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Flow-injection determination of catechol with a new tyrosinase/DNA biosensor[1]

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Abstract

Biosensors find application in flow analysis due to their high selectivity and sensitivity. Decrease in the response during extended use, originated by degradation, inhibition or structural changes of the enzyme or leaching of active components by the flow, is the prevailing problem. As an alternative to additives and preparation techniques cited in the literature, it is proposed to use DNA as a matrix for improving preservation of the activity of a diphenol-sensor-based tyrosinase, ***Tyr***, (EC 1.14.18.1). The Tyr–DNA mixture was incorporated into carbon paste, ***CP–DNA–Tyr***, or applied on glassy carbon, ***GC–DNA–Tyr***. The ***CP–DNA–Tyr***, covered by a membrane -of Cuprophan, presented superior performance in amperometric operation under flow conditions (electroreduction of the products of the enzymatic oxidation of diphenols in the presence of O_2). In comparison with paste electrodes without DNA, ***CP–Tyr***, a current increase of one order of magnitude was observed for catechol FIA peaks, with good repeatability during several hours of operation. The response decayed ca. 50% after every 3 to 4 days of use (with dry storage at 4°C overnight). Original performance was recovered by simply substituting the used paste for a new portion of stock paste, stable for 2 months under refrigeration. Evaluation of 18 different substrates and potential interferents indicated that, at the adopted potential of −0,15 V vs. Ag/AgCl, only *p*-cresol gives a response comparable to catechol. Flow-injection determination of catechol samples was conducted at a frequency of 30 injections/h, with linear response from the detection limit of 1×10^{-6} up to 5×10^{-5} mol l^{-1}. © 1998 Elsevier Science B.V.

Keywords: DNA biosensor; Tyrosinase; Catechol determinations; Amperometric flow analysis

1. Introduction

The growing interest of using biosensors as quantitative detectors in flow-injection analysis (FIA) [1,2] derives from their excellent selectivity, high sensitivity and good repeatability and response times compatible with FIA. Decrease in the sensitivity during extended use, originating in the degradation, inhibition or structural changes of the enzyme or leaching of active components by the flow, is not unusual and its mitigation is being actively researched [2,3]. Most electrochemical biosensors rely on oxidoreductase enzymes, because they involve electron transfer during the catalytic reaction. Since few enzymes can

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exchange electrons with unmodified electrode surfaces [4], electroactive reactants or products of the catalytic process are preferentially measured when using amperometric detection. The principle of the primordial biosensor [5] – immobilization of an enzyme near a Clark cell acting as a transducer of oxygen consumption – continues to generate new applications (e.g. [6]). When the potentials for the oxidation or reduction of electroactive species are high, the use of mediators becomes advantageous, providing better selectivity. Some mediators also protect the enzyme-active center from inhibition caused by the products of catalytic reaction [7–9]. Eventually, well-selected mediators can bridge electron transfer between the enzyme and the electrode [10,11].

Carbon-paste electrodes became very popular in the development of biosensors because they are inexpensive, easy to prepare and show good performance, generally without memory effects. Existing applications have been extensively reviewed [3,10]. A judicious formulation of the paste is required since, beside the above-mentioned problems, a decrease in the observed electron-transfer rate of the electrochemical process as well as in the effective enzymatic activity can occur, e,g, by lowering the ration of graphite to mineral oil in the paste [3,12,13]. To avoid the denaturing of the enzyme in organic media or improve sensitivity and operational stability, researchers have used additives such as polyethylene glycol [13], cationic antibiotics, polymers, small uncharged molecules and negatively charged proteins [14].

Authors of the present paper were able to modify glassy-carbon electrodes with DNA and pioneer their application to the electroanalysis of drugs [15,16] and to the study of the interaction of drugs with DNA [16–18]. Recently, DNA has also been used for other sensing purposes [2].

