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Bismuth nanoparticles for phenolic compounds biosensing application

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ABSTRACT

The rapid determination of trace phenolic compounds is of great importance for evaluating the total toxicity of contaminated water samples. Nowadays, electrochemical tyrosinase (Tyr) based biosensors constitute a promising technology for the *in situ* monitoring of phenolic compounds because of their advantages such as high selectivity, low production cost, promising response speed, potential for miniaturization, simple instrumentation and easy automatization. A mediator-free amperometric biosensor for phenolic compounds detection based on the combination of bismuth nanoparticles (BiNPs) and Tyr for phenol detections will be hereby reported. This is achieved through the integration of BiNPs/Tyr onto the working electrode of a screen printed electrode (SPE) by using glutaraldehyde as a cross-linking agent. BiNPs/Tyr biosensor is evaluated by amperometric measurements at −200 mV DC and a linear range of up to 71 μM and 100 μM and a correlation coefficient of 0.995 and 0.996 for phenol and catechol, respectively. The very low DC working potential ensures the avoidance of interferences making this biosensor an advantageous device for real sample applications. In addition, the response mechanism including the effect of BiNPs based on electrochemical studies and optical characterizations will be also discussed. The obtained results may open the way to many other BiNPs applications in the biosensing field.

1. Introduction

The determination of trace phenol compounds released into the ground and surface water is of special importance. Phenolic compounds are widely used in petrochemical products and in wood preservatives, textiles, plastics, dyes, paper, herbicides and pesticides (Shan et al., 2009; Lu et al., 2010). Most of them can be highly toxic and many health problems are related to them, as they could be absorbed through oral, dermal, or respiratory tracts (Shan et al., 2009; Apetrei et al., 2011). The development of methods to identify and quantify phenolic compounds in various samples to evaluate their total toxicity in environmental and human health problems is of great relevance. Therefore, numerous analytical methodologies for detecting phenolic compounds, such as, ultraviolet spectrophotometric analyses, gas chromatography, liquid chromatography, or capillary electrophoresis have been developed. These methods offer good limits of detection (LODs) and wide working concentration ranges. However, they need the use of sophisticated and relatively costly apparatus and they generally require complicated pretreatment procedures. Recently, large efforts have been focused on the development of simple and effective analytical methods for the determination of phenolic compounds (Tsai and Chiu, 2007; Lu et al., 2010).

Nowadays, electrochemical enzyme-based biosensors constitute promising technology for the *in situ* monitoring of phenolic compounds because of the advantages that they present, such as high selectivity, low production cost, promising response speed, potential for miniaturization, simple instrumentation and easy automation (Apetrei et al., 2011; Lu et al., 2010). Among the developed electrochemical methods, amperometric biosensors based on tyrosinase (Tyr) and polyphenol oxidase (PPO) have proved to be sensitive and convenient for the determination of phenolic compounds (Song et al., 2011; Tsai and Chiu, 2007; Shan et al., 2009). In particular, tyrosinase (Try) enzyme catalyzes the hydroxylation of monophenols to o-diphenols (monophenolase activity) by oxygen, and also catalyzes the oxidation of o-diphenols to o-quinones (catecholase activity). O-quinones can be later electrochemically reduced to catechol without any mediator on the electrode surface. The detection of phenol derivates thus relies on monitoring the catechol product (Tsai and Chiu, 2007; Apetrei et al., 2011; Cosnier and Popescu, 1996). The effective immobilization of tyrosinase on the electrode surface is considered a key step in the development of tyrosinase biosensors for phenolic compounds determination (Lu et al., 2010). More recently, various supporting materials have been successfully used to immobilize tyrosinase (Tyr) on the electrode. In addition, there are several related works reporting the development of

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biosensors where tyrosinase is immobilized onto the screen printed electrode (SPE) (Song et al.,2011) that can be implemented in portable systems as an alternative detection method for the direct on-site analysis.

