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Highly sensitive amperometric enzyme biosensor for detection of superoxide based on conducting polymer/CNT modified electrodes and superoxide dismutase

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ABSTRACT

A novel highly sensitive electrochemical biosensor for the direct determination of the reactive oxygen species superoxide, $O_2^{\bullet-}$, using superoxide dismutase has been developed, by incorporating multiwalled carbon nanotubes (MWCNT) together with the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) in different configurations. After characterisation, the experimental conditions have been optimized and the analytical parameters of superoxide dismutase biosensors based on PEDOT/CNT or CNT/PEDOT modified glassy carbon electrodes, as well as those with one component only (MWCNT, PEDOT), have been determined. The biosensor with CNT on top of PEDOT presented the best analytical performance due to synergistic effects, with fast, and selective response to $O_2^{\bullet-}$, a high sensitivity of ~1115 μA cm^{-2} mM^{-1} and a low detection limit of 1 μM, and was applied to the determination of the antioxidant capacity of beverages. The biosensor exhibited outstanding stability over a period of 2 months, with a slight increase in the initial sensitivity.

1. Introduction

Reactive oxygen species (ROS), which include hydroxyl radicals, superoxide radicals and hydrogen peroxide, mediate chemical reactions that are involved in various pathogenic processes, being the initiators of the oxidative process and playing an important role in the development of certain diseases, including cancer and neurodegenerative diseases [1,2]. Organisms produce antioxidants, such as catalase, superoxide dismutase, and uric acid, as part of a defence system against ROS-mediated cellular injury. Recently, exogenous antioxidants introduced through diet or by other means are becoming popular. In this context, developing sensitive and selective superoxide, $O_2^{\bullet-}$, sensors has received increasing attention in recent years, with application for determining $O_2^{\bullet-}$ in healthy and diseased tissues, and also the antioxidant activity of food, beverages and pharmaceutical formulations.

Different technologies have been used for $O_2^{\bullet-}$ anion determination, such as chemiluminescence [3], fluorimetry [4], electron spin resonance [5], conductometric analysis [6], mass spectrometry [7] and spectrophotometry [8,9]. Recently, electrochemical methods have been investigated because of the possibility for direct, real-time measurements and capability of in vivo detection [10]. An electrochemical sensor has been developed based on the dismutation of $O_2^{\bullet-}$ by Mn^{2+}/MnO^{2+} [11] as well as several biosensors which rely on cytochrome c [12–14], or the enzyme superoxide dismutase (SOD) [15–17], the last having the advantage of higher specificity [18,19]. SOD converts $O_2^{\bullet-}$ to O_2 and H_2O_2 via a cyclic oxidation-reduction electron-transfer mechanism [17]. Due to the excellent selectivity and high sensitivity of SOD, third generation $O_2^{\bullet-}$ biosensors based on the direct electron transfer of SOD have been achieved, despite the difficult electronic communication between the enzyme and the electrode, since regardless of the immobilization method, the redox centre of SOD is buried deeply inside the proteic shell [20].

In order to improve electron transfer and prolong the life of enzyme biosensors, the use of nanomaterials as carriers/hosts to immobilize enzymes is promising [21]. Nanomaterials including conducting polymers, redox polymers, metallic/polymeric nanoparticles and carbon nanotubes (CNT) are ideal for the immobilization of enzymes due to their large surface area, good bio-

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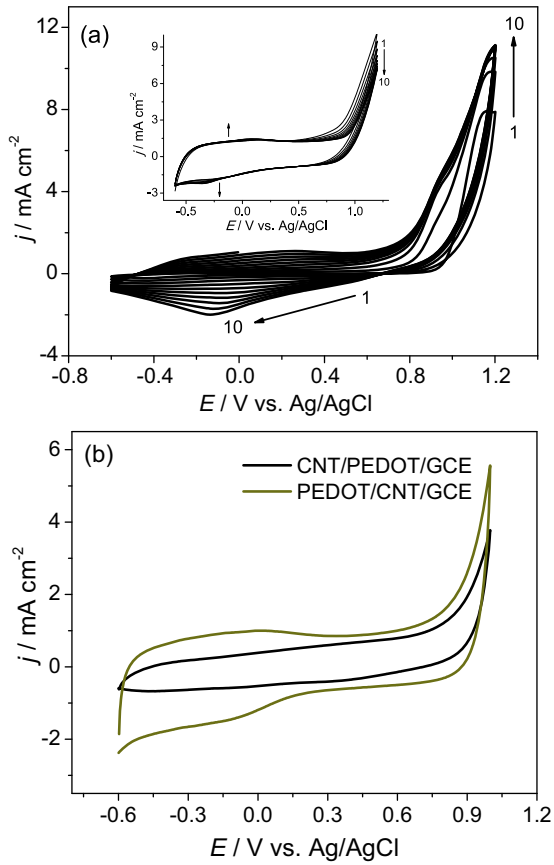


Fig. 1. (a) CVs recorded during the electropolymerisation EDOT on GCE (inset on CNT/GCE) and (b) CVs of GCE modified with CNT and PEDOT in different configurations in 0.1 M KCl, $\nu = 50\,mV\,s^{-1}$.

compatibility and excellent stability. The combination of CNT with polymers can give rise to polymer-CNT hybrids with higher electronic conductivity and a three-dimensional nanostructure with a large electroactive area, which can lead to biosensors with superior performance [22–26].

The combination of CNT with conducting/redox polymers is beneficial, since CNT can improve the electrical conductivity and mechanical strength of the resulting polymer-CNT hybrids, apart from the possibility of presenting superior analytical performances due to synergistic effects. Redox and conducting polymers, such as poly(methylene blue) (PMB) and poly(3,4-ethylenedioxythiophene) (PEDOT) have both been recently and successfully used in our group together with multiwalled CNT (MWCNT) for the development of new (bio)sensors, e.g. [23].

