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Laccase biosensor based on screen-printed electrode modified with thionine–carbon black nanocomposite, for Bisphenol A detection

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ABSTRACT

The relevance of Bisphenol A (BPA) in human health is well-known. For this reason we designed and developed a biosensor based on a bionanocomposite (laccase–thionine–carbon black)-modified screen-printed electrode. Thionine, a commercially available dye, was used as electrochemical mediator coupled with a nanostructured carbon black. By means of cyclic voltammetry, the interaction of thionine adsorbed on modified screen printed electrode with laccase/BPA reaction products has been studied. In addition, the immobilization of laccase by physical adsorption on the surface of thionine–carbon black modified screen printed electrodes was investigated. The response of the biosensor has been optimized in terms of enzyme loading, pH and applied potential reaching a linear concentration range of 0.5–50 μM, a sensitivity of 5.0 ± 0.1 nA/μM and a limit-of-detection (LOD) of 0.2 μM. The developed biosensor has been also challenged in tomato juice samples contained in metallic cans where release of BPA due to the epoxy resin coating can be assumed. A satisfactory recovery value comprised between 92% and 120% was obtained.

1. Introduction

Bisphenol A [BPA, 2,2-(4,4-dihydroxydiphenyl) propane; CAS Registry No. 80-05-7] is produced by combining acetone and phenol and is an important organic chemical owing to its wide use as intermediate in the manufacture of polycarbonate plastics, epoxy resins, and flame retardants [1]. Its main use is as coating of metallic cans, powder paints, dental fillings, and antioxidant in plastics. It has also been used as an inert ingredient in pesticides, antioxidants and polyvinyl chloride stabilizer [2]. Recently, BPA has received considerable attention due to its endocrine disrupting activity and possible toxic impact on environment [3–5]. BPA levels in the μg/L range have been found in biological, food and water samples [6,7]. The presence of BPA is still a "hot topic": the European Food Safety Authority (EFSA) is still investigating on new risk assessment of BPA and, very recently, in its newsletter, invited "*. . .Member States, research institutions, academia, food business operators, packaging business operators and other stakeholders to submit data on BPA, in particular its occurrence in food and beverages, migration from food contact materials, occurrence in food contacts materials. Deadline: 31 July 2012*". The above findings suggest that it is necessary to determine the BPA presence also in trace amounts.

Traditional methods for BPA detection include chromatographic techniques coupled with mass spectrometry, capillary electrophoresis and solid phase microextraction, methods that are time consuming, cannot be performed on-site and require sample pre-treatment [8]. Electrochemical sensors can provide rapid and on-site BPA detection. For this purpose many researchers have developed electrochemical sensors using tyrosinase as biological element and different immobilization procedures [9–13]. Recently, the use of different tyrosinase-functionalised nanoparticle systems has shown interesting results [14].

All the developed electrochemical sensors for BPA use the enzyme tyrosinase, differently from the approach used for other phenol compounds for which other enzymes, such as peroxidase [15] and laccase [16], have been successfully employed. In particular the use of laccase combined with a properly chosen redox-active compound could represent a potential good candidate for designing and fabricating novel BPA sensors.

Laccases (E.C. 1.10.3.2) are dimeric or tetrameric glycoproteins, containing four copper atoms per monomer distributed in three redox sites: one in each T1 and T2 sites, and two in T3 site. It is assumed that the catalysis firstly involves T1 Cu reduction by the substrate, followed by internal electron transfer from T1 Cu to T2 and T3 Cu and, finally, dioxygen reduction at T2 and T3 sites

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[17]. Laccases catalyze the oxidation of ortho- and para-diphenols, aminophenols, aryl diamines, polyphenols, polyamines, lignin as well as some inorganic ions, coupled to the reduction of molecular dioxygen to water [18,19]. In the case of BPA not all the reaction products have been characterized. For instance, an interesting study was performed by Fukuda et al. [20], in which they demonstrated that the BPA was metabolized by laccase in two kinds of compounds: one kind composed by high molecular weight compounds and another kind composed by low molecular weight compounds, one of which was identified as 4-isopropenylphenol by means of gas chromatography–mass spectrophotometry. The 4-isopropenylphenol as oxidative degradation product has been identified using chromatography mass–spectrometry also in other works reported in literature [21,22].

It has been also reported that some redox-active compounds, known as "mediators", allow enlarging the range of compounds that can be targeted for oxidation by laccase [23]. The mediator interacts with the enzyme or with the reaction product. In some cases, typically the enzyme firstly oxidizes the mediator, which diffuses away from the enzyme active site, and sequentially oxidizes a compound that may be or not to be substrate of the enzyme. The mediator then returns to its original form and can subsequently be used to accomplish the conversion of more target species. The employment of mediators makes the use of laccase more attractive because it increases the number of pollutants that can be targeted [24]. When mediators are involved in laccase-assisted processes, the electron transfer from the mediator to the enzyme is followed by electron donation from the target molecule to oxidized mediator, which gives rise to the regeneration of the mediator [25–27]. In other cases, instead, the mediator reacts directly with the reaction products, as it occurs for tyrosinase interacting with phenols [10].

