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New CNT/poly(brilliant green) and CNT/poly(3,4-ethylenedioxythiophene) based electrochemical enzyme biosensors

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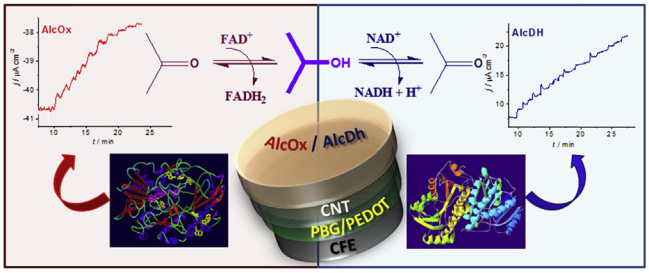
HIGHLIGHTS

- New biosensor platforms using the synergistic combination of polymers and CNT.
- CNT co-immobilized with poly(-brilliant green) (PBG) and PEDOT.
- Glucose and alcohol biosensors based on CNT/PBG and CNT/PEDOT.
- CNT/PBG platforms are best substrates for alcohol oxidase/dehydrogenase enzymes.
- Alcohol biosensors successfully used for ethanol detection in alcoholic beverages.

GRAPHICAL ABSTRACT



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ABSTRACT

A combination of the electroactive polymer poly(brilliant green) (PBG) or conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) with carbon nanotubes to obtain CNT/PBG and CNT/PEDOT modified carbon film electrodes (CFE) has been investigated as a new biosensor platform, incorporating the enzymes glucose oxidase (GOx) as test enzyme, alcohol oxidase (AlcOx) or alcohol dehydrogenase (AlcDH). The sensing parameters were optimized for all biosensors based on CNT/PBG/CFE, CNT/PEDOT/CFE platforms. Under optimized conditions, both GOx biosensors exhibited very similar sensitivities, while in the case of AlcOx and AlcDH biosensors, AlcOx/CNT/PBG/CFE was found to give a higher sensitivity and lower detection limit. The influence of dissolved O_2 on oxidase-biosensor performance was investigated and was shown to be different for each enzyme. Comparisons were made with similar reported biosensors, showing the advantages of the new biosensors, and excellent selectivity against potential interferents was successfully demonstrated. Finally, alcohol biosensors were successfully used for the determination of ethanol in alcoholic beverages.

1. Introduction

In recent years, the field of biosensors is growing in importance since these devices permit a simple and fast detection of many biological analytes, which are considered key compounds in medical, pharmaceutical and food areas. Biosensors are generally based on enzymatic reactions, which are very selective for a particular substrate, and require, in order to find applications, to be low-cost, rapid, inert and stable platforms [1]. Good enzyme immobilization [2,3] and fast electron transfer between the enzyme and the electrode are the two key points to be considered during the planning

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of a new biosensor. In this context, the choice of the electrode material is the best way to control this aspect. Different materials can be used to build biosensors. Nanomaterials [2], such as nanoparticles [4–7], graphene [8,9], carbon nanotubes [10] and conducting polymers [11,12], permit the production of biosensors with such properties.

The use of polymers for enhancing electrode performance is already documented [13–15]. The use of conducting polymers, which have a conjugated π-bond structure, in electroanalysis is very promising owing to their unique properties, bringing speed, sensitivity and versatility enhancement [16]. Moreover, electroactive polymers are very attractive, since they add the properties of electrocatalysis and redox-mediation, thus increasing sensor performance [17]. Polyphenazines are a class of electroactive polymers successfully applied not only for the detection of a vast number of organic and inorganic species, but also for the production of very sensitive and stable biosensors [13]. Triphenylmethane dyes, very similar to phenazines, but characterized by an open and ionized structure can further improve electrode performance. Their use in the field of sensors is up to now very limited, and related to only two dyes: Malachite Green (MG) and Brilliant Green (BG), and no examples of biosensor applications are reported in the literature. Polymerized films of BG (PBG), in particular, are very promising for the construction of biosensors, considering PBG's recently reported excellent performance in the detection of hydrogen peroxide [18], an ubiquitous product of enzymatic biological reactions.

To further improve electrode performance, electroactive polymers can be associated with other conducting polymers, such as poly(3,4-ethylenedioxythiophene) (PEDOT) [19–21], or with carbon nanotubes (CNT) [22]. PEDOT shows high conductivity, moderate band gap, low oxidation potential, high chemical stability in aqueous solutions and good biocompatibility with biological media [23–25], while CNT possess high conductivity and high surface to mass ratio, high chemical and thermal stability, high elasticity and high tensile strength [26,27]. The resulting composites can offer better stability, higher rates of electron transfer, better sensitivity and lower detection limits in sensing applications.

Among all the types of biosensor, those for the detection of glucose [28] are ideal to evaluate the performance of a newly-developed platform, showing also potential application in the food and medical areas [29]. Glucose oxidase (GOx) can be considered a model enzyme, since it permits the production of simple and cheap biosensors, which allow a fast investigation of electrode performance.

Ethanol is another important analyte and its detection is required in many different areas: clinical and forensic analysis, food, pulp and beverage industries, agricultural and environmental measurements. Many analytical methods have been developed for the determination of ethanol [30] and include the use of chemical methods, colorimetric methods, specific gravity and refractive index measurements, chromatographic and spectroscopic methods. These methods present different drawbacks, such as complexity, time-consuming procedures, separation pre-processes, expensive instrumentation and trained operators. Enzymatic methods could overcome these problems, two enzymes being extensively used in the determination of ethanol, *i.e.* alcohol dehydrogenase (AlcDH), which requires the cofactor NAD^+, and alcohol oxidase (AlcOx), which already contains the FAD cofactor. Ethanol biosensors usually require a redox mediator, in order to facilitate electron communication between the immobilized enzyme and the electrode [31] or to lower the potential required for the oxidation of NADH, generated in the AlcDH catalysed reaction [32].

In this work, two different electrode architectures were taken into consideration for the construction of new biosensors, CNT/PBG and CNT/PEDOT modified carbon film electrodes (CFE), chosen on the basis of the results of a previous paper concerning the determination of hydrogen peroxide, these two configurations showing the best performances for H_2O_2 detection [18]. The working conditions of GOx, AlcOx and AlcDH based biosensors, *i.e.* working potentials and the solution pH were optimized, and the influence of dissolved oxygen on the oxidase based biosensor's performance was evaluated, by testing in the absence of O_2, in regular O_2 content and in O_2-saturated buffer solution. Sensitivities and detection limits were compared with other, similar biosensors reported in the literature and, finally, alcohol biosensors were applied to the detection of ethanol in alcoholic beverages.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and used as received.

The electrolyte for the electropolymerisation of BG (Fluka) was 0.1 M McIlvaine buffer pH 4.0 from (Aldrich), containing 1 mM of monomer. The solution for the polymerisation of EDOT (Aldrich) was prepared with 10 mM of monomer dissolved in 0.1 M 4-styrenesulfonic acid sodium salt hydrate (NaSS) (Aldrich).

Multiwalled CNT (NanoLab, USA) were functionalized in 5 M nitric acid (65%) and were dispersed in a solution of 1% chitosan (low molecular weight, degree of deacetylation 80%), prepared in 1% glacial acetic acid, both from Aldrich.

Glucose oxidase from *Aspergillus niger*, alcohol oxidase from *Hansenula* sp, alcohol dehydrogenase from *Saccharomyces cerevisiae*, nicotinamide adenine dinucleotide, albumin from bovine serum (BSA) and glutaraldehyde solution (25% in water) were purchased from Aldrich.

