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Amperometric sensors based on tyrosinase-modified screen-printed arrays

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Abstract

This paper describes the design, development and characteristics of a tyrosinase (polyphenol oxidase) modified amperometric screen-printed biosensor array, with the enzyme cross-linked in a redox–hydrogel namely the PVI_{13}-dmeOs polymer. Two types of Au-screen-printed four-channel electrode arrays, differing in design and insulating layer, were compared and investigated. Au-, graphite-coated-Au- and Carbopack C-coated-Au-surfaces, serving as the basis for tyrosinase immobilisation, were investigated and the performances of the different arrays were evaluated and compared in terms of their electrocatalytic characteristics, as well as operational- and storage stability using catechol as model substrate. It was found that the Carbopack C-coated array was the best choice for tyrosinase immobilisation procedure mainly due to a higher mechanical stability of the deposited enzyme layer, combined with good sensitivity and stability for up to 6 months of use. In the batch mode the biosensors responded linearly to catechol up to 30 μM with limits of detection from 0.14 μM. Parameters from cyclic voltammograms indicated that the reversibility of the direct electrochemical reaction for catechol on the three types of electrode surfaces (no tyrosinase modification) was not the limiting factor for the construction and performance of tyrosinase biosensors.

Keywords: Screen-printed arrays; Tyrosinase; Amperometric biosensor

1. Introduction

Environmental contaminations by toxic and other hazardous compounds have become a public health concern that has led to a vast expansion of the area of environmental analytical chemistry [1]. To develop fast and reliable pollution monitoring instruments, the potential of biosensors has frequently been emphasised and there is no doubt that considerable progress has been achieved in the development of biosensors for phenols including chloro- and bromo-phenols, polychlorinated biphenols, organophosphoric and carbamate insecticides, metal ions, and recently surfactants [2].

The area of biosensors is driven towards development of small, hand held and battery-operated instruments suited for on-site de-centralized biomedical- and industrial-analysis, or in the field

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environmental control [3]. An electrochemical instrument would be ideal for this purpose, because electrochemical instrumentation has the potential to be compact, inexpensive, rugged, and versatile [4]. Some barriers, however, must be overcome before commercial instruments based on biosensors become available and the first barrier involves the storage stability of the sensor. Commercially viable biosensors must have a shelf life of at least 4–6 months in order to allow reasonable production, inventory, shipping, and customer test schedules [5]. Screen-printed electrodes are potentially promising in these aspects because they are economic and easily mass-produced [6–9]. The practical utility of screen-printed electrodes has been developed despite the fact that little is known about the nature of electrode reactions at these microfabricated sensors [10]. The application of screen-printed biosensors is favourable due to some generally claimed advantages such as miniaturisation and easy automation, and for the construction of simple portable device for fast screening purposes and in-field/on-site monitoring [11]. Screen-printed multi-channel arrays have the potential possibility for monitoring several different compounds simultaneously. Hence, differences in ink composition (e.g. type, size or loading of carbon particles), and in the printing and curing conditions, may strongly affect the electron transfer reactivity and overall analytical performance of the resulting sensor. The designs of such sensors are still the object of discussion as well. Different arrangements, i.e. linear [12] or circular, maintain different dispersion of the analyte zone. In [12] the general reduction in peak heights between channels on one array was explained by the increasing dispersion of the sample zone pumped through the flow channel.

The most frequently studied phenol biosensors are based on carbon type electrodes with immobilised tyrosinase [4]. In the presence of oxygen, tyrosinase catalyses the oxidation of phenolic compounds in two steps, to the corresponding quinones, which are further reduced by the electrode, reforming the original phenol, thus forming a bio-electrocatalytic amplification cycle [13]. Both direct and mediated electron transfer pathways for this bio-electrocatalytic reaction have been at focus [14–19]. Nevertheless tyrosinase-based electrochemical biosensors suffer from low stability and significant inhibition of the enzyme by reaction products; both these factors deteriorate electrode characteristics in phenol determination and one of the more efficient ways to circumvent this seems to be through the use of osmium based hydrogels, which are reported to lead to high current densities and also stable biosensor on a long-term basis [15,20–22].