Several organic and inorganic compounds have been reported as effective mediators for the above purposes. The laccase reduction of dioxygen to water in the presence of different redox mediators or nanomaterials [28] has been studied in other electrochemical applications [29–34].

It has been also demonstrated that the use of redox mediators coupled with carbon nanomaterials can further improve the electrochemical performances of the developed sensor. For example carbon nanotubes were used together with ferrocenedicarboxylic acid [35], azure dye [36], thionine [37]. Among the carbon nanostructured materials such as carbon nanotubes and graphene, it was recently demonstrated the useful use of carbon black (CB). CB is an industrially manufactured colloidal material that consists of approximately spherical carbon primary particles with diameter comprised from 15 to 100 nm, which typically forms fused aggregates with sizes below 1000 nm. CB was demonstrated: (i) to have electrocatalytic properties towards many compounds such as ascorbic acid, dopamine, NADH, benzoquinone, epinephrine, cysteine, thiocholine, hydrogen peroxide [38–43], and (ii) that it can be used as the basis for construction of a tyrosinase biosensor for catechol detection [44]. In addition, as highlighted in our papers [41–43], this material allows the production of a stable dispersion using a cheap carbon nanostructured material, as recently confirmed by Compton's group [45].

In this paper we describe a disposable BPA biosensor based on laccase. To our knowledge, this is the first report in literature concerning biosensor for BPA detection based on laccase coupled with disposable sensor. By means of cyclic voltammetry (CV), the effect of thionine as a mediator has been investigated when the immobilized laccase interacts with BPA. In order to increase the analytical performance and the easiness of the proposed biosensor, a screen printed electrode (SPE) modified by using a nanocomposite formed by CB and thionine has been used. Also in this case, to our knowledge this is the first time that the CB was used coupled with a redox mediator in developing an electrochemical sensor.

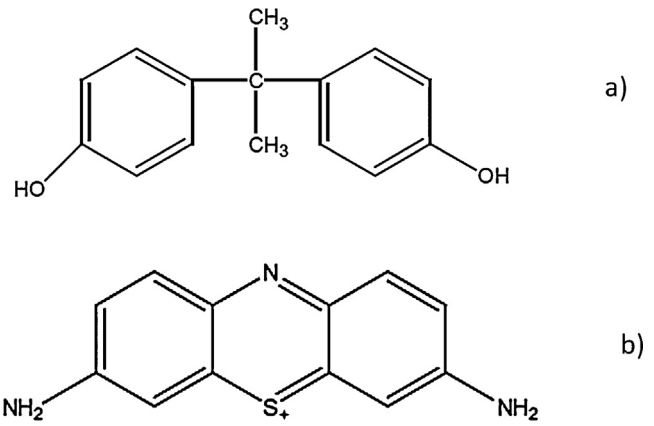

Fig. 1. Chemical structure of BPA (a) and of thionine (b).

The biosensor response was examined in terms of immobilized enzyme units, applied electrical potential and pH. Finally the optimized biosensor has been used for the amperometric determination of BPA in aqueous buffer solutions and in peeled tomatoes stored in metallic cans.

2. Materials and methods

2.1. Materials

Laccase (20 Units/mg) from *Trametes Versicolor*, Bisphenol A (see chemical structure in Fig. 1a), Thionine acetate (see chemical structure in Fig. 1b), Nafion® and all the other chemicals were purchased from Sigma (Sigma–Aldrich, Milan, Italy) and used without further purification. Carbon Black N220 (CB) was obtained from Cabot Corporation (Ravenna, Italy). Screen-Printed Electrodes G-Sensor (SPEs) was obtained from Ecobioservice and Research (Firenze, Italy).

The working electrode was made of graphite and its diameter was 0.3 cm.

2.2. Methods

The enzymatic working electrode was prepared by modifying the surface of a screen printed electrode with 6 μL of a dispersion of CB 1 mg/mL in acetonitrile [41]. After solvent evaporation, 5 μL of thionine 0.4 mM aqueous solution were added and then the electrode was put in an oven at 60 °C for about 15 h [13]. After this treatment, the electrode was further modified by adding 5 μL of laccase solution at different concentrations, in order to obtain enzymatic unit values ranging from 0.59 U to 6.52 U. After drying, the electrodes were covered with 3 μL of a neutralized aqueous solution of Nafion® 2.2% (v/v) and left to dry at room temperature for 30 min in order to avoid rapid enzyme leaking. The whole scheme is reported in Fig. 2a.

The BPA/laccase reaction products were separately obtained by the following procedure: 500 μL of 0.05 M citrate buffer at pH 4.5 were added to 100 μL of 1 mM BPA and 100 μL of laccase (40 mg/mL) both in the same buffer and allowed to react at 37 °C for 1 h to obtain total conversion of BPA in product.

To obtain a thionine–CB-modified SPE, two steps were carried out: (1) 6 μL of a dispersion of CB 1 mg/mL in acetonitrile were placed on SPE as described in our previous work [41]; (2) thionine was made to adsorb on the modified SPE as previously described.

