Construction of an Electrochemical Xanthine Biosensor Based on Graphene/Cobalt Oxide Nanoparticles/Chitosan Composite for Fish Freshness Detection

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Abstract: A xanthine biosensor based on glassy carbon electrode (GC) modified with graphene (GR), Co_3O_4 nanoparticles, and chitosan (CH) composite was fabricated. Xanthine oxidase (XAO) solution was dropped on the surface of $Nafion/Co_3O_4/CH/GR/GC$ and the electrode was placed in saturated glutaraldehyde vapor for the crosslinking of the enzymes. The modified electrode was characterized by scanning electron microscopy, cyclic voltammetry and electrochemical impedance spectroscopy. Under the optimized experimental conditions, xanthine was detected in the concentration range from 5.0×10^{-4} to 8.0×10^{-2} mM with a detection limit of 2.0×10^{-4} mM. The low Michaelis–Menten constant (0.17 mM) suggested enhanced enzymic affinity for the immobilized enzyme as compared to previously reported xanthine biosensors. Moreover, the biosensor exhibited some advantages, such as short response time (10 s), high sensitivity (6.58 µA/mM or 74.8 µA/mMcm^2), and good reproducibility (RSD = 1.2%). The suitability of the device was verified by xanthine assay in a fish meat sample. The biosensor provided to be a reliable, easy, fast and economical method for the evaluation of fish freshness.

INTRODUCTION

Estimation of fish freshness is important in the food industry for the manufacture of high quality products. After the death of fish, ATP (adenosine triphosphate) decomposition proceeds according to the sequence ATP $\rightarrow$ ADP $\rightarrow$ AMP $\rightarrow$ IMP$\rightarrow$ HxR $\rightarrow$ Hx $\rightarrow$ X$\rightarrow$ U where ADP is adenosine diphosphate, AMP is adenosine 5'-phosphate, IMP is inosine 5'-phosphate, HxR is inosine, Hx is hypoxanthine, X is xanthine, and U is uric acid [1]. Among various indicators of freshness such as total volatile basic nitrogen [2], ammonia [3], volatile acids [4] and pH [5], nucleotides produced by ATP decomposition are considered the most reliable and useful indicators for estimating fish freshness. IMP is one of the major contributing factors to the pleasant flavor of fresh fish whereas, its degradation product hypoxanthine/xanthine contributes to the "off-taste" [6]. In general, enzymatic decomposition of hypoxanthine is the rate-determining step, so hypoxanthine and xanthine accumulate in fish tissue. Thus, the quantification of both hypoxanthine and xanthine may be used to indicate fish freshness [7]. Moreover, determination of xanthine level in blood and tissue is essential for diagnosis and medical management of various diseases like hyperuricemia, gout, xanthinuria, and renal failure [8]. Obviously a rapid and reliable method for the detection of xanthine is of great significance in clinical assay and food quality control.

According to the literature, several analytical methods are available for the analysis of xanthine. Among these are capillary column gas chromatography [9], HPLC [10], chemiluminesence [11] and spectrophotometry [12]. In general, the methods already in use are time-consuming and tedious or expensive and difficult to operate. They are also inadequate for real time monitoring. Biosensors possess the characteristics of being simple for operation, highly sensitive and selective. Various electrochemical biosensors have been reported for xanthine analysis [13-16] notwithstanding the fact that most of these biosensors have drawbacks such as being relatively less stable, less sensitive and their reproducibility being somewhat poor. So, some improvement is still needed. On the other hand, graphene has attracted tremendous research interest in recent times because of its unique physicochemical properties, such as high surface area, strong mechanical strength, excellent thermal and electric conductivity, and ease of functionalization. It comprises a single layer of sp^2 hybridized carbon atoms joined by covalent bonds to form a flat hexagonal lattice. The idealized structure of graphene is completely two dimensional [17]. In the field of electrochemistry, graphene is considered

as an ideal material for sensing application since it can play an important role in improving the performance of sensors due to its high specific surface area and good electrical conductivity [18-19].

Recently, metal or metal oxide nanoparticles have been widely used in the fabrication of nanocomposites. They have many excellent properties such as large surface-to-volume ratio, good electrical properties, high surface reaction activity, high catalytic efficiency, chemical stability, and strong adsorption ability [20]. Among various types of nanoparticles, Co_3O_4 nanoparticles are of special interest in recent years as the material is cheap, biocompatible, easily available, and has highly prospective electrocatalytic properties [21]. Up to now, various metal or metal oxide nanoparticles and graphene composites such as Pd–graphene [22], Fe_3O_4–graphene oxide [23], TiO_2–graphene [24], Pt nanoparticles–graphene [25], and Au–graphene [26] have been investigated for biosensor construction and synergistic effects of these composites in electrocatalytic applications have been reported. With its extraordinary properties such as film forming capability, superior mechanical strength, high permeableness, and nontoxicity, chitosan (CH) appears to be the biocompatible polymer of choice. As such, it serves as a matrix for the assembly of biomolecules, nanoparticles and other substances [27]. Combinations of chitosan with graphene or metal/metal oxide nanoparticles have also been tested as the electrochemical biosensing platforms [25-26]. In this paper, the construction of a new amperometric xanthine biosensor for fish freshness detection based on immobilization of xanthine oxidase on Nafion/Co_3O_4/CH/GR composite film modified GC is presented. The experimental conditions related to the preparation and the characterization of the biosensor were studied in detail. Optimum parametric studies such as enzyme loading, pH, and working potential were conducted. The analytical performance parameters of the biosensor including the linear range, detection limit, sensitivity, response time, repeatability and stability were also reported. The biosensor developed was also employed in the analysis of xanthine in fish samples as a means for the estimation of fish freshness.