Phosphate buffer saline (PBS) at different pH, containing 0.1 M NaPB ($Na_2HPO_4 + NaH_2PO_4$ from Riedel-de-Haën) and 0.05 M NaCl (Aldrich) were used for the optimization of operative conditions of biosensors and for the detection of glucose (Aldrich) and ethanol (Aldrich).

Millipore Milli-Q nanopure water (resistivity $\geq$ 18 MΩ cm) was used for the preparation of all solutions.

2.2. Instrumentation

All experiments were performed in a conventional electrochemical cell with a modified carbon film electrode (CFE, geometric area 0.20 cm^2, resistance 2 Ω, film thickness 15 μm) as working electrode, a Pt wire as counter electrode and a saturated calomel electrode (SCE) as reference. The carbon film electrodes were made from carbon film resistors, as described in Ref. [33]. All currents were normalised by the electrode geometric area.

Electrochemical experiments were performed with a computer-controlled μ-Autolab type I potentiostat-galvanostat with GPES software (Metrohm-Autolab, Utrecht, The Netherlands).

The pH-measurements were done with a CRISON 2001 micro pH-meter.

All experiments were carried out at room temperature (25 ± 1 °C).

2.3. Modified electrode preparation

The modification of carbon film electrodes with PBG, PEDOT and CNT was optimized in previous work [18]. Two types of electrodes showed the best results in the determination of hydrogen peroxide, namely CNT/PBG/CFE, when PBG was deposited on the CFE and covered with CNT, and CNT/PEDOT/CFE, PEDOT being deposited on the CFE and covered with CNT. These were therefore chosen for the construction of the enzyme biosensors.

The CFEs were pre-treated in 0.2 M NaCl by cycling the potential 10 times between −1.0 and +1.0 V vs. SCE at a scan rate of 100 mV s^{-1} in order to obtain a reproducible surface.

2.3.1. Brilliant green and EDOT electropolymerisation

Before electropolymerisation, the electrodes were pre-treated in 0.1 M sulphuric acid for 10 cycles from −1.0 V up to +1.2 V vs. SCE, at 100 mV s^{-1}.

For BG electropolymerisation, a 1 mM monomer solution was prepared by dissolving the appropriate quantity of BG in 0.1 M McIlvaine buffer pH 4.0. Then, BG was electropolymerised by cycling the potential for 10 scans between −1.0 V and +1.2 V vs. SCE at 100 mV s^{-1}.

EDOT was electropolymerised as reported in Ref. [20]. A solution of 10 mM monomer was dissolved in 0.1 M NaSS and heated for 10 min until complete dissolution of the monomer. EDOT was then electropolymerised by cycling the potential for 10 cycles between −0.6 V and +1.2 V vs. SCE, at 50 mV s^{-1}.

2.3.2. Carbon nanotube functionalization

Multiwalled carbon nanotubes were purified and functionalised by stirring them in 5 M nitric acid for one night, collecting using a filter paper (pore size 11 μm) and washing with water until neutral pH. The powder was dried in the oven at 80 °C for one night. This procedure allowed removal of metal catalysts and amorphous carbon, derived from the synthetic process, and functionalization of the ends of the CNT with −OH containing groups.

A solution containing 1% chitosan and 1% acetic acid was prepared and used to form a 1% CNT suspension, sonicating in an ultrasound bath for 3 h. A volume of 10 μL was placed on the electrode by drop-casting and left to dry for one hour, before dropping another 10 μL. Afterwards, electrodes were left to dry in air, for at least 24 h.

2.3.3. Biosensor preparation

GOx, AlcOx and AlcDH were immobilised using cross-linking with glutaraldehyde (GA) after electrode modification. An enzyme solution was prepared by mixing the enzyme together with BSA in 0.1 M NaPBS pH 7.0 in concentration 1% w/v GOx/AlcDH + 4% w/v BSA or 5% w/v AlcOx + 10% w/v BSA, as reported in previous papers [19,21,31]. A volume of 10 μl of enzyme solution was then mixed with 5 μl GA (2.5%, v/v diluted in water). 10 μl of this mixture was dropped on the electrode surface and left to dry at room temperature for 4 h before use.

The electrodes were kept at 4 °C in their electrolyte when not in use.

3. Results and discussion

3.1. Polymer synthesis and characterization

The polymerization of the monomers brilliant green and EDOT was performed on CFE, by cycling the potential between −1.0 V and +1.25 V vs. SCE in 1 mM BG + 0.1 M McIlvaine buffer pH 4.0, for PBG formation and between −0.6 and 1.2 V vs. SCE in 10 mM EDOT + 0.1 M NaSS, SS acting as counterion. Fig. 1a and b shows cyclic voltammograms recorded during the ten cycles of polymerization for both monomers. In the case of BG, the first four cycles revealed the formation of cation radicals at high positive potentials, that promote the formation of the polymer, which, being very compact with a closed polymeric structure, impedes monomer species reaching the electrode surface, evidenced by the decrease in current beginning with cycle 5. Regarding PEDOT, deposition is shown by the continuous increase in current during potential cycling, up to the last cycle of polymerization.

The PBG or PEDOT modified CFE was afterwards covered with CNT, as described in the experimental section, and further characterized by cyclic voltammetry (Fig 1c), which shows high capacitive currents at both modified electrodes, being highest for the CNT/PEDOT/CFE, due to the contribution of both CNT and the PEDOT film. Fig. 1d shows cyclic voltammograms for CNT/PEDOT/CFE at different scan rates. Plots of oxidation and reduction peak current with square root of scan rate reveal that the electrochemical process at both CNT/PBG/CFE and CNT/PEDOT/CFE is controlled by diffusion of the counterions from solution, i.e. K^+, H^+, which are inserted during reduction and expelled during oxidation. The similarity in the slopes of the linear plots of peak current vs. square root of scan rate of 23.3 and 25.0 μA cm^{-2} (mV $s^{-1})^{1/2}$ for the CNT/PBG and CNT/PEDOT modified CFE respectively, indicate similar diffusional rates at these two modified electrodes.

3.2. Oxidase based biosensors

The applied potential in fixed potential chronoamperometry and pH conditions play an important role in biosensor activity, since both can influence the sensitivity of detection and the possible interferences.

3.2.1. Optimization of applied potential for GOx and AlcOx biosensors

Optimisation of the applied potential, from −0.4 V up to 0.0 V vs. SCE, for the glucose biosensor was carried out in 0.1 M NaPBS pH 7.0 electrolyte, taking into account the good sensitivities of other similar biosensors at this pH [19,21]. For both CNT/PEDOT/CFE and CNT/PBG/CFE electrodes the current response is higher at −0.4 V, closer to the formal potential of the $FAD/FADH_2$ couple, and decreases rapidly when the potential approaches 0.0 V, similarly to other GOx-based biosensors [19,21]. A potential of −0.3 V was chosen for further amperometric experiments with the aim of minimising possible interferences, as well as ensuring good sensitivity.

In the case of AlcOx biosensors, the influence of the buffer solution pH and the applied potential on biosensor behaviour was different, depending on the redox mediator. Fig. 2a1 and b1 shows the change in current after addition of 0.4 mM ethanol to buffer solutions of pH from 6.5 to 9.0, at AlcOx/CNT/PEDOT/CFE and AlcOx/CNT/PBG/CFE, applied potential −0.3 V vs. SCE, demonstrating that in the case of AlcOx/CNT/PEDOT/CFE the optimum pH is 7.0, while in the case of AlcOx/CNT/PBG/CFE it is 8.5. As presented in Fig. 2a2 and b2, where the current is shown as a function of applied potential, for AlcOx/CNT/PEDOT/CFE the current reaches its highest value close to the formal potential of the $FAD/FADH_2$ couple, as already reported for another AlcOx biosensor [31]. However, at AlcOx/CNT/PBG/CFE, the behaviour is completely different, the current increasing from −0.4 V to −0.2 V, followed by a small decrease at −0.1 V. The high signals at potentials closer to 0.0 V vs. SCE at this latter modified electrode assembly is attributed to the presence of the redox polymer PBG, which exhibits good redox activity even at these potentials. Taking into account the results obtained, the potentials chosen were −0.3 V vs. SCE for AlcOx/CNT/PEDOT/CFE and −0.1 V vs. SCE for AlcOx/CNT/PBG/CFE.

