Novel enzyme-based electrochemical and colorimetric biosensors for tetracycline monitoring in milk

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Abstract

Recently, there has been a growing demand to develop portable devices for the fast detection of contaminants in food safety, healthcare, and environmental fields. Herein, two biosensing methods were designed by the use of NAD(P)H-dependent TetX2 enzyme activity and thionine as an excellent electrochemical and colorimetric mediator/probe to monitor tetracycline (TC) in milk. The nano-porous glassy carbon electrode (NPGCE) modified with

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polythionine was first prepared by electrochemically and then TetX2 was immobilized onto the NPGCE using polyethyleneimine. The prepared biosensor provided a high electrocatalytic response toward NAD(P)H by significantly reducing its overpotential. The proposed biosensor exhibited a detection limit of 40 nM with a linear range of 0.1 to 0.8 μM for TC determination. Besides, the thionine probe was used to develop a novel colorimetric assay using a simple enzymatic color reaction within a few minutes. The limit of detection for TC was experimentally achieved as 60 nM, which was lower than the safety levels established by the WHO (225 nM). The correlation between change in the color of the solution and the concentration of TC was used for quality control of milk samples, as confirmed by the standard HPLC method. The results show the great potential of the proposed assays as portable instruments for on-site TC measurements.

Keywords: colorimetric detection, electrochemical biosensor, milk, on-site measurement, Tetracycline, TetX2, thionine probe

1. Introduction

Tetracyclines (TCs) are broad-spectrum antimicrobial substances most frequently used in the animal husbandry for infectious disease prevention and therapy, as well as growth promoter additives in animal feed [1]. As a result, human exposure to TC residues in animal source food can cause adverse health effects, including the development of bacterial resistance, allergic reactions, and permanent teeth discoloration [2]. Therefore, there is a need for severe regulations to be established by the food safety authority's surveillance systems. In line with these requirements, the Food and Agricultural Organization (FAO), World Health Organization (WHO), and the European Union (EU) have set a maximum residue limit (MRL) of 100 μg/kg (225 nM) of TC in milk [3].

To ensure food safety, various analytical methods have been developed for detection of TC. Nowadays, TC residues assay has been mostly carried out in laboratory settings with large and expensive instruments such as liquid chromatography coupled with mass spectroscopy (LC-MS) [4], capillary electrophoresis (CE) [5], and high-performance liquid chromatography (HPLC) [6]. Although quantification of TC residues by HPLC and LC/MS methods is reliable and quite sensitive for enforcement of the maximum residue limits or tolerance levels of antibiotic residues in milk, the operational processes are complicated, time-consuming, and require multi-steps and extensive sample preparation procedures [7]. To address these issues, it is crucial to develop a simple, rapid, and on-site analysis method with desired sensitivity and selectivity in order to monitor TC residues in food and environmental samples by untrained personnel.

Recently, electrochemical biosensors (EBs) have been widely developed for the detection of antibiotic residues including sulfamethoxazole, trimethoprim, erythromycin, penicillin, and TC by virtue of low-cost equipment and excellent analytical performance [8-11]. Different EBs have been so far designed by using modifications with incorporating enzymes, aptamers, polymers, metals, nanomaterials, and mediators [12, 13]. Among them, enzymes-based EBs provide remarkable advantages over non-enzymatic sensors due to their selectivity, specificity, sensitivity, potential for miniaturization and portability, as well as less interfering of the possible endogenous species that may coexist in the food matrix [14]. However, enzyme-based EBs have drawbacks such as the low loading capacity of the immobilized enzyme [15], the poor electron transfer between the active redox center of the enzyme and the electrode surface [16, 17], as well as the large overvoltage related to the electrochemical oxidation of the electroactive species in the solution [18, 19].

Glassy carbon (GC) is one of the most commonly used electrodes for electrochemical analysis. However, unmodified GC electrodes (GCEs) typically exhibited sluggish electron

transfer kinetics, and thus require appropriate surface treatments to improve their electrochemical performance [20]. So, several methods have been employed for the pretreatment of GCE including heat treatment, laser activation, radio-frequency plasma, and electrochemical treatment [21, 22]. The electrochemical approach was commonly used to create a nano-porous film at the electrode surface. The nano-porous GCE (NPGCE) has many advantages such as 3D structure, easy fabrication, good reproducibility, and high surface area [22, 23].

NAD(P)H is a redox couple in many enzymatic reactions that can be electrochemically oxidized on the electrode surface [24]. The inherent problems related to the high overvoltage for the electrochemical oxidation of NAD(P)H as well as the fouling of the working electrode have been minimized using different electrode materials and conducting polymers, such as redox mediators that accelerate the rate of heterogeneous electron transfer between the electrode and redox species in solution [25, 26]. Polythionine (PTH), which can be synthesized electrochemically and deposited from thionine (TH), has been extensively used as a mediator to prepare modified electrodes in electrochemical sensors and biosensors such as H_2O_2 biosensor [27, 28], NADH biosensor [29], glucose sensor [30], and for DNA hybridization detection [31].

