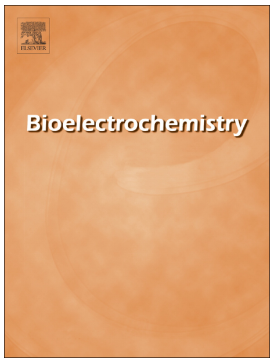


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# Laccase stabilized on β-D-glucan films on the surface of carbon black/gold nanoparticles: A new platform for electrochemical biosensing

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Abstract

In this study, (1→3)(1→6)-β-D-glucan (botryosphaeran) from *Botryosphaeria rhodina* MAMB-05 was used, for the first time, to immobilize laccase on a carbon black paste electrode modified with gold nanoparticles. The physicochemical characterization of the proposed laccase-biosensor was performed using transmission electron microscopy and electrochemical impedance spectroscopy. The performance of this novel bio-device was evaluated by choosing hydroquinone as a typical model of a phenolic compound. For hydroquinone determination, experimental variables such as enzyme concentration, pH and operational parameters of the electroanalytical technique were optimized. From square-wave voltammograms, a linear dependence between the cathodic current peak and the hydroquinone concentration was observed within the range 2.00-56.5 μmol $L^{-1}$, with a theoretical detection limit of 0.474 μmol $L^{-1}$. The proposed method was successfully applied to determine hydroquinone in dermatological cream, and samples from biological and environmental niches. The proposed biosensor device presented good selectivity in the presence of uric acid, various inorganic ions, as well as other phenolic compounds, demonstrating the potential application of this biosensing platform in complex matrices. Operational and analytical stability of the laccase biosensor were evaluated, and demonstrated good intra-day (SD = 0.3 %) and inter-day (SD = 3.4 %) repeatability and long storage stability (SD = 4.9 %).

Keywords: *Botryosphaeria rhodina* MAMB-05, bioelectrochemistry, botryosphaeran, (1→3)(1→6)-β-D-glucan, gold nanoparticles, phenolic compounds.

## 1. Introduction

Sustainability has become a central theme that influences contemporary society in many aspects. In this context, *green* chemistry aims to develop chemical products and processes that are less harmful to human and environmental health. Green chemistry is based on twelve principles, including the use of catalysts and biocatalysts, and renewable materials and compounds that degrade harmlessly after use [1]. Biocatalysts, such as enzymes, are synthesized by biological systems and are relatively inexpensive to produce by fermentation. In addition, they are non-toxic, renewable, and biodegradable. The major advances associated with biotechnology can be attributed to the exploitation of these biomaterials.

Laccases (*p*-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) are enzymes within the class of multicopper oxidases that contain copper ($Cu^{+}/Cu^{2+}$) (T1/T2/T3 cluster) atoms in their catalytic active center. Laccases act selectively in the oxidation of phenolic compounds in the reduced form that occurs at the T1 copper center, while simultaneously reducing molecular oxygen, which takes place at the T2 and T3 copper sites [2]. In recent research, the laccase toolbox has expanded greatly, as this enzyme can be easily produced by diverse sources such as bacteria, fungi, algae, plants and insects, and has a wide spectrum of applications. Examples include decolorization of textile dyes in industrial effluents, uses within the pharmaceutical and food sectors [3,4], in bioremediation [5], and in the development of biosensors [6].

In laccase catalysis, the kinetics of the redox process is determined by the potential difference between the T1-copper centre and the substrate. The development of an electrochemical biosensor requires the best electronic transfer kinetics during the oxidation of the substrate, ensuring the formation of a large amount of the oxidized species that will be reduced electrochemically on the biosensor surface. Consequently,

the unique redox properties of laccases have been widely applied for bioelectrochemical purposes, and especially those from fungi due to their greater oxidation potential when compared to laccases from bacterial and plant systems [7]. The use of crude enzyme extracts in the biosensor architecture may present selectivity problems. However, it reduces the cost of building the device on a production scale, in addition to being simpler and ensuring a longer lifetime of activity when compared to the purified enzyme [8].

The ligninolytic ascomyceteous fungus *Botryosphaeria rhodina* MAMB-05 produces an extracellular laccase, and induction by veratryl alcohol results in high titers over the constitutive levels of the enzyme produced [9]. *B. rhodina* MAMB-05 also produces an exopolysaccharide of the (1→3)(1→6)-β-D-glucan type named botryosphaeran [10]. This exocellular biopolymer is produced when the fungal isolate was grown on glucose as sole carbon source, and consists of a backbone chain bound by β-(1→3)-glucosidic linkages, with 22% side branches of β-(1→6)-linked glucose and gentiobiose units. Furthermore, botryosphaeran exists in a triple helix conformation [11]. Both products from this fungal isolate were used for the first time in the fabrication of the carbon based electrochemical-sensing device as described in this work.

The immobilization of enzymes on the surfaces of different materials to constitute the biosensing platform is a critical factor in the development of bioelectrochemical sensing devices. The use of enzymes in their natural environment provides greater stability in catalytic activity, as well as protection against inactivation; factors that directly affect the analytical performance of the biosensor, such as selectivity, stability and the ability to analyze real samples. The transducing phase of an

electrochemical biosensor acts as a support for the immobilization of biomaterials that constitute the chemically-selective phase of the device.

Carbon black (CB) is a low-cost material, extensively used in modern electrochemistry as a transducer in electrochemical sensors and biosensors [12]. It consists of spherical nanoparticles, with diameters ranging between 10 and 100 nm, which gives it an extensive surface area and high conductivity, providing high sensitivity and lower detection limits to the sensor [13]. It is customary to modify the surface of carbon electrodes with species that promote changes in physicochemical conditions of the electrode/solution interface, as a way of imposing and controlling their reactivity and selectivity. Metal nanoparticles, due to their high conductivity and electrocatalytic properties, are a recurrent approach associated with nanoscience and nanotechnology in the construction of new electrical devices [14]. Gold nanoparticles (AuNPs) have been described in the architecture of electrochemical biosensors as a modifier, mainly due to their biocompatibility, promoting electrocatalysis without compromising the activity of the biomolecules [15].

Phenolic compounds, the natural substrates of laccases, are mostly organic pollutants frequently present in aquatic environments. These compounds, even at low concentrations, can affect the physiology of contaminated aquatic organisms and can impact on human health when present in the food chain [16]. Hydroquinone (HQ), for example, has been used in skin bleaching formulations for the treatment of hyperpigmentation. There is concern that HQ, being a derivative of benzene, may also be associated with the carcinogenicity of benzene [17]. Around 45 % of the total dose of hydroquinone is absorbed directly into the skin and part is excreted in the urine [18]. Thus, the detection and quantification of hydroquinone with an electrochemical biosensing device has merit and facilitates clinical diagnosis associated with its

monitoring in biological fluids, environmental samples, as well as laboratory control of the dermatological products available to the public.

Different methodologies have been developed for the determination of hydroquinone, including high performance liquid chromatography (HPLC) [19] and spectrophotometry [20]. However, these methods have their pitfalls in that the reagents used can be toxic, expensive instrumentation is required, and extensive sample preparation can be involved. In this context, electrochemical biosensors are promising alternative analytical procedures, since these devices are chemically selective, have relatively low implementation costs, and are amenable to routine chemical analyses.

Taking the above features into account, the work reported herein describes the development of a new platform for electrochemical biosensing based upon carbon black paste electrode (CBPE) modified with AuNPs as the transducing phase. The performance of the β-glucan (botryosphaeran) film was evaluated as a natural microenvironment for laccase immobilization on the surface of CBPE/AuNPs. Hydroquinone was chosen as substrate to evaluate the performance of this architecture in a new biosensor device for its determination in dermatologic cream, and environmental and biological samples, by square-wave voltammetry (SWV). Parameters of interest, such as electrical resistance, selectivity, sensitivity, precision and accuracy of the proposed device, were determined from electrochemical and electroanalytical studies in addition to statistical analysis based on a comparative method reported in the literature [19]. Experimental factors that affect the analytical performance of the proposed biosensor, such as pH, SWV operating parameters and concentration of the modifiers were also evaluated and optimized.