The particular advantages of using PEDOT in sensing are, apart from its high electrical conductivity, that it can be used over a wide pH range, it is highly stable, is easy to prepare by electropolymerisation in aqueous solution and can be made into composite materials [27]. Although there are many examples of the use of PEDOT and CNT together for sensing purposes, to our knowledge none of these investigate the measurement of superoxide by SOD biosensors.

In the present work, electrochemical $O_2^{\bullet -}$ biosensors were developed involving immobilization of SOD onto a glassy carbon electrode (GCE) modified with poly(3,4-ethylenedioxythiophene) (PEDOT) together with MWCNT in a chitosan dispersion in different configurations. Initial experiments carried out with PMB and CNT, as in [23], showed that in this case PEDOT and CNT would lead to better analytical parameters, so PMB-CNT based biosensors were not studied further.

Scanning electron microscopy (SEM) enabled the characterization of the PEDOT/MWCNT, MWCNT/PEDOT and PEDOT surface morphology. The modified electrodes with different architectures were characterized electrochemically by cyclic voltammetry and electrochemical impedance spectroscopy (EIS). Experimental conditions for SOD biosensors, after immobilisation of SOD by cross-linking, were optimized and the performance of the developed SOD biosensors was tested by fixed potential amperometric measurements, in order to determine the best combination of polymer and CNT for further application. Interferences and stability studies were performed using the biosensor with best performance, and the antioxidant capacity of some food samples was determined utilizing the SOD biosensor.

2. Experimental

2.1. Reagents and solutions

The enzyme bovine Cu-Zn superoxide dismutase (SOD), potassium dioxide, chitosan (low molecular weight), 3,4-ethylenedioxythiophene (EDOT), glutaraldehyde (GA), potassium superoxide (KO_2), monobasic and dibasic sodium phosphate, ascorbic acid, citric acid, xanthine, tyrosine, H_2O_2, sodium chloride, sodium styrene sulfonate (NaSS) were from Sigma-Aldrich. The MWCNT were from NanoLab, USA.

For electrochemical experiments, the supporting electrolyte was sodium phosphate buffer saline (NaPBS) (0.1 M phosphate buffer + 0.05 M NaCl, pH = 7.0). Polymerisation of EDOT was performed in 0.1 M NaSS containing 10 mM EDOT.

Standard solutions of KO_2 were prepared in dimethylsulphoxide, and were used as the source of superoxide ions.

Millipore Milli-Q nanopure water (resistivity $\geq$ 18 MΩ cm) was used for the preparation of all solutions. All experiments were performed at room temperature (25 ± 1 °C).

2.2. Instrumentation

Electrochemical experiments were performed with a computer-controlled μ-Autolab type I potentiostat-galvanostat with GPES software (Metrohm-Autolab, Utrecht, Netherlands). EIS experiments were carried out with a potentiostat/galvanostat/ZRA, (Gamry Instruments, Reference 600). A sinusoidal voltage perturbation of 10 mV rms amplitude was applied over the frequency range 65 kHz–0.1 Hz, with 10 frequency values per decade.

A conventional three-electrode cell was used, containing a modified GCE as working electrode, a platinum wire as counter electrode and a Ag/AgCl (3 M KCl) electrode as reference.

The pH-measurements were carried out with a CRISON 2001 micro pH-meter (Crison Instruments SA, Barcelona, Spain) at room temperature.

SEM (JEOL, JSM-5310, Japan) was used to characterize the morphology of the different polymer/CNT developed in this work, which were deposited on indium tin oxide (ITO, resistivity 30–60 Ω/sq, Sigma-Aldrich, Madrid, Spain) electrodes, for SEM imaging.

2.3. Preparation of modified electrodes and biosensors

Functionalised MWCNT were obtained by treating the MWCNT in 3 M HNO_3 under continuous stirring for 24 h, afterwards being thoroughly washed with Milli-Q water and dried at 60 °C for 12 h.

Two types of GCE modified by MWCNT and the conducting polymer PEDOT were prepared: PEDOT/CNT/GCE and CNT/PEDOT/GCE.

For MWCNT deposition, a suspension of 0.1% MWCNT was prepared in 1% (w/v) chitosan dissolved in 1% (v/v) acetic acid. The solution was sonicated for 4 h and used for drop casting on the GCE

