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BIOSENSOR FOR AMPEROMETRIC DETECTION OF CATECHOL BASED ON MUSHROOM TISSUE AND A METHYLENE GREEN-NAFION MODIFIED ELECTRODE

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ABSTRACT. An enzyme based biosensor for the amperometric detection of catechol is described. The bioelectrode architecture consists of a Nafion® film, as immobilization matrix, phenothiazine dye (Methylene Green, MG) as mediator and mushroom tissue as tyrosinase source. The electrochemical and electrocatalytical behavior of the bioelectrode was investigated by cyclic voltammetry and amperometry. The biosensor has a sensitivity of 2 $mA*M^{-1}$, with a detection limit of 20 μM catechol and reaches 95 % of the steady-state current within 50-100 s, when the applied potential was -0.05 V *vs*. Ag/AgCl, KCl_{sat}.

Keywords: Methylene Green, mushroom tissue, catechol, Nafion, amperometric biosensor.

INTRODUCTION

Most of the phenolic compounds are generated artificially and are present in wastewaters of chemical plants, exhaust gases of incinerators, sidestream smoke of cigarettes etc. [1]. They have toxic effects on animal and plants because of their skin and membrane penetration, determining a broad spectrum of genotoxic, mutagenic, carcinogenic and hepatotoxic effects, affecting also the biocatalysed reaction rates in respiration and photosynthesis [2]. The European Community directive (80/778/EEC) sets the maximum concentration permitted for all phenols in aquatic environments at 0.5 μg/l and at 0.1 μg/l for individual phenols [2].

The detection of mono- and polyphenols is usually performed by means of chromatography and/or spectrometry [2]. In particular, catechol could also be detected by inhibited chemiluminescence method [3]. Among the electrochemical methods, those using enzyme electrodes incorporating tyrosinase [4], lacasse [5] or peroxidase [1] are relative sensitive. For *in vivo* detection of catechol, the inhibitor effect of ascorbic acid could be eliminated using a Nafion film or a self-assembled monolayer [3]. There has been considerable interest in replacing isolated enzymes with tissue materials as the biocatalytic entity of electrochemical sensors. The major advantages resulting from the use of tissue materials are high stability and bioactivity (associated from the presence of the enzyme in large quantities in its natural environment), generally associated with low cost [6].

Tyrosinase (Ty) is a phenol oxidase present in microorganisms, animals and plants and is responsible for the browning of exposed surfaces on some fruits (i.e. banana) and tubers (i.e. potato) [7]. The two steps mechanism involving tyrosinase consists in the oxidation of monophenols or o-diphenols into their

corresponding o-quinones, with the expense of reducing oxygen to water [2]. The catalytic cycle of tyrosinase with catechol as substrate and subsequent electrochemical reduction of o-quinones occurring at the solution-electrode interface is presented in figure 1 [8].

Nafion is a linear copolymer derived from tetrafluoroethylene and perfluoro-sulphonic acid monomers. Over the last few years one important application of ion-exchange polymers was their use as immobilization matrices for different mediators or enzymes involved in the construction of amperometric biosensors [2].

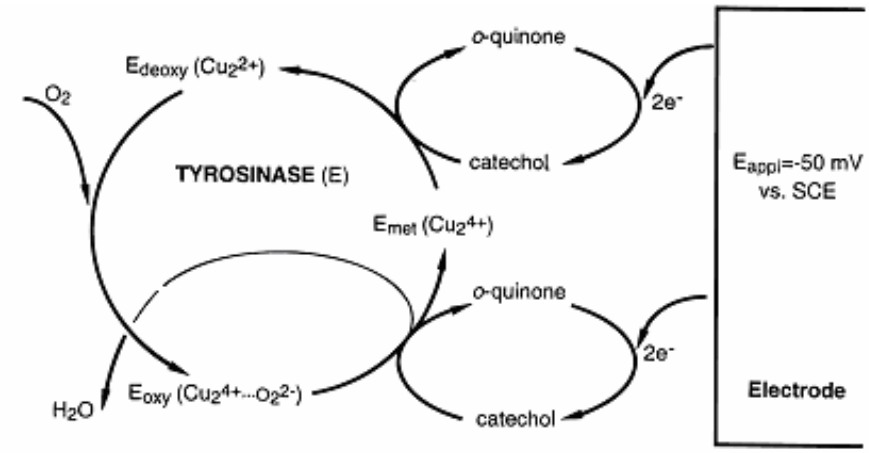

Figure 1. Catechol oxidation catalysed by tyrosinase at an enzyme-modified electrode [8].

