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# Application of a Thiadiazole-derivative in a Tyrosinase-based Amperometric Biosensor for Epinephrine Detection

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**Abstract:** Enzyme-based amperometric biosensors are proving to be important analytical tools in several fields such as food, environmental and, in recent years, the biomedical one. This work describes the use of 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT) in the development of a tyrosinase-based biosensor for epinephrine detection. The modifying agent was obtained as a film by electrochemical oxidation of TBT on a gold disk electrode. Electrochemical characterization and scanning electrode microscopy (SEM) images suggest

the formation of a conducting film on the electrode surface. Tyrosinase from *mushroom* was then immobilized by a mixed technique of adsorption and cross-linking. Glutaraldehyde was used as a coupling agent. The obtained device shows a very good linear response (0.1–50  $\mu\text{M}$ ) with a LoD value of 0.06  $\mu\text{M}$  and a LoQ of 0.09  $\mu\text{M}$ . Moreover, good selectivity towards some typical interferents (namely, ascorbic acid, tryptophan, uric acid and L-cysteine) and satisfactory recoveries have been observed.

**Keywords:** Biosensor · Epinephrine · Tyrosinase · Thiadiazole-derivative · Amperometric detection

## 1 Introduction

Epinephrine (also known as adrenaline or “fight or flight hormone”, EP) (Figure S1) belongs to the catecholamine or biogenic amines group, and works both as a chemical neurotransmitter and as a hormone [1]. Most of it is biosynthesized in the adrenal medulla and in sympathetic nerve terminals, and it is also secreted by the suprarenal gland, as well as norepinephrine [2]. It plays an essential role in the functioning of renal, cardiovascular, hormonal and central nervous systems, both of animals and of humans. Epinephrine regulates blood pressure, immune system, heartbeat rate, lipolysis and glycogen metabolism and it is a potent vasoconstrictor. Moreover, it is released in response to stressful, exciting, dangerous or threatening situations. It is a drug used in medicine in the treatment of allergic emergencies, heart attack (being a cardiac stimulant), bronchial asthma (being a bronchodilator) and cardiac surgery [1,3–5]. Nevertheless, EP, in combination with dopamine (DOP) and norepinephrine (NOR), is wrongly and illegally used by some athletes as energy stimulators, resulting in an increase in the concentration of DOP and EP in urine [6].

Abnormalities of EP levels can represent symptoms of several diseases: low levels can be associated with Parkinson's disease and orthostatic hypotension, whereas slightly high levels are observed in stress and thyroid hormone deficiency, or can be connected with the presence of tumours (e.g. pheochromocytoma) [5,7].

Usually, endogenous EP concentration in plasma is lower than 10 ng/L (0.054 nM). This value can significantly increase or decrease due to physical stress or prolonged intense periods. Specific pathologies, as pheochromocytoma or acute-care cardiac, can also cause an

increasing of 2–4 magnitude order in EP concentration [8]. It is, therefore, clearly evident the importance of monitoring changes in the concentration levels of EP in biological fluids (blood plasma, urine and extracellular fluid of the brain) in order to make possible the detection of potential pathologies, but also for the doping control of the athletes [6]. Moreover, when EP is used in pharmaceutical formulations, especially in therapeutic treatments, it is important to control its concentration and production process, as well as prevent side effects of overdose [8,9].

According to the European Pharmacopoeia, determination of epinephrine is performed by potentiometric titration in anhydrous acetic acid with perchloric acid [10]. Due to the low physiological concentration of epinephrine, a highly sensitive method to conduct a quantitative analysis in biological samples is necessary. In this regard,

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several determination methods, including liquid chromatography [11], flow injection analysis [12], capillary electrophoresis [13], and fluorimetry [14] are used. With this type of methods, LoD values in the range of  $10^{-7}$ – $10^{-9}$  M have been reported. Nevertheless, methods with higher LoDs (in the micromolar range,  $10^{-6}$  M) are commonly used for pharmacological samples investigations [5,8]. Most of these techniques are characterized by high sensitivity and selectivity but tend to be expensive, complicated, sometimes time-consuming, due to the need of derivatization or extraction steps, and often they require high-cost equipment or analysis and cannot be used to perform measurements out of the laboratory. Hence, it is very useful to realize commercial, rapid, low-cost and portable analytical tools, possibly with the high sensitivity and selectivity of the aforementioned methods. A proper solution can be represented by electrochemical (bio)sensors [8,9].