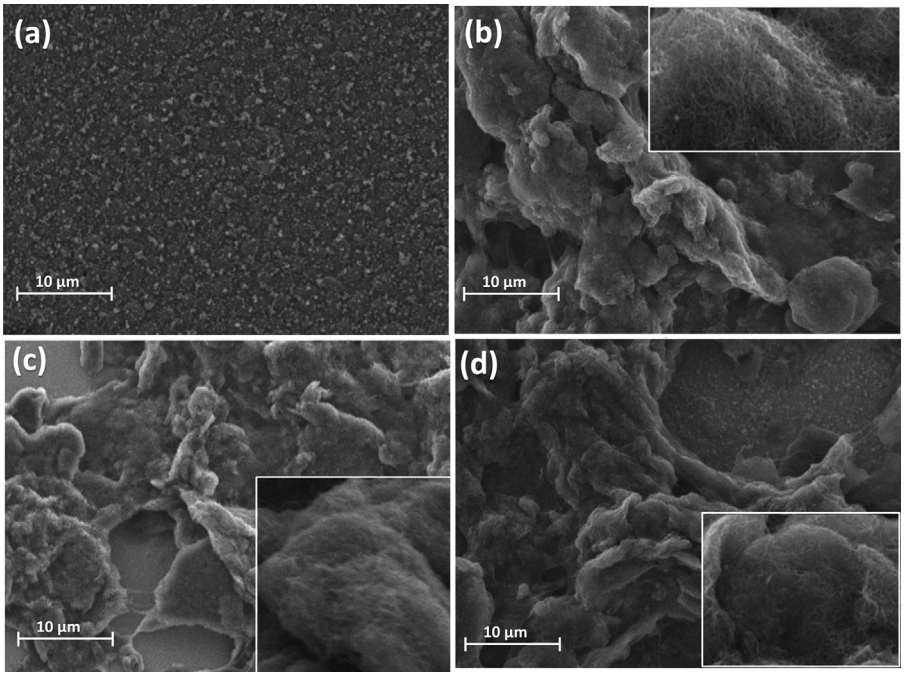
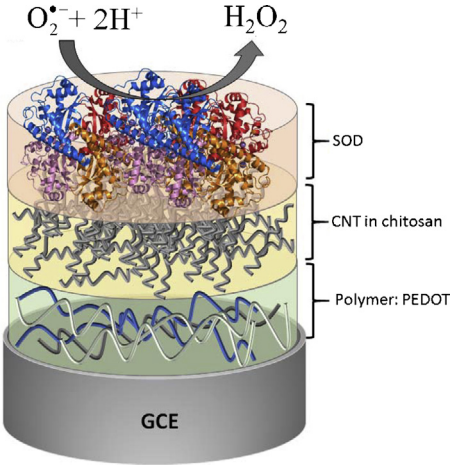


Fig. 2. SEM images of (a) PEDOT, (b) CNT, (c) PEDOT/CNT, (d) CNT/PEDOT; insets are 5 times magnifications.

Table 1
Equivalent circuit element values obtained by fitting the spectra from Fig. 3 a).

Modified electrode	$Z_w/\Omega\,s^{0.5}\,cm^2$	τ/ms	α_1	CPE/mF cm^{-2} s$^{\alpha-1}$	α_2
CNT/PEDOT/GCE	700	60	0.45	6.4	0.95
PEDOT/CNT/GCE	33	7.0	0.25	19.1	0.95



Scheme 1. Schematic representation of SOD biosensors based on PEDOT and CNT.

with 1 µL per 0.01 cm^2. The modified electrodes were left to dry at room temperature for 1 h, being dried afterwards under N_2 stream, before further use.

PEDOT was deposited on GCE or on CNT/GCE during 10 scans at 50 mV s^{-1} between −0.6 and 1.2 V vs. Ag/AgCl in 10 mM EDOT + 0.1 M NaSS [23].

For the preparation of biosensors, enzyme solutions containing bovine serum albumin (BSA) were prepared, 0.1% SOD (2698 units per mg solid) + 2% BSA. The enzyme solution was mixed with a 2.5% GA solution, in a volume ratio of 2:1 enzyme:GA, and 1 µL per 0.01 cm^2 of this mixture was dropped onto the GCE, and allowed to dry for at least 4 h in ambient air. Biosensors were kept in 0.1 M NaPBS pH 7.0, at 4 °C.

2.4. Preparation of samples of beverages for determination of the relative antioxidant capacity

Samples of red and white wines, port wines and berry juice were used directly without any prior dilution or purification. For the determination of relative antioxidant capacity (RAC), two fixed potential chronoamperometric measurements were performed for five injections of superoxide, corresponding to final concentration increments of 40 µM KO_2 in the cell, one without and after addition of 8 µL of the antioxidant sample in a 2 mL volume of solution in the electrochemical cell. Following the injection of superoxide, the disproportionation reaction of the superoxide radical, catalysed by SOD, produces oxygen and hydrogen peroxide, the latter being reduced at a negative potential and giving rise to a cathodic change in current. The determination of RAC was based on the decrease of this signal, since the antioxidant sample reacts with the injected superoxide, decreasing its concentration in solution, and thence the enzymatically produced hydrogen peroxide decreasing the amperometric biosensor signal corresponding to hydrogen peroxide reduction.

3. Results and discussion

3.1. Electrode modification by electropolymerisation of EDOT and CNT deposition

Two polymer-CNT hybrids were formed by using MWCNT together with the conducting polymer PEDOT. The polymerisation

of the monomer EDOT was done after or prior to the modification of GCE with MWCNT.

Electropolymerisation profiles of EDOT onto GCE and MWCNT/GCE are shown in Fig. 1a, in which can be seen the formation of radical cations at positive potentials, higher than 1.0 V vs. Ag/AgCl, and the increase in the capacitive currents upon cycling. The polymerisation of EDOT on bare GCE leads to a significant increase in the capacitive currents with each cycle, which was less visible on top of CNT, since MWCNT/GCE already possesses high capacitive currents, as also observed in [25].

3.2. Characterization of polymer-CNT modified electrodes

3.2.1. Scanning electron microscopy characterization

SEM images of ITO electrodes modified with the two polymers and MWCNT alone, as well as with their combinations in different architectures are presented in Fig. 2. The polymer PEDOT presents a rough/porous structure, with globular structures between 100 to 450 nm in diameter, Fig. 2a. The SEM images of CNT in chitosan, Fig. 2b, revealed their typical tubular structures, with diameters between 19 and 38 nm, which correspond with the values declared by the producer (30 ± 10 nm). The CNT curvy tubular structures are observed in the SEM images of polymer-CNT hybrids, polymer/CNT and CNT/polymer, similar to what was observed in [28], where the chitosan concentration was much lower. When PEDOT polymer is formed on the chitosan layer containing CNT, PEDOT is deposited in between the pores of chitosan CNT material, filling those holes and leading to a smoother PEDOT/CNT surface (Fig. 2c). On the other hand, when PEDOT is deposited on GCE, prior to CNT modification, the rough/porous PEDOT structure is visible beneath the CNT network, in the large pores of the chitosan CNT film, see Fig. 2d.