Despite the high analytical performance, the EBs are usually evaluated based upon changing a single signal caused by the target. Nevertheless, the response can be affected by external interferences comprising nonstandard test process, different personnel operating, and various experimental condition, which results in the uncertainties. An approach to address this issue is using dual-signal method rather than single-signal readout. For instance, Wei's group reported a dual-modal split-type immunosensor for detection of microcystin-LR based upon the photocurrent change of CdS/ZnO hollow nanorod arrays (HNRs) and the blue-shift of surface plasmon resonance peak from Au nanobipyramids@Ag [32]. Additionally,

Apilux's group constructed a lab-on-paper device combining the electrochemical and colorimetric detection for the screening of Au(III) in the presence of Fe(III) as an interference, Fe(III) [33].

The colorimetric is a solution-based assay method that can estimate the concentration of the analyte just by observing a color change with the naked eye; thus, has attracted significant attention due to the simplicity and low cost. In this regard, integration of different enzymes such as horseradish peroxidase (HRP) [34], glucose oxidase (GOx) [35], phenylalanine dehydrogenase (PheDH) [36], β-galactosidase (β-Gal) [37], and acetylcholinesterase [10, 38] in conjugation with antibody, aptamer, nanomaterials, and dye probes have been reported for the colorimetric assay of various target analytes.

Herein, we designed and optimized two novel enzyme-based electrochemical and colorimetric biosensors to detect TC based on TetX2, NAD(P)H and TH as an enzyme, cofactor and mediator, respectively. The TetX2 enzyme, originally isolated from transposon CTnDOT in *Bacteroides thetaiotaomicron*, specifically catalyzes the conversion of the TC to 11-a-hydroxytetracycline, leading to the consumption of NAD(P)H [39]. The electrochemical biosensor was fabricated by successively applying a positive and negative potential by amperometry to fabricate a NPGCE followed by electropolymerization of PTH and immobilization of TetX2 using positively charged polyethyleneimine (PEI). To the best of our knowledge, the use of a NPGCE modified with PTH has not been yet reported for TC detection. Reasonable results obtained using the colorimetric assay corroborate the proposed electrode. This is the first report in which TH-TetX2 has been utilized to develop a colorimetric assay for the detection of TC in milk samples. The application of this design by using TH as a signal tag can be further expanded to other NAD(P)H-dependent systems for on-site analysis.

2. Experimental

2.1. Reagents and Materials

Recombinant TetX2 was purified from a heterogeneous expression system in *E. coli* BL21 (DE3) using a simple procedure as previously described [40]. The purified protein was concentrated using an Amicon centrifugal filter unit (Millipore, MWCO = 10 kDa) and stored at -20 °C until use. Bradford assay was used to quantify the protein concentration. NAD(P)H (>95% purity) was purchased from Sigma Aldrich. TC (purity, 98%) was obtained from Hakim pharmaceutical company (Tehran, Iran). All other reagents used in this work were purchased from Merck or Sigma Aldrich. A TC stock solution of 2.5 mM was prepared in deionized water and stored at 4 °C for no longer than one month. TH solution was prepared by dissolving 1 mg of TH bulk powder in 1 ml of phosphate buffer (Na_2HPO_4, 0.1 M, pH 8.5). Phosphate buffer (Na_2HPO_4, 0.1 M) was used as a buffer solution for enzyme activity assay and an electrolyte for all electrochemical experiments. Deionized water used for the preparation of aqueous solutions throughout the experiment.

2.2. Electrochemical Measurements

Electrochemical measurements were carried out on a μAutolab potentiostat-galvanostat analyzer (Type III, The Netherlands) at room temperature (25 °C) and data were acquired by using GPES Ver. 4.9 software, (Metrohm Autolab B.V., The Netherlands). A conventional three-electrode configuration was employed for all electrochemical measurements, in which Platinum (Pt), Ag|AgCl|KCl_{sat}, and modified GCE (2 mm diameter) served as the auxiliary, reference, and working electrodes, respectively. All of the potentials were referred to this reference electrode.

2.3. Enzyme Electrode Preparation

All steps for designing the electrochemical biosensor is exhibited in Figure 1a. To prepare the enzyme-modified electrode, GCE was polished with aqueous slurries 1.0, 0.3 and 0.05 μm alumina powder sequentially (Struers, Copenhagen, Denmark), then subjected to ultrasonic bath vibration in absolute ethanol and finally washed thoroughly with distilled water. NPGCE electrode was prepared using an electrochemical procedure, as described previously, without further modification [41]. PTH electropolymerization onto the NPGCE was accomplished using chronoamperometry by applying a constant potential of +1.2 V to the NPGCE for the 40 s in 0.1 M Na_2HPO_4 solution containing 1 mg/ml TH [42]. The electrode modified with the electropolymerized PTH (PTH/NPGCE) was washed with deionized water before the enzyme immobilization.

TetX2 immobilization on the modified electrode was performed using the drop-casting method. A suspension (3 μl) of the purified TetX2 of desired concentration (4 mg/ml) was drop-coated on the PTH/NPGCE surface and left to dry at ambient temperature. To prevent enzyme loss, the electrode surface was covered with PEI, chitosan, and Nafion, separately. PEI 0.25 % (v/v), chitosan 0.5 % (w/v), and Nafion 0.5 % (v/v) were prepared in deionized water, acetic acid 1.0 % (v/v) and ethanol, respectively, and 3 μl of each solution was drop casted on the TetX2/PTH/NPGCE surface. The immobilization step was conducted at room temperature for approximately 30 min. These electrodes coated with a PEI/chitosan/Nafion thin film were evaluated for biosensing of TC. The surface morphology of the activated electrode surface was characterized using a DME DualScope Scanner DS 95-200 (DME, Herlev, Denmark) atomic force microscopy (AFM).

