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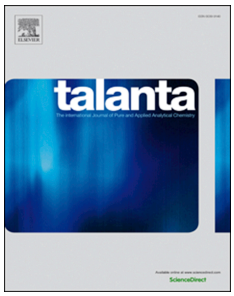

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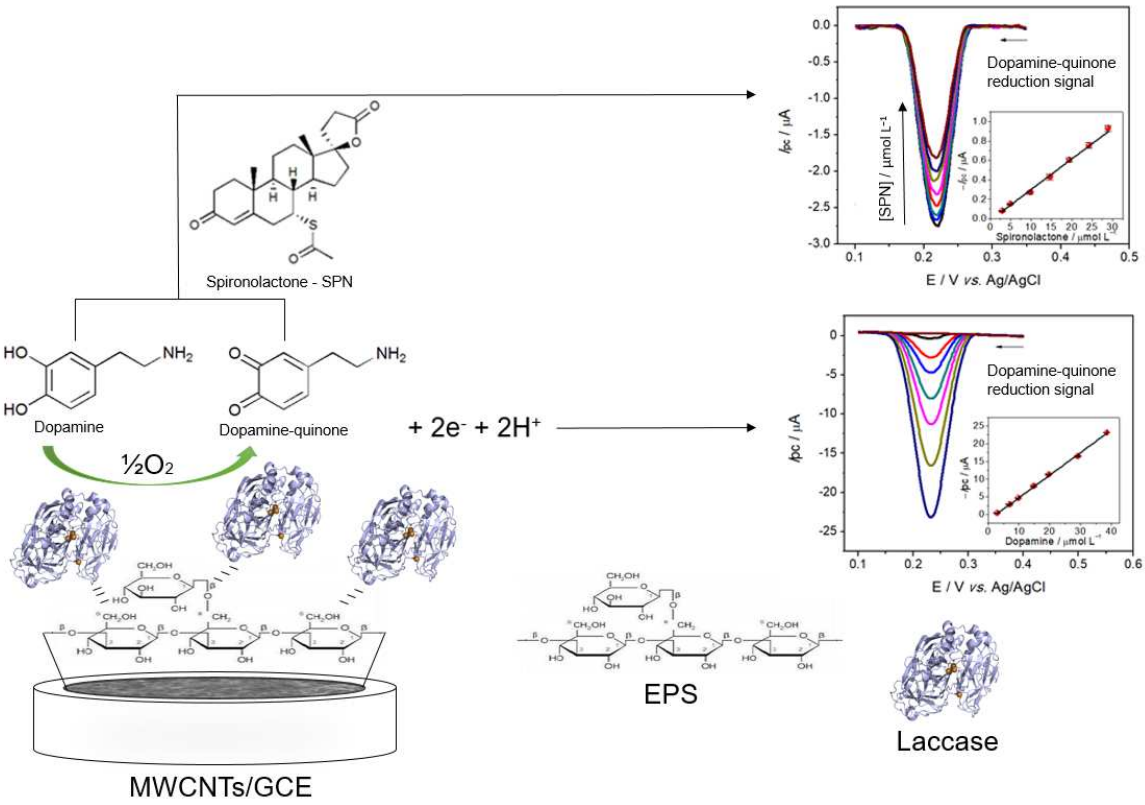
GRAPHICAL ABSTRACT



Exploring the exocellular fungal biopolymer botryosphaeran for laccase-biosensor architecture and application to determine dopamine and spironolactone

João H. Coelho[1], Ana P. P. Eisele[1], Camila F. Valezi[1], Gabriel J. Mattos[1], Jéseka G. Schirmann[1], Robert F. H. Dekker[2], Aneli M. Barbosa-Dekker[1], Elen R. Sartori[1,*]

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Abstract

Laccase was immobilized on a glassy carbon electrode layered with multi-walled carbon nanotubes using a film of botryosphaeran, a fungal (1→3)(1→6)-β-D-glucan. This novel biosensing platform was characterized by electrochemical impedance spectroscopy and scanning electron microscopy, and applied for the determination of dopamine. Experimental variables such as enzyme concentration, pH value and operational parameters of the electroanalytical technique were optimized. Using square-wave voltammetry, there was a linear dependence of peak current and dopamine concentration within the range of 2.99 – 38.5 µmol L^{-1} with a limit of detection of 0.127 µmol L^{-1}. The biosensor was successfully applied in the determination of dopamine in pharmaceutical injection and synthetic biological samples, and presented good selectivity even in the presence of uric acid and ascorbic acid, as well as other phenolic compounds. The different aspects regarding the operational stability of the laccase biosensor were evaluated, demonstrating good intra-day and inter-day repeatability, and long-storage stability. Furthermore, this biosensor was evaluated in the indirect determination of spironolactone by using the analytical signal of dopamine, presenting a limit of detection of 0.94 µmol L^{-1}. The results obtained in the analysis of spironolactone in commercial pharmaceutical samples were satisfactory.

Keywords: *Botryosphaeria rhodina* MAMB-05, (1→3)(1→6)-β-D-glucan, electrochemical biosensors, dopamine, indirect determination, spironolactone.

1. Introduction

Natural polysaccharides have been extensively explored in the development of new architectures of electrochemical biosensors. Chitosan and carboxymethyl cellulose are some of the most investigated polysaccharides for this purpose [1,2], considering their remarkable characteristics that include biocompatibility, biodegradability, ability to form adherent thin films on traditional electrochemical surfaces, renewable, abundant in nature and generally nontoxic [3,4].

The ascomyceteous fungus *Botryosphaeria rhodina* MAMB-05 produces an exopolysaccharide (EPS) named botryosphaeran (a (1→3)(1→6)-β-D-glucan), when cultivated by submerged fermentation on glucose medium [5]. The chemical structure of this EPS consists of a backbone of β-(1→3)-linked glucose residues to which are linked glucose and diglucose (gentiobiose) residues via β-(1→6) bonds with approximately 22% side-branching through C-6 along the backbone chain. An attractive characteristic of polysaccharides such as botryosphaeran is that they form a film upon which enzymes can assemble providing a matrix for maintaining enzyme stability. This feature provides a favorable biochemical environment for the enzyme, and was of interest to exploit to immobilize laccase onto botryosphaeran for the purpose of fabricating an electrochemical transducer. Other polysaccharides have been used to adhere laccase to electrochemical transducers and facilitate electron transfer between the enzyme and the electrode [6,7].

B. rhodina MAMB-05 also produces a laccase (EC 1.10.3.2) constitutively at low enzyme titers, but can be induced to higher activity levels by veratryl alcohol [8,9]. Laccases are glycoproteins that contain a cluster of copper atoms at the enzyme's active centre, which are involved in shuttling electrons in Redox reactions involving the oxidation of phenolic compounds in the reduced form, and the reduction of molecular oxygen to

water [10]. Several electrochemical sensors and biosensors are described in the literature for the determination of dopamine with high sensitivity [11-18]. However, laccase-based biosensors allow the direct determination of a broad range of phenolic compounds in complex matrices due to their sensitivity and selectivity. Besides, they have advantages of being low cost, simplicity of fabrication, rapid responses and possibility of miniaturization and potential ability for real-time and on-site analysis.