All the measurements were carried out using SPEs connected to a PalmSens instrument (Palm Instruments, the Netherlands) coupled to a PC and were performed at room temperature (25.0 ± 0.5 °C). The potentials were referred to the internal Ag

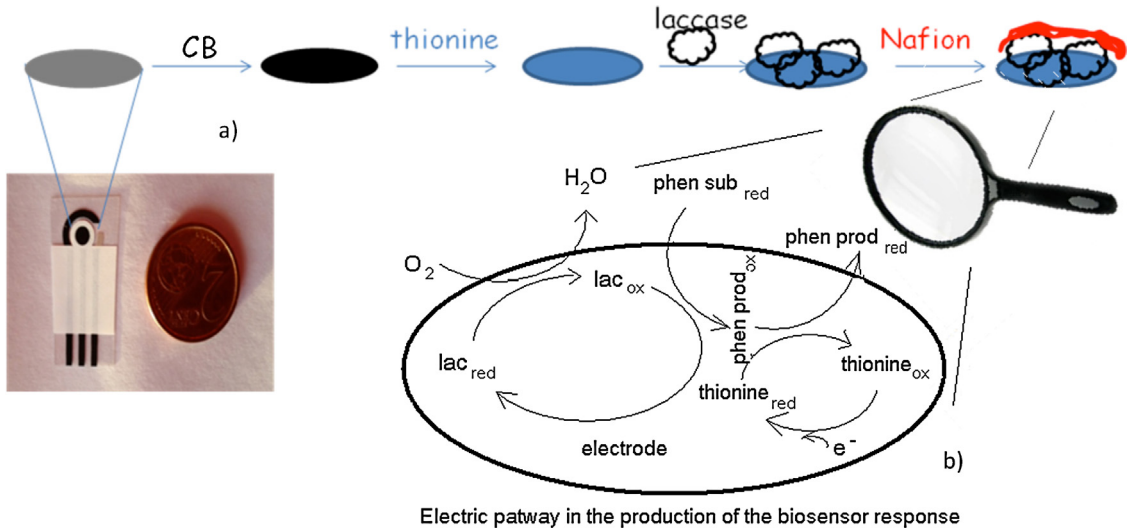

Fig. 2. (a) Fabrication process of laccase biosensor based on screen-printed electrode modified with thionine–carbon black and Nafion. (b) Scheme of biosensor electrocatalytic mechanism.

pseudo-reference electrode of the SPE. The cyclic voltammetry experiments were carried out in 0.1 M phosphate buffer plus 0.1 M KCl, pH = 6.5 and at scan rate of 100 mV/s.

For amperometric experiments, a magnetic stirrer and a stirring bar provided the convective transport in the electrolytic cell filled with a volume of 10 mL. These measurements were carried out in the absence of KCl in the reaction volume, because salinity affects the solubility of the reaction products, leading to inactivation or changes in the enzymatic processes as a result of interactions with the product. In particular, chlorinated compounds substantially inhibit the conversion of BPA and then chlorine ion is the main cause of low conversion [24].

Each experimental point in the figures is the average of four experiments carried out under the same conditions. The experimental error never exceeded 5%.

Micrographs of CB-SPE, thionine modified CB-SPE and laccase biosensor based on thionine modified CB-SPE were obtained by scanning electron microscopy (SEM) using a Carl Zeiss model SUPRA 40 microscope.

3. Results and discussion

3.1. Cyclic voltammetry measurements using thionine in solution

Preliminary experiments were performed in order to study the electrocatalytic properties of thionine towards BPA/laccase reaction products. Thionine is an artificial organic dye derivative of phenothiazine, and it is used as a mediator since its formal potential falls between 0.08 and −0.25 V, near the redox potential of many biomolecules [13,37].

Initially 100 μL of 0.4 mM thionine were poured on the SPE without any modification and the typical thionine voltammogram obtained is shown in curve 1 of Fig. 3a, with E_{pa} = −330 mV, E_{pc} = −360 mV, I_{pa} = 1.47 μA e I_{pc} = 1.33 μA and *a* I_{pa}/I_{pc} ratio equal to 1.10.

Afterwards, 20 μL of BPA/laccase reaction product, obtained as reported in Section 2.2, were added to the 100 μL of 0.4 mM thionine and poured on the screen printed electrode. The CV in presence of BPA/laccase reaction product and thionine is reported in curve 2 of Fig. 3a. It is easy to notice a relevant change in the voltammogram shape after the addition of BPA/laccase reaction product. The current signal due to thionine reduction process increases in respect to the one in absence of BPA/laccase reaction product. Conversely, the anodic peak of current signal due to the thionine oxidation process decreases, as expected for mediated reactions. The enzymatic reaction caused an increase of the amount of $thionine_{ox}$ on the working electrode surface, with the consequent increase of cathodic current peak. On the contrary, the anodic current peak is proportional to the amount of $thionine_{red}$ and this quantity decreases after the enzyme/BPA reaction. These results confirm that thionine has an electrocatalytic activity towards the reduction of BPA/laccase oxidation products as shown by the electrocatalytic mechanism scheme reported in Fig. 2b. Then the SPEs are an useful tool for monitoring the electrocatalytic effect with the advantage, in respect to the conventional electrodes such as glassy carbon electrode, of using a few microliters of solution.