In this paper, the voltammetric behavior of some diphenols is evaluated on the previously described [16] DNA-modified glassy-carbon electrode, and DNA is proposed and investigated as an additive to improve the response and stability of a carbon-paste biosensor for phenols applied under flow conditions. With this intent, the enzyme tyrosinase, ***Tyr***(EC 1.14.18.1), is simply mixed with DNA before preparing the paste. ***Tyr*** is a metalloprotein with copper as the prosthetic group, that, besides other polyphenoloxidases, catalyzes the hydroxylation of monophenols to *o*-dihydroxy phenols (cresolase activity) and the further oxidation to *o*-quinones (catecholase activity), sustained by molecular oxygen [19,20]. They have been widely used in the construction of biosensors, many of them with ***Tyr*** incorporated into carbon paste [[10,14,21–27] and references cited therein]. In relation to these, the proposed carbon-paste sensor containing ***DNA-Tyr*** associates attractive features, specially in flow operation.

2. Experimental

2.1. Chemicals and solutions

Acheson 38 graphite (Cat. No. G67-500) was obtained from Fischer (Springfield, USA) and glassy carbon GC20, in form of 3 mm o.d. cylinder, from Tokai (Japan). Tyrosinase EC 1.14.18.1 from mushroom (Cat. No. T-7755) and DNA (Cat. No. D1501) from CALF thymus were supplied by Sigma (St. Louis, USA) and Cuprophan hemodialysis membranes, by Akzo Nobel (Wuppertal, Germany). All other reagents were of analytical grade, from Merck (Darmstadt, Germany) and used without further purification.

All solutions were prepared with water purified with a Barnstead Nanopure system. Dilution of stock solutions was made with 0.05 mol l^{-1} phosphate buffer, pH 7.0, just before use. The same buffer was used as carrier electrolyte in flow systems.

2.2. Apparatus

All electrochemical measurements were made with a polarographic Analyzer – stripping voltammeter Model 264A from EGϵG PAR (Princeton, USA) connected to a X-t recorder 6512 B from Linseis (Princeton, USA) for the amperometric measurements and to an X-Y Plotter RE 0089 from EGϵG PAR, for voltammetry. The flow-injection system comprised a peristaltic pump model MS Reglo model 7331-10 from Ismatec, obtained from Cole–Parmer (Chicago, USA), adjusted to deliver carrier electrolyte at a flow of 1.3 ml min^{-1}, in series with a commutator for sample introduction, provided with a 100 µl loop, ending at the electrochemical flow cell fitted with the biosensor under investigation.

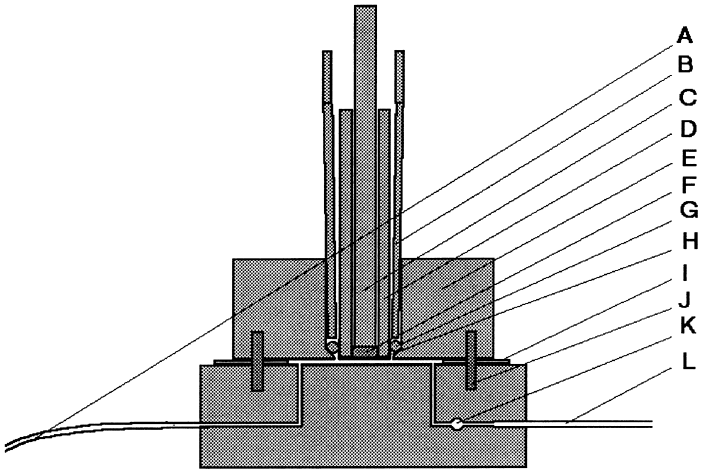

Fig. 1. Schematic diagram of the amperometric thin-layer flow. A, flow in; B, polypropylene tube (cut from a disposable 1 ml pipette tip) to pressure fit the working electrode; C, brass electric contact; D, Teflon body of the working electrode; E, counter block in acrylic; F, carbon paste (can be replaced for GC electrode); G, O-ring to fasten Cuprophan membrane; H, Cuprophan membrane; I, Teflon spacer; J, Teflon pin to align cell counterparts; K, electrolytic connection with mini-reference electrode; and L, steel needle serving as outlet and counter electrode.

