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ORIGINAL PAPER

Design of a new hypoxanthine biosensor: xanthine oxidase modified carbon film and multi-walled carbon nanotube/carbon film electrodes

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Abstract A new and simple-to-prepare hypoxanthine biosensor has been developed using xanthine oxidase (XOD) immobilised on carbon electrode surfaces. XOD was immobilised by glutaraldehyde cross-linking on carbon film (CF) electrodes and on carbon nanotube (CNT) modified CF (CNT/CF). A comparison of the performance of the two configurations was carried out by the current response using amperometry at fixed potential; the best characteristics being exhibited by XOD/CNT/CF modified electrodes. The effects of electrolyte pH and applied potential were evaluated, and a proposal is made for the enzyme mechanism of action involving competition between regeneration of flavin adenine dinucleotide and reduction of hydrogen peroxide. Under optimised conditions, the determination of hypoxanthine was carried out at −0.2 V vs. a saturated calomel electrode (SCE) with a detection limit of 0.75 μM on electrodes with CNT and at −0.3 V vs. SCE with a detection limit of 0.77 μM on electrodes without CNT. The applicability of the biosensor was verified by performing an interference study, reproducibility and stability were investigated, and hypoxanthine was successfully determined in sardine and shrimp samples.

Keywords Hypoxanthine biosensor · Xanthine oxidase · Carbon film electrodes · Carbon nanotubes · Flavin adenine dinucleotide

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Introduction

Hypoxanthine is formed during the degradation of adenosine triphosphate (ATP); the full degradation pathway being:

$$ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow HxR \rightarrow Hx \rightarrow X \rightarrow U$$

where ADP is adenosine diphosphate, AMP is adenosine 5'-monophosphate, IMP is inosine 5'-monophosphate, HxR is inosine, Hx is hypoxanthine, X is xanthine and U is uric acid. The understanding of this pathway is needed to study the diseases correlated with high levels of uric acid in blood. Food enriched in purines, such as beer, liver or fish, leads to an increase in the amount of uric acid in the human body, which deposits in joints in urate crystals causing gout, i.e. inflammation, intense pain, and even disability to patients [1]. Thus, knowing the concentration of hypoxanthine and uric acid in food is very important in gout prophylaxis [2].

In comparison with other analytical techniques used for hypoxanthine determination, such as high performance liquid chromatography [3, 4], spectrophotometry [5], mass spectrometry [6], capillary electrophoresis [3, 7], or chemiluminescence [8], electrochemical sensors possess simplicity of operation and enzyme substrate selectivity. In recent years, various electrochemical methods have been employed to measure the concentration of hypoxanthine in clinical and food samples, including voltammetric [9–12], potentiometric [13], and amperometric with [14–16] and without [16, 17] redox mediator. More recently, xanthine and hypoxanthine amperometric biosensors have used graphene [18], gold nanoparticles [19], or carbon nanotube [20, 21] modified electrodes.

Carbon nanotubes (CNTs) are among the nanomaterials that have received most attention in recent years [22, 23].

They possess one of the simplest chemical compositions and atomic bonding configuration; multi-walled carbon nanotubes (MWCNTs) have more electrochemically active sites (e.g. edge plane-like carbons), compared with, for example, glassy carbon and graphite, which makes them very attractive for electrochemical determinations at low potentials [24]. The presence of pentagonal defects produces regions with charge density higher than those observed in the region of hexagonal graphite, either in planar or in tubular structures [25]. The area of contact between enzymes immobilised on MWCNT where electron exchange occurs is greater than on a smooth electrode [26]. This characteristic can make MWCNT very attractive for the development of biosensors where selectivity is increased since the effect of interferent oxidation or reduction is less at potentials close to zero [12, 27]. Previous reports show that architectures using multi-walled carbon nanotubes are favourable, due to the simple construction and to the higher sensitivity and stability compared with other biosensors, e.g. [28–30].

The aim of this work was to design and develop a novel sensitive and easy-to-prepare biosensor for hypoxanthine. Biosensors were prepared on carbon film electrodes, without and with deposited CNT, onto which xanthine oxidase was immobilised. The two types of biosensor were evaluated and compared with other biosensors in the literature. There are only a few reports on xanthine oxidase biosensors based on carbon nanotube modified electrodes, most of them for xanthine detection. Studies include carbon nanotubes in combination with cyclodextrin [20], single-walled carbon nanohorns with gold nanoparticles for hypoxanthine [31] or single-walled carbon nanotubes for direct electrochemistry of xanthine oxidase [21]. However, to our knowledge, none of them used the simple configuration proposed here: xanthine oxidase immobilised onto MWCNT, for the determination of hypoxanthine.

Experimental

Reagents and solutions

Xanthine oxidase (E.C. 1.1.3.22, from buttermilk 0.068 U/mg) was purchased from Fluka. Hypoxanthine and xanthine were from Sigma. Neutral red (NR), with 65 % dye content was purchased from Aldrich.