Carbon-based nanostructured materials, such as multi-walled carbon nanotubes (MWCNTs), have been extensively applied to modify electrode surfaces to construct new architectures of biosensors [6,19]. They are well suited for transduction of electric signals generated upon recognition of a biological response resulting from a biochemical reaction between the enzyme immobilized on the sensor surface and the target analyte. The small size of the MWCNTs enhances overall electrochemical performance of the bulk electrode, promoting a high transfer electronic signal between the electrode/solution interface, and so, increases the sensitivity and lowers the detection limits. MWCNTs are insoluble in water or any of the common solvents. A wide variety of strategies have been proposed to solve these problems, including chemical oxidation with strong acids, which helps to eliminate metallic impurities, removes the end-caps and adds oxygenated functional groups to the tube-ends and defective sites, facilitating their handling, and may also offer special opportunities for the adsorption of biological molecules [20,21].

Electrochemical biosensors containing laccase can be employed in indirect drug determinations that refer to parallel reactions that can occur with the substrate or product of the enzymatic reaction. Sartori *et al.* [22] determined sulfite in wine samples using a voltammetric biosensor based on a crude preparation of polyphenol oxidase from an extract of sweet potato (*Ipomoea batatas* (L.) Lam.). In this case, sulfite, which is a nucleophile, reacts with *o*-quinone leading to the formation of *o*-quinone sulfite. Similarly,

Santhiago and Vieira [23] employed a carbon-paste electrode modified with laccase from *Aspergillus oryzae* for the determination of cysteine. When cysteine was added, the *p*-benzoquinone formed in the enzymatic step reacted through a nucleophilic 1,4-Michael addition forming the corresponding thiol compound. From the foregoing examples, biosensors appear as suitable alternative strategies to develop simple and precise analytical methods for the indirect determination of spironolactone (used in the therapy of essential hypertension, congestive heart failure, and liver cirrhosis [24]). Previous studies have described the direct electrochemical determination of spironolactone employing mercury-based electrodes [25–28]. These electrodes require special care due to the toxicity of mercury, reinforcing the importance of the development of alternative analytical procedures that employ materials that are non-toxic and sustainable.

With the foregoing in mind as our objective, we report the fabrication of a novel voltammetric biosensing platform obtained by immobilizing a crude preparation of laccase onto a glassy carbon electrode (GCE) modified with MWCNTs and the EPS botryosphaeran (denoted as laccase-EPS-MWCNTs/GCE). Dopamine was chosen as the phenolic compound to evaluate the performance of this novel biosensing platform for its determination in pharmaceutical and synthetic-prepared biological samples. The sensitivity and selectivity towards the factors affecting the analytical characteristics of this biosensor, such as pH, buffer and enzyme activity is reported. The advantages and performance of this novel biosensor are described and are compared to those of similar biosensors reported in the scientific literature. After establishing the best operating conditions of this biosensor, this fabricated electrochemical device was used to evaluate the indirect determination of spironolactone in pharmaceutical samples.

2. Experimental

2.1. Reagents and solutions

Dopamine, spironolactone and MWCNTs (20-30 nm in diameter and 0.5-2.0 µm in length; purity: ≥ 95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, uric acid, $K_4[Fe(CN)_6]$ and N,N-dimethylformamide (DMF) were obtained from Synth (São Paulo, SP, Brazil). All reagents were of analytical grade and used as received. The MWCNTs was purified before use with 2.0 mol L^{-1} HCl solution followed by its functionalization by acid treatment using a mixture of HNO_3:H_2SO_4 (3:1, v/v), as described in the literature [20,29]. Commercial pharmaceutical samples (pharmaceutical injection-type dopamine, spironolactone tablets) were purchased from a local pharmacy in the city of Londrina, Brazil.

All reagent solutions were prepared with ultra-purified water (resistivity greater than 18 MΩ cm) produced by a Milli-Q system (Millipore®, Milford, MA, USA). Potassium phosphate buffer solution (PBS; 0.10 mol L^{-1}, pH 6.0) was used as the supporting electrolyte throughout the experiments. A stock solution of dopamine (10 mmol L^{-1}) was freshly prepared in 0.10 mol L^{-1} PBS (pH 6.0) prior to use, while a stock solution of spironolactone (10 mmol L^{-1}) was prepared daily in 50% (v/v) ethanol and 0.10 mol L^{-1} PBS (pH 6.0). Standard solutions of these analytes were prepared from the stock solutions by appropriate dilutions.

2.2. Apparatus

Square-wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements used with the constructed biosensor were performed on an FRA2

μAutoLab Type III potentiostat/galvanostat (Eco Chemie, The Netherlands) controlled with GPES software (www.ecochemie.nl). A conventional three-electrode single-compartment glass-cell assembly containing a biosensor as the working electrode, an Ag/AgCl (3.0 mol L^{-1} KCl) reference electrode, and a platinum plate as auxiliary electrode were used at room temperature (25.0 ± 0.5 °C). A stirring time of 30 s was used to homogenize the supporting electrolyte containing the analytes in the electrochemical cell. The square-wave voltammograms were baseline-corrected by the moving average method (peak width: 0.001). The EIS experiments were performed within the range of 10 mHz to 100 kHz (10 points per decade) and with a 10 mV (r.m.s.) ac perturbation, for a solution of 1 mmol L^{-1} $K_4[Fe(CN)_6]$ in 0.10 mol L^{-1} KCl.

Microscopy images were obtained with a field emission gun/scanning electron microscope (FEG/SEM), model JOEL FEG-SEM JSM 6330-F (JEOL, USA), operated at 5 kV. A cut disk from a glassy carbon was used to obtain the different modifications, using the same drop-coating method employed for electrochemical analysis, and dried under ambient conditions.

Spectrophotometric determinations were carried out in a 1 cm quartz cell using a ThermoSpectronic UV-visible spectrophotometer, model Genesys (Waltham, MA, USA) coupled to a computer.

2.3. Laccase immobilization on botryosphaeran and fabrication of the biosensor

A mass of 1.0 mg of MWCNTs was added to 1.0 mL of DMF and subjected to sonication for 60 min to give a stable black suspension. A GCE (Tokai Carbon Co. Ltd., Japan, 3-mm diameter) was used as the base electrode. Prior to modification, the GCE was

carefully polished with 0.3 µm alumina slurry on a polishing cloth and then ultrasonically cleaned with ultrapure water for three minutes, and allowed to dry at room temperature.

For the fabrication of the biosensor, laccase and botryosphaeran were obtained from *B. rhodina* MAMB-05 as previously described [5,30]. The biosensor was prepared by first dropping a suspension of 5 µL of MWCNTs onto the cleansed surface of the GCE using a micropipette and allowing this to dry at room temperature for 2 h. This procedure was repeated twice. Thereafter, botryosphaeran solution (3.5 µg) and 0.14 units of the crude laccase extract (CLE) were dropped simultaneously onto the surface of GCE modified with MWCNTs and allowed to dry for 2 h at 4 °C. Fig. 1 shows the schematic of the steps involved in the preparation of the biosensor. The constructed biosensor was stored in a flask containing PBS (pH 6.0) at 4 °C (refrigerator) when not in use.