This paper describes the investigation of two types of four-channel screen-printed Au electrode arrays, differing in the design and insulating layer, with immobilised tyrosinase. The Au electrode base was manually modified with different carbon coatings and tyrosinase was immobilised on top in a redox–hydrogel based on osmium. The sensitivity and long-term operational stability of the tyrosinase biosensors were investigated. In this paper, we report for the first time on a Carbopack C-coated screen-printed array as a basis for tyrosinase immobilisation. This coverage provides substantial improvement in long-term stability of the catechol biosensor. The difference between electrode design, type of carbon, and enzyme layer modification procedures, are discussed. The electrode surface property before addition of enzyme was performed with cyclic voltammetry.

2. Experimental

2.1. Chemicals

Tyrosinase (EC 1.14.18.1, 6050 U mg^{-1}) from Sigma was used without further purification. Synthetic graphite powder (1–2 μm), cellulose acetate both from Sigma-Aldrich, and Carbopack C (80/100 mesh) from Supelko were used to prepare carbon-coated arrays. Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE), from Polysciences, Warrington, PA (cat. No. 08210), was used as the cross-linker and {poly[(1-vinylimidazolyl)osmium (1,4′-dimethylbipyridine)$_2$Cl]}$^{+/2+}$ (PVI_{13}-dmeOs) was synthesized according to a previously published protocol [23].

Catechol (99%) was purchased from Sigma and 30% H_2O_2 from Merck (Darmstadt, Germany).

Phosphate buffer (pH 7.0, 0.02 mol l^{-1} with 0.1 mol l^{-1} potassium chloride) was prepared by mixing sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate and potassium chloride of analytical-reagent grade (Merck), in water obtained from Millipore Milli-Q purification system (Bedford, MA).

A stock solution of 0.01 mol l^{-1} catechol was prepared daily in phosphate buffer (pH 7.0, 0.02 mol l^{-1} with 0.1 mol l^{-1} potassium chloride).

2.2. Screen-printed arrays

Two types of screen-printed electrode arrays were produced by Krejci Engineering (Tisnov, Czech Republic), see Fig. 1. Type A: (size: 13 × 38 mm) four working electrodes (1 mm diameter) based on printed Au-paste as well as one common reference electrode in the middle, based on printed Ag-paste. Ag paste was used for contacts and conducting paths, protected by a ceramic-based insulating coating on top of a ceramic support. Type B: (size: 8.5 × 50 mm) four working electrodes (1 mm diameter) based on printed Au-paste with Ag-paste used for contacts and conducting paths, and a rugged blue polymeric coating was deposited as the insulating layer on top of a ceramic support. As this type of electrode array did not include any reference electrode, an external hand-made Ag/AgCl (3 M KCl) reference electrode was used.

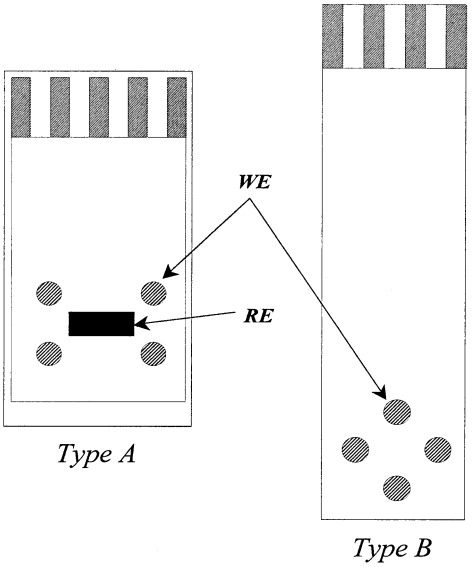

Fig. 1. Schemes of the two types of screen-printed electrode arrays, type A: four working Au-electrodes (WE) and one common Ag-reference (RE) electrode in the middle; type B: four Au-WE with an external reference used during measurements. The arrays differ by their size and design, and type of insulating layer.

2.3. Preparation of carbon-coated arrays

A volume of 0.32 ml of a solution of acetyl cellulose (1.5%) in acetone/cyclohexanone (1:1) was added to 100 mg of graphite powder or Carbopack C and homogenized by intensive shaking and stirring for 20 min. The end of a common micropipette tip (0.1–20 μl) was filled with the carbon mixture and the area of the working electrode of the screen-printed sensor was covered by gently touching the surface with the tip just to receive a carbon spot on the electrode's surface (exact amount of used ink could not be specified).