Methylene Green, a water soluble phenotiazine derivative, has been used successfully as mediator either in solution, or immobilized on the electrode surface for H_2O_2 detection [9], NADH [10-11].

In the present work, a modified electrode was realized by incorporating Methylene Green into a Nafion thin layer on a graphite surface. The corresponding biosensor, based on tyrosinase was obtained by immobilizing on the tip of the modified electrode a thin layer of mushroom tissue using nylon net. The electro-chemical behavior of the modified electrode and its operational stability have been studied and the kinetic aspects of the catechol detection process were also investigated. The analytical parameters of the biosensor were evaluated.

EXPERIMENTAL

Reagent and Materials

Methylene Green (MG) was purchased from *Janssen Chimica (Beerse, Belgium*), Nafion® solution (5% in methanol with equivalent weight of about 1100) from *Fluka (Buchs, Switzerland)* and the phenol from "*Reactivul*" *Bucuresti.* A phosphate buffer solution (PBS, pH 7, ionic strength 0.1 M) was prepared from a 0.4 M NaH_2PO_4 and 0.4 M Na_2HPO_4 (*Sigma*) solutions and was always employed as supporting electrolyte. The mushrooms used throughout this study were purchased from a local grocery store and were stored at 5°C until use.

The 1 mM **MG** solution was prepared by dissolving its chloride salt in a 0.1 M phosphate buffer (pH 7). The Nafion 2.5 % solution was obtained by diluting in ethanol a 5% Nafion solution. The solution of 0.05 M PBS was prepared with distilled water. All chemicals were of analytical grade and were used without further purification. The buffer solutions were kept refrigerated to minimize bacterial growth.

Apparatus

Cyclic voltammetric investigations were carried out on a computer controlled Autolab voltammetric analyzer (*PGSTAT 10, EcoChemie, Netherlands*).

All measurements were done using a standard single-compartment three electrode cell equipped with a platinum counter electrode, an Ag/AgCl, KCl_{sat} reference electrode (*Radiometer*, *France*) and a spectral graphite (3 mm diameter) (*Ringsdorff-Werke, Gmbh, Bonn-Bad Godesberg, Germany*) working electrode. All experiments were performed at room temperature.

Modified electrode preparation

Before modification, the graphite electrodes were polished with fine emery paper and then were rinsed thoroughly with distilled water. 5 µl of 2.5% Nafion solution was dropped onto the electrode surface. After solvent evaporation, the Nafion-coated graphite electrode was immersed into a 1 mM MG solution and the MG incorporation into Nafion film was electrochemically controlled by scanning the electrode potential in the range -0.7 to +0.7 V *vs*. Ag/AgCl, KCl_{sat}, for a specified number of cycles. The obtained modified electrodes were symbolized by G/Nafion-MG25, G/Nafion-MG50 and G/Nafion-MG100 where the associated number indicates the number of CV cycles used for MG incorporation.

The Ty modified electrode was obtained by immobilizing of a thin layer mushroom tissue on the tip of the graphite or of the MG modified electrode, using a Nylon membrane. The obtained bioelectrode was symbolized by G/Ty or G/Nafion-MG/Ty, respectively.

RESULTS AND DISCUSSION

Electrochemical behavior of G/Nafion-MG modified electrodes

The cyclic voltammograms recorded at G/Nafion electrode, in contact with a 1 mM MG/phosphate buffer, showed two couples of redox peaks at a formal standard potentials ($\varepsilon^{\circ\prime}$; calculated as $(\varepsilon_{pa} + \varepsilon_{pc})/2$, where ε_{pa} is the potential of the anodic peak and ε_{pc} is the potential of the cathodic peak) of -0.325 V *vs*. Ag/AgCl, KCl_{sat} (peak A1/C1) and -0.105 V *vs*. Ag/AgCl,KCl_{sat} (peak A2/C2) (Figure 2.A). Both peaks pairs correspond to a single electron transfer, involved in the MG electrode reaction [10].

The increase of the peak currents for A1/C1 couple, with the increase of the number of cycles proves an increase of the MG concentration in the Nafion film. This immobilization/inclusion of MG within Nafion matrix is supposed to be facilitated by the electrostatic attraction between the negatively charged sulfonic acid groups of Nafion and the positively charge of the oxidized state of MG [9], as well as to the partial electropolymerization of MG. The second hypothesis may be advanced because after the first cycle, which shows two distinct pairs of peaks, in the following cycles the A2/C2 peaks progressively disappear and at the same time, the $W_{1/2}$ value of A1/C1 peaks increase [10].

