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Biosensors for phenolic compounds: The catechol as a substrate model

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Abstract

The behaviour of three different laccase-based graphite biosensors was studied in view of their use in agricultural or industrial waters polluted by phenolic compounds. Catechol was used as a substrate model. Laccase from *Trametes versicolor* was immobilized on one biosensor (type A electrode) by adsorption while, on the other two biosensor types, laccase was covalently bound through the carboxylic groups created on the graphite by means of treatment with an electric potential difference (type B electrode) or with nitric acid (type C electrode). In the latter two cases, hexamethylenediamine and glutaraldehyde were used as the spacer and the coupling agent, respectively. The extension of linear response range and the sensitivity and time stability of each biosensor type were investigated. The type C biosensor gave the best results and its electrochemical properties proved comparable to those reported by other authors.

Keywords: Catechol; Biosensors; Enzyme immobilization; Graphite electrode; Phenolic compounds

1. Introduction

Phenolic compounds have been recognised as toxic substances and endocrine disruptors [1–4]. This definition has been used by the scientific community to classify certain chemicals of natural or synthetic origin which are capable of interfering with the endocrine system, modulating it or mimicking natural hormones [5]. The result of this interaction for humans and wildlife is the induction of serious pathologies such as developmental abnormalities and carcinogenesis [6,7]. For these reasons, the determination of phenolic compounds in environmental matrices, including tap and surface water, has become a matter of great concern and scientific interest. Determinations are usually carried out in centralised laboratories using liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), or capillary electrophoresis (CE). Recent research activity has focused on the design and construction of biosensors which are capable of improving the efficiency of site monitoring and can be used for the necessary remediation activities.

Tyrosinase or laccase-based enzyme electrodes [8–17] were designed for the selective determination of phenolic compounds in environmental matrices. Their functioning is based on the reductive amperometric detection of the produced quinone species [8,18,19]. The reaction can be schematized as

$$\text{Phenol} \overset{\text{Enzyme},O_2}{\longrightarrow} \text{catechol} \overset{\text{Enzyme},O_2}{\longrightarrow} o\text{-quinone} + H_2O \quad (1)$$

according to which the enzymatic *o*-hydroxylation of phenolic compounds to catechols is followed by dehydrogenation to *o*-quinones [8,16,20].

In this paper, we will discuss the functioning of three different laccase-based enzyme electrodes, obtained by immobilizing the laccase from *Trametes versicolor* on graphite electrodes via absorption or via covalent bond. Our attention was focused on the dehydrogenation process, which is the bottleneck of the electric detection system. For this reason, we used catechol as a substrate.

Sensitivities, calibration curves and stability of the three biosensors will also be compared to similar results obtained by other authors.

Our results confirm the effectiveness of laccase-based biosensors in the determination of phenolic compounds in polluted

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waters which derive from agricultural activity (including the partial degradation of phenoxy herbicides), or from the petrol chemicals and textile industries.

2. Experimental

2.1. Materials

Laccase (EC 1.10.3.2; 26.8 U mg^{-1}) from *T. versicolor* was used as a catalyst. Laccases are cuproproteins which belong to the group of blue oxidase enzymes [21,22]. Laccase is a polyphenol oxidase that catalyzes the reaction of several inorganic substances such as phenol, with concomitant reduction of oxygen to water. The reduction of oxygen to water is accompanied by the oxidation of the phenolic substrate. Laccases have four neighbouring copper atoms which are distributed among different binding sites and classified into three types: copper types 1, 2 and 3. Copper type 1 is involved in electron capture and transfer, copper type 2 activates molecular oxygen, while copper type 3 is responsible for oxygen uptake. Substrate oxidation using laccase is a one-electron reaction which generates a free radical [23].

Nitric acid, *N*,*N*′-dicycloexylcarbodiimide, hexamethylenediamine (HMDA) and glutaraldehyde (GA) were employed for the process of enzyme immobilization. In particular, HMDA and GA were used as the spacer and coupling agent, respectively, while the carbodiimide was used as activator of the carboxylic groups produced on the graphite electrode surface. Catechol was the substrate model for phenolic compounds.