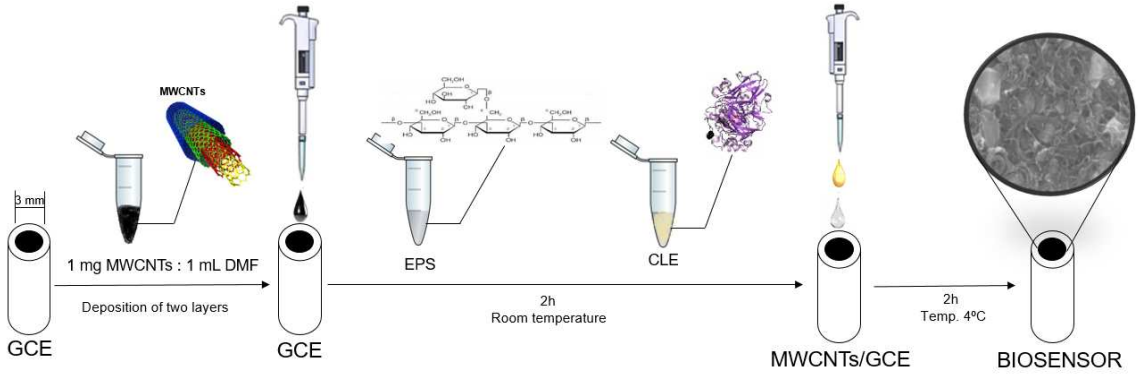

Fig. 1. Schematic illustration of the different steps employed in the construction of the biosensor.

2.4. Measurement procedures

The analytical curve for dopamine was constructed by adding small volumes of a standard solution of this phenolic compound to a clean and dried electrochemical cell containing 10 mL of PBS (pH 6.0), in which the measurements were performed in triplicate. Square-wave voltammograms were recorded by scanning the potential from 0.40

to 0.10 V after successive additions of dopamine. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formula: LOD = 3*S* / *M* and LOQ = 10*S* / *M*, where *S* is the standard deviation of three and ten measurements, respectively, of the blank solution, and *M* is the slope of the analytical curve

The same scanning potential (0.40 to 0.10 V) was employed to construct the analytical curve for spironolactone. For this purpose, a first analytical signal (I_o) was measured relative to PBS (pH 6.0) containing 7.40 µmol L^{-1} dopamine in the absence of spironolactone. Then, a second analytical signal (I) was generated, which was smaller than I_o, when spironolactone was added in the solution. Thus, the addition of spironolactone leads to a decrease in the base signal of dopamine, which is proportional to the varied concentration of spironolactone added.

2.5. Preparation of pharmaceutical and synthetic biological samples for the determination of dopamine

To prepare the sample, pharmaceutical injection-type dopamine (5.0 mg mL^{-1}), a volume of 307 µL of this sample was pipetted into a 10 mL volumetric flask containing PBS (pH 6.0). Then an aliquot of 150 µL of the prepared solution was transferred to the electrochemical cell containing the supporting electrolyte, and the square-wave voltammograms were obtained. For comparison, this sample was also analyzed, in triplicate, by a spectrophotometric method, as recommended by the British Pharmacopoeia [32].

Synthetic cerebrospinal fluid was prepared as described by Zhang *et al.* [33] and Toledo *et al.* [34], and was used immediately after its preparation to avoid any hydrolysis of urea. A synthetic urine sample was prepared as described by Laube, Mohr and Hesse

[35], and was also used immediately after its preparation. An aliquot of 100 µL of each of the biological samples was spiked with 3.98 and 7.94 µmol L^{-1} of dopamine. The analyses of these samples were performed using the standard addition method, and SWV experiments were conducted directly on these samples without any pretreatment procedures.

2.6. Preparation of pharmaceutical samples for the indirect determination of spironolactone

The samples of spironolactone were prepared as follows: ten tablets of each formulation were weighed and pulverized to a homogeneous fine powder using a pestle and mortar. An accurate amount equivalent to that of one tablet of each pharmaceutical powdered sample was transferred into a 10 mL volumetric flask and PBS (pH 6.0) added. Afterwards, 17 and 33 µL of these solutions were transferred to the electrochemical cell containing 10 mL of the supporting electrolyte and 7.4 µmol L^{-1} of dopamine, and quantified after successive additions of the spironolactone stock solution, and the square-wave voltammograms obtained. The concentration of spironolactone in each sample solution was determined in triplicate by the standard addition method. A reference spectrophotometric method described by Brazilian Pharmacopoeia [36] was used to compare the analytical results obtained for spironolactone using the biosensor.

3. Results and discussion

3.1. Immobilization of laccase on modified GCE via botryosphaeran

Enzyme immobilization on a solid matrix is a key factor in the preparation of a biosensor, and efforts have concentrated on the control over enzyme activity and stability, which are important. Direct adsorption of enzymes onto a solid surface may lead to a rapid and simple immobilization procedure, retaining the enzyme's specific biological function.

In this work, laccase was immobilized onto modified GCE by physical adsorption using the fungal EPS, botryosphaeran. At the same time as the EPS is adsorbed onto the MWCNTs film on the GCE surface, the EPS film adsorbs the laccase maintaining the native enzyme's activity, without restricting access to phenolic compounds at the enzyme's catalytic site.

The proposed mechanism for the immobilization of laccase onto botryosphaeran is based upon non-covalent interactions, mainly through hydrogen bonding between the hydroxyl and carboxyl groups of the enzyme and hydroxyl groups in the structure of botryosphaeran. In addition, carboxyl groups present in the functionalized MWCNTs can also interact through hydrogen bonding with the hydroxyl groups in the EPS resulting in an adherent film on GCE with high stability. The possible reason may be due to the good biocompatibility and the stabilizing property provided by botryosphaeran. This EPS when dried forms a film that is an attractive matrix for enzyme immobilization, and consequently an excellent material for the construction of novel biosensor architectures. It is worth mentioning that a homogeneous dispersion of MWCNTs in botryosphaeran was not observed, thus botryosphaeran was added directly to the MWCNTs film formed on the GCE surface.

3.2. Morphological and electrochemical characterization of the proposed biosensor

The characterization of the following fabricated electrodes: GCE, EPS/GCE, MWCNTs/GCE, EPS-MWCNTs/GCE and laccase-EPS-MWCNTs/GCE was performed by SEM analysis. As shown in Fig. 2B, the surface of the GCE modified with only EPS film does not show a significant difference when compared to the GCE clear surface (Fig. 2A), indicating the formation of a transparent film on the GCE surface. On the other hand, Fig. 2C indicates that MWCNTs were well distributed on the surface of the GCE. In Fig. 2D, a thin layer of EPS covered the MWCNTs, while Fig. 2E shows the proposed biosensor morphology, with agglomerates of different sizes, indicating immobilization of the laccase together with EPS on the surface of MWCNTs/GCE.