Conducting polymers (CPs) [15–18] proved to be excellent materials for immobilization of biomolecules and to promote rapid electron transfer, thus showing to be particularly suitable for the development of enzyme-based biosensors. Among different CPs, polythiophenes (PThs), polypyrrole (PPy), polyaniline (PANI) and their derivatives are widely employed, thanks to their high conductivity and stability [19]. Here we report the use of a pyridine-thiophene derivative, 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT, Figure 1), that by anodic oxidation on a gold disk electrode results in a film acting as immobilizing agent for tyrosinase in an amperometric biosensor.

The film has proved to efficiently work in the immobilization of tyrosinase, avoiding denaturation or misfolding processes. Furthermore, the sensing mechanism of the biosensor is discussed, according to the role of the analyte and of the modifying film.

## 2 Experimental

### 2.1 Chemicals

Tyrosinase from *mushroom* (lyophilized powder  $\geq 1000$  U/mg solid protein, stored at  $-20^{\circ}\text{C}$ , Tyr), (–)-epinephrine (EP), tetrabutylammonium tetrafluoroborate

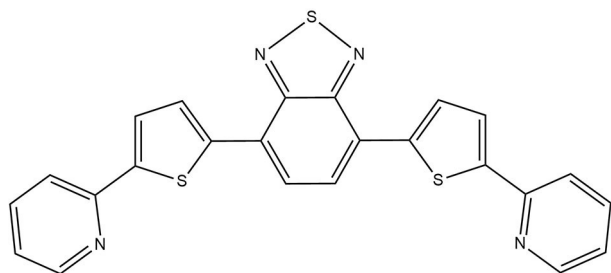


Fig. 1. Structure of 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT).

(TBABF<sub>4</sub>) 99%, methylene chloride (CH<sub>2</sub>Cl<sub>2</sub> anhydrous  $\geq 99.8\%$ ), glutaraldehyde (technical 50% in H<sub>2</sub>O, 5.6 M), hydrochloric acid 37%, uric acid (UA) and Tris (Trizma<sup>®</sup> base) were from Sigma-Aldrich. Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), sodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>), acetic acid and sodium acetate were from Carlo Erba, citric acid from Riedel-De Haën, ascorbic acid (AA) from Lancaster, L-cysteine (L-cys) from Fluka and tryptophan (Trp) from Alfa Aesar. Epinephrine (ADRENALINA S.A.L.F. 0.5 mg/mL, stored at  $2-8^{\circ}\text{C}$ ) was purchased from a local pharmacy. All chemicals were used without any further purification. 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT) was synthesized by some of us in Wrocław University of Science and Technology, Poland [20].