MATERIALS AND METHODS

Chemicals

Graphene solution (2 mg/mL) was obtained from Dropsense (Llanera, Spain). Xanthine oxidase (from microbial source with a specific activity of 8 Units/mg solid), Co_3O_4 nanoparticles (<50 nm particle size (TEM), 99.5% trace metals basis) $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, uric acid, ascorbic acid, and glutaraldehyde were obtained from Sigma (St. Louis, MO, USA). Chitosan (90% deacetylation) was from Aldrich. Xanthine and glucose were from Fluka (Buchs, Switzerland). All other chemicals were purchased from Merck (Darmstadt, Germany).

Apparatus and measurements

Electrochemical measurements were carried out with IVIUM electrochemical analyzer (Ivium Technologies, Netherlands). A three-electrode configuration was employed, consisting of a modified glassy carbon electrode (3 mm in diameter) serving as the working electrode, and Ag/AgCl (BAS MF 2052) and platinum wire (BAS MW 1034) serving as the reference and counter electrodes, respectively. Scanning electron microscope (SEM) images were obtained using SEM from Carl Zeiss AG, EVO® 50 Series. ORION Model 720A pH/ion meter and ORION combined pH electrode (Thermo Scientific, USA) was used to check the pH of test solutions. Double distilled water was used for preparing all solutions (ELGA PURELAB). Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements were performed in the presence of 5.0 mM $K_3[(Fe(CN)_6]$, 5.0 mM $K_4[(Fe(CN)_6]$ and 100 mM KCl solution. EIS measurements were performed at the frequency range of 10^5 Hz–0.05 Hz with 10 mV amplitude. The amperometric measurements were performed at an applied potential of +0.70 V in 50 mM phosphate buffer solution (PBS pH 7.5) under stirring at room temperature. All amperometric measurements were performed in oxygen-saturated solutions.

Biosensor preparation and modification

Prior to coating, firstly the unmodified GC was polished with 0.05 μm alumina slurry, and then rinsed throughly with double distilled water, followed by ultrasonication in ethanol for 5 minutes. 0.025 g of chitosan (CH) was dissolved in 5.0 mL of acetate buffer solution

(pH 5.0) via magnetic stirring at room temperature. Co_3O_4 nanoparticles were dispersed in chitosan solution with 4 h of ultrasonication to obtain a concentration of 1.0 mg mL^{-1}.

5 μL of GR solution (2 mg/mL) was drop-casted on the cleaned surface of GC. After being dried at room temperature, 5 μL of Co_3O_4/CH solution was drop-casted on the surface of graphene modified GC to prepare the Co_3O_4/CH/GR/GC electrode. 10 μL of XAO solution (0.0248 Units μL^{-1}) was dropped on the surface of Co_3O_4/CH/GR/GC to fabricate XAO/Co_3O_4/CH/GR/GC. XAO modified electrode was placed in 2.5% glutaraldehyde vapor for 15 min in order to induce chemical cross-linking and then dried at room temperature. Finally, 7.5 μL of nafion solution (0.5%) was dropped on the enzyme electrode and the solvent was allowed to evaporate. The biosensor was dipped in 50 mM PBS (pH 7.5) to wash off the unbound constituents from the electrode surface. The fabrication process of the biosensor is presented in Scheme 1.
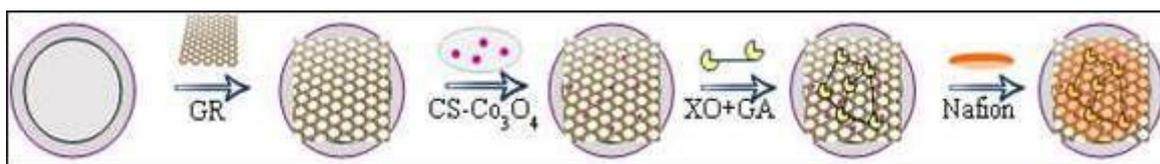
Scheme 1. Stepwise fabrication processes of the modified electrode.
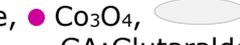
(GR:Graphene, ● Co_3O_4, CH:Chitosan, XAO:Xanthine oxidase, GA:Glutaraldehyde, Nafion).

Preparation of fish sample for real sample analyses

The trout fish was cut into small pieces by chopping and homogenized. The homogenate was then divided into three parts. One part was stored at room temperature, the second part was stored at +4 °C and the last part was stored at -18°C. Before each experiment, the homogenate preparation was mechanically stirred and then centrifuged. This homogenate suspension was filtered through a Whatman filter paper to obtain fish meat extract. Distilled water was then added into the extract producing a total volume of 25.0 mL of homogenized sample solution.

