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A novel electrochemical biosensor based on TetX2 monooxygenase immobilized on a nano-porous glassy carbon electrode for tetracycline residue detection

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ABSTRACT

Different carbon-based nanostructures were used to investigate direct electron transfer (DET) of TetX2 monooxygenase (TetX2), and an enzyme-based biosensor for sensitive determination of tetracycline (TC) also fabricated. A polyethyleneimine (PEI) with positive charge groups was used for immobilization of TetX2 on modified glassy carbon electrodes. Cyclic voltammetry (CV) was employed to study the electrochemical characteristics of the immobilized enzyme and the performance of the proposed biosensor. Amongst multiple carbon-modified electrodes, nano-porous glassy carbon electrode (NPGCE) was selected because of its amplified signal response for flavin adenine dinucleotide (FAD) and superior electrocatalytic behavior toward oxygen reduction. The cyclic voltammogram of PEI/TetX2/NPGCE showed two couple of well-defined and quasi-reversible redox peaks of FAD, consistent with the realization of DET. The prepared electrode was then successfully introduced as a biosensing interface based on the oxygen reduction peak current, resulting in a linear range response from 0.5 to 5 μM with a good detection limit of 18 nM. The as-fabricated electrode demonstrates a fast response and excellent stability for the detection of TC. The results indicate that this simple, rapid, eco-friendly and economic strategy of PEI/TetX2/NPGCE preparation has potential for the fabrication of an enzyme-based biosensor for the practical detection of TC in food products.

1. Introduction

Tetracyclines (TCs) are protein-synthesis inhibitors with a broad antibacterial spectrum which have been extensively employed in agriculture, aquaculture, and animal husbandry because of their effective antibacterial and growth promotion properties [1]. However, the high usage of TCs has led to their accumulation in food products such as eggs, chicken, milk, and meat [2], causing adverse human health effects such as liver damage and allergic reactions. Besides this, an increase in bacterial antibiotic resistance in both veterinary and human due to the extensive usage of TCs is inevitable. Thus, developing simple, selective, and accurate methods for the detection and determination of TCs in food products and pharmaceutical samples is of great importance [3]. To achieve this, various methods including microbiological assay [4], enzyme-linked immunoassay (ELISA) [5], high performance thin-layer chromatography (HPTLC) [6], capillary electrophoresis (CE) [7], liquid chromatography-mass spectrometry (LC/MS) [8] and high-performance chromatography (HPLC) [9] have been widely applied fulfilling the above-stated requirements. However, most of these need sophisticated and expensive instrument, different sample pre-treatments, professional operators, and involve a lengthy turnaround time; limiting their potential for fast, on-site, and real-time TC determination.

Recently, Electrochemical methods, especially electrochemical biosensors, have attracted significant attention thanks to their advantages, such as ease of operation, low cost, amenability to miniaturization, rapid response, capability of on-site analysis, high sensitivity, and selectivity [10]. To fabricate a typical electrochemical biosensor, biological receptors such as antibody, aptamer, DNA, and enzyme are employed as the recognition element. For instance, Xu et al. [11] have developed an electrochemical aptasensor based on modified glassy carbon with antimony tin oxide nanoparticle-chitosan to determine TC with a detection limit of 6.7 nM. Also, Liu et al. [12] immobilized anti-TC monoclonal antibody on gold electrode-modified carboxyl-Fe_3O_4 nanoparticle to fabricate an electrochemical immunosensor for the detection of TC. However, to the best of our knowledge, there have been no reports on the use of an enzymatic electrochemical biosensor for TC detection. Use of enzyme-

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based biosensors provides some remarkable advantages over non-enzymatic sensors, including high selectivity and sensitivity, and less interference from of the possible endogenous species that may coexist in the food matrix.

TetX2 monooxygenase (TetX2), a flavin-containing enzyme with a molecular mass of 44-kDa found in bacteria, catalyzes the regioselective hydroxylation of TC to 11a-hydroxytetracycline via the participation of NADPH as a cofactor and O_2 as electron acceptor [13]. A tightly bound flavin adenine dinucleotide (FAD) cofactor acts as an electroactive species in the active site of the enzyme, which can be electrochemically tracked to investigate the hydroxylation of TC, and thus the determination of TC. Nevertheless, some drawbacks need to be overcome to fabricate enzyme-based biosensors using this strategy. One of these is the weak biostability and low loading capacity of immobilized enzyme. The other is the poor electron transfer between the flavin adenine dinucleotide (FAD) and the electrode surface due to the presence of the protective protein shell which covers the active redox center FAD, preventing direct electron transfer (DET) [14,15]. Therefore, it is necessary to increase the stability of the enzyme on the electrode and to improve DET between the electroactive sites and the electrode surface to fabricate a sensitive and selective enzyme-based biosensor to determine TC with a low detection limit and high reproducibility.