### 2.2 Methods

All electrochemical tests were carried out using a CHI-650 potentiostat interfaced with a PC using the specific software CHI-650 in a conventional three-electrodes voltammetric cell. A gold disk electrode (disk diameter = 2 mm) (properly modified in voltammetric analysis) or an indium tin oxide (ITO) glass was the working electrode, an aqueous Ag/AgCl with suitable salt bridge the reference electrode and a platinum wire the counter electrode. The working electrode was well polished with alumina (Al<sub>2</sub>O<sub>3</sub> 0.3  $\mu\text{m}$ ), washed with water and sonicated for 15 minutes, and finally washed with anhydrous acetone before the electrodeposition. The electrochemical characterization of TBT and the film deposition and characterization on the gold disk electrode were performed by cyclic voltammetry in CH<sub>2</sub>Cl<sub>2</sub> solvent containing TBABF<sub>4</sub> 0.1 M as supporting electrolyte, with a potential scan rate equal to  $100\text{ mV s}^{-1}$ . A potential range between 0.4 and 1.7 V was used for the TBT characterization and film deposition, and between 0.4 and 1.6 V for the film characterization. The deposition of the film on the ITO electrode was performed in CH<sub>2</sub>Cl<sub>2</sub>/TBABF<sub>4</sub> 0.01 M by applying a 1.4 V constant potential. The charge density of the films was measured by integrating the current passed during the electrochemical polymerization process. All the electrochemical measurements for the quantitative epinephrine determination were carried out in a phosphate buffer solution (0.1 M, pH = 7.0) in open-air atmosphere. The voltammetric characterizations of EP solutions were carried out in a potential scan rate range between 10 and  $350\text{ mV s}^{-1}$ . The chronoamperometric curves were recorded at 0.3 V. All potential values are referred to aqueous Ag/AgCl electrode. UV-Vis spectra were recorded on a Hitachi U-2010 spectrophotometer. Scanning electron microscopy (SEM) analysis was performed on a SEM Fei Quanta 200 instrument.

## 2.3 Electrode Preparation

The electrochemical tests on TBT were carried out on a gold disk and on ITO electrode, in anhydrous  $\text{CH}_2\text{Cl}_2$  solution containing 0.01 M TBABF<sub>4</sub> as supporting electrolyte and 0.002 M TBT. Cyclic voltammetry investigation was performed scanning the potential between 0.4 and 1.7 V, at a potential scan rate equal to  $100 \text{ mV s}^{-1}$ , for 50 scans, in order to obtain a film with an adequate thickness, corresponding to a charge density  $Q_{\text{dep}}$  around  $60 \text{ mC cm}^{-2}$ . The modified electrode was characterized by cyclic voltammetry in the same experimental conditions (solvent/supporting electrolyte system and potential scan rate), and finally washed with phosphate buffer solution (pH 7.0) before enzyme immobilization. UV-Vis spectra and SEM analysis were performed on a film ( $Q_{\text{dep}} = 10$  and  $13 \text{ mC cm}^{-2}$ , respectively) deposited on ITO coated-glass from a 0.002 M TBT solution in  $\text{CH}_2\text{Cl}_2/\text{TBABF}_4$  0.01 M by chronoamperometry at 1.4 V and neutralized at 0.0 V for 60 s.

## 2.4 Tyrosinase-Based Biosensor Preparation

Tyrosinase biosensor was prepared through physical absorption and cross-linking between enzyme molecules using glutaraldehyde as coupling agent, according to the procedure reported by Baluta et al. [7] for the immobilization of laccase, that has proved to be effective also in the case of tyrosinase. 40  $\mu\text{L}$  of tyrosinase solution (1.5 mg/1 mL in phosphate-citrate buffer solution pH 5.2) were dropped on the modified electrode surface, taking care that the surface of the electrode did not dry out. After 2 hours, 40  $\mu\text{L}$  of 40 % glutaraldehyde solution in phosphate buffer were added on the electrode surface to crosslink the enzyme. After 10 minutes the unbound protein was washed away dipping the electrode in phosphate buffer at pH 7.0 ( $2 \times 15$  minutes), acetate buffer at pH 5.2 ( $2 \times 15$  minutes), Tris-HCl buffer at pH 7.2 (45 minutes). The sensor was stored at  $4^\circ\text{C}$  in phosphate buffer when not in use.

## 2.5 Epinephrine Sensing

The epinephrine sensing tests were carried out at room temperature in open-air atmosphere, in a cell containing 30 mL of phosphate buffer (0.1 M, pH 7.0). A constant potential of 0.3 V was applied while the solution was gently and constantly stirred. When the background current reached a constant value, incremental amounts of a  $2.7 \times 10^{-4}$  M epinephrine in phosphate buffer solution were injected into the cell with time intervals of about 200 s, and the current/time response was recorded. Epinephrine detection was investigated in the range 0.1–50  $\mu\text{M}$ , where the current response was proportional to the concentration. Stability tests were performed storing the biosensor in phosphate buffer 0.1 M, pH 7.0, at  $4^\circ\text{C}$  between a measurement and another one.