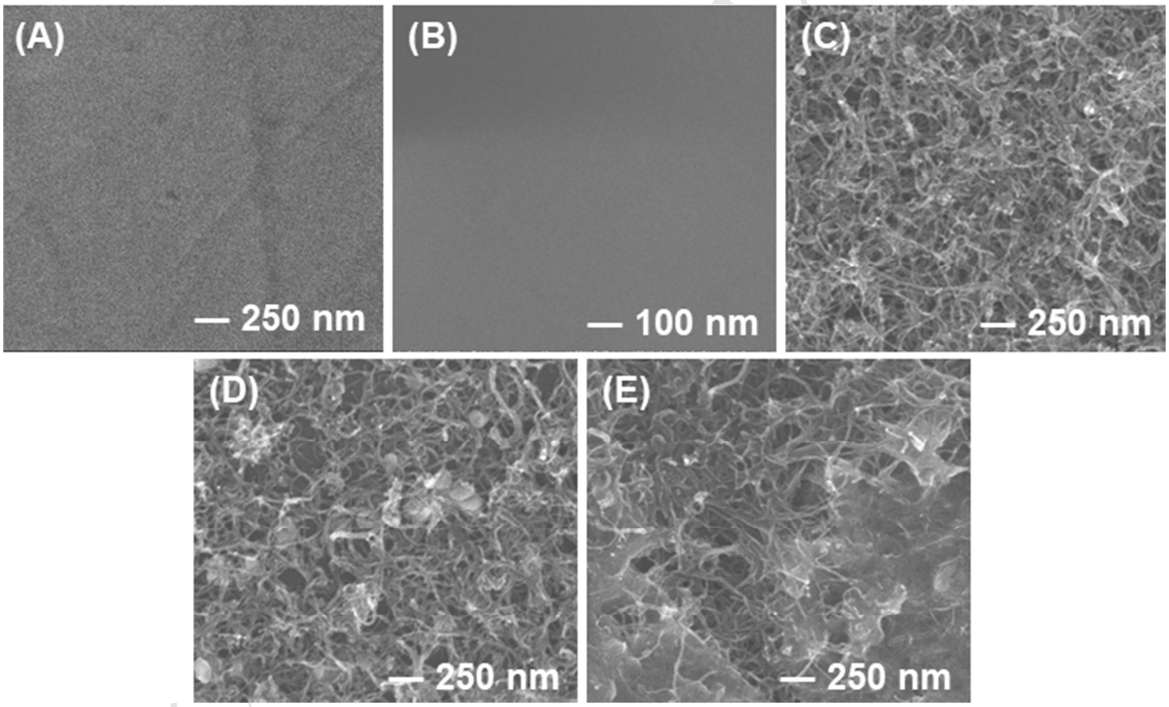

Fig. 2. Scanning Electron Microscope images of the surfaces of (A) GCE, (B) EPS/GCE, (C) MWCNTs/GCE, (D) EPS-MWCNTs/GCE and (E) laccase-EPS-MWCNTs/GCE.

The electrochemical characterization of GCE, EPS/GCE, EPS-MWCNTs/GCE and laccase-EPS-MWCNTs/GCE was performed based upon electrochemical impedance

spectroscopy (EIS) using the well-known $[Fe(CN)_6]^{4-}$ as a redox probe (Fig. 3). This study was employed to evaluate the properties of the different electrodes with respect to the kinetics of electron transfer. Fig. 3 presents the Nyquist plot of the Randles impedance (inset circuit) with the semicircle at higher frequencies corresponding to the charge transfer resistance (R_{CT}), and the straight line at an angle of 45° to the real axis at lower frequencies representing the diffusion process (mass transfer control). Differences in the electron-transfer resistances were observed according to the electrode surface modification. The addition of botryosphaeran caused a significant increase in R_{CT} (4.06 kΩ) compared to the bare GCE (R_{CT} 0.880 kΩ), since, in general, macromolecules can block the surface of the electrode, hindering electronic transference in the inorganic system ($[Fe(CN)_6]^{4-}$). As can be seen in Fig. 3, the modification of MWCNTs (EPS-MWCNTs/GCE), provided a decrease in the semi-circle ratio (R_{CT} 1.42 kΩ), suggesting that the nanomaterial increase the electron-transfer rate on the electrochemical system, as expected. In addition, when laccase (a protein) was deposited onto the modified surface of GCE (laccase-EPS-MWCNTs/GCE), the resistance of the electrochemical device reached its highest value (R_{CT} 14.8 kΩ). This phenomenon can be attributed to the presence of the biocatalytic film without charge, structured by botryosphaeran and laccase, which introduces a high resistance to electron-transfer and retards the diffusion mechanism of the inorganic species ($[Fe(CN)_6]^{4-}$) to the surface of the electrode.

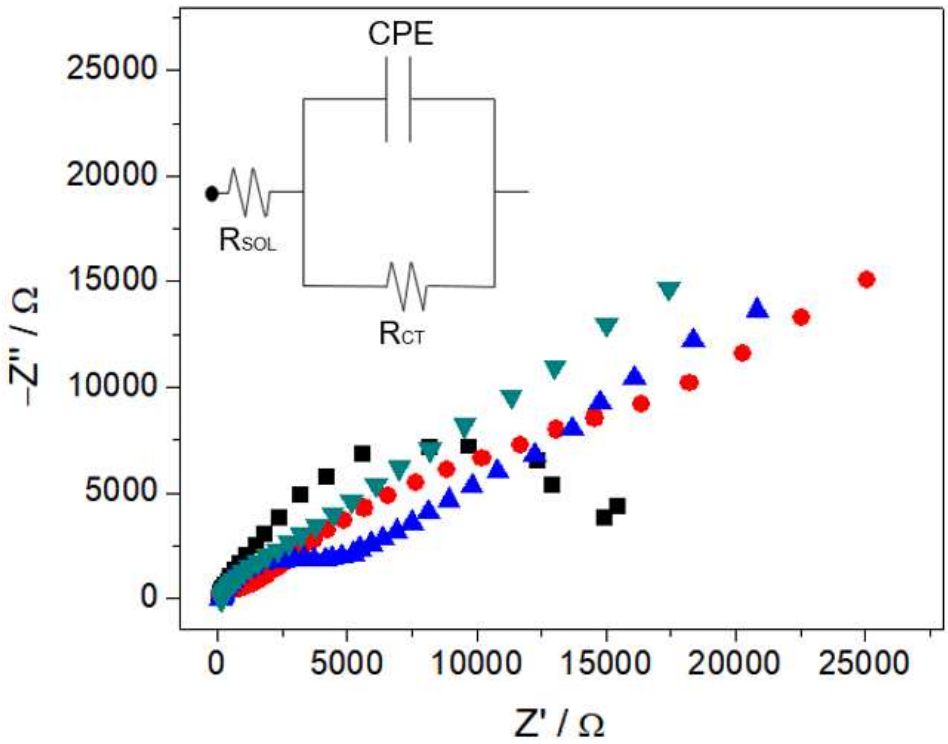

Fig. 3. Electrochemical impedance spectra for GCE (●), EPS/GCE (▲), EPS-MWCNTs/GCE (▼) and laccase-EPS-MWCNTs/GCE (■) in 0.10 mol L^{-1} KCl solution containing 1.0 mmol L^{-1} $[Fe(CN)_6]^{4-}$. EIS conditions - open circuit mode, 10 mV amplitude and frequency range of 10 mHz to 100 kHz. *Inset*: Randles circuit model; R_{SOL} - solution resistance, R_{CT} - charge transfer or polarization resistance, and CPE – constant phase element.

3.3. Electrochemical response of the laccase biosensor to dopamine

Although the EPS exerted little influence on the redox current of $[Fe(CN)_6]^{4-/3-}$, its use significantly influenced dopamine, presenting a stable and well-defined voltammetric response. The performance of this novel biosensing platform was evaluated by choosing dopamine as the typical model of a phenolic compound. It is known that laccase catalyzes the oxidation of dopamine to dopamine-quinone as a primary product while molecular oxygen in solution is reduced to water [10]. Subsequently, the dopamine-quinone formed is

electrochemically reduced on the proposed biosensor surface at 0.23 V back to dopamine. The peak current obtained in the electrochemical reduction of dopamine-quinone on the proposed biosensor was directly proportional to the dopamine concentration in the solution and was used for the determination of this analyte in real samples.