Nanomaterials have also been used to fabricate tyrosinase-based biosensor, especially nanoparticles, such as ZnO nanoparticles (Li et al., 2006), calcium carbonate nanoparticles (Shan et al., 2007), gold nanoparticles (Song et al., 2011; Pingarrón et al., 2008), or hydroxyapatite nanoparticles (Lu et al., 2010). On the other hand bismuth (Merkoçi et al., 2010), and bismuth oxide (Shan et al., 2009) films have recently been reported for the detection of phenolic compounds by using amperometric biosensors. These materials applied to electroanalysis have opened a new range of possibilities for the construction of phenolic electrochemical biosensors based on these nanomaterials.

In addition to its regular size bismuth nanoparticules (BiNP) shows other advantages such as a larger specific surface area and biocompatibility. These properties provide an excellent platform for enzyme and protein immobilizations.

In the current study, we propose the development of an amperometric BiNP/Tyr-based biosensor. This is achieved through BiNP/Tyr integration onto the working electrode of a screen printed electrode (SPE) by using glutaraldehyde as a cross-linking agent. The resulting BiNP/Tyr-based biosensor exhibited high sensitive response toward phenol and catechol detection with very low detection limits (26 nM for catechol and 62 nM for phenol) and showing a linear response up to 100 μM and 71 μM for catechol and phenol, respectively.

2. Materials and methods

2.1. Apparatus and reagents

Electrochemical measurements were performed with a model CH-Instrument potentiostat 660 A electrochemical workstation from CH Instruments Inc., Austin, TX. Impedance analysis of the prepared SPE modified electrodes was performed by using an Autolab302 potentiostat/galvanostat/frequency-response analyzer PGST30, controlled by GPES/FRA Version 4.9. Magnetic stirrer was used to provide the convective transport during the amperometric measurements. Scanning electron microscope (SEM) analysis were performed by using a EVO (Carl Zeiss NTS GmbH. Germany). Transmission electron microscope (TEM) images were taken with a JEM-2011 (Jeol, Ltd., Japan). X-ray powder diffraction patterns were collected on a Siemens D-5000 diffactometer (German) by using Cu Kα radiation. Patterns were recorded over the 2θ range 20–65° at 40 kV and 40 mA. Z-Potential measurements were made with a Malvern ZetaSizer Nano ZS instrument operating at a light source wavelength of 532 nm and a fixed scattering angle of 173° for detection.

Glutaraldehyde (at 25%), tyrosinase from mushroom ($\geq$ 1000 unit/mg), phenol, potassium dihydrogen phosphate, potasium monohydrogen phosphate dehydrate and potassium chloride were purchased from Sigma Aldrich. For the synthesis of bismuth nanoparticles, bismuth (III) nitrate pentahydrate (reagent grade, 98%) from Aldrich was used. Potassium hydroxide, ACS-ISO-For analysis and isopropanol (synthesis grade) were obtained by Carlo Ebra reagents. Poly (vinyl pirrolidone) (PVP, MW$\sim$30.000) was acquired from Aldrich. J. T. Baker Chemicals B. V. supplies ethylene glycol. All solutions were prepared with ultra-pure water from a Millipore-MilliQ system.

2.2. Synthesis of bismuth nanoparticles

One-step bismuth (III) nitrate reduction method has been used in order to obtain bismuth nanoparticles (Wang and Kim, 2008). In this synthesis, 0.1 g of $Bi(NO_3)_3$, 0.19 g KOH and 0.05 g of Poly(vinyl pirrolidone) (PVP, MW $\approx$ 30.000) were dissolved in 25 mL of ethylene glycol (EG). The mixture was heated up to 185 °C and kept under nitrogen atmosphere and constant stirring during 2 h. The obtained nanoparticles were cleaned with isopropanol and milli-Q water by centrifugation. The obtained nanoparticles were dried by a nitrogen airflow. These nanoparticles can also be partially dispersed in water and ethanol.