## 2. Material and methods

### *2.1. Reagents and solutions*

The synthesis of gold nanoparticles (AuNPs) required the following chemicals: $HAuCl_4 \cdot 3H_2O$ (hydrogen tetrachloroaurate trihydrate (chloroauric acid), 48% in gold), PVP (polyvinylpyrrolidone, 55,000 g $mol^{-1}$), and ascorbic acid (99.0%). They, as well as hydroquinone and $H_2SO_4$, were obtained from Sigma-Aldrich (St Louis, MO, USA), Carbon black VXC72R/LOT-3945501 was kindly provided by Cabot Brasil Indústria e Comércio Ltda., São Paulo-SP, Brazil. 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. Commercial dermatologic cream samples were acquired from a local pharmacy in Londrina, Brazil.

All solutions were prepared using ultra-purified water supplied by a Milli-Q system (Millipore®) with resistivity greater than 18 MΩ cm. Phosphate buffer solutions (0.1 mol $L^{-1}$) were prepared from potassium monobasic phosphate with dibasic potassium phosphate with the pH adjusted to pH 6.0 and pH 7.0, and were used as the supporting electrolyte throughout the electroanalytical experiments. A stock solution of 10 mmol $L^{-1}$ hydroquinone was freshly prepared daily in 0.1 mol $L^{-1}$ phosphate buffer solution at pH 6.0/7.0. Standard solutions of hydroquinone were prepared from the stock solutions by appropriate dilution.

### *2.2. Apparatus*

#### *2.2.1 Physical-chemical characterization*

For the characterization of AuNPs, scanning electron microscopy (SEM) images were collected using a JEOL microscope (FEG-SEM JSM 6330F; JEOL, USA)

operated at 5 kV. The preparation of SEM samples was performed by drop-casting an aqueous suspension of the washed particles on a silica support film wafer, followed by drying under ambient conditions. UV-VIS spectra were obtained using a Shimadzu UV-2600 UV-VIS spectrophotometer (Pleasanton, CA, USA) and quartz cuvettes. The concentration of gold (Au) was determined by flame atomic absorption spectrophotometry (FAAS) performed on a Shimadzu AA-6300 instrument (Pleasanton, CA, USA).

Transmission electron microscopy (TEM) images were obtained on a transmission electron microscope, model Fei Tecnai $G^2$ (FEI, NY, USA) operated at 80 kV. Images of isolated AuNPs and in the presence of biomaterials (botryosphaeran and laccase) were evaluated. The materials for examination were deposited on formvar coated grids to obtain the images.

*2.2.2 Electrochemical characterization*

Square-wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements using the biosensor were performed on an FRA2 μAutoLab Type III potentiostat/galvanostat (Metrohm Autolab B.V., The Netherlands) controlled with NOVA 2.1 software (www.metrohm-autolab.com/Products/Echem/Software). A conventional three-electrode single-compartment glass cell was used at room temperature (25.0 ± 0.5 °C) for all experiments, and comprised a biosensor as the working electrode, an Ag/AgCl (3.0 mol $L^{-1}$ KCl) reference electrode, and a platinum plate as auxiliary electrode. A stirring time of 30 s was used to homogenize the solution of hydroquinone in the electrochemical cell.

SW voltammograms were baseline-corrected by the moving average method (peak width: 0.001). The EIS experiments were performed in the range of 10 mHz to

100 kHz (10 points per decade) and with a 10 mV (r.m.s.) ac perturbation for a 1 mmol $L^{-1}$ $K_4[Fe(CN)_6]$ in 0.1 mol $L^{-1}$ KCl solution.

pH measurements were made at 25.0 ± 1.0 °C using a combined glass electrode with an Ag/AgCl (3.0 mol $L^{-1}$ KCl) external reference electrode connected to a pH meter, model W3B (BEL, SP, Brazil).

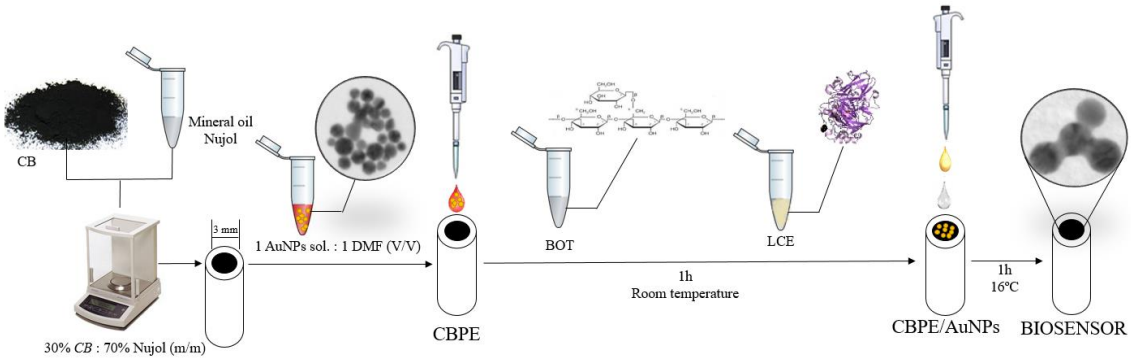
*2.3. Construction of the biocatalytic film of LCE and BOT on the nanostructured platform: biosensor architecture*

The carbon black paste electrode (CBPE) was prepared by mixing carbon black (CB) and mineral oil (Nujol®) at a ratio of 30:70% (w/w). We observed that the paste obtained could be used over a five-month period without any variation in the values of the current obtained in the electrochemical analyses. The CB paste was carefully packed into the cavity of a Teflon® tube supported with a copper plate, and the contents compacted resulting in a smooth surface. The CBPE presented an exposed geometric area of 0.0707 $cm^2$. Sequentially, a dispersion of the AuNPs (synthesized as previously described in the literature [21]) in DMF was prepared (ratio 1:1, v/v) and vigorously stirred for 2 min on a Vortex® mixer), and an aliquot (10 µL) was taken with the aid of a micropipette and deposited onto the surface of the carbon black paste electrode (CBPE/AuNPs). This was allowed to dry (1 h) prior to the next steps.

For the construction of the biosensor, laccase and botryosphaeran were obtained from *B. rhodina* MAMB-05 as previously described in the literature [9,22] . The CBPE/AuNPs/BOT/LCE electrode was constructed by depositing 12 µL of crude laccase extract (0.33 Units) and simultaneously 8 µL of BOT (0.113 g $L^{-1}$), onto the surface of the carbon black paste electrode that had been modified with AuNPs (Schematic 1 shows the steps involved in the construction of the biosensor device).

After this step, the biosensor was kept at 16 °C for one hour, and finally, the electrochemical analysis can be used as a working electrode. The developed biosensor was stored at 4 °C (refrigerator) when not in use.

For comparison, the following electrodes were used: carbon black paste electrode (CBPE), CBPE surface with gold (CBPE/AuNPs) and CBPE/AuNPs layered with botryosphaeran (CBPE/AuNPs/BOT).




**Schematic 1.** Scheme of the steps involved in the construction of the proposed laccase-biosensor device. CB – carbon black; AuNPs – gold nanoparticles; DMF – dimethylformamide; CBPE – carbon black paste electrode; BOT – botryosphaeran and LCE – laccase.

*2.4. Measurement procedures*

SW voltammograms were obtained for the different sensors constructed (see above), and in the presence of hydroquinone to evaluate the electrochemical contribution of each modifying procedure of the electrode architecture developed. The voltammetric measurements were performed using an electrochemical cell containing 10 mL of phosphate buffer (pH 6.0), and scanning the potential from 0.5 to −0.5 V. Measurements were carried out in triplicate to ensure the repeatability of our results.

The calibration curve (2.00-56.5 μmol $L^{-1}$) for hydroquinone was constructed by adding small volumes of a standard solution of hydroquinone (1.0 mmol $L^{-1}$) in the electrochemical cell containing 10 mL of phosphate buffer solution (pH 7.0), and measurements were performed in triplicate. SW voltammograms of hydroquinone were recorded scanning the potential from 0.5 and −0.5 V, after successive additions of hydroquinone. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the formulae: 3 x *S / M* and *10 x S / M*, respectively; where *S* is the standard deviation of ten measurements of the blank solution, and *M* is the slope of the calibration curve [23].