Graphite rods (4 mm in diameter) were purchased from Agar Scientific (Agar Scientific Limited, 66a, Cambridge Road Stansted, Essex CM24 8DA, England). The platinum and the Ag/AgCl electrodes were purchased from Radiometer-Analytical (Radiometer-Analytical.SAS, Villeurbanne CEDEX, Lyon, France).

All chemicals, including the enzyme, were purchased from SIGMA (Sigma, Milan, Italy) and used without further purification.

2.2. Apparatus

The electrochemical cell (Fig. 1a) was a three electrode cell where the enzyme modified graphite electrode acted as a working electrode and the platinum electrode (type M241Pt) as a counter electrode. All measurements were carried out versus an Ag/AgCl reference electrode (type REF321), kept at −100 mV versus the working electrode. The potential difference was ensured by means of a low current potentiostat/galvanostat model 2059 from Amel (Amel, Milan, Italy) interfaced to a PC through a board (PCI-6221) purchased from National Instruments Corporation (National Instruments, Austin, TX, USA).

Electric current measurement was performed by means of a flow injection analysis (FIA) system, shown in Fig. 1b. A continuous flow of the carrier (the washing buffer solution: 0.1 M sodium acetate, pH 5.0; $T = 25\,^{\circ}C$) or of the mixture containing the catechol was injected through the electroanalytic cell under the control of an electrovalve from RS Components (RS Components s.p.a., Cinisello Balsamo, Milan, Italy). The injected volume was 200 μL and the electrical response, which constituted the output signal from the biosensor, was acquired using the Labview software package, purchased from the National Instrument Corporation (National Instruments, Austin, TX, USA). The software accounted for the values of the background current, which was continuously subtracted from the subsequent value of the measurement. The electrical current produced by the oxidation of the substrate by the immobilized enzyme according to

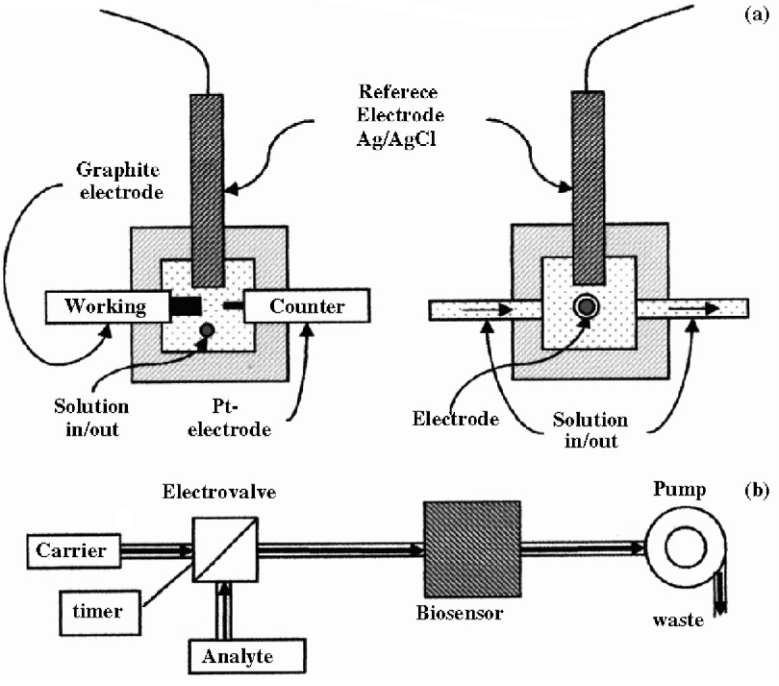
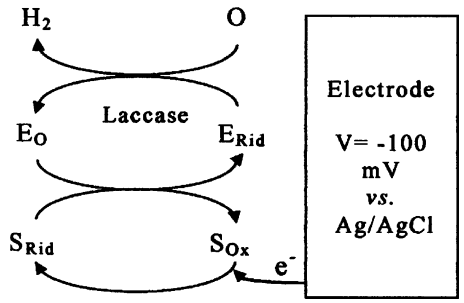


Fig. 1. (a) The electrochemical cell and (b) the FIA architecture.