Glutaraldehyde (GA) (25 % *v*/*v*) aqueous solution was acquired from Fluka. The phosphate-buffered (PB) solution is constituted by monobasic sodium phosphate (NaH_2PO_4), purchased from Sigma-Aldrich and disodium hydrogen phosphate (Na_2HPO_4), from Fluka.

Bovine serum albumin (BSA), uric acid and ascorbic acid were obtained from Sigma. MWCNTs were purchased from NanoLab. Nafion (5 % *v*/*v*) was obtained from Aldrich.

Electrochemical measurements and apparatus

Measurements were made in a one-compartment cell. Working electrodes were made from carbon film resistors (2 Ω nominal resistance, 15 μm film thickness) of 6 mm length and 1.5 mm diameter. The resistors were fabricated from ceramic cylinders by pyrolytic deposition of carbon from methane in a nitrogen atmosphere [32]. One of the two tight-fitting metal caps, linked to an external contact wire, was removed and the other one covered in plastic and protected by normal epoxy resin. The geometric area of the electrodes is 0.20 cm^2. The other electrodes were a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. Voltammetric and amperometric experiments were carried out using a PalmSens potentiostat (Palm Instruments BV).

Graphite epoxy–resin composite electrodes were also used. These electrodes were constructed using graphite powder and Araldit epoxy resin/hardener by hand-mixing in the ratio 70:30 (*m*/*m*), as described elsewhere [33]. The resulting paste was placed into the tip of a 1 mL insulin plastic syringe. The final electrodes had 5 mm diameter, geometric area of 0.196 cm^2, and their thickness was 5–7 mm.

Carbon film electrode pre-treatment

Since carbon film electrode surfaces cannot be renewed by polishing or other mechanical methods, electrochemical pre-treatment was carried out in order to obtain a reproducible electrode response. The electrochemical pre-treatment was always performed, by potential cycling between −1.0 and +1.0 V vs SCE, at a scan rate of 100 $mV s^{-1}$, until a stable voltammogram was obtained.

Functionalisation of the carbon nanotubes

A mass of 60 mg of MWCNT was stirred in 10 mL of a 5 M nitric acid solution for 24 h. The solid product was collected, filtered and washed several times with pure water until the filtrate solution became pH 5.0. The activated MWCNTs obtained were then dried in an oven at 100 °C for 24 h. Nitric acid usually causes significant destruction of carbon nanotubes and introduces –COOH groups at the end and sidewall defects of the nanotube structure [34].

An aqueous solution of 1 % (*v*/*v*) acetic acid was prepared in which chitosan was dispersed by agitation during 2 h. The final chitosan concentration was 1 % (*w*/*v*). The functionalised MWCNTs were dissolved in this chitosan solution by spreading 4 mg in 400 μL of chitosan to get 1 % (*w*/*v*) carbon nanotube solution. The CNT solution was then sonicated for 2 h to ensure a homogeneous mixture.

The surface of the carbon film electrode was modified with 10 μL of this solution. After drying for 1 h, another 10 μL of the carbon nanotubes was immobilised and left to dry for 24 h before enzyme immobilisation [35]. The scanning electron microscopy (SEM) images shown in Fig. 1a on indium tin oxide (ITO) electrodes (on a glass slide support) show a fully covered surface.

Enzyme immobilisation

The method for enzyme immobilisation was the same for the two types of electrode: CF and CNT/CF. An enzyme solution was prepared containing 5 mg of XOD and 5 mg of BSA in 50 μL phosphate buffer. To this solution, 2 μL of the cross-linking agent glutaraldehyde (25 % *v*/*v* in water) was added. From this mixture, an aliquot of 7 μL was placed onto the electrodes and then dried for at least 2 h. The resulting biosensors were XOD/CF and XOD/CNT/CF. When not in use, enzyme electrodes were kept at 4 °C in 0.05 M phosphate buffer, pH 7.0.

Sample preparation

For measurement in natural samples, sardine and shrimp were chosen. They were prepared according to the procedure described in [19]: A portion of the samples was triturated, and 4 g of each was added to 10 mL of distilled water plus a few drops of 5 mM NaOH. After 2 h, the samples were centrifuged at 4,000 rpm for 30 min. The extract was removed and then filtered. This filtered solution was analysed by the standard addition method.

Results and discussion

The new biosensor was optimised regarding its response to hypoxanthine on carbon film electrodes. Optimisation included applied potential, buffer pH, concentration of bovine serum albumin, inclusion of redox mediator or Nafion polymer, and influence of carbon nanotubes.

Biosensor for hypoxanthine development and optimisation

The major aim was the development of a simple and easy to prepare biosensor. All measurements in this section were carried out in phosphate buffer pH 7.0 at a fixed potential of −0.3 V vs SCE for the XOD/CF biosensor and −0.2 V vs SCE for the XOD/CNT/CF biosensor.

With the aim of achieving the most simple biosensor construction, the first experiments consisted in direct enzyme immobilisation on the CF electrode by physical adsorption from a solution containing only the enzyme, but the enzyme layer did not adhere sufficiently well to the surface.