2.4. Colorimetric Detection of TC.

Colorimetric experiments were carried out at ambient temperature (25 °C) in a reaction volume of 60 µl composing of 0.75 mg/ml TH (1 mg/ml, pH 8.5) as a probe and 20 µg enzyme TetX2 (4 mg/ml). Then, 1 mM NAD(P)H (10 mM) was added to the previous mixture to achieve a colorless solution. When TC (at a final concentration of 7 µM) as the detection target was added into the solution, the color turned to blue within 2 min. The color change analysis was evaluated by UV-vis spectroscopy. TC concentration-dependent color variations were observed by the naked eye and digitized pictures obtained using a phone camera. The time-dependent mechanism of the colorimetric assay was further investigated by pipetting the solution immediately and after 2, 6 and 10 min in the reaction condition as described above. Then, for quantitative analysis of the images, the absorbance values were obtained using the ImageJ software by measuring the red (R), green (G), blue (B), and average RGB values (See page S2 for more details) [43, 44]. Absorbance (A), which is the negative log of the transmittance (I_n/I_{blank}) was calculated according to the following formula [45]:

Eq. 2: $$A = -log\ (I_n/I_{blank})$$

Where I_n is the R, G, B, or average RGB value for the sample and I_{blank} is the R, G, B, or average RGB value for the blank that can be obtained from the images collected using a camera.

To further characterize the selectivity of TetX2-based calorimetric biosensor, the control experiments were taken using 10 times higher concentrations of glucose, lactose, kanamycin, and ampicillin (70 µM) than TC (7 µM) under the same condition as described above. All experiments were conducted three times.

2.5. Milk Analysis

The assay capability for testing milk sample was also conducted in this work. The pasteurized milk samples used in this study were purchased from a local market and stored at 4 °C before further preparation. First, the acid extraction pretreatment with 1% trifluoroacetic acid was applied to the milk sample and centrifuged two times at 12.000 rpm (4 °C) for 20 min to remove any insoluble and larger particles, which may affect the measurements. Then, the supernatant was collected (pH set to 8.5), spiked with TC at different final concentrations of 1, 10, and 20 μM, and added to Na_2HPO_4 buffer containing TH/NAD(P)H/TetX2 (pH 8.5). Also, the milk samples without pretreatment (just diluted 12 times in the final assay volume) spiked with TC at final concentrations of 1, 10, and 20 μM, were used. The obtained results and intrinsic TC residues in the commercial milk samples were validated and proved with HPLC method similar to the previous report [46] (See Page S4 and S5 in Supporting information).

3. Results and Discussion

In this section, first, the enzyme-based electrochemical biosensor will be fully presented, then the colorimetric assay will be mentioned in details.

3.1. Electrode Characterization

The electrochemical biosensor fabrication is demonstrated in Figure 1a. The AFM micrographs of the bare GCE and the NPGCE surfaces are shown in Figure 1b, and c, respectively. As shown, the surface of NPGCE showed more porosity than that of the bare GCE. This porosity acts as conductive cores which facilitates the transfer of electrons by

providing an enhancement in the active surface area of NPGCE, and accumulating more PTH film on its surface [47].

Figure 1d illustrated the cyclic voltammogram (CV) of the NPGCE decorated with TH using amperometry to create a stable PTH film on the electrode surface. The PTH-NPGCE demonstrated a couple of well-defined redox peaks with the anodic (E_{pa}) and cathodic (E_{pc}) peak potential of -320 and -345 mV (at 50 mV s^{-1}), respectively, which could be ascribed to the redox reaction of the TH electropolymerized on the surface of NPGCE.

The immobilization of TetX2 with different membranes including Nafion, chitosan, and PEI was also investigated, and the results were depicted in Figure 1e, f, and g, respectively. The coating of the electrode with PEI film improved the biosensor response to oxidation of NAD(P)H largely and also provided direct electron transfer between electroactive species of TetX2 (FAD) and the electrode surface. This result has been attributed to the positive charges of the PEI that facilitated the entrance of NAD(P)H molecules to the proximity of the catalytic site of the enzyme. Also, the PEI film avoided the release of enzyme molecules from the electrode surface and preserved their three-dimensional active structure needed for the enzyme activity [48]. Thus, PEI was selected for further work. The electrochemical responses of the bare GCE and the NPGCE to NAD(P)H were also investigated (Figure S1). As seen, no NAD(P)H oxidation signals observed in the potential range from -0.7 to +0.4 V.

Figure 1.