RESULTS and DISCUSSION

Surface morphologies of composite electrodes

The surface morphologies of GR/GC, Co_3O_4/CH/GR/GC, and XAO/Co_3O_4/CH/GR/GC were investigated by SEM (Fig. 1). Image a shows wavy and crumpled shapes of graphene. Image b indicates that the Co_3O_4 nanoparticles are distributed throughout the porous chitosan network. The resulting porous film is suitable for immobilization. The surface of the composite (XAO/Co_3O_4/CH/GR) shows globular structures (image c), indicating that enzymes were successfully immobilized on the surface of Co_3O_4/CH/GR/GC.
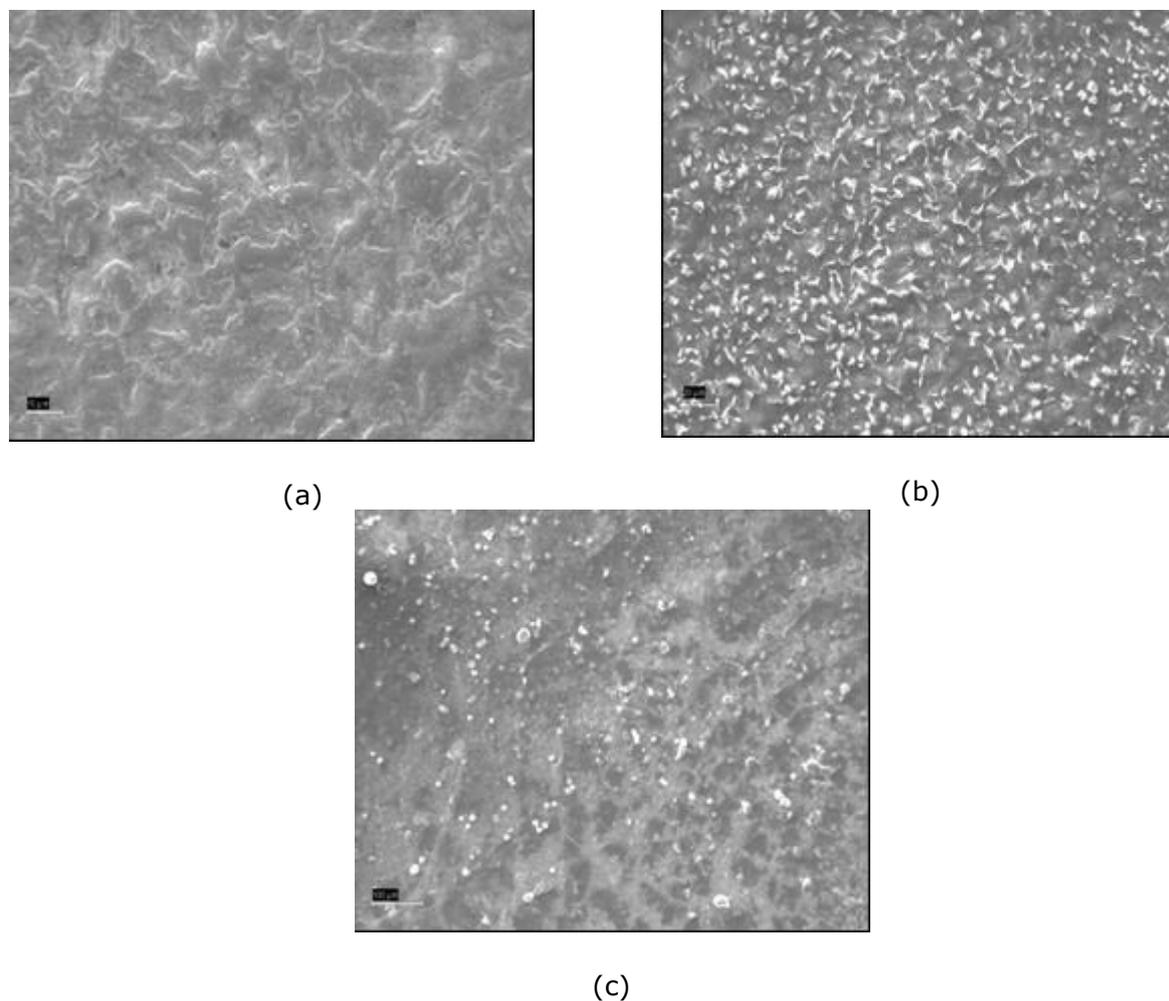

(a)

(b)

(c)

Fig. 1 SEM images of (a) GR/GC, (b) Co_3O_4/CH/GR/GC and (c) XAO/Co_3O_4/CH/GR/GC.

Electrochemical characteristics of composite electrodes

$[Fe(CN)_6]^{3-/4-}$ solution containing 100 mM KCl was used as redox probe to evaluate the electrochemical characteristics of modified electrodes. Figure 2A compares the CVs of unmodified GC, CH/GC, GR/GC, Co_3O_4/CH/GC and Co_3O_4/CH/GR/GC in this probe. The CV curve obtained with unmodified GC indicates a well-defined reduction and oxidation peak (curve b) that belongs to $[Fe(CN)_6]^{3-/4-}$ redox couple. After the modification of the GC with CH, a decrease is observed in the peak current (curve a); leading to the conclusion that CH is acting as an inert blocking layer making the electron transfer more difficult. CH is known to have a relatively poor conductivity and some decrease in the peak current has also been reported previously [14]. GC coated with a layer of GR produce a CV (curve c), with a remarkable enhancement in the peak currents compared to those with unmodified GC and CH/GC. These changes could be ascribed to the enhanced surface area of graphene. The CV obtained with Co_3O_4/CH/GC (curve d) shows an increase in the peak current compared to the ones with unmodified GC and CH/GC. We suggest that the Co_3O_4 nanoparticles play some role in the enlargement of the electroactive surface area of the electrode.