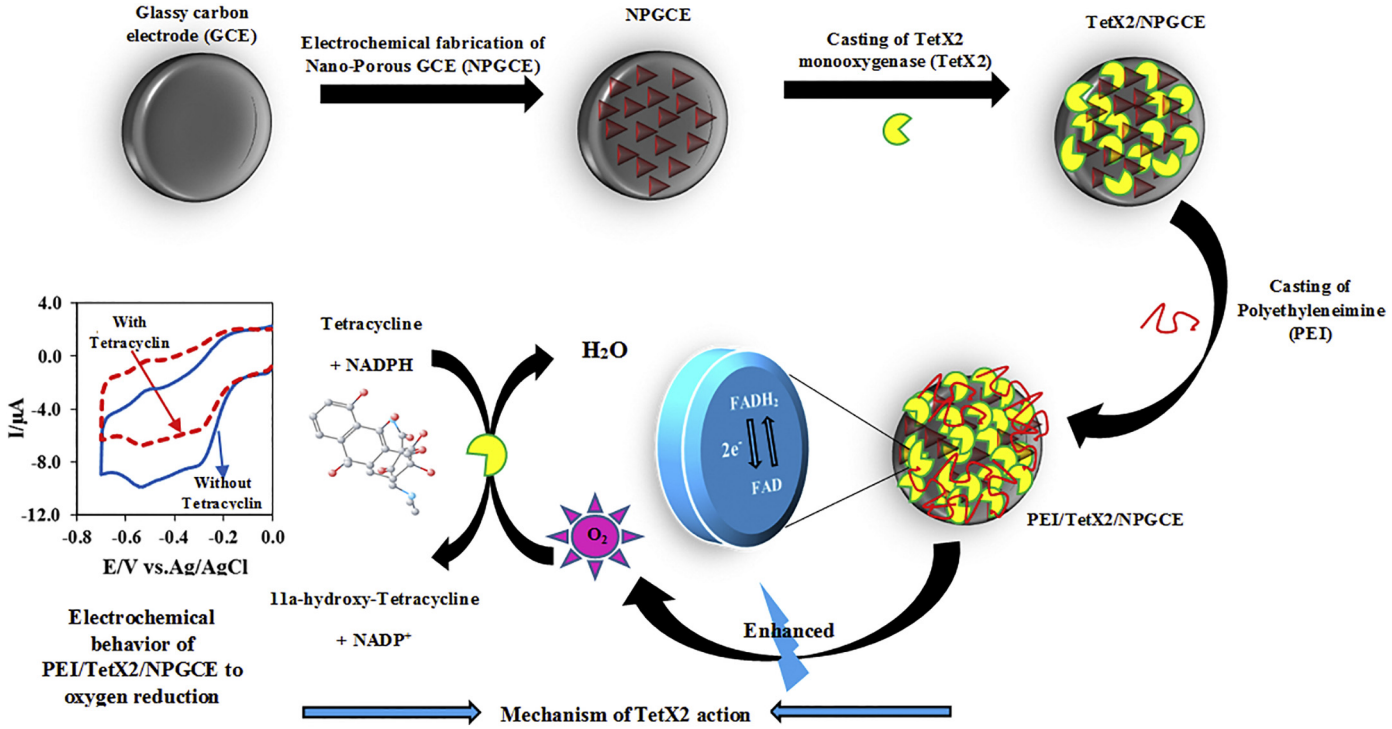
Nanomaterial-based electrodes have shown a number of advantages in electroanalysis such as an improved catalytic reaction, more efficient electron transfer, increased surface area, excellent biocompatibility and good control over the electrode microenvironment [16]. Carbon-based nanomaterials are superior to precious metals (e.g., Pt or Au) due to their low cost, large surface area, excellent mechanical and electrical properties, sufficient stability and high functionality [17,18].

Herein we describe the development of a novel electrochemical biosensor, comprising a TC degrading enzyme, TetX2, immobilized on nano-porous glassy carbon electrode (NPGCE) using polycation polyethyleneimine (PEI), for the specific and fast detection of TC (Scheme 1). For this purpose, cyclic voltammetry was used as an informative method to monitor DET between TetX2 and the electrode surface, as well as the enzymatic reaction through detection of the electroactive substrates and cofactors. In this way, comparison of redox signals on various carbon nanostructures, i.e., multi wall carbon nanotubes (MWCNTs) and reduced graphene oxide (rGO), revealed the suitability of NPGCE with enhanced electrocatalytic activity as an electrochemical sensing substrate for TC detection. Also, the satisfactory conductivity of the nano-porous glassy carbon electrode resulted in the amplification of electrochemical signals, better sensitivity, more reduction of oxygen, and significantly improved the loading capacity for the enzyme, such that this biocompatible composite could serve as a protective substrate to sustain the bioactivity of the anchored enzyme. In the sensor design presented here, indirect quantification of TC was achieved by measuring the decrease in oxygen concentration which was consumed in the enzymatic reaction proportional to the TC concentration. This advanced sensing strategy indicated qualified storage stability, good reproducibility and excellent selectivity, offering a desirable approach with a wide range of applications in the food, environmental and clinical monitoring of TC.

2. Experimental section

2.1. Materials and reagents

E. coli BL21 (DE3) strain bearing an expression vector, pET-28b (+) containing *Bacteroides thetaiotaomicron tetX2* gene was received as a gift from Prof. G.D. Wright (McMaster University, Canada). Purification of His6-tagged fusion enzyme TetX2 from this strain was performed using affinity chromatography. An enzyme concentration of 0.1 mg/ml was used in all spectrophotometric experiments. TC (purity, 98%) was received from Hakim Pharmaceutical Co. (Tehran, Iran). NADPH (in the form of tetra-sodium salt, >95% purity) was obtained from Sigma Aldrich. Isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma Co. (St. Louis, Missouri, US). All other reagents were analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma Aldrich. Na_2HPO_4 solution (0.1 M, pH 8.5) was used as supporting electrolyte, and deionized water was used throughout the experiments.



Scheme 1. The schematic illustration for fabrication of enzyme-based nano-biosensor.

2.2. Apparatus for electrochemical measurements

All electrochemical tests were performed by an Autolab potentiostat-galvanostat model (μ TYPE III, Netherlands). A conventional three-electrode configuration with PEI/TetX2/NPGCE as the working electrode, an Ag|AgCl|saturated KCl as the reference electrode and a platinum rod as the auxiliary electrode was used. The working electrode was a glassy carbon disk electrode (GCE) (diameter 2 mm, geometric surface area 0.03 cm^2). All applied potentials were measured versus Ag/AgCl/saturated KCl and the output signals acquired by GPES software (Metrohm Autolab, Netherlands). Cyclic voltammetry was utilized for electrochemical experiments. The surface morphology of the electrodes was characterized using a DME DualScope Scanner DS 95–200 (DME, Herlev, Denmark) atomic force microscopy (AFM). All experiments were accomplished at ambient temperature.