Fig. 4 displays the square-wave voltammograms obtained using the electrodes GCE, EPS/GCE, EPS-MWCNTs/GCE and laccase-EPS-MWCNTs/GCE in PBS (pH 6.0) in the presence of 39.8 µmol L^{-1} dopamine. As shown in Fig. 4, the electrode response for dopamine is slightly increased by EPS modification (curve b) compared with GCE (curve a). These results suggest that the presence of EPS promotes a pre-concentration of the analyte on the electrode surface, possibly due to hydrogen interactions between hydroxyl groups in the EPS structure and the amino group of the dopamine molecule. When GCE was modified with functionalized MWCNTs and EPS (curve c), an increase in the response occurred due to the high electronic conductivity and high surface area presented by the nanomaterial. The presence of laccase on the electrode (curve d) caused an increase in the peak current for dopamine, which was almost 81-fold higher than that observed signal for GCE alone. The difference between the analytical signals was due to laccase acting as a catalyst in the redox reaction occurring on the electrode surface combined with the properties of MWCNTs and EPS.

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dopamine molecule. The analytical signal for dopamine using MWCNTs/GCE, in relation to the unmodified GCE and EPS/GCE electrodes (curves a and b), can be improved (curve c). However, the surface of GCE modified with MWCNTs alone did not exhibit good stability in relation to the other electrode modifications, and did not present good definition of a peak for dopamine. When GCE was modified with functionalized MWCNTs and EPS (curve d), an increase in the response occurred due to the high electronic conductivity and high surface area presented by the nanomaterial. The presence of laccase on the electrode (curve e) caused an increase in the peak current for dopamine, which was almost 81-fold higher than that observed signal for GCE alone. The difference between the analytical signals was due to laccase acting as a catalyst in the redox reaction occurring on the electrode surface combined with the properties of MWCNTs and EPS.

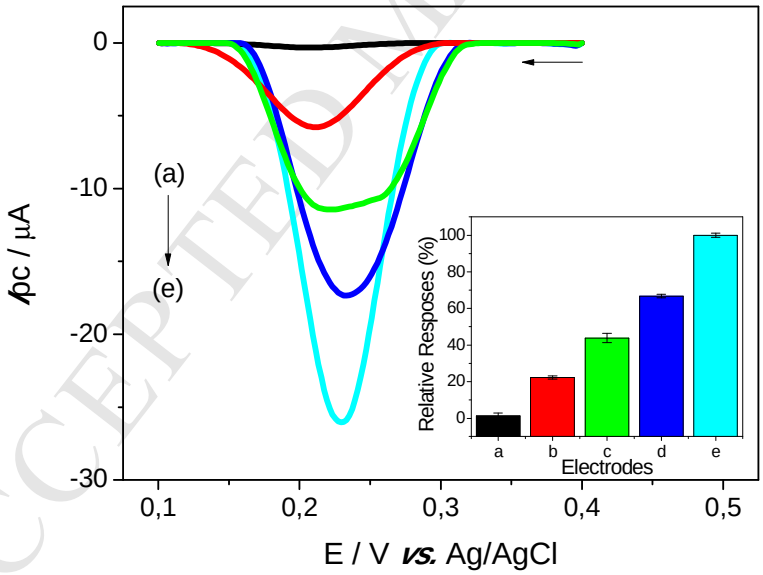

Fig. 4. Square-wave voltammograms obtained using the electrochemical devices GCE (a), EPS/GCE (b), MWCNTs/GCE (c), EPS-MWCNTs/GCE (d), and laccase-EPSMWCNTs/GCE (e) in PBS buffer (pH 6.0) containing 39.8 µmol L^{-1} dopamine. SWV conditions: f = 30 Hz, a = 50 mV and ΔEs = 2 mV. *Inset*: relative response.

The stability of the laccase-EPS-MWCNTs/GCE biosensor was assessed with regard to long-term storage. For this purpose, its response was monitored in 39.8 μmol L^{-1} dopamine solution by repetitive measurements over several days. After each measurement, the biosensor was rinsed with water and stored at 4 °C in PBS (pH 6.0) in a closed environment. From these experiments, we observed that after 60 days (the longest period tested) 86.2% of the initial cathodic current response was maintained, with stability being retained for up to 200 measurements without loss of activity. We conclude that the excellent stability observed for the fabricated electrode system was attributable to the effective protection of laccase bestowed by the electrode microenvironment, and that the enzyme was firmly immobilized on the surface of modified GCE via EPS. Thus, botryosphaeran provided a suitable matrix for laccase immobilization and biosensor fabrication, maintaining enzyme activity and promoting the stability, and prolonging lifetime of the biosensor.

In a previous study from this laboratory, we demonstrated that laccase from *B. rhodina* MAMB-05 fabricated on a nanostructured carbon black electrochemical electrode was successful in determining epinephrine by differential pulse voltammetry, and demonstrated this biosensor was a promising analytical platform for drug analysis [37].

3.4. Selection of experimental conditions of the biosensor response to dopamine

Several parameters related to the fabrication of biosensor and experimental conditions of the proposed method were investigated by SWV. Initially, the effect of laccase concentration in the biosensor preparation was investigated in the analytical response for dopamine by using 0.08 U, 0.14 U and 0.19 U of enzyme. A higher analytical signal (cathodic peak current) was found for 0.14 U of laccase immobilized on EPS. An

increase in the laccase concentration (as protein) can cause agglomeration on the EPS film formed on the modified GCE, reducing the number of active sites, and is accompanied by a decrease of conductive surface on the biosensor. Based upon these findings, 0.14 U of laccase was optimal for the construction of further biosensors for this platform.

The effect of the number of layers of deposited MWCNTs (1 – 3) in the development of this biosensor platform was investigated. A higher analytical signal was observed when two layers were employed, which provided a relatively homogeneous film on the GCE surface. The influence of pH of the PBS in the range 4.0 – 8.0 on the reduction peak of dopamine was investigated for biosensor responses. The pH of the medium affects the ionizable groups of the enzyme structure, and pH variation was found to strongly influence the electrocatalytic response of the biosensor. From the results shown in Fig. 5A, the highest voltammetric response for dopamine was registered at pH 7.0. At pH 8.0, the OH^- binds to the T2/T3 sites of laccase, decreasing its enzymatic activity [10,38], and contributing to the eventual descent of the pH profile. Due to better repeatability of the analytical signals, a pH value of 6.0 was chosen for further biosensing experiments. In addition, Fig. 5B shows the linear variation between the peak potential and the PBS pH with a slope of −55.8 mV/pH (r^2 = 0.995); a value closer to that expected for a Nernstian system with two electrons and two protons involved as described in other studies reported the literature [39,40].