The cell was designed and constructed in the laboratory and presents low internal dead volume. It can be assembled with ***GC*** and ***CP*** electrodes, covered or not by a permeable film such as Cuprophan, as depicted in Fig. 1. The Ag/AgCl (sat KCl) reference electrode was of the miniaturized type [28] and the auxiliary electrode was a stainless-steel hypodermic needle that served simultaneously as flow outlet. The body of the paste electrode was made from a PTFE cylinder and presents a circular reentrance of 3 mm i.d. and 2 mm depth, to be filled with the carbon paste. The electric contact was provided by an internal brass wire. The body of the solid electrode was similar, possessing a ***GC*** disk of 3 mm o.d. embedded at the tip.

Stationary voltammetric measurements were made with a conventional three-electrode cell, containing 10 ml of solution. A stainless-steel wire served as counter electrode and a miniaturized Ag/AgCl (KCl_{sat}) electrode [28], as reference.

Hydrodynamic voltamograms were constructed from the amperometric peak heights of flow injections of the analytes at different potentials, changed manually.

2.3. Preparation of electrodes

The DNA–glassy-carbon modified electrode, ***GC–DNA***, was prepared by a previously established method [16], consisting in recovering the ***GC*** surface with double strand DNA dissolved in pH 4.7 acetate buffer. The dried electrode was polarized at 1.4 V for 5 min in the buffer solution. After that successive differential pulse voltammograms were recorded from 0 to 1.4 V in a solution containing 78 μg/ml of a mixture of denaturated and hydrolyzed DNA until stabilization of guanine and adenine oxidation current peaks. This modified electrode was used to observe the direct oxidation of phenols in the absence of the enzyme.

The following types of biosensors were considered:

1. carbon paste with tyrosinase, ***CP–Tyr***;
2. carbon-paste–DNA–tyrosinase, ***CP–DNA–Tyr***; and
3. glassy carbon with DNA and enzyme, ***GC–DNA–Tyr***.

The ***GC–DNA–Tyr*** biosensor was prepared by dissolving 1 mg of tyrosinase in previously prepared DNA solution (0.5 mg of DNA in 100 μl of phosphate buffer). The bare surface of the glassy carbon was polished with alumina down to 1 μm, thoroughly washed and sonicated. One drop of the ***DNA–Tyr*** solution was applied on electrode and dried overnight at room temperature.

To prepare the ***CP–Tyr*** electrodes, 1 mg of tyrosinase was dissolved in 50 μl in buffer solution and

mixed with 13 mg of graphite powder. Once dry, the powder was pasted with 6 mg of mineral oil (Nujol). This gave a ratio of 5:65:30 w/w.

The ***CP–DNA–Tyr*** electrodes were prepared in a similar way, but the tyrosinase (1 mg) was previously mixed with 100 μl of buffer containing 0.5 mg DNA solution (ratio, w/w, 5:2.5:65:27.5). A higher content of DNA was also considered (ratio, w/w, 5:5:65:25).

3. Results and Discussion

3.1. Voltammograms of catechol, dopamine and hydroquinone solutions at a *GC*- and *GC–DNA* modified electrodes

Voltammograms carried out in phosphate buffer solution, pH 7.0, containing 1×10^{-3} mol l^{-1} catechol at bare ***GC*** and ***GC–DNA*** electrodes presented nearly the same anodic peak potential of 0.45 V (Fig. 2(a)). However, the peak of the reduction wave was shifted from 0.20 to 0.12 V in the presence of DNA. Additionally, the current levels were ca. 50% higher at the ***GC–DNA*** electrode. During sequences of 30 cycles, the current levels increased about 0.5% per cycle at the DNA-modified electrode and decreased ca. 0.2% per cycle at a bare electrode (Fig. 2(b)).

