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Analytical Methods

Construction of novel xanthine biosensor by using polymeric mediator/MWCNT nanocomposite layer for fish freshness detection

Muamer Dervisevic [a], Esma Custiuc [a], Emre Çevik [a], Mehmet Şenel [b,*]

[a] Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, B.Cekmece, Istanbul 34500, Turkey
[b] Department of Chemistry, Faculty of Arts and Sciences, Fatih University, B.Cekmece, Istanbul 34500, Turkey

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ABSTRACT

A novel nanocomposite host matrix for enzyme immobilization of xanthine oxidase was developed by incorporating MWCNT in poly(GMA-co-VFc) copolymer film. In the food industry fish is a product with a very low commercial life, and a high variability as well elevated level of xanthine is an important biomarker as a sign of spoilage. The fabricated process was characterized by scanning electron microscopy (SEM), and the electrochemical behaviors of the biosensor were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The prepared enzyme electrodes exhibited maximum response at pH 7.0 and 45 °C +0.35 V and reached 95% of steady-state current in about ~4 s and its sensitivity was 16 mAM^{-1}. Linear ranges (2–28 μM, 28–46 and 46–86 μM), analytical performance and a low detection limit 0.12 μM obtained from the xanthine biosensor gives reliable results in measuring xanthine concentration in the fish meat. All the results indicating that the resulting biosensor exhibited a good response to xanthine that was related to the addition of MWCNT in the polymeric mediator film which played an important role in the biosensor performance. In addition, the biosensor exhibited high good storage stability and satisfactory anti-interference ability.

1. Introduction

Freshness of fish meat is the most important raw material for food industries being used for manufacturing safe and quality products. Immediately after fish death, when respiration and biosynthesis of adenosine triphosphate (ATP) ceases, the existing nucleotide in muscle undergoes degradation, according to the following sequence: adenosine di phosphate (ADP), adenosine 5′ phosphate (AMP), inosine 5′ phosphate (IMP), inosine (HxR), hypoxanthine (Hx), xanthine (X), and uric acid (UA) (Kassemsarn, Sang, Murray, & Jones, 1963; Nakatani et al., 2005; Venugopal, 2002), among which the IMP is the major contributing factor to for flavoring freshness fish and its degraded product hypoxanthine imparts the bitter taste of fish meat (Mulchandani, Luong, & Male, 1989). On the other hand, determination of xanthine in blood sample and tissue is essential for the diagnosis of different diseases, such as gout, hyperuricemia, xanthinuria, and renal failure as well as number of mild stimulants derived from xanthine like caffeine and theobromine are present in coffee and tea (Devi, Thakur, & Pundir, 2011). Thus, xanthine determination is of clinical and industrial importance. Meat and its products are crucial in human diet due to its protein reaches (Paula & Ana, 2013) as well meat consumption increases from day to day, and having it under control is of big significance. Xanthine and hypoxanthine concentration can be consequently measured by xanthine biosensors which will provide better control over fish and meat freshness control (Abdulazeez & Samuel, 2012). The most commonly used methods for analyzing xanthine are: high pressure liquid chromatography (HPLC) (Kock, Delvoux, & Gresiling, 1993), enzymatic fluorometric, fluorometric mass spectrometry fragmentography (Olojoa, Xiab, & Abramsona, 2005), capillary column gas chromatography (Renata, Pagliarussi, Luis, Freitas, & Bastos, 2002), and enzymatic colorimetric (Berti, Fossati, Tarenghi, Musitelli, & Melzideril, 1988). However, this method suffers from some drawbacks such as required time for sample preparation, expensive apparatus which needs skilled person to operate with it, lack of high sensitivity and specificity (Devi, Batra, Lata, Yadav, & Pundir, 2013) etc., which hardly fits in the time of technological explosion, where we can produce a small compact devices such as biosensors which easily overcome these limitations. Amperometric detection of xanthine by biosensors is more sensitive, simple, requires no complex sample preparation, and fast analysis with lower cost (Shah, 1996). The basic principle and electrochemical reaction of

* Corresponding author. Tel.: +90 2128663300; fax: +90 2128663402.
E-mail address: msenel@fatih.edu.tr (M. Şenel).

