess amino groups which can compete for binding with the carbonyl group of glutaraldehyde, resulting in weaker enzyme immobilisation, as well as poor contact between CNT and GOx [18].

The results with the biosensors combining CNTs and polymers might be explained as follows. It was observed that the PNR/GOx biosensor exhibited a higher response than PBCB/GOx and that the presence of CNT influences positively the polymerisation of the two azines, more in the case of PNR than PBCB. Thus, one could expect that biosensors with CNT and PNR would have a higher response than those with CNT and PBCB, but this was only observed in the case of CNT/PNR/GOx vs CNT/PBCB/GOx. In the case of PNR/CNT/GOx and PBCB/CNT/GOx, since CNTs cover the polymer films they could impede their redox activity, which may explain why PNR/CNT/GOx biosensors exhibited such a low response.

If there is a synergic effect of polymer and nanotubes, as reported in [23], with the addition of CNT the response would always increase compared with only polymer or only CNT. There is always an increase compared with polymer alone but compared with CNT alone, only by the CNT/PNR/GOx and PBCB/CNT/GOx combinations. If the PNR film is more compact and adherent than PBCB (observed in independent studies) it is probable that PNR and CNT form distinct layers, but PBCB and CNT mix to some extent so that the properties of PBCB would be improved by the presence of CNT. In the case of PNR, the mediator properties are improved most when CNT are deposited first, due to the increased growth rate and amount of polymer formed on top.

Conclusions

Novel redox-mediated glucose oxidase biosensors have been investigated, involving combinations of two azine polymers, PNR and PBCB, and MWCNT. The two polymers investigated exhibited different characteristics, leading to different biosensor performances. PNR was formed more easily on top of CNT than PBCB. The mechanism of action was investigated in the absence and presence of oxygen and by comparison with bienzymatic devices containing glucose oxidase and catalase. The overall response was in most cases greater than that of either polymer-based or CNT-based biosensors. The highest response was exhibited by the PBCB/CNT/GOx biosensor, due to the synergic effect of polymer and nanotubes as well as a possible mixing of the polymer and CNT layers, followed by CNT/PNR/GOx; the lowest response was that of PNR/CNT/GOx. Further investigations will be performed to elucidate these different behaviours, including using other polyazines. Enzyme biosensors will be developed exploiting these polymer/CNT combinations for application to analytes in foods and beverages.

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