3.2. Cyclic voltammetry measurements using thionine adsorbed on the working electrode surface

In order to develop a ready-to-use biosensor modified with thionine, this mediator was adsorbed on the working electrode surface, accordingly to our previously optimized procedure in the case of thionine modified carbon paste electrode [13]. The thionine-modified SPE was thus checked by cyclic voltammetry in order to test its working stability. As shown in Fig. 3b, the redox peaks of thionine can be observed at E_{pa} = −320 mV and at E_{pc} = −360 mV with a large increase in the peak currents. However, after 10 scans, a decrease of about 70% in anodic and cathodic intensity current of thionine is observed, demonstrating that the simple modification by adsorption gives an unsatisfactory working stability.

In order to avoid this drawback, we constructed a biosensor modified with CB–thionine nanocomposite. The approach used in this work was the bilayer one, in which firstly, a layer of CB was deposited on the working electrode surface by “drop” coating, followed by another layer of thionine. In this way, a thionine–CB–SPE was obtained. This latter was tested by cyclic voltammetry in order to investigate its working stability. As shown in Fig. 3c, the potential of anodic and cathodic peaks were E_{pa} = −220 mV and E_{pc} = −260 mV, respectively, and stable cyclic voltammograms were obtained. The difference of the experimental ΔE_p from the ideal surface redox process (with $\Delta E_p = 0$) is due to limitations associated with charge propagation in the film, the chemical interaction between the ions and the modified film, and the polarizability of the cation influencing its penetration in or out of film. Using thionine–CB–SPE, after 10 scans only a small decrease of about

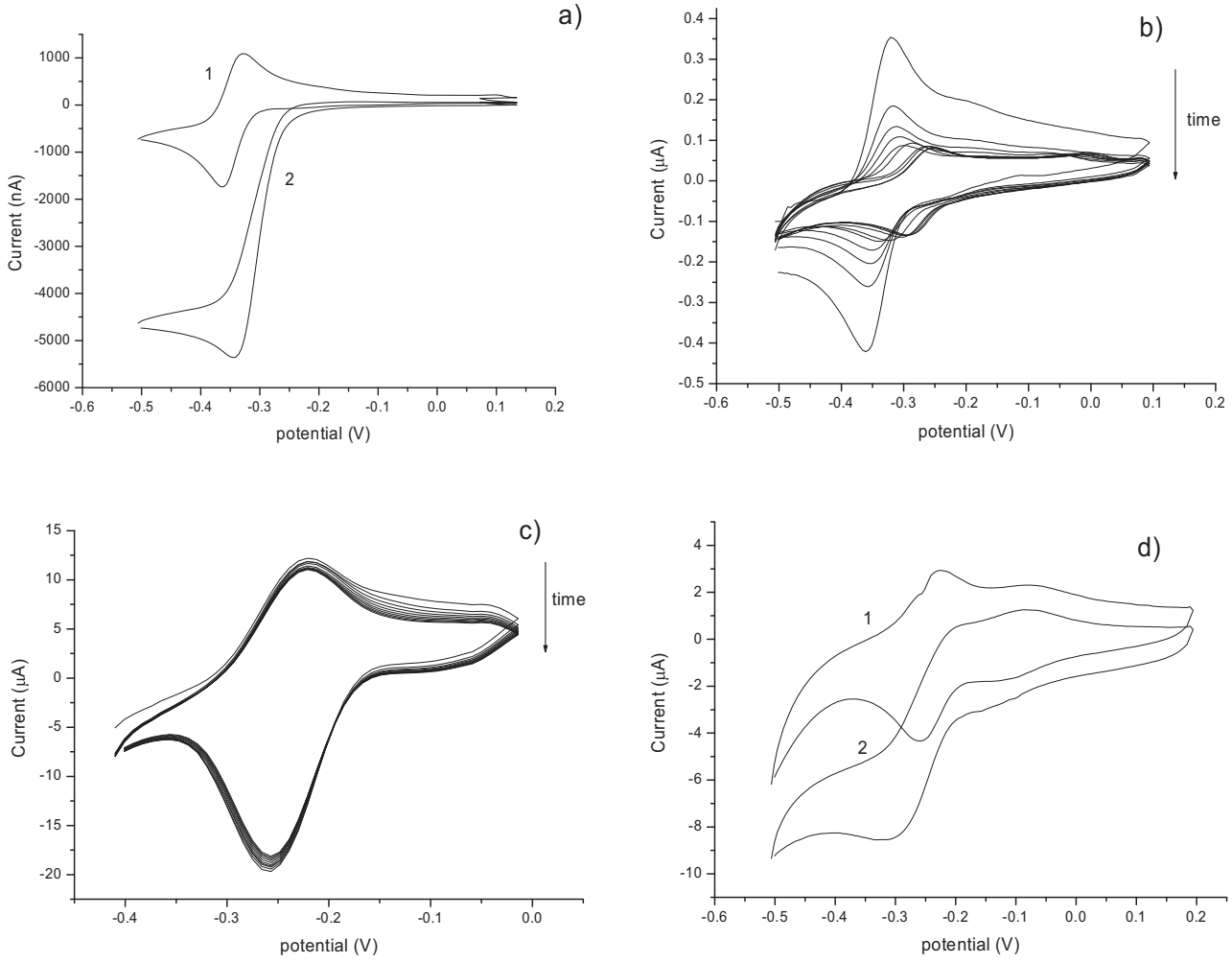

Fig. 3. (a) Cyclic voltammetry for SPE with thionine solution in the absence (curve 1) and in the presences (curve 2) of the laccase/BPA reaction products. (b) Cyclic voltammetry for an adsorbed thionine modified SPE. (c) Cyclic voltammetry for an adsorbed thionine and CB-SPE. (d) Cyclic voltammetry for an adsorbed thionine and CB-SPE in the absence (curve 1) and in the presences (curve 2) of the laccase/BPA reaction products. For all the measurements v_{scan} = 100 mV/s.