2.4. Enzyme immobilisation

Tyrosinase was immobilised on the surface of plain or carbon-coated Au-screen-printed electrode arrays by the entrapment in a redox–hydrogel composition. Hydrogel mixtures were prepared from stock solution of 10 mg ml^{-1} (60 500 IU ml^{-1}) tyrosinase, 5 mg ml^{-1} PVI_{13}-dmeOs, and 10 mg ml^{-1} PEGDGE (mixture freshly prepared and used within 15 min). Biosensors were prepared as follows: different volumes of the tyrosinase-, redox polymer-, and cross-linker solutions were mixed and deposited on top of the working electrodes of the arrays to get different compositions of the redox–hydrogel. After drying at room temperature for 20 h in a dessicator the film was equilibrated for at least 1 h in a phosphate buffer in order to allow hydrogel formation.

2.5. Measurements and apparatus

Cyclic voltammetry experiments were performed using a BAS potentiostat (Model CV-50W, Bioanalytical Systems, West Lafayette, USA) in a three-electrode cell with the screen-printed array electrodes as working electrodes, an external Pt wire as a counter and an external saturated calomel electrode as a reference electrode.

Amperometric measurements were realized in a two electrode system with a multi-purpose four-channel hand-held electrochemical detector 'Multichannel Electrochemical Biosensor' (supplied by P. Skladal, Masaryk University, Brno, Czech Republic), which was created within the INCO Copernicus project MEBFOOD (Multichannel Electrochemical Biosensors for Rapid Food Control, project no. ERB-IC 15-CT98-0910). The signals were recorded with a portable computer using the MEBTOOLS software (created by P. Skladal) and used for graphic display of the measured current and storage of data. An applied potential of -50 mV vs. Ag/AgCl was used throughout all measurements. All electrochemical experiments were performed in batch mode at room temperature.

3. Results and discussions

Tyrosinase was immobilised within a redox polymer matrix on plain or carbon-coated screen-printed Au-electrode arrays, as shown in Fig. 1 (type A array). The mediator ($Os(bpy)_2Cl$) was covalently bound to a non-conducting polymer backbone (poly(1-vinylimidazole), resulting in a redox polymer, PVI_{13}-dmeOs (the subscript indicates that every 13th vinylimidazole mer is complexed to an osmium redox center). The redox polymer was further cross-linked with the enzyme using a long chain cross-linker, i.e. PEGDGE, resulting in a three-dimensional polymeric network, referred to as a 'redox hydrogel' attached at the electrode surface [14]. The action of tyrosinase proceeds via the hydroxylation of monophenols into *o*-diphenols followed by the oxidation of the *o*-diphenols into the corresponding quinones. The bio-electrocatalytic amplification cycle proceeds at an osmium hydrogel entrapped tyrosinase electrode [21].

3.1. Tyrosinase-redox–hydrogel composition

It has been reported that different curing procedures of similar hydrogels can lead to more reproducible and stable electrodes [18] with the best results achieved by storing the biosensors at room temperature for 20 h in desiccated conditions. The thickness of the enzymatic layer constitutes another important parameter, which governs the biosensor response time, sensitivity and stability [18,24–26]. The biosensor response can be expected to increase with increasing amounts of deposited redox–hydrogel, an effect that however, is counteracted by limitations imposed by the rates of substrate diffusion and charge propagation across thick coatings [18]. High catechol sensitivity can, thus, be obtained when the enzymatic layer exhibits a high catalytic activity and good permeability. In addition, the optimum thickness must be at least three times the reaction length [26]. Thicker enzymatic layers are not only unnecessary, but will have the drawback of increasing the response time. Keeping this in mind the amount of immobilisation mixture applied to each electrode was optimised.

The effect of the layer thickness was studied by application of different volumes of the same enzyme redox–hydrogel composition, i.e. 0.5–2.0 μl. The best results in terms of mechanical stability were achieved using the lowest volume, i.e. 0.5 μl, since larger volumes lead to the crackelation of the layer after only a few measurements.

One of the most significant variables during the electrode modification process is the concentration of the enzyme, osmium-polymer and the cross-linking agent [18,21]. Thus, the composition of the tyrosinase-redox–hydrogel mixture was varied in a rather wide range. The amperometric response of the biosensor to catechol was examined at steady-state conditions and the cathodic current was directly related to the concentration of catechol in the solution. Preliminary results demonstrated that the highest signal to noise (S/N) ratio was obtained with a composition that contained 5–