3.2.2. Influence of buffer solution oxygen content on GOx and AlcOx biosensors/mechanism

Oxygen is a very important parameter to be considered for the evaluation of an oxidase enzyme based biosensor, since it can strongly influence its performance, firstly due to the fact that it can be involved in the biosensor mechanism and secondly because it can directly influence the enzyme activity. The influence of oxygen on enzyme activity may depend on the type of microorganism,

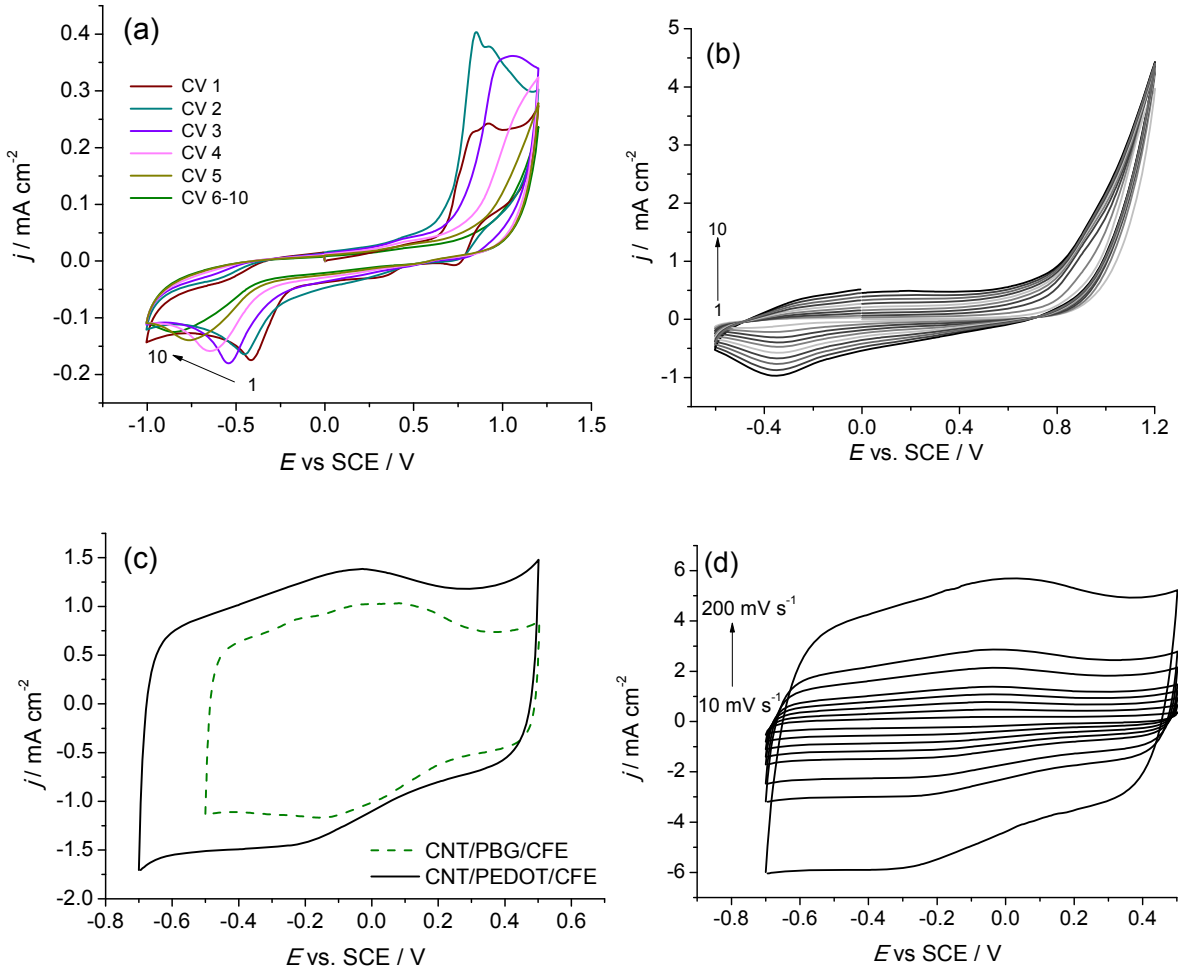

Fig. 1. Cyclic voltammograms obtained during polymerization of (a) BG (b) EDOT; CVs in 0.1 M KCl of (c) CNT/PBG/CFE and CNT/PEDOT/CFE at 50 mV s^{-1} and (d) CNT/PEDOT/CFE at different scan rates.

aerobic or anaerobic species, from which the enzyme is extracted. Another key point is the possibility that oxygen can also influence the behaviour of the electroactive polymer during its redox process, as already discussed for PBG in Ref. [18].

For these reasons, the influence of oxygen was studied for both glucose and alcohol biosensors with the best architecture (CNT/PBG/CFE). Fixed potential chronoamperograms were recorded in buffer solution without oxygen (saturated with N_2), normal oxygen content (air saturated) and saturated with oxygen. The calculated sensitivities for both GOx and AlcOx biosensors in the above specified media are presented in Table 1, which shows that concentration of dissolved oxygen significantly influences the GOx biosensor, and less the AlcOx biosensor performance.

Using GOx biosensors, the sensitivity of 44 ± 3 (RSD = 6.4%, n = 3), in air saturated buffer solution, increases to 57 ± 4 μA cm^{-2} mM^{-1} (RSD = 6.3%, n = 3) in buffer saturated with O_2, while in N_2 saturated buffer it decreases by a factor of two, to 21 ± 1 μA cm^{-2} mM^{-1} (RSD = 5.6%, n = 3). The fact that GOx biosensor performance is best in O_2 saturated buffer can be explained by two facts: first that enzyme has a high O_2 affinity, since GOx belongs to a highly aerobic species *A. niger* [34] and secondly, considering the fact that charge transfer at PBG modified electrodes is faster when O_2 is in solution [18].

The proposed mechanism at phenazine-based biosensors reported in Ref. [35] involves a competitive reaction at the electrode, where there are simultaneous oxidation and reduction currents. These correspond, respectively, to oxidation of PBG(red), in the case that PBG acts as electron acceptor from $FADH_2$, being reduced after interaction with enzyme and re-oxidized at the electrode, and to reduction of PBG(ox) following reaction of PBG(red) with H_2O_2, formed in the enzymatic reaction if O_2 is the electron acceptor.