2.3. Preparation of Screen Printed Electrode (SPE) and modification with BiNP and Tyr

Screen printing electrodes fabrication is based on the sequential deposition of a graphite ink, Ag/AgCl ink and insulating ink on a polyester substrate. After the deposition of each layer, a drying process is followed by keeping the polyester substrate at 120 °C for 45 min (graphite) and 30 min (Ag/AgCl and insulating). A 5 μL BiNP at 1 mg/ml solution drop was deposited onto the working SPE electrode surface and allowed to dry at room temperature for 20 min. 1 mg of tyrosinase (Tyr) enzyme was dissolved in 50 μL of 0.1 M phosphate buffer at pH 6.5. A 7 μL drop of Tyr solution was deposited onto the working SPE/BiNP electrode surface and allowed to dry at room temperature for 3 h. Finally, 5 μL drop of glutaraldehyde (Glu) solution at 1% was casted onto the SPE/BiNP/Tyr electrode surface and let to dry at 40 °C for 30 min. The prepared SPE/BiNP/Tyr/Glu sensor was kept at 4 °C.

2.4. O-quinone synthesis

As to determine phenol detection mechanism o-quinone was synthesized following the method described by Laird et al. (Aebisher et al., 2007). A solution composed of catechol (0.1 g), acetone (10mL) and silver oxide (0.4 g) was stirred during 10 min. The obtained solution was immediately filtered and dried. To purify this product it was dissolved in water and extracted again by using chloroform. After the extraction, the product is dried under vacuum.

2.5. Electrochemical experiments

All electrochemical experiments were carried out at room temperature. In order to study the characteristics of the BiNPs/Tyr modified electrodes, electrochemical impedance spectroscopy (EIS) studies were performed in 1 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) mixture with KCl 0.1 M as redox probe. The AC frequency range studied was from 0.1 Hz to 100 kHz, with logarithmic scale of 10 points per decade and an applied potential of 50 mV of AC Potential. Nyquist (imaginary impedance versus real impedance) diagrams were also recorded. CV measurements were carried out at the potential range of -0.8 to 0.8 V vs. Ag/AgCl with an scan rate of 50 mv/s. All electrochemical experiments were carried out in 0.1 M phosphate buffer (PB) at pH 6.5 with 0.1 M KCl.

3. Results and discussion

3.1. Morphological studies

A homogeneous distribution of the BiNP on the electrodes surface can be observed by Scanning Electron Microscopy (SEM) image (see Fig. 1A). Morphological and structural studies of bismuth nanoparticles were performed by Transmission Electron Microscopy (TEM). Highly regular and uniform average diameter of around 244 nm (see Fig. 1B) can be measured.

Z-potential (surface charge) measurements are a commonly used tool to determine the stability of a colloidal suspension of

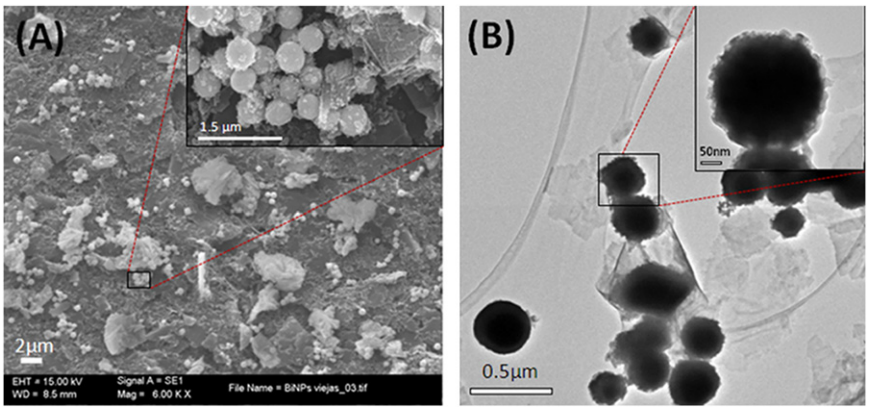

Fig. 1. SEM micrographs of BiNP modified SPE (A). TEM image of BiNP dispersed in milli-Q water.