In order to improve adhesion, 2 μL of glutaraldehyde (GA, 25 %) was added to the enzyme solution, as in [36], but no biosensor response was obtained. One explanation could be deactivation of the enzyme by GA. It is well known that GA acts by binding to the amine groups of the enzyme (see Fig. 1b) and could block its active centre [37], a drawback that is normally overcome by introducing BSA, a carrier protein. BSA possesses amine groups, which bind with the carbonyl group of the GA, leaving the active centres of the enzyme free to interact with enzyme substrate. Indeed, when using BSA, the biosensor exhibited response to hypoxanthine. In order to optimise the concentration of BSA for a good biosensor response, three different concentrations of BSA in the enzyme solution were tested: 2.5, 5.0 and 10.0 mg. The highest response was obtained with 5.0 mg BSA, it being 49 % lower when using 2.5 mg and 36 % lower with 10.0 mg. Hence, 5.0 mg of BSA was used for further tests.

In order to increase the biosensor robustness, the enzyme layer was covered by a thin layer of Nafion polymer (Nafion/XOD/CF). Normally, Nafion is used due to its film hydrophobicity and enzyme-favoured environment as well as to enhance selectivity of the sensor by electrostatic

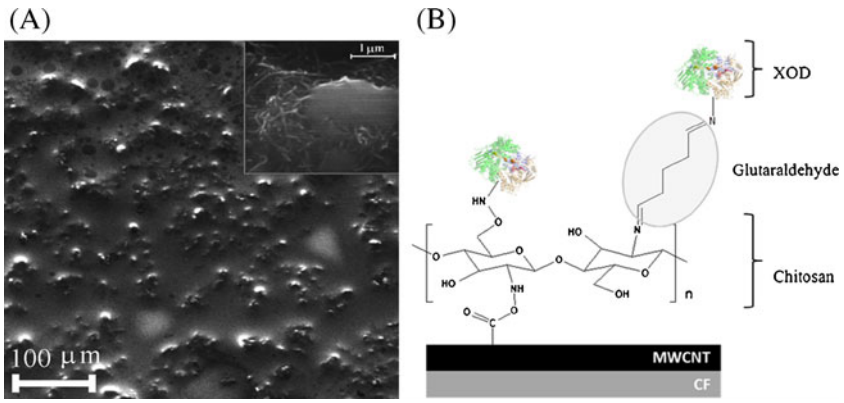

Fig. 1 **A** SEM images on indium tin oxide-modified electrodes of CNT in chitosan; *inset* at higher magnification. **B** Schematic representation of xanthine oxidase immobilisation: binding through glutaraldehyde and chitosan

repulsion of unwanted species [27]. However, although the linear range increased, the sensitivity of the sensor decreased drastically, by about 40 times and the detection limit was higher (see Table 1). This is different to the results reported in [27], but the experimental conditions were different, since there the enzyme was directly immobilised in Nafion and polyphenol during phenol electropolymerisation. The decrease in response at the electrode with Nafion might be due to the slow substrate diffusion through the Nafion layer. According to [38], the enzyme XOD has a net charge of +50 at pH 5.0 (the pH used in [27]) and of +6 at pH 7.0 (the pH used here). This may be due to the lower positive charge the enzyme is not retained in the Nafion layer, which leads to such decrease in biosensor response.

To investigate whether enzyme adhesion is related to the identity of the electrode surface, graphite epoxy-resin composite electrode substrates were also tested for enzyme immobilisation (XOD/GrEC). In this case the response was lower, by about a factor of 15, and no improvement in adhesion was verified, so the carbon film electrode was retained for further experiments.

The possibility of using a redox mediator, poly(neutral red) (PNR) [39], to increase sensitivity, was also investigated (XOD/PNR/CF). The response was seven fold lower with mediator than without, verified in several measurements. The decrease in response can be attributed mainly to the fact that PNR film enhances hydrogen peroxide reduction, which leads to a decrease in the net current response.

All the results obtained for these different biosensor configurations obtained at −0.3 V vs. SCE are summarised in Table 1. The highest response was obtained with the biosensor developed on CF without any mediator and no Nafion (XOD/CF), the sensitivity being 97.4 nA cm^{-2} μM^{-1}.

In order to improve the analytical parameters, particularly the sensitivity, CF electrodes were first modified with carbon nanotubes before immobilising the enzyme: XOD/CNT/CF. With this configuration, the response to hypoxanthine was ~6 times higher (see Fig. 2), mainly due to an increase in electroactive area and also due to a better enzyme immobilisation procedure (Fig. 1b), which, in the case of XOD/CNT/CF, occurs by GA and Chit (the medium used for CNT dispersion), whilst for XOD/CF, only by GA. The linear range and detection limit are similar. Besides the higher sensitivity, another advantage of using CNT-based biosensors is that the applied potential is less negative than without nanotubes: −0.2 V instead of −0.3 V (see next section); thus, more interferences can be avoided.