20% w/w of cross-linker, >60% w/w of tyrosinase and <27% w/w of redox polymer. Higher concentrations of redox-polymer lead to extremely high noise and lower S/N ratio. For further optimisation, six different mixtures of cross-linker, enzyme and redox polymer were prepared in the optimal range and the calibration graph characteristics for each composition is shown in Table 1. Fig. 2a illustrates typical amperometric responses (current–time recordings) as a function of catechol concentration for a four-channel tyrosinase biosensor array, in all the cases with a response time within 30 s. Even though all electrodes in one array were modified in the same way, they all gave different responses for catechol, making it necessary to calibrate each electrode on the array. It should be mentioned that in almost all the cases when working with type A arrays there were differences in the responses depending on the position of the electrodes relative to the stirrer, i.e. top electrodes gave lower signals than electrodes at the bottom. This fact is probably due to different hydrodynamics near the electrodes on the array. Fig. 2b displays the calibration curves over a wide catechol concentration range, corresponding to the data seen in Fig. 2a, with a linear relationship for catechol up to 35 μM [7,27–29]. A curvature is observed for higher concentrations as a consequence of the progressive saturation of the enzyme active sites by the substrate as well as limitations due to the consumption of molecular oxygen. Although all mixtures in Table 1 produced good sensitivities for catechol, it can be mentioned that a higher enzyme and lower redox polymer content resulted in higher sensitivities and lower detection limits.

3.2. Operational and storage stability of sensors

The long-term stability of the sensors was investigated by recording the response for catechol for the electrode arrays during 6 months, as shown in Fig. 3a. The response to 17 μM catechol decreases significantly for all arrays during the first 15 measurements after which the signal remained relatively stable during 3 months. After 6 months only 95% of the current response was lost as compared to the response received after 12 days of running.

The operational stability was investigated by recording repetitive current response for 17 μM catechol solutions. The results in Fig. 3b show that the reduction current remained stable through 10 repetitive measurements, producing, depending on the enzyme redox–hydrogel composition, an average current from 81.1 to 327.6 nA with relative standard deviations (R.S.D.) compared between measurements 2.4–10.3% (Table 1).

3.3. Differences between type A and type B electrode arrays

Another type of array was tested (type B, Fig. 1) and compared with type A (Fig. 1) and the result is presented in Table 2. The absolute signals and sensitivities for catechol were always higher and the detection limits lower with type B compared to type A arrays under the same conditions.

The graphite composite layer, applied on the surface of screen-printed electrodes, as the basis for further modification, was previously shown to be mechanically unstable [1], and the same was experienced here for type B arrays. The two types of arrays used in this work have different thickness of the insulating layer, seemingly different hydrophobic properties, and the polymer insulator of type B arrays appears to have a rougher surface. Probably, due to the thicker insulating layer of type B arrays, the graphite 'ink' cannot form a thin and stable film in the electrode cavities. When the graphite coating covers the edge of the thicker insulating layer, the graphite is quickly washed away from the surface of the Au-electrodes possibly because of capillary effect of the rough insulator surface. By using Carbopack C, which as opposed to graphite is characterised by a chemically and geometrically uniform plane surface, this problem was partly avoided and relatively stable Carbopack C coatings could be achieved on type B arrays. Carbopack C is a conductive, non-polar graphitised carbon black powder, which is usually used as an adsorbent in gas chromatography. To improve the strength of the powder granules and to cover non-uniform parts, they are coated with pyrocarbon. Such materials were successfully used

Table 1
The calibration graph and electroanalytical characteristics of tyrosinase-modified graphite-coated screen-printed Au-electrode arrays (type A) with different hydrogel compositions

Hydrogel	Composition (% w/w)			Linear range (μM)	Sensitivity (μA mM^{-1})	LOD (μM)	Reduction current, nA	R.S.D. (%) ($N = 10$)	S/N
	Tyrosinase	Redox polymer	Cross-linker						
I	66.7	26.6	6.7	0.5–35 $R = 0.999$	12.8 ± 0.2	2	156.4	10.3	8
II	62.5	25	12.5	0.5–35 $R = 0.999$	8.6 ± 0.2	1.7	81.1	8	8
III	72.7	21.8	5.5	0.5–30 $R = 0.997$	37 ± 1	0.35	327.6	4.6	16.7
IV	74.1	18.5	7.4	0.5–30 $R = 0.997$	27 ± 1	0.34	227.1	4.8	21.2
V	57.7	23.1	19.2	0.5–35 $R = 0.999$	22.2 ± 0.5	0.88	273	2.4	11.7
VI	66.6	16.7	16.7	0.5–35 $R = 0.999$	16.0 ± 0.3	0.93	132.5	3.4	9.7

Limit of detection (LOD): calculated as three times the S.D. of the background signal. Average currents from 10 measurements for 17-μM catechol at steady-state were calculated for one electrode in the array. Applied potential: −50 mV vs. Ag/AgCl; buffer: 20 mM phosphate buffer pH 7.0, containing 0.1 M KCl.