*2.5. Preparation of diverse samples for the determination of hydroquinone*

The dermatological cream was prepared by weighing 1.0 g of the commercial sample, which was transferred to a 100 mL volumetric flask, and the volume completed with the phosphate buffer solution (pH 7.0). As hydroquinone is water soluble, the analyses were performed directly on the resulting solution without further treatment. To evaluate the accuracy of the proposed method, the dermatological cream sample was also analyzed by a spectrophotometric method for determining hydroquinone [19]. All determinations were carried in triplicate using a Thermo Spectronic spectrophotometer UV-VIS, model Genesys (Waltham, MA, USA) coupled to a computer, and using a 1-cm quartz cell.

As an example of a biological sample, urine was selected to determine whether concomitant substances interfered with the analysis of HQ. A urine sample was acquired from a healthy adult male immediately prior to the experimental measurements. For analysis, 0.1 mL of fresh urine was placed into the electrochemical cell containing 10 mL of phosphate buffer solution (pH 7.0). This solution was then

spiked with a standard solution of hydroquinone to achieve a required concentration. Addition and recovery tests were performed on the urine sample to verify the accuracy of the method in this biological sample.

As an example of an environmental sample, river water was selected and was taken from Rio Tibagi, Paraná (Brazil). The river water was first filtered, and then 0.1 mL was transferred to the electrochemical cell containing 10 mL of phosphate buffer solution (pH 7.0). This solution was then doped with a standard solution of hydroquinone of known concentration. Recovery tests were performed to evaluate the accuracy of the method.

## 3. Results and discussion

### *3.1. Characterization of isolated AuNPs and when dispersed on the surface of CBPE*

Firstly, the physicochemical characterization of the synthesized AuNPs was performed, which were later used in the construction of the proposed biosensor device. Based upon the results of atomic absorption spectrometry (flame atomization), the concentration of AuNPs corresponded to 0.376 mg $mL^{-1}$. Fig. 1A shows the SEM image for an AuNPs aqueous solution deposited on a silicon wafer. TEM images were also obtained and can be found on the supporting information. Based on these images, the diameter distribution of the synthesized nanomaterial was determined by individually measuring the size of 230 entities. When the repetition frequency of a given size is considered as a function of diameter, a histogram is obtained whose profile is presented in Fig. 1B. Based on these results, it was possible to conclude that the AuNPs synthesized have an apparent average diameter of 12.7 ± 2.8 nm. The UV-VIS spectrum for the AuNPs obtained from aqueous suspensions presented a peak centered at 528 nm (Fig. 1C). According to Linic et al. [24] this band can be assigned to the dipole mode of

the Localized Surface Plasmon Resonance (LSPR) excitation for AuNPs. LSPR is an optical phenomenon that characterizes plasmonic nanoparticles when light interacts with the electron cloud in nanoparticles that are smaller than the incident wavelength [25].

The electrochemical characterization of the AuNPs on the CBPE surface was carried out by cyclic voltammetry in $H_2SO_4$ solution (0.5 mol $L^{-1}$) at a scan rate of 50 mV $s^{-1}$. In the case of AuNPs, the mechanism of electro-oxidation can be observed in Fig. 1D according to Wang et al. [26]. As shown, when using the modified AuNPs/CBPE electrode in acidic medium, the system presents a classic voltammetric gold profile with an anodic peak at 1.32 V and a cathodic peak at 0.90 V, suggesting that the AuNPs are stably distributed on the CBPE surface. The observed electrochemical signals are attributed to processes involving gold oxide species that form on the electrode surface when sulfuric acid was used as supporting electrolyte as shown in Fig. 1D.

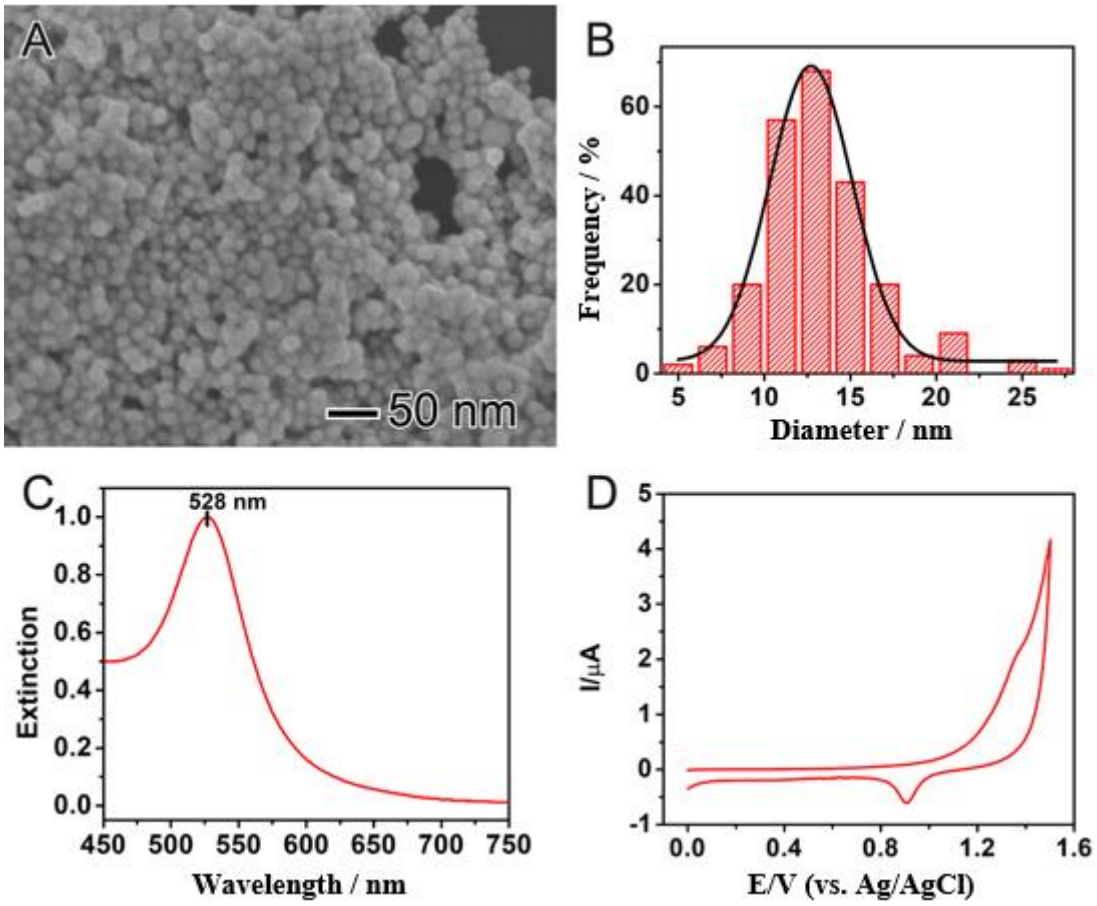



**Fig. 1. (A)** SEM image of AuNPs deposited onto a silicon wafer, **(B)** Histogram corresponding to the diameter distribution of the synthesized AuNPs. **(C)** UV-VIS spectrum of an aqueous suspension containing the AuNPs, and **(D)** Cyclic voltammogram of the AuNPs/CBPE system in $H_2SO_4$ solution (0.5 mol $L^{-1}$) at the scan rate of 50 mV $s^{-1}$.

*3.2. Exploring botryosphaeran as a matrix for immobilization of laccase on the CBPE/AuNPs electrode surface*

The construction of the biocatalytic film through the physical adsorption of the enzyme is one of the most simple and functional ways of immobilizing enzymes. In this study, the immobilization of laccase was based upon simultaneous physical adsorption with botryosphaeran (BOT) on the surface of the carbon black electrode modified with

gold nanoparticles. As aforementioned, both laccase and botryosphaeran are from the same microorganism. The BOT film formed on the transducer phase acts as a binding agent through the natural stabilization of the biocatalytic macromolecule. Since both are exocellular compounds from the same fungal system, we assume that no loss of conformation of the enzyme active center occurs, thus preserving its selective activity towards phenolic compounds. Gold nanoparticles used in this work not only promote electrocatalysis, but it is also expected that AuNPs will promote an increase in the availability of adsorption sites, being distributed in a stable way on the carbon black paste surface [27, 28]. Thus, the interaction between laccase and botryosphaeran, physically adsorbed on the support of nanostructured materials, is based upon hydrogen bonding between the hydroxyl groups on BOT and the oxygen groups on amino acids that constitute the structure of laccase. This exopolysaccharide is a very promising natural matrix for the immobilization of fungal enzymes. It is stable in an environment constituted by inorganic nanomaterials, allowing the development of new devices for electrochemical purposes.