Hence, the following study is based on these two biosensors: without (XOD/CF) and with (XOD/CNT/CF) carbon nanotubes on carbon film electrodes.

Table 1 Comparison of analytical parameters of different architectures of hypoxanthine biosensors developed in this work

Electrode type	Upper limit linear range (μM)	Sensitivity (nA cm^{-2} μM^{-1})	Detection limit (μM)
Nafion/XOD/CF	400	2.4	1.1
XOD/GrEC	200	6.4	0.27
XOD/PNR/CF	200	12.4	0.23
XOD/CF	130	97.4	0.77
XOD/CNT/CF	135	1,235	0.75

Effect of the applied potential on biosensor response

In order to optimise the response to hypoxanthine, the two types of biosensors, XOD/CF and XOD/CNT/CF, were tested by applying different fixed potentials between −0.4 and +0.2 V.

The electrode configuration XOD/CF presented a maximum response at −0.4 V (Fig. 3a) above, which the response begins to decrease. A 6 % decrease in the response was observed at −0.3 V, and 15 % at −0.2 V. No response was obtained in the range 0.0–0.2 V. Thus, in order to reduce interferences, but to ensure a reasonable response, the potential chosen was −0.3 V.

In the case of the XOD/CNT/CF electrode, the maximum response was obtained at a potential of −0.2 V (Fig. 3b). At −0.3 V, the current is 36 % lower and 12 % lower at −0.1 V. At more positive potentials the response decreases further.

Effect of pH on biosensor response

Each enzyme has an optimum pH in its natural environment (not immobilised). However, when immobilised this pH may change, depending on the immobilisation procedure, which may lead to changes in enzyme conformation. In order to evaluate the effect of pH on the hypoxanthine

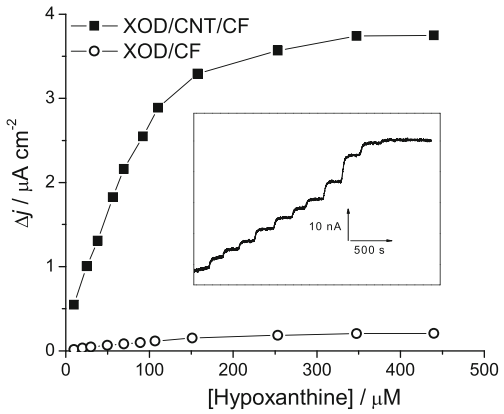

Fig. 2 Calibration curve for hypoxanthine in 0.05 M PB obtained at −0.3 V for XOD/CF (*square*) and −0.2 V for XOD/CNT/CF biosensor (*circle*). *Inset* show the typical response for hypoxanthine at −0.3 V

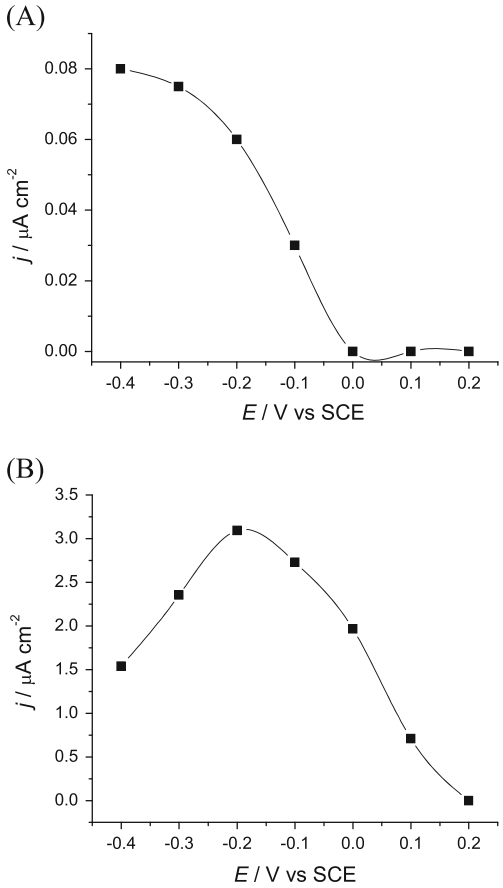

Fig. 3 Effect of the applied potential on the amperometric response of hypoxanthine at **A** XOD/CF and **B** XOD/CNT/CF biosensor