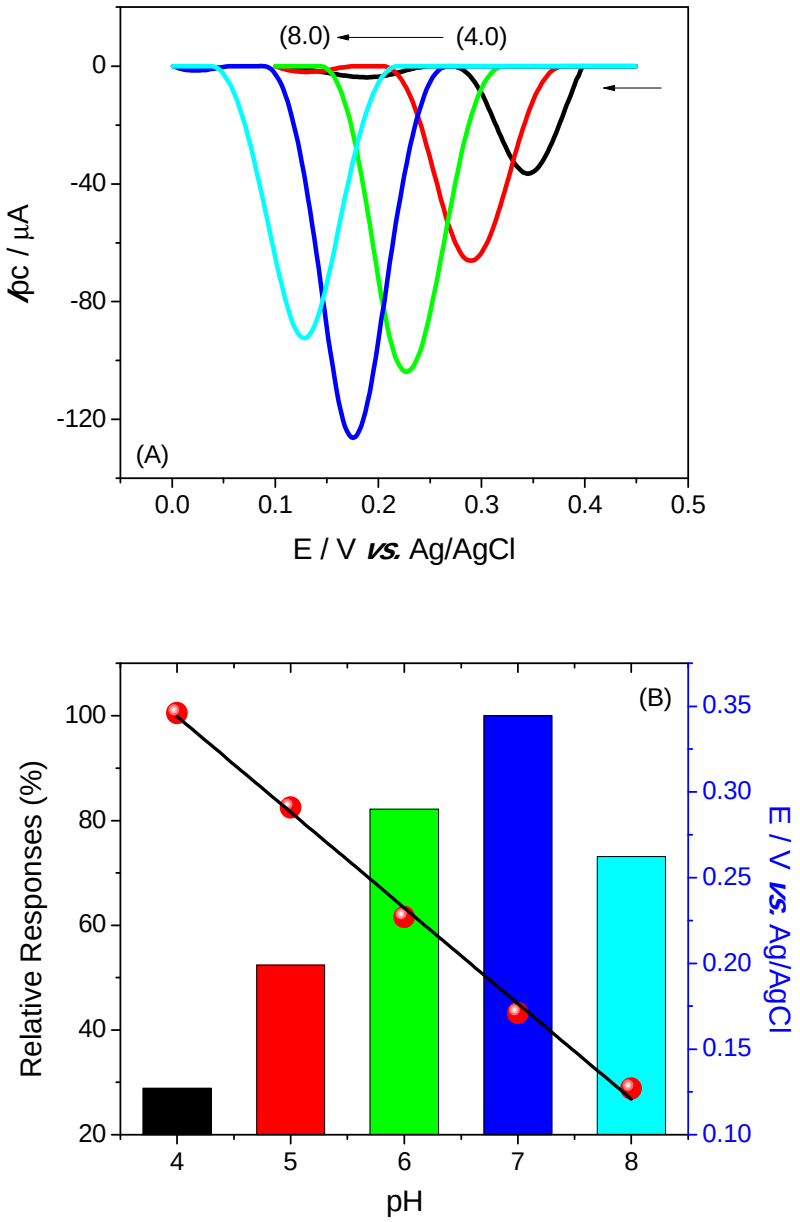

Fig. 5. (A) Square-wave voltammograms of the laccase-EPS-MWCNTs/GCE biosensor for 0.10 mmol L^{-1} dopamine in PBS at different pH values: 4.0 to 8.0; (B) Influence of pH on the cathodic peak current (expressed as relative response) and the peak potential (black line) of the proposed biosensor. SWV conditions were the same as indicated in Fig. 4.

The SWV operating parameters of the biosensor were also studied in order to obtain a defined peak and a higher peak current for the determination of dopamine. The

corresponding ranges investigated for these parameters were f = 10 – 60 Hz, a = 10 – 100 mV and ΔE_S = 1 – 3 mV. The optimum values observed (f = 20 Hz, a = 75 mV and ΔE_S = 2 mV) were selected for all further voltammetric determinations of dopamine. Under the selected conditions, the analytical curve was recorded in PBS (pH 6.0).

The analytical curves were constructed from the resultant cathodic peak current vs. dopamine concentration. The response time of the biosensor towards dopamine was calculated as 2 s, indicating fast electrocatalytic oxidation of this phenolic compound by laccase. The electrochemical reduction of dopamine-quinone at the biosensor surface was obtained at +0.23 V (Fig. 6). There was a linear relationship between current (µA) and dopamine concentration ranging from 2.99 – 38.5 µmol L^{-1} (inset of Fig. 6), with the following linear regression equation Ipc (µA) = –1.6 + 0.639 [c (µmol L^{-1})] (r = 0.999); where Ipc is the cathodic peak current and c the dopamine concentration, with a LOD and LOQ of 0.127 µmol L^{-1} and 0.423 µmol L^{-1}, respectively.

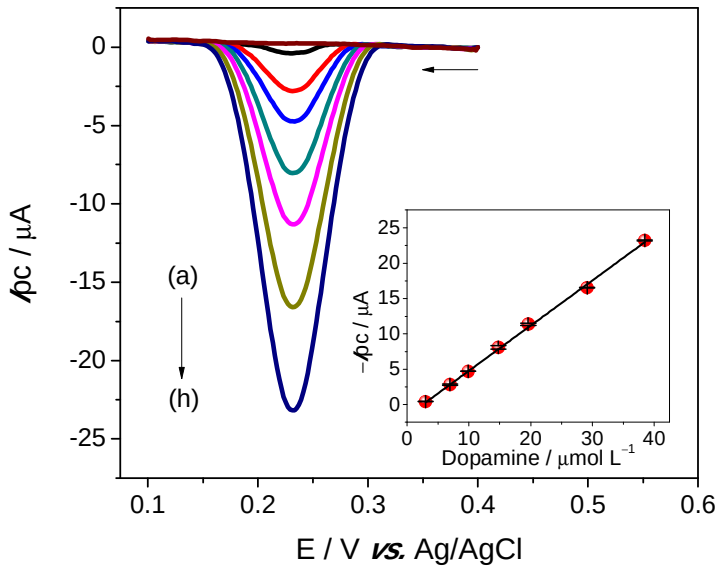

Fig. 6. Square-wave voltammograms obtained in PBS (pH 6.0) using the proposed laccase-EPS-MWCNTs/GCE biosensor at the following concentrations of dopamine: (b – h): 2.99 – 38.5 µmol L^{-1}. *Inset*: Corresponding analytical curve.

The analytical performance of the proposed biosensor was compared with that of previously reported laccase-based biosensors for dopamine determination as shown in Table 1. The comparative data suggested excellent performance of the proposed biosensor in terms of a lower LOD with a broad linear range of dopamine concentrations and comparable stability over the other biosensors reported in the scientific literature. Moreover, the proposed biosensor has advantages over earlier literature described enzyme sensors in that it was not necessary to use a cross-linking reagent to immobilize the laccase, there was no leaching of enzyme from the biosensor during use, and the laccase activity was unaffected by the external environment due to the protective influence of the microenvironment containing the EPS. The proposed biosensor is thus an excellent alternative for dopamine determination in real samples.

Table 1. Comparison of the proposed biosensor with results those of other biosensors reported in the literature for the determination of dopamine.

Biosensor (reference)	**Technique**	**Linear range** (μmol L^{-1})	**LOD** (μmol L^{-1})	**Stability**
AuNPs-PAH-laccase/CPE [41]	SWV	0.49 – 23.0	0.260	95% after 60 days
laccase/Si/MWCNTs/SPE [17]	DPV	1.30 – 85.5	0.420	86% after 30 days
laccase/(h-SiO_2-PA)/GCE [15]	Amperometry	0.99 – 138.4	0.170	89% after 20 days
laccase-CDBA-rGO/SPE [16]	DPV	0.10 – 70.0	0.0300	87% after 30 days (56% after 60 days)
Au-MPA-laccase/SAM [12]	DPV	0.50 – 13.0 and 47.0 – 430	0.0290	97.3 – 96.5% after 7 days
MWCNT/PPy/laccase [13]	DPV	0.50 – 4.75	0.140	90.4% after 30 days
laccase/SiO_2NPs-PA [14]	Amperometry	0.99 – 103.1	0.260	80% after 40 days
laccase-EPS-MWCNTs/GCE (This work)	SWV	2.99 – 38.5	0.127	86.2% after 60 days

DPV: differential pulse voltammetry; AuNPs: gold nanoparticles; PAH: poly(allylamine hydrochloride); CPE: carbon paste electrode; Si: silica spheres; SPE: screen-printed electrode; h-SiO_2-PA: helical nanostructured silica-phytic acid; CDBA-rGO: β-cyclodextrin and benzaldehyde-reduced graphene oxide; MPA: mercaptopropionic acid; SAM: self-assembled monolayers; PPy: polypyrrole; PA: phytic acid.