In the case of GR/GC and Co_3O_4/CH/GC the peak potential separation ($\Delta E = E_{pa}-E_{pc}$) was found to be smaller than for the GC and CH/GC indicating that faster electron transfer reaction. The treatment of the GR/GC surface with Co_3O_4/CH composite leads to an increase in the redox peak currents of $[Fe(CN)_6]^{3-/4-}$ system (curve e) relative to the other four-electrode systems. This observation assures that the Co_3O_4/CH composite have been successfully combined onto the electrode surface, and the electron transfer of $[Fe(CN)_6]^{3-/4-}$ at the electrode surface was made easier. The peak potential separation ΔE at Co_3O_4/CH/GR/GC was smaller than for the GC, CH/GC and Co_3O_4/CH/GC demostrating faster electron transfer behavior due to the synergistic action of the Co_3O_4 nanoparticles and graphene.

The effective surface area of the GC, GR/GC, Co_3O_4/CH/GC and Co_3O_4/CH/GR/GC was estimated according to the Randles-Sevcik equation [28]. The effective surface area of the GC, GR/GC, Co_3O_4/CH/GC, and Co_3O_4/CH/GR/GC was 0.071 cm^2, 0.100 cm^2, 0.122 cm^2 and 0.210 cm^2, respectively. In comparison with the unmodified GC, the effective surface area of the GR, Co_3O_4/CH and Co_3O_4/CH/GR/GC modified electrodes was increased about 1.5, 1.2 and 2.9 times, respectively. These results were expected, as

Co_3O_4 nanoparticles and GR facilitate enhanced electron transfer for the redox process of $Fe(CN)_6^{3-/4-}$ [18; 29-30], thus increasing the effective surface area. The surface area of the Nafion/XAO/Co_3O_4/CH/GR/GC was also calculated as 0.088 cm^2 and further used for the sensitivity estimation of the enzyme electrode.
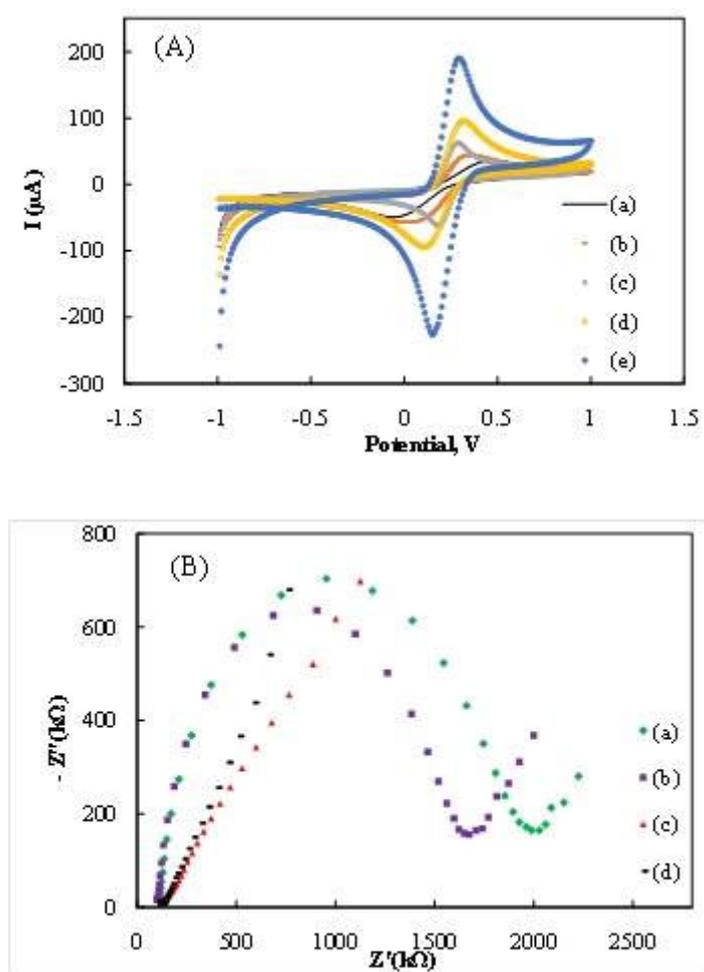
Fig. 2 (A) Cyclic voltammograms of (a) CH/GC, (b) GC, (c) GR/GC, (d) Co_3O_4/CH/GC and (e) Co_3O_4/CH/GR/GC at 50 mVs^{-1}, in 100 mM KCl solution containing 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ (B) The Nyquist curves of in (a) CH/GC, (b) GC, (c) GR/GC, and (d) Co_3O_4/CH/GR/GC 100 mM KCl solution containing 5.0 mM$Fe(CN)_6^{3-/4-}$

In this study, EIS was employed to probe the electron transfer kinetics at the unmodified GC, CH/GC, GR/GC and Co_3O_4/CH/GR/GC (Fig. 2B). The Nyquist plots of the EIS consist of a semicircular portion and a linear portion, which correspond to the electron transfer limited process and the diffusion limited process, respectively. The electron transfer resistance (R_{ct}) at electrode surface is equal to the semicircle diameter, which can be used to describe the interface properties of the electrode [31]. With unmodified GC, a semicircle curve (Fig. 2B (b)) was obtained. When the electrode was modified with CH

film (Fig. 2B (a)), the diameter of the semicircle is larger indicating that a layer of CH coated the electrode surface supposedly, this layer blocks the diffusion of the electroactive species to the electrode surface. With GC covered with GR, the diameter of the semicircle decreases (Fig. 2B (c)), relative to the ones obtained with unmodified GC and CH/GC. The smallest semicircle was observed with Co_3O_4/CH/GR/GC in accordance with the expectation (Fig. 2B (d)) suggesting that Co_3O_4 and GR have a synergetic effect and this composite can make the electron transfer easier. The results of EIS study is constistent with the results reported in the literature for Co_3O_4 and graphene modified electrodes [30; 32].

