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Novel amperometric xanthine biosensor based on xanthine oxidase immobilized on electrochemically polymerized 10-[4*H*-dithieno(3,2-*b*:2′,3′-*d*)pyrrole-4-yl]decane-1-amine film

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ABSTRACT

In this paper, a novel amperometric xanthine (X) biosensor is constructed by xanthine oxidase (XOx) immobilization on the pencil graphite electrode (PGE). Xanthine oxidase is immobilized using glutaraldehyde (GA) on the electrochemically polymerized conducting polymer film. The detection of xanthine is based on its consumed amount due to the enzymatic reaction of xanthine oxidase. The effects of polymer thickness, applied potential, pH, and temperature were investigated and optimum parameters were found to be five cycles, +5 V, +0.5 V and 30 °C, respectively. Storage stability, operation stability of the enzyme electrode, and effect of interferant substances on the amperometric response were also studied. In order to verify the applicability of proposed biosensor, fabricated electrode was used to measure the xanthine concentration in chicken meat samples. The present xanthine biosensor with high selectivity, sensitivity, and stability is promising for practical applications.

1. Introduction

Biosensors are the subject of extensive research for the development of wide fields of application including medical diagnostics, environmental monitoring, and food analysis [1]. Two staged procedures including preparation of biocompatible support and enzyme immobilization are followed in the production of the enzyme based biosensors where the immobilization of enzyme is the most important part. Immobilization of enzymes on the desired biosensor surface and maintaining their activity during a desired application are important factors in constructing qualified enzyme based biosensors. Enzymes are complex and expensive molecules; hence, it is difficult and costly to separate them from the reaction mixture conserving their full activity. On the other hand, immobilized enzymes have advantages of repeated usage, easy separation from the product environment, enhanced stability, and reduction in the cost of operation [2]. Enzyme immobilization is strongly dependent on the properties of the support. Providing simplicity and high reproducibility in preparation, easiness in arranging the thickness of the film, tunable physical and optical properties, compatibility with biological molecules, and possibility to produce at room temperature make electrochemically synthesized conducting polymers charming in designing biosensors [3,4]. The usage of polymers in the production of biosensors brings the advantages of minimizing the access of interfering compounds to the sensor surface and preventing biofouling [5]. Polymeric conducting dithieno(3,2-*b*:2′,3′-*d*)pyrrole (DTP) derivatives are becoming prominent as useful structures for both molecular and polymeric materials in the biosensor design. They have planar structures, fused ring systems, electron releasing groups, and conjugation properties to increase the electron transfer rate in the enzyme based biosensors. Therefore, these properties make them very important in overcoming the difficulty of the direct electron transfer between enzyme and the electrode [6].

Conducting polymers which are the π-conjugated organic materials are the rapid and considerably growing area of organic materials [7]. Synthesis and characterization of variety of these π-conjugated organic materials have been done to be applied practically in fabrication of batteries [8,9], electronic devices [10], sensors and capacitors [11–14], and electromagnetic devices

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[15] because of beneficial optical and electronic properties, and unique advantages such as having low-cost, simple manufacturing processes, and light weight [16]. In recent years, in this area, DTP moiety received much attention. The synthesis of DTP type conducting polymers is very important due to thiophene–pyrrole–thiophene fused ring system. Having good planar structure, extended conjugation, and strong electron-donating ability, and giving the opportunity to be very easily substituted by functional groups are very well-known and useful properties of DTP type materials [17]. They have been used beneficially in OLED [18], OTFTs [19], FET [20], and photovoltaic cells [21].

Meat and meat products are currently one of the most important components of a healthy and well balanced diet due to their nutritional richness [22]. Therefore, the need for keeping freshness of meat on acceptable quality level is of highly importance in food industries to manufacture safe and qualified products, especially when meat consumption rapidly increases as population increases. Xanthine is an intermediate of the purine nucleotide and deoxynucleotide metabolism [23]. As the metabolic precursor of uric acid, xanthine is the first indicator of an abnormal purine profile and can serve as a marker of various diseases [24]. In the food industry elevated levels of hypoxanthine and xanthine are important biomarkers as a sign of meat spoilage [25]. Xanthine is generated from adenosine triphosphate (ATP) degradation occurring in muscle according to the following sequence;