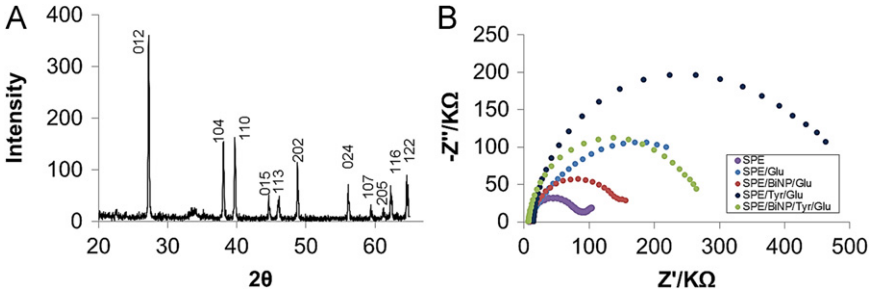

Fig. 2. (A) X-ray diffraction pattern recorded from the synthesized BiNP. (B) Argand diagram of: bare SPE, SPE/Glu, SPE/BiNP/Glu and SPE/BiNP/Tyr/Glu modified electrodes in 1 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) containing 0.1 M KCl.

electrostatically stabilized nanoparticles. Dynamic light scattering (DLS) allows the determination of the hydrodynamic diameter of colloidal particles and conjugates, which is the diameter of the sphere with the same Brownian motion of the analyzed particle. According to the DLS measurements, particles with 460 nm of hydrodynamic range with high homogeneous sizes have been synthetized, although z-potential analysis shows poor electrostatic stabilization of this nanoparticles in water medium. If we compare the values obtained by TEM and by Z-potential studies, BiNP diameters are similar.

The crystalline structure and the phase composition of bismuth nanoparticles were characterized by X-ray diffraction (XRD). Fig. 2A shows the typical XRD patterns of the bismuth nanoparticle powder synthesized. All diffraction peaks can be perfectly indexed to the rhombohedral phase of bismuth (Bi°), which is consistent with the already reported phase for bismuth nanopowders (López-Salinas et al., 2010). Phase identification was performed by comparing the obtained XRD with the standard data facilitated by Joint Committee on Powder Diffraction Standards (JCPDS no. 04-006-7762, R3m).

3.2. EIS characterization of bismuth nanoparticles SPE/BiNP/Tyr biosensor

Electrochemical impedance spectroscopy (EIS) is a well known method used to study the surface features of modified electrodes. The curve of EIS presented as Nyquist plot consists in two parts: one part is the semicircle section, located at higher frequencies corresponding to the electron transfer limited process. The electron-transfer resistance (Rct) can be obtained by measuring its diameter. The other part of Nyquist plot is the linear section, which brings the information related to the diffusion process held in solution and located at lower frequencies. This second section is used as to analyze the detailed electrochemical response of the modified electrodes by using individual or mixed Nyquist components. EIS of bare SPE (with and without glutaraldehyde), and SPE/BiNP, SPE/Tyr and SPE/BiNP/Tyr, all surfaces modified with glutaraldehyde were firstly evaluated in a 1 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) with 0.1 M KCl as a redox probe by using (EIS) as to confirm the BiNP/Tyr immobilization (see Fig. 2B). The Rct of the glutaraldehyde modified SPE increased in comparison with the bare SPE, which indicates that glutaraldehyde film was an obstacle for the electron transfer at the interface region. The result is consistent with the already reported ones by using similar systems (Alarcón et al., 2010; Mayorga-Martinez et al., 2012). With BiNP assembled within the glutaraldehyde film, the obtained Rct decreased. The lower charge transfer resistance is probably due to the faster ion diffusion/migration in the BiNP/Glu film in comparison with the glutaraldehyde film only. On the other hand, when the enzyme was immobilized through glutaraldehyde the charge transfer resistance increased, which not only indicates the efficient enzyme absorption but at the same time that both materials hinder the electron transfer. Finally it can be observed that the adsorption of Tyrosinase onto BiNP is related to the decrease of the semicircle, indicating that Tyrosinase had been successfully immobilized onto de SPE modified with BiNP.