Evaluation of the repeatability of the proposed biosensor was performed by measuring the cathodic peak current using SWV in PBS (pH 6.0) containing 14.8 µmol L^{-1} dopamine, and resulted in a relative standard deviation (RSD) of 3.60 % for 10 successive measurements on the same biosensor device. The findings demonstrated the viability of the biosensor over repeated determinations. The repeatability of three independent fabricated biosensors (laccase-EPS-MWCNTs/GCE) was also evaluated by measuring the cathodic peak current of 14.8 µmol L^{-1} dopamine in PBS (pH 6.0) resulting in a RSD value of 4.30 % among the three biosensors, confirming that the results were highly reproducible.

3.5. Selectivity and analytical application for the determination of dopamine

In order to assess the selectivity of the proposed analytical method, the influence of some concomitant compounds on the biosensor response were investigated. Phenolic compounds such as acetaminophen, epinephrine, guaiacol, catechol and hydroquinone were chosen. Other compounds such as ascorbic acid and uric acid were chosen from the group of substances commonly found with dopamine in biological samples, and which present one of the main interference problems in the electrochemical determination of this phenolic compound in this sample types. The selectivity was thus evaluated by the addition of these possible interfering compounds in a standard solution containing 19.6 µmol L^{-1} dopamine, in the concentration ratios (standard solution: interfering chemicals) of 1:1 and 1:10, and 1:1000 (standard solution: ascorbic acid), and the current signals obtained under these conditions compared with those arising from the standard solution using SWV measurements (data not shown). From the results obtained, the phenolic compounds investigated displayed peak potentials of similar values to those obtained for dopamine, which suggests that the laccase-EPS-MWCNTs/GCE biosensor was not specific, but

selective. In addition, no significant changes were observed in the peak current of dopamine in the presence of uric acid, indicating that it does not present significant interference (< 1.0%) in determining dopamine. According to the literature, ascorbic acid can chemically reduce dopamine-quinone to dopamine, and consequently decreases the cathodic peak current [42]. Ascorbic acid can, however, be eliminated by treating the sample solution with cucumber extract (*Cucumis sativus* L.), and under such conditions the biosensor can be used for dopamine determination in the presence of these compounds without significant interference in the analytical signal produced.

To verify the applicability of the proposed biosensor for dopamine determination in real samples, pharmaceutical, synthetic urine and synthetic cerebrospinal fluid were employed in these experiments. Dopamine concentration in a commercial pharmaceutical injection sample (5.0 mg mL^{-1}) was determined by the proposed biosensor, and was compared to that determined spectrophotometrically [32]. The analyses were performed in triplicate, and the dopamine concentration found was 4.92 ± 0.02 mg mL^{-1} using the proposed biosensor, and 4.88 ± 0.03 mg mL^{-1} by the spectrophotometric procedure. The recovery obtained for dopamine in the analyzed pharmaceutical sample was around 101 %, indicating that there was no matrix interference in determining dopamine in the pharmaceutical sample.

The proposed biosensor was also used for determining dopamine in synthetic samples of urine and cerebrospinal fluid using the standard addition method (Table 2). Two different dopamine concentrations (3.98 and 7.94 µmol L^{-1}) were spiked in each of the samples. As can be seen, the proposed biosensor exhibited adequate recoveries; thus, one may conclude that this method is potentially applicable for dopamine determination in biological samples, without any significant effects of matrix interference.

Table 2. Determination of dopamine in synthetic samples of urine and cerebrospinal fluid by the proposed biosensor.

Samples	**Dopamine concentration** (μmol L^{-1})		**Recovery**
	Added	**Found**	(%)
Urine			
1	3.98	3.89 ± 0.04	97.7
2	7.94	7.99 ± 0.02	101
Cerebrospinal fluid			
1	3.98	4.05 ± 0.05	102
2	7.94	7.87 ± 0.07	99.1

3.6. Application of the proposed biosensor for the indirect determination of spironolactone

After fabrication and optimization of the proposed biosensor using dopamine as analyte, the device was used to develop an analytical method for determining spironolactone in commercial pharmaceutical samples. In this study, we observed that in the presence of spironolactone, the cathodic peak current of dopamine decreased proportionally with an increase in spironolactone concentration. This finding therefore led to the idea of developing an indirect method to measure spironolactone.

Fig. 7 illustrates the schematic of the reaction between dopamine-quinone (laccase-catalyzed oxidized product from dopamine) and spironolactone occurring on the biosensor surface. Firstly, dopamine (compound **1**) is enzymatically oxidized to dopamine-quinone (**2**) by the biosensor. In the next step, nucleophilic attack between the primary amine group

on dopamine-quinone and the carbonyl group bonded to the sulfur atom of spironolactone (**3**) can occur when the latter is added to the solution in the electrochemical cell of the biosensor, and consequently an imine (**4**) is formed. The blocking of cycling of dopamine-quinone to dopamine cannot occur, and a decreasing cathodic peak current was consequently observed that was proportional to an increase in the concentration of spironolactone.

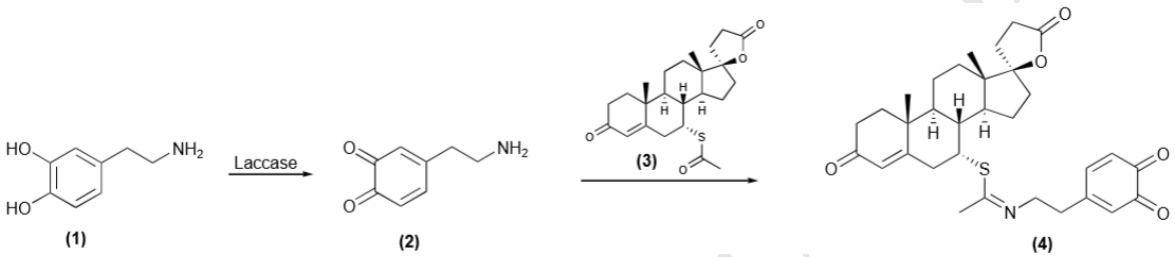

Fig. 7. Schematic of the laccase-catalyzed oxidation of dopamine (**1**) to dopamine-quinone (**2**) on the biosensor surface, and its subsequent reaction with spironolactone (**3**) to form the imine (**4**).

Spironolactone at concentrations of 3.0, 7.4 and 9.9 µmol L^{-1} were selected based upon the dopamine analytical curve (Fig. 8), and resulted in better analytical responses (sensitivity and linearity) at a dopamine concentration of 7.40 µmol L^{-1}. When the current reached a steady-state after the addition of 7.40 µmol L^{-1} dopamine to PBS (pH 6.0), square-wave voltammograms were recorded in the presence of various concentrations of spironolactone. We found that the cathodic current peak for dopamine decreased with increasing concentrations of spironolactone as shown in Fig. 8. The inset (Fig. 8) depicts the respective analytical curve obtained for spironolactone concentrations within the range 2.97 to 28.9 µmol L^{-1}, for which the corresponding regression equation was:

$$-I\text{pc}\ (\mu\text{A}) = -0.014 + 0.032\ [c\ (\mu\text{mol L}^{-1})]\ (r^2 = 0.995),$$

where *I*pc is the cathodic peak current and *c* the spironolactone concentration. The LOD value was 0.94 µmol L^{-1}. This analytical curve was used for the determination of spironolactone in pharmaceutical samples at different doses.