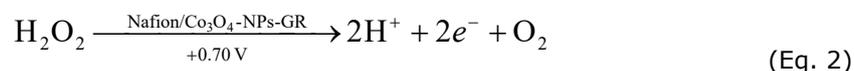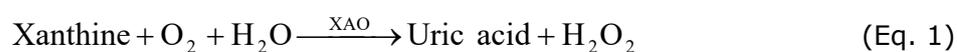## Optimization of experimental parameters

A study was carried out to assess the optimum GR amount on the GC. 5; 7.5 and 10 μL of GR solution (2 mg/mL) was casted on the cleaned surface of GC and dried at room temperature. The electrode response was determined by CV in 100 mM KCl solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$. The highest peak currents were obtained with 10 µL graphene (data not shown). However, the peak currents of three different graphene concentrations were close to each other. Thus, 5 µL GR solution was used for further experiments to prevent the waste of this expensive reagent. The effect of the Co_3O_4 amount for the preparation of Co_3O_4/CH composite was investigated by dispersing in the range of 0.5 to 1.5 mg Co_3O_4 in 1 mL of CH solution. The electrode response was determined by CV in 100 mM KCl solution containing 5 mM $Fe(CN)_6^{3-/4-}$. The highest peak currents were obtained with 1.0 mg Co_3O_4 (data not shown). Thus, a 1.0 mg Co_3O_4 amount was selected for the preparation of Co_3O_4/CH composite.

The effect of varying the concentration of XAO (0.1–0.5 U) during preparation was studied. The optimal amount of 0.3 Unit of XAO was obtained for the immobilization on the working electrode. This loading was therefore used for all biosensors in further experiments. Higher enzyme loadings caused the current decrease which is most likely due to the blocking of the electrode surface area by the large amount of immobilized protein. On the other hand, lower enzyme quantities caused the decrease of biosensor response.

Since enzymic activity is extremely affected by pH, the influence of pH on the biosensor response was investigated with 0.06 mM xanthine by using 50 mM PBS between pH 5.5 and 8.5. The response increases from pH 5.5 to 7.5. At pH values higher than 7.5, a decrease of the response was found. This results indicated that the maximum response of the biosensor occured at pH 7.5. Thus, pH 7.5 was selected as optimum pH value for further experiments. The optimum pH value found is within the pH between (7.0–8.5) reported for free XAO from microbial sources [33]. These results indicate that this immobilization procedure does not significantly effect the optimum pH of XAO.

The optimum working potential was investigated after the optimal composition of electrode was established. We have determined the optimum working potential by evaluating the xanthine response of the enzyme electrode at different potentials. The best response was obtained at +0.70 V and this potential was selected as the optimum working potential of the enzyme electrode.

The electrochemical reactions involved are known to be as follows at +0.70 V [14]:

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{XAO}} \text{Uric acid} + H_2O_2 \qquad \text{(Eq. 1)}$$

$$H_2O_2 \xrightarrow[\text{+0.70 V}]{\text{Nafion/Co}_3\text{O}_4\text{-NPs-GR}} 2H^+ + 2e^- + O_2 \qquad \text{(Eq. 2)}$$

The electron transfer mechanism of the biosensor developed also involves the H_2O_2 formed from xanthine by XAO immobilized on the Co_3O_4/CH/GR composite. H_2O_2 molecules are then oxidized on the biosensor surface at +0.70 V. The response current of the biosensor is directly proportional to xanthine concentration (Fig. 3A).
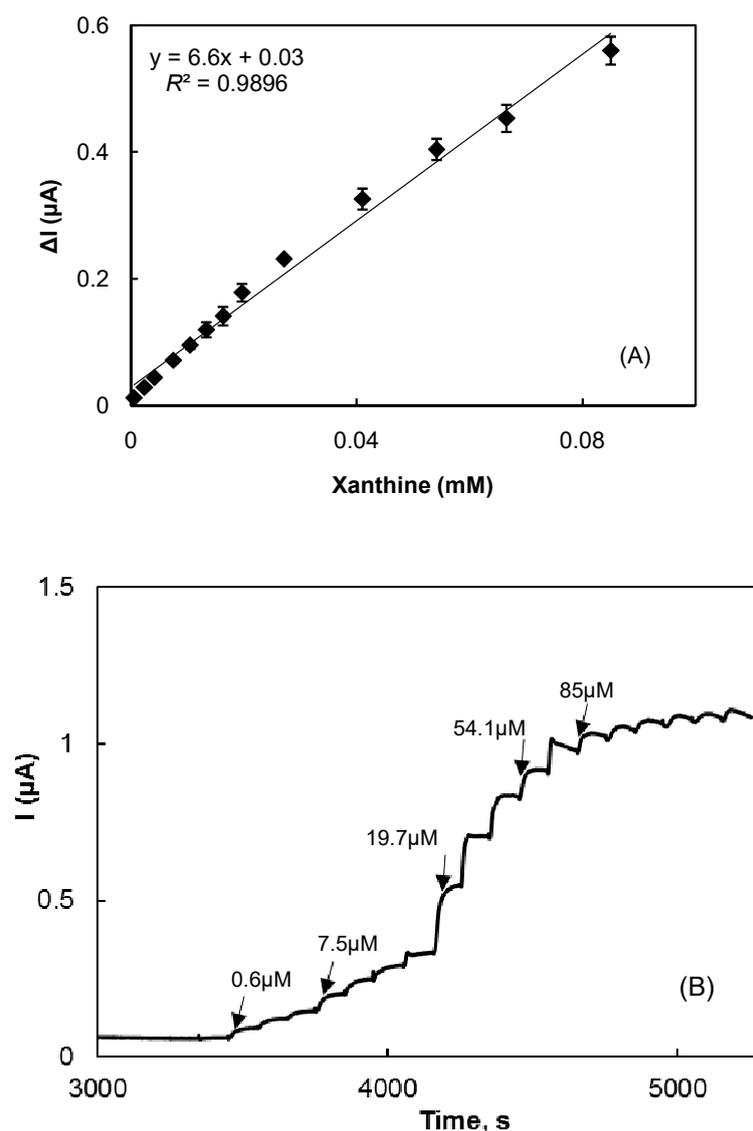
Fig. 3. (A) Calibration curve of the Nafion/XAO/Co_3O_4/CH/GR/GC. (B) Typical current–time response of the on successive injection of xanthine into a stirred solution of 50 mM PBS (pH 7.5).