3.2. Analytical Performance of the Enzyme Modified Electrode

To evaluate the electrocatalytic activity of PTH/NPGCE for NAD(P)H oxidation, the cyclic voltammograms of the modified electrode was measured within the potential window of -0.7 to +0.5 V. Figure 2a demonstrated the CVs of the modified electrode in the absence and presence of NAD(P)H in a phosphate buffer solution (0.1 M, pH 8.5). As NAD(P)H was added, a pronounced anodic peak current emerged which is indicative of electrochemical oxidation of NAD(P)H on the electrode surface. It was observed that the potential of NAD(P)H anodic peak at -0.05 V was better than that of the previously reported enzyme-based biosensors such as 0.46 V for alcohol dehydrogenase-Al_2O_3/GO nanocomposite modified carbon paste electrode [49], 0.3 V for urease producing *Bacillus sphaericus* modified carbon paste electrode [50] and 0.15 V for formate dehydrogenase (FDH)-modified membrane with 3,4-dihydroxybenzaldehyde-coated glassy carbon electrode [51], confirming the strong effect of PTH/NPGCE. The decrease in overvoltage and increase in peak current of NAD(P)H oxidation confirmed that PTH film coated on the NPGCE surface had a high catalytic ability for NAD(P)H oxidation. Therefore, PTH film was suitable as a mediator to transfer electron between NAD(P)H and the surface of the working electrode.

The analytical response of the model bioelectrode was then examined using linear sweep voltammetry (LSV) at a scan rate of 0.01 V/s after a 2 min delay with a successive injection of TC to the cell, and the corresponding calibration curve was depicted (Figure 2). The catalytic anodic peak current decreased linearly with TC concentration within the range of 0.1 to 0.8 μM (Figure 2b), most likely as a consequence of the enzymatic consumption of NAD(P)H by the TetX2 confined on the electrode, causing a decrease in the oxidation signal related to NAD(P)H molecules remained in the solution. The calibration plot was depicted for TC concentrations between 0.1 and 0.8 μM for the proposed biosensor system with R^2 =

0.9162 (Inset in Figure 2b). The LOD was measured to be 40 nM through multiplying the quotient of the standard deviation of the mean intensity of the blank (σ) and slope of the calibration (S) graph by 3 (3σ/S). This good response was attributed to nano-size effects and specifically to the change in nanomaterial surface chemistry/physics of the electrode. This value is an acceptable detection limit below the permissible limit of TC in milk. Moreover, the acceptable linearity and analytical performance of the proposed electrochemical biosensor makes it promising for potential applications in food, environmental, and clinical samples.

Figure 2.

3.3. TH-Based Probe and Colorimetric Strategy

The principle of the enzyme-based colorimetric assay was shown in Figure 3. The system was made of a mixture of two primary components, TH/TetX2, in Na_2HPO_4 solution (0.1 M, pH 8.5) having a purple/blue color (Figure 3a and b). The third component was NAD(P)H that is a cofactor for the enzyme activity. In the absence of TC, NAD(P)H (1 mM) as an electron donor reacts with TH oxide (TH-ox) and produces reduced TH, resulting in the change of the solution color from blue to colorless (Figure 3c). As a result of adding TC into detection solution, NAD(P)H enzymatically converted into $NAD(P)^+$, thus the reducing agent activity decreased dramatically, and the color of solution returned to the original blue color (Figure 3d). This suggested that TC can successfully promote the formation of TH-ox.

Figure 3.

To further study the above color changes, the resulting solutions were assayed using UV-visible spectroscopy (Figure 3e). TH-ox has a maximum absorbance at 600 nm. When TetX2 was added to the TH solution no change in absorbance at 600 nm was observed, while upon exposure to NAD(P)H, the intensity of the 600-nm peak decreased that is due to

producing reduced TH. Addition of TC to TH/NAD(P)H/TetX2 containing solution induced the enzyme activity and produced the TH-ox with the initial absorbance intensity at 600 nm (Figure 3e).

3.4. Performance of the Colorimetric TC Biosensor

The catalytic activity of TetX2 depends on the solution pH, in which TetX2 catalytic activity was higher in basic solutions comparing with neutral and acidic solutions [39, 52]. Thus, considering the catalytic activity and stability of TetX2, pH=8.5 was set as the solution pH value.

To get a better insight into the system response time, a kinetic study of the TH/NAD(P)H/TetX2-catalyzed reaction in the presence of TC was performed. In this way, images were taken by pipetting the solution after the TC addition at different time intervals. As shown in Figure 4a, the colorimetric assay responded in a time-dependent manner. The absorbance values, which were quantified using ImageJ and the data correspond to the red values of RGB are shown in Figure 4b. The results of the other channels (mean RGB, green and blue) are also shown in Figure S2a .It was found that 6 min pipetting time was enough for better detection of TC (no significant color change was detected after 6 min) (Figure 4b).

Figure 4c displays the photographs of the calorimetric assay in the presence of different concentrations of TC. As shown in Figure 5a, the color progression from pale blue to purple could be observed by increasing the concentration of TC from 0.1 to 10 μM. Plot of absorbance versus concentration was obtained using ImageJ based on the red value is shown in Figure 4d, which has more linearity compared to other channels (Figure S2b). Under the above-mentioned condition, TC was quantified from 0.1 to 7 μM. The limit of detection (LOD) was estimated to be 60 nM using the $3\sigma/S$ criteria, indicating that the current assay can potentially be used for the detection of TC in terms of the assay sensitivity and short

response time (～6 min). In agreement with the electrochemical biosensing system, calorimetric assay allowed the detection of TC down to 60 nM, accordingly electrochemical biosensor is approximately two times more sensitive than the proposed colorimetric method. Thus, the proposed colorimetric biosensor provided semi-quantitative results (naked eye observation) and quantitative results when using a simple RGB analysis of images collected with a digital camera.