2.3. Preparation of NPGCE modified by TetX2

Before surface modification, the GCE was polished in turn with alumina powder (0.3, 0.1 and 0.05 μm), rinsed thoroughly with deionized water and subjected to ultrasonic vibration in ethanol and deionized water. NPGCE was fabricated using an electrochemical procedure as described previously [19]. Briefly, a freshly polished and cleaned GCE was anodized at a positive potential of 1.8 V (versus Ag/AgCl/saturated KCl) in phosphate buffer solution (0.1 M) for 10 min to achieve oxidized GCE (GCE-ox). Then, the GCE-ox was reduced at −1.0 V (versus Ag/AgCl/saturated KCl) in the same solution for 1 min to achieve the NPGCE.

The procedures for the expression, purification and concentration and activity determination of TetX2 are described in detail in the Supporting Information (Section 1.1). TetX2/NPGCE was prepared by dropping 3 μl of a 5 mg/ml TetX2 stock solution onto the NPGCE surface, and allowed to dry at room temperature for two hours. Subsequently, to prevent the leakage of the enzyme from the electrode surface, its possible immobilization was investigated using polyethyleneimine (PEI) (20% *V*/V in deionized water) and Nafion (0.2% V/V in light alcohol) as positively and negatively charged polymers, respectively. This step was achieved by casting 3 μl of PEI or Nafion on the surface of the TetX2/NPGCE, separately. Finally, the constructed biosensor was dried at room temperature for approximately 30 min and stored at 4 °C when not in use.

3. Results and discussion

The mechanism for the enzymatic hydroxylation of TC is illustrated in Fig. S1. Enzyme purity and relative molecular weight were examined by SDS-PAGE, shown in Fig. S2. Moreover, the results of TetX2 activity and optimum pH are shown in Fig. S3 and Fig. S4, respectively.

3.1. Direct electrochemistry of the TetX2 on different carbon nanostructures-based electrodes

The FAD moiety, as a tightly bound part of the TetX2 molecule, undergoes a redox reaction. Cyclic voltammograms (CVs) of TetX2 immobilized on different carbon nanostructures, including rGO, NPGCE, MWCNT and a combination of rGO/MWCNT to observe the direct electrochemical behavior of TetX2, are shown in Fig. 1. As shown, no redox peaks were observed at GCE, indicating that GCE was not a suitable substrate to access TetX2 direct electron transfer. However, a couple of well-defined and reversible redox peaks were observed for NPGCE and MWCNT. The result indicates that FAD, the active interior redox center of TetX2, was able to transfer electrons directly to the electrode surface. Furthermore, the higher peak current and lower difference in potential of the redox peaks, ΔE_p, of the NPGCE compared to those of MWCNT and rGO confirmed a better NPGCE capacity for surface modification. The better electrochemical response for NPGCE could be due to adsorption and trapping of TetX2 inside the porous surface of

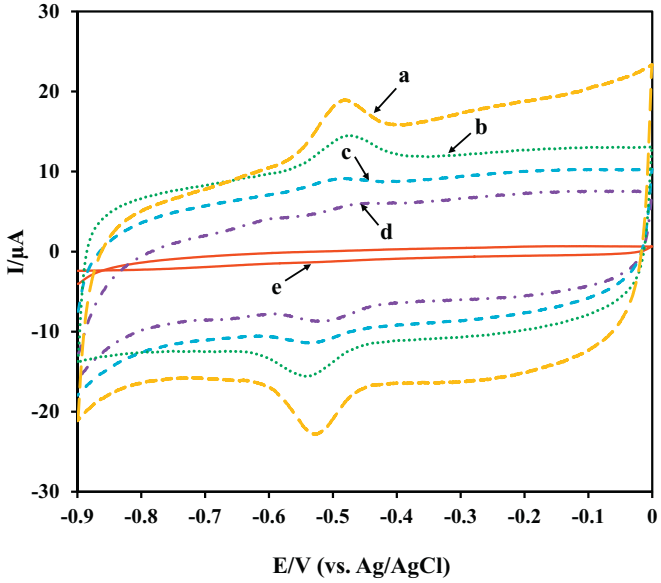


Fig. 1. Cyclic voltammograms of the TetX2 immobilized on the different carbon nanostructures in a N_2-saturated phosphate buffer solution (0.1 M, pH 8.5) at a scan rate of 50 mV s^{-1}. NPGCE (a), MWCNT/GCE (b), rGO/MWCNT/GCE (c), rGO/GCE (d) and GCE (e). 5 μl of the enzyme (conc. 6 mg/ml) was used.

the NPGCE, which decreases the electron transfer distance between the redox site (FAD) and the electrode surface. Therefore, NPGCE was selected as the effective enzyme immobilization substrate for further analysis. The CV spectrum of PEI/TetX2/NPGCE shows a quasi-reversible electrochemical behavior with anodic and cathodic peak potentials at approximately −472 and − 523 mV, respectively, which is in accordance with TetX2 (FAD) to TetX2 ($FADH_2$) conversion [20]. There is no electrochemistry study reported on the possibility of DET between TetX2 and different carbon nanostructures. Achieving DET of redox proteins and enzymes, provides a basis for the fabrication of new kinds of electrochemical biosensors, bio-fuel cells and biomedical devices without the use of artificial electron transfer mediators [21,22]. Ikegami et al. utilized [23] a naphthalenethiol-coated gold electrode to immobilize microsomal human flavin-containing Monooxygenases 1 and 3 (hFMO1 or hFMO3) and observed DET between immobilized enzymes and the modified electrode by the use of cyclic voltammetry. Castrignanò et al. [24] constructed a modified glassy carbon electrode with graphene oxide and didodecyldimethylammonium bromide (DDAB) for immobilization of hFMO3 and carried out electrochemical characterization by cyclic voltammetry. DET was observed between immobilized hFMO3 and the modified electrode and its biotechnological application was successfully demonstrated in the screening of two therapeutic drugs, benzydamine and tamoxifen.