Performance parameters

Fig. 3 shows the calibration curve and the chronoamperometric response of the biosensor the sequential additions of xanthine in 50 mM PBS (pH 7.5). In Figure 3A, Δi is the difference between the response current of the biosensor (i) and background current. After the addition of xanthine, the anodic current increased and reached a stationary state within 10 s. The linear response range of the biosensor to xanthine concentration was from 5.0×10^{-4} mM to 8.0×10^{-2} mM with a correlation coefficient of 0.9896 and

sensitivity of 6.6 µA/mM or 74.8 µA/mMcm^2. The limit of detection (LOD) of the biosensor, which is the minimum concentration at which the ratio of signal to noise is not less than 3, is 2.0×10^{-4} mM ($S/N = 3$). This detection limit is lower than that of ferrocene modified polypyrrole coated Pt electrode (1.0×10^{-6} M) [34] and mesoporous graphite material micro-structured with palladium-platinum deposits (1.5×10^{-6} M) [35] but comparable to silver nanoparticles/cysteine modified Au electrode (1.5×10^{-7} M) [36] and poly(glycidyl methacrylate-co-vinylferrocene)/MWCNT nanocomposite layer modified pencil graphite electrode (1.2×10^{-7} M) [37]. In order to evaluate the effect of direct oxidation of xanthine the response of Nafion/Co$_3$O$_4$/CH/GR/GC and Nafion/XAO/Co$_3$O$_4$/CH/GR/GC to xanthine were determined. The sensitivity of Nafion/XAO/Co$_3$O$_4$/CH/GR/GC ($y=6.6x+0.03$ R^2=0.9896) was found to be four times higher than Nafion/Co$_3$O$_4$/CH/GR/GC ($y=1.7x+0.06$ R^2=0.9896). Hence we can decide that the direct oxidation of xanthine has a contribution to biosensor response

Lineweaver–Burk plot gave the apparent Michaelis–Menten constant (K_m^{app}) value of 0.17 mM for immobilized enzyme, and this value is lower than those of a CuPtCl$_6$/glassy carbon chemically modified electrode based xanthine biosensor (1.11 mM) [38] polypyrrole and ferrocene modified Pt electrode based xanthine biosensor (1.33 mM) [34], and a platinum electrodeposited polyvinylferrocenium perchlorate matrix modified Pt electrode based xanthine biosensor (3.45 mM) [39]. These results show that the current biosensor possesses higher affinity to xanthine compared with some of earlier biosensors.

As a measure of the repeatability of the Nafion/XAO/Co$_3$O$_4$/CH/GR/GC, five successive calibration curves were prepared using the same biosensor. The relative sensitivity standard deviation (RSD) was around 1.0%. The reproducibility was also good (use of three different electrodes resulted in a relative standard deviation of 1.2%).

Long-term stability is one of the most important properties of biosensors due to the tendency of the enzymes to lose their activity when not stored in appropriate conditions. The long–term stability of the biosensor was tested by storing it in a dry atmosphere at +4 °C when not in use. There was no significant change in the amperometric response within the first 15 days. At the end of a 30 day storage period, some 90% of the original sensitivity was retained. Even after 2 months, the biosensor was found to have reserved

(83%) of its initial activity. The high stability of this biosensor may be attributed to the chemical and mechanical stability of Co_3O_4/CH/GR composite and thus it can be used as a useful platform for immobilising enzymes to enhance biosensor performances.

The common issue in the electrochemical determination of xanthine is the interference from species, such as glucose, uric acid, and ascorbic acid. The interference of glucose (0.02 mM), uric acid (0.02 mM), ascorbic acid (0.02 mM), and sodium benzoate (0.02 mM) were investigated. The response of the biosensor to 0.02 mM xanthine was found to have changed by −3%, 15%, 20% and −4% respectively. The high interferences due to uric acid (15%) and ascorbic acid (20%) are no surprise as the working potential is fairly high.