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amperometric xanthine biosensors are based on the reaction below (Pundir & Devi, 2014):

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{XOD} \text{Uric acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{V \text{ vs } (Ag/AgCl)} 2H^+ + O_2 + 2e^-$$

$$2e^- \rightarrow \text{Working electrode}$$

Xanthine oxidase has been widely used for biosensor designing that is being used for fast quantitative analysis of xanthine in real samples (Madalina, Krishna, Xueliang, & Christopher, 2014; Palraj, Silke, & Paul, 2012; Pundir & Devi, 2014), by immobilizing it on various supports, such as nafion membrane (Nakatani et al., 2005), polypyrrole (PPy) film (Lawal & Adeloju, 2012), polyvinyl chloride (PVC) membrane (Pundir, Devi, Narang, Singh, & Shewta, 2012), self-assembled phospholipids membrane (Rehak, Snejdarkova, & Otto, 1994), cellulose acetate membrane (Basu, Choudhury, & Chakraborty, 2005), silk membrane (Mao, Xu, Xu, & Jin, 2001), and silk fibroin membrane (Mulchandani et al., 1989). However, this supports have big drawbacks such as slow electron transfer, poor stability, reusability, non-conducting and non-elastic, fragile, and poor absorption ability (Devi et al., 2011), which made requirement for introducing a system modified by electron transfer mediator providing highly desirable lower detection limit and faster electron transfer (Çevik, Şenel, & Abasıyanık, 2010). Ferrocene mediators are widely used to construct mediated amperometric biosensors since they are excellent in electron transfer (Cass et al., 1984; Rajesh, Pandey, Takashima, & Kaneto, 2005). The essentiality of development of polymeric mediators for applications in biosensors is because it allows the incorporation of reagents (Çevik et al., 2010). Some widely used redox copolymers trialing the covalent attachment of ferrocene are poly(vinylferrocene-co-hydroxyethyl methacrylate) (Saito & Watanabe, 1998), poly(glycidyl methacrylate-co-vinylferrocene) (Şenel, Çevik, & Abasıyanık, 2010), acryl amide copolymers (Kuramoto, Shishido, & Nagai, 1994), poly(N-acryloylpyrrolidine-co-vinylferrocene) (Koide & Yokoyama, 1999), ferrocene-containing polythiophen derivatives (Abasıyanık & Şenel, 2010), and multiwalled carbon nanotubes (Kandimalla, Tripathi, & Ju, 2006). Carbon nanotubes were one of the first new nanomaterials with huge impact on amperometric biosensors, with ability to blend into a number of formulation to improve current densities and overall performance of enzyme-labeled immunosensors and enzyme electrodes (Turner, 2013; Wang, 2005). The unique electronic properties of nanomaterials has been widely exploited in electrochemistry as a mean of promoting the electron transfer reaction for a wide range biosensors applications (Lowrence, Deo, & Wang, 2005). Therefore, biosensors incorporated with carbon nanotubes are generally characterized by fast electron transfer, lower limit detection, and high sensitivity compared to traditional biosensors (Periasamy, Chang, & Chen, 2011). As an example, Dalkiran, Kaçar, Erden, and Kiliç (2014) reported a study on amperometric xanthine biosensors based on chitosan Co_3O_4 multiwalled carbon nanotube modified glassy carbon electrode, and Devi, Yadav, and Pundir (2012) reported amperometric xanthine biosensor based on immobilization of xanthine oxidase on a composite film of zinc oxide/chitosan/multiwalled carbon nanotube/polyaniline composite film.

Herein, we report a study on constructing amperometric xanthine biosensor, which is based on immobilization of xanthine oxidase on poly(GMA-co-VFc)/MWCNT nanocomposite mediator film. Presented biosensors important considerations were focused on: (i) electrode construction, electrochemical conditions such as current density and faster electron transfer, (ii) analytical utilization of biosensor, interference, shelf life, analytical performance, (iii) optimum parameter studies such as pH, temperature, reusability, and (iv) application of the biosensor in real sample by measuring xanthine concentrations in the fish meat.

2. Experimental part

2.1. Materials

Xanthine oxidase, xanthine, vinylferrocene (VFc) and Glycidyl methacrylate from was purchased from Sigma–Aldrich. Reagent-grade purchased from Aldrich Co, and was used without purification. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of poly(GMA-co-VFc)

The redox random copolymer, having different compositions, was prepared according to our earlier study (Şenel & Abasıyanık, 2010). A mixture of VFc and GMA at a known molar ratio was injected into a Pyrex tube, AIBN (1 mol% based on the total monomer concentration was identical for all samples, 5 mol/dm^3). The mixture was degassed with Argon gas and vacuum sealed. Next, the tubes were placed in constant temperature baths, controlled to 70 °C. After 2 days, the reaction mixture was added drop wise, while rapidly, stirring the diethyl ether to precipitate the copolymer. The precipitated copolymer was washed with diethyl ether and re-precipitated in this manner, twice and finally vacuum dried. The prepared copolymer were characterized by using FTIR and NMR spectroscopy and the data are no given.