In dopamine solutions, the anodic peak potential at the ***GC–DNA*** electrode was anticipated by 0.16 V and presented nearly twice the peak current, in comparison with a bare electrode, as can be seen in Fig. 3(a). In the reversed scan direction, the two reduction peaks became better defined and more intense at the modified electrode, however, displaced to negative potentials, with no improvement in terms of reversibility. Such behavior is in agreement with experiments of ^{32}P-postlabeling of DNA in the presence of dopamine (with, or without ***Tyr***) showing that dopamine can covalently bind to DNA, presumably after autooxidation [29]. Repeated cycling results in gradual decrease of the peak's currents, ca. 2.3% each cycle for the anodic peak, slightly faster then the 1.3% observed at the bare electrode (Fig. 3(b)).

In hydroquinone solutions, results were similar to those obtained with dopamine: improved sensitivity and resolution with the ***GC–DNA*** electrode. This favorable electrochemical behavior with the diphenols led to the idea of investigating the possibility of incorporating ***Tyr*** in a DNA matrix, in analogy with other additives and modifiers proposed in the literature [14,30], in the development of glassy-carbon and carbon-paste biosensors.

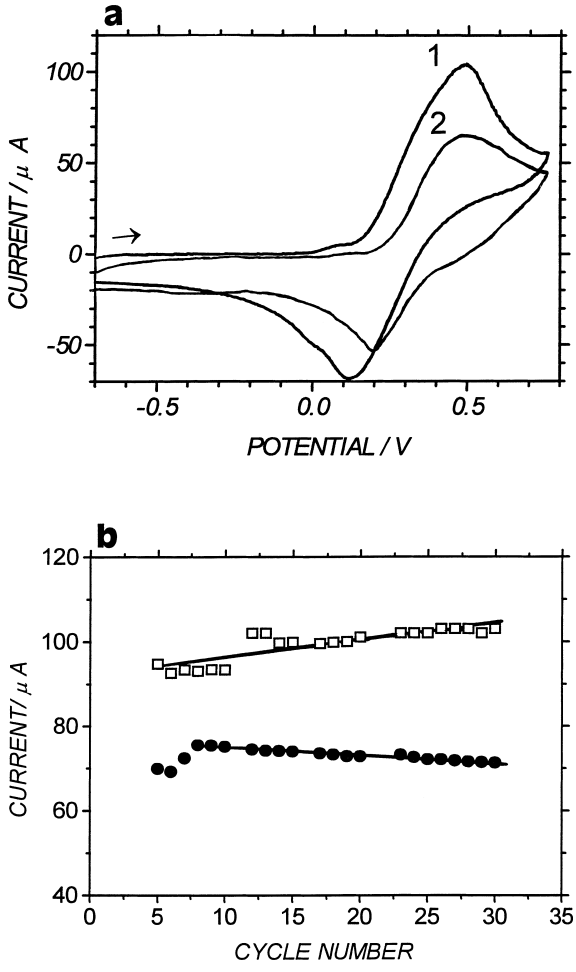

Fig. 2. (a) Cyclic voltammograms of catechol on a ***GC–DNA*** electrode (curve 1) and bare ***GC*** electrode (curve 2). Scan rate, 50 mV.s $^{-1}$, phosphate buffer, pH 7.0, 1×10^{-3} mol l^{-1} in catechol, and (b) decrease of I_{pa} during repeated cycling. Same conditions as (a), (□) ***GC–DNA*** and (b), (●) bare ***GC*** electrode.

3.2. FIA at *GC–DNA–Tyr* electrode

Fig. 4 shows a series of measurements obtained with ***GC–DNA–Tyr*** for catechol flow-injection determinations. Repeatable current signals were obtained