ATP → ADP → AMP → IMP → HxR → Hx → X → UA

adenosine di phosphate (ADP), adenosine 5′ phosphate (AMP), inosine 5′ phosphate (IMP), inosine (HxR), hypoxanthine (Hx), xanthine (X), and uric acid (UA) [26–29]. Food industry, evaluates the quality of a product through periodic chemical and microbiological analyses, using conventional techniques such as chromatography, spectrophotometry, electrophoresis, titration and others, which do not allow an easy continuous monitoring because they are expensive, time consuming, they have need for well trained personnel and in some cases, require steps of extraction or sample pretreatment which additionally increases analysis time [30]. Application of aptamers in biosensors has been taken a lot of attention lately because of their high chemical stability [31]. However, RNA aptamers are susceptible to degradation by endogenous ribonucleases therefore biosensor based on RNA aptamers are used only for single shot measurement [32] which is not sufficient enough to apply this type of biosensors in biological surroundings. In the time of unceasing technological development, construction of small compact amperometric biosensor for cheap and continuous monitoring, with short response time, low detection limit, high sensitivity and simple usage, can easily overcome above limitations. Combination of complex chemical, microbiological, and physical processes leads to the loss of freshness and finally spoilage when the most important criterion in meet quality is to ensure that it is fresh enough in daily routine basis until reaching the consumers [33].

The present work describes a novel amperometric xanthine biosensor which can determine xanthine simply, fastly, and with high sensitivity by using a DTP type monomer, DTP-alkyl-NH_2 (10-[4*H*-dithieno(3,2-*b*:2′,3′-*d*)pyrrole-4-yl]decane-1-amine), which was electrochemically polymerized on pencil graphite electrode and used as an immobilization matrix for the enzyme. Xanthine oxidase enzyme was finally immobilized on P(DTP-alkyl-NH2) coated PGE by using glutaraldehyde. DTP-alkyl-NH_2 was synthesized and characterized in the previous study [30]. This work was focused on: (i) electrode construction, electrochemical properties 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 chicken meat.

2. Experimental part

2.1. Materials

Xanthine oxidase and xanthine were purchased from Sigma–Aldrich. The synthesis of monomer DTP-alkyl-NH_2 (10-[4*H*-dithieno(3,2-*b*:2′,3′-*d*)pyrrole-4-yl]decane-1-amine) was reported in our previous study [34]. All the chemicals employed in the study were analytical grade and used as received without a purification.

2.2. Preparation of enzyme electrode

The xanthine biosensing electrode was prepared according to following procedure as illustrated in Fig. 1. Prior to any modifications on pencil graphite electrodes (PGEs), PGE was rinsed with distilled water (DW) and acetone to remove all adsorbed materials from the electrode surface. Electropolymerization of DTP-alkyl-NH_2 on PGE was performed in conventional three electrode cell system where PGE is working, platinum wire auxiliary, and Ag/AgCl is reference electrode by means of cyclic voltammogram technique in the range of −1.5 V to 2.5 V applied potential and at 100 mV/s scan rate on CHI6005D electrochemical workstation at room temperature (RT). In the sequel, polymer coated PGE was immersed in 2.5% glutaraldehyde for 3 h. Xanthine oxidase (XOx) integration into the biosensing compartment of the electrode as final step of working electrode preparation was done by immersing PGE in 2 U/mL XOx ammonium sulfate suspension (X1875, Sigma) for 48 h on orbital shaker set at 150 rpm at 4 °C.

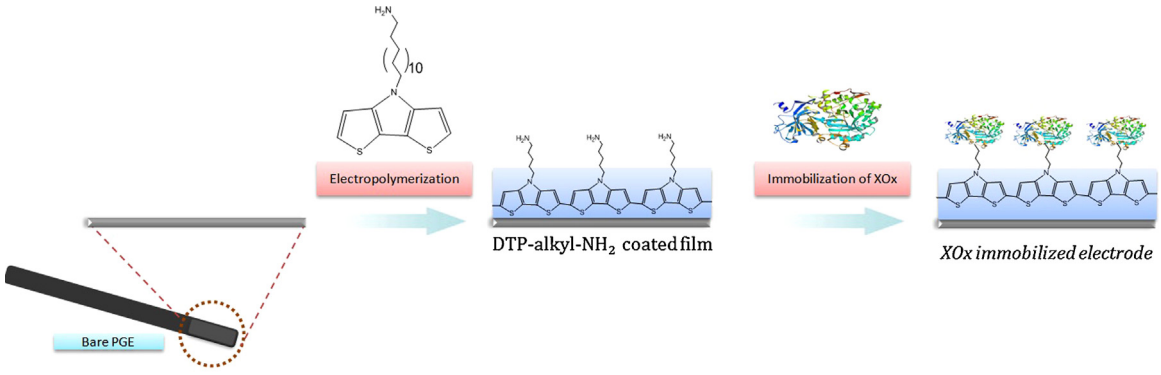

Fig. 1. Schematic illustration of preparation of XOx immobilized biosensing electrode.