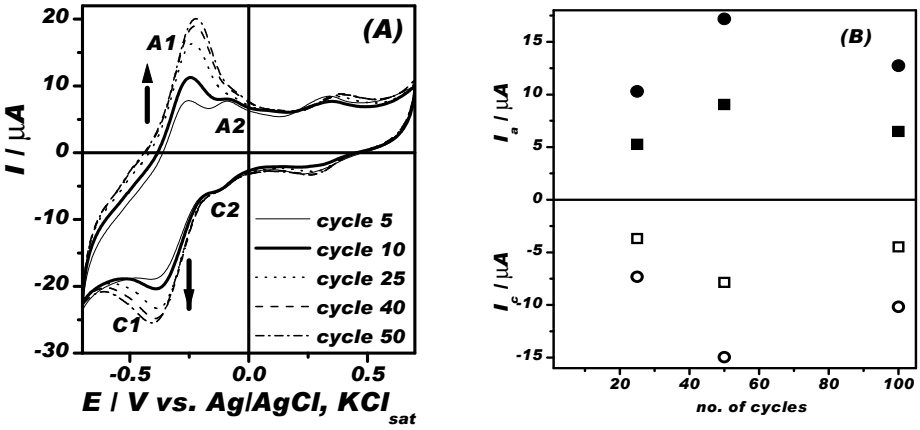

Figure 2. Cyclic voltammograms of G/Nafion electrode (**A**) and the dependence of the peak intensity for A1/C1 couple on the number of potential cycles (**B**). Experimental conditions: 1 mM MG in phosphate buffer 0.1 M (pH 7); starting potential, -0.5 V *vs*. Ag/AgCl/$KCl_{sat.}$; potential scan rate, 50 $mV*s^{-1}$ (**A**) 25 $mV*s^{-1}$ (■,) or 50 $mV*s^{-1}$ (●, ○) (**B**).

After 50 cycles of potential scanning, the concentration of MG in the Nafion matrix reaches a maximum (figure 2.B).

The electrochemical behaviour of the G/Nafion-MG modified electrode was studied with respect to the potential scan rate (Figure 3A). In the investigated potential range, irrespective the modified-electrodes preparation, only one couple of peaks (A1/C1) appears at approximately the same formal standard potential: -0.110 V, -0.130 V and -0.125 V *vs*. Ag/AgCl, KCl_{sat}, for G/Nafion-MG25, G/Nafion-MG50 and G/Nafion-MG100 respectively. Comparing the average formal standard potential with that reported for MG in aqueous solution (-0.070 V *vs*. SCE [11]), a negative potential shift was observed, indicating a stability increase of the MG incorporated in the Nafion matrix. In literature, this peak was attributed to the MGH/MG^+ couple involved in the global reaction [9]:

$$MGH \rightarrow MG^+ + H^+ + 2e^- \quad (1)$$

As showed by the peak separation ($\Delta\varepsilon_p = \varepsilon_{pa} - \varepsilon_{pc}$), which is significantly different from zero even at a low scan rate, the rate of the electron transfer between MG and the electrode is an important aspect to be considered in the investigated system [11]. For all type of investigated modified-electrodes, the peak potential separation is almost constant in the range of investigated potential scan rates (from 10 to 200 $mV*s^{-1}$) and does not exceed 100 mV [12], suggesting that the electron transfer process is not fully reversible.

This behaviour can be assigned to the presence of a small resistance at the electrode/solution interface [11], which could be due either to the difficulty of the supporting electrolyte species to diffuse through the Nafion matrix [11], or to a slower electron transfer rate between MG-incorporated into Nafion matrix than that of MG adsorbed on the glassy carbon electrode [9].