5% in the measured anodic and cathodic intensity current was observed. Moreover, the peak currents (I_{pa} = 11 μA and I_{pc} = 16 μA) were much higher than the ones previous found in the case of thionine–SPE (I_{pa} = 0.39 μA and I_{pc} = 0.29 μA), demonstrating the improvement due to the presence of carbon black, because the presence of the nanomaterial CB N220, characterized by a high surface area (120 m^2/g), allows the adsorption in a stable way of a higher amount of thionine on the working electrode surface in a stable way.

In order to test the electroactivity towards the BPA/laccase reaction product, cyclic voltammetry was carried out in absence and in presence of BPA/laccase reaction products. Fig. 3d shows that the current due to the thionine reduction increases with respect to that obtained in the absence of BPA/laccase reaction products, while in the reverse scan the current of the anodic peak due to the thionine oxidation decreases, as expected for mediated oxidative reactions. These results confirm the electrocatalytic activity of thionine adsorbed on CB-SPE towards the reduction of BPA/laccase reaction products. The thionine–CB–SPE was thus chosen for further studies.

3.3. Electrochemical characterization of thionine–CB–SPE

Then the thionine–CB–SPE was electrochemically characterized. The current of the anodic (□) and cathodic (■) peaks increases linearly with increasing scan rate up to 200 mV/s (Fig. 4), demonstrating that there is a surface controlled electrochemical reaction. In fact, for thin films and low scan rates (if finite diffusion occurs within the film), thin-layer behaviour predominates, and the peak

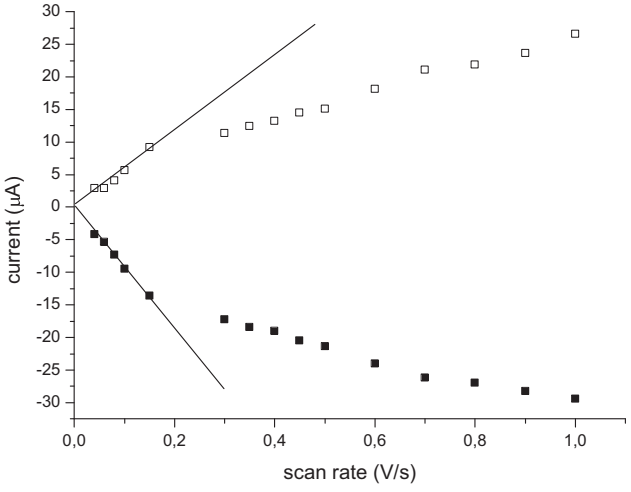

Fig. 4. Electric current as a function of scan rate.

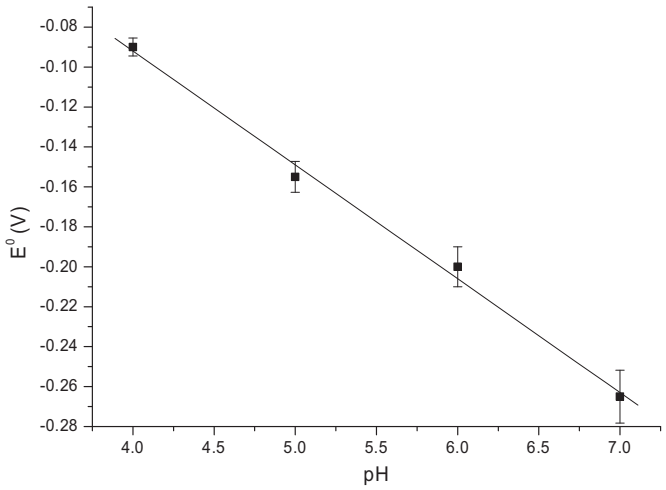

Fig. 5. Formal redox potential as a function of pH.

current (I_p) is proportional to the scan rate υ according to the following equation:

$$I_p = \left(\frac{n^2F^2}{4RT}\right)\upsilon A_\Gamma$$

where n is the number of the electrons involved in the redox reaction, F is the Faraday constant, R is the gas constant, T is the absolute temperature, A is the surface of the working electrode assuming that its surface corresponds to the geometrical area and Γ is surface coverage. At high-scan rates it can be observed that the anodic and cathodic peak currents deviate from a linear trend.