3.3. Evaluation of the electrochemical sensing capability of SPE/BiNP/Tyr/Glu biosensor.

Fig. 3B depicts the chrono-amperometric responses for the different enzyme modified sensors, and using 0.1 M phosphate buffer (PB) at pH 6.5 with 0.1 M KCl upon successive addition of 2 µM phenol. The SPE/BiNP/Tyr biosensor showed a higher current change than the SPE/Tyr one (Fig. 3B). The observed reduction current was attributed to the direct reduction of o-quinone to cathecol, released from the enzyme-catalyzed reaction on the

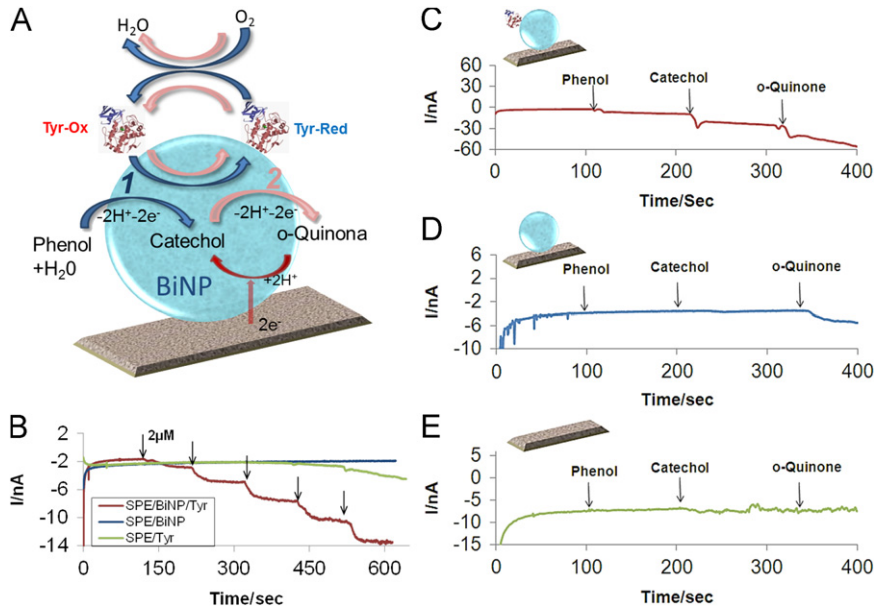

Fig. 3. Proposed mechanism for the phenol and catechol electrocatalytic detection by using BiNP based biosensor (A). Current–time response curves of bare SPE, SPE/BiNP and SPE/BiNP/Tyr modified electrodes upon successive additions of 2 μM phenol (B). Chronoamperometric responses of SPE/BiNP/Tyr (C), SPE/BiNP (D), SPE (E) biosensors upon successive additions of 10 μM Phenol, catechol and o-quinone. All the experiments were carried at −200 mV, without nitrogen saturation.

electrode surface (see Fig. 3A). Only a background current was observed in the SPE/Glu sensor.