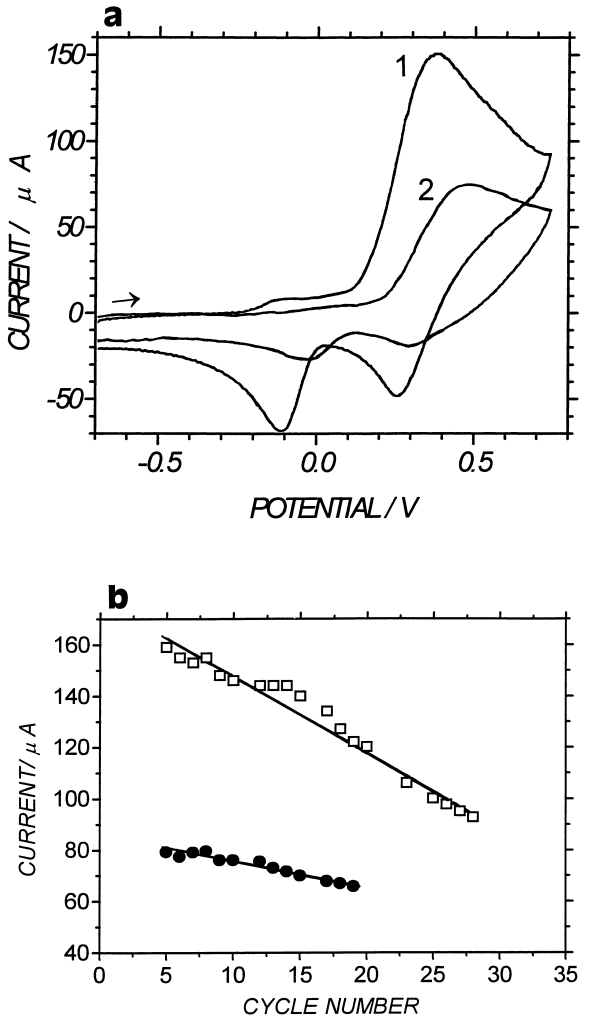

Fig. 3. (a) Cyclic voltammograms of dopamine on a ***GC–DNA*** electrode (curve 1) and bare ***GC*** electrode (curve 2). Scan rate, 50 mV.s^{-1}, phosphate buffer, pH 7.0, 1×10^{-3} mol l^{-1} in dopamine, and (b) decrease of I_{pa} during repeated cycling. Same conditions as (a): (□), ***GC–DNA*** and (●), bare ***GC*** electrode.

during 3 h of continuous operation. Linear response to concentration covered the 1–30$\times10^{-5}$ mol l^{-1} range. On the next day, a drastic decrease in performance was observed. Application of a Cuprophan membrane on the modified surface was of limited help. The pressure exerted by the membrane on the ***Tyr–DNA*** mixture spread it away from the ***GC*** surface. Due to these difficulties, graphite paste was examined as an alternative to the ***GC*** electrode.

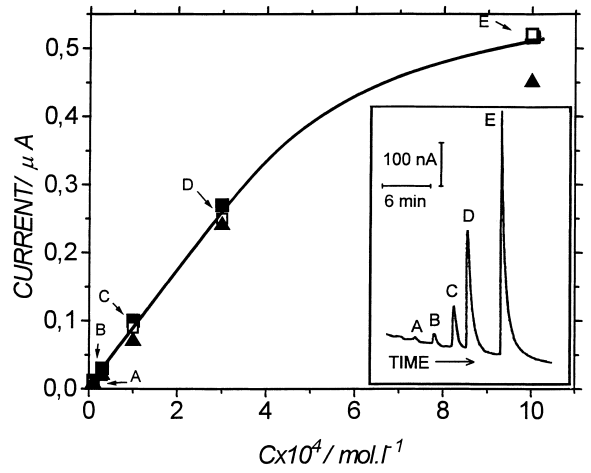

Fig. 4. Stability of analytical curves of catechol at the ***GC–DNA–Tyr*** electrode. Applied potential=−0.15 V vs. Ag/AgCl, flow rate=1.3 ml min^{-1}, phosphate buffer, pH 7.0: (□), initial measurements; (■), measurements after 1 h of flow; and (▲), measurements after 3 h of flow. A, 1×10^{-5} mol l^{-1}; B, 3×10^{-5} mol l^{-1}; C, 1×10^{-4} mol l^{-1}; D, 3×10^{-4} mol l^{-1}; and E, 1×10^{-3} mol l^{-1}. Inset corresponds to the recorded signal after 1 h of flow.