2.3. Xanthine determination in chicken samples

Chicken was purchased from regional market, cut into pieces, homogenized, and mixed with DW with a ratio of 1:3 (w/w), chicken and DW, respectively. Homogenate was strained trough filter paper then analyzed in terms of xanthine content with working PGE in 10 mM (pH 7.0) PBS at optimum conditions with the exception of temperature being RT. The xanthine measurements were carried out among samples awaited for 5, 11, 16, 20, and 27 days at 4 °C while data were interpolated using standard curve (concentration vs. current) prepared under optimum working conditions.

2.4. Instrumentations

Electropolymerization experiments were performed using CHI6005D Electrochemical Analyzer (CH Instruments) in traditional three electrode system comprised of Ag/AgCl-saturated KCl as reference, Platinum wire as auxiliary and bare PGE as working electrode all immersed in electrochemical cell containing polymer. Electrochemical measurements were carried out using CompactSoft portable electrochemical interface and impedance analyzer (*Ivium Technologies*) in usual three electrode system where working electrode was PGE/DTP-alkyl-NH_2/glutaraldehide/XOx in electrochemical cell containing phosphate buffer solution (PBS).

2.5. Electrochemical measurements

Electrochemical measurements of xanthine with PGE/DTP-alkyl-NH_2/glutaraldehide/XOx was accomplished by adding known amounts of xanthine after the system reaches the steady state current into three-electrode-electrochemical cell containing 10 mM PBS at pH 7.0, 0.5 V applied potential, and continuously stirred at RT. The substrate solution was freshly prepared by dissolving xanthine in 2:8 (v/v) 1 M NaOH and PBS, respectively, and the amperometric responses of working electrode to known amounts of xanthine additions were recorded.

3. Results and discussion

3.1. Morphological characterization

The morphology of the prepared biosensing electrodes was monitored by using scanning electron microscopy (SEM). Fig. 2 shows SEM images of the PGE, PGE/DTP-alkyl-NH_2, and PGE/DTP-alkyl-NH_2/XOx. The surface of the electrochemically polymerized DTP-alkyl-NH_2 coated PGE showed porous granular morphology which indicates the formation of polymer on PGE surface. After immobilization of xanthine oxidase with glutaraldehyde, the porous granular morphology changed to regular form indicating the successful immobilization of XOx on the surface of the modified electrode. The enzyme molecules were utterly bound at the outer layer owing to their relatively larger size. By this way, the substrate has a higher possibility to access to the biolayer.

3.2. Determination of experimental variables

The thickness of the polymer interface between enzyme and the electrode surface is very important parameter due to the electron transfer function of that polymer layer. The effect of polymer film thickness was investigated by measuring the amperometric response of biosensing electrode. Polymers with different thicknesses were deposited on the working electrodes with 1, 3, 5, 10, 15 and 20 scans to find the optimum thickness. The thickness can be measured in terms of the charge passing through the cell. The charges and film thicknesses for 1, 3, 5, 10, 15 and 20 cycles polymer films were calculated as 0.06 mC (8 nm), 0.62 mC (17 nm), 0.86 mC (25 nm), 1.43 mC (48 nm), 2.12 mC (74 nm) and 2.83 mC (86 nm), respectively. As shown in Fig. 3A, the apparent activity of biosensing electrode did not increase linearly with increased composite film thickness. *I* increased with increasing composite film thickness until the electropolymerization cycle number is 5. However, *I* clearly decreased with polymer deposition more than five cycles resulting mainly due to the possible diffusion problems which may arise from high polymer layer thickness.

The effect of applied potential on biosensor's amperometric response to substrate concentration was investigated over the range of −0.3 V to 0.7 V as shown in Fig. 3B. In comparison with high applied potentials, low potentials −0.3, 0.0, and 0.3 V had negative effect on amperometric response of enzyme electrode. Maximum current response was obtained at 0.5 V after which increase of applied potential decreased electrodes' responses almost for 40%. Therefore, optimum working potential for biosensor was selected as 0.5 V and it was used in further experiments.