3.2. Characterization of surface morphology and electrochemical properties of the electrodes

The morphological change of the GCE by electrochemical treatment was confirmed by AFM (Fig. S5). These images illustrate the more porous surface of the activated GCE (NPGCE) compared to the smooth surface of the GCE. Quantitatively, the average roughness and the maximum height of the roughness for the NPGCE were 76.1 nm and 88.8 nm, respectively, which consistent with the 10-time higher porosity compared with freshly polished glassy carbon. This result is in line with morphological features reported in other studies [25–27]. Cyclic voltammetry was also employed to investigate the charge transfer property of the $Fe(CN)_6^{3-/4-}$ redox couple at the electrodes (Fig. S6).

3.3. Electrochemical behavior of PEI/TetX2/NPGCE

CVs of the PEI/TetX2/NPGCE were recorded in phosphate buffer solution (0.1 M, pH 8.5) at different scan rates (v) (Fig. 2). The peak current intensities (I_p) increased linearly with the scan rate in the range 10 and 400 mV s^{-1}, revealing a surface-limited redox process. As scan rate was increased, the oxidation and reduction peaks shifted toward more positive and more negative potentials respectively with a gradual increase in the ΔE_p value.

The average surface concentration (Γ^*) of the electroactive TetX2 was estimated by integrating the oxidation peak current of the TetX2 in phosphate buffer solution (0.1 M, pH = 8.5) according to the following equation:

$$Q = nFA\Gamma^* \quad (1)$$

Where Q is the integrated charge, A is the effective surface area and the other symbols have their standard meanings. The apparent surface area (A) of the NPGCE was estimated using cyclic voltammetry at different scan rates in a solution containing 5 mM $Fe(CN)_6^{3-/4-}$ redox couple (1:1) and 0.1 M KCl (Fig. S7).

The value of Γ^* was calculated to be 1.07×10^{-11} mol cm^{-2} for $n = 2$ and $A = 0.069$ cm^2. The obtained Γ^* value was apparently smaller than that of a cholesterol oxidase (ChOx)/gold nanoparticle-MWCNT modified electrode (2.04×10^{-10} mol cm^{-2}) [28], and a GOx/amine terminated ionic liquid functionalized CNT-gold nanoparticle modified electrode (1.27×10^{-10} mol cm^{-2}) [29]; while being close to a GOx/MWCNT-ionic liquid modified electrode (3.77×10^{-11} mol cm^{-2}) [30]. This low value of Γ^* showed that only a small quantity of the TetX2 molecules had the orientation and diameter corresponding to the NPGCE pores to penetrate and thus access the pore cavities. Achieving this, however, resulted in interaction between TetX2 and the pores and allowing the DET [26].

Based on Laviron's theory [31], the values of the electron transfer coefficient (α) and the apparent heterogeneous electron transfer rate constant (k_s) of the immobilized TetX2 at the PEI/TetX2/NPGCE were calculated as 0.52 and 1.9 s^{-1}, respectively. The estimated value of k_s was higher than the value observed for PEI/TetX2/rGO/GCE (1.35 s^{-1}), and PEI/TetX2/MWCNT/GCE (1.02 s^{-1}) in this work, suggesting that NPGCE is more efficient than rGO and MWCNT in facilitating the DET of TetX2. Other k_s values reported for FAD-containing enzymes are1.5 s^{-1} for GOx immobilized on a carbon nanotube modified glassy carbon electrode [32], 0.75 s^{-1} for ChOx immobilized on a conductive polymer electrode [33], and 1.55 s^{-1} for ChOx immobilized on gold nanoparticles-decorated MWCNT [28]. The relatively high k_s value indicates that the nano-porous structure of the NPGCE provides a nano-sized environment which connects the redox center of the enzyme to the electrode and facilitates interfacial electron transfer between FAD of TetX2 and the electrode surface.

3.4. The effect of TetX2 on the electrochemical behavior of PEI/TetX2/NPGCE

The effect of different concentrations of TetX2, ranging from 1.5 to 6.0 μl (conc. 6 mg/ml) on the electrochemical response of FAD is shown in Fig. 3A. The calibration curves resulting from three different amounts of enzyme are drawn in Fig. 3B-D and the limit of detection (LOD) for TetX2 calculated to be 0.7, 0.25, and 0.24 μM for 1.5, 3, and 6 μl, respectively. The negligible difference between the electrochemical responses and corresponding LODs for 3 and 6 μl volumes of the immobilized enzyme could be due to the amount of active enzyme that was available on the electrode surface, i.e., the amount of enzyme on the electrode surface is saturated by using 3 μl of the enzyme. Thus, by considering a minimum usage of the enzyme and maximum electrocatalytic activity, 3 μl of TetX2 was selected for further investigations.