3.2.2. Electrochemical characterization

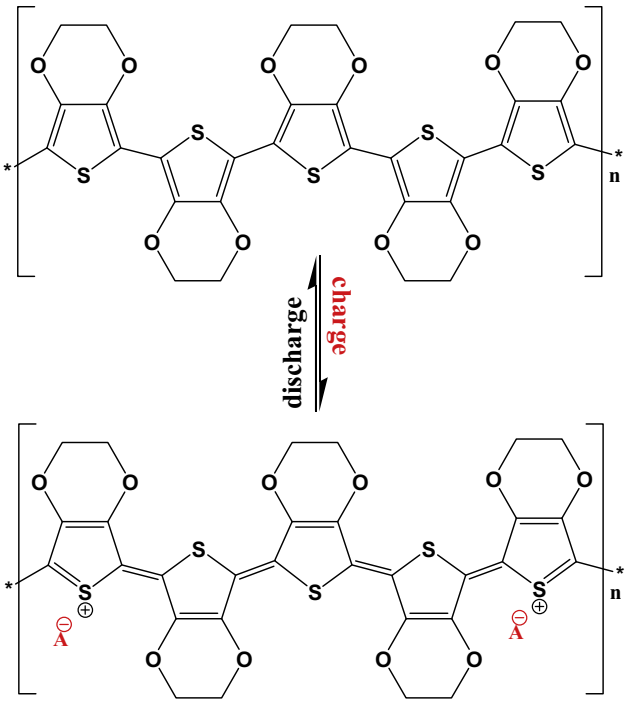
Electrode modification is an important step in the biosensor development, because the surface state has a strong effect on the sensing properties of the biosensor. The GCE modifications were followed by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements.

3.2.2.1. Cyclic voltammetry. Fig. 1b shows CVs of PEDOT/CNT and CNT/PEDOT modified electrodes. It was observed that, even though the polymer formed better on bare GCE, the peak currents of PEDOT/CNT/GCE are higher than those of CNT/PEDOT/GCE. This is probably due to a more open structure of the polymer-CNT hybrid, when PEDOT is formed on top of the CNT, when the polymer can be formed inside the CNT 3D network, allowing easier access/expulsion of the counter-ions in solution during the process of doping/dedoping [29], as presented in Scheme 1.

Analysis of the influence of scan rate for the PEDOT-CNT modified electrodes reveals a diffusion-controlled electrochemical process, for scan rates between 10 and 200 mV s^{-1} (results not shown), demonstrating that counter-ion diffusion is the rate-determining step of the redox process (Scheme 2).

3.2.2.2. Electrochemical impedance spectroscopy. EIS was used to study the physical and interfacial properties of the polymer-CNT modified electrodes. Spectra were recorded in 0.1 M NaPBS at −0.30 V vs. Ag/AgCl, the potential at which the biosensors will be tested. Complex plane plots recorded at the two different polymer-CNT architectures are shown in Fig. 3a, with the electrical equivalent circuits used to fit the impedance spectra in Fig. 3b. The parameter values obtained by fitting the spectra are presented in Table 1.

In the equivalent circuit, R_Ω is the uncompensated cell resistance, Z_W is the Warburg element, with $Z_W = R_W \mathrm{cth}[(\tau i\omega)\alpha](\tau i\omega)^{-\alpha}$, where $\alpha < 0.5$, with R_W the diffusional resistance and τ the diffusional time constant. CPE_{pol} is a constant phase element representing both the charge separation of the double layer and the polarization of the polymer-CNT film, defined as $CPE = [(Ci\omega)^\alpha]^{-1}$, a pure capacitor in the case of $\alpha = 1$ or as a non-ideal capacitor, due to the porosity and non-homogeneity of the surface, for $0.5 < \alpha < 1$. The value of R_Ω is between 0.2 to 0.9 kΩ. The equivalent circuit used to



Scheme 2. Representation of doping/dedoping of PEDOT polymer.

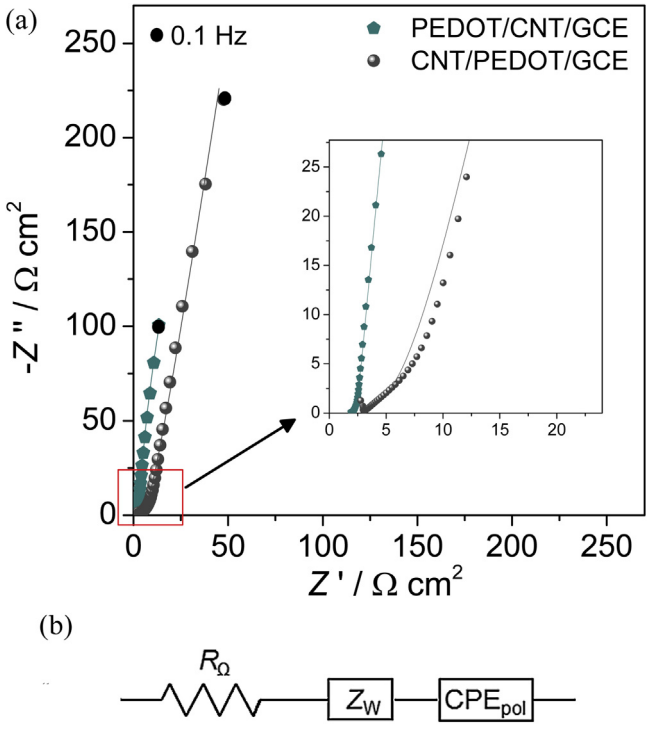


Fig. 3. (a) Complex plane impedance plots recorded at GCE modified with the PEDOT-CNT architectures in 0.1 M NaPBS pH 7.0, at −0.3 V vs. Ag/AgCl and (b) equivalent circuit used to fit the spectra.

fit the spectra was similar to that used in [25], for electrodes with similar architectures.