In optimizing parameter for pH dependence of biosensor, experiments were performed over the range of pH 5.0–8.0 in the 10 mM phosphate buffer solution (PBS) with an applied potential of 0.5 V. In Fig. 3C experimental results of current response are represented. As it can be seen, biosensor's response slightly increased as pH increased reaching maximum response at pH 7.0 after which response drastically decreased almost for 30% at pH 7.4 and continued to decrease as pH increased. From results obtained it can be concluded that acidic systems are reducing electrodes response slightly less when compared with basic systems. As well it can be clearly seen strong effect of different pH environments to electrodes response which probably might be occurred due to enzyme deformations which leads to loss of catalytic capability. These optimization experiments also showed that there is no big influence on the xanthine oxidase enzyme's optimum pH when immobilized on proposed biosensor, which is also reported in literature [35–38]. Therefore, pH 7.0 was selected as the working pH for biosensor in subsequent experiments.

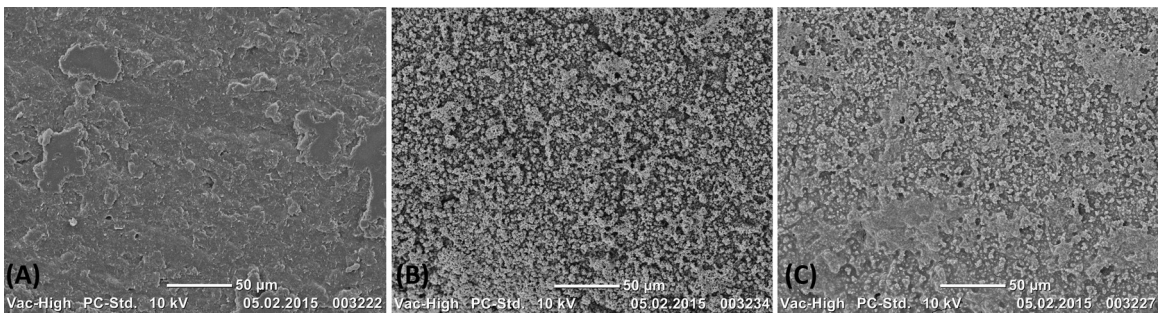

Fig. 2. SEM images of the (A) PGE, (B) PGE/DTP-alkyl-NH_2, (C) PGE/DTP-alkyl-NH_2/XOx.