2.3. Preparation of enzyme electrode

Pencil graphite electrode (PGE) fabrication was initiated by rinsing meticulously the 10–2 mm area from the tip of the electrode with acetone followed by distilled water (DW). After DW drops completely dried in air, 0.4 P(GMA-co-VFc)/MWCNT was carefully dropped and spread out on all of PGE surfaces. The electrode was placed in drying-oven set at 60 °C for a faster surface drying process and kept until the polymer was sufficiently adsorbed. Subsequently, it was first rinsed with 10 mM PBS at a pH of 7.0 and then immersed in 9 mg/ml of xanthine oxidase dissolved in PBS solution for 48 h on the orbital shaker adjusted at 200 rpm and placed at 4 °C as the last step for coming up with the ready working electrode (Fig. 1). The working electrode was washed carefully with few drops of PBS prior to be used and stored in PBS at 4 °C while not used.

2.4. Xanthine determination in fish sample

Fish was purchased from local market, cut into pieces, homogenized, and mixed with DW with a ratio of 1:3 (w/w), fish and DW respectively. Afterwards the homogenate was filtered with filter paper, and content of xanthine was determined by producing biosensors under optimal conditions. Xanthine measurements were performed with the samples awaited for 5, 8, 10, 13, 15 and 20 days at room temperature, and data was interpolated using standard curve (concentration vs current) prepared under optimal working conditions.

2.5. Instrumentations

Electrochemical measurements were performed using CompactSoft portable electrochemical interface and impedance analyser (*Ivium Technologies*). The electrochemical measurements were recorded via a three electrode system set at a working potential of +0.35 V that was composed of Ag/AgCl as reference,

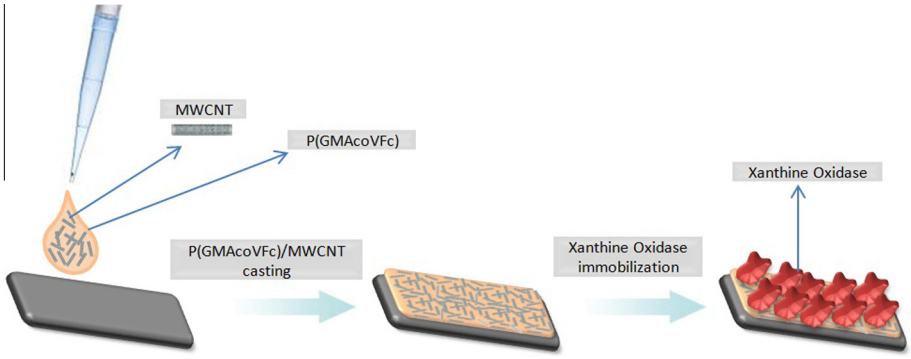

Fig. 1. The schematic illustration of fabrication process of the biosensor.

Platinum wire as auxiliary, and PGE/P(GMA-co-VFc)/MWCNT as working electrodes all immersed in an electrochemical cell filled with 10 mM PBS (pH = 7.0). Conventional three-electrode electrochemical cell were purchased from CH Instruments. After allowing the steady-state current to be reached, the Xanthine catalysis were triggered by Xanthine additions to the buffer solution from working cell being continuously stirred at room temperature. The substrate solution was freshly prepared by dissolving Xanthine in 30/70% 0.1 M NaOH and PBS, and the amperometric responses were recorded with the addition of known amounts of xanthine solution, respectively.

Electrochemical impedance spectroscopy (EIS) measurements were carried out using a CHI Model 6005 electrochemical analyzer in a background solution of 5 mM Fe^{3+}/Fe^{2+} phosphate buffer (pH 7.0) at a formal potential. The alternating voltage was 5 mV and the frequency range was 5.0×10^{-2} to 1.0×10^{6} Hz.

3. Results and discussion

3.1. Electrochemical characterizations

The electrochemical behavior of the bare PGE, P(GMA-co-VFc) and P(GMA-co-VFc)/MWCNT electrodes were investigated using CV. As shown in Fig. 2A, the redox peak current of the ferrocene unit of the copolymer is increased with the addition of MWCNT in the polymer solution when compared with the CV of the copolymer itself. The addition of MWCNT copolymer-coated PGE surface leads to increase in current intensity as MWCNT provided a conductive path for electron transfer and promoted electron transfer reactions at a low potential.