As previously observed, CNT have a dominant influence when placed together with polymer modifiers, especially in the low-frequency region, the polymer-CNT hybrids presenting similar spectral profiles as CNT/GCE [24,25]. In the low frequency region, the capacitance is ascribed to charge separation inside the pores of the material, which is directly influenced by the penetration of ions inside the pores, therefore being strongly influenced by the pore size and their distribution. Well-distributed pores with similar average size as the penetrating ions give rise to higher pseudo-capacitance values, pores larger than around 1 nm leading to a decrease in capacitance value according to the equation $C = \varepsilon A/d$, where A is the surface area, d is the separation between carbon and ions, and ε is the local dielectric constant of the electrolyte [30]. The presence of CNT leads to an increased CPE for PEDOT modified GCE, in both architectures, due to an increase in surface area and material porosity. It was observed that higher CPE values were obtained with polymer deposited on top of the CNT. With the polymer underneath the CNT layer, the diffusional region at high frequency was broader with higher diffusional resistance, being attributed to a more compact structure, which slows down the diffusion.

3.3. Superoxide detection

3.3.1. Effect of applied potential and dissolved oxygen

The determination of superoxide at SOD/GCE, SOD/CNT/GCE, SOD/CNT/PEDOT/GCE was performed using fixed potential amperometry in neutral solution (0.1 M NaPBS pH 7.0). The effect of applied potential on the amperometric responses of the biosensors was evaluated by comparing the change in current following the addition by injection of 0.2 mM superoxide, at potentials from −0.4 to +0.3 V vs. Ag/AgCl. Typical responses for SOD/GCE and SOD/CNT/GCE are shown in Fig. 4a and 4b. SOD/CNT/PEDOT/GCE exhibited very similar response profiles with SOD/CNT/GCE. While the SOD/GCE only senses superoxide at more negative potentials, −0.4 and −0.3 V, the CNT-containing ones respond over a broader potential range from −0.4 to 0.1 V vs. Ag/AgCl, being more sensitive at negative potentials. Since the sensor response was already sufficiently sensitive at −0.3 V vs. Ag/AgCl, this was chosen in further experiments, in order to minimize interferences from other electroactive compounds.

The response of the developed sensors SOD/GCE and SOD/CNT/GCE in the presence of the same amounts of superoxide as a function of potential, Fig. 4a, revealed a significant increase of the change in current on incorporating CNT in the biosensor architecture.

Amperometric studies were performed at SOD/CNT/GCE, SOD/CNT/PEDOT/GCE and in solutions with different O_2 content: normal oxygen content, saturated (O_2 bubbled) and deoxygenated (N_2 bubbled). The best conditions for biosensor functioning were in deoxygenated solution, regardless of biosensor architecture, when the response was very clear and stable, also with higher sensitivity. For normal O_2 content, the current decreased upon substrate injection, but over time the current increased leading to a very difficult interpretation of the response, as exemplified in Fig. 4c. For these reasons, the changes in current were not reproducible for the same substrate concentration. When O_2 was bubbled into the solution, to ensure O_2 saturation, no response was detected upon injection of biosensor substrate. Therefore, the amperometric measurements were performed in deoxygenated buffer solution.

3.3.2. Analytical parameters of biosensor and enzymatic mechanism

The amperometric response of the developed biosensors was recorded in 0.1 M NaPBS, pH = 7.0 at −0.3 V vs. Ag/AgCl. Current