The result of the analytical determination of spironolactone by the proposed method showed a low LOD, allowing the method to be applied as an alternative to other analytical procedures described in the literature for this purpose (Table 3). These techniques, however, suffer from some disadvantages, such as high cost of acquisition and maintenance, derivatizing preparations, extraction and pre-concentration steps, and also the use of toxic components such as mercury and organic solvents (methanol, acetonitrile, hexane) that are expensive. Altogether, these features make the other analytical methods less attractive as they involve lengthy procedures and are relatively not environmentally friendly compared to our proposed biosensing device. The proposed biosensor showed excellent performance for spironolactone determination, with simplicity of use, stability, rather rapid analysis times and the use a minimal amount of ethanol for sample/analyte dissolution.

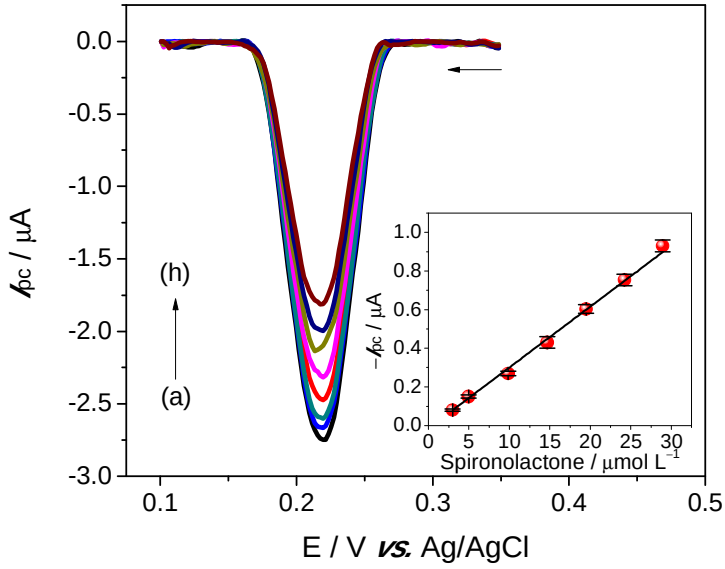

Fig. 8. Square-wave voltammograms obtained by the laccase-EPS-MWCNTs/GCE biosensor using a dopamine concentration of 7.40 μmol L^{-1} at increasing concentrations of spironolactone. Curves: (a) 0 (control), (b) 2.97, (c) 4.94, (d) 9.83, (e) 14.7, (f) 19.5, (g) 24.2 and (h) 28.9 μmol L^{-1} in PBS (pH 6.0). *Inset:* analytical curve for spironolactone.

Table 3. Comparison of the analytical parameters of the proposed biosensor with other methods for the determination of spironolactone

Analytical technique	**Linear range** ($\mu mol\ L^{-1}$)	**LOD** ($\mu mol\ L^{-1}$)	**Reference**
HPLC – Photolysis – Electrochemical detection	2.40×10^{-4} – 12.0×10^{-4}	3.60×10^{-6}	[43]
HPLC – Solid-phase extraction – UV detection	0.015 – 0.960 and 0.075 – 4.80	–	[44]
HPLC – Mass Spectrometry detection	0.0066 – 0.440	–	[45]
UV – Derivative Spectrophotometry	12.0 – 84.0	–	[46]
Fluorescence – CdSe quantum dots	6.00 – 1680	0.480	[47]
Polarography – Dropping mercury working electrode	2.50 – 100	0.100	[25]
DPCSV – Hanging mercury drop electrode	1.20×10^{-4} – 0.960	1.10×10^{-5}	[27]
DPAdSV – Silver based mercury film electrode	0.0150 – 3.00	0.00470	[26]
SWV – Biosensor (indirect determination)	2.97 – 28.9	0.94	This work

HPLC: High Performance Liquid Chromatography; DPCSV: differential pulse cathodic stripping voltammetry; DPAdSV: differential pulse adsorptive stripping voltammetry.

Table 4 presents the values of amounts of spironolactone determined in pharmaceutical samples employing the proposed biosensor, and is compared with a reference spectrophotometric method [36]. Three determinations were carried out for each sample, and the standard deviations were calculated. As shown in Table 4, no significant differences were observed between the spironolactone concentration found in the pharmaceutical samples employing the SWV and the spectrophotometric method. Applying the paired *t*-test [48] to the data obtained for both methods, the calculated *t* values (2.38) were smaller than the critical value (4.30, $\alpha = 0.05$). From this we conclude that the results obtained with the proposed procedure are not statistically different from the comparative method used at the 95.0 % confidence level. The advantage of the electrochemical method is that less time is consumed during analysis, and that the solid suspension of the sample has no influence on the analysis.

Table 4. Indirect determination of spironolactone in pharmaceutical samples by the SWV method employing the proposed biosensor and compared to a spectrophotometric method.

Sample	**Spironolactone** (mg/tablet)			**Relative error** [b] (%)
	Labeled amount	**Spectrophotometric method** [a]	**SWV method** [a]	
A	25.0	25.4 ± 0.4	26.5 ± 0.2	4.30
B	25.0	25.6 ± 0.3	25.9 ± 0.4	1.20
C	50.0	52.3 ± 0.6	51.9 ± 0.8	–0.760

[a] Average of 3 measurements. [b] Relative error (%) = 100 × (SWV method – spectrophotometric method / spectrophotometric method).

4. Conclusion

The proposed laccase-EPS-MWCNTs/GCE biosensor was constructed with laccase immobilized on botryosphaeran on GCE modified with MWCNTs. This novel biosensing platform was successfully applied to the quantification of dopamine in pharmaceutical and biological samples. The good analytical performance presented by this biosensor for dopamine (sensitivity, selectivity, rapid response time and stability) demonstrated that botryosphaeran was an efficient matrix for the immobilization of laccase, presenting a biocompatible microenvironment without affecting enzymatic activity, and without enzyme leaching from the fabricated sensing device when in solution. The results further proved that the proposed immobilization method was effective, and it was not necessary to cross-link enzyme to support, showing advantages for the fabrication of this kind of biosensor. In addition, an indirect method was developed for measuring spironolactone. In this case, spironolactone can react with the dopamine-quinone product formed from the laccase-catalyzed oxidation of dopamine on the surface of the biosensor thereby blocking the electrochemical reduction of the oxidized quinone to dopamine. Spironolactone successfully decreased the cathodic current peak allowing this drug to be quantified in commercial pharmaceutical products. The results for the quantification of dopamine and spironolactone in pharmaceuticals were in agreement with those obtained by spectrophotometric methods [32,36]. Furthermore, the proposed biosensor displayed excellent analytical performance for phenolic compounds in a rapid, simple to operate, recyclable, and cost-effective manner.

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HIGHLIGHTS

- Botryosphaeran was used as laccase protector for biosensor development.

- A novel biosensor platform was developed using GCE modified with MWCNTs.

- Dopamine was determined in pharmaceutical injection and synthetic biological samples.

- Spironolactone was indirectly determined in pharmaceuticals using the laccase biosensor.