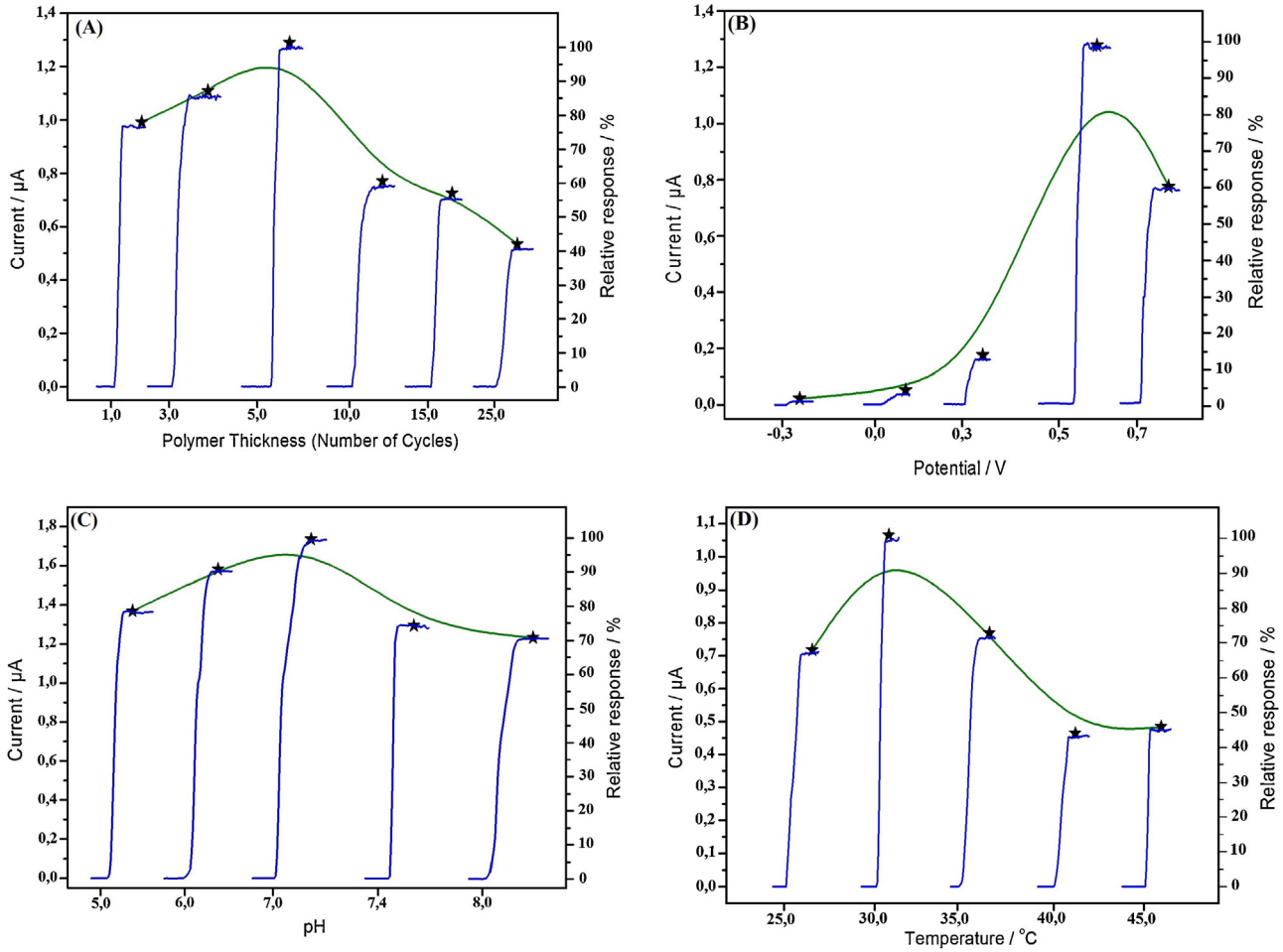

Fig. 3. (A) Amperometric response of enzyme electrode with different thickness of electropolymerized polymer on the xanthine substrate. (B) Effect of applied potential on the response of biosensor on the xanthine substrate (10 mM PBS pH 7.0). (C) Effect of amperometric response of biosensor to xanthine in PBS at different pHs at an applied potential +0.5 V in 10 mM. (D) Influence of temperature on the amperometric response to xanthine at an applied potential +0.5 V in 10 mM pH 7.0 PBS.

Influence of temperature on amperometric response of biosensor was studied across a range of 25–45 °C and given in Fig. 3D. The current response of enzyme electrode significantly increased giving maximum response at 30 °C after which decrement has occurred in response. There was almost 30% at 35 °C and 55% at 45 °C loss in current response comparing to the one at 30 °C making 30 °C as optimum working temperature for proposed biosensor. Loss of amperometric response at higher temperatures might occur because of thermal deactivation of enzyme at higher temperatures and because of decrement of molecular oxygen in solution [39], in this case above 30 °C. From experience in our early studies on xanthine biosensors [27,35] in which optimum temperature was much higher than work presented can be attributed to immobilization materials used. However in this work optimum temperature of 30 °C is much more closer to room temperature and as such it is easier to apply biosensor on-site detections, and save extra time and effort to heat up sample in order to get reliable response.

3.3. Amperometric response of enzyme electrode

Fig. 4A shows the amperometric response plot obtained with a XOx immobilized conducting polymer coated electrode during the successive addition of xanthine in a 10 mL of PBS (10 mM, pH 7.0) at the optimized detection potential of 0.5 V and pH of buffer obtained from above studies. The modified electrode achieved 95% of steady-state current within ~5 s and the sequential wise increase in xanthine addition is observed. Fig. 4B shows the current vs. xanthine concentration based on the data from Fig. 4A. Deviation from linearity is observed at higher (>25 µM) xanthine concentration, representing a typical characteristic of the Michaelis–Menten model. The current response increased linearly with the increase of xanthine concentration ranging from 0.3 to 25 µM with a correlation coefficient of 0.99894 (insets of Fig. 4B). The sensitivity and the detection limit were 124 mA M^{-1} and 0.074 µM, respectively, (S/N = 3). Linear range and sensitivity along with detection limit and response time for each electrode are summarized in Table 1. The proposed biosensor exhibited a good hydrodynamic range. The detection limit was much lower than previously described xanthine oxidase-based electrochemical xanthine biosensors [27,35,33,41]. The sensitivity of the biosensor is higher than that in iron oxide nanoparticles decorated REGO and polyaniline [27,33].