3.5. The effect of pH of the reaction medium on the electrochemical behavior of PEI/TetX2/NPGCE

It is well known that the electrochemical behavior of proteins strongly depends on the pH of the electrolyte solution [34]. In this study, CV measurement of the direct electron transfer reaction of the immobilized TetX2 on the modified electrode demonstrated a linear dependence to pH values (3.0–9.0). As displayed in Fig. 4A, both anodic and cathodic peak potentials shift toward a negative direction by increasing in pH. The plotted curve of formal potential ($E^{0\prime}$) versus pH results in a straight line with a slope of 55.1 mV pH^{-1} (Fig. 4B), which is close to the theoretical value (59.0 mV pH^{-1}) at 25 °C for a two-electron redox reaction coupled with the two-proton exchange shown in Eq. (2) [35]:

$$TetX2\ (FAD) + 2e^- + 2H^+ \rightarrow TetX2\ (FADH_2) \quad (2)$$

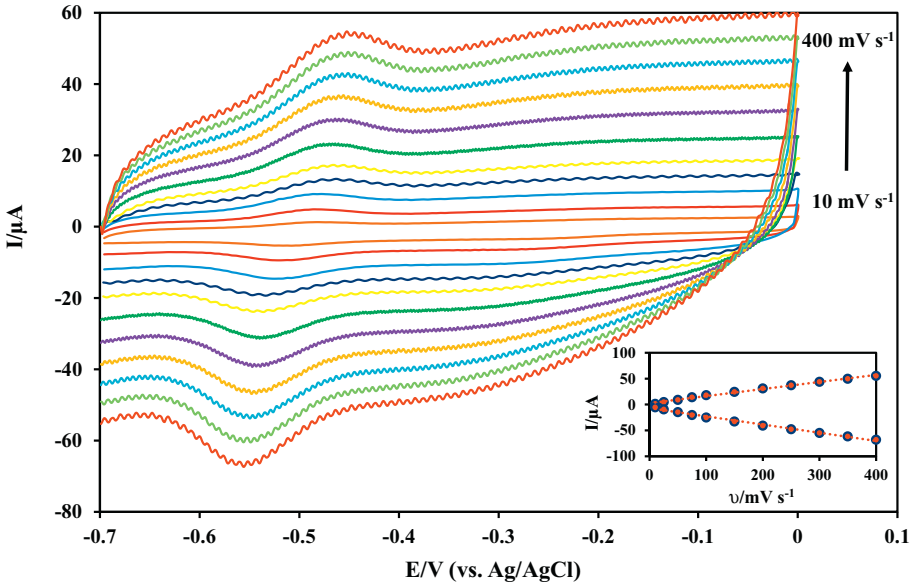


Fig. 2. Cyclic voltammograms of the PEI/TetX2/NPGCE in an air-saturated phosphate buffer solution (0.1 M, pH 8.5) at various scan rates (from inner to outer: 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, and 400 mV s^{-1}). Inset: plot of the peak current (I_p) vs. scan rate (υ).

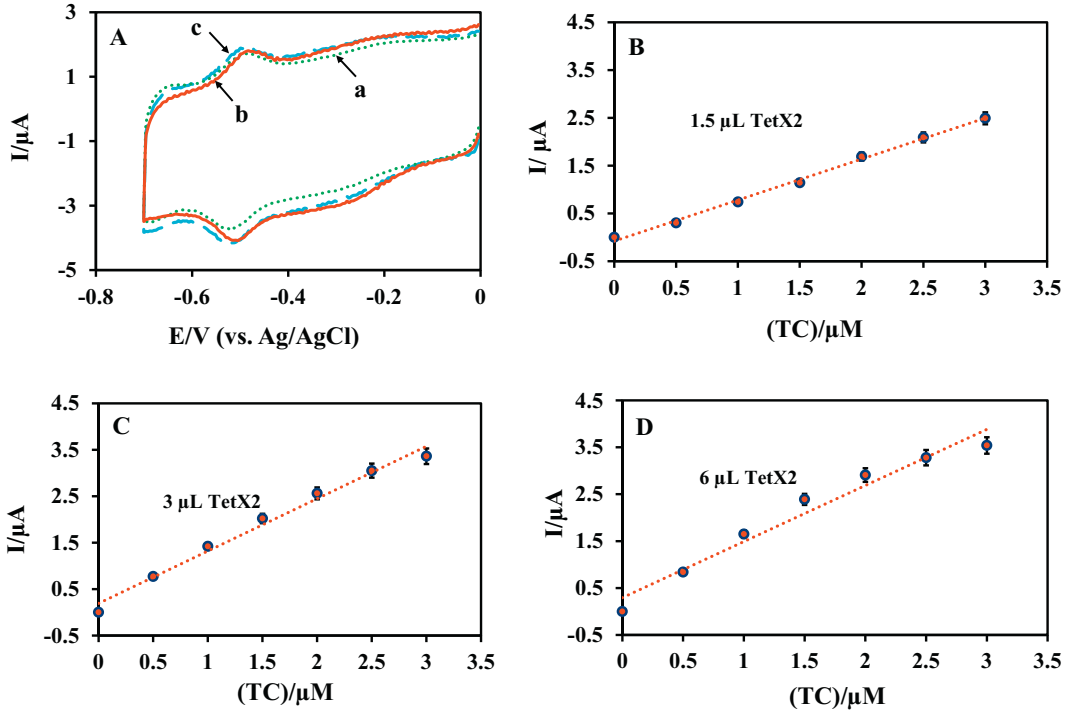


Fig. 3. Cyclic voltammograms of the PEI/TetX2/NPGCE in an air-saturated phosphate buffer solution (0.1 M, pH 8.5) at different enzyme contents of 1.5 (a), 3 (b) and 6 µl (c) (conc. 6 mg/ml) at a scan rate of 10 mV s^{-1} (A). The dependence of the calibration curve (LOD) on different amounts of enzyme in the electrode substrate. 1.5 µl ($y = 0.8571x - 0.0786$, $R^2 = 0.9965$) (B), 3 µl ($y = 1.1271x + 0.1921$, $R^2 = 0.986$) (C) and 6 µl ($y = 1.2179x + 0.2432$, $R^2 = 0.9807$) (D).