Tyrosinase (Tyr) has hydroxylase activity, by which phenol can be hydroxylated to catechol using molecular oxygen (Eq. 1), and also oxidase activity that can catalyze the oxidation of catechol to o-quinone (Eq. 2). At moderately negative potential (−200 mV) the o-quinone product of phenol oxidation may be electrochemically reduced to catechol (Eq. 3). Oxidation process by the enzyme, followed by the reduction at the electrode surface, may result in cycling between catechol and o-quinone yielding a catalytically amplified current. Therefore, what is occurring at the electrode surface is the reduction of o-quinone to catechol (working electrode is acting as a cathode) (Guix et al., 2010).

$$\text{Phenol} + \text{Tyr}(ox) + \text{H}_2\text{O} \rightarrow \text{Catechol} + Tyr\ (red) + 2\text{H}^+ + 2\text{e}^- \quad (1)$$

$$\text{Catechol} + \text{Tyr}(ox) \rightarrow \text{o-quinone} + Tyr\ (red) + 2\text{H}^+ + 2\text{e}^- \quad (2)$$

$$o\text{-quinone} + 2\text{e}^- + 2\text{H}^+ \xrightarrow{-200\ mV} 200\ mV\ \text{catechol} \quad (3)$$

For a better elucidation of the phenolic compounds detection mechanism (Fig. 3A), amperometric measurements were carried out. Measurements were performed under different experimental conditions. The first one (Fig. 3C) was carried out using a SPE/BiNP/Tyr, while the second case (Fig. 3D) corresponded to SPE sensor modified only with BiNP. Finally, a bare SPE was also checked (Fig. 3E). The current responses of each electrode toward 10 μM phenol, 10 μM catechol and 10μM o-quinone (synthesized in our laboratory by the method already described in the materials and methods section) additions were recorded. A decrease of current of 1.72 and 15.4 nA was recorded for SPE/BiNP and SPE/BiNP/Tyr, respectively. This behavior shows an appropriate current response to o-quinone (see Fig. 3C and D). In addition, the mentioned current response in both electrodes may be attributed to the presence of BiNP in the surface, which seems to bring an electron transfer kinetic enhancement in the reduction current of o-quinone.

SPE/BiNP/Tyr biosensor is the only one which exhibits response to phenol and catechol addition, showing a current increase of 4.34 and 12.43 nA, respectively (see Fig. 3C). This sensor can detect phenolic compounds because of the presence of both tyrosinase and BiNP. Using this sensor the following reactions can be coupled: phenol hydroxilation to catechol occurs first, followed by the catechol oxidation to o-quinone and o-quinone reduction to catechol due to the presence of BiNP. SPE modified only with glutaraldehyde (Fig. 3E) doesn't show any response toward either phenol, catechol or o-quinone.

3.4. SPE/BiNP/Tyr biosensor calibration and evaluation of interferences

The amperometric response of the SPE/BiNP/Tyr to successive additions of phenol and catechol were further evaluated under the optimized experimental conditions (data not shown). Fig. 4A and B show the typical current–time dynamic responses of the SPE/BiNP/Tyr towards phenol and catechol, respectively. The electrode showed a rapid and sensitive bioelectrocatalytic response, reaching about 95% of the steady-state current within 15 and 50 s after each addition of catechol and phenol, respectively. The different response times are due to the fact that phenol has to be firstly hydroxilated to catechol, which in turn needs then to be oxidized to o-quinone and this last one to be reduced afterwards to catechol in presence of BiNP (Fig. 4A). In the case of catechol detection the first step is omitted (Fig. 4B).

Fig. 4C and D show the typical calibration curves of the SPE/BiNP/Tyr toward phenol and catechol, respectively. The biosensing performance present two linear response ranges at low and high concentrations for both phenolic compounds. The phenol detection shows a linear response range from 0.2 to 1 μM (r^2=0.974) and 3–71 μM (r^2=0.995) for low concentration and high concentration ranges, respectively. While for catechol detection the linear response ranges are from 0.5 to 2.5 μM for low concentration and 3–100 μM for the high concentration range, both with a similar linearity. On the other hand SPE/BiNP/Tyr biosensor shows LOD of 26 nM and 62 nM for catechol and phenol, respectively. The sensitivities in the linear calibration regions for low concentration shows the following order: 44.056 nA/μM (catechol) > 14.025 nA/μM (phenol). The difference in sensitivity might depend on the catalytic selectivity of tyrosinase for different phenolic compounds (Lu et al., 2010). Moreover, the Michaelis–Menten constants (K_M^{app}) obtained for