*3.3. Morphological and electrochemical characterization of the proposed laccase biosensor*

TEM images of AuNPs isolated and in the presence of biomaterials (BOT and LCE) were evaluated. As shown in Fig. SM1A, the isolated AuNPs displayed a regular distribution on the formvar support film grid, as expected for their behavior on the carbon black surface. Since the resolution of these images displayed a better contour to them, we were able to confirm that their actual diameters are within a range of 13.0 ± 3.1 nm, about 8 nm smaller than that observed by SEM. Based on the images of the AuNPs in the simultaneous presence of BOT and laccase (Fig. SM1B), as would appear

in the electrochemical device, an agglomeration of the metallic nanoparticles was observed, that were joined through a film constituted of the macromolecules. This behavior indicated the direct contact between the transducing phase and the biocatalytic phase, demonstrating that BOT as a new biomaterial, is associated with the metallic nanoparticles and opening up potential applications (See Supplementary Material – SM for more details).

Electrochemical devices can be studied with methods based on impedance measurements, associated with electronic transfer kinetics [29]. In the work reported herein, the electrochemical characterization of the following fabricated electrodes: CBPE, CBPE/AuNPs, CBPE/AuNPs/BOT and CBPE/AuNPs/BOT/LCE was performed by electrochemical impedance spectroscopy (EIS) using the classical electrochemical probe, hexacyanoferrate $[Fe(CN)_6]^{4-}$.

The Randles circuit is one of the most common models used for impedance measurements [30]. This system includes only solution resistance ($R_{SOL}$), a parallel combination of a double-layer capacitor (CPE – constant phase element) and a charge transfer or polarization resistance ($R_{CT}$), as represented in the circuit model shown in Fig. 2 (inset **A**). As can be seen, the Nyquist plot of a Randles cell is always a semicircle, and at high frequencies, the diameter represents the charge transfer resistance, and the straight line at an angle of 45° to the real axis at lower frequencies represents the diffusion process (mass transfer control).

Variations in the electron transfer resistance were observed according to the difference of the chemical nature among the surface modified electrodes evaluated in this study. As can be seen in Fig. 2, the introduction of AuNPs (CBPE/AuNPs) caused a physicochemical alteration at the electrode/solution interface, resulting in a decrease in the semi-circle ratio ($R_{CT}$ 7.6 kΩ) compared to the CBPE alone ($R_{CT}$ 8.6 kΩ). This

result suggests that the nanomaterial increased the electron transfer rate in the electrochemical system, as expected. The addition of the exopolysaccharide (CBPE/AuNPs/BOT) caused an increase in $R_{CT}$ (8.7 kΩ) compared to the nanostructured materials. This behavior can be predicted theoretically [15], since, in general, uncharged organic macromolecules can block the diffusion of the inorganic electroactive species on the surface of the electrode, such as hexacyanoferrate demonstrated in this case. Finally, when the biocatalyst (laccase) was immobilized on the modified carbon black electrode surface (CBPE/AuNPs/BOT/LCE), an organic macromolecular film (BOT/LCE) was formed, and the resistance of this device reached its highest value ($R_{CT}$ 21.6 kΩ). Based on studies reported in the literature, laccases commonly present an isoelectric point between 3 and 5 [5]. The impedance experiment presented was performed at pH 7.5, above the laccase isoelectric point. In this condition, the enzyme presents negative charge and electric repulsion with the ferrocyanide group, resulting in the high measured resistance.

Based on the impedance complex-plane plots, the $R_{CT}$ value obtained was used to calculate the heterogeneous electron transfer rate constant ($k^0$) (Table SM1), according to equation (1):

$$k^0 = \frac{RT}{F^2 R_{CT} A C n^2} \qquad (\mathbf{1})$$

where $R$ is the universal gas constant (8.314 J $K^{-1}$ $mol^{-1}$), $T$ is the thermodynamic temperature (298.15 °K), $F$ is Faraday's constant (96 485 C $mol^{-1}$), $A$ is the electrode geometrical area (0.07 $cm^2$), $C$ is the concentration of the electroactive inorganic entity, $[Fe(CN)_6]^{4-}$ (1 µmol $mL^{-1}$), and $n$ is the number of electrons.

For a given electrochemical system, the higher the $k^0$ value, the lower the time required for equilibrium to be established [31]. Therefore, the order of magnitude of this

constant is related to the measurement of the kinetics associated with the redox mechanism of the electroactive system under study. In this case, based upon the $k^0$ values presented in Table SM1, it is evident that the presence of AuNPs promotes an increase in the electron transfer rate. The biocatalytic enzyme film, on the other hand, delays the diffusion of inorganic species and impairs the kinetics of the system. The constant found ($1.7 \times 10^{-4}$ cm $s^{-1}$) was the lowest value for the proposed LCE-biosensor. The fabricated electrochemical sensing device displays high selectivity and, therefore, for the organic species of interest in this study, promotes high electronic transfer rates, as presented for hydroquinone (see later in the text).

The inset B in Fig. 2 shows the cyclic voltammograms obtained using different sensors in 0.1 mol $L^{-1}$ KCl solution containing 1 mmol $L^{-1}$ $[Fe(CN)_6]^{4-}$ at a scan rate of 50 mV $s^{-1}$. A pair of well-defined redox peaks related to $[Fe(CN)_6]^{4-}$ can be observed by employing the electrodes: CBPE, CBPE/AuNPs, CBPE/AuNPs/BOT and CBPE/AuNPs/BOT/LCE. An increase in the peak current was observed for CBPE/AuNPs when compared to the CBPE, indicating that the electron transfer reaction was kinetically favored, as previously discussed. It was also observed that the electrodes with immobilized biomaterials (CBPE/AuNPs/BOT and CBPE/AuNPs/BOT/LCE) presented lower current responses, reinforcing the results obtained by EIS.

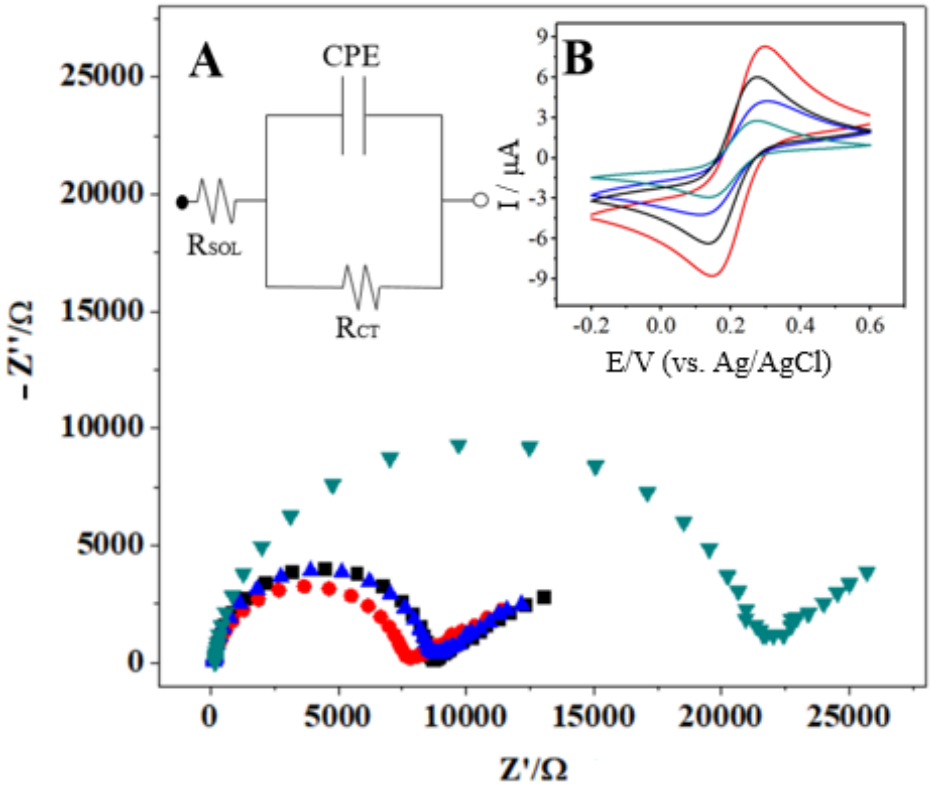



**Fig. 2.** Electrochemical impedance spectra for CBPE (■), CBPE/AuNPs (●), CBPE/AuNPs/BOT (▲) and CBPE/AuNPs/BOT/LCE (▼) in 0.1 mol $L^{-1}$ KCl solution containing 1.0 mmol $L^{-1}$ $[Fe(CN)_6]^{4-}$ (pH 7.5), open circuit mode (inset **A** circuit model), 10 mV amplitude and frequency range of 10 mHz to 100 kHz. *Inset:* (**A**) Randles circuit model, and (**B**) Cyclic voltammograms (50 mV $s^{-1}$) obtained using the corresponding electrodes in 0.1 mol $L^{-1}$ KCl solution containing 1.0 mmol $L^{-1}$ $[Fe(CN)_6]^{4-}$.