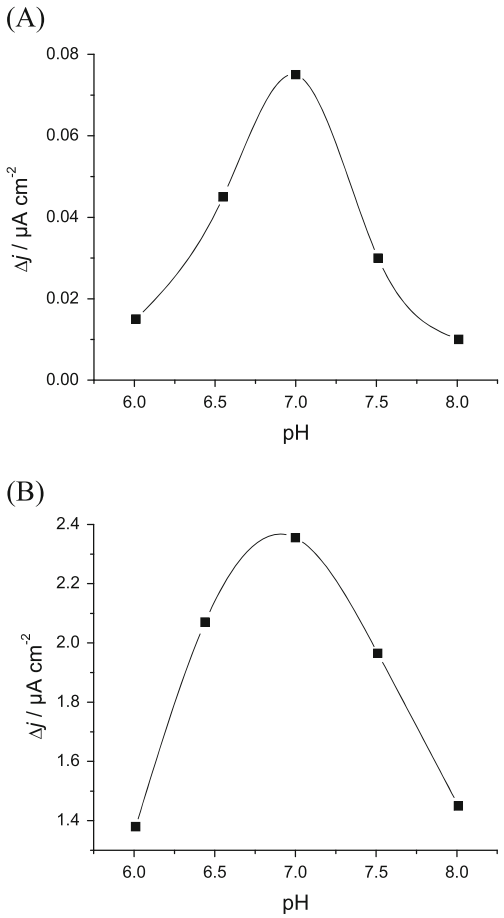

Fig. 4 Effect of pH on the amperometric response of hypoxanthine at **A** XOD/CF and **B** XOD/CNT/CF biosensor

biosensor, the response was examined in phosphate buffer solution in the pH range from 6.0 to 8.0. Figure 4 shows the dependence of current response with pH for both biosensors, XOD/CF and XOD/CNT/CF, a larger response being obtained with CNT present.

For the two biosensor configurations, the response increases with increase in pH from 6.0 to 7.0 and then decreases, so the response maximum is exhibited in pH 7.0 electrolyte. Thus, further measurements were performed in 0.05 M phosphate buffer pH 7.0. These results are consistent with others previously obtained [10, 19, 30] and is also closer to the isoelectric point found in [38] for xanthine oxidase of 6.9–7.4.

Mechanism proposal

The enzyme, xanthine oxidase, has three cofactors: flavin adenine dinucleotide (FAD), molybdenum (Mo) and ferredoxin iron–sulphur (Fe_2S_2) [40, 41]. It is not necessary to use a redox mediator in biosensors with xanthine oxidase, since the redox centres can perform direct electron transfer with the electrode. This was observed not only at gold electrodes modified with single-walled CNT [21] and at glassy carbon electrodes modified with MWCNT [42] but also at bare glassy carbon and mercury electrodes [43]. According to [44], the mechanism of action of xanthine oxidase consists of hypoxanthine being oxidised to xanthine, the oxidised form of the molybdenum centre is reduced and there is then an intraenzymatic electron transfer from molybdenum to FAD, followed by its reduction to $FADH_2$, this last being reoxidised at the electrode (Fig. 6).

In all cases in this work, an increase in anodic current was observed on addition of hypoxanthine. Various experiments were carried out to clarify the mechanism, including differential pulse voltammetry and fixed potential amperometry in the absence of dissolved oxygen.

Differential pulse voltammetry from −0.8 to −0.2 V at 5 $mV\,s^{-1}$ (amplitude, 50 mV; step potential, 1 mV) was carried out at CF and CNT/CF both modified with xanthine oxidase, and in both cases, there were two peaks one at around −0.55 V for XOD/CNT/CF (Fig. 5) and −0.53 V for XOD/CF, ascribed to molybdenum and another at −0.41 and −0.44 V, respectively, which corresponds to FAD. Hence, similarly to what was found in previous work [21, 42, 43, 45], direct electron transfer at XOD/CF and XOD/CNT/CF occurs.

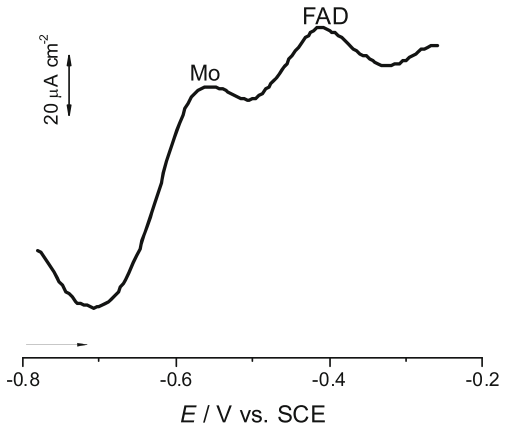

Fig. 5 Differential pulse voltammetry at XOD/CNT/CF electrode between −0.8 and −0.2 V at 5 $mV s^{-1}$, potential step of 1 mV, pulse amplitude of 50 mV

Measurements with addition of hypoxanthine in the absence of dissolved oxygen were also carried out. At XOD/CF electrodes, it was observed that the response decreased by 10 %, but continues to be oxidation. At XOD/CNT/CF electrodes, very small reduction currents were observed. Hydrogen peroxide determination was also performed at both types of biosensor as well as at the corresponding electrodes without enzyme; in all cases, reduction was observed. Thus, under the experimental conditions in this work, i.e. −0.3 V (XOD/CF) and −0.2 V (XOD/CNT/CF), the biosensor response could result from competition between regeneration of FAD and hydrogen peroxide reduction (Fig. 6). When bubbling N_2, a small amount of dissolved O_2 always remains, and at CNT-based biosensors, it is most probably more difficult to remove oxygen, since it can be trapped inside the tubes or within the tube network (see Fig. 1a). Hence, there is always some hydrogen peroxide produced (more for XOD/CNT/CF), which can be further reduced. On the other hand, oxidase enzymes' response can be decreased in the absence of O_2.