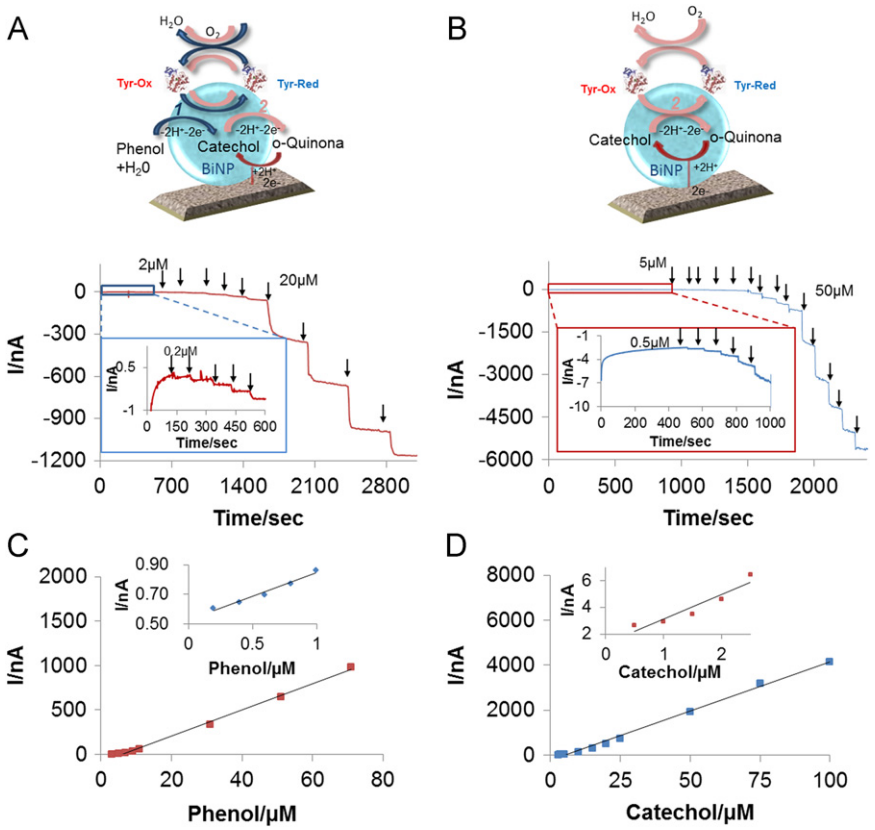

Fig. 4. Typical current–time response curves for the successive additions of 0.2, 2 and 20 μM of phenol (A) and 0.5, 5 and 50 μM of catechol (B); inset: magnification of the initial steps. Sensor calibration given as current versus phenol (B) and catechol (D) concentration; inset: magnification at low concentration range for phenol and catechol.