## 3 Results and Discussion

### 3.1 Electrochemical Behaviour of 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT)

The CV response of a 0.002 M TBT solution between 0.4 and 1.7 V showed a broad peak centred at 1.45 V, with a pre-peak at 1.24 V (Figure 2a), showing a current increase at increasing scans typical of an anodic-polymerization behaviour (Figure 2b).

The deep-red deposit visible on the gold surface after 50 scans strengthens the occurrence of a polymerization process. The voltammetric characterization of the film evidenced a highly broad process at about 1.2–1.4 V and a corresponding cathodic process between 1.1 and 0.5 V, according to a doping/de-doping feature typical of a conducting polymer (Figure S2).

A deep thinking could be carried on the nature of the film formed on the electrode surface. Actually, pyridine is known to give electrochemical cathodic polymerization when properly functionalized, as in the case of vinylpyridine [21]. On the other hand, few examples of anodic polymerization of a pyridine ring are reported. A very interesting case concerns the electrochemical behaviour of acridine [22], that gives an oxidation product explained as an oligomer species resulting from C–C and C–N linkages between units of acridine. According to this reference, we propose an analogue mechanism explaining the results of the electrochemical investigation of TBT. In particular, we assume that the oxidation of TBT causes the activation of the pyridine ring (maybe facilitated from the presence of the thiophene fragment), forming a pyridinium radical cation that results in an oligomerization product with a C–N linked structure (Figure 3).

The nature of the film was investigated by UV-Vis spectroscopy and Scanning Electrode Microscopy.

The UV-Vis spectrum (Figure S3) of an ITO electrode coated with a TBT-film evidences a broad absorption band centred at 532 nm ascribable to  $\pi-\pi^*$  transitions due to a high electron delocalization in the organic structure. As a matter of fact, the band-gap value ( $E_g$ ) of the polymer evaluated from the onset-wavelength is significantly lower (1.61 eV) compared to the band-gap of the monomer TBT (2.32 eV).

Scanning Electron Microscopy was performed in order to examine the morphology and the surface features of

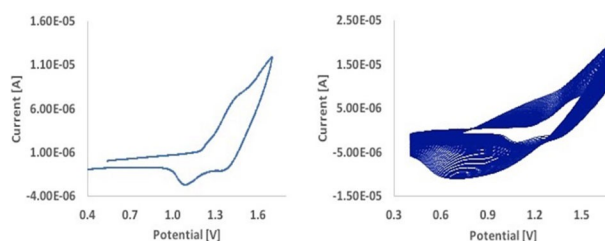


Fig. 2. Cyclic voltammograms of TBT on a gold-disk electrode in  $\text{CH}_2\text{Cl}_2/\text{TBABF}_4$  solvent system: (a) first scan, (b) 50 subsequent scans; potential scan rate:  $100 \text{ mV s}^{-1}$ .

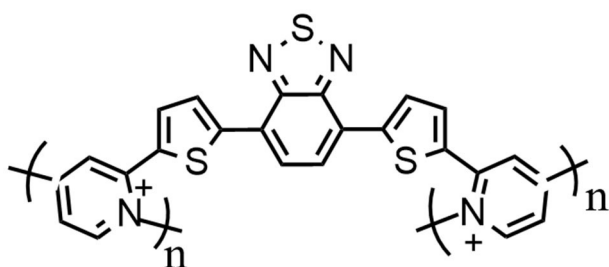


Fig. 3. Proposed growing unit of TBT.

the obtained film (Figure S4). The homogeneous distribution and the porous structure of the film are proved to be advantageous in the development of biosensors. As a matter of fact, a porous structure can increase the specific surface area, thus determining an increase in the sensitivity of the biosensor, as well as facilitate the electron transfer between the active sites of the enzyme and the electrode surface [23].