Thus, initially:

$$\text{Substrate} + \text{oxidase(FAD)} \rightarrow \text{Product} + \text{oxidase(FADH}_2\text{)}$$

followed by either

$$\text{FADH}_2 + \text{PBG(ox)} \rightarrow \text{FAD} + \text{PBG(red)}$$

$$\text{PBG(red)} \rightarrow \text{PBG(ox)} + e^-$$

or

$$\text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{PBG(red)} \rightarrow \text{H}_2\text{O} + \text{PBG(ox)}$$

$$\text{PBG(ox)} + e^- \rightarrow \text{PBG(red)}$$

According to this mechanism, the absence of oxygen should eliminate the competitive reduction reaction, so that the sensitivity

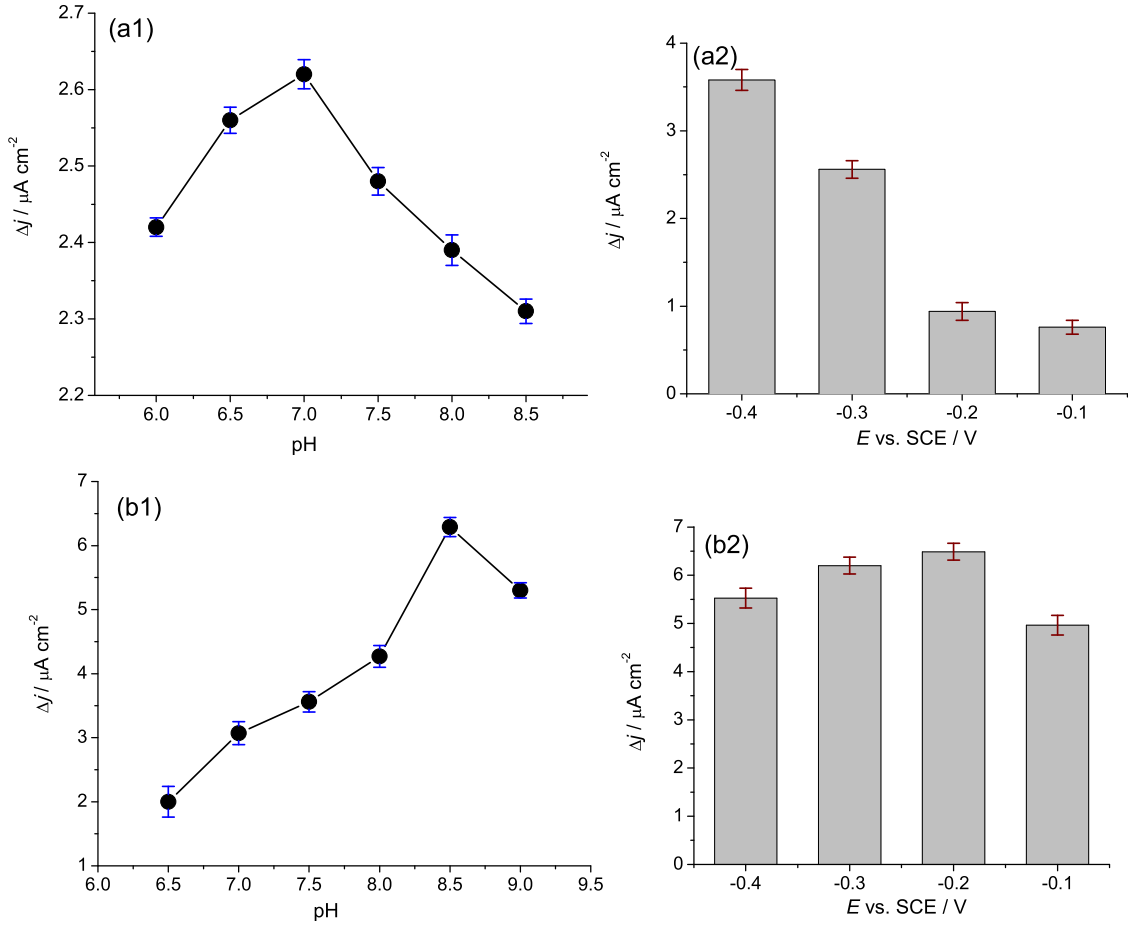

Fig. 2. Fixed potential amperometric response at AlcOx biosensors. Influence of (1) solution pH at −0.3 V vs. SCE and (2) potential at optimum pH of 7.0 and 8.5, respectively, for (a) AlcOx/CNT/PEDOT/CFE and (b) AlcOx/CNT/PBG/CFE.

Table 1
Sensitivities obtained at GOx and AlcOx CNT/PBG/CFE in 0.1 M NaPBS buffer solutions for different oxygen contents (GOx: pH 7.0, −0.3 V vs. SCE and AlcOx: pH 8.5, −0.1 V vs. SCE).

Solution	S/(μA cm^{-2} mM^{-1})	
	GOx	AlcOx
O_2 saturated	57 ± 4	9.8 ± 0.5
Air saturated	44 ± 3	12 ± 1
N_2 saturated	21 ± 1	14 ± 1

of the biosensor should be increased. However, since this did not occur, we deduce that dissolved O_2 does not make an important contribution in the mechanism, the polymer conductivity and enzyme activity having more influence, both increasing with increase in dissolved O_2 concentration.

In the case of the ethanol biosensor, the best sensitivity was obtained in the absence of oxygen, being 14 ± 1 (RSD = 5.6%, n = 3), higher than the sensitivities of 12 ± 1 (RSD = 5.6%, n = 3) and 9.8 ± 0.5 μA cm^{-2} mM^{-1} (RSD = 5.1%, n = 3), recorded in air and in O_2-saturated solutions. The better performance of the AlcOx based biosensor in N_2-saturated buffer, can be attributed to the fact that the enzyme is extracted from *Hansenula* sp, an anaerobic bacteria [36] and that it has a low oxygen affinity. Indeed, biosensor sensitivities were less influenced by the O_2 concentration for the AlcOx based biosensor, than in the case of GOx.

The influence of oxygen on the enzymatic activity of GOx and AlcOx was assessed by recording fixed potential amperograms under the same experimental conditions, but with the enzyme in the solution (0.1% enzyme w/v) instead of immobilized on the electrode surface. It was confirmed that oxygen had the same influence on enzyme activity, being higher for GOx in oxygenated solution (saturated with O_2) and for AlcOx in deoxygenated solution (saturated with N_2).

Thus, the biosensor performance in media containing different amounts of dissolved O_2 appears to be mainly dictated by the influence of O_2 affinity to the enzyme and on mediator performance.

3.3. Analytical parameters of GOx and AlcOx biosensors under optimized experimental conditions

Amperometric measurements were made under the optimised experimental conditions for both GOx and AlcOx biosensors based on CNT/PBG/CFE platforms, see Figs. 3a and 4a. Before performing amperometric measurements, a series of cyclic voltammograms was recorded at both GOx and AlcOx biosensors based on CNT/PBG/CFE platforms, for increasing concentrations of glucose and ethanol, respectively, see insets of Figs. 3a and 4a. As observed, in the negative potential region there is a decrease of the cathodic current and a slight increase in the anodic current following substrate injection.

The amperometric biosensor signals shown in Figs. 3a and 4a for the addition of substrate show an anodic change in current, as expected from the CVs, due to polymer oxidation, which acts as

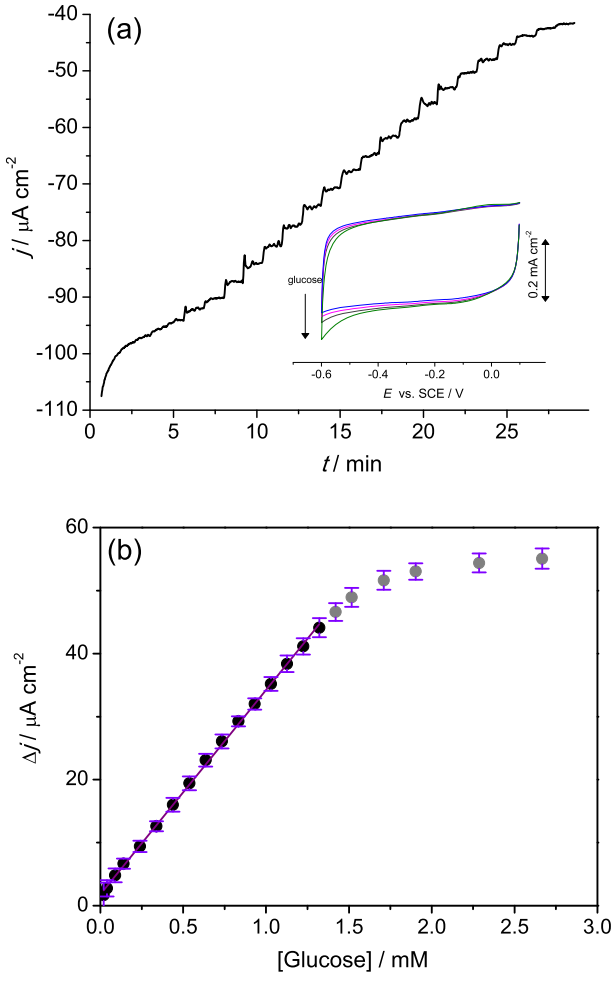

Fig. 3. a) Amperometric response of GOx/CNT/PBG/CFE at −0.3 V vs. SCE to successive injections glucose in 0.1 M NaPBS pH 7.0 (air saturated) and b) the corresponding calibration plot; inset in a) CV-s recorded before and after successive injections of glucose.