*3.4. Electrochemical behavior of the hydroquinone molecule on the laccase biosensor surface*

As described above, laccases catalyze the oxidation of phenolic compounds in their reduced form. In our work hydroquinone was selected as the model phenolic compound, which is oxidized to the corresponding *p*-benzoquinone by laccase [32]. Subsequently, *p*-benzoquinone is reduced electrochemically at the surface of the

proposed laccase-biosensor at 0.04 V *vs.* Ag/AgCl for hydroquinone, according to the mechanism previously described in the literature [02] and shown in Fig. SM2. The monitored cathodic peak current of the electrochemical reduction of *p*-benzoquinone on the biosensor surface was directly proportional to the concentration of hydroquinone in the solution, thus allowing the determination of this compound in real samples.

The oxidation product of hydroquinone through laccase catalysis was evaluated. For this, HQ was incubated with crude laccase extract containing 33 U of enzyme, in phosphate buffer solution (pH 7.0) at ambient temperature (same conditions as with the electrochemical cell). During a 4-h incubation period, a gradual change was observed in the solution color going from colorless to yellow/orange (See Fig. SM3A). The product was evaluated by UV-VIS spectrophotometry. A weak absorption band was observed in the visible region with a maximum around 500 nm. This result confirms the formation of *p*-benzoquinone, which has a yellowish coloration and characteristic absorption band at this wavelength [33]. Fig. SM3B presents the UV-VIS absorption spectrum.

The different electrodes (CBPE, CBPE/AuNPs, CBPE/AuNPs/BOT, CBPE/AuNPs/BOT/LCE) constructed were evaluated to determine the contribution of each modifier at the electrode/solution interface. Fig. 3 shows the SW voltammograms obtained in phosphate buffer solution (pH 6.0) resulting from the electrochemical reduction of *p*-quinone on the surface of the different working electrodes at a hydroquinone concentration of 99.0 µmol $L^{-1}$. Based on the presented behavior, the electronic transfer is favored by the electrocatalysis of AuNPs (signal B, Fig.3) in relation to the unmodified CBPE (signal A, Fig.3). After the deposition of the exopolysaccharide (BOT), a thin film of this biomaterial is formed on the surface of the electrode. Organic uncharged polymers may interfere with the electronic transfer at the interface, as well as delaying the diffusibility of the electroactive species, resulting in a

decrease in the current signal, as observed in the presence of BOT (signal C, Fig.3). The highest cathodic current obtained (signal D, Fig.3) was with the working electrode CBPE/AuNPs/BOT/LCE employing laccase as a biocatalyst. Its activity based on a stereospecific reaction confers selectivity on the proposed device and promotes the formation of large amounts of the oxidized species (*p*-quinone) in solution, which results in a greater signal of current arising from the electrochemical reduction. To prove the contribution of the AuNPs, a CBPE/BOT/LCE biosensor (electrode E) was constructed minus AuNPs, which presented a lower peak current than the proposed biosensor. These observations confirmed the success in the association of the nanomaterials (CB and AuNPs) with the laccase enzyme using the BOT film as an intermediate for immobilization.

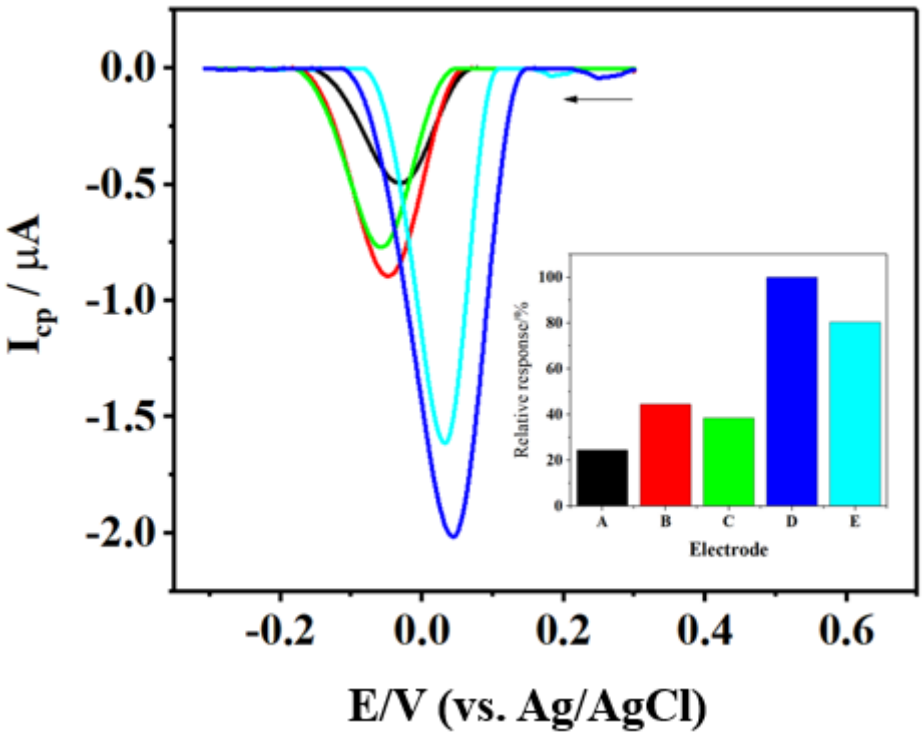



**Fig. 3.** SW voltammograms obtained using the (**A**) CBPE, (**B**) CBPE/AuNPs, (**C**) CBPE/AuNPs/BOT, (**D**) CBPE/AuNPs/BOT/LCE and (**E**) CBPE/BOT/LCE in phosphate buffer solution (pH 6.0) containing 99.0 µmol $L^{-1}$ hydroquinone. SWV

conditions: $a$ = 50 mV, $\Delta E_s$ = 2 mV and $f$ = 30 Hz. *Inset*: relative responses for the different electrodes evaluated.

The stability and maintenance of the CBPE/AuNPs/BOT/LCE electrode were tested following the evaluation of the electrochemical responses of the proposed biosensor after a long period in storage. For this, the response was monitored using a solution of hydroquinone (30.0 μmol $L^{-1}$) by repetitive measurements over several days. After each measurement, the biosensor was rinsed, and stored at 4.0 °C (refrigerator). After 25 days of these sequential experiments, current signals showed a standard deviation (SD) of 4.90 %, providing 250 stable and reproducible measurements taken without loss of laccase activity, as determined by responses obtained from the analysis of hydroquinone by the electrode. The maintenance of the laccase activity on the electrode is attributed to the preservation of the three-dimensional structure of the polypeptide chain at all its organizational levels (primary, secondary, tertiary and quaternary) due to the efficient immobilization onto the nanostructured platform of the BOT film. Thus, this exopolysaccharide showed itself as a biological matrix suitable for the immobilization of laccase and biosensor manufacture, maintaining enzyme activity, and promoting the stability and a long lifetime for the biosensor.

*3.5. Selection of the experimental parameters for the best performance of the proposed laccase biosensor*

Several parameters associated with the construction of the biosensor device and the experimental/instrumental conditions of the proposed method were investigated to guarantee the best analytical performance. Initially, the effect of laccase concentration on the analytical response for hydroquinone was investigated by using 0.22, 0.28, and

0.33 U of enzyme. The higher cathodic peak current ($I_{CP}$) was found for 0.33 U of laccase immobilized on BOT. As expected, the higher concentration of deposited laccase increased the number of active sites for biocatalysis to occur. Therefore, the concentration of the oxidized species of the analyte is highly favored, resulting in a highly analytical signal in the electrochemical reduction at the biosensor surface. Based on these results, 0.33 U of enzyme solution was used as optimum for the construction of further devices.