Tyrosinase was finally immobilized on the modified gold electrode, as described in the experimental section, giving an Au/TBT-film/Tyr biosensor, that was tested in detection of epinephrine.

### 3.2 EP Sensing Mechanism

Tyrosinase belongs to the “type III copper” family. The overall structure can be divided in three domains: the central domain, and the N-terminal and C-terminal domains. The active site is placed in the central domain and it consists of a binuclear type III copper protein containing two copper centres ( $\text{Cu}_A$  and  $\text{Cu}_B$ ) with six histidine residues located in a four helical bundle, coordinating the two metal centres [24,25]. The properties of tyrosinase are determined by four oxidation states of its active site: met, oxy, deoxy and deactivated forms. Using molecular oxygen, tyrosinase catalyses two sequential enzyme reactions: orthohydroxylation of monophenols and oxidation of *o*-diphenols to the corresponding *o*-quinones (Figure S5).

When EP is present, the tyrosinase mechanism can be described as follows: the EP binds to the  $\text{Tyr}_{\text{oxy}}$  (oxidised form of tyrosinase) form to give  $\text{Tyr}_{\text{oxy}}\text{-EP}$ , which oxidises the EP to the corresponding quinone through two steps, involving the  $\text{Tyr}_{\text{oxy}}$  and the  $\text{Tyr}_{\text{met}}$  form, respectively, finally resulting in a deoxy form  $\text{Tyr}_{\text{deoxy}}$  [26].  $\text{Tyr}_{\text{deoxy}}$  can be reactivated by the action of molecular oxygen or, like in our case, by the application of a suitable potential value. When Tyr is immobilized on the electrode surface through a conducting film, direct electronic coupling between the enzyme and the electrode surface could occur, allowing the system to behave as a so-called “third-generation” biosensor. Nevertheless, at least a further requirement has to be satisfied in order to favour a direct communication between enzyme and transducer, that is a short distance between the biomolecule and the conduct-

ing surface [27]. Some examples of redox enzymes (such as tyrosinase, laccase and peroxidase) acting according to such a mechanism are reported [28–31]. However, usually for such redox enzymes a different mechanism is accepted, where the analyte (a phenolic compound) acts as mediator [32]. Furthermore, a key role is played also by the conducting film used to modify the electrode surface. Hence, the behaviour of the biosensor here reported, where tyrosinase enzyme is immobilized on a conducting film covering a gold disk electrode and EP plays the double role of analyte and mediator, can be viewed as a specific case between a mediated electron transfer (where usually the mediator is an “artificial” electron acceptor added to the solution containing the analyte) and a direct electron transfer (without mediator added in solution).

### 3.3 Electrochemical Detection of EP

The ability of the Au/TBT-film/Tyr biosensor to detect EP was initially checked by cyclic voltammetry. Figure 4 reports voltammetric curves recorded on a bare gold electrode and on the modified Au/TBT-film and Au/TBT-film/Tyr electrodes in a  $20\ \mu\text{M}$  EP solution. Two broad peaks, with low current intensity, are observed with the bare electrode, attributable to the oxidation of EP to the open-chain quinone (adrenalinequinone), which can cyclize to leucoadrenochrome, and to the oxidation of this last one to adrenochrome, respectively [33–35]. Conversely, the voltammetric responses obtained with film-modified as well as tyrosinase-based electrodes evidence a peak intermediate between the peaks of the Au bare electrode. This feature suggests that the double-step oxidation process of EP occurs in a single-step on the modified electrodes. The voltammetric responses appear