3.4. Operational and storage stability

Operational stability of amperometric biosensor was investigated by measuring current response of same electrode by 20 consequent measurements represented by number of use (*x*-axis), as shown in Fig. 5A. Measurements were performed in 10 mM pH 7.0 PBS at 0.50 V applied potential. Biosensing electrode recovery experiment was performed by storing it at 4 °C for 5 min after each measurement. Amperometric response was relatively close for the first six measurements after which response diminished

Table 1
Comparison of analytical performance of the prepared biosensor with previously prepared biosensing electrodes.

Electrode material	RT (s)	LR (μM)	DL (μM)	Sensitivity (mA M^{-1})	Ref.
P(DTP-alkyl-NH_2)	5	0.3–25	0.074	140	This work
P(GMA-co-VFc)/REGO-Fe_3O_4	3	2–36	0.17	0.17	[27]
P(GMA-co-VFc)/MWCNT	4	2–48	0.12	16	[35]
ZnO-NPs-PPy	8	0.8–40	0.80	NR	[40]
CHT/Pt/PANI/Fe_3O_4	8	0.2–36	0.10	1.0	[33]
Multi-wall carbon nanotubes	2	0.2–10	0.1	NR	[41]

LR, linear range; DL, detection limit; NR, not reported.

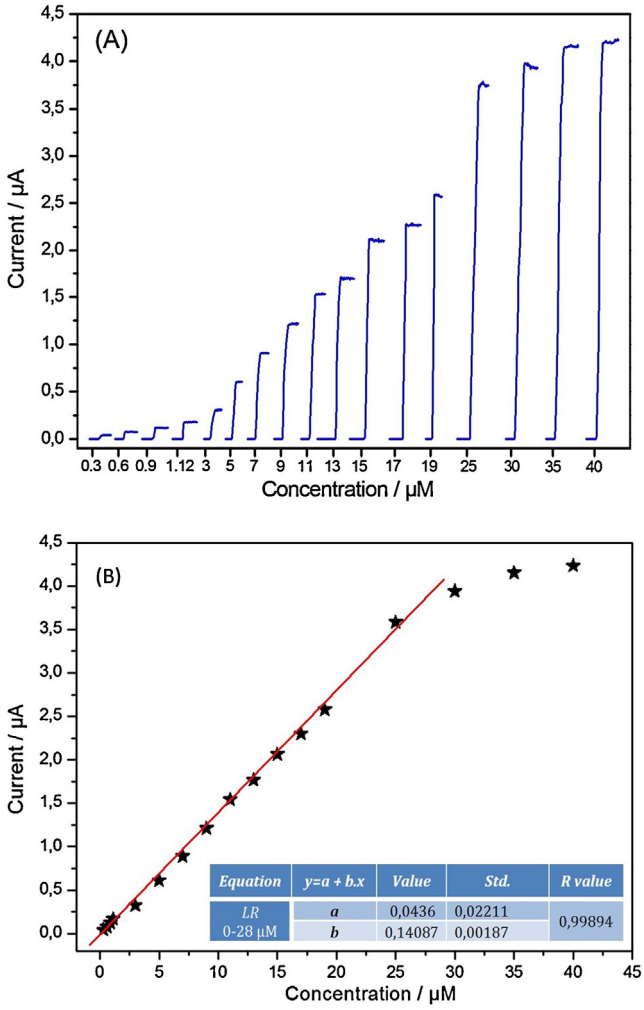

Fig. 4. (A) Amperometric response of xanthine biosensor on known xanthine concentration at 10 mM (pH 7.0) PBS and applied potential of +0.5 V. (B) Calibration curve of xanthine concentration obtained from the amperometric response of the xanthine oxidase based electrodes.