The cyclic voltammograms of the P(GMA-co-VFc)/MWCNT composite-coated electrode at different scan rates ranging from 10 to 500 mV s^{-1} are shown in Fig. 2B. The relationship between peak current and scan rate was found to be linearly proportional to the square root of scan rates, which indicated that the electrochemical kinetics of the biosensor was a typical diffusion-controlled process (Fig. 2C). Initially, the ferric ion in the VFc units exists in both the reduced form (Fe (II)) and oxidized form (Fe (III)). The voltammetric behavior also indicates that the ferrocene has been immobilized on the surface of the glassy carbon electrode (Şenel, Nergiz, & Çevik, 2013).

Electrochemical impedance spectroscopy (EIS) is an effective technique to study the interface properties of surface-modified electrodes. Fig. 2D shows EIS of bare PGE, P(GMA-co-VFc), and P(GMA-co-VFc)/MWCNT electrodes, respectively. The diameter of the semicircle is equal to the electron transfer resistance, R_{ct}, which corresponds to the electron transfer limited process. The electron transfer resistance (R_{ct}) of P(GMA-co-VFc) coated electrode was estimated to be 802 Ω. For P(GMA-co-VFc)/MWCNT-coated electrode, the value of R_{ct} was found to be 317 Ω. The decrease of R_{ct} suggested when MWCNT added copolymer film was prepared on the bare electrode surface, a barrier of the polymer film would increase the electron transfer efficiency and decrease the R_{ct} value. This kind of electrode surface, without pretreated with a binding agent EDC/NHS, when a few drops of enzyme solution were added, it resulted into immobilization of enzyme on it for amperometric biosensing electrode construction.

3.2. Optimum pH and temperature

The optimum pH of the biosensor was investigated in the range of pH 5.0–9.0 with freshly prepared enzyme electrodes and the results are illustrated in Fig. 3A. The best amperometric response of the enzyme electrode was obtained at pH 7.0 to a solution of 2 μM. The steady-state current increases with increasing pH starting from pH 5.0 to 7.0 and further increase in pH caused a decrease in the current response. This result demonstrated that acidic and basic systems reduce the activity of biosensing electrodes. Therefore, pH 7.0 was chosen for further experiments and for the determination of xanthine.

Due to the sensitivity of the enzymes are to thermal denaturation, the operating temperature was optimized for all conditions. The influence of temperature of the supporting electrolyte on the electrochemical behaviors of biosensor were investigated over the range of 25–50 °C. Fig. 3B shows that the steady-state current response of the electrode, depending on the temperature, increased up to 45 °C. Further increase in temperature caused a decrease in the current response of the electrode, which is caused by the thermal degradation of the enzyme. Although best current response obtained at 45 °C (Shengshui & Liu, 1997), considering the possible evaporation and easy preparation, all experimentation were performed at room temperature (25 °C).

3.3. Amperometric response of bionanocomposite electrode

The amperometric response of the Xo immobilized biosensing electrodes was given in Fig. 3C on successive injection of 2 μM xanthine into the stirring 0.01 M PBS (pH 7.0) at an applied potential of +0.35 V. In order to demonstrate the better behavior of the MWCNT addition in polymer layer, the amperometric response of the Xo immobilized P(GMA-co-VFc) coated electrode was also studied on successive injection of 2 μM xanthine at the same condition. The results indicate that the MWCNT can dramatically increase the current response of the biosensing electrode when compared with the P(GMA-co-VFc) coated biosensing electrode. With the increase in substrate concentration, the P(GMA-co-VFc)/MWCNT biosensing electrode responded rapidly and 95% steady-state current was reached within ~4 s, which indicated a very fast diffusion process.