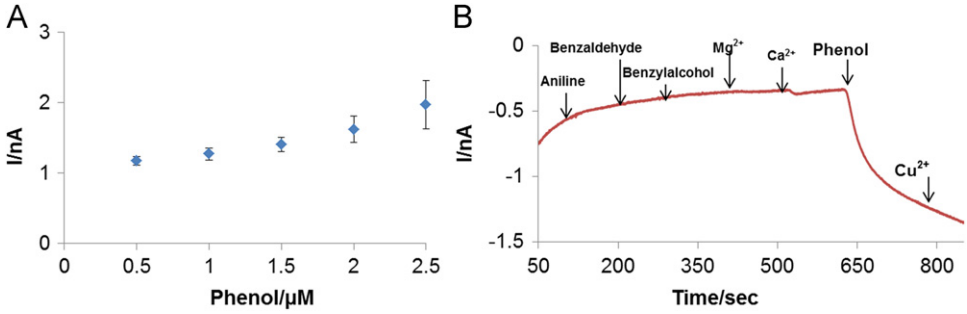

Fig. 5. Triplicate sets, indicated by error bars, of the measurements with a relative standard deviation (RSD) less than 15% for 0.5–2.5 μM phenol concentration (A). SPE/BiNP/Tyr biosensor response upon successive additions of 50 μM aniline, benzaldehyde, benzylalcohol, Mg^{2+}, Ca^{2+}, 5 μM of phenol and 25 μM Cu^{+2} (B).

phenol and catechol are 0.063 mM and 0.083 mM, respectively. The lower K_M^{app} is estimated from the data obtained from the amperometric response, indicating that the immobilized tyrosinase is strongly adsorbed onto the working electrode surface of the SPE modified with BiNPs, and a good affinity of the enzyme for phenol and catechol is shown.

The lower K_M^{app} value may be related to an efficient recycling process of the substrate process, by electrochemically reducing the o-quinone to catechol, which serves as tyrosinase substrate (Shan et al., 2009).

Analytical performance of the proposed biosensor was compared with other reported tyrosinase biosensors based in SPE. The proposed SPE/BiNP/Tyr biosensor exhibited improved analytical performances in terms of linear range of response and limit of detection in comparison with other reported biosensors, such as SPE/MWCNT/Bi/Tissue (Merkoçi et al. 2010), SPE/MWCNT/Tyr/Glu (Alarcón et al., 2010) and Tyr–Au/PASE–GO/SPE (Song et al. 2011). All these results indicate that the developed BiNP based electrochemical biosensor is an excellent candidate for phenol and catechol detection.

The reproducibility of the fabrication process was evaluated by the comparison of the current responses obtained for 0.5 μM phenol using 3 different biosensors. The results of the triplicate sets, indicated by error bars, reveal the repeatability and reproducibility (Fig. 5A) of the measurements with a relative standard deviation (RSD) of less than 15% within a 0.5–2.5 μM phenol concentration range.

Selectivity is a very important parameter to be considered for biosensors detecting phenol derivates. To evaluate the selectivity of the biosensor, chrono-amperometric response toward aniline, benzaldehyde, benzylalcohol, Mg^{2+},Ca^{+2} (each one in a concentration 10 times higher than phenol concentration) and Cu^{+2} (5 times higher than phenol concentration) were measured (see Fig. 5B). The selected species for interference studies seem to be the ones usually present with the phenolic compounds in contaminated samples (Cosnier et al., 1999). A good selectivity of the

SPE/BiNP/Tyr biosensor toward phenol detection is evident by the neglected responses toward interfering species.

4. Conclusions

A novel electrochemical biosensor based on bismuth nanoparticles and tyrosinase for the determination of phenolic compounds is developed. The suggested bionanocomposite matrix provides mild and efficient immobilization process for tyrosinase and BiNPs ensuring at the same time an enhanced sensibility and fast response. These qualities may be ascribed to an efficient process of enzyme substrates recycling (electrochemical reduction of the enzymatically generated o-quinone by producing catechol, the tyrosinase substrate). It must be noted that the enzyme recycling of the phenolic compounds is dependent on the electrode matrix used. The biosensor exhibited good analytical performances to phenolic compounds in terms of sensitivity, low detection limit, reproducibility and fabrication simplicity and can be used for the accurate determination of several phenolic compounds without the need to use extra mediators. This sensor uses a very low DC potential, decreasing this way the effect of interferences such as aniline, benzaldehyde, benzylalcohol, Mg^{2+}, Ca^{2+} and Cu^{+2}. Phenol and catechol detections mechanisms associated to the designed biosensor are also evaluated.

Acknowledgments

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