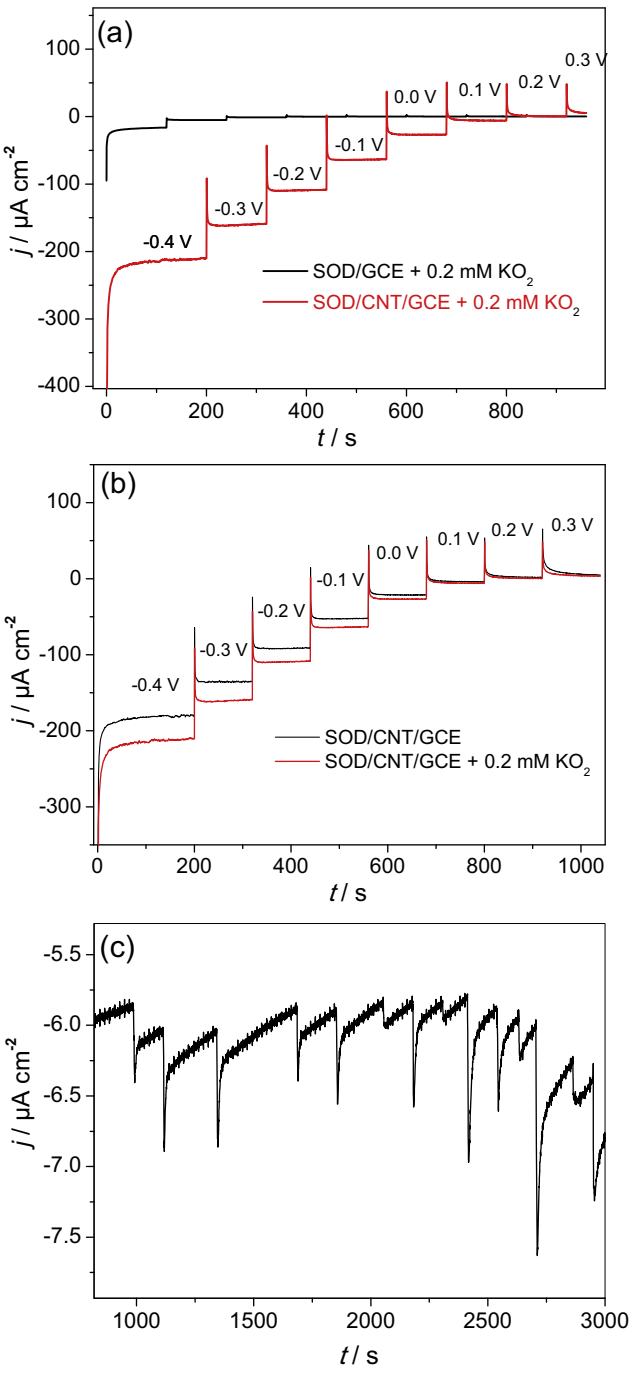


Fig. 4. Effect of applied potential on (a) SOD/GCE and SOD/CNT/GCE, (b) on SOD/CNT/GCE response in the absence and presence of 0.2 mM KO_2; (c) example of biosensor amperometric response in normal O_2 content 0.1 M NaPBS pH 7.0 at −0.3 V vs. Ag/AgCl.

versus time responses with different electrode architectures for the successive addition of superoxide concentrations are shown in Fig. 5. As observed, the current becomes more negative upon superoxide addition, explained as follows. The detection mechanism of $O_2^{\bullet-}$ is explained by a redox and reduction cycle of the SOD enzyme. SOD can efficiently catalyse the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 via a redox cycle of the copper complex moiety ($Cu^{I/II}$) couple in Cu–Zn SOD. Two $O_2^{\bullet-}$ ions are stoichiometrically converted to one O_2 molecule and one H_2O_2 molecule with consumption of two H^+

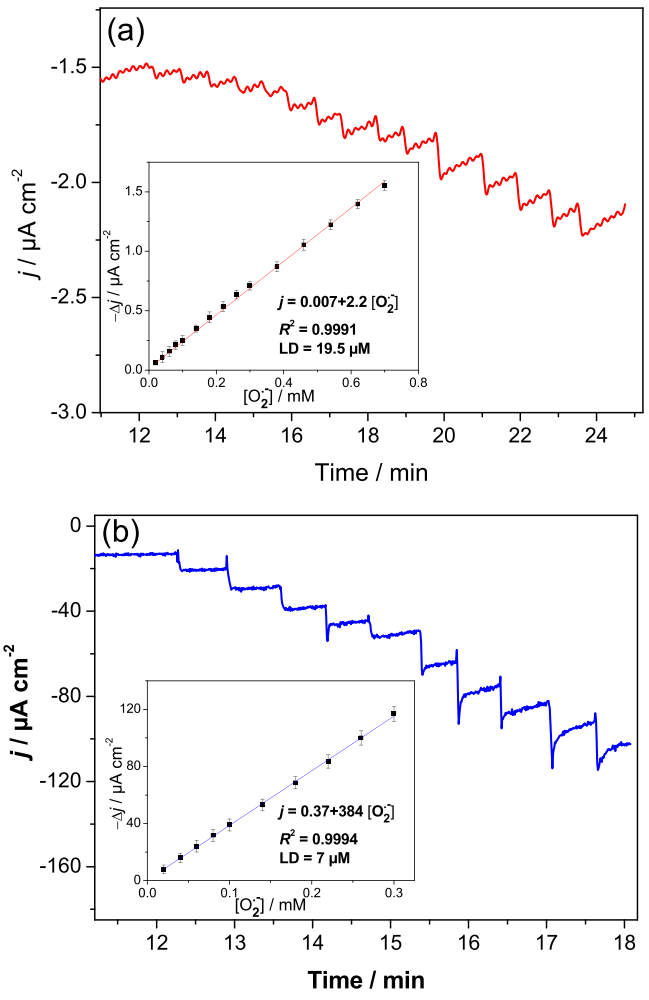


Fig. 5. Typical amperometric responses of the (a) SOD/GCE (b) SOD/CNT/GCE modified electrodes to successive addition of KO_2 at applied potential −0.3 V vs. Ag/AgCl in NaPBS 0.1 M pH 7.0; inset corresponding calibration plots.

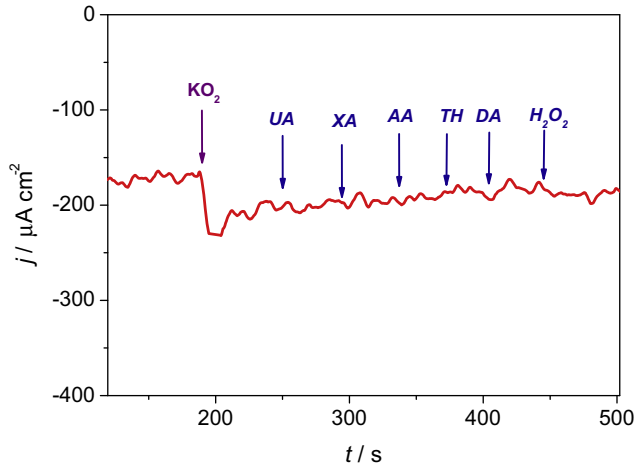


Fig. 6. Influence of electroactive interfering compounds on the response to superoxide at SOD/CNT/PEDOT/GCE biosensor at −0.3 V vs. Ag/AgCl in 0.1 M NaPBS pH 7.0; molar ratio KO_2: interferent is 1:2.

ions during the dismutation. The mechanism follows the following reaction sequence [17]:

$$\mathrm{SOD\,(Cu\,(I))} + \mathrm{O_2^{\bullet -}} + 2\mathrm{H^+} \rightarrow \mathrm{SOD\,(Cu\,(II))} + \mathrm{H_2O_2} \quad (1)$$

$$\mathrm{SOD\,(Cu\,(II))} + \mathrm{O_2^{\bullet -}} \rightarrow \mathrm{SOD\,(Cu\,(I))} + \mathrm{O_2} \quad (2)$$

At −0.30 V vs. Ag/AgCl the first reaction occurs, equation 2 being exclusively for positive potentials. Therefore, the working principle of the biosensors developed here is based on the amperometric signal linked to equation 1, which is that the current becomes more cathodic upon addition of superoxide, proportional to the change in concentration of superoxide radical in solution.