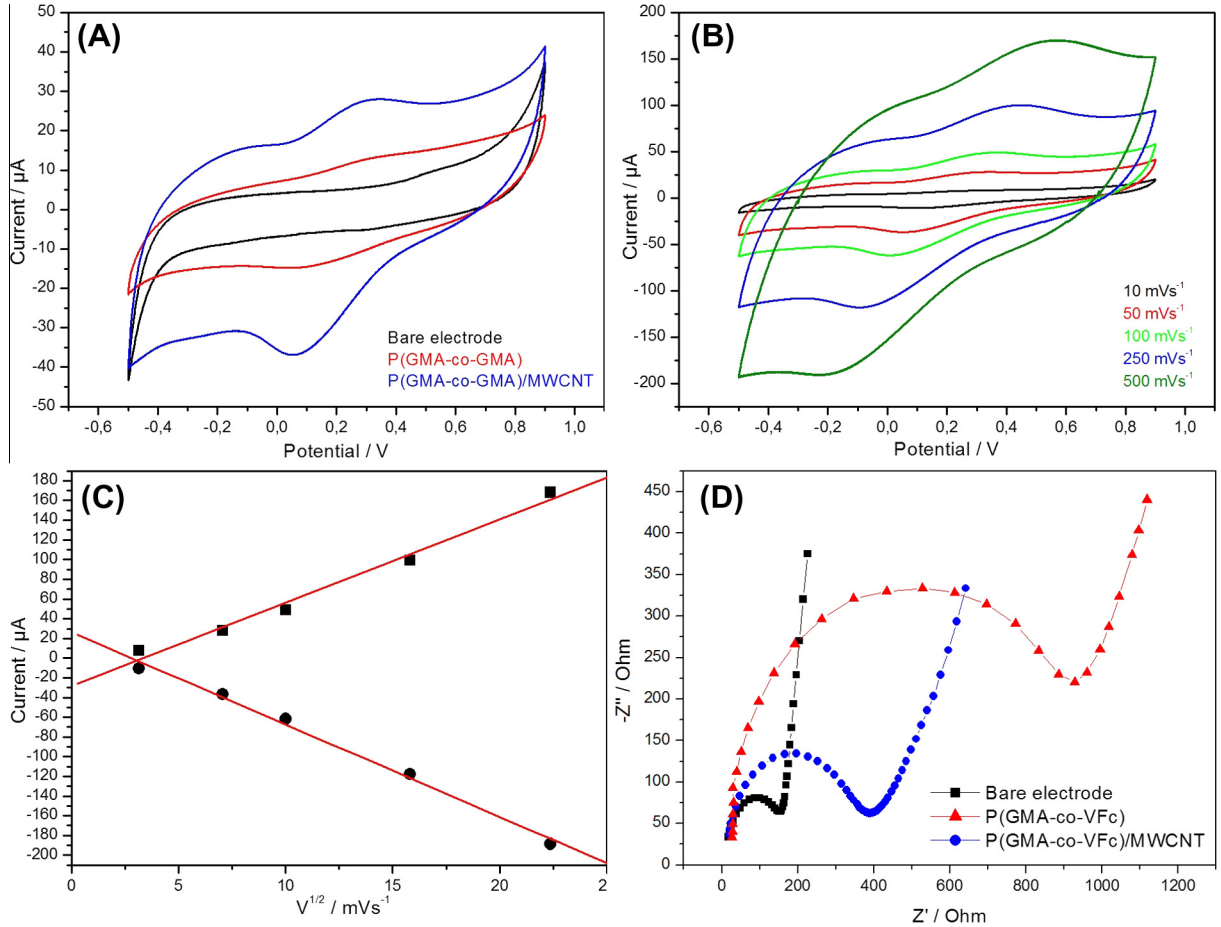

Fig. 2. (A) Cyclic voltammograms of the electrode modified with different components scan rate, 100 mV s^{-1} in 0.1 M PBS (pH 7.0) at scan rate of 100 mV/s. (B) Cyclic voltammograms of P(GMA-co-VFc)/MWCNT coated PGE electrode in 0.1 M PBS (pH 7.0) at scan rates of 10, 50, 100, 250 and 500 mV/s (from internal to external). (C) Plot of the anodic (Ip,a) and cathodic (Ip,c) current with the square root of scan rate. (D) Electrochemical impedance spectra of PGE, P(GMA-co-VFc) and P(GMA-co-VFc)/MWCNT coated electrodes in 100 mM PBS solution, pH 7.0.

Such a fast response could be attributed to the cooperation of MWCNT and polymeric mediator host matrix layer. The current response increased linearly with the increase of xanthine concentration ranging from 2 to 28 µM with a correlation coefficient of 0.99897, 28–46 µM with a correlation coefficient of 0.99904 and 46–86 µM with a correlation coefficient of 0.99908 (insets of Fig. 3D). The sensitivity and the detection limit were 16 mAM^{-1} and 0.12 µM, respectively, (S/N = 3). The prepared xanthine biosensor showed high sensitivity, low detection limit, fast response, which could be attributed to good electrocatalytic activity, high conductivity, and large surface-to-volume ratio of P(GMA-co-VFc)/MWCNT nanocomposite film. A further comparison of the performance of different electrochemical biosensors for the determination of xanthine was summarized in Table 1. Compared with some reported amperometric xanthine biosensors, the LODs in this work was lower than that in ferrocene modified polypyrrole coated Pt/crosslinking (1 µM) (Arslan, Yaşar, & Kiliç, 2006) and cobalt oxide nanoparticle/chitosan/multiwalled carbon nanotube/composite film/crosslinking (2 µM) (Dalkiran et al., 2014) and the sensitivity of the biosensor higher than that in Gold coated iron nanoparticles/chitosan composite/covalent immobilization (0.001169 mAµ M^{-1} cm^{-2}) (Devi et al., 2013).