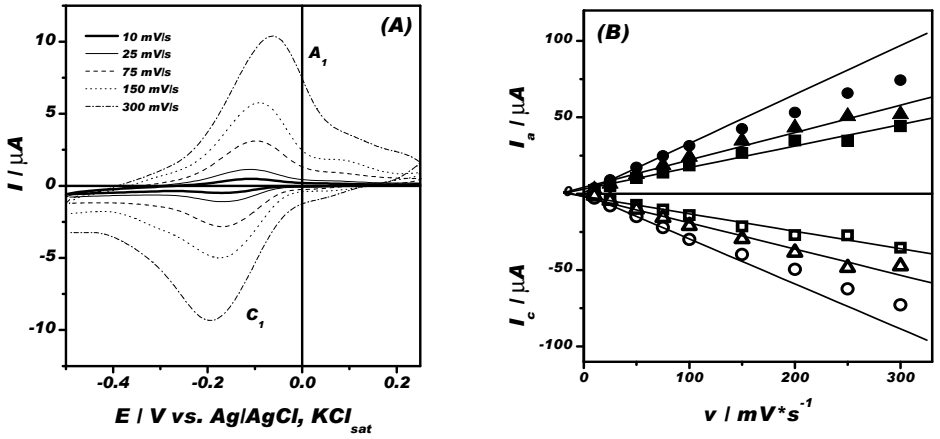

Figure 3. Influence of the scan rate on the cyclic voltammograms recorded at G/Nafion-MG50 electrodes (**A**) and the relationship between the peak current and the scan rate (**B**). Experimental conditions: phosphate buffer 0.1 M, pH 7; starting potential, -0.5 V *vs*. Ag/AgCl/ KCl_{sat}; G/Nafion-MG25 (■, □), G/Nafion-MG50 (●, ○) and G/Nafion-MG100 (▲, Δ).

Another aspect that should be emphasised is the linear correlation observed between the peak currents and the potential scan rate (figure 3B and table 1). This behaviour suggests that MG is confined on the electrode surface and the redox process involving MG is not affected by the mediator diffusion [11].

Table 1.

The parameters of the linear regression for the log (I_p/μA) *vs*. log(v/mV*s^{-1}) dependence.

Electrode	slope	R/n
G/Nafion-MG25	0.91 ± 0.01	0.9997/ 7
G/Nafion-MG50	0.87 ± 0.01	0.9991/ 9
G/Nafion-MG100	0.93 ± 0.01	0.9996/ 7

The stability of the G/Nafion-MG modified electrodes

The stability of the modified electrode was tested, simulating the operational conditions, by repetitive potential cycling in the potential range from -0.5 to 0.25 V. Using the surface coverage (Γ_0^* /mol cm^{-3}), estimated from the integrated anodic peak recorded at low scan rate (10 mV s^{-1}) [12], the activation constants for G/Nafion-MG25 ($4.82*10^{-12}$ s^{-1}) and for G/Nafion-MG50 ($3.02*10^{-11}$ s^{-1}), as well as the deactivation constant for G/Nafion-MG100 ($1.37*10^{-11}$ s^{-1}) were evaluated. This estimation was done form the plots $ln\Gamma_0^*$ *vs*. time, supposing a first order kinetic for both processes. The activation process could be due to the MG rearrangement into the Nafion matrix during the electrode potential scanning. Contrarily, the deactivation process could be assigned to the loss of MG excess, incorporated in the polymer matrix.

In conclusion, because the activation constant and the sensitivity of the MG inclusion in Nafion matrix are the highest for the G/Nafion-MG50 electrode, this type of electrode was used for further experiments**.**

Bioelectrocatalytic detection of catechol at G/Nafion-MG/Ty biosensor

The presence of MG mediator could modify the detection scheme of catechol (figure 4A) as is presented in figure 4B.

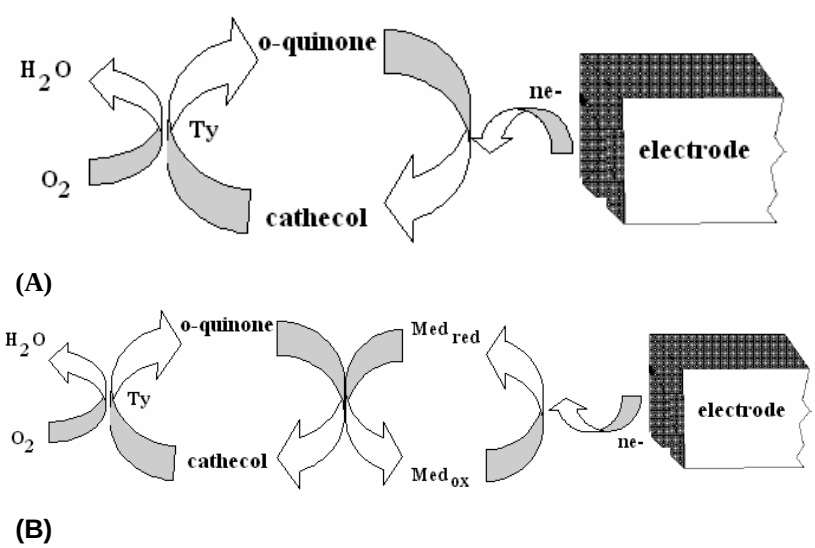

Figure 4. Catechol detection scheme in the absence (**A**) and in the presence of a mediator (**B**).