The influence of pH of the phosphate buffer solution, in the range 4.0 – 8.0, on the reduction peak of hydroquinone was checked for biosensor response. With the fabricated biosensing device, we demonstrated that by increasing the pH it also increased the electrochemical response for hydroquinone as shown in Fig. 4. The highest voltammetric response for hydroquinone was registered at pH 8.0. However, at higher pH values, the internal transfer of electrons from the T1Cu ion to the trinuclear cluster is inhibited by the binding of $OH^-$ anion to the T2 and T3 Cu ions [34]. This phenomenon, therefore, can severely hinder the catalytic stability of the enzyme, so that, at pH 8.0, repeatability of the cathodic current may not always be observed. Due to the best reproducible intensity of the analytical signals, phosphate buffer of pH 7.0 was used in further studies.

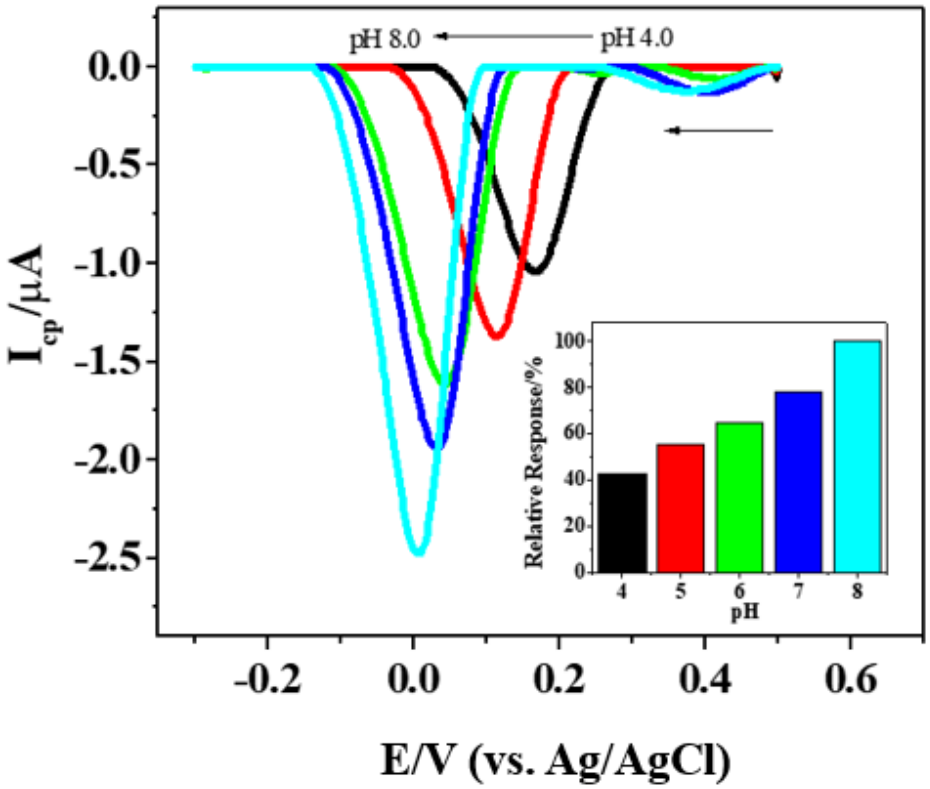



**Fig. 4.** (A) SW voltammograms obtained using the CBPE/AuNPs/BOT/LCE biosensor with 99.0 μmol $L^{-1}$ hydroquinone in phosphate buffer at different pH values: 4.0 to 8.0. SWV conditions are the same as indicated in Fig. 3. *Inset*: relative responses.

Prior to the construction of the analytical curve by SWV, the instrumental parameters of this technique were optimized in order to obtain a higher current value and a defined peak for hydroquinone determination. The parameters evaluated included pulse amplitude (*a*), square-wave frequency (*f*) and scan increment ($\Delta E_S$), within the ranges of 10 – 100 mV, 10 – 100 Hz, and 1 – 6 mV, with optimum values obtained with 50 mV, 60 Hz, and 3 mV, respectively. These parameters were used to record the analytical curve by addition of aliquots of a standard solution of hydroquinone, in phosphate buffer solution (pH 7.0).

The analytical curve was obtained from the resultant cathodic peak current, recorded in the voltammograms of Fig. 5, as a function of the concentration of

hydroquinone. There was a linear relationship between peak current (μA) and hydroquinone concentration ranging from 2.00 – 56.5 μmol $L^{-1}$ (Fig. 5, inset), with the following linear regression equation $I_{CP}$ (μA) = 0.025 + 0.069 [$c$ (μmol $L^{-1}$)] ($r^2$ = 0.9996), at pH 7.0; where $I_{CP}$ is the cathodic peak current, and $c$ the hydroquinone concentration. The theoretical LOD and LOQ were 0.474 μmol $L^{-1}$ and 1.58 μmol $L^{-1}$, respectively. As observed in Fig. 5 (inset), a higher sensitivity was obtained at pH 7.0 than at pH 6.0 (0.033 μA.μmol$^{-1}$.L). This result is attributed to the increase of the catalytic performance of the enzyme under conditions of higher hydrogenionic potential, as discussed above.

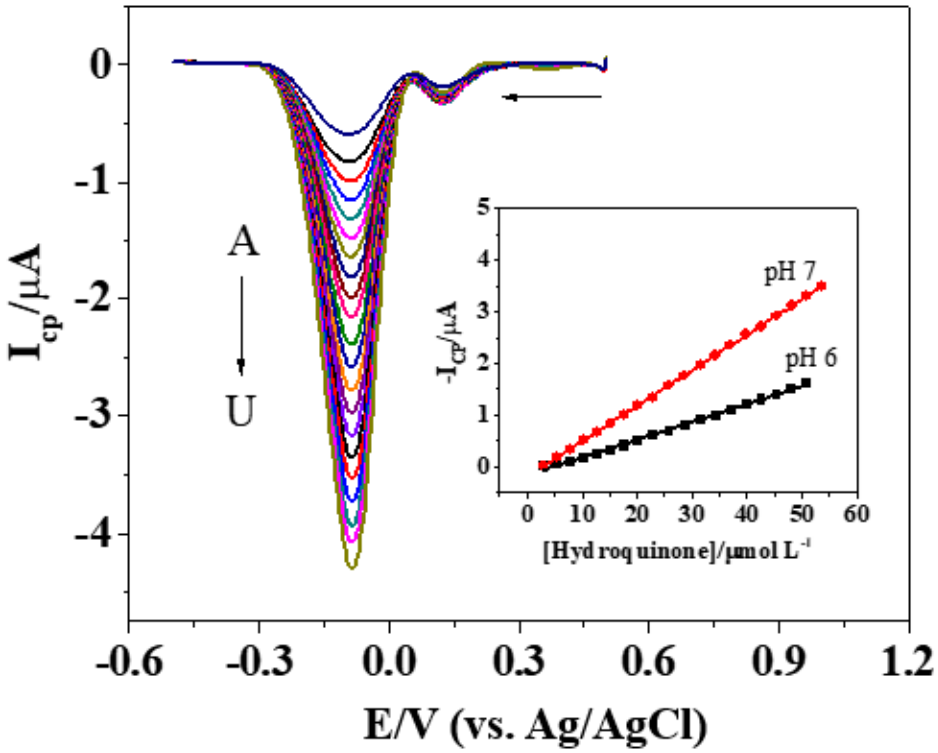



**Fig. 5.** SW voltammograms obtained in phosphate buffer solution (pH 7.0) employing the CBPE/AuNPs/BOT/LCE biosensor as working electrode for the following concentrations of hydroquinone: (B – U): 2.0 – 56.5 μmol $L^{-1}$. *Inset*: Corresponding calibration curve.

The repeatability of the electrochemical measurements performed using the proposed biosensor was evaluated by monitoring the cathodic current by SWV in phosphate buffer solution (pH 7.0) using 14.8 μmol $L^{-1}$ hydroquinone. After ten successive measurements using the same device, without any superficial renovation, the current signals showed a 0.3% SD. This confirmed the stability of the biocatalytic surface, and that the biosensor can be used in repetitive analyzes providing accurate measurements.