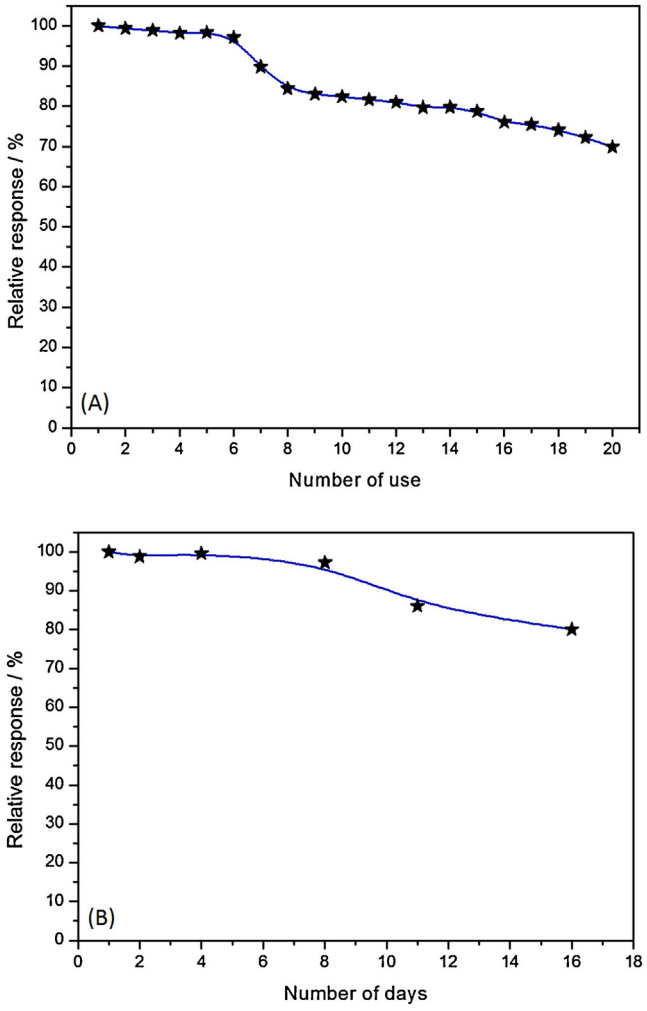

Fig. 5. (A) Operational stability of proposed biosensing electrodes to consequent xanthine additions. (B) Storage stability of the prepared biosensing electrodes to xanthine additions (stored at 4 °C in 10 mM pH 7.0 PBS).

to 80% of its initial response. As measurements were prolonged, current response started slowly to decrease and eventually after 20 repeated measurements electrode retained almost 70% of its initial response. Relative response represents amperometric current response difference in percentages (%), as well it provides easy comparison among electrodes responses which are studied under different conditions such as pH, applied potential, temperature and particularly in operational stability.

The long-term stability of enzyme electrode towards xanthine was assessed by regular experiments over 16 days. Electrode was kept in 10 mM pH 7.0 PBS at 4 °C while being not used. As demonstrated in Fig. 5B, the amperometric current response of biosensor is observed to have 4% loss during the first 8 days of its initial response while the current decreased by ~15% in the 2nd week of long-term storage stability experiments. The reduction in electrocatalytic activity as a function of time of proposed biosensor may be due to denaturation of XOx or leakage of enzyme from the inner membrane. Finally, biosensor retained 80% of its initial activity after 16 days of storage.

3.5. Interference, recovery, and real sample measurements

Analytical recovery experiment of the polymer electrode was performed by recording amperometric current response to known xanthine concentrations as shown in Table 2 (10 mM, pH 7.0 PBS; room temperature, applied potential 0.50 V). Xanthine amount was

