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Short communication

CYP450 biosensors based on gold chips for antiepileptic drugs determination

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Abstract

Three-electrode configuration chips containing a Pt, Au and a screen-printed Ag/AgCl as counter, working and reference electrode, respectively, have been developed. Selective determination of Phenobarbital (PB) has been carried out by Cytochrome P450 2B4 (CYP450) immobilization into a polypyrrole matrix onto the gold working electrode. Chronoamperometric experiments show a PB diffusion coefficient of 2.42×10^{-6} cm^2 s^{-1}, a reproducibility and repeatability in terms of residual standard deviation (RSD) of 13% and 5.51%, respectively, and a limit of detection (LOD) of 0.289 μmol dm^{-3} ($\alpha = \beta = 0.05$) for the developed CYP450-biosensor chip. Its performance has been showed by the determination of PB in pharmaceutical drugs. HPLC has been used as reference technique.

Keywords: Biosensor; Microelectronic techniques; Screen-printed Ag/AgCl reference electrode; Phenobarbital; Cytochrome P450 2B4

1. Introduction

Epilepsy is a common chronic neurological disorder, suffered from millions people worldwide. Epilepsy is usually controlled with medication; therefore, several anticonvulsant drugs (Stefan and Feuerstein, 2007) have been developed.

Anticonvulsant drugs of the first generation – phenobarbital (PB), primidone (PRD), phenytoin (PHT), carbamazepine (CBZ) and valproic acid (VPA) – have an increased potential for interactions and side effects due to enzyme induction and/or inhibition. Long-term use can give rise to changes in bone and connective tissue or hormonal-metabolic disturbances leading to sexual dysfunction (Stefan and Feuerstein, 2007). Therapeutic drug monitoring can increase the clinical efficacy while minimizing adverse effects (Zhang et al., 1999).

In 1949, a colorimetric method for the determination of PB was described (Jones and Howland, 1949). Since then, hundreds of PB determinations have been described based on spectrophotometric (Boeris et al., 2000), chromatographic (Cavazos et al., 2005; Vermeij and Edelbroek, 2007), phosphorimetric (Wineford and Tin, 1965), liquid–liquid extraction (Kelsey and Connors, 1965), electrophoresis (Jiang et al., 2007), and immunoassay methods (Sayo et al., 1988). In spite of the high sensitivity, simplicity and inherent miniaturization of electrical assays, only a few electrochemical determinations have been reported (Bordes et al., 1999; Ni et al., 2004; Romer et al., 1977; Temizer and Solak, 1986; Zhang et al., 1999). Moreover, the combination of an electrochemical transducer and a biological element, which confers high specificity to the sensor, has only been shown in the case of electrochemical immunoassays. Due to their specificity, speed, portability and low cost, biosensors offer exciting opportunities for numerous decentralized clinical applications (e.g., home self-testing) (Wang, 2006). Thus, a gold chip biosensor based on cytochrome 450 (CYP450) has been developed in this research work for the determination of PB in different kind of samples, such as synthetic samples (model solution) and pharmaceutical preparations.

CYP450 is an important family of heme-containing hydroxylases, found in most organisms from bacteria to human beings (Metzler, 2001). PB is one of the preferred CYP450 2B substrates. The general characteristic of this enzyme substrates

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include non-planar molecular geometries couple with a relatively high lipophilicity, together with the presence of one or two key hydrogen bond-forming groups and, usually, at least one aromatic ring adjacent to a tetrahedral carbon often imparting a V-shaped conformation to the structure (Lewis, 2001). In essence, the CYP450-mediated reaction involves the combination of oxygen with the organic substrate (RH) to produce a molecule of water and a mono-oxygenated metabolite (ROH) (Metzler, 2001; Shumyantseva et al., 2004).

CYP450 2B4 can be immobilized onto the working electrode surface by electropolymerization. Thus, native structure and appropriate orientation can be achieved, increasing electron transfer between the enzyme and the electrode (Bistolas et al., 2005). Here, CYP450 2B4 has been immobilized onto a gold working electrode by pyrrole (py) electropolymerization. Polypyrrole (PPy) is one of the most studied conducting polymers because of its easy preparation, high conductivity and relative stability (Sabah et al., 2006).