The maximum CV response of TetX2 was achieved at a pH near 8–8.5. At lower and higher pH values, the reaction gradually decreased that was likely to be because of enzyme denaturation and loss of the redox component (FAD). Therefore, subsequent TC determination was carried out in pH 8.5 phosphate buffer solution to retain the highest catalytic efficiency of TetX2.

3.6. Electrocatalytic activity of the TetX2 modified electrode

Oxygen is consumed in the enzymatic reaction for TC inactivation, producing H_2O [13]. In this reaction, two electrons are transferred from NADPH to FAD and ultimately to H_2O. A detectable change in oxygen in solution is due to enzyme activity in the presence of TC. To obtain better insight into the mechanism, the electrocatalytic behavior of PEI/TetX2/NPGCE toward oxygen reduction was further investigated in 0.1 M phosphate buffer solution (pH 8.5) alternatively saturated with N_2 and O_2 at a scan rate of 50 mV s^{-1} (Fig. 5). The obtained result showed the remarkable capacity of the PEI/TetX2/NPGCE structure to effectively catalyze an oxygen reduction reaction. Moreover, the response of the MWCNT and rGO as the most widely investigated carbon nanostructures for oxygen reduction was studied in O_2-saturated condition and compared with the NPGCE. As depicted in Fig. 5, the magnitude of the oxygen reduction current is higher at the PEI/TetX2/NPGCE than at PEI/TetX2/MWCNT/GCE or PEI/TetX2/rGO/GCE, further confirming a preference for NPGCE as a substrate for TetX2 immobilization.

3.7. Bioelectroanalytical characteristics of PEI/TetX2/NPGCE toward TC

Fig. 6a shows the CVs of PEI/TetX2/NPGCE to successive increments of TC concentration in a 0.1 M phosphate buffer solution (pH 8.5) containing an excess concentration of NADPH (0.5 mM) under the optimized condition of oxygen saturation at a scan rate of 10 mV s^{-1} after a 2 min delay. PEI was preferred for enzyme immobilization, because it is a positively charged polymer that facilitates the absorption of

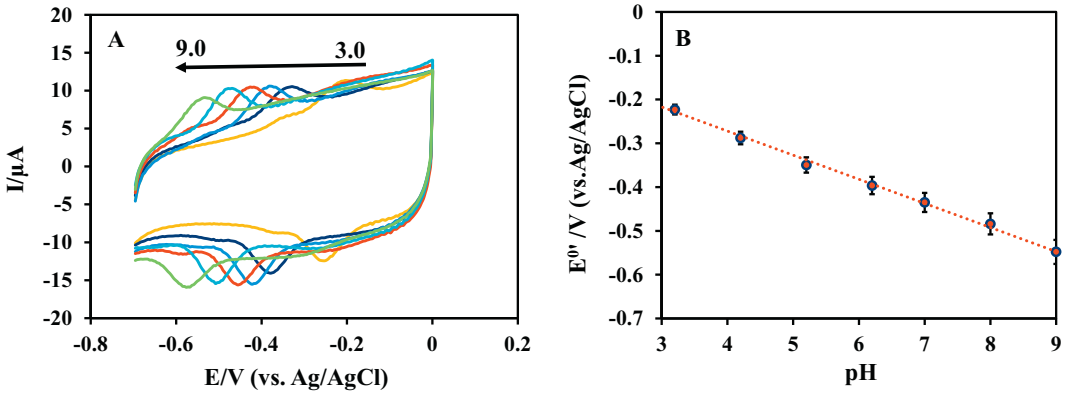


Fig. 4. Cyclic voltammograms of the PEI/TetX2/NPGCE in an air-saturated Na_2HPO_4 solution (0.1 M) at different pH values from 3.0 to 9.0 at a scan rate of 50 mV s^{-1} (A). Plot of the formal potential ($E^{0\prime}$) vs. pH ($y = -0.0551x - 0.0517$, $R^2 = 0.9975$) (B).

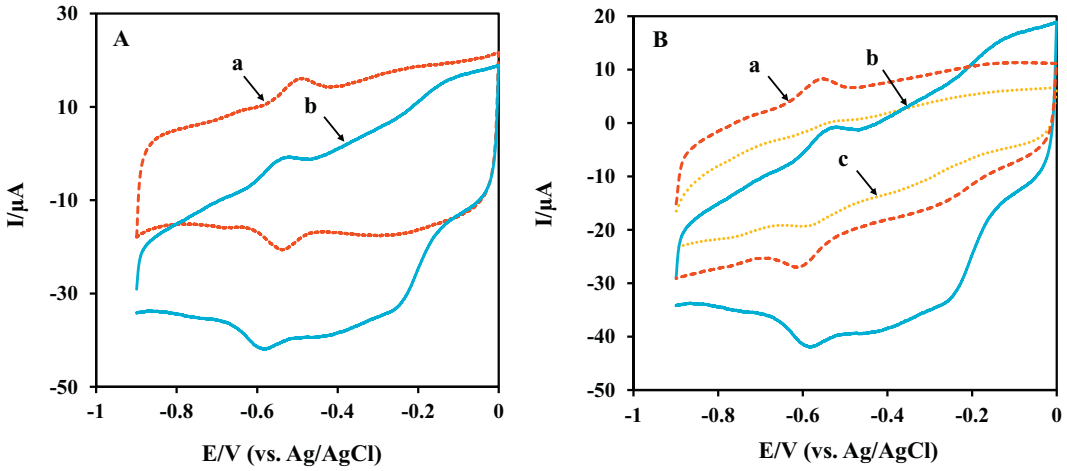


Fig. 5. Cyclic voltammograms of the PEI/TetX2/NPGCE in a N_2- (a) and O_2-saturated (b) 0.1 M phosphate buffer solution (pH 8.5) at a scan rate of 50 mV s^{-1} (A). CVs of PEI/TetX2/MWCNT/GCE (a), PEI/TetX2/rGO/GCE (b) and PEI/TetX2/NPGCE (c) to oxygen reduction reaction in O_2-saturated 0.1 M phosphate buffer solution (pH 8.5) at a scan rate of 50 mV s^{-1} (B).