The first step was to determine the SOD/GCE and SOD/CNT/GCE biosensor response in order to examine the influence of CNT in biosensor analytical properties (the amperometric response and corresponding calibration plots are presented in Figs. 5a and b). It was observed that no saturation is reached up to 3 mM superoxide, but for the determination of analytical parameters the upper limit was chosen to be 300 µM. The biosensor sensitivity increased significantly from 2.2 µA cm^{-2} mM^{-1} to 384.0 µA cm^{-2} mM^{-1} on modifying with CNT, attributed to the increased surface active area in the presence of the CNT (determined using hexacyanoferrate (II) as electroactive standard probe) and to the porosity of the CNT modified electrode [31].

Table 2 contains the analytical parameters of all biosensor configurations tested, in order to assess the contribution of each component to the analytical properties of the final assembly and whether architectures with CNT on top or beneath the polymer are the better ones. The SOD/CNT/PEDOT/GCE biosensor had a sensitivity of 1115 µA cm^{-2} mM^{-1}, and a detection limit of 1.0 µM. When PEDOT was formed on the CNT modified electrode, the sensitivity of the biosensor was very similar to that of PEDOT alone, 176 compared to 184 µA cm^{-2} mM^{-1}. Thus, with CNT as the top layer, the sensitivity was higher than when it was beneath the polymer, even though the peak currents and capacitance of the modified electrodes with polymer on top of the CNT layer were superior. This is clear evidence of the important role of CNT in enzyme immobilization and maintenance of catalytic activity, as they are biocompatible and create very good conditions for a porous layer of the enzyme to be immobilized on top. Moreover, the chitosan biopolymer, in which CNT are dispersed, is biocompatible, with amino groups that are beneficial for cross-linking of the enzyme via glutaraldehyde, in this way allowing more enzyme to be immobilized when chitosan was in the outer layer covering the polymers [32].

Some analytical parameters of SOD biosensors based on nanostructured materials, reported since 2009, are summarised in Table 3 [33–41]. As observed, the SOD/CNT/PEDOT/GCE biosensor developed in this work had by far the highest sensitivity, only two of those reported having comparable sensitivities, SOD/Pt-Pd_NP/PDARGO and SOD/Pt-Pd_NP/CNT/SPEG, with 909.7 [40] and 601 µA cm^{-2} mM^{-1}, respectively [38]. Three biosensors had significantly lower detection limits, all based on metal nanoparticles, SOD/Fe_3O_4_NP/Au [33], SOD/chitosan_AuNP/GCE [36] and SOD/cys/denAu/GCE [39], with values within the nanomolar range. Beside the high sensitivity, the new biosensor has the advantage to work at negative potentials, where interferences from other electroactive compounds are minimal, as will be seen in Section 3.3.3, and a large linear range, up to at least 3 mM KO_2.

3.3.3. *Biosensor selectivity and stability*

One of the most important problems to solve in the practical application of amperometric sensors is to minimize the effect of interfering species that may be present in real samples together with the analyte. Possible compounds that may interfere in the amperometric determination of superoxide, such as uric acid (UA), xanthine (XA), ascorbic acid (AA), thiazine (TH), dopamine (DA) and peroxide were investigated. Chronoamperometric measurements at a fixed potential of −0.3 V are shown in Fig. 6. Superoxide was injected before the above-mentioned interferent compounds, so

Table 2
Analytical parameters of different SOD biosensors.

Biosensor	Sensitivity/μA cm^{-2} mM^{-1}	RSD (n = 3)/%	LoD/μM	R^2
SOD/GCE	2.1 ± 0.1	4.8	81	0.9910
SOD/PEDOT/GCE	184 ± 11	6.0	8.2	0.9994
SOD/CNT/GCE	384 ± 18	4.7	7.7	0.9994
SOD/PEDOT/CNT/GCE	176 ± 12	6.8	4.1	0.9980
SOD/CNT/PEDOT/GCE	1115 ± 20	1.8	1.0	0.9999

Table 3
Comparison of recently reported SOD biosensors based on nanostructured materials (2009-present).

Modified electrode		E/V vs. Ag/AgCl	Sensitivity μA cm^{-2} mM^{-1}	Linear range/μM	LoD/μM	Reference
SOD/Fe_3O_4_NP/Au		+0.50	183.1	0.2–1.4	3.5 e-3	[33]
SOD/CNT/PPy/Pt		+0.07	190.0	0.1–750	0.1	[34]
PMMA/PANI-AuNP/SOD-ESCFM		+0.30	42.5	0.5–2.4	0.3	[35]
SOD/chitosan_AuNP/GCE		−0.19	6.7[a]	0.006–2.7	1.7 e-3	[36]
SOD/Cys- SG/AuNP/ITO		+0.17	185	0.08–0.64	0.2	[37]
SOD/Pt-Pd_NP/CNT/SPEG		−0.1	601	40–1550	0.71	[38]
SOD/cys/denAu/GCE	+0.26		6.3	0.01–540	2.1 e-3	[39]
	−0.24		7.0	0.05–440	0.03	
SOD/Pt-Pd_NP/PDARGO		−0.30	909.7	16–240	2.0	[40]
MeSOD/AuFs/GCE	Mn	−0.30	4.2	158–2030	92.1	[41]
	Mn(N_3^-)		8.8	97–1020	9.1	
	Fe		11.8	58–655	3.1	
SOD/CNT/PEDOT/GCE		−0.30	1115	20–3000	1.0[b]	Present work