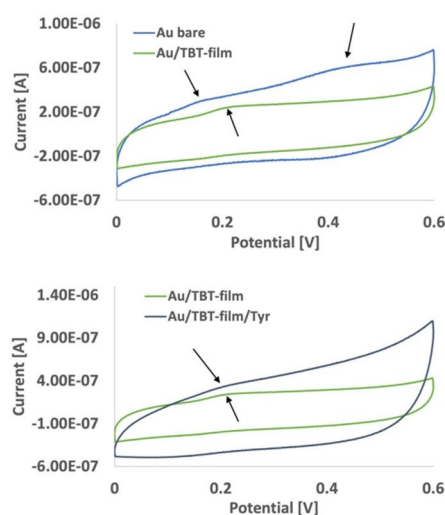


Fig. 4. Comparison between bare Au and modified Au/TBT-film (top) and between Au/TBT-film and Au/TBT-film/Tyr (bottom) electrodes in presence of  $20\ \mu\text{M}$  EP. Potential range: 0–0.6 V; potential scan rate:  $30\ \text{mV s}^{-1}$ .

clearer at increasing potential scan rate, with an increase in peak current and a shift to more anodic potential values.

In order to investigate the response of the sensor to EP, chronoamperometric experiments were performed at 0.3 V, according to CV characterization. As evidenced in Figure 5a, addition of known amounts of EP solution produced a jump-like increase of current. The current response was proportional to the concentration in the range 0.1–50  $\mu\text{M}$  with a very good correlation factor ( $R^2 = 0.996$ ) (Figure 5b).

Limit of detection (LoD) of the Au/TBT-film/Tyr sensor has been calculated as:

$$LoD = (3.29\sigma_B)/b \quad (1)$$

where  $\sigma_B$  is the standard deviation of blank response (11 measurements) and  $b$  is the slope of the regression line. Using the equation (1), LoD has been determined as

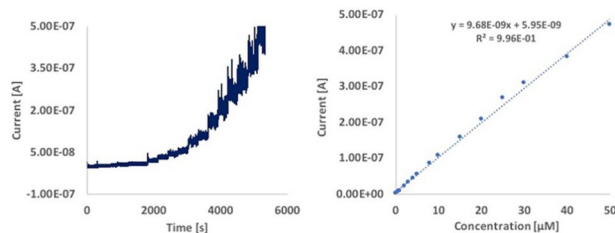


Fig. 5. (a) Current/time response in 0.1 M phosphate buffer (pH 7.0) with EP concentration between 0.1 and 50  $\mu\text{M}$  and (b) corresponding calibration curve. Working potential: 0.3 V vs Ag/AgCl.

0.06  $\mu\text{M}$ . Limit of quantification (LoQ) was determined using equation (2):

$$LoQ = (5\sigma_B)/b \quad (2)$$

LoQ value was calculated as 0.09  $\mu\text{M}$ . Sensitivity was calculated as the ratio of the slope of the calibration curve to the area of the electrode surface and was found to be  $3.08 \cdot 10^{-7} \text{ A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-2}$ . The enzyme sensor was tested for stability by repeated measurement every week on a 20  $\mu\text{M}$  EP solution, showing a stability for times higher than 30 days.

All analytical parameters are reported in table 1.

The comparison to other tyrosinase biosensors (table 2) evidences that the performances of the reported biosensor in terms of linear range and LoD are comparable to (or better than) literature data.

In addition, it is possible to calculate the apparent Michaelis-Menten constant ( $K_M$ ) using the adapted Lineweaver-Burk equation (3) valid for an immobilized enzyme onto an electrode surface [9,39]:

$$1/I = 1/I_{max} + K_M/(I_{max}[C]) \quad (3)$$

where  $I$  is the anodic current,  $I_{max}$  is the steady-state current and  $[C]$  is the concentration of EP.  $I_{max}$  ( $4.5 \cdot 10^{-7} \text{ A}$ ) and  $K_M$  (35  $\mu\text{M}$ ) values have been calculated from the intercept and slope, respectively. In particular, the small  $K_M$  value, measuring the affinity between the enzyme and its substrate, suggests a strong affinity between the immobilized tyrosinase and EP.