The selectivity of the TetX2-based colorimetric method was investigated and shown in Figure 4e. As indicated, none of the control samples, including glucose, lactose, kanamycin, and ampicillin with the concentrations of 10 times greater than TC, generated the blue color compared with TC solution. This corroborates the results collected from ImageJ with the red value (Figure 4f) (See Figure S2c for other channels analysis). It showed that these compounds couldn't be catalyzed with TetX2, suggesting the strong enzyme selectivity toward the target analyte. Therefore, this enzyme-based colorimetric biosensor exhibited attractive selectivity and short-time response, which gives insight into the growing practical applications in the real sample analysis.

Figure 4.

Several recently published colorimetric assays for determination of TC are shown in Table 1. As can see, different colorimetric agents were used and some response characteristics of the biosensors, including, color change, LOD and detection time listed in Table 1. Compared with other studies, the proposed biosensing method showed faster detection time and acceptable LOD.

Table 1.

Milk sample, as an essential food of animal origin, was then analyzed to demonstrate the capabilities of the proposed biosensor where interfering species are present. Using this

colorimetric method, the low concentrations of TC (1 μM) in the spiked pasteurized milk sample was detected by a short and straightforward pretreatment (Figure 5a), and also with no sample pretreatment (10% milk) (Figure 5c). The data shown in Figure 5b and d correspond to the red values of RGB collected using ImageJ (See Figure S2d and e for other channels analysis). It can be seen that the color intensities in the pretreated milk samples were more obvious than that of the samples without pretreatment. The recovery percentages of 92.97% and 103.46% were calculated at the TC concentration of 10 μM in the pretreated and non-pretreated milk samples, respectively, which were in good agreement with the value of 98.72% measured by HPLC at concentration of 10 μM according to Eq. 2 [53]. The obtained results suggest that the as-designed biosensor is has promising potential for detecting low concentrations of TC in real samples.

Eq. 2: $$R\ (\%) = \left(\frac{[TC]buffer}{[TC]real-sample}\right) \times 100$$

Figure 5.

4. Conclusions

In summary, we have demonstrated the development of two electrochemical and calorimetric assays based on a bacterial enzyme TetX2 and TH probe for a facile and rapid determination of TC. TH was used as a mediator that electropolymerized on the modified surface of the electrode (PTH film), and a probe in a color change reaction. The advantages of our proposed biosensors are the high selectivity for the target analyte using TC degrading enzyme TetX2, and low working potential (- 0.05 V), which reduces interference signals from other analytes present in the real sample matrix. Besides, the color change can be detected easily by the naked eye. The electrochemical and colorimetric showed the LODs of 40 and 60 nM, respectively, along with the short response times (2-6 min). Therefore, the designed biosensors can provide a powerful platform for dual-signal detection of TC.

Moreover, the colorimetric assay can be used as a simple tool to detect TC in milk samples without complicated sample pretreatment and sophisticated instruments.

For future development, the colorimetric assay can be applied on a solid substrate such as a paper for commercial application. Furthermore, the proposed sensing scheme, in which TH was used as a mediator/probe, can be extended to the development of NAD(P)H-based biosensors for point-of-care environmental and biological sample analysis.

Supporting Information

Supplementary material including more detailed on colorimetric experimental method and results, HPLC analysis are presented in supporting information.

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Table 1. Comparison of the colorimetric sensors for TC with respect to the colorimetric agent, response time and LOD

Colorimetric agent	Biorecognition agent	Color change	Time (min)	LOD (nM)	Ref.
TMB[a]	Aptamer	Colorless-blue	45	0.22	[54]
TMB	-	Colorless-blue	30	45	[55]
Au NPs[b]	-	Colorless-red	10	45	[56]
CS[c]-Au NPs	Aptamer	Red-blue	30	87.7	[57]
Aptamer-Au NPs	Aptamer	Red-blue	30	0.26	[58]
Aptamer-Au NPs	Aptamer	Red-purple	25	45.8	[59]
TH	Enzyme	Colorless-blue	6	60	This study

[a] Tetramethylbenzidine [b] Nanoparticles [c] Cysteamine

Figures legends:

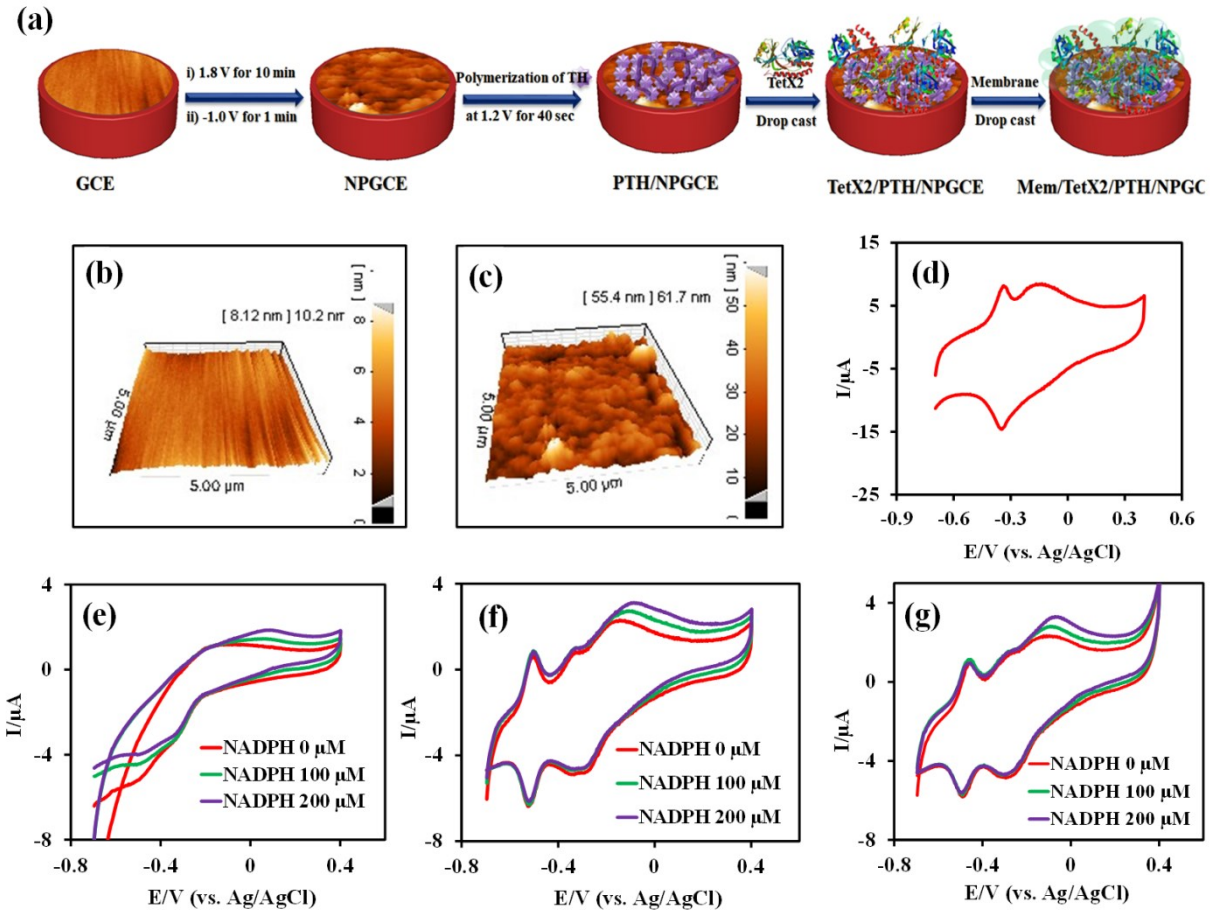

Figure 1. (a) Schematic illustration of electrochemical biosensor fabrication. The AFM images of the surface of the GCE (b) before and (c) after the electrochemical treatment. (d) Cyclic voltammogram obtained after the electropolymerization of PTH on the NPGCE in 0.1 M Na_2HPO_4 (pH 8.5) at a scan rate of 0.05 Vs^{-1}. Cyclic voltammograms obtained after the enzyme immobilization with (e) chitosan, (f) Nafion and (g) PEI in 0.1 M Na_2HPO_4 (pH 8.5) at a scan rate of 0.01 Vs^{-1}

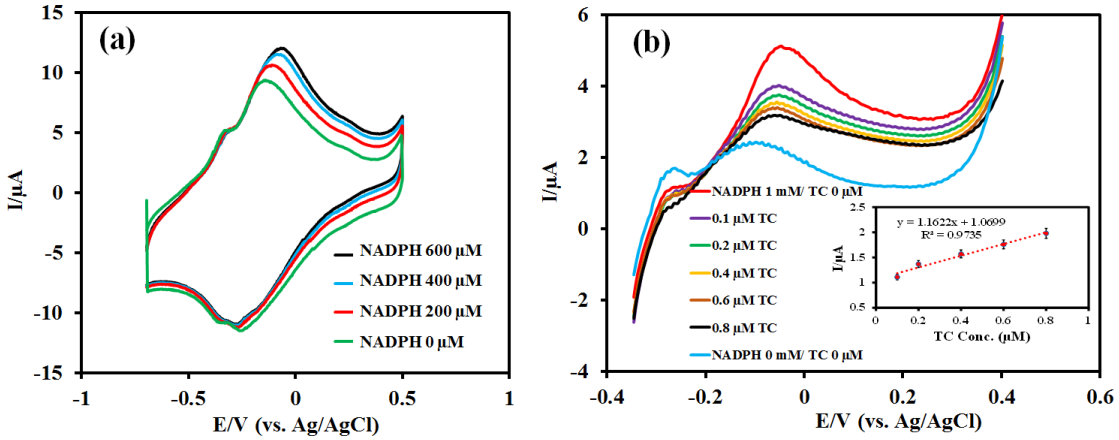

Figure 2. (a) Cyclic voltammograms obtained for the modified electrode (PEI/TetX2/PTH/NPGCE) at varying NAD(P)H concentrations of 200, 400 and 600 µM in 0.1 M Na_2HPO_4 (pH 8.5) at a scan rate of 0.01 Vs^{-1}. (b) Cyclic voltammograms obtained for the successive injection of TC on the modified electrode of the proposed biosensor system in 0.1 M Na_2HPO_4 (pH 8.5) at a scan rate of 0.01 Vs^{-1}. Inset: Corresponding calibration curve of electrochemical biosensor toward different concentrations of TC