negatively charged NADPH molecules as cofactor near immobilized enzyme on the surface of an electrode, thereby improving the analytical performance of the proposed biosensor more than with Nafion (data not shown). From Fig. 6A, it could be deduced that the baseline of the reduction current decreases corresponding to an increase in TC concentration due to the oxygen consumption. This result demonstrated a characteristic of the catalytic reaction of the TC with TetX2 protein and suggests a means of developing a technique for the quantitative determination of TC. It was found that oxygen consumption increased linearly with an increase in TC concentration ranging from 0.5 to 5 μM with a correlation coefficient (R^2) of 0.9913 and a high sensitivity of about 37.56 μA μM $^{-1}$ cm^{-2}. Furthermore, for an increase in the concentration of TC to values >5 μM, led to a response plateau of the peak current, representing the characteristics of a Michaelis–Menten kinetic process (Fig. 6B). The apparent Michaelis–Menten constant (K_M^{app}), an indication of enzyme–substrate kinetics, was calculated to be 6.2 μM according to the Lineweaver–Burk equation (Fig. 6C) [36]. This obtained K_M^{app} is lower than the 54.0 μM as reported by Yang et al. [37] where the enzyme was assayed in solution. The low K_M^{app} revealed that TetX2 immobilized in NPGCE retained its high affinity properties and prominent biological activity for TC catalysis. The detection limit of the biosensor was found to be 18 nM at a signal to noise ratio of 3 according to the curve plotted in Fig. 6D. This value is lower than the maximum residue level (MRL) of 100 ng/g

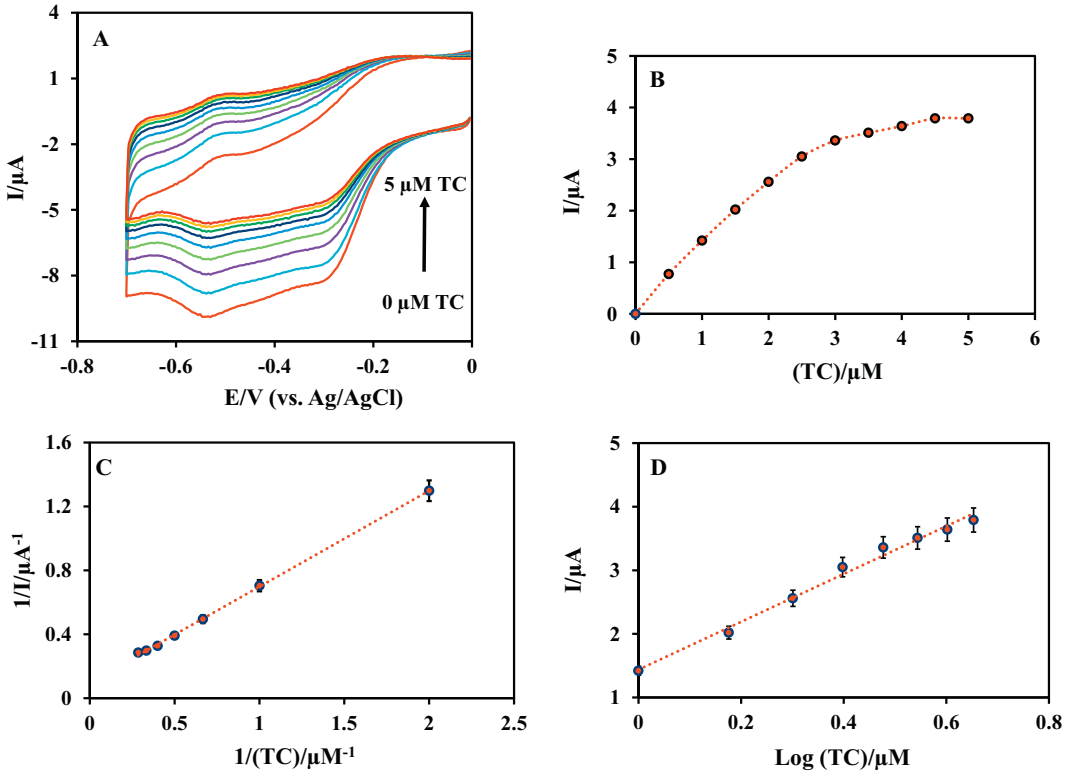


Fig. 6. Cyclic voltammograms of the PEI/TetX2/NPGCE in an O_2-saturated phosphate buffer solution (0.1 M, pH 8.5) containing different amounts of TC (from outer to inner: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 μM) at a scan rate of 10 mV s^{-1} (A). The relationship between catalytic reduction peak currents vs. the TC concentrations (B). Lineweaver–Burk plot ($y = 0.6005x + 0.0979$, $R^2 = 0.9994$) (C). The relationship of catalytic reduction peak currents vs. the log of TC concentrations ($y = 3.7619x + 1.4368$, $R^2 = 0.9897$) (D).