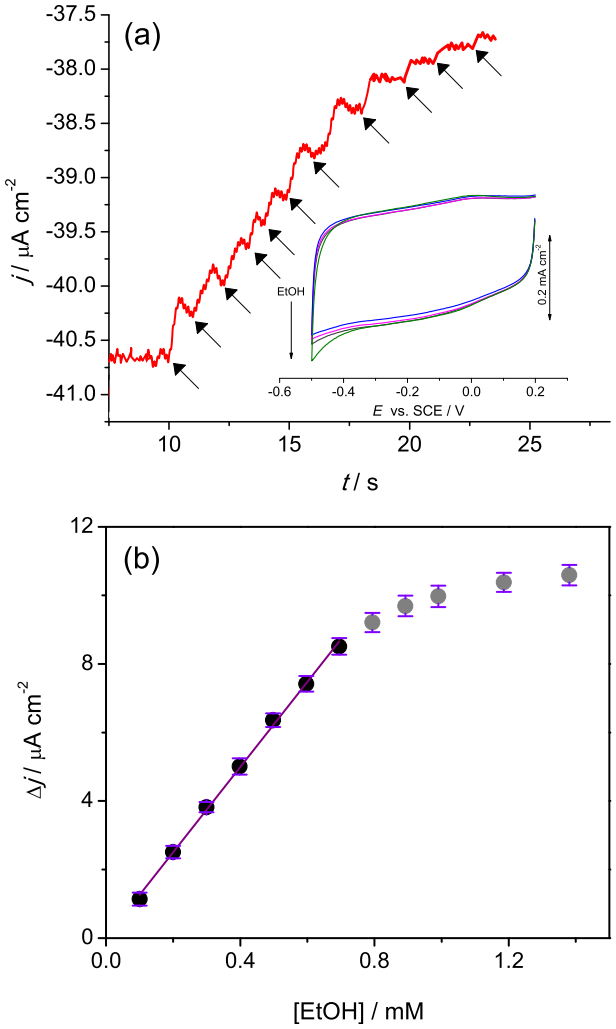

Fig. 4. a) Amperometric response of AlcOx/CNT/PBG/CFE at −0.1 V vs. SCE to successive injections of ethanol in 0.1 M NaPBS pH 8.5 (air saturated) at and b) the corresponding calibration plot; inset in a) CV-s recorded before and after successive injections of ethanol.

electron acceptor for the enzyme cofactor, and is afterwards oxidized at the electrode.

For glucose detection, calibration plots were obtained for both enzyme electrodes in 0.1 M NaPBS pH 7.0 at −0.3 V vs. SCE. A typical amperometric signal is shown in Fig. 3a with the corresponding calibration plot, with a linear range from 0.05 mM to 1.25 mM, above this value both CNT/PEDOT/CFE and CNT/PBG/CFE biosensors reaching saturation.

In the case of ethanol determination, the best experimental conditions chosen for the two enzyme electrodes were different in the light of the optimisation studies reported in Section 3.2.1. For CNT/PEDOT/CFE, the supporting electrolyte was 0.1 NaPBS pH 7.0 and the working potential was −0.3 V vs. SCE, while for CNT/PBG/CFE the supporting electrolyte was 0.1 M NaPBS pH 8.5 and the working potential was −0.1 V vs. SCE. Fig. 4a shows the amperometric signal for AlcOx/CNT/PBG/CFE, which increases continuously for consecutive additions of ethanol solution, with not very distinguishable current steps, similar to the AlcOx biosensor in Ref. [31]. The corresponding calibration plot (Fig. 4b) shows linearity in the interval 0.1–0.7 mM, before reaching saturation.

Table 2 shows sensitivities and detection limits for all the biosensors tested. As observed by comparing the sensitivity values from Table 2, GOx and AlcOx biosensor sensitivities evaluated the first day (after 4 h of drying) increase significantly, almost by a factor of 2 in the case of GOx biosensors, on the second day (after storage at 4 °C in the buffer electrolyte), probably due to hydration and rearrangement of the enzyme in the enzymatic layer.

As expected, GOx biosensors based on CNT/polymer modified electrodes show higher sensitivity values and lower LOD with respect to the biosensor without CNT/polymer. Sensitivities were very similar for both CNT/PEDOT/CFE and CNT/PBG/CFE, being 42 ± 2 (RSD = 5.5%, $n = 3$) and 44 ± 3 µA cm^{-2} mM^{-1} (RSD = 6.4%, $n = 3$), respectively, but the LOD was lower for the PBG containing biosensor, having the value of 13 ± 1 µM.

The enzyme AlcOx did not show any electronic communication with the bare CFE substrate, no response towards ethanol being recorded. Both CNT/PEDOT and CNT/PBG allowed electrons to be shuttled between the AlcOx cofactor and the electrode substrate, the biosensor based on CNT/PBG/CFE exhibiting the best analytical properties, with a sensitivity of 12 ± 1 µA cm^{-2} mM^{-1} (RSD = 5.6%, $n = 3$) and a lower LOD of 29 µM.

These results are in agreement with those obtained for the detection of hydrogen peroxide [18], where the electrode with PBG shows the higher sensitivity and lower detection limit, probably due to its redox nature, which can amplify the signal. Moreover, biosensors based on both polymer and CNT had higher sensitivities

Table 2
Sensitivities and detection limits obtained at glucose and ethanol biosensors under the optimized experimental conditions.

Electrode	S/(μA cm^{-2} mM^{-1})		LOD/μM
	First day	Second day	
GOx/CFE	$0.44 \pm 0.00_4$	$0.43 \pm 0.00_1$	105
GOx/CNT/PEDOT/CFE	23 ± 2	42 ± 2	37
GOx/CNT/PBG/CFE	24 ± 1	44 ± 3	13
AlcOx/CFE	–	–	–
AlcOx/CNT/PEDOT/CFE	4.4 ± 0.2	9.3 ± 0.6	70
AlcOx/CNT/PBG/CFE	9.2 ± 0.5	12 ± 1	29
AlcDH/CFE	–	–	–
AlcDH/CNT/PEDOT/CFE	2.7 ± 0.2	2.7 ± 0.2	100
AlcDH/CNT/PBG/CFE	4.1 ± 0.03	4.1 ± 0.2	100

than biosensors with only one component. As also observed in Ref. [18], the CNT play a major role in improving biosensor performance.

Relative standard deviations were lower than 6.5% for all the developed and tested biosensors, demonstrating their reliability for both biosensor construction and application as analytical tools.