3.4. Operational and storage stability

The reusability of the biosensor was performed successfully using the same biosensing electrode. All measurements were performed in 10 mM PBS at +0.35 V applied potential and after each measurement electrode was stored for recovery itself (4 °C for 10 min). Relatively closed current response was obtained from the first five measurements and a decrease of electrode response was observed for further measurements (Fig. 4A). The enzyme electrode was, after 15 successful measurements, retained almost 70% of its activity. This may be explained as good interaction between enzyme and the conductive composite material to stabilize the enzyme.

As a performance parameter of the biosensor long-time stability needs to be tested. As shown in Fig. 4B, the stability of the biosensor was investigated by regular experiments for 25 days. During the period of storage, the same enzyme electrode was used and when not in use it was stored in 10 mM PBS pH 7.0; 4 °C. Clearly understood from the figure, the biosensor gave very close results for the first four measurements (17 days) and retained almost all its original activity which is possibly related to enzyme immobilization method and the biological compatibility of composite film. After that point, electrochemical response quickly reduced was observed. This reduction of the enzyme activity as a function of time can be explained by denaturation and or some of the immobilized enzymes were being dropped. At the end of 25 days biosensor was retained 70% of its activity.

3.5. Interference, recovery and real sample measurements

Substances which can have an interference effect on the current response of the biosensor to xanthine were tested. 1:1 ratio four different interfering substances which are glucose, uric acid (UA),