Scheme 1. Electric pathway in the production of the biosensor response.

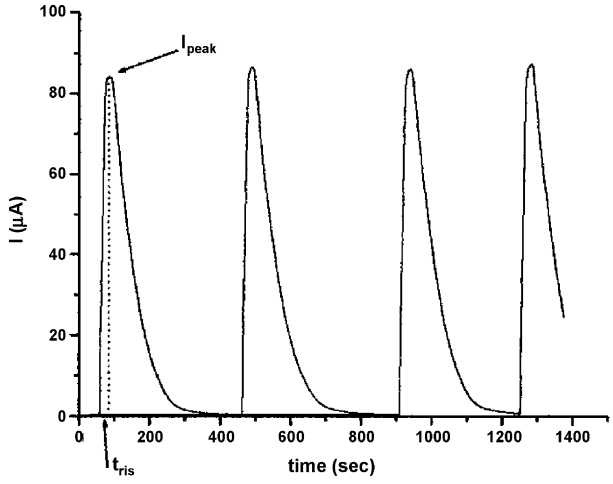


Fig. 2. A typical electrical response of the laccase-based graphite biosensors. Experimental conditions: catechol 1 mM in 0.1 M sodium acetate buffer solution, pH 5.0 and $T = 25\,^{\circ}C$, type C electrode (see in the following).

Scheme 1 is proportional to the reaction rate, which is function of the substrate concentration. A typical electrical response is illustrated in Fig. 2, where the reproducibility of the measurements and the small "response time" of the biosensor can also be appreciated.

2.3. Preparation of the laccase-based graphite electrodes

Three different electrode types, hereafter referred to using the code A, B and C, were prepared. The enzyme immobilization was carried out on the electrode A by absorption, while it was performed on electrodes B and C using a covalent bond. In the latter case, a spacer (HMDA) was used to bind the enzyme to the functional carboxylic groups induced on the graphite electrode by treatment under a potential difference (electrode B) or with nitric acid (electrode C). The enzyme immobilization phase for all electrode types was preceded by a cleaning phase of the electrode surface using gamma alumina powder, after which the electrode was washed and sonicated in 5% (v/v) ethanol aqueous solution.

The carboxylic groups on the electrode B were obtained by placing the graphite electrode for 2 h under a 3 V potential difference with respect to the Ag/AgCl electrode in the presence of a 0.1 M phosphate buffer solution, pH 7.2 and $T = 25\,^{\circ}C$. At the end of this procedure, the graphite electrode was then washed in deionized water.

The carboxylic groups on the electrode C were obtained by treating the graphite electrode for 24 h with an aqueous solution of nitric acid (20% v/v). At the end of the treatment the graphite electrode was washed in deionized water.

2.3.1. Preparation of electrode A

The cleaned electrodes were dipped for 30 min at room temperature in a glutaraldehyde (10% v/v) aqueous solution containing 2 mg/mL of enzyme. The enzymatic solution was prepared 30 min before the electrode immersion.

2.3.2. Preparation of electrodes B and C

The treated electrodes were dipped for 2 h in a 0.1 M sodium acetate buffer solution, pH 4.8 and $T = 60\,^{\circ}C$, containing 14 mg/mL of carbodiimide. At the end of this treatment, the graphite electrodes were dipped for 30 min at room tempera-