Moreover, the calibration data allow to evaluate the Hill coefficient ( $n_H$ ) by graphing  $\log [I/(I_{max}-I)]$  vs.  $\log [C]$ .

Table 1. Analytical parameters of the calibration curve for the determination of epinephrine with Au/TBT-film/Tyr biosensor.

Linearity [ $\mu\text{M}$ ]	LoD [ $\mu\text{M}$ ]	LoQ [ $\mu\text{M}$ ]	$R^2$	Slope [ $\text{A}/\mu\text{M}$ ]	Intercept [A]	Sensitivity [ $\text{A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-2}$ ]	Stability [days]
0.1-50	0.06	0.09	0.996	$9.68 \cdot 10^{-9}$	$5.95 \cdot 10^{-9}$	$3.08 \cdot 10^{-7}$	> 30

Table 2. Comparison of biosensors for EP detection.

(Bio)sensor	Technique	Linear Range [M]	LoD [ $\mu\text{M}$ ]	Ref.
CPE/PPO	FIA	$0.5-3.5 \cdot 10^{-4}$	15	[2]
GCE/GQDs/Lac	CV	$1-120 \cdot 10^{-6}$	0.083	[7]
CPE/PtNPs in BMI.PF <sub>6</sub> /Lac	SWV	$9.99 \cdot 10^{-7}-2.13 \cdot 10^{-4}$	0.29	[1]
CPE/MWCNT/Nafion/Tyr	DPV	$5.0-500 \cdot 10^{-6}$	0.3	[5]
GCE/SWCNT/Tyr	CA	$10-110 \cdot 10^{-6}$	2.54	[9]
Nanostructured Au electrode	LSV and DPV	$10-150/60-600 \cdot 10^{-6}$	2.8/7.3	[8]
MnO <sub>2</sub> /GCE/Nafion	CV and DPV	$0.03-10/10-100 \cdot 10^{-6}$	0.005	[36]
CPE/MWCNT	DPV	$0.05-1/1-10 \cdot 10^{-5}$	0.029	[37]
MWCNT/EPPGE	SWV	$0.5-100 \cdot 10^{-9}$	0.00015	[38]
Au/pol/Tyr	CA	$0.1-50 \cdot 10^{-6}$	0.06	This work

CPE: carbon paste electrode; PPO: polyphenol oxidase; FIA: flow injection analysis; GCE: glassy carbon electrode; GQDs: graphene quantum dots; Lac: laccase; PtNPs: platinum nanoparticles; SWV: square wave voltammetry; MWCNT: multi-walled carbon nanotubes; Tyr: tyrosinase; CA: chronoamperometry; LSV: linear sweep voltammetry; DPV: differential pulse voltammetry; EPPGE: plane pyrolytic graphite electrode; pol: poly(4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole).

Table 3. Recovery tests results for Au/TBT-film/Tyr biosensor (n = 5).

EP in Samples ( $\mu\text{M}$ )	EP found ( $\mu\text{M}$ )	RSD	Recovery%
25.0	24.2	$\pm 4.89$	96.8
40.0	39.5	$\pm 2.60$	98.7

This dimensionless parameter gives information about the cooperativity of ligand binding. The obtained value is  $1.09 \pm 0.01$  ( $R^2 = 0.984$ ), which, being very close to unity, suggests that the kinetics of enzyme reaction is in agreement with a Michaelis-Menten type kinetics. Furthermore, the value slightly higher than 1 shows that there is a positive cooperative effect between the occupied active centres, that is the affinity of the tyrosinase for EP increases after the binding of an EP molecule with the enzyme.

The effect of scan rate on biosensor response was also investigated for  $50 \mu\text{M}$  EP at different scan rates (Figure S6a). A linear graph peak current vs. square root of scan rate with  $R^2 = 0.997$  was obtained in the range  $10\text{--}350 \text{ mVs}^{-1}$  (Figure S6b), typical of a diffusion-controlled mechanism.