3.3. Comparison of FIA with *CP–Tyr* and *CP–DNA–Tyr* electrodes

Although favorable results have been reported in the literature for a ***CP–Tyr*** electrode operated in batch conditions and protected with a dialysis membrane [23], such a sensor was not satisfactory under flow conditions. Sequences of 100 injections of 1×10^{-4} mol l^{-1} catechol, at a frequency of 30 h^{-1}, showed a relative peak current decrease of ca. 23% each hour. This decay could be alleviated to nearly 10% per hour by covering the sensor with a layer of Cuprophan membrane. After prolonged use, by scratching off the outmost layer of the paste, recuperation of sensitivity was not complete. These results show that loss of enzyme by lixiviation is hindered by the membrane, but that other factors, like progressive inactivation of the enzyme in the paste, are also brought into action. During storage of the electrode overnight under refrigeration, loss of enzymatic activity continues and sensitivity may be depressed to 5% of the original one, impairing the use of the biosensor, at least, near detection limit.

Results obtained with the ***CP–DNA–Tyr*** electrode were quite superior. An increase of one order of

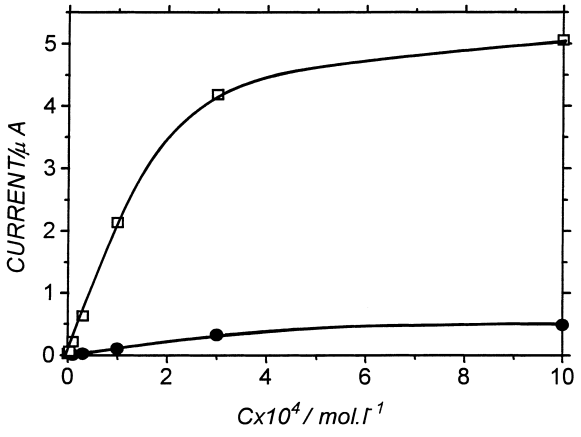

Fig. 5. Analytical curves of catechol at the electrodes: (□), ***CP–DNA–Tyr*** and (●), ***CP–Tyr***. Applied potential=−0.15 V vs. Ag/AgCl and flow rate=1.3 ml min^{-1}, phosphate buffer, pH 7.0.

magnitude was observed for the FIA amperometric peak of catechol on the ***CP–DNA–Tyr*** electrode in comparison with the ***CP–Tyr*** one, both covered with Cuprophan (Fig. 5) and prepared with tyrosinase from the same flask (effective activity of tyrosinase from different shippings to Brazil was found to vary considerably on arrival at the laboratory). Good peak repeatability was obtained during routines of 3 h of daily operation under flow conditions. The decrease of the sensitivity was slow, reaching 50% after 3 to 4 days of use. The ***CP–DNA–Tyr*** electrode was still usable after a week when overnight storage was carried out in a refrigerator. The original performance of the biosensor was recovered by simply substituting the outermost layer of the used paste for a new portion taken from the stock paste, stable for at least 2 months under refrigeration.

In short, the presence of DNA in the biosensor improves the durability and greatly increases the sensitivity of the sensor, in comparison with the ***CP–Tyr***. The clarification of the role of DNA in the biosensor is beyond the scope of this paper, but some comments and speculations will be presented. When the ***CP–DNA–Tyr*** is first exposed to the electrolyte, some swelling is observed, as a result of hydration of the DNA. In comparison with the enzyme dispersed in Nujol and carbon powder, the presence of DNA provides a more hydrophilic environment for the enzyme. It is known that this condition favors the acceleration of the catalysis. For example, when HY-zeolites are added to the tyrosinase–carbon paste, the more hydrophilic the zeolite is, the higher and sharper the FIA peaks obtained for catechol and phenol [30]. Conversely, the catalytic action of the enzyme ceases when it becomes dehydrated in organic media [31]. The direct interaction of DNA with the functional groups of amino acids of proteins occurs through hydrogen bonding, with partial displacement of the well-ordered water shell of DNA, sometimes, with individual water molecules acting as bridges of the hydrogen bonding [33]. Through such interactions, DNA can improve the stabilization of the tertiary structure of the enzyme in comparison with other additives. However, an overwhelming DNA/tyrosinase ratio probably led the DNA to hinder the access of the substrate to the prosthetic center (Cu(II)/Cu(I)), imparting the biocatalytic activity. With regard to the decrease of the enzymatic activity, it is well known that ***Tyr*** is gradually inactivated by *o*-semibenzoquinone, an intermediate free radical, which reacts with the imidazole group of one or more histidyl groups bound to the copper ions, in the active site [22,32]. In a higher hydrophilic medium, the life time of the free radicals decreases, possibly slowing down the inactivation of the enzyme.