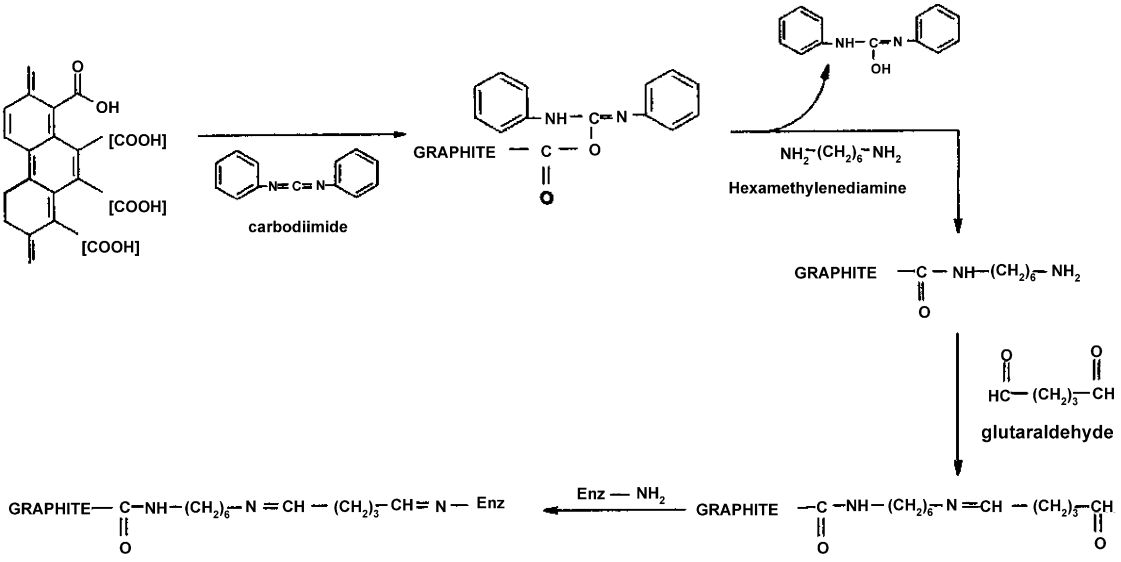


Fig. 3. Enzyme immobilization process on type B and C electrodes.

ture in an aqueous solution of HMDA (5% v/v), then washed in deionized water for 1 min. After this treatment the electrode was dipped for 30 min at room temperature in a 10% (v/v) GA aqueous solution containing 2 mg/mL of enzyme.

The whole process of enzyme immobilization is shown in Fig. 3.

2.4. Electrode stability

Electrode stability was tested daily by measuring the electrical response under standard conditions: 1 mM catechol in 0.1 M sodium acetate buffer solution, pH 5.0 and $T = 25\,^{\circ}C$. After a 2-day period, during which the response decreased by 20% with respect to the first set of measurements, fairly stable conditions were obtained. All the experiments reported in the following section were carried out under similarly stable conditions. The electrodes were discarded when the daily measure values differed by 10% from the standard reference measure. When not in use, the biosensors were stored in 0.1 M sodium acetate buffer, pH 5.0 at 4 °C.

2.5. Treatment of the experimental data

Each experimental point in the figures is the average of six independent measurements performed under the same experimental conditions. The experimental error never exceeded 4.2%.

3. Results and discussion

Since an enzyme reaction is strongly dependent on the pH of the solution, especially when the catalyst is immobilized [24–28], the biosensor response was studied at different values of the pH in the range from 3.5 to 6.5. In Fig. 4, the results of this investigation are reported as relative values of the I_{peak} as a function of pH for each of the three biosensor types. The catechol concentration was 1 mM and the temperature 25 °C. The results in the figure clearly show that: (i) the best electrical response for each of the three biosensor types occurs at pH 5.0; (ii) the width of the bell-shaped response curves depends on the enzyme immobilization method.