Stability was also evaluated, testing electrodes, by recording a 5 point calibration plot, three times a week for five weeks. GOx electrodes were stable for the whole 40-day period, maintaining 90% of the initial sensitivity, while AlcOx electrodes showed a decrease in sensitivity of 20% after 5 days, after 14 days of storage the biosensor response falling to zero.

3.4. AlcDH biosensors

AlcDH biosensors are NAD^+ dependent, so that the applied potential was chosen after evaluating the oxidation potential of NADH at both CNT/PBG/CFE and CNT/PEDOT/CFE, using CV and DPV, and results are displayed in Fig. 5. As observed in Fig. 5a, an irreversible peak is observed at both CNT/PBG/CFE and CNT/PEDOT/CFE in the presence of 1.0 mM NADH, located at 0.48 and 0.43 V vs. SCE at CNT/PBG/CFE and CNT/PEDOT/CFE, respectively. The peak is better defined in DPV as shown in Fig. 5b, at 0.33 V vs. SCE, similar for both electrode architectures. Thus, the fixed potential amperometric responses of the AlcDH biosensors were tested at applied potentials of 0.2, 0.3 and 0.4 V vs. SCE, in 0.1 M NaPBS pH 7.0 + 3.0 mM NAD^+, the response increasing with the applied potential. However, in order to avoid a high potential which could compromise selectivity owing to the oxidation of interferent species in complex matrices, a potential of 0.3 V vs. SCE was chosen to evaluate the AlcDH biosensors.

The effect of solution pH was also evaluated, for several pH values between 7.0 and 9.0, *i.e.* 7.0, 8.0, 8.6 and 9.0, the maximum response of the biosensor to 0.4 mM ethanol being achieved for pH 8.6. Calibration plots of measurements made in pH 7.0 and 8.6 buffer solution are shown in Fig. 6, and it was observed that the biosensor responses were very different. Linear ranges were between 0.2 and 1.8 mM, and sensitivities were 4.1 and 2.7 μA cm^{-2} mM^{-1}, at the optimum pH 8.6, while at pH 7.0 the linear ranges were broader between 0.5 and 10 mM, and sensitivities were lower, of 0.6 and 0.3 μA cm^{-2} mM^{-1}, recorded, for the PBG and PEDOT containing AlcDH biosensors, respectively. K_m^{app} values, estimated as the concentration corresponding to half of the maximum Δj in the calibration plots, also varied being smaller in pH 8.6 solution, of ≈3 mM, while in pH 7.0 solution, it was much higher at ≈13 mM. The analytical parameters of AlcDH biosensors based on both electrode architectures are displayed in Table 2,

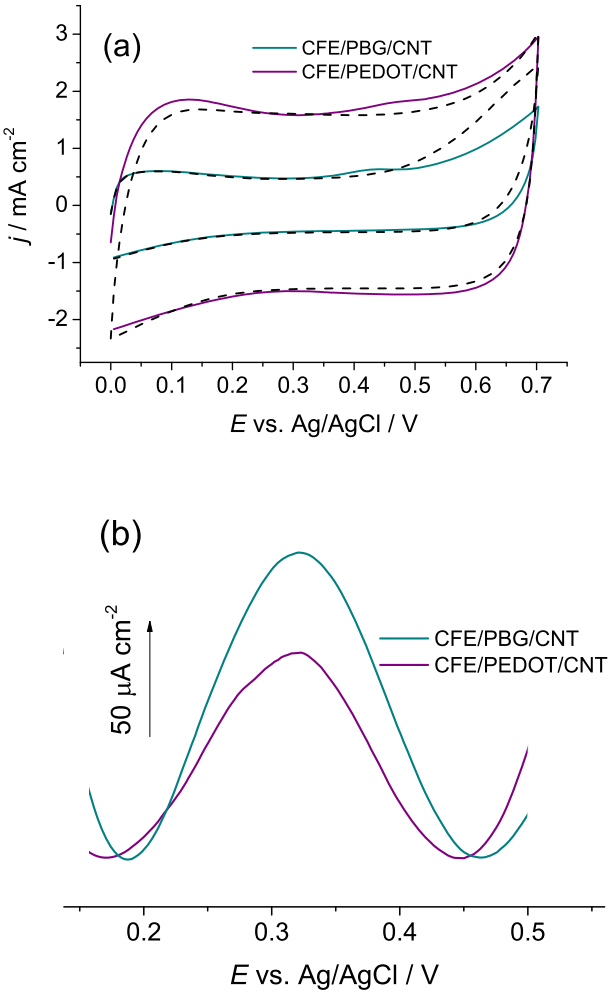

Fig. 5. a) CV and b) DPV recorded at CNT/PBG/CFE and CNT/PEDOT/CFE in 0.1 M NaPBS pH 7.0 after addition of 1.0 mM NADH.

where it can be seen that the biosensor based on CNT/PBF/CFE showed better analytical parameters, thus being chosen for interference studies and applications. Similarly to the oxidase biosensors, the stability of the AlcDH biosensor was also tested. It was observed that the initial sensitivity decreased 15% during the first 5 days, and 50% after 14 days of storage, which represents a significantly better stability in relation to AlcOx, for which zero response was recorded after 14 days.

3.5. Selectivity studies

The amperometric responses of the CNT/PBG/CFE-based biosensors were evaluated in the presence of acetic acid, ascorbic acid, tartaric acid, dopamine, catechol, fructose and uric acid, which are electroactive compounds possibly interfering in the determination of glucose or ethanol in real samples (Fig. 7). The applied potentials were −0.3 V for glucose and −0.1 and +0.3 V vs. SCE, for ethanol at AlcOx and AlcDH, respectively. Glucose or ethanol was injected before and after addition of the interfering compounds, using the ratio 2:1 interferent:substrate in these experiments.

For the oxidase biosensors, which operate at negative potentials, the tested compounds did not interfere significantly with the enzyme substrate response, a slight decrease of 8% in glucose response in the presence of all the interfering compounds being observed.