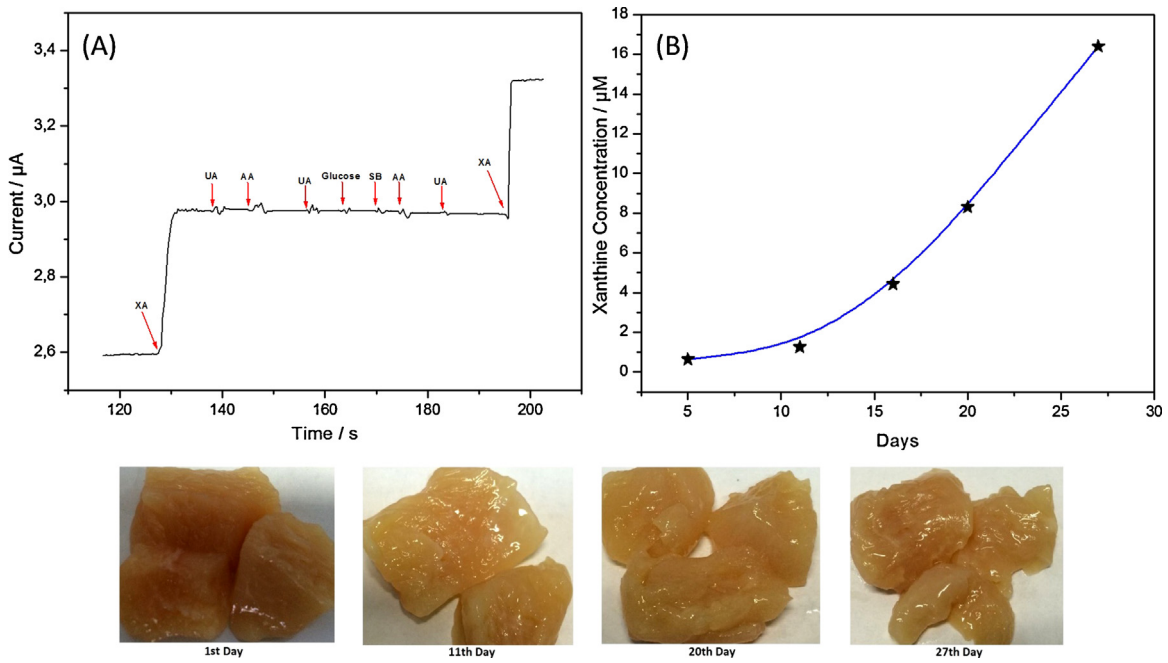

Fig. 6. (A) Effect of potential interferants (uric acid (UA), ascorbic acid (AA), glucose, and sodium benzoate (SB)) to the amperometric current response of biosensing electrode (10 mM PBS pH 7.0). |(B) Determination of xanthine concentration in chicken samples stored in fridge during 27 days. Visual demonstration of chickens meat colour change during 27 days of storage at 4 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calculated using calibration curve shown in Fig. 3B and effectiveness was calculated by obtaining error rates from which can be observed that proposed method is of high reliability. After proving the working concept and reliability of the biosensor, study was continued with interference and real sample applications.

In order to use the biosensor for determination of xanthine level in real samples, a study of the discrimination against possible interferants was carried (uric acid, ascorbic acid, glucose, and sodium benzoate). Fig. 6A examines the effect of interferants on the current response of xanthine biosensor. The response current for a constant concentration of xanthine was compared with the current value that was obtained in the presence of the variable concentrations of the interfering species. The value of I_{max} does not vary significantly in the presence of interferants indicating that those compounds do not interfere with the determination of xanthine.

The practical application by using real samples is very important to show the usefulness of the constructed biosensing electrodes. For this purpose, chicken samples were tested. Chicken samples were stored at different time ranging from 5 to 20 days at room temperature. Before each measurement, samples were homogenized in distilled water and filtered through a membrane, after which filtered sample was added into electrochemical cell containing PGE/DTP-alkyl-NH_2/glutaraldehide/XOx electrode in PBS. This procedure was done according to reported studies in literature [36,33,42,43]. The concentration of xanthine was measured as the storage time was increasing and the results were interpreted using previously obtained optimum parameters. The results obtained with the biosensor for the analysis of all samples were given in Fig. 6B. Linearity was obtained for samples of 10–25 days-old, after which the linearity was found to be lost.

Table 2
Analytical performance of xanthine biosensor.

Xanthine added, µM	Xanthine found, µM	Error, %
3	2.87	4.52
8	7.9	1.26
15	14.85	1.01

4. Conclusions

In this study, XOx is immobilized on the surface of the modified pencil graphite electrode by the electrochemical polymerization of DTP-alkyl-NH_2 polymer. Fabricated biosensor showed high sensitivity, low detection limit, broad dynamic range, fast response time, no significant reduction in signal caused by selected interferants, high stability, and long term reproducibility by retaining enzyme activity. This biosensor provides a simple and rapid method for the determination of xanthine in chicken meat samples. Therefore, xanthine determination can be made in a variety of different samples with this biosensor. Represented DTP type conducting polymer can supply a biocompatible and electrochemical microenvironment for immobilization of the enzyme, making it to be a good candidate for the fabrication of other amperometric biosensors.

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