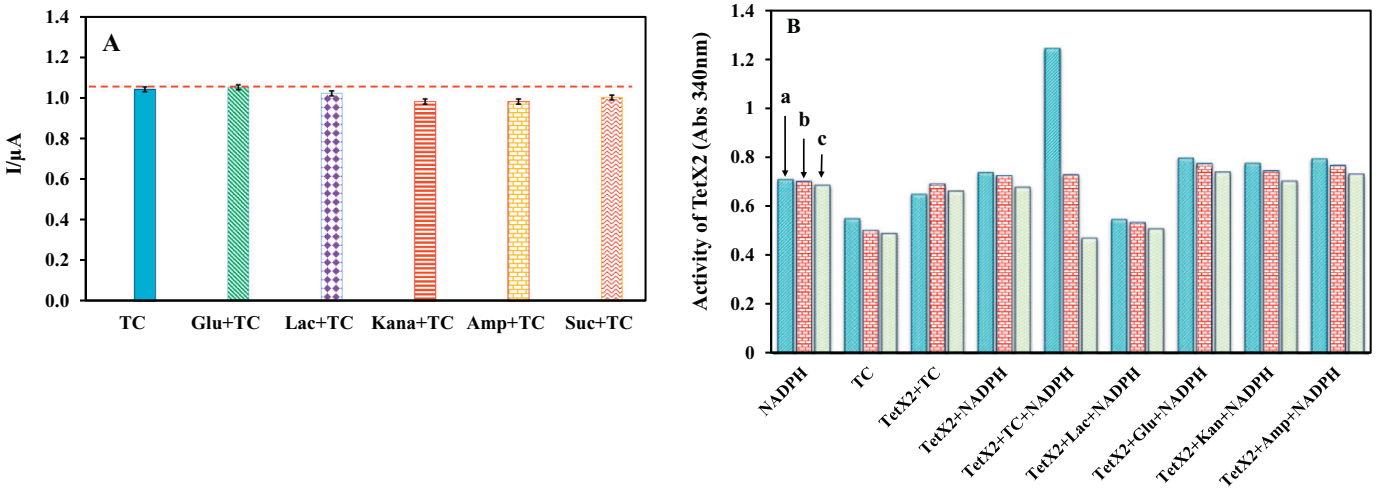


Fig. 7. Biosensor electrochemical response to TC (1 μM) in the presence of the possible interfering substances, lactose (Lac), glucose (Glu), kanamycin (Kan), ampicillin (Amp) and sucrose (Suc) at the 10-fold quantities (10 μM) (A). The activity of TetX2 to TC and the possible interfering substances, Lac, Glu, Kan, Amp and Suc (50 μM) as decreasing of the absorbance at 340 nm after 30 (b) and 60 min (c) (B).

(0.22 μM) that was established in the European Union Regulation (EU) for TC in milk [38]. Therefore, the PEI/TetX2/NPGCE could be used as a sensitive TC biosensor in food analysis.

3.8. Selectivity, stability, reproducibility of the biosensor

The evaluation results of the selectivity of the proposed nano-biosensor, in the absence and presence of five possible interfering substances in food samples including lactose, glucose, kanamycin, ampicillin and sucrose are shown in Fig. 7. As observed in Fig. 7A, no significant interference was shown by the proposed nano-biosensor when 10-fold greater concentrations of the above-mentioned interferences were added to the reaction medium containing TC. This observation is consistent with the results obtained by spectrophotometer (Fig. 7B), suggesting that TetX2 could catalyze TC selectively in food and biological samples.

The operational stability of the proposed TC biosensor was also examined (Fig. S8A). The change in the current of biosensor after 30 successive cycles was insignificant, demonstrating the substantial stability of the bioelectrode. Also, the evaluation result relating to the long-term stability of the PEI/TetX2/NPGCE following storage of the same biosensor for one month at 4 °C shows only a 6.2% decrease in initial current (Fig. S8B). This exceptional stability could be attributed to the biocompatibility of the NPGCE substrate, providing a suitable environment for TetX2 immobilization.

Moreover, the relative standard deviation (RSD) as 5.6% was obtained with six repeated measurements separately, where the same prepared PEI/TetX2/NPGCE was used to detect 1 μM TC, revealing an acceptable reproducibility of the TC analysis.

3.9. Analytical application in a real food sample

Finally, to evaluate the applicability of the proposed enzymatic biosensor for real sample analysis, the TC content in pasteurized milk was determined. For this, a 100 μl of a milk sample mixed with 900 μl O_2-saturated Na_2HPO_4 solution (0.1 M and pH 8.5) containing 0.5 mM NADPH spiked with 2 or 3 μM TC was analyzed using the fabricated biosensor. The recoveries for the spiked samples were estimated to be 97.7% and 98.1% (Table S1). This result again indicates that PEI/TetX2/NPGCE could be used to determine TC in a real food sample.

4. Conclusions

In the current study, we described the fabrication and characterization of a novel electrochemical biosensor using a bacterial monooxygenase enzyme immobilized at an activated glassy carbon electrode for the preliminary screening of TC in an animal food product. The as-fabricated bioelectrode showed an enhanced direct electron transfer rate of FAD and excellent electrocatalytic properties toward oxygen reduction provided. Furthermore, based on the ease of the fabrication process, selective recognition of TC, fast response, the proposed biosensor could effectively avoid expensive assays and the complex pretreatment procedures related to the traditional analytical methods employed in core facilities. Our findings showed that TC could be detected at levels lower than the values set by food control agencies in milk with minimal sample pretreatment. Also, no or negligible interference in the milk analysis was noted, showing potential for use as a portable diagnostic tool for TC detection in real samples.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2019.02.010.

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