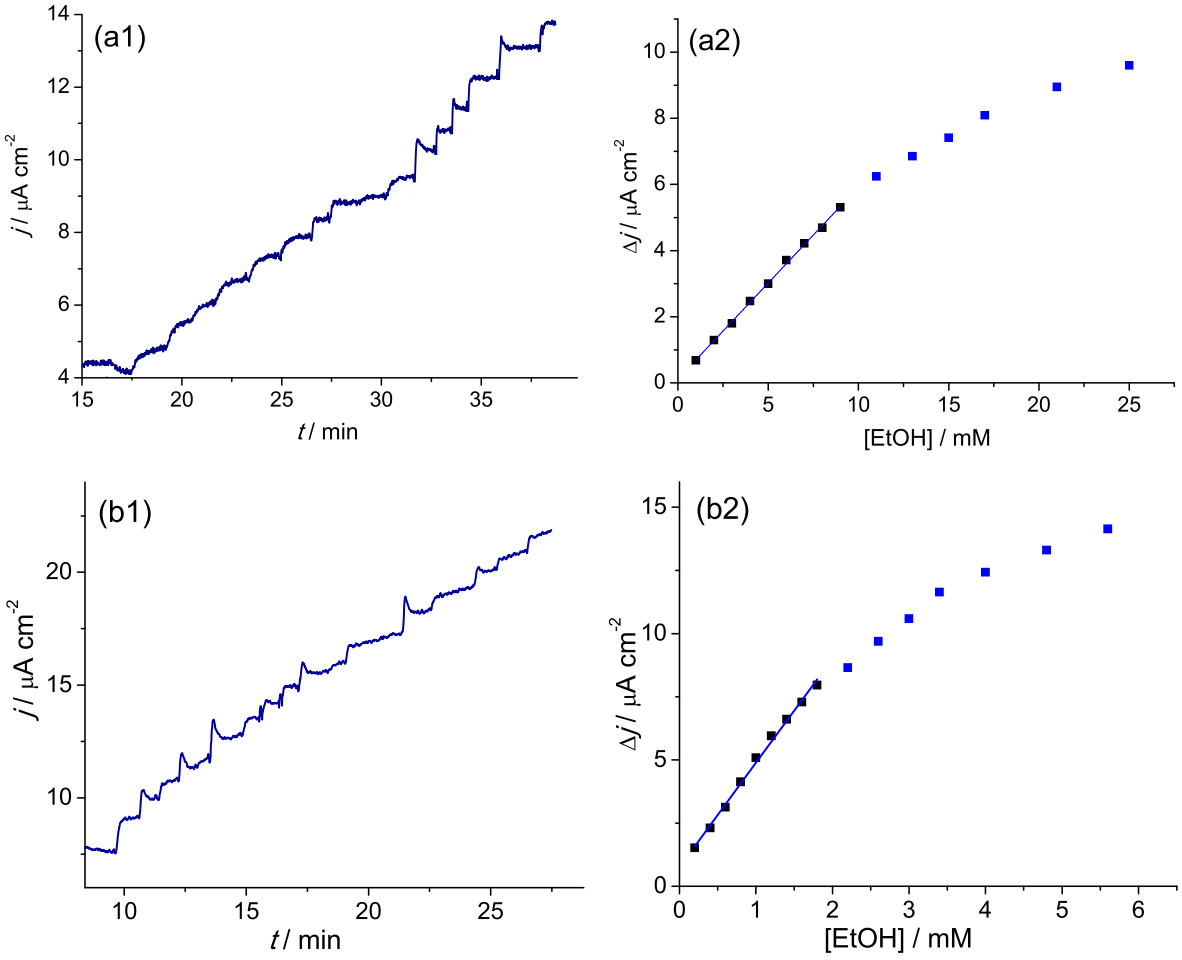

Fig. 6. 1) Amperometric response of AlcDH/CNT/PBG/CFE at +0.3 V vs. SCE in (a) pH 7.0 and (b) pH 8.6 buffer solution to successive injections of ethanol in 0.1 M NaPBS + 3.0 mM NAD^+ (air saturated) and 2) corresponding calibration plots.

The AlcDH biosensor sensed ascorbic acid and dopamine, its response in the presence of these electroactive species being of 129% and 131%, respectively, which limits its applicability for ethanol detection in beverages with small or no ascorbate, as for example beers, wines, and distilled alcoholic beverages. The response to ethanol in the presence of interferents at the AlcDH biosensor was 94%, after the injection of all other possible interfering compounds.

These results demonstrate the applicability of the developed biosensors for the determination of glucose or ethanol in real complex matrices.

3.6. Comparison with other biosensors

In Tables 3 and 4 a comparison with recent (2011–2015) literature data of modified electrodes for glucose and ethanol sensing is presented.

For glucose determination, the values of operation ranges and limits of detection are comparable with the other types of sensors, some reporting lower detection limits [37,38,40,41]. However, the sensitivity was higher, only the biosensors based on a gold nanoparticle network reported in Ref. [37] exhibiting comparable sensitivity values for the GOx monoenzymatic system and superior for the HRP-GOx bienzymatic system, respectively.

In the case of ethanol, only two other publications reported new AlcOx biosensors, with smaller or comparable sensitivities [45,46], the biosensor developed here having the advantage to operate at −0.1 V vs. SCE, which allows avoidance of the majority of interferences, as shown in Section 3.3. Several AlcDH biosensors have been reported during the past 4 years, which operate under similar experimental conditions as the one developed in this work. The majority had lower sensitivities [48–50,52,53], three comparable sensitivities [55–57] and only three higher sensitivities [47,51,54] than that reported.

3.7. Application of AlcOx and AlcDH biosensors to the determination of ethanol in alcoholic beverages

AlcOx and AlcDH biosensors based on CNT/PBG/CFE were both applied to ethanol determination in alcoholic beverages by using the standard addition method, and results were consistent with the values obtained by the linear fitting of the calibration plot, performed prior the standard addition method. The calculated values are displayed in Table 5, and as observed, with the exception of two beverages that contained ascorbate, both biosensors accurately detected the ethanol concentration in complex matrices. As expected and shown in the interference study, the AlcDH biosensor detected more than the real quantity of ethanol in sangria and vermouth, attributable to ascorbate interferences, while AlcOx biosensor detected a concentration value close to the labelled one. Results show the applicability of both biosensors, underlying the important advantage of using the AlcOx biosensor in matrices that

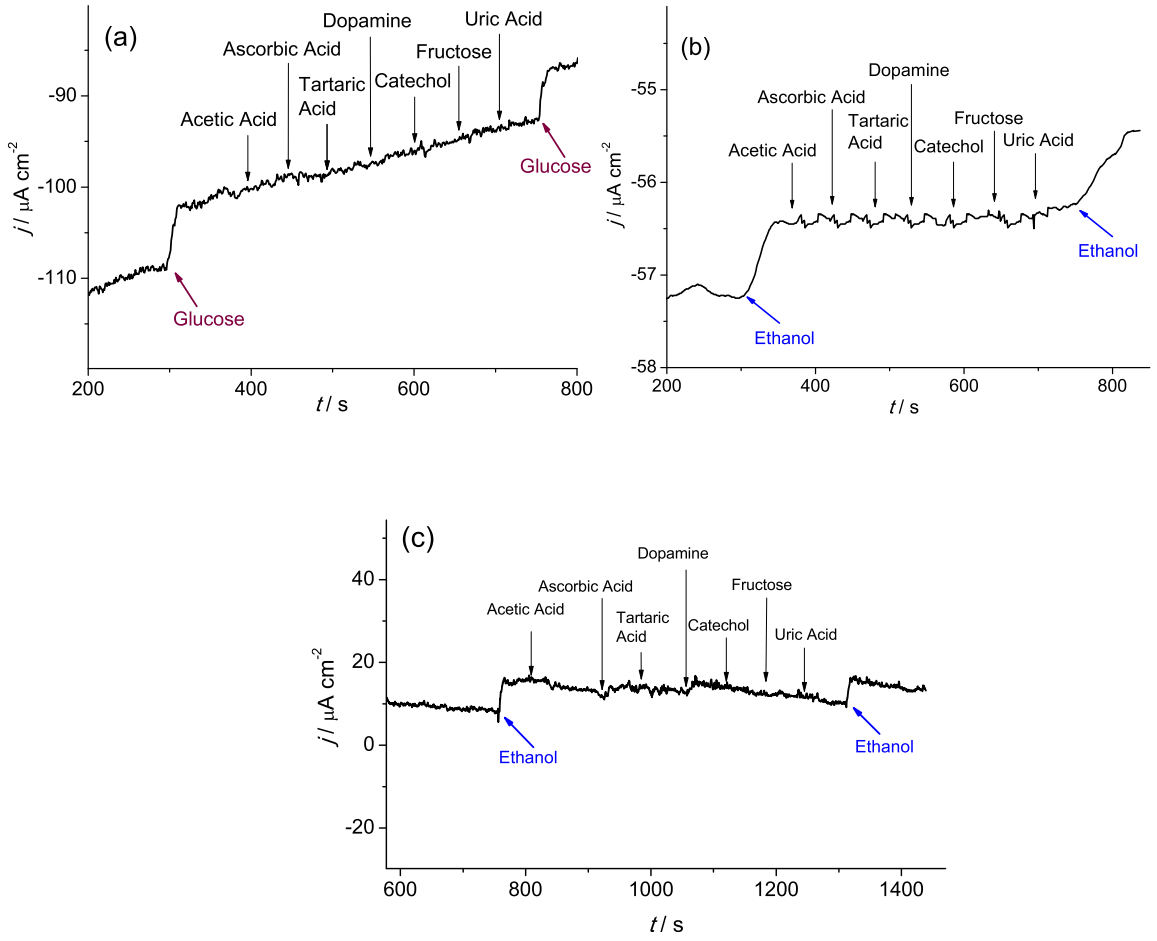

Fig. 7. Interference studies at CNT/PBG/CFE based biosensors in 0.1 M NaPBS for a) GOx (−0.3 V vs. SCE, pH 7.0), b) AlcOx (−0.1 V vs. SCE, pH 8.5) and c) AlcDH (+0.3 V vs. SCE, pH = 8.6); interferent:substrate concentration ratio 2:1.