The explanation is the following. At the XOD/CF electrode at −0.3 V, the FAD regeneration response may be lower than in the presence of O_2 since a stoichiometric amount is needed for its regeneration. Some peroxide is produced and can still be reduced, but very little compared with oxygenated media. This leads to a decrease in the total observed anodic current.

At the XOD/CNT/CF electrode, at −0.2 V, peroxide reduction occurs more easily than at electrodes without nanotubes. Thence, when summing the two currents, it can be that the reduction due to peroxide is greater than that of $FADH_2$ oxidation, which leads to a net cathodic current.

Analytical performance of the hypoxanthine biosensor

The best results were obtained with the XOD/CNT/CF electrode: linear range, 10–135 μM; detection limit, 0.75 μM; and sensitivity, 1,235 $nA cm^{-2}\ \mu M^{-1}$.

Table 2 shows a comparison of this biosensor with others in the literature. There are only a few reports concerning hypoxanthine sensors that include the use of carbon nanomaterials. One uses MWCNT-dicetyl phosphate (DCP) at open circuit potential (OCP) [12] and another functionalised reduced graphene oxide (RGO) with conducting polypyrrole graft copolymer, poly(styrenesulfonic acid-g-pyrrole) (XOD/PSSA-g-PPy/RGO) on platinum [18], but the sensor exhibited a shorter linear range and low sensitivity at a high applied potential of +0.55 V. Other CNT-based biosensors exist for xanthine determination [41, 46], which in the present work was determined with lower sensitivity than hypoxanthine (see "Interference studies"). Further hypoxanthine sensors are based on gold nanoparticle modified carbon paste electrodes (XOD/nAu/CPE) [19] or on gold nanoparticles–single-walled carbon nanohorn (XOD-AuNP/SWCNH) [31], but both have a smaller linear range, and lower sensitivity. However, their detection limits are a little lower than here, most likely due to the gold nanoparticles.

Analytical parameters that are more similar were obtained in [9] with a carbon paste electrode modified with Nafion and methyl viologen (XOD/Nafion-MV/CPE): linear range up to 200 μM, detection limit of 0.8 μM and sensitivity of 1,930 $nA cm^{-2}\ \mu M^{-1}$, but the applied potential is very negative (−0.68 V).

A sensitivity of 971 $nA cm^{-2} \mu M^{-1}$ (lower than in this work) was obtained in a sensor employing XOD immobilisation in a silica–graphite matrix by the sol–gel technique (UM/Hx–S/Graphite), and a higher detection limit of 1.3 μM was recorded [16]. Other biosensors have higher detection limits, 1.5 μM in a biosensor for XOD immobilised on carbon fibre microelectrodes (CFME) using a composite film of Nafion and polymerised phenol (PPh) (Nafion/XOD/PPh/CFME) [19] and 5.3 μM using a glassy carbon paste electrode (GCPE) modified with xanthine oxidase (XOD/GCPE) [30], as well as lower sensitivities.

Thus, and as shown in Table 2, the biosensor developed here has a better overall performance than those prepared in the previous studies in the literature. It is used at a potential close to zero and is simpler to prepare than all except that in [30], but the latter has significantly inferior analytical characteristics of shorter linear range, lower sensitivity and higher detection limit.

Repeatability and reproducibility

The repeatability of measurements with both types of biosensor was evaluated by measuring the response to 50 μM hypoxanthine for six successive additions and comparing the results. The relative standard deviation (RSD) between measurements was 3.1 % for electrodes without CNT and 3.9 % for electrodes modified with CNT.