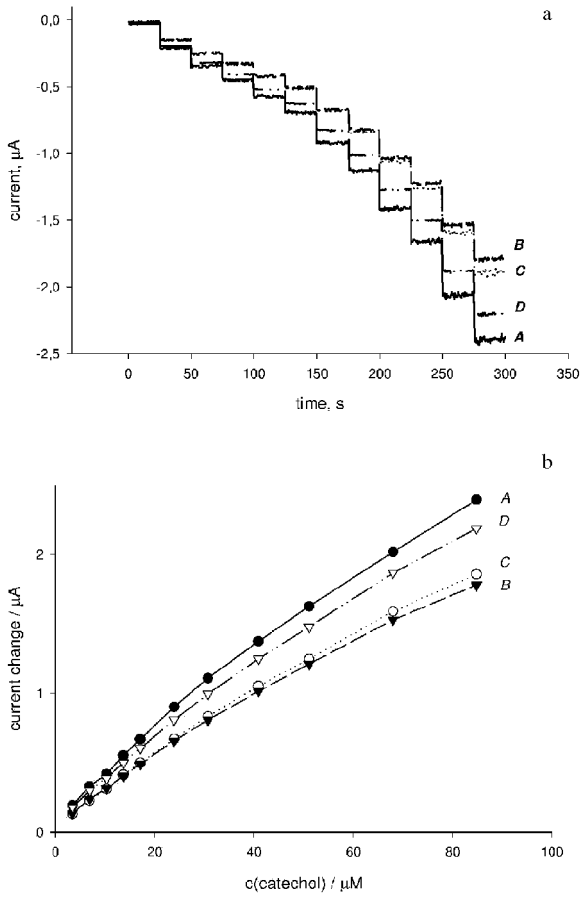

Fig. 2. (a) Current–time recordings and (b) calibration graphs for successive additions of catechol obtained with tyrosinase-modified screen-printed graphite-coated four-channel Au-array (12 days after preparation, type A array). Tyrosinase-redox–hydrogel compositions: (A, B)-III; (C, D)-IV (as indicated in Table 1). The array was immersed in 15 ml of phosphate buffer solution (pH 7.0) with an applied reducing potential of −0.05 V vs. pseudo-Ag/AgCl. Once a steady background current was obtained aliquots of catechol stock solution were added to a magnetically stirred solution and the steady-state response was recorded.

for the construction of carbon composite electrodes for voltammetric determination of substituted phenols [30,31], but never for biosensors. The comparison of the calibration curve characteristics of these sensors is shown in Table 3. Even after half a year of storage in buffer the Carbopack C-coated array remained mechanically stable on the top of the electrode surfaces. The long-term stability and reproducibility of Carbopack C- and graphite–tyrosinase-modified Au-electrode is shown in Fig. 4. Despite better electroanalytical characteristics of the graphite-coated surface, the graphite-enzymatic layer had worse stability (it was washed away after 36 days). It can be concluded that the Carbopack C-coated Au-array is the best choice for tyrosinase immobilisation due to a higher mechanical stability, combined with good sensitivity and stability of the response.

3.4. Reversibility of catechol oxidation on electrodes without tyrosinase

When working with screen-printed electrodes very often the reversibility of ferricyanide is used as a means to examine the suitability of a surface as a base for biosensor development where good reversibility is usually required before biosensor manufacturing [8,32]. Previously it was found that there is a direct correlation between the reversibility of ferricyanide oxidation at screen-printed graphite electrodes and sensitivity for the detection of phenols at cellobiose dehydrogenase modified electrodes [33]. The correlation was explained by the fact that facile electrochemistry of phenolic compounds at the graphite surface enables their rapid recycling between the enzyme and the surface of graphite electrode. This finally leads to high sensitivity for phenols at cellobiose-modified electrodes. In our study some experiments were carried out with cyclic voltammetry to check if there exists a correlation between the reversibility of the catechol reaction on the different electrodes, unmodified with tyrosinase, and the biosensors characteristics. Cyclic voltammograms for catechol using a graphite–Au-array, a bare Au-array and a Carbopack–Au-array are shown in Fig. 5a–c. Two features are evident: (1) there was a substantial increase in the cathodic and anodic currents in the order bare Au, Carbopack–Au, and graphite–Au, (2) the electrochemical reversibility was improved in the same order. The electrochemical behaviour of catechol on the graphite–Au array is characterised by $i_{pc}/i_{pa} = 1.21$ and $\Delta E_p = 51$ mV and can be compared to the theoretical values of $i_{pc}/i_{pa} = 1$ and $\Delta E_p = 30$ mV for a $2e^-$ redox process. For carbopack the same parameters varied from 1.25 to 1.34 for i_{pc}/i_{pa}