*3.4. Evaluation of the analytical performance of the **CP–DNA–Tyr** electrode*

When starting a new working day, after half-an-hour stabilization of the base line under phosphate-buffer electrolyte flow, some increase in sensitivity is experienced during initial exposition of the biosensor to catechol (≅10–15%), as can been seen comparing the peak heights of the increasing- and decreasing-concentration branches of the analytical curve presented in Fig. 6. After this first cycle of the day, the response remains quite stable. For a different system, it has been postulated in the literature that the observed hysteretic behavior of an enzyme as a sensor can be explained by changes in the conformational states of the enzyme [34,35]. Further investigation is needed to verify if such hypothesis is applicable for the present system.

The analytical curve of Fig. 6 was obtained after renewal of the electrode refilling it with a ***CP–DNA–Tyr*** mixture prepared 17 days before. It illustrates the

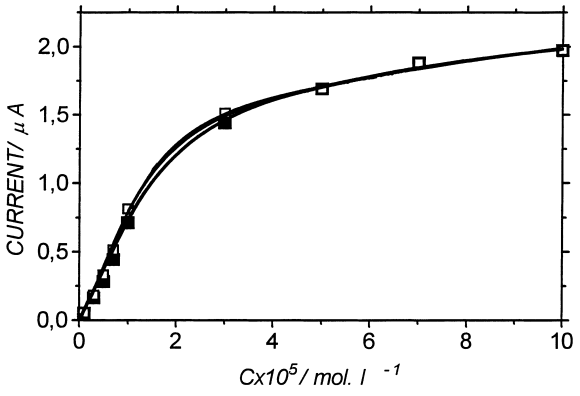

Fig. 6. Analytical curves of the ***CP–DNA–Tyr*** electrode (worst case, electrode renewed with 17-day old paste). Same conditions as in Fig. 5. Superimposition of peak heights obtained in: (■), the increasing concentration direction; and (□), decreasing concentration direction.

worst case in terms of the upper limit of the linear region, restricted to 1×10^{-5} mol l^{-1}. The typical values are 3 and 5×10^{-5} mol l^{-1}, as can be observed in Table 1, that summarizes the results of 51 analytical curves obtained along 2 months.

The estimated detection limit (3×SD of base line noise) was ca. 1×10^{-6} mol l^{-1}, as a rule, as indicated in Table 1. Although detection limits as low as 1×10^{-8} mol l^{-1} of catechol have been reported for ***CP–Tyr*** electrodes in stationary use [23], when the biosensor is used in FIA, typical values fall in the 1×10^{-7}–2×10^{-5} mol l^{-1} range [6,7,24,26] or even higher [36].

After weekly renewal, the sensor has always recovered the original sensitivity, allowing to anticipate that the ***CP–DNA–Tyr*** mixture kept under refrigeration will last much longer than the 2 months evaluation period. This achievement compares favorably with the literature [6,22,37] and affords the simultaneous preparation of sets of sensors with a shelflife, at least, of several months.

The FIA system was operated at a frequency of ca. 20 injections h^{-1}. Admitting a less complete decay of the current after each peak, a frequency of 30 injections h^{-1} is affordable. Since the rise and decay times are determined mainly by the diffusion of reactants/products through the Cuprophan membrane and the surface layer of the paste, the flow rate has little influence on the results and was kept constant at 1.3 ml min^{-1}. It was verified that the decay rate is influenced by the content of DNA in the paste. By increasing it to 5%, instead of the adopted 2.5% of the paste weight, the peaks for catechol became broadened and sluggish, impairing the sensitivity and the determination frequency.