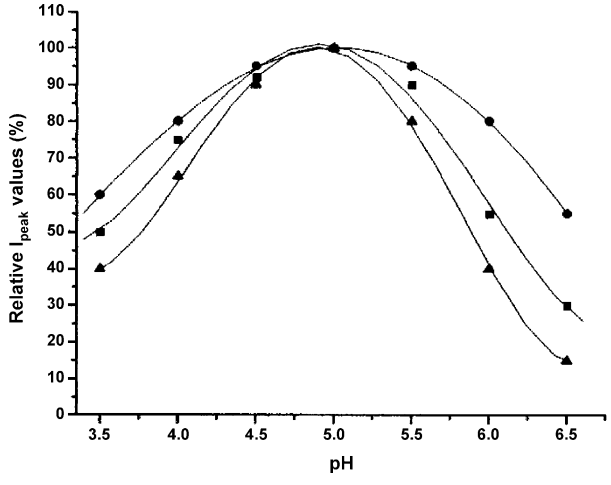


Fig. 4. Relative I_{peak} values as a function of pH. Experimental conditions: catechol 1 mM and $T = 25\,^{\circ}C$. Symbols: (■) type A electrode; (●) type B electrode; (▲) type C electrode.

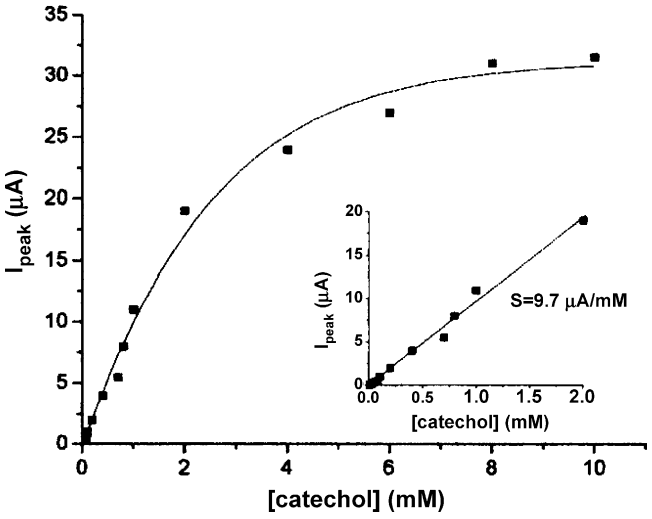


Fig. 5. Type A electrode: I_{peak} currents as a function of catechol concentration.

Once established that the optimum value of the peak current for our biosensors occurs at pH 5.0, experiments were conducted under these conditions in order to obtain calibration curves for each biosensor type. The results of the experiment are shown in Figs. 5–7, where the peak currents are reported as a function of catechol concentration. Fig. 5 refers to biosensor A, Fig. 6 to biosensor B, and Fig. 7 to biosensor C. In each of these figures, the inset represents the linear range of the electrical response, i.e. the calibration curve, of the corresponding biosensor type. The results in Figs. 5–7 clearly indicate that: (1) the electrical response for each of the three biosensor types resembles Michaelis–Menten behaviour; (2) there are remarkable differences in the extension of the calibration curves and in the sensitivities. Sensitivities are the slopes of the calibration curves.

The first observation is based on the circumstance that in each of the three figures, the following equation provides a good fit

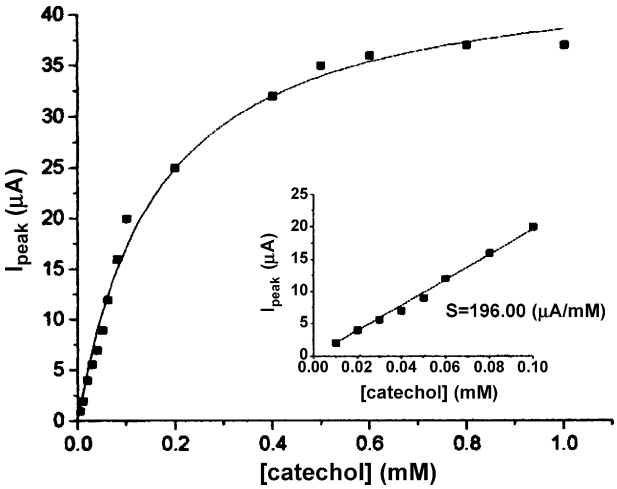


Fig. 6. Type B electrode: I_{peak} currents as a function of catechol concentration.