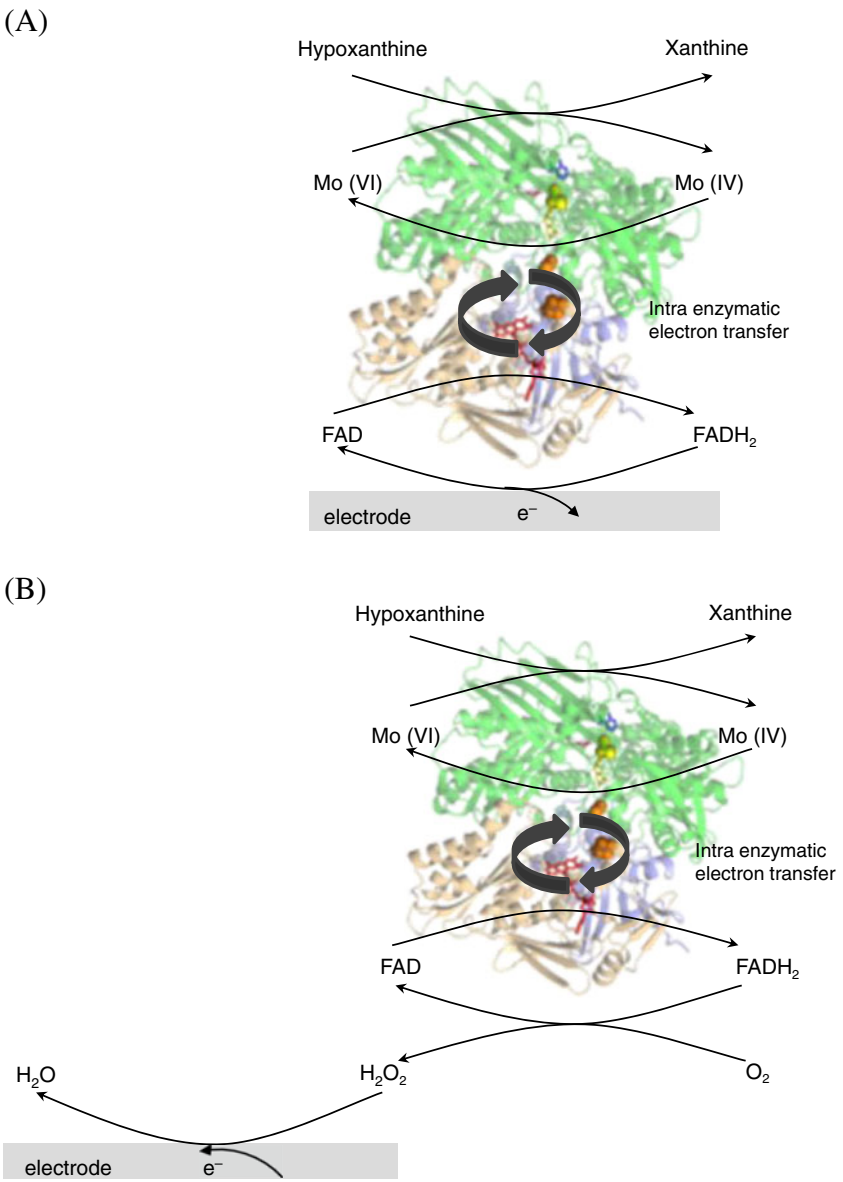

Fig. 6 Proposed mechanism for biosensor functioning based on the competition between the regeneration of FAD by oxidation (**A**) and H_2O_2 reduction (**B**)

Table 2 Comparison of analytical parameters of various hypoxanthine biosensors with different surface modifications (see text for explanation of abbreviations)

Electrode type	Linear range (μM)	Sensitivity (nA cm^{-2} μM^{-1})	LOD (μM)	*E* (V)	References
XOD/Nafion-MV/CPE	1.0–200	1,930	0.8	−0.68	[9]
MWCNT-DCP/GCE	0.5–200	–[a]	0.2	OCP	[12]
UM/Hx–S/Graphite	5.6–950	971	1.3	0.58	[16]
XOD/PSSA-g-PPy/RGO	0.03–28	0.673	0.01	0.55	[18]
XOD/nAu/CPE	0.5–10	327	0.10	0.60	[19]
XOD/nAu/CPE	0.5–10	22.8	0.22	0.00	[19]
Nafion/XOD/PPh/CFME	5.0–1,800	142	1.5	0.60	[27]
XOD/GCPE	20–80	17.1	5.3	0.90	[30]
XOD-AuNP/SWCNH	1.5–35.4	203	0.61	0.40	[31]
XOD/CNT/CF	10–135	1,235	0.75	−0.20	This work

[a] Not specified

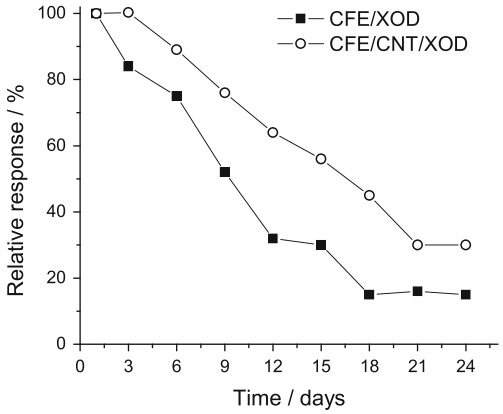

Fig. 7 Stability of hypoxanthine biosensors: *square* XOD/CF and *circle* XOD/CNT/CF

The reproducibility was evaluated by measuring the sensitivity of three different electrodes prepared in the same way, without and with CNT. The RSD was 4.9 % for XOD/CNT/CF electrodes and 5 % for XOD/CF electrodes.