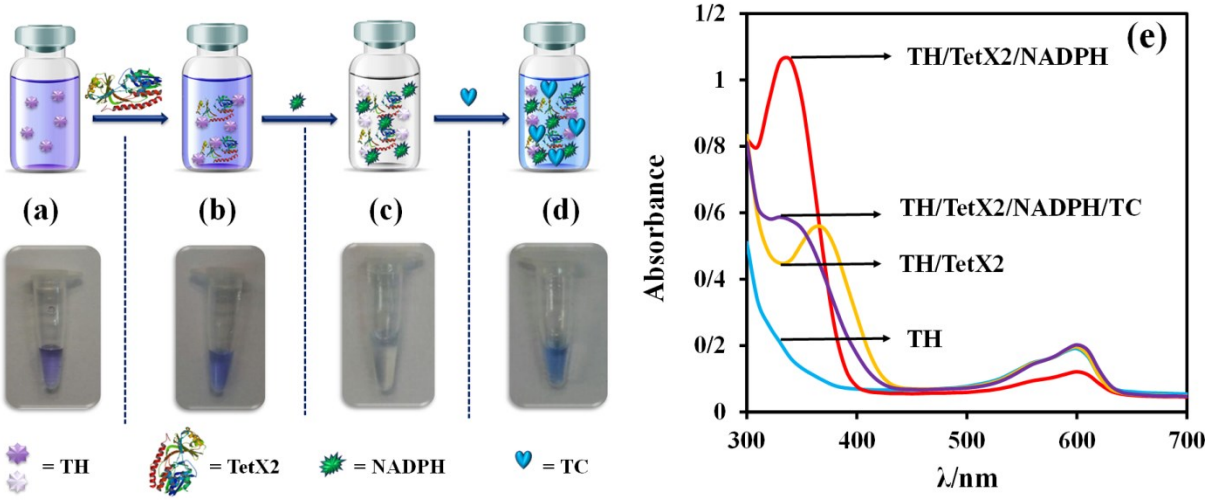

Figure 3. Sensing mechanism for the TetX2-induced TH-based colorimetric assay in Na_2HPO_4 solution (0.1 M, pH 8.5). Color change reaction by naked eyes and corresponding schematic: (a) TH-ox solution (1 mg/ml) which displayed purple/blue color. (b) Adding 3 µl of TetX2 (Conc. 4 mg/ml) to TH-ox solution which results in no color change. (c) The addition of NAD(P)H (1 mM) led to the color change of TH/TetX2 solution from purple/blue to colorless. Image was taken at 30 s pipetting after the NAD(P)H addition. (d) The color change of

the detection solution after incubation with 7 µM TC which generated a blue color in <2 min. (e) UV-Vis spectra of the resulting solutions of TH-ox, TH/TetX2, TH/TetX2/NAD(P)H, and TH/TetX2/NAD(P)H/TC immediately after imaging