The effect of pH on the redox reaction of thionine adsorbed on the CB-SPEs was also evaluated as reported in Fig. 5. The thionine resulted to be electroactive when it is working in the pH range from 4.0 to 7.0 (Britton–Robinson buffer). The relationship between E° (V) and pH is described by the equation E° (V) = (-0.057 ± 0.003) pH + (0.136 ± 0.016) (R = 0.997). The slope value is equal to (-57 ± 3) mV/pH and it is in good agreement with the theoretical value of −59 mV/pH (at 20 °C) for a reversible electron transfer process coupled with identical number of protons and electrons [46]. In order to evaluate the number of electrons involved, the peak width at half height of the cathodic peak (Fig. 3c) was measured and resulted equal to of 92 mV [47], thus means that the redox reaction of thionine involves a transfer of one electron coupled to the transfer of one proton.

3.4. SEM micrographs

In order to understand the morphological changes due to different steps in electrode preparation (see Fig. 2a), SEM images of bare SPE (Fig. 6a), CB-SPE (Fig. 6b), thionine modified CB-SPE (Fig. 6c) and laccase biosensor based on thionine modified CB-SPE (Fig. 6d) are reported. The comparison between Fig. 6a and b shows that the presence of CB is well evidenced; the nanostructure material confers to electrode surface a rough topography, while the presence of the thionine layer on the CB-SPE causes only a different contrast in respect to the SPE-CB SEM image. A relevant surface change was observed in Fig. 6d when Nafion membrane is present on the enzymatic electrode.

3.5. Laccase-CB-SPE biosensor

In order to develop a laccase based CB-SPE biosensor characterized by satisfactory sensitivity, several parameters affecting the analytical performances of the biosensor were investigated and optimized.

3.5.1. Effect of enzyme loading

The amount of enzyme immobilized onto the electrode surface is a key parameter to obtain a high sensitivity of the biosensor. In detail we investigated the electrochemical response of the

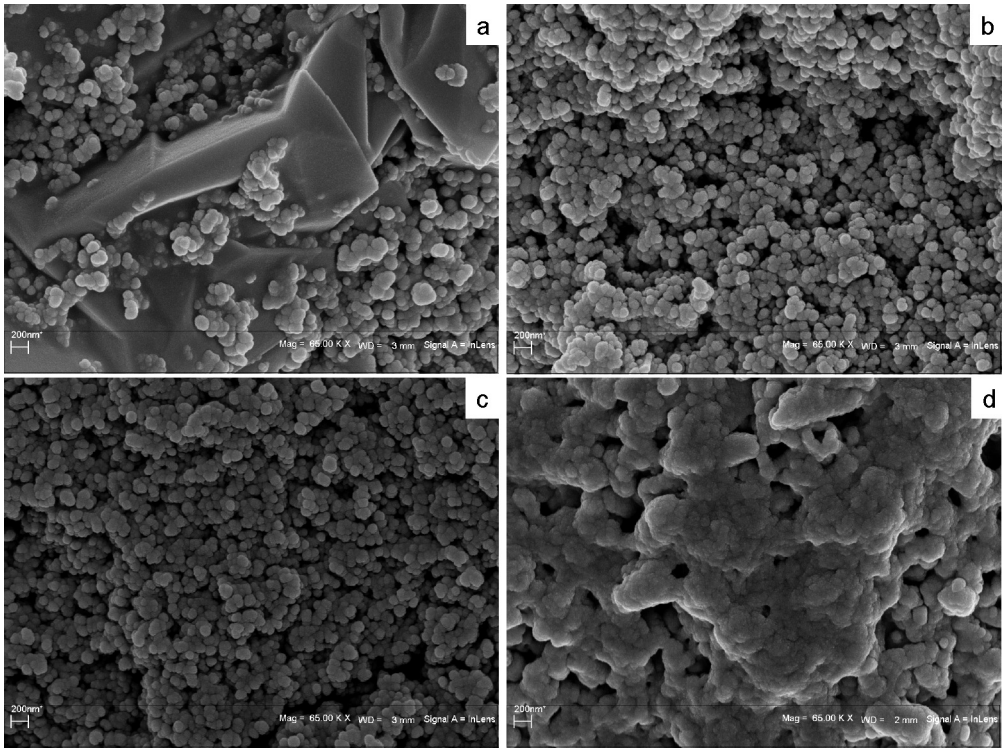

Fig. 6. SEM images of: bare SPE (a); CB-SPE (b); CB-SPE modified with thionine (c); laccase biosensor based on SPE modified with CB and thionine and Nafion (d).

electrode as a function of the amount of immobilized enzyme (0.59 U, 1.19 U, 2.36 U, 4.74 U and 6.52 U of laccase at a potential of −200 mV in 0.05 M citrate buffer, pH 4.5). As shown in Fig. 7a, the electric response increases with the amount of immobilized laccase, reaching a maximum value when using 1.19 U of enzyme. For greater amounts, there is a significant decrease in the current value. This behaviour could be attributed to the increase of film thickness, leading to an increase of interfacial electron transfer resistance, making the electron transfer more difficult [48]. For this reason, subsequent experiments were carried out using 1.19 U of laccase immobilized on the working electrode covered by a layer of Nafion to avoid the leakage of the enzyme during the measurement.