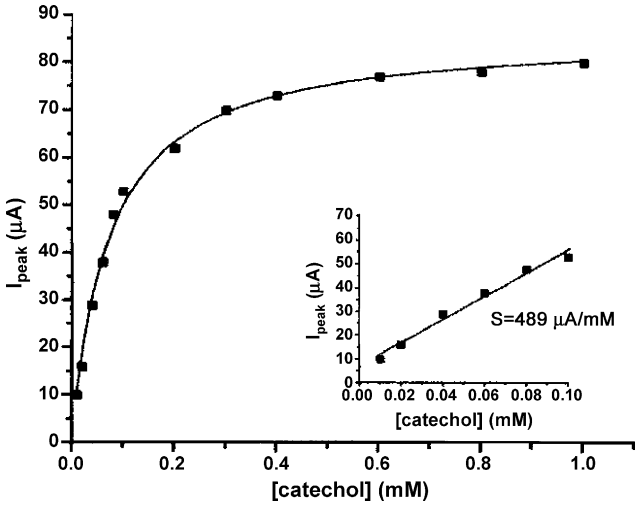


Fig. 7. Type C electrode: I_{peak} currents as a function of catechol concentration.

for the experimental points:

$$I_{peak,C} = \frac{I_{peak,max} C}{K^{app}_{m,el} + C} \quad (2)$$

where $I_{peak,C}$ is the peak current at the catechol concentration C, $I_{peak,max}$ is the peak current at saturating substrate conditions, and $K^{app}_{m,el}$ is a constant with the same meaning as the Michaelis–Menten constant K^{app}_{m}. The apex "app" was used to differentiate this value from that of the free enzyme. Indeed, when an enzyme is immobilized, the kinetic constant K_m changes owing to the diffusional limitations introduced by the graft carrier. The value of the resulting K^{app}_{m} may be higher or lower than the K_m value for the free enzyme. For example, K^{app}_{m} is greater than K_m when the carrier and the substrate have the same electrical charge, while the opposite occurs when the carrier and the substrate have opposite electrical charge.

The kinetic parameters, obtained for each biosensor type from expression (2), or from the equivalent electrochemical Eadie–Hofstee equation, which linearize the Michaelis–Menten equation:

$$I_{peak,C} = I_{peak,max} - K^{app}_{m,el}\left(\frac{I_{peak,C}}{C}\right) \quad (3)$$

are reported in Table 1. Some interesting observations can be made about the values of $K^{app}_{m,el}$, which are indicative of the electrochemical affinity of the immobilized enzyme for the catechol. The data in Table 1 imply that the laccase covalently bound to the graphite support displays a greater affinity towards catechol,

Table 1
Electric kinetic parameters

Biosensor type	$K^{app}_{m,el}$ (mM)	$I_{peak,max}$ (μA)	$I_{peak,max}$/mg$_{enz}$ (μA/mg)
A	3.07	42	105
B	0.18	45	98
C	0.07	86	95

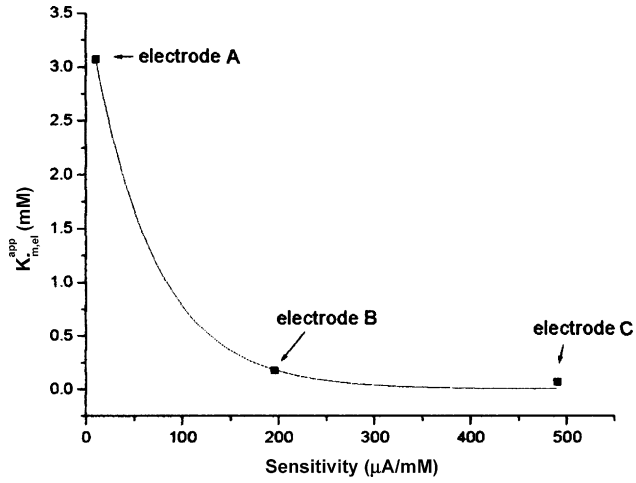


Fig. 8. $K^{app}_{m,el}$ values as a function of sensitivity.

the smaller affinity being displayed by the adsorbed laccase. On the contrary, when the maxima peak currents are normalized taking the amount of the immobilized enzymes into account, no differences appear between the different electrode types.