Table 1 lists the response characteristics of the proposed xanthine biosensor compared to the some other xanthine biosensors previously reported. The results presented in Table 1 demonstrate that the different characteristics of the developed xanthine biosensor based on Nafion/Co_3O_4/CS/GR modified GC are better in some situations or comparable with earlier sensors reported.

Analysis of the fish sample

The analytical reliability and application potential of the presented biosensor was evaluated for the determination of the concentration of xanthine accumulated in fish continuously after death, which attracts much interest as an indicator for estimating the freshness of fish [37]. After freshly killed and stored for 6, 12, 24 and 48 h at room temperature, the concentration of xanthine in fish meat was determined by Nafion/XAO/Co_3O_4/CH/GR/GC. Xanthine level of fish samples were determined by the standard addition method. The concentrations of the analyte were arranged so as to fall into the linear working range; using fish meat extract as a matrix. The multiple addition calibration curve was found to be linear and the original xanthine concentration in the fish meat sample was 62.8±0.2 mg L^{-1} (n=3). After 6, 12, 24, and 48 h, the concentration of xanthine increased from 62.8 to 113.7, 119.3, 123.3, and 253.5 mg L^{-1}, respectively. The level was doubled after 6 h, increases slowly up to 24 h of storage and beyond 24 h it increases very rapidly (Fig. 4a). The increase in xanthine accumulation with storage time is an expected result. To investigate the effect of storage conditions on

fish freshness, the change of xanthine concentration with time was also investigated in fish meat samples stored at +4 °C and -18 °C. In the fish sample stored at +4 °C xanthine level was increased from 62.6 to 245.8 and 340.2 mg L^{-1} after 8 and 15 days, respectively. (Fig. 4b). In the fish sample stored at room temperature xanthine level was found to be 253.5 mg L^{-1} after 2 days. Thus, it can be concluded that degradation is very high at room temperature compared to +4°C. In the fish sample stored at -18 °C xanthine level was only increased from 62.4 to 71.7 mg L^{-1} after 15 days. (Fig. 4c). The results obtained demonstrated that the degradation is higher at 4°C than at -18 °C. In the fish sample stored at -18 °C xanthine level was nearly constant after 3 days. On the basis of the measurements of the fish sample stored at -18 °C, one can conclude that no significant degradation occurred over this time period and storage temperature is very important to reduce the spoilage of fish meat.

Table 1: Various amperometric xanthine biosensors reported in recent years.

No	Enzyme /Working potential	Immobilization matrix/ Immobilization technique	Linearity/Detection limit	pH	Response time/Repeat ability	Storage stability	Reference
1	XAO/+0.55 V vs.SCE	Nano $CaCO_3$ particles and XAO modified electrode/cross-linking	2.0×10^{-6}–2.5×10^{-4} M/2.0×10^{-6} M(XAO-NanoCaCO$_3$ electrode)	7.5	<5 s/RSD 4.9% (XAO-NanoCaCO$_3$)	15% loss after 28 days (XAO-Nano CaCO$_3$)	[33]
	XAO-HRP/-0.05V vs.SCE	Nano $CaCO_3$ particles, XAO and HRP modified electrode/cross-linking	4.0×10^{-7}–5.0×10^{-5}M/1.0×10^{-7} M(XAO-HRP NanoCaCO$_3$ electrode)				
2	XAO/-0.05 V vs. Ag/AgCl	Mesoporous graphite material micro-structured with palladium-platinum deposits/adsorption	1.5– 70 µM/1.5 µM	8.4	60 s	-	[35]
3	XAO/+0.70 V vs.Ag/AgCl	Co_3O_4 nanoparticle/chitosan/multiwalled carbon nanotube/ composite film/cross-linking	2.0×10^{-7}–1.6×10^{-5} M/2.0×10^{-7}	7.0	5 s/2.9%	35% loss after 1 month	[14]
	XAO/HRP/−0.30 V		2.0×10^{-5}–3.6×10^{-4} M/2.0×10^{-5}			(XAO/Co$_3$O$_4$/M WCNTs/CH/GC)	
4	XAO/+0.38 V vs. Ag/AgCl	Zinc oxide nanoparticles–polypyrrole composite/physiosorption	0.8 µM–40 µM /0.8 µM	7.0	5 s/<5.1%	40% loss after 100 days	[40]
5	XAO/+0.60 V vs. Ag/AgCl	Supramolecular assembly between single walled nanotube surface and an adamantane-modified xanthine oxidase via β-cyclodextrin-modified pyrene derivative	5.0–600 µM/2 µM	7.0	10 s/6.1%	67%loss after 1 month	[15]
6	XAO/+0.35 V vs. Ag/AgCl	Reduced *expanded* graphene oxide sheets decorated with iron oxide nanoparticles into poly(glycidyl methacrylate-covinylferrocene)/covalent immobilization	2–36 µM /0.17 µM	7.0	3 s	30% loss after 25 days	[13]