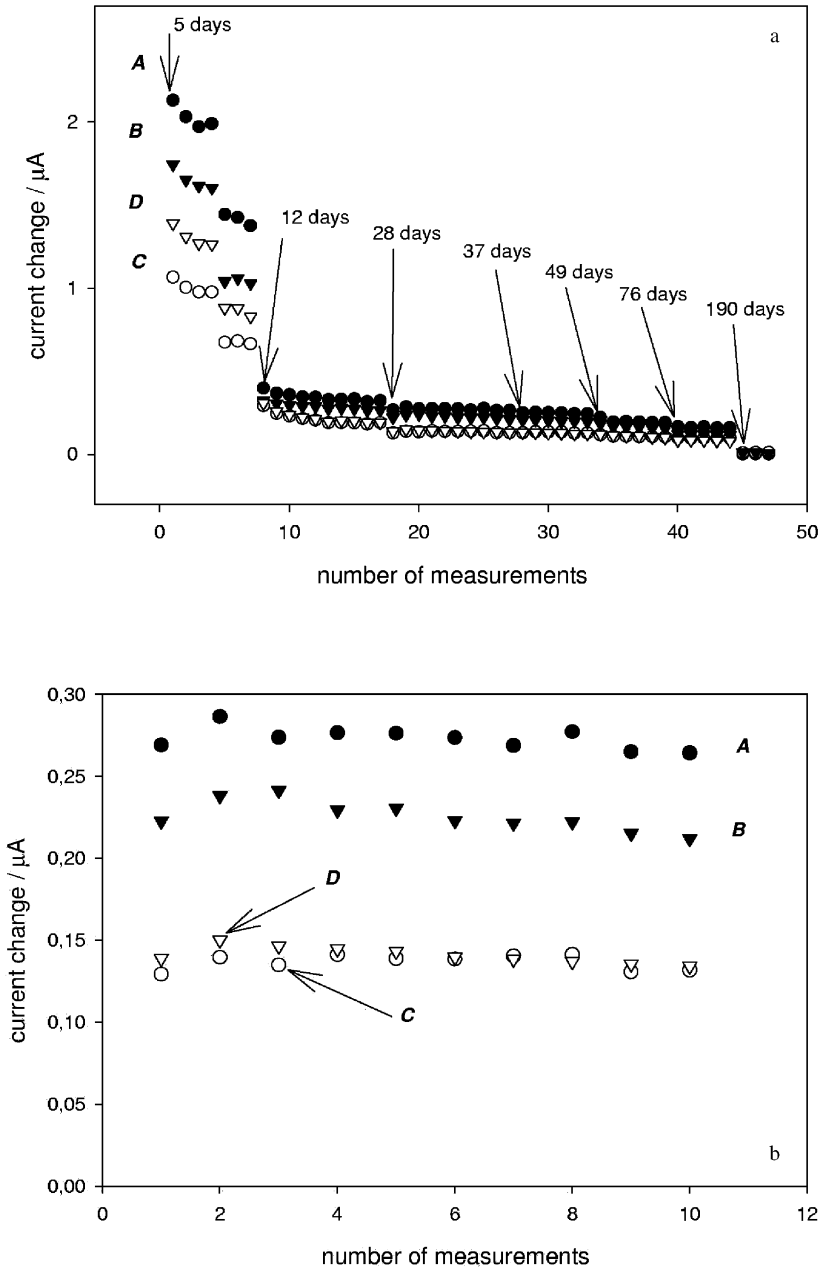

Fig. 3. Long-term stability (a) and reproducibility (b) of tyrosinase-modified graphite-coated screen-printed four-channel Au-array (type A) for 17 µM catechol; tyrosinase-redox–hydrogel compositions: (A, B)-V; (C, D)-VI (as indicated in Table 1). Other conditions are as given in Fig. 2.