At this point, it is interesting to have quantitative informations about the physical parameters related to the functioning of a biosensor, such as the sensitivity and the extension of the linear range. All these parameters, which were obtained from the insets in Figs. 5–7, are listed in Table 2. The data show that sensitivies are higher when the laccase is covalently bound to the electrode in comparison to the value obtained with the adsorbed laccase. The opposite is true when the extension of the linear range is considered. Similar results were obtained using another system [24].

When the $K^{app}_{m,el}$ values are reported as a function of the sensitivities, the results reported in Fig. 8 emerge. The data in Fig. 8 clearly show how small values of sensitivity correspond to small affinity values, i.e. to high values of the electrical constant $K^{app}_{m,el}$, and vice versa, how high values of sensitivity correspond to high values of the electrical affinity, i.e. to small values of $K^{app}_{m,el}$. It is therefore evident that, in order to design laccase-based

Table 2
Characteristics of laccase-based biosensors

Biosensor type	Sensitivity (μA/mM)	Linear range extension (mM)	Stability days	Reference
A	9.7	up to −2	10	This paper
B	196	up to −0.1	30	This paper
C	490	up to −0.1	60	This paper
Laccase from *Trametes versicolor* on carbon fibres	16.1	1–90	60	[9]
Laccase from *T. versicolor* on graphite electrode	68.6	1–10	100	[15]
Laccase from *Coriolus versicolor* on Pt electrode	0.2	10–100	40	[17]

biosensors for the determination of phenolic compounds (such as catechol), one must adapt the immobilization methods on the basis of the interest in having high sensitivity or extension of the calibration curve.

4. Conclusions

It has been demonstrated that it is possible to modulate the electrical response of laccase-based graphite biosensors by using different immobilization methods. Laccase immobilization using covalent bonds ensures higher sensitivities with respect to immobilization obtained by absorption. This provides clear indications for the design of biosensors obtained by covalent enzyme immobilization, provided that the time stability is interesting. The time stability for each of the three biosensor types is reported in Table 2. These values confirm that the biosensor C is the most interesting type among those tested in this work, even when compared to similar biosensors described by other authors [9,15,17].

A final observation concerns the actual "on-line" exploitation of our biosensors. The pH ranges in which biosensors B and C exhibit current values higher than 80% of their maximum response (i.e. between pH 4.0 and 6.0 for biosensor B, and between pH 4.25 and 5.5 for biosensor C) make the proposed biosensors useful for direct application in waste waters from agricultural and industrial activities which are known to be acidic. In order to use a single calibration curve in "on-line" measurements when samples with different pH values have to be analyzed, an automatic pH adjustment of the sample can be programmed before any measurement.