The cyclic voltammograms recorded at G/Nafion-MG50/Ty bioelectrode in the absence and in the presence of catechol are displayed in figure 5. It can be seen that, in the presence of Ty and of catechol, there is a great increase in the current intensity of peaks A1/C1, corresponding to the MGH/MG+ couple, comparatively to those observed in their absence. This current increase was supposed to be due to the restricted diffusion of MG towards solution side. The A3/C3 pair of peaks at a formal potential of -0.045 V *vs*. Ag/AgCl, KCl_{sat} was attributed to the catechol / quinone redox couple, existing in the mushroom tissue.

Also, in the presence of MG, a slight increase of the current intensity for C3 peak was noticed: from $–3.978*10^{-6}$ A in the absence of catechol to $–4.964*10^{-6}$ A in the presence of 0.56 mM catechol.

The amperometric response of the G/Nafion-MG50/Ty bioelectrode for different catechol concentration is described by a Michaelis-Menten type calibration curve. The steady-state current was measured at a fixed potential of -0.05 V *vs*. Ag/AgCl, KCl_{sat}. The analytical parameters calculated in the absence and in the presence of MG are presented in table 2.

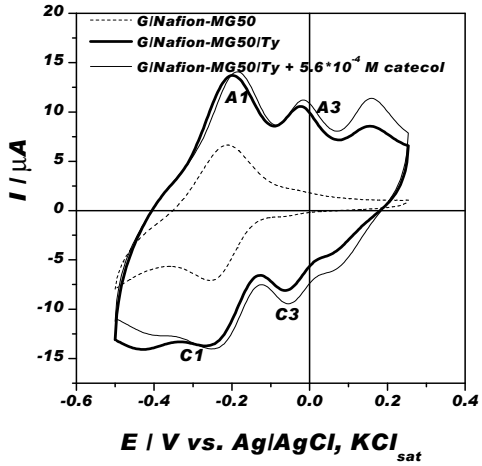

Figure 5. Cyclic voltammograms of the G/Nafion-MG/Ty bioelectrode in the absence (▬) and in the presence of 0.56 mM catechol (——). Experimental conditions: phosphate buffer 0.1 M, pH 7; scan rate, 50 mV/s.

Table 2.

Analytical parameters for G/Ty and G/Nafion-MG50/Ty.

Parameter	G/Ty	G/Nafion-MG50/Ty
Sensitivity / mA*M^{-1}	22	2
Linear range / μM	5 – 50	100 – 300
Detection limit* / μM	2.5	20
Response time ($t_{95\%}$) / s	50 - 100	50 - 100

* The detection limit was calculated considering the signal as three times the noise.

The biosensor response time could be influenced by one of the following rate limiting steps: (i) the diffusion and solubility of substrates (catechol and oxygen) into the vegetal tissue; (ii) the enzymatic reaction kinetics; (iii) the electrochemical reduction of the o-quinone at the electrode surface [2]. Generally, the biosensor response is kinetically controlled by the biochemical reaction rate. However, the presence of the polymer layer and the vegetal tissue on the surface of the working electrode acts as diffusion barrier for the substrates and/or the products, inducing an increase of the biosensor response time [2, 9].

If the solution stirring rate is sufficiently fast and the pore size of the vegetal tissue is relatively large, the diffusion through the vegetal tissue is not critical for the overall reaction rate. The presence of the polymer layer on the working electrode surface and/or the strong electrostatic forces existing between MG^+ and SO_3^- groups [9] will hinder the diffusion of the substrates (oxygen and catechol) as well as the diffusion of the reaction products, resulting in increase of the biosensor response time [2].

The kinetic parameters values for the G/Ty bioelectrode without MG were estimated by fitting the experimental data with the Michaelis-Menten equation as well as by different linearizations. Almost identical values for the apparent Michaelis-Menten constant ($K_{M,app}$ ~ 0.17 mM) and maximum current ($I_{max,app}$ ~ 3.7 μA) were obtained in all cases. The sensitivity (defined as the ratio $I_{max,app}$/ $K_{M,app}$) was found of 21.8 mA/M. The values are in good agreement with those reported in literature for similarly architectures [2].

CONCLUSIONS

A biosensor using a simple immobilization method for enzyme and a low cost enzyme source from a vegetal tissue was developed. The obtained results suggest that the MG molecules were anchored into the Nafion polymer film either by electrostatic force between MG^+ and HSO_3^- [9], either by a partial electropolymerization of the MG monomer [10]. The high ionic conductivity, the high solubility of oxygen, the biocompatibility and the high stability of Nafion make it a particularly good matrix for MG immobilization [2]. Also, it was demonstrated that this type of positively charged mediator can be used to improve the electron transfer in the investigated system. The design of such architecture for the biosensor construction is promising for rapid low-cost toxicity testing.

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