3.5.2. *Effect of applied potential*

In order to obtain a high sensitivity in the electrochemical detection of enzymatic product, the biosensor response towards BPA has been studied as a function of the applied potential in 0.05 M citrate buffer, pH 4.5. We investigated the biosensor response varying the applied potential in the range between −300 and −100 mV vs internal Ag pseudo-reference electrode. As evidenced in Fig. 7b, the highest current peak is obtained at the potential of −200 mV, so this working potential has been applied in the successive amperometric measurements.

3.6. *Effect of pH*

It is well known the influence of pH on the biosensor response, thus this influence on the biosensor response to BPA at a concentration of 10 μM has been studied in a pH range between 3 and 5.8 in 0.05 M citrate buffer. The results are reported in Fig. 7c, showing that current values are pH dependent and that the optimum pH occurs at 4.5. For lower or higher pH values, a current decrease occurs, probably due to loss or inactivation of the enzyme activity. These results are in agreement with the optimum pH range of 3.5–5 reported for free laccase [24,49] and indicate that the immobilization procedure did not alter the laccase activity. Once established that the optimum value of the peak current occurs at pH 4.5, the experiments henceforward were carried out under these conditions.

3.7. *BPA concentration determination*

Once optimized the biosensor response in terms of enzyme loading (1.19 U), pH (0.05 M citrate buffer at pH 4.5) and applied potential (−200 mV), the signal current was studied as a function of the BPA concentration (Fig. 8).

In the inset (a) the amperometric signal obtained with BPA at a concentration equal to 20 μM is reported and it shows the result of two successive additions of BPA standard.

As can be seen from Fig. 8, the electrical response as a function of BPA concentration is similar to a Michaelis–Menten behaviour, with a $K_m^{app} = 161 \pm 18$ μM and $I_{max} = 1073 \pm 57$ nA. The K_m^{app} value is similar to K_m biochemical one reported in [50] and obtained from experiment with BPA and free laccase.

Linearity was obtained in a BPA concentration range of 0.5–50 μM, while the sensitivity was 5.0 ± 0.1 nA/μM (see inset (b)). The limit-of-detection (LOD) was equal to 0.2 μM. The LOD has been calculated as ratio signal/noise (S/N) = 3. The intra-electrode repeatability was around the 3% (n = 5), while the inter-electrode reproducibility was around 5%. Concerning the stability, the electrode had an operational stability of about two weeks.

The LOD obtained is comparable with the one (0.15 μM) obtained in our previous study in which a tyrosinase–thionine carbon paste electrode was developed, but better than the one (23 μM) found by Dempsey et al. [10] using tyrosinase-thionine glassy carbon electrode. Moreover, in our case there is the advantage of