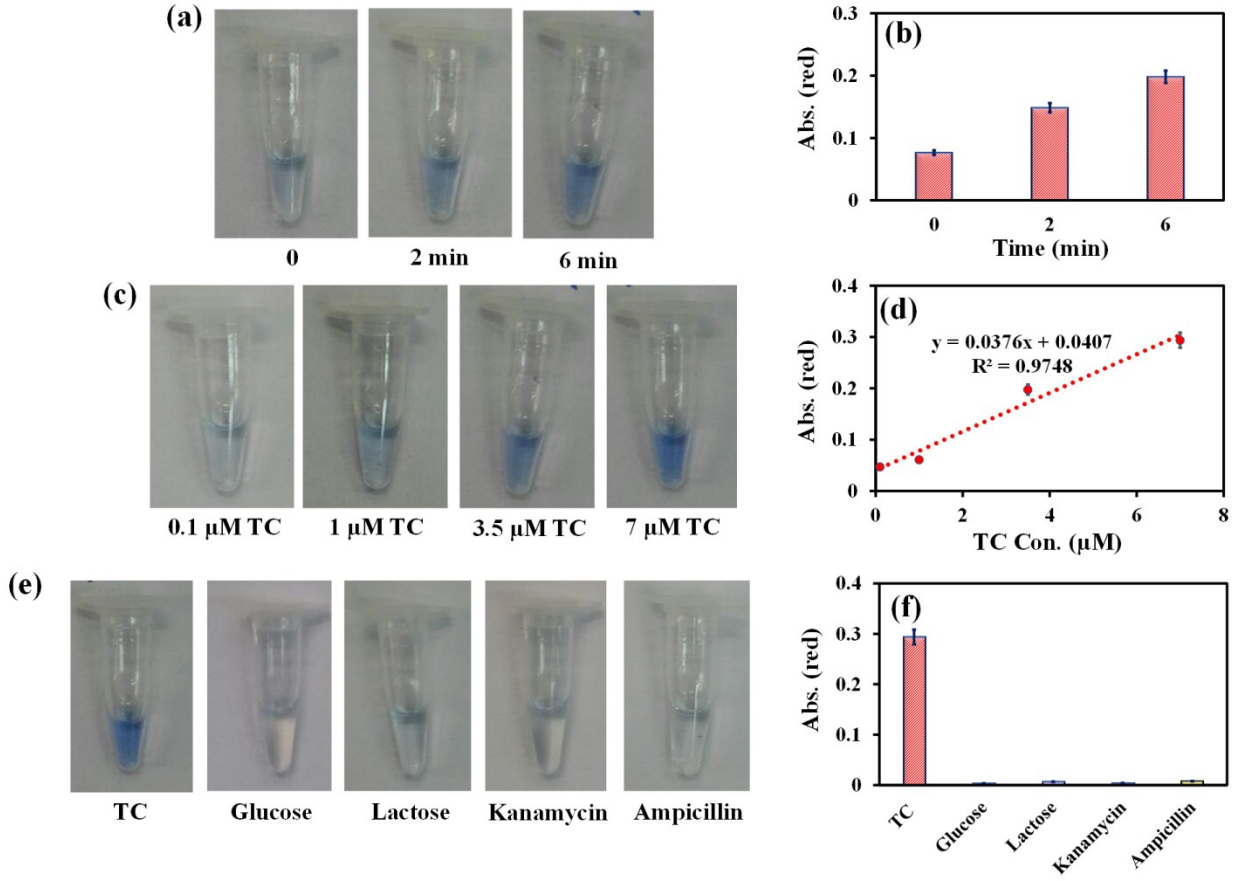

Figure 4. (a) Photograph of the calorimetric assay after TC addition (3.5 µM) and pipetting the detection solution for 2 min time intervals. (b) Bar graph of absorbance at the different time intervals obtained using ImageJ with the red value. (c) Photograph of semi-quantitative determination for different concentrations of TC (0.1, 1, 3.5 and 7 µM) in terms of the color change of TH. (d) Plot of absorbance versus concentration obtained using ImageJ with the red value. (e) The selectivity of TC detection in terms of color change with the various interferences at the concentrations of 10 times greater than TC (from left to right: TC, glucose, lactose, kanamycin, and ampicillin). (f) Bar graph of absorbance in the presence of TC and tested interferences obtained using ImageJ with the red value

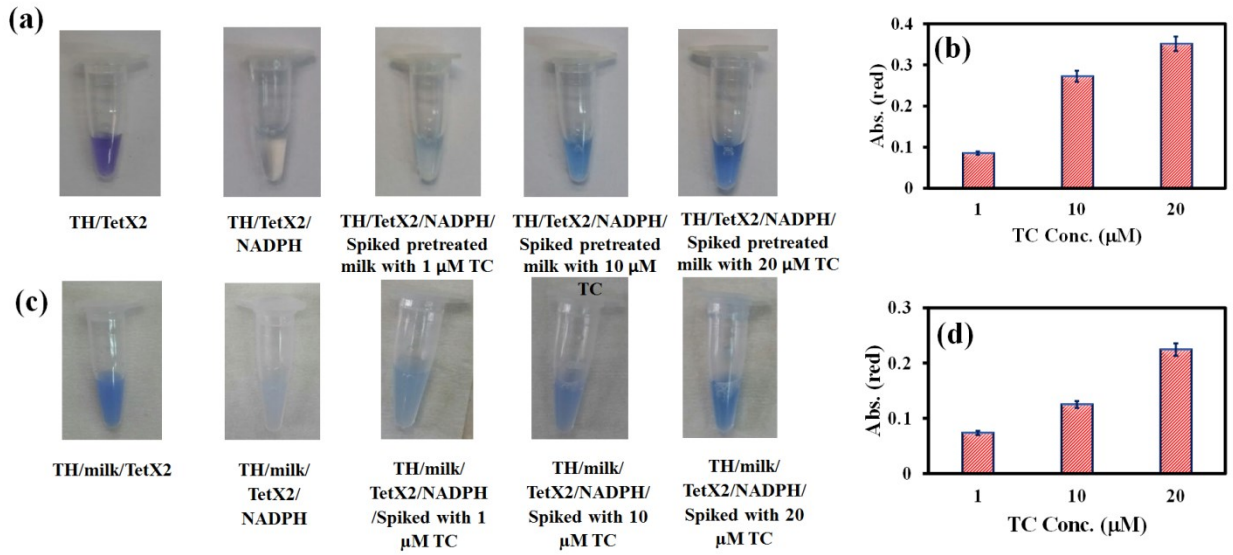

Figure 5. Photograph of the color change in pretreated pasteurized milk spiked with different concentrations of TC (1, 10 and 20 µM). (b) Bar graph of absorbance obtained using ImageJ with the red value in pretreated pasteurized milk spiked with different concentrations of TC (1, 10 and 20 µM). (c) Photograph of the color change in pasteurized milk without any pretreatment (diluted 12 times in the final assay solution) spiked with different concentrations of TC (1, 10 and 20 µM). (d) Bar graph of absorbance obtained using ImageJ in pasteurized milk without any pretreatment (10% milk) spiked with different concentrations of TC (1, 10 and 20 µM)

Graphical abstract