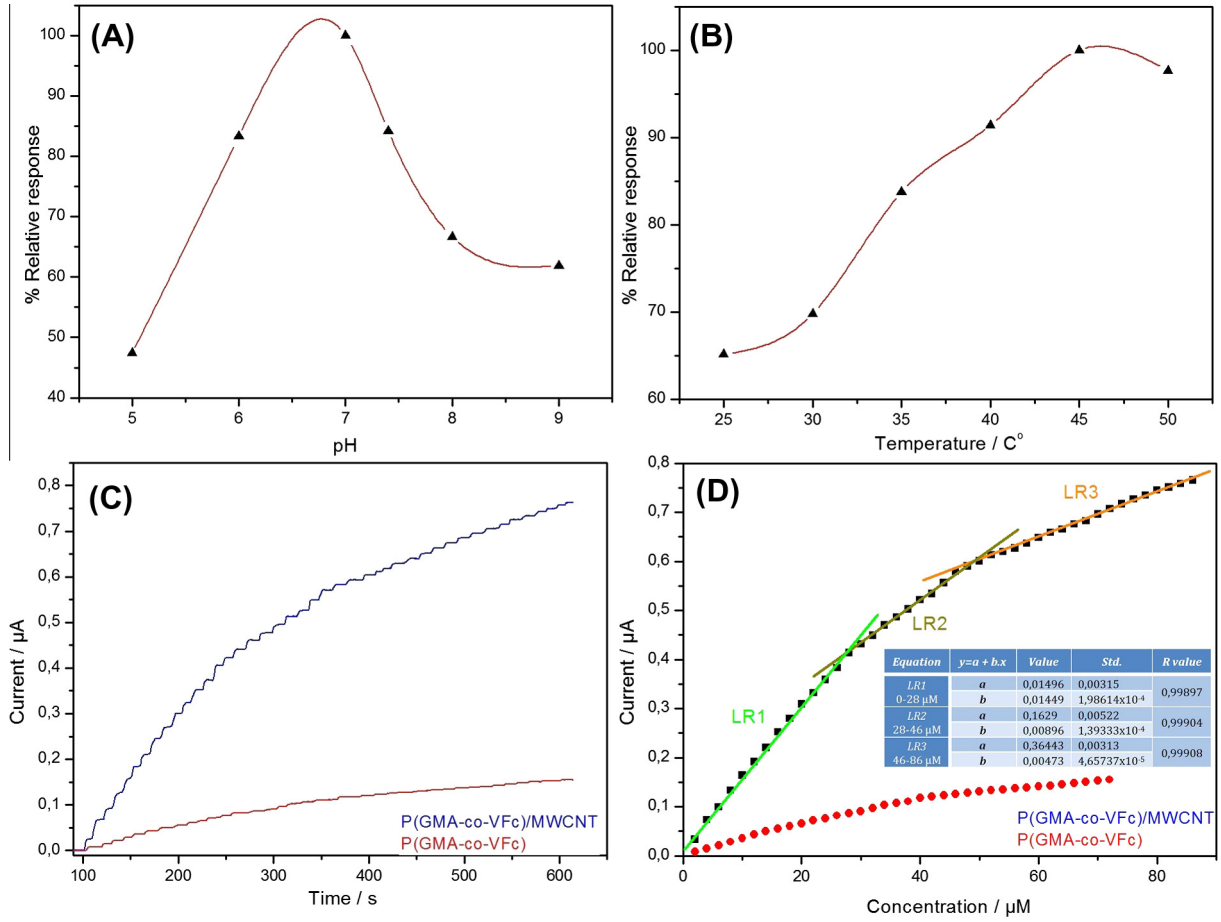

Fig. 3. (A) Effect of buffer pH on the amperometric response to xanthine at an applied potential +0.35V in 10 mM phosphate buffer system (PBS). (B) Influence of temperature on the amperometric response to xanthine at an applied potential +0.35 V in 10 mM PBS. (C) Amperometric response of P(GMA-co-VFc) and P(GMA-co-VFc)/MWCNT coated electrodes to successive addition of 2 μM xanthine at an applied potential +0.35 V in stirred 10 mM PBS (pH 7.0; ~25 °C). (D) Calibration curves for the amperometric response of the biosensing electrodes.

Table 1
Analytical performance of the xanthine biosensors reported in literature.

Electrode	RT(s)	linear range (μM)	Sensitivity	DL (μM)	Refs.
XO/Co_3O_4/MWCNT/CS/GCE	5	0.2–16	–	0.2	Dalkiran et al. (2014)
XO/ZnO-NPs-PPy/Pt	5	0.8–40	–	0.8	Devi et al. (2011)
Modified graphite/gelatin	120	0–40	0.210 μA μM^{-1}	4.5	Dimcheva, Horozova, and Jordanova (2002)
Prussian blue/SPTE	–	0.1–4.98	13.83 mAM^{-1}	–	
XOD/CHT/Pt/PANI/Fe_3O_4 NPs/CPE	8	0.2–36	1.0 μA μM^{-1}	0.10	Sadeghi, Fooladi, and Malekaneh (2014)
XOD/AgNPs/l-Cys/Au	5	2–16	0.20 μA μM^{-1} cm^{-2}	0.15	Devi et al. (2013)
P(GMA-co-VFc)/MWCNT/XO	4	2–48	16 mAM^{-1}	0.12	This work

Note: Response time (RT), detection limit (DL).

ascorbic acid (AA), sodium benzoate (SB), were used to determine the effect of interference to the biosensor performance respectively (Fig. 4C). It is understood from the Fig. 4C, between two additions of 5 μM xanthine, very little effect of the interfering substance on the amperometric response of the biosensor was observed.

After the death of fish, the freshness of fish products has to be protected at all times until it reaches the end consumer. Within this period, depending on the time, the increase in the amount of xanthines (Kassemsarn et al., 1963) affects the quality (lowers) of the meat. The concentration of xanthine was measured and the results interpreted using previously obtained optimum parameters. Detection limit (0.12 μM) and linear range (2–26 μM) of biosensors were taken into consideration while performing real sample measurements. When compared to other xanthine biosensors in which real sample values are out of the linear range of biosensor (Kavitha, Sakthivel, Swaminathan, John, & Uma, 2013). However, in this study real sample values are found to be inside the limitations of proposed P(GMA-co-VFc)/MWCNT based xanthine biosensor and as such, very reliable. Fig. 4D shows increments in the amount of the xanthine as the storage time was increasing. Xanthine value in the first days of measurements are matching values reported by Zen, Lai, Yang, and Kumar (2002) which reports xanthine concentration 0.5–8.8 μM after first days of measurements and the results are including values previously reported for common fish fillet by Balladin, Narinesingh, Stoute, and Ngo (1997) (1.54–24.7 μM). Fish samples were stored at