Table 3
Performance parameters of glucose biosensors for different modified electrodes recently reported in the literature.

Electrode	Linear range/mM	S/(μA cm^{-2} mM^{-1})	LOD/μM	Potential/V	Ref.
GOx/PMB/PEDOT/GCE	0.02–1.4	31.4	7.20	−0.3	[21]
GOx/CNT/Au fibres	0.0–30.0	0.5	4.00	+0.8	[37]
GOx-Cys/SG/AuNPs/ITO	0.05–4.0	55.7	0.02	−0.1	[38]
GOx-HRP-Cys/SG/AuNPs/ITO	0.02–3.2	132	0.01	−0.1	[38]
GOx/PtNPs/PANI-CNT/GCE	0.003–8.2	16.1	1.0	+0.6	[39]
GOx/GO/Chit-Fc/GCE	0.02–6.8	10.0	7.6	+0.3	[40]
GOx-G/PDA/AuE	0.0–4.0	28.4	0.1	+0.7	[41]
GOx/MCPGNs/GCE	0.00005–1.0	12.5	0.005	−0.3	[42]
GOx/Chit-PB-G/GCE	0.025–3.2	0.9	11.0	−0.2	[43]
GOnanofibers/GOx/Chit/PVA/Pt	0.005–3.5	12.0	5.0	+0.4	[44]
GOx/CNT/PBG/CFE	0.013–1.5	57	13	−0.3	This work

PMB – poly(methylene blue); Cys – cysteine; SG – silica sol–gel; AuNPs – Au nanoparticles; ITO – indium thin oxide; HRP – horseradish peroxidase; PtNPs – Pt nanoparticles; PANI – polyaniline; GO – graphene oxide; Chit-Fc – chitosan-ferrocene; G – graphene; PDA – polydopamine; AuE – Au electrode; PDA – polydopamine; MCPGNs – metal coordination polymer-graphene nanosheets; Chit-PB-G – chitosan-Prussian Blue-graphene; PVA – polyvinylalcohol.

possibly contain ascorbate.

4. Conclusions

Poly(brilliant green) and PEDOT as polymer films together with carbon nanotubes on carbon film electrode substrates both demonstrate good performance as redox/conducting polymers for the design of new biosensors for glucose and ethanol. For application as biosensors, the electrolyte pH and the operating potential were optimized. For the PBG-based biosensor, the best operating potential was found to be lower than that of the PEDOT-based biosensor, showing the importance of the redox polymer in the electrode process. The influence of oxygen was also studied, showing a significant decrease in sensitivity for glucose detection under nitrogen atmosphere whereas there is a slight increase for ethanol determination. The electrode with the redox polymer PBG, showed the best performance for both glucose and ethanol determination, with good sensitivity and detection limits, including in comparison with other biosensors in the recent literature. Selectivity studies showed the applicability of CNT/PBG based biosensors

Table 4
Performance parameters of ethanol sensors for different modified electrodes recently reported in the literature.

Electrode	Linear range/μM	S/(μA cm^{-2} mM^{-1})	LOD/μM	Potential/V	Ref.
AlcOx/SPPBCE	0.05–0.5	14.3	20.0	+0.4	[45]
AlcOx/SPFcCE	0.1–1.0	6.0			
AlcOx/SPCPCE	0.05–1.0	9.6			
AlcOx/PEI/Nafion/CNT/Au	0.008–0.05	1.0	5.0	+0.3	[46]
AlcOx/CNT/PBG/CFE	0.05–0.7	14	29	−0.1	This work
AlcDH/PCV/CNT/PGE	0.001–0.3	146	1.3	+0.2	[47]
AlcDH-NAD$^+$-GrPE	1.0–10.0	0.3	56	+0.5	[48]
AlcDH/PDAMS/PtNP/Pt AlcDH/PMDUS/PtNP/Pt	0.9–18.0	0.2	452	+0.3	[49]
	1.4–30.0	0.3	547		
AlcDH-NAD$^+$/AuNP/PDRGO/GCE	Up to 3.0	0.1	88	+0.3	[50]
AlcDH/G-AuNR/GCE	0.005–0.4	102	1.5	+0.4	[51]
AlcDH/MB/GMC/SPCE	0.5–15.0	1.0	80	−0.2	[52]
AlcDH/PTH-ERGO/GCE	0.05–1.0	2.8	0.3	+0.4	[53]
AlcDH/{G-COO$^-$/CNT-NH$_3^+$}5/PSS$^-$/PDDA$^+$/Gr	0.03–0.2	83	25	+0.1	[54]
AlcDH/CNT-IL/GCE	5–60	13	–	+0.1	[55]
AlcDH/PDDA-CNT/GCE	–	13	–	+0.5	[56]
AlcDH/PEDOT/AuNP/SPCE	0.005–0.1	5.8	2.0	+0.3	[57]
AlcDH/CNT/PBG/CFE	0.2–2.0	4.1	100	+0.3	This work

SPPBCE, SPFcCE SPCPCE – screen printed carbon electrodes modified with Prussian blue, ferrocyanide, Co-phthalocyanine; PEI – Polyethylenimine; PCV – pyrocatechol violet; PGE – pencil graphite electrode; PDAMS – polydiallylmethylsilane; PDMUS – polymethyldiundecenylsilane; PtNP – Pt nanoparticles; AuNP – Au nanoparticles; PDRGO – polydopamine modified reduced graphene oxides; G-AuNR – graphene-Au nanorods; MB – Meldola's Blue; GMC-graphitized mesoporous carbons; SPCE – screen printed carbon electrodes; PTH polythionine; ERGO – electroreduced graphene oxide; PDDA – poly(diallyldimethylammoniumchloride); PSS – polystyrenesulphonate; IL – ionic liquid.

Table 5
Application of AlcOx and AlcDH biosensors based on CNT/PBG/CFE for determination of ethanol in alcoholic beverages.

Sample	Labelled/%	Found/%		Apparent recovery/%	
		AlcOx	AlcDH	AlcOx	AlcDH
White wine 1	10.0	9.5 ± 0.1	10.4 ± 0.0	95.0	104.0
White wine 1	10.5	10.3 ± 0.1	10.5 ± 0.0	98.1	100.0
Red wine 1	13.0	13.2 ± 0.1	13.4 ± 0.1	101.5	103.1
Red wine 2	14.0	14.6 ± 0.2	14.5 ± 0.2	104.3	103.6
Red vermouth	14.0	13.8 ± 0.1	17.3 ± 0.2	98.6	123.6
Sangria	7.0	7.4 ± 0.5	10.2 ± 0.5	105.7	145.7
Vodka	40.0	39.0 ± 1.3	40.3 ± 1.5	97.5	100.8
Whisky	40.0	39.2 ± 1.5	40.3 ± 1.0	98.0	100.8

to real samples, which was exemplified by the accurate detection of ethanol in several alcoholic beverages, which augurs well for the future application of these biosensor platforms.

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