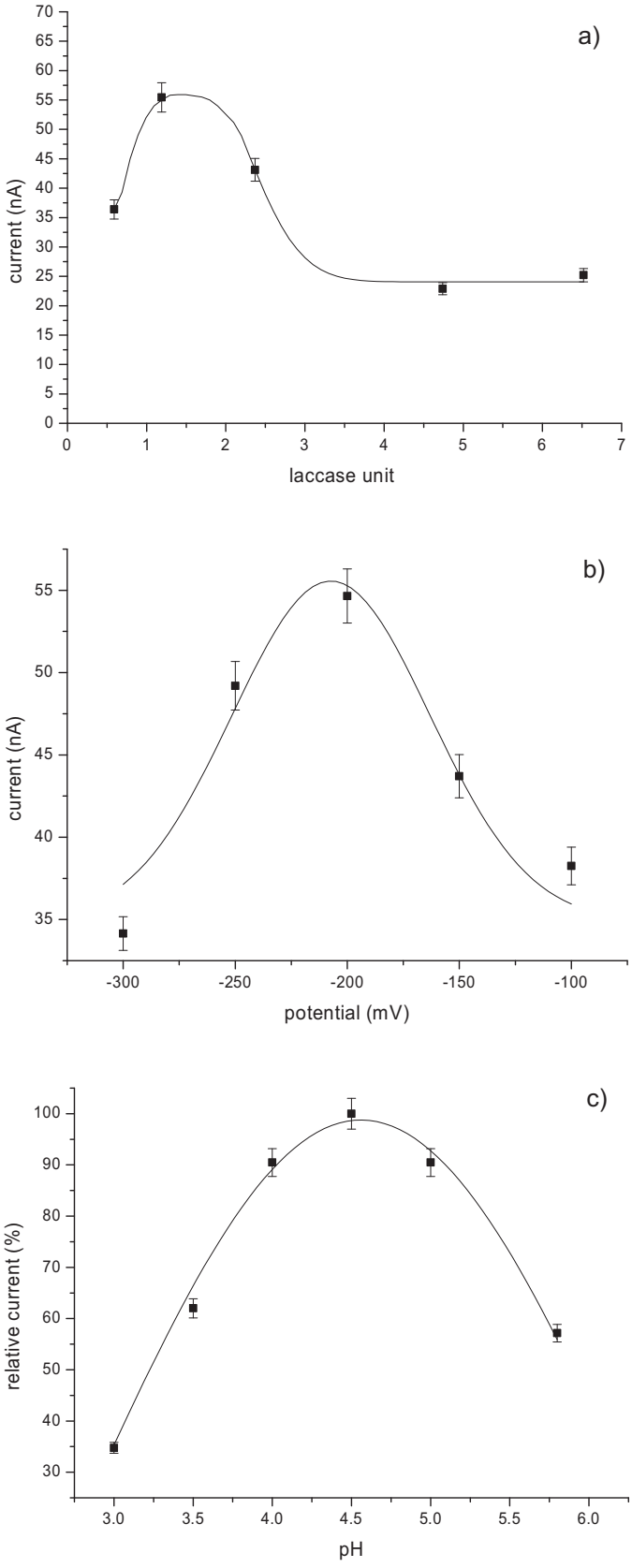

Fig. 7. (a) Effect of enzyme units on the biosensor response in presence of 10 μM BPA in 0.05 M citrate buffer, pH 4.5, applied potential equal to −200 mV, T = 25 °C. (b) Dependence of biosensor response on the applied potential in the presence of 10 μM BPA in 0.05 M citrate buffer, pH 4.5, T = 25 °C and 1.19 U laccase. (c) Effect of pH on the biosensor response in presence of 10 μM BPA in 0.05 M citrate buffer, applied potential equal to −200 mV, T = 25 °C and 1.19 U laccase.

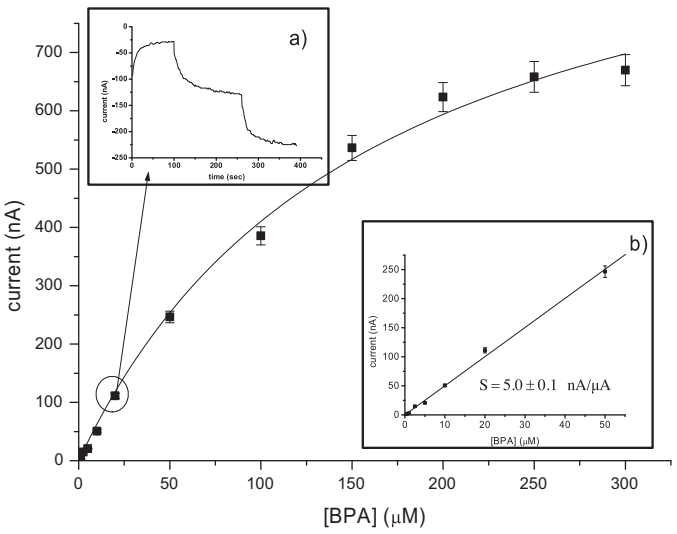

Fig. 8. Dependence of biosensor response on the concentration of BPA in 0.05 M citrate buffer, pH 4.5, applied potential of −200 mV, 1.19 U of laccase. Inset: (a) amperometric signal obtained with two successive addition of BPA standard, of 20 μM; (b) linear range for biosensor response.

Table 1
Recovery studies of spiked tomato juice samples.

Sample	BPA concentration added (μM)	BPA concentration found (μM)	Recovery (%)
A	10	12	120
A	20	18.5	92
B	10	9	90
B	20	19	95

Experimental conditions: 0.05 M citrate buffer, pH 4.5, applied potential of −200 mV, 1.19 U of laccase. All the values are mean of triplicate measurements.

a cost-effective biosensor, because we have used (i) disposable, cost-effective and miniaturized screen-printed electrode instead of carbon paste or glassy carbon electrodes (ii) the carbon black, which is a cost-effective nanostructured material and (iii) the laccase enzyme, which is cost-effective than the tyrosinase enzyme.

3.8. BPA measurements in real samples

To challenge the biosensor with real samples, the biosensor was then tested using tomato juice coming from metal cans coated with epoxic resins, which are known to contain and leak BPA. Two different commercial brands were investigated. The juices were properly treated [51] and each sample (A and B) was examined with our biosensor using the procedure previously described. No appreciable current signals were obtained, according to the circumstance that the same samples analyzed with HPLC gave a BPA concentration smaller than the value of our LOD. To evaluate the accuracy of our biosensor, the samples were spiked with known amount of BPA. Two different concentrations of BPA were used for each sample and Table 1 shows the obtained results. A recovery varying between 92% and 120% was obtained. The relative standard deviation (RSD) of three successive measurements of the same sample was around 5%. These results suggest a good recovery values and a low matrix effect on the biosensor samples.

4. Conclusion

In this paper it has been reported, for the first time to our knowledge, the design and development of a biosensor based on a bionanocomposite laccase–thionine–carbon black for BPA detection.

In particular the interaction with laccase/BPA reaction products by thionine adsorbed on screen-printed electrodes modified with nanostructured carbon black was investigated. The thionine seems to be an effective electrochemical mediator towards the BPA detection in both tyrosinase [10,13] and laccase based biosensors.

The response of the biosensor was optimized in terms of enzyme loading, pH and applied potential reaching a detection limit of micromolars level.

The biosensor proposed here shows better results when compared with laccase carbon paste biosensor [52] and tyrosinase–thionine glassy carbon electrode [10]. The coupling of thionine with nanostructured materials (carbon black in our case) thus allows the development of a stable, miniaturized and low cost device for BPA detection.

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