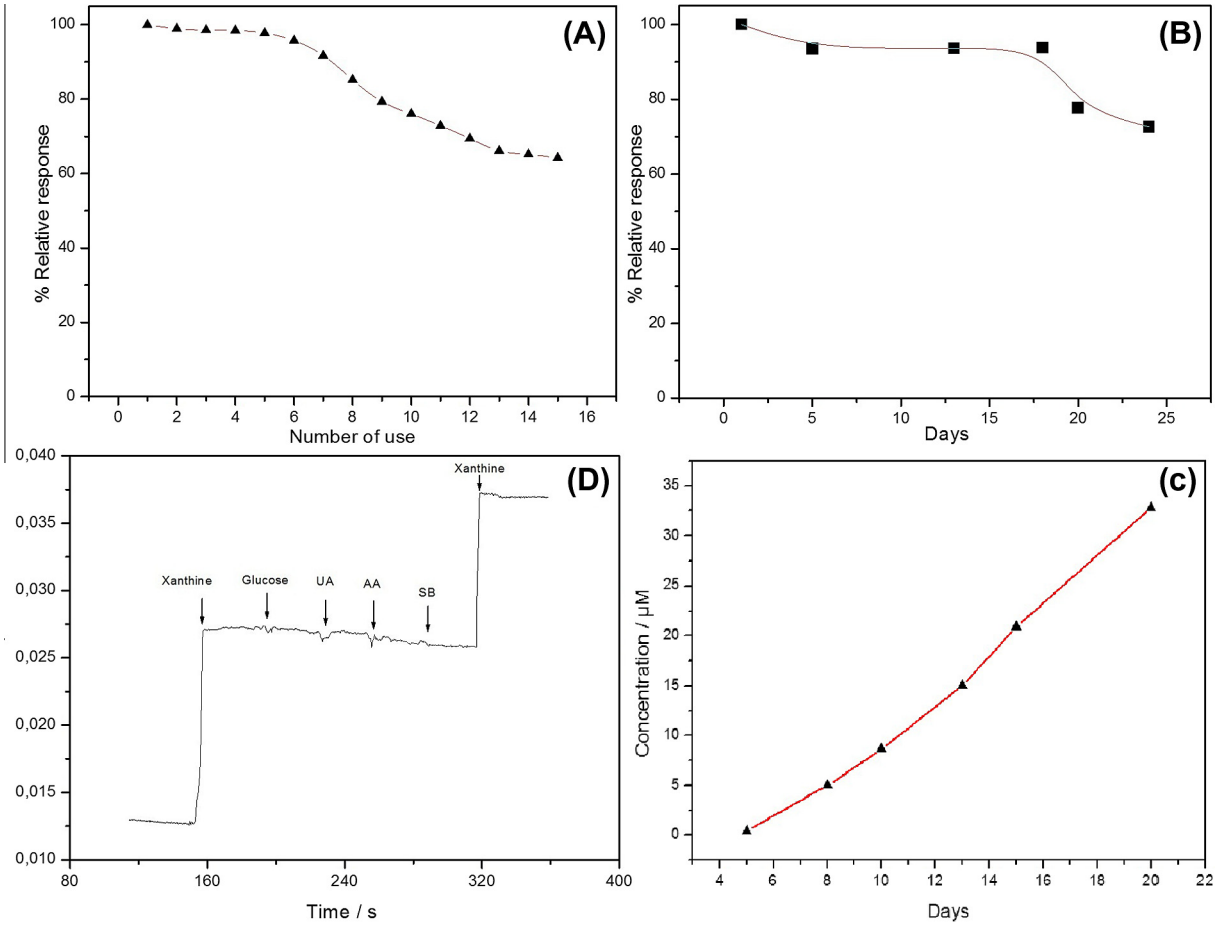

Fig. 4. (A) Operational stability of the prepared biosensing electrodes to xanthine additions (pH 7.0; 25 °C). (B) Storage stability of the prepared biosensing electrodes to xanthine additions (pH 7.0; 25 °C). (C) Interference effect of glucose, uric acid (UA), ascorbic acid (AA), sodium benzoate (SB) to the current response of xanthine in 10 mM pH 7.0 PBS at +0.35 V. (D) Determination of xanthine in fish samples using the prepared biosensing electrode (10 mM pH 7.0; 25 °C; +0.35 V).

Table 2
Analytical recovery of the xanthine biosensor.

Xanthine added µM	Xanthine found µM	Error %
2.5	2.32	6.32
5	4.67	7.06
10	9.87	1.31

different time ranging from 5 to 20 days at room temperature. Before each measurement, samples were homogenized in 10 mL of distilled water and filtered through a membrane. The filtrate was used for amperometric detection of xanthine level.

Analytical performance of the biosensor was performed by the additions of a known concentration of the xanthine as shown in Table 2. The amperometric current response obtained from each xanthine edition (known amount) (in 10 mM pH 7.0 PBS; 25 °C; 0.35 V), and using the calibration curve (Fig. 4B) biosensor effectiveness was tested by calculating the errors. Error rates show that biosensor measuring accuracy is quite high.

4. Conclusions

Herein, a novel bionanocomposite was prepared and used, construction of xanthine biosensor. The enzyme, xanthine oxidase, was covalently immobilized on the nanocomposite support matrix without using any coupling reagent. The use of a MWCNT resulted in the improved analytical performance of the xanthine biosensor in terms of low response time (~4 s), good sensitivity (16 mAM^{-1}),wider linear ranges (2–28 µM, 28–46 and 46–86 µM), and no interference by compared with previously reported biosensors. Attending to these results, this nanocomposite mediator film provides a new electrochemical platform for preparing new amperometric enzyme biosensors.

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