|							
7	XAO/+0.20 V vs. Ag/AgCl	Cadmium oxide nanoparticles/carboxylated multiwalled carbon nanotube (c-MWCNT) composite film electrodeposited on the surface of Au electrode/covalent immobilization	0–120 µM/0.05 µM	7.5	4 s	50% loss after 120 days	[41]
8	XAO/+0.50 V vs. Ag/AgCl	Silver nanoparticles/cysteine modified Au electrode/covalent immobilization	2 μM–16 μM/0.15 μM	7.0	5 s/<2.6%	20% loss after 60 days	[36]
9	XAO/+0.35 V vs. Ag/AgCl	Poly(glycidyl methacrylate-co-vinylferrocene)/MWCNT nanocomposite layer modified pencil graphite electrode/adsorption	2–48 µM 28–46µM 46–86µM/0.12 µM	7.0	4 s	30% loss after 25 days	[37]
10	Nafion/XAO/ +0.70 V vs.Ag/AgCl	Cobalt oxidenanoparticle/ chitosan/graphene/ composite/cross-linking	5.0×10^{-4}–8.0×10^{-2} mM/2.0×10^{-4} mM	7.5	10 s/1.0%	17% loss after 2 months	This work
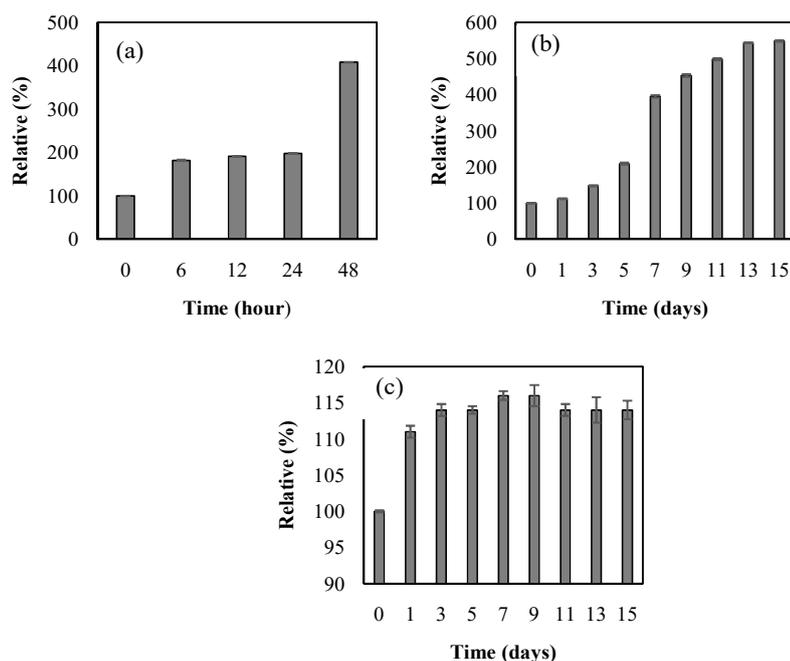
Fig. 4. Change of xanthine concentration in fish meat with time at different storage conditions: (a) room temperature (b) +4°C and (c) -18°C.

CONCLUSIONS

With the electrocatalytic synergy of graphene and Co_3O_4 nanoparticles to xanthine, a sensitive xanthine biosensor was achieved. Nafion/XAO/Co_3O_4/CH/GR composite of known composition was coated on GC to act as a biosensor for xanthine. The use of Nafion/Co_3O_4/CH/GR composite resulted in the improved analytical performance of the xanthine biosensor in terms of low response time (10 s), high reproducibility (1.2%), good sensitivity (6.58 µA/mM), wider linear range (5.0×10^{-4}–8.0×10^{-2} mM), and low detection limit (2.0×10^{-4} mM) compared with some of the previously reported biosensors. The presented biosensor was shown to be adequate for the determination of xanthine in fish samples. The results are used to evaluate the freshness. The purposed strategy may be to be applicable to the development of enzyme-based biosensors for other bioactive species.

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Türkçe Öz ve Anahtar Kelimeler

Balıkta Tazelik Tayininin Grafen/Kobalt Oksit Nanoparçacıkları/Kitosan Kompozitine Dayanan Elektrokimyasal Ksantin Biyosensörü ile Gerçekleştirilmesi

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Öz: Camsı karbon elektrodu (GC) grafen (GR), Co_3O_4 nanoparçacıkları ve kitosan (CH) kompoziti üretilmiştir. Ksantin oksidaz (XAO) çözeltisi Nafion/Co_3O_4/CH/GR/GC yüzeyine damlatıldı ve elektrot doymuş glutaraldehit buharında enzimlerin çapraz bağlanması için tutuldu. Modifiye elektrot taramalı elektron mikroskopisi, döngülü voltammetri ve elektrokimyasal impedans spektroskopisi ile karakterize edildi. Optimize deneysel koşullar altında, ksantinin $5,0 \times 10^{-4}$ ile $8,0 \times 10^{-2}$ mM arasında, tayin sınırı $2,0 \times 10^{-4}$ mM olmak üzere tayin edildiği bulundu. Düşük Michaelis-Menten sabiti (0,17 mM), daha önce bildirilen ksantin biyosensörleriyle karşılaştırıldığında immobilize enzimin artmış aktivitesini gösterdiği tespit edildi. Bunun dışında, biyosensör bazı avantajlar da sunmaktadır, bunlar arasında kısa tepki zamanı (10 s), yüksek hassasiyet (6,58 µA/mM ya da 74,8 µA/mMcm^2), ve iyi tekrarlanabilirlik (RSD = %1,2) öne çıkmaktadır. Cihazın uygunluğu balık eti örneğinde ksantin tayini yapılarak doğrulandı. Biyosensör güvenilir, kolay, hızlı ve ekonomik bir şekilde balık tazeliğini değerlendirilmesi için bir yol sunmaktadır.

Anahtar kelimeler: Amperometrik biyosensör, Co_3O_4 nanoparçacık, grafen, ksantin, balık tazeliği.