3.5. *Response of the sensor prepared with **CP–DNA–Tyr** to various compounds*

Besides catechol, the response of 17 other compounds, including potential substrates for tyrosinase, was evaluated at the ***CP–DNA–Tyr*** sensor through hydrodynamic voltammograms obtained for a concentration of 3×10^{-5} mol l^{-1}. At a potential of −0.15 V, adopted for the amperometric FIA measurements throughout the work, *p*-cresol was the only substrate that presented signals of similar magnitude, as shown by the relative sensitivities given in Table 2. Significant response to *p*-cresol has been reported for other biosensors based on ***Tyr*** [23,30], revealing the cresolase activity. Table 2 shows an increased *p*-cresol/phenol signal ratio in comparison to the literature [27,30]. This result, formerly observed for a laccase biosensor [25], cannot simply be attributed to the presence of DNA since the preparation process of tyrosinase from mushroom can dramatically influence this ratio [32].

The recuperation of the sensitivity of the biosensor after repeated exposure to the above listed compounds was also evaluated. The analytical curve for catechol, comprising seven distinct concentrations, was

Table 1
Upper linear limit and estimated detection limit of 51 analytical curves obtained during 2 months of evaluation of the *CP–DNA–Tyr* electrode, with weekly substitution of the paste

	Upper linear limit of analytical curve				Estimated detection limit			
Concentration (mol l^{-1} catechol)	1×10^{-5}	3×10^{-5}	5×10^{-5}	7×10^{-5}	1×10^{-4}	1×10^{-6}	3×10^{-6}	7×10^{-6}
Number of cases	1	22	23	2	3	48	2	1

Table 2
Relative sensitivity of the *CP–DNA–Tyr* biosensor to various phenols and other compounds at 3×10^{-5} mol l^{-1} level and recoverage of the response for catechol, taken as the ratio of the slope of the calibration curve for catechol obtained after, and before the 40–50 injections of the compound under examination

Compound	Relative sensitivity (%)	Recovery of sensitivity (%)
Catechol	100	95
p-Cresol	100	80
Hydroquinone	10	95
Dopamine	10	90
Caffeic acid	8	82
Phenol	5	31
Pyrogallol	5	28
Chlorogenic acid	5	62
o-Cresol	2	77
l-Tyrosine	2	64
Benzoic acid	2	32
Guaiacol	2	56
Salicylic acid	1	44
α-Naphtol	1	52
Caffeine	1	28
p-Phenylenediamine	1	95
o-Phenylenediamine	0	42
Resorcinol	0	28

obtained before, and after the construction of the hydrodynamic voltammogram for each compound. By comparing the ratio of those slopes (slope after/slope before), it comes out that after 40–50 injections of pyrogallol or caffein, the response of the biosensor becomes more significantly impaired then for the other compounds. As mentioned before, when required, substitution of the paste for a new portion restores initial performance.

4. Conclusions

The finding that diphenols such as catechol, hydroquinone and an important neurotransmitter (dopamine) can be oxidized at a ***GC–DNA*** electrode prepared by a previously described procedure [16], is very relevant. The achievement of voltammograms with higher currents and better defined oxidation and reduction peaks, in comparison to the bare ***GC*** electrode, deserves deeper investigation in the future.

The utilization of DNA as a matrix to aid enzyme immobilization is a promising alternative for the development of new biosensors and their application in flow systems. The increase of one order of magnitude of the measured current with virtually no memory effects are attractive characteristics of this new ***CP–DNA–Tyr*** biosensor, as are his good stability (one week as a FIA detector for catechol), straightforward renewal by substitution of the outermost layer of the paste and the long shelflife of the stock paste (at least 2 months under refrigeration). It can be envisioned that the proposed biosensor will possibly find practical application in FIA and also that other enzymatic biosensors can similarly benefit from the presence of DNA.

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