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References

[1] R. Elsby, J.L. Maggs, J. Ashby, B.K. Park, J. Pharmacol. Exp. Ther. 297 (2001) 103.
[2] K. Howdeshell, A.K. Hotchkiss, K.A. Thayer, J.G. Vandenbergh, F.S. vom Saal, Nature 401 (1999) 762.
[3] P.A. Hunt, K.E. Koehler, M. Susiarjo, C.A. Hodges, A. Ilagan, R.C. Voigt, S. Thomas, B.F. Thomas, T.J. Hassold, Curr. Biol. 13 (2003) 546.
[4] A. Soares, K. Jonasson, E. Terrazas, B. Guieysse, B. Mattiasson, Appl. Microbiol. Biotechnol. 66 (2005) 719.
[5] R.J. Kavlock, G.P. Daston, C. De Rosa, L.E. Gray, S. Kaattari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, H.A. Tilson, Environ. Health Perspect. 104 (1996) 715.
[6] D.L. Davis, H.L. Brandlow, Sci. Am. 273 (1995) 166.
[7] L.A. Haighton, J.J. Hlywka, J. Doull, R. Kroes, B.S. Lynch, I.C. Munro, Regul. Toxicol. Pharm. 35 (2002) 238.
[8] A.I. Yaropolov, A.N. Kharybin, J. Emneus, G. Marko-Varga, L. Gorton, Anal. Chim. Acta 308 (1995) 137.
[9] R. Freire, N. Duran, L.T. Kubota, Talanta 54 (2001) 681.
[10] A.I. Yaropolov, A.N. Kharybin, J. Emneus, G. Marko-Varga, L. Gorton, Biochem. Bioenerg. 40 (1996) 49.
[11] D. Quan, W. Shin, Mater. Sci. Eng. 24 (2004) 113.
[12] F. Vianello, A. Cambria, S. Ragusa, M.T. Cambria, L. Zennaro, A. Rigo, Biosens. Bioelectron. 20 (2004) 315.
[13] A. Jarosz-Wilkolazka, T. Ruzgas, L. Gorton, Enzyme Microb. Technol. 35 (2004) 238.
[14] R. Freire, N. Duran, L.T. Kubota, Anal. Chim. Acta 463 (2002) 229.
[15] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jonsson, Anal. Chim. Acta 487 (2003) 3.
[16] N. Duran, M.A. Rosa, A. D'Annibale, L. Gianfreda, Enzyme Microb. Technol. 31 (2002) 907.
[17] S.A.S.S. Gomes, M.J.F. Rebelo, Sensor 3 (2003) 166.
[18] J. Wang, Y. Lin, A.V. Eremenko, A.L. Ghindilis, I.N. Kurockin, Anal. Lett. 26 (1993) 197.
[19] G. Hall, D. Best, A.P.F. Turner, Anal. Chim. Acta 213 (1988) 113.
[20] J.N. Rodriguez-Lopez, J. Tudela, R. Varon, F. Garcia-Carmona, F. Garcia-Canovas, J. Biol. Chem. 267 (1992) 3801.
[21] B. Reinhammar, B.G. Maldrom, in: T.G. Spiro (Ed.), Copper Proteins Metal Ions in Biology, vol. 3, Wiley and Sons, New York, 1981, p. 109.
[22] H. Claus, Micron 35 (2004) 93.
[23] A. Yaropolov, O.V. Scorobogako, S.S. Vartonov, S.D. Varfolomeyer, Appl. Biochem. Biothechnol. 49 (1994) 257.
[24] M. Portaccio, M. El-Mansry, S. Rossi, N. Diano, A. De Maio, V. Grano, M. Lepore, P. Travascio, U. Bencivenga, N. Pagliuca, D.G. Mita, J. Mol. Catal. B: Enzym. 18 (2002) 49.
[25] D. Durante, R. Casadio, L. Martelli, G. Tasco, M. Portaccio, P. De Luca, U. Bencivenga, S. Rossi, S. Di Martino, V. Grano, N. Diano, D.G. Mita, J. Mol. Catal. B: Enzym. 27 (2004) 191.
[26] A. De Maio, M. El-Masry, S. Di Martino, S. Rossi, U. Bencivenga, V. Grano, N. Diano, P. Canciglia, D.G. Mita, Biotechnol. Bioeng. 86 (2004) 308.
[27] A. De Maio, M.M. El-Masry, M. Portaccio, N. Diano, S. Di Martino, A. Mattei, U. Beuncivenga, D.G. Mita, J. Mol. Catal. B: Enzym. 21 (2003) 239.
[28] A. Attanasio, N. Diano, V. Grano, S. Sicuranza, S. Sergio, U. Bencivenga, L. Fraconte, S. Di Martino, P. Canciglia, D.G. Mita, Biotechnol. Prog. 21 (2005) 806.