The diffusion coefficient ( $D$  [ $\text{cm}^2 \cdot \text{s}^{-1}$ ]) of EP can be calculated from the slope of the graph S6b using the Randles-Sevcik equation (4):

$$i_p = 2.69 \cdot 10^5 n^{3/2} A D^{1/2} C v^{1/2} \quad (4)$$

where  $i_p$  is the maximum anodic peak current [A],  $n$  is the number of exchanged electrons,  $A$  is the electrode area [ $\text{cm}^2$ ],  $C$  is the EP concentration [ $\text{mol} \cdot \text{cm}^{-3}$ ], and  $v$  is the potential sweep rate [ $\text{V} \cdot \text{s}^{-1}$ ]. Diffusion coefficient was estimated as  $5.1 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ .

#### 4 Interference Study

The selectivity of the TBT-based biosensor was investigated by differential pulse voltammetry towards some typical components of biological fluids, namely ascorbic acid (AA), tryptophan (Trp), uric acid (UA), and L-cysteine (L-cys). The effect of each interferent was tested for EP:interferent ratios equal to 1:1, 1:5 and 1:10 in phosphate buffer solutions containing  $10 \mu\text{M}$  EP. As evidenced in Figure S7, AA, Trp and UA appear to have a low or very low effect in these experimental conditions. In particular, AA does not interfere in the determination of EP when present with the same concentration or with a ratio EP:AA = 1:5, and causes a slight increase (4%) in the value of EP when EP:AA = 1:10. A slightly high effect is observed in the case of Trp (−2%, −5% and −11%) and UA (−4%, −7%, −11%) at increasing EP:interferent ratios. A higher effect is observed when L-cys is present, producing an underestimation of 14% and 22% when EP:L-cys is equal to 1:1 and 1:5, respectively. Higher concentrations of L-cys cause the disappearance of the voltammetric peak of EP, making so impossible its determination. This specific behaviour of L-cys is reason-

ably ascribable to its chemical structure containing -SH groups, that likely deactivate the enzyme.

#### 5 Real Samples

EP concentration levels in biological fluids are reported to be very low ( $< 10 \text{ ngL}^{-1}$ ) in physiological conditions [8]. For this reason, the calculated LoD and LoQ values for the Au/TBT-film/Tyr biosensor seem suitable for determination of EP in pharmacological formulations rather than in biological samples. Therefore, recovery tests were performed on commercially available injectable solutions containing EP  $0.5 \text{ mg mL}^{-1}$  ( $2.7 \text{ mM}$ ) diluted to  $25.0 \mu\text{M}$  and  $40.0 \mu\text{M}$ . Recovery values were calculated as percent ratio of the detected EP concentration to the real value, and relative standard deviation (RSD) values were calculated on five replicated measurements for each sample. Recovery and RSD values reported in Table 3 show an acceptable accuracy in detecting EP.

#### 6 Conclusion

A film was obtained from the electrochemical oxidation of 4,7-bis(5-(pyridin-2-yl)thiophen-2-yl)benzo[c][1,2,5]thiadiazole (TBT) on a gold electrode surface. Tyrosinase from *mushroom* has successfully been immobilized on the modified electrode, leading to development of a biosensor for EP detection. The highly conjugated structure of the film, as suggested from the UV-Vis characterization, as well as its homogeneous and porous structure shown by SEM characterization, are reasonably the main features responsible for the performances of the biosensor. In particular, the here-reported biosensor has shown very good electrocatalytic activity in the linear concentration range of  $0.1\text{--}50 \mu\text{M}$  with a LoD value of  $0.06 \mu\text{M}$  and LoQ of  $0.09 \mu\text{M}$  that are comparable (or better than) to those reported in literature data for other (bio)sensors for EP determination. Furthermore, interference study shows a high selectivity of the reported biosensor in presence of some typical components of biological fluids. These features make the device a potential useful tool for the quantification of EP in pharmaceutical formulations.

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#### Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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