Data are all obtained under physiological conditions (PBS pH 7.0–7.4)
SOD: superoxide dismutase, Fe_3O_4_NP: Fe_3O_4 nanoparticles; PPy: polypyrrole, SA: sodium alginate, PMMA: poly(methyl methacrylate), PANI: polyaniline, AuNP: gold nanoparticle, ESCFM: electrospun composite functional membrane, Cys: L-cysteine, SPEG: Screen printed gold electrode, SG:silica sol gel, denAu: dendritic Au nanostructure.; PDARGO-polydopamine deposited on reduced graphene oxide; Me SOD – artificially SOD with metal centres, AuFs-nanostructured Au flowers.
[a] Electrode area not specified.
[b] Linear range up to 300 μM used to calculate LoD.

that its final concentration (40 μM in the cell) was a factor of two lower than that of the interferents, which were injected sequentially. None of these compounds oxidises or reduces at −0.3 V, and it was confirmed that they have no interference in superoxide determination. Sensor recovery was determined to be close to 100%.

The SOD/CNT/PEDOT/GCE biosensor performance was monitored during a period of 2 months, being tested 2–3 times per week (a full calibration curve). When not in use, the biosensor was kept in 0.1 M NaPBS pH 7.0, at 4 °C. The biosensor sensitivity increased by 10% during the first week and by another 5% during the second week. After this period, the biosensor sensitivity remained constant up to 5 weeks, after which it gradually decreased, maintaining, however, a slightly higher response, of ≈105%, after 8 weeks, compared to the biosensor initial response. These results indicate excellent preservation of enzyme activity. The increase in sensitivity during the first two weeks of storage/usage is probably due to conformational changes providing better accessibility of the substrate to the active centres of the enzyme.

3.3.4. Determination of antioxidant activity

Natural antioxidants, such as vitamins and polyphenols, play an important role in the prevention of diseases associated with free radicals. Among such natural antioxidant compounds, it has been confirmed that resveratrol, a natural polyphenol, is an effective scavenger of hydroxyl, superoxide, and metal-induced radicals [42] having protective effects against cardiovascular diseases [43]. Some of the most popular food sources of resveratrol are the skin of grapes, blueberries, raspberries, and mulberries. Therefore, samples of red, white and Port wine were evaluated for their antioxidant capacity together with a juice containing a mixture of berries.

The determination of the antioxidant capacity of a sample possessing antioxidant properties is based on the fact that by reacting with the superoxide radical, the concentration of these species in solution is lowered. Consequently, both the amount of released H_2O_2 and thence the amperometric current of the biosensor is diminished.

Table 4
RAC of wine and juice determined by the SOD/CNT/PEDOT/GCE biosensor.

Beverage	RAC/%
Port wine (ruby)	70.0
Port wine (tawny)	68.3
Red wine 1	82.7
Red wine 2	80.3
White wine 1	66.0
White wine 2	62.5
Berry juice	10.0

The value of the antioxidant capacity was expressed in "Relative Antioxidant Capacity" (RAC) units, defined by the following equation:

$$RAC = \left[1 - \frac{m_{antiox}}{m}\right] \times 100$$

where m is the slope of the calibration curve of change in amperometric response vs. KO_2 concentration in the absence and m_{antiox} is the slope in the presence of the sample containing antioxidant [44].

The RAC values were determined for 2 port wines, 2 red wines, 2 white wines and berry juice, and the values obtained are given in Table 4. Superoxide was injected prior to and after the addition of the beverage sample, and sensitivities and corresponding RAC calculated, as exemplified in Fig. 7. As observed, red wines had the highest RAC values of 80.3 and 82.7, followed by the port wines (both red varieties) and white wines. The lowest RAC of 10% was obtained for berry juice.

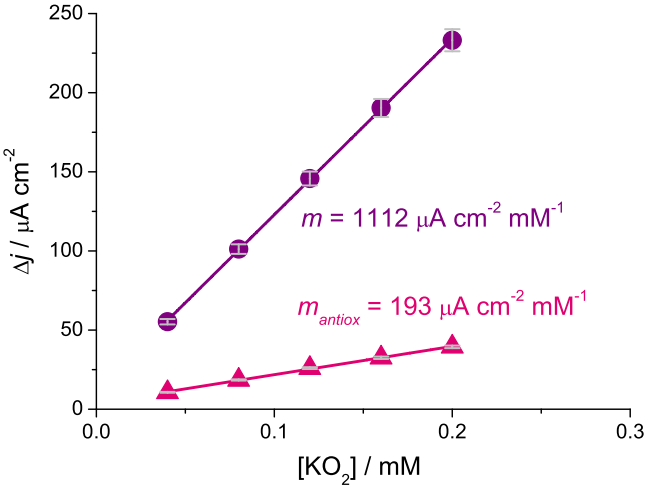


Fig. 7. Example of determination of RAC at SOD/CNT/PEDOT/GCE biosensor for red wine by addition of increasing amounts of KO_2 in the absence (●) and presence (▲) of red wine (8 μL added to 2 mL solution). Experimental conditions as in Fig. 6.

4. Conclusions

Superoxide dismutase biosensors have been developed based on PEDOT-CNT hybrid nanocomposites, among which the one based on the conducting polymer PEDOT with CNT on top of the polymer layer exhibited the best analytical parameters. The biosensor operated at −0.3 V vs. Ag/AgCl, and exhibited excellent selectivity and stability over a period of 2 months, with a considerably higher sensitivity than other recently-reported SOD biosensors. The developed biosensor was successfully applied to the determination of the relative antioxidant capacity of red and white wines, port wines and berry juice, which augurs well for future widespread application.

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