In order to evaluate the reproducibility of the biosensing platform assembly, the cathodic currents of three biosensors were monitored for 14.8 μmol $L^{-1}$ HQ in phosphate buffer solution (pH 7.0). The SD between measurements of the three devices for the same concentration was 3.4 %, indicating excellent reproduction in the biosensor construction.

*3.6. Selectivity and analytical application of the proposed laccase biosensor for the determination of hydroquinone*

In order to evaluate the selectivity of the proposed laccase biosensor, the effect of the presence of different chemical species on electrochemical measurements of hydroquinone was assessed by SWV (data not shown). This procedure was performed to check for possible interference in determining hydroquinone. A standard solution of 20.0 μmol $L^{-1}$ hydroquinone at the concentration ratios (hydroquinone:other chemical species) of 1:1 and/or 1:10, was used for this purpose.

Substrate specificity was examined in the presence of other phenol-like model compounds: e.g., epinephrine (EPN), dopamine (DPM), guaiacol (GUA) and catechol (CAT). Although the biosensor showed a response in the presence of all of these

compounds, no significant interference was observed in the cathodic current signal corresponding to hydroquinone (SD < 3%). Based upon these results, the biosensor was not specific, but could have potential for the simultaneous determination of these phenolic compounds, as the reduction signals did not overlap with the hydroquinone peak (Fig. SM4).

In the presence of uric acid and several inorganic ions ($Ca^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $SO_4^{2-}$ and $NO_3^-$), the proposed biosensor also presented a stable response to hydroquinone measurements (RSD < 2 %). However, when a solution of ascorbic acid (AA) was added, a decrease in the cathodic current signal for hydroquinone was observed. AA has been described in the literature as an inhibitor of laccases [35]. Its reducing electrochemical character is responsible for the conversion of *p*-benzoquinone to hydroquinone, causing a decrease in the cathodic current signal.

In order to evaluate its applicability in real and complex samples, the biosensor was used to determine hydroquinone in commercials samples of dermatological cream, as well as samples of human urine and river water. Hydroquinone in two samples (A and B) of dermatological cream (4 %, w/w) was determined comparing a spectrophotometric method [19] with the developed laccase biosensor to evaluate the accuracy of the proposed method. The possibility of interference by tretinoin and fluocinolone acetonide (two ingredients used with hydroquinone in topical creams), was also evaluated, but no changes were observed in the electrochemical behavior of the hydroquinone molecule with the biosensor (data not shown).

The analyses were performed in triplicate, and the concentration of hydroquinone in samples A and B found was 3.98 ± 0.03 % (w/w) and 4.08 ± 0.02 % (w/w), respectively, using the proposed biosensor. The values provided by the spectrophotometric comparative method were respectively, 4.02 ± 0.01% (w/w) and

4.01 ± 0.03 % (w/w). Applying the student's *t* test ($\alpha = 0.05$) to the results obtained, no statistical differences were observed between the values for the laccase biosensor and the comparative spectrophotometric method.

The proposed biosensor was also employed to determine hydroquinone when contained in a biological specimen (human urine), and an environmental sample (river water). Both samples were doped with two different concentrations of hydroquinone: 4.0 and 8.0 μmol $L^{-1}$ for human urine, and 8.0 and 20.0 μmol $L^{-1}$ for the water river. As shown in Table SM2, excellent recovery results were obtained, with values close to 100 %. Based on these results, the proposed bioelectrochemical device ensured a high degree of accuracy in the quantitative analysis of hydroquinone.

## 4. Conclusions

The proposed electrochemical biosensor (CBPE/AuNPs/BOT/LCE) was successfully fabricated. The excellent analytical performance of this device can be attributed to the stable environment for enzyme immobilization. The laccase biosensor device maintained its catalytic activity at the interface electrode/solution, providing selectivity and stability to the proposed analytical method. In view of this, botryosphaeran was demonstrated to be a promising biomaterial in the construction of a bioelectrochemical sensor device, eliminating the need for reagents that promote covalent linking of the enzyme to the electrode support materials. Furthermore, the inorganic nanomaterials of the transducing phase promoted electrocatalysis in the biological environment without affecting the activity or conformation of the bound bioactive macromolecules. The determination of hydroquinone was successfully demonstrated in complex samples (dermatological cream, urine and river water) without any prior treatment being required. Based upon the addition and recovery studies, no

interference from the matrices was observed, confirming the biosensor selectivity in complex chemical environments. The results for the quantitation of hydroquinone were in agreement with that obtained by the comparative spectrophotometric method. The biosensing platform fabricated presented in this study appears to have wide applications in the detection and determination of phenolic compounds, with fast response times, selectivity, based on renewable and non-polluting biological recognition elements for large-scale production.

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References

[1] V. Polshettiwar, R.S. Varma, Green chemistry by nano-catalysis, Green Chem. 12 (2010) 743. doi:10.1039/b921171c.

[2] M.D. Cannatelli, A.J. Ragauskas, Two decades of Laccases: Advancing sustainability in the chemical industry, Chem. Rec. 17 (2017) 122–140. doi:10.1002/tcr.201600033.

[3] C. Pezzella, L. Guarino, A. Piscitelli, How to enjoy laccases, Cell. Mol. Life Sci. 72 (2015) 923–940. doi:10.1007/s00018-014-1823-9.

[4] T. Robinson, G. McMullan, R. Marchant, P. Nigam, Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative, Bioresour. Technol. 77 (2001) 247–255. doi:10.1016/S0960-8524(00)00080-8.

[5] B. Viswanath, B. Rajesh, A. Janardhan, A.P. Kumar, G. Narasimha, Fungal laccases and their applications in bioremediation, Enzyme Res. 2014 (2014) 163242. doi:10.1155/2014/163242.

[6] M.M. Rodriguez-Delgado, G.S. Alemán-Nava, J.M. Rodríguez-Delgado, G. Dieck-Assad, S.O. Martínez-Chapa, D. Barceló, R. Parra, Laccase-based biosensors for detection of phenolic compounds, TrAC - Trends Anal. Chem. 74 (2015) 21–45. doi:10.1016/j.trac.2015.05.008.

[7] F. Xu, W. Shin, S.H. Brown, J.A. Wahleithner, U.M. Sundaram, E.I. Solomon, A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability, Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1292 (1996) 303–311. doi:10.1016/0167-4838(95)00210-3.

[8] I.R.W.Z. De Oliveira, I.C. Vieira, Construção e aplicação de biossensores usando diferentes procedimentos de imobilização da peroxidase de vegetal em matriz de quitosana, Quim. Nova. 29 (2006) 932–939. doi:10.1590/S0100-40422006000500009.

[9] R.F.. Dekker, A.M. Barbosa, The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete Botryosphaeria sp., Enzyme Microb. Technol. 28 (2001) 81–88. doi:10.1016/S0141-0229(00)00274-X.

[10] A.M. Barbosa, R.M. Steluti, R.F.H. Dekker, M.S. Cardoso, M.L.C. Silva, Structural characterization of Botryosphaeran: a (1→3;1→6)-β-d-glucan produced by the ascomyceteous fungus, *Botryosphaeria* sp., Carbohydr. Res. 338 (2003) 1691–1698.

[11] E.C. Giese, J. Gascon, G. Anzelmo, A.M. Barbosa, M.A.A. da Cunha, R.F.H. Dekker, Free-radical scavenging properties and antioxidant activities of botryosphaeran and some other β-D-glucans, Int. J. Biol. Macromol. 72 (2015) 125–130. doi:10.1016/J.IJBIOMAC.2014.07.046.

[12] T.A. Silva, F.C. Moraes, B.C. Janegitz, O. Fatibello-Filho, Electrochemical biosensors based on nanostructured carbon black: A review, J. Nanomater. (2017) 1–14. doi:10.1155/2017/4571614.

[13] R. Alcántara, J.M. Jiménez-Mateos, P. Lavela, J.L. Tirado, Carbon black: a promising electrode material for sodium-ion batteries, Electrochem. Commun. 3 (2001) 639–642. doi:10.1016/S1388-2481(01)00244-2.