and from 234 to 310 mV for ΔE_p. The formal redox potential, taken as $E^{0\prime} = 1/2(E_{p,a} + E_{p,c})$ was found at 158 mV for graphite–Au and 162 mV for Carbopack–Au surfaces. Despite a more reversible redox reaction at the graphite–Au array and an almost irreversible redox reaction at the bare

Table 2
Comparison of two types screen-printed arrays used for biosensor preparation (12 days after preparation)

Array	Reduction current (nA)	S/N	Sensitivity (μA mM^{-1})	LOD (μM)
Type B	917±42	45.4	48.8±0.9	0.28
Type A	225±10	12.4	12.8±0.2	1.95

Tyrosinase-redox–hydrogel composition I (as indicated in Table 1). Otherwise, conditions as in Table 1.

Table 3
The calibration graph characteristics of tyrosinase-modified electrode-arrays, based on different surface modifications

Basic surface	Linear range (μM)	Correlation coefficient	Relative sensitivity (%)[a]	LOD (μM)
Graphite paste coated screen-printed golden array (36 days old)[b]	0.5–30	0.999	100	0.14
Carbopack C paste coated screen-printed golden array (36 days old)[b]	0.5–30	0.999	83	0.16
Screen-printed golden array (12 days old)[c]	0.5–30	0.997	71	0.18

Tyrosinase-redox–hydrogel composition I (as indicated in Table 1). Otherwise, conditions as in Table 1.
[a] Calculated as the ratio of sensitivity-to-sensitivity of graphite-coated array of the same type.
[b] Array type B.
[c] Array type A.

Au-array, the tyrosinase biosensors developed on these electrodes showed similar sensitivities and detection limits for catechol, as seen in Table 3. It can thus be concluded that the reversibility of electrochemical reaction for catechol at the electrodes is not the limiting factor for the construc-

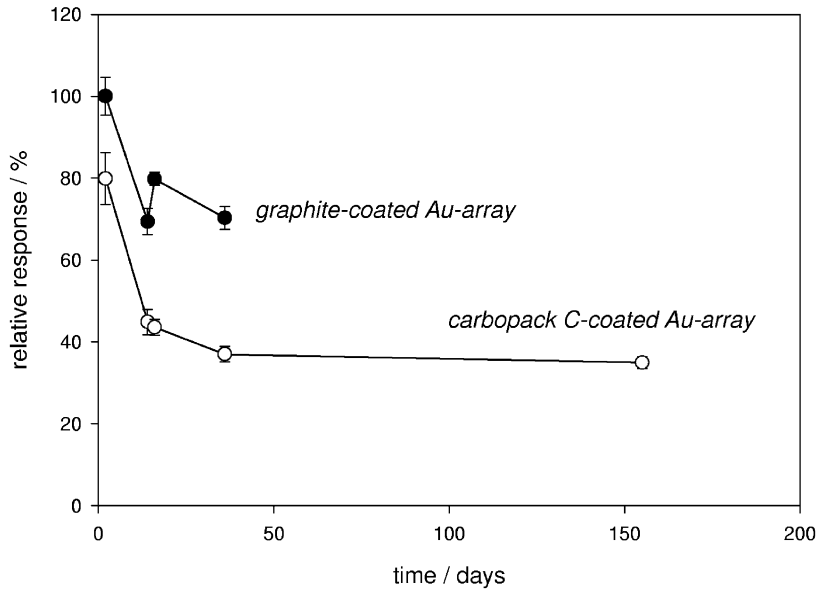

Fig. 4. Long-term stability of tyrosinase-modified four-channel biosensor arrays, based on graphite- and Carbopack C-coated Au-electrodes (type B array). Continuous measurements of 17 μM catechol are presented as the mean of 10 successive measurements for one electrode; tyrosinase-redox–hydrogel composition I (as indicated in Table 1). The average initial signal from the graphite-coated Au-electrode was chosen as the reference signal that all other signals were considered relative to. Other conditions are as given in Fig. 2.

tion and performance of tyrosinase biosensors. Cyclic voltammograms for catechol using a tyrosinase–hydrogel-modified Au-array is shown in Fig. 5d. The involvement of the Os-redox polymer in the chemical reactions at the electrode possibly relaxes the requirements for good reversibility of catechol.