Electrode stability

Five additions of hypoxanthine were performed each day during 4 days. For both biosensors, an increase in response was observed between the first and the last measurement on each day, which is probably due to increase in enzyme membrane permeability and enzyme reorganisation, as observed also in [47]. This increase was 6 % for XOD/CNT/CF and 10 % for XOD/CF. The initial response decreased from the first to the fourth day, to 97 % for the XOD/CNT/CF and 88 % for XOD/CF. The long-term stability of the xanthine oxidase biosensor was studied by recording calibration curves every 3 days. When not in use, biosensors were kept in phosphate buffer at 4 °C. As seen in Fig. 7, the biosensor with CNT presents a better stability, since, after 3 days, the response is still 100 %, whereas for the biosensor without CNT the enzyme activity decreases to about 84 % on day 3 and then more rapidly; by day 9, the biosensor having only 40 % of its initial response. For electrodes modified with CNT, the response decreases approximately 50 % after 2 weeks. The measurements were continued during 24 days, when the electrode responses decreased to 16 % of the initial response for electrodes without CNT and 30 % for electrodes with CNT. The better stability of the response at the XOD/CNT/CF electrode compared with XOD/CF is most probably due to stronger enzyme binding, since at CNT/CF electrodes, the XOD is immobilised through glutaraldehyde and chitosan, as well as direct attachment to nanotubes. At CF electrodes, the enzyme is cross-linked only by GA.

Interference studies

Some species, present in natural samples, can potentially change the biosensor selectivity towards hypoxanthine. An interference study is thus critical, since pre-treatment involves separation of the sample components, in this way making measurements in real time impossible.

Compounds tested as possible interferents for the xanthine oxidase biosensor were ascorbic acid, uric acid and xanthine since the structure of these compounds is similar to hypoxanthine and the enzyme response might undergo some alteration. Since xanthine oxidase also metabolises xanthine to form uric acid, it is also very important to evaluate and quantify its possible interference. After stabilisation of the baseline, 40 μM of hypoxanthine was added to the buffer solution and the response was measured. The same concentration of the three interferents in a 1:1 ratio of hypoxanthine to interferent was added and after that, 40 μM of hypoxanthine was again added and the response was measured again. The relative responses were calculated as an average of three measurements, performed under the same conditions.

The hypoxanthine response undergoes a small change when the interferents ascorbic acid and uric acid are added, decreasing by only 5.1 % in the case of XOD/CF electrodes and 5.8 % for XOD/CNT/CF electrodes. When xanthine was also added, the xanthine oxidase response signal increased by 25 % for electrodes without CNT and by 15 % for electrodes modified with CNT. The interference by xanthine was also observed in [27] where xanthine shows around 22 % of the current response to hypoxanthine. In [10], a 10 % change in signal was obtained in the presence of xanthine.

Table 3 Concentrations of hypoxanthine found in sardine and shrimp samples (average of three measurements, made under the same conditions)

Biosensor	Sardines				Shrimps			
	Added (μM)	Expected (μM)	Found (μM)	Recovery (%)	Added (μM)	Expected (μM)	Found (μM)	Recovery (%)
XOD/CF	0		69		0		95	
	30	99	101	102	30	125	120	96
XOD/CNT/CF	0		67		0		94	
	30	97	92	95	30	120	124	103

Independent xanthine determination was carried out under the same conditions as for hypoxanthine with both XOD/CF and XOD/CNT/CF electrodes. The results showed a much lower response for xanthine than for hypoxanthine. In the case of XOD/CF electrodes the response to xanthine is 116-fold lower (0.84 $nA\,cm^{-2}\,\mu M^{-1}$) than for hypoxanthine and for XOD/CNT/CF electrode it is 55 times lower (29.34 $nA\,cm^{-2}\,\mu M^{-1}$). All these results lead to the conclusion that the presence of xanthine should not be a problem for determinations in natural samples

Measurement in natural samples

With the aim of checking the applicability of the developed biosensor for the determination of hypoxanthine, sardine and shrimp samples were chosen, based on the fact that they cause or worsen gout [48]. The samples were prepared as described in the "Experimental". An aliquot of the filtered sample solution was injected into buffer electrolyte followed by known amounts of hypoxanthine according to the standard addition procedure.

The concentration of hypoxanthine in shrimps is expected to be higher than in sardines, taking into account the value found in [49], and this was also found here (see Table 3). Small variations were observed between the values obtained with the two types of biosensor: 4 % for hypoxanthine in sardines and only 2 % for the shrimp samples. Recovery studies were also performed by adding a known concentration of hypoxanthine in sardine and shrimp samples and then measuring again using standard addition. The values obtained showed recoveries between 95 and 103 %, which leads us to conclude that the biosensor is suitable for measurements in natural samples.

Conclusions

A novel and easy to construct biosensor for the determination of hypoxanthine based on fixed potential amperometric detection was developed. Carbon film electrodes with and without CNT were investigated, and the electrodes modified with CNT exhibited much higher sensitivity and better selectivity, reflecting the intrinsic characteristics of this material in electrochemical analyses and the potentialities that CNT offer in the construction and development of biosensors.

The measurement of hypoxanthine was performed by XOD/CNT/CF electrodes with a very high sensitivity and low detection limit, better than all reports in the literature except for one biosensor, which includes more steps in the preparation procedure and uses a redox mediator [9]. A small change in the hypoxanthine response due to interferences was observed with and without CNT, and the determination of hypoxanthine in sardine and shrimp food samples, without specific pre-treatment, was successfully carried out.

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