[14] T.R. Silva, D. Brondani, E. Zapp, I. Cruz Vieira, Electrochemical Sensor Based on Gold Nanoparticles Stabilized in Poly(Allylamine hydrochloride) for Determination of Vanillin, Electroanalysis. 27 (2015) 465–472.

http://doi.wiley.com/10.1002/elan.201400517.

[15] T.R. Silva, I.C. Vieira, SILVA, T. R.; VIEIRA, I. C. A biosensor based on gold nanoparticles stabilized in poly(allylamine hydrochloride) and decorated with laccase for determination of dopamine. The Analyst, v. 141, n. 1, p. 216–224, 2016. A biosensor based on gold nanoparticles, Analyst. 141 (2016) 216–224. doi:10.1039/C5AN01784J.

[16] F.J. Enguita, A.L. Leitão, Hydroquinone: environmental pollution, toxicity, and microbial answers., Biomed Res. Int. 2013 (2013) 542168. doi:10.1155/2013/542168.

[17] M. Gaskell, K.I.E. McLuckie, P.B. Farmer, Comparison of the repair of DNA damage induced by the benzene metabolites hydroquinone and p-benzoquinone: a role for hydroquinone in benzene genotoxicity, Carcinogenesis. 26 (2004) 673–680. doi:10.1093/carcin/bgi007.

[18] N. Rothman, W.E. Bechtold, S.N. Yin, M. Dosemeci, G.L. Li, Y.Z. Wang, W.C. Griffith, M.T. Smith, R.B. Hayes, Urinary excretion of phenol, catechol, hydroquinone, and muconic acid by workers occupationally exposed to benzene., Occup. Environ. Med. 55 (1998) 705–11. doi:10.1136/OEM.55.10.705.

[19] P. López García, M.I. Rocha Miritello Santoro, E.R.M. Kedor-Hackman, A. Kumar Singh, Development and validation of a HPLC and a UV derivative spectrophotometric methods for determination of hydroquinone in gel and cream preparations, J. Pharm. Biomed. Anal. 39 (2005) 764–768. doi:10.1016/J.JPBA.2005.04.016.

[20] E.S. Elzanfaly, A.S. Saad, A.-E.B. Abd-Elaleem, Simultaneous determination of retinoic acid and hydroquinone in skin ointment using spectrophotometric

technique (ratio difference method), Saudi Pharm. J. 20 (2012) 249–253. doi:10.1016/J.JSPS.2012.03.004.

[21] E.C.M. Barbosa, J.L. Fiorio, T. Mou, B. Wang, L.M. Rossi, P.H.C. Camargo, Reaction Pathway Dependence in Plasmonic Catalysis: Hydrogenation as a Model Molecular Transformation, Chem. - A Eur. J. 24 (2018) 12330–12339. doi:10.1002/chem.201705749.

[22] T.V. Theis, G.C. Calegari, V.A.Q. Santos, H.E.Z. Junior, A.M. Barbosa, R.F.H. Dekker, M.A.A. da Cunha, Exocellular (1→6)-β-D-Glucan (Lasiodiplodan): Carboxymethylation, Thermal Behavior, Antioxidant and Antimicrobial Activity, Am. J. Immunol. 13 (2017) 19–33. doi:10.3844/ajisp.2017.19.33.

[23] G.L. Long, J.D. Winefordner, Limit of detection. A closer look at the IUPAC definition, Anal. Chem. 55 (1983) 712A–724A. doi:10.1021/ac00258a001.

[24] S. Linic, P. Christopher, D.B. Ingram, Plasmonic-metal nanostructures for efficient conversion of solar to chemical energy, Nat. Mater. 10 (2011) 911–921. doi:10.1038/nmat3151.

[25] K.A. Willets, R.P. Van Duyne, Localized Surface Plasmon Resonance Spectroscopy and Sensing, Annu. Rev. Phys. Chem. 58 (2007) 267–297. doi:10.1146/annurev.physchem.58.032806.104607.

[26] Y. Wang, E. Laborda, A. Crossley, R.G. Compton, Surface oxidation of gold nanoparticles supported on a glassy carbon electrode in sulphuric acid medium: contrasts with the behaviour of “macro” gold, Phys. Chem. Chem. Phys. 15 (2013) 3133. doi:10.1039/c3cp44615h.

[27] A. Sassolas, L.J. Blum, B.D. Leca-Bouvier, Immobilization strategies to develop enzymatic biosensors, Biotechnol. Adv. 30 (2012) 489–511. doi:10.1016/j.biotechadv.2011.09.003.

[28] M.H. van de (Marcel H.. Voorde, B. Sels, Nanotechnology in catalysis : applications in the chemical industry, energy development, and environment protection, n.d.

[29] C. Brett, A. Brett, Electrochemistry: principles, methods, and applications, Springer. (1993) 427. doi:10.1002/anie.199419892.

[30] V.F. Lvovich, Impedance Spectroscopy: applications to electrochemical and dielectric phenomena, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2012. doi:10.1002/9781118164075.

[31] C. Zanardi, E. Ferrari, L. Pigani, F. Arduini, R. Seeber, Development of an Electrochemical Sensor for NADH Determination Based on a Caffeic Acid Redox Mediator Supported on Carbon Black, Chemosensors. 3 (2015) 118–128.

[32] M. Mogharabi, M.A. Faramarzi, Laccase and Laccase-Mediated Systems in the Synthesis of Organic Compounds, Adv. Synth. Catal. 356 (2014) 897–927. doi:10.1002/adsc.201300960.

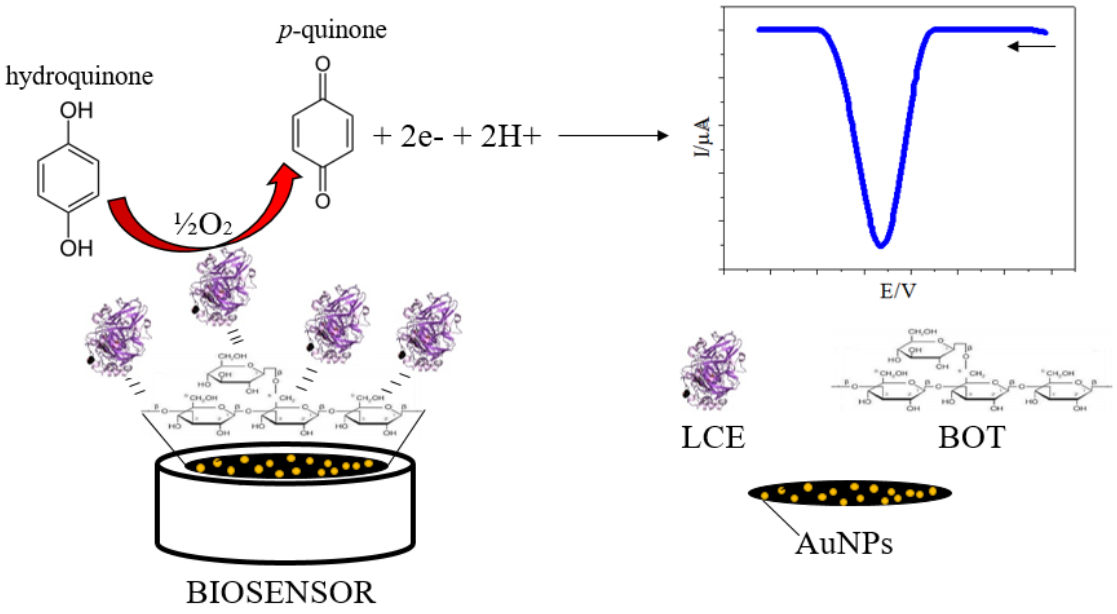
[33] R.A. MOPTON, Biochemistry of quinones., Academic Press, Inc., London: New York, 1965.

[34] F. Xu, Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases, J. Biol. Chem. 272 (1997) 924–928. doi:10.1074/JBC.272.2.924.

[35] P. Baldrian, Fungal laccases – occurrence and properties, FEMS Microbiol. Rev.

30 (2006) 215–242. doi:10.1111/j.1574-4976.2005.00010.x.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- A novel biosensor platform was developed using carbon black electrode/AuNPs.
- Laccase from *B. rhodina* was immobilized on the biopolymer, (1→3)(1→6)-β-D-glucan.
- The voltammetric biosensor presented good selectivity in chemically complex samples.
- HQ was successfully determined in several samples using this bio-analytical device.