4. Conclusions

The use of four-channel electrode arrays with tyrosinase entrapped in an osmium-based redox–hydrogel for the detection of catechol was shown. The concentration of the enzyme, redox polymer and cross-linking agent was optimised. After comparison of bare Au-, graphite coated Au-, and Carbopack coated Au-surfaces, it was concluded that the Carbopack C-coated electrode array was the best choice due to the higher mechanical stability of the resulting enzyme layer. It was also concluded that the reversibility of the electrochemical reaction for catechol on unmodified electrodes is not a limiting factor for the construction and performance of tyrosinase biosensors. The constructed sensors remained very stable under storage and could be used up to 6 months.

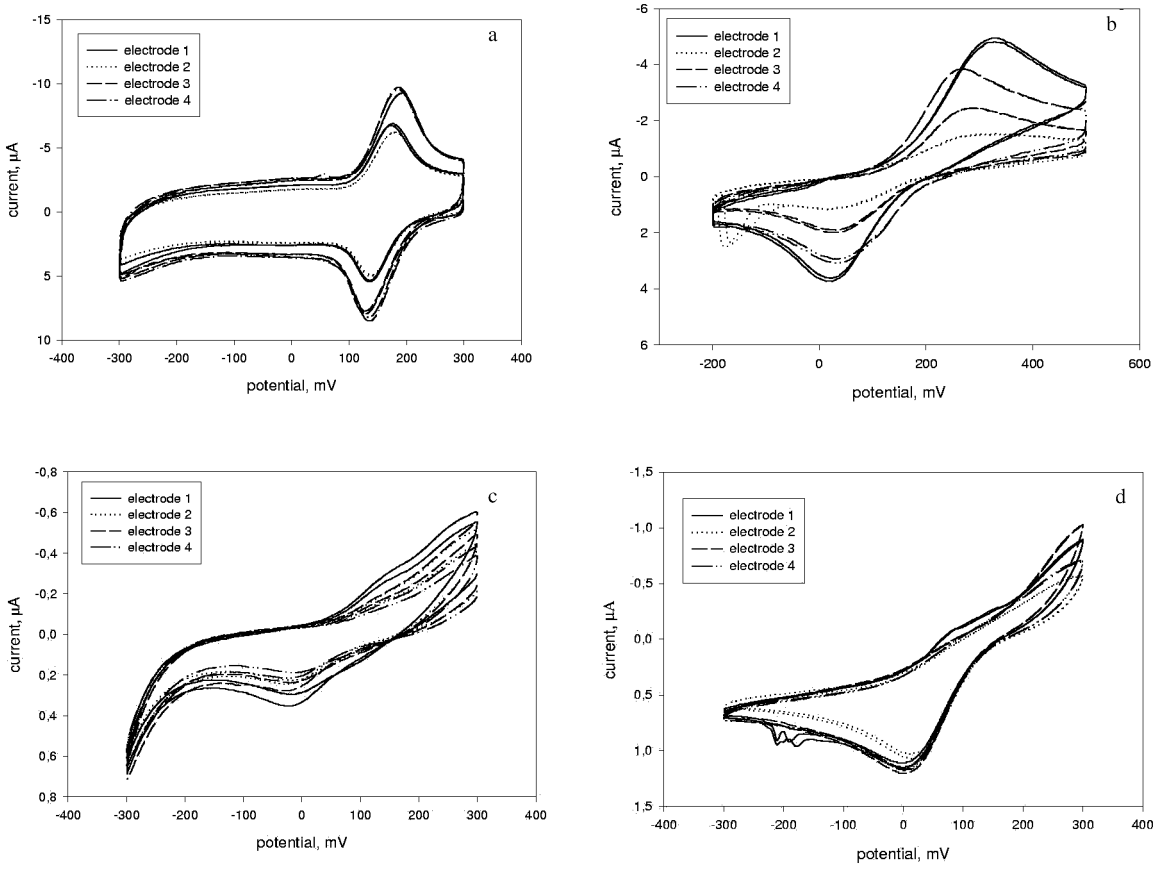

Fig. 5. Cyclic voltammograms of (a) graphite-coated Au-array, (b) Carbopack C- coated Au-array, (c) bare Au-array, and (d) tyrosinase-modified Au-array, tyrosinase-redox–hydrogel composition I (as indicated in Table 1). Conditions: 0.5 mM catechol (in 20 mM phosphate buffer with 0.1 M KCl pH 7.0). Scan rate 100 mV s^{-1}, sensitivity (a, b) 10 µA/V^{-1}, (c, d) 1 µA V^{-1}. Electrode numbers are corresponding to four electrodes on one array.

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