As only those biomolecules present in the vicinity of the electrode surface are incorporated in the growing polymer (Cosnier et al., 1998), high concentrations of biomolecules in the aqueous electrolyte during the electropolymerization process are required. Consumption of large quantities of biological reagents can be avoided using electrochemical cells based on microelectronic configurations. Therefore, miniaturized amperometric silicon-based transducers (3 mm × 3 mm), containing the three-electrode configuration, have been developed by means of photolithographic techniques (Alonso-Lomillo et al., 2005). Au and Pt have been used for the working and counter electrodes, respectively. In order to get the reference electrode, Ag/AgCl has been printed at wafer level by screen-printing techniques.

Experimental variables in the chronoamperometric determination of PB have been optimized by means of the experimental design methodology. Reproducibility, repeatability and limit of detection of the CYP450 biosensor have been analyzed. In order to check the performance of the proposed method, the sensor has been applied to the PB determination in pharmaceutical drugs.

2. Experimental

2.1. Reagents, equipment and software

2.1.1. Reagents

In the fabrication of the screen-printed reference electrode at wafer level, Ag/AgCl ink (Electrodag 6037) from Acheson Colloids Company (Port Huron, USA) was used.

CYP450 2B4 (C9095-15Y, USBiological, Swamscott, Massachusetts, USA) was used as received.

Solutions of 0.05 mol dm^{-3} pyrrole (Sigma, Steinheim, Germany), 0.1 mol dm^{-3} $LiClO_4$ (Panreac, Barcelona, Spain) were prepared by dissolving the adequate amount of each one in Milli-Q water.

0.05 mol dm^{-3} phosphate buffer pH 9, 0.1 mol dm^{-3} KCl was used as supporting electrolyte.

For the chromatographic analysis, acetonitrile–water (25:75, v/v) adjusted to pH 2.5 with H_3PO_4 was used as mobile phase.

Stock solutions were prepared by dissolving the appropriate amount of PB (Sigma, Steinheim, Germany) in ethanol–water (10:90, v/v) and in mobile phase for the electrochemical and chromatographic measurements, respectively.

2.1.2. Equipment

Voltammetric and amperometric measurements were carried out in bulk solution using a μAutolab Type II Potentiostat (Eco Chemie, BV, The Netherlands).

HPLC analysis was performed on a chromatographic system equipped with a HPLC-Pump K-120 (Knauer, Berlin, Germany), a Knauer injector and a 254 nm fixed wavelength detector K-200 (Knauer, Berlin, Germany). The HPLC column (VERTEX column, Eurosphere 100 C18, 5 μm, 125 mm × 4 mm) was also bought from Knauer (Berlin, Germany).

2.1.3. Software

Amperograms and voltamperograms were acquired and processed by using the General Purpose Electrochemical System Software. A EuroChrom 2000 software was used to collect, integrate and analyse the chromatographic data.

STATGRAPHICS PLUS (STATGRAPHICS, Copy 1994–2001) was used for experimental design and data analysis, PROGRESS for the robust regressions (Rousseeuw and Leroy, 1989) and DETARCHI for estimation of the detection limit (Blanco et al., 1994; ISO11843-2, 2000).

2.2. Electrode preparation

Three-electrode configuration chips (3.0 mm × 3.0 mm) were fabricated over a silicon wafer by photolithographic and screen-printing techniques (Fig. 1).

Thermal oxide layer (800 nm thick) was grown as insulating substrate on a 4 in, diameter silicon wafer. This was followed by a metallization step, carried out by electron gun system. A titanium layer acting as adhesion promoter (20 nm) was followed by a platinum layer (150 nm). Subsequent patterning was done by lift-off procedure in order to form the platinum electrodes. Following this step, Ti (20 nm), Ni (20 nm) and Au (20 nm) were deposited by physical vapour deposition (PVD) and patterned by wet etching to define the gold working electrode and the contact paths of the electrochemical sensor. Finally,

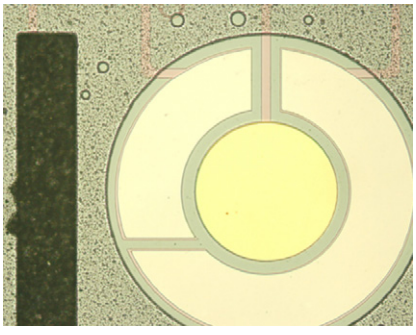

Fig. 1. Microscopic image of a three-electrodes configuration chip developed by photolithographic and screen-printing techniques.

a passivation layer was deposited onto the wafer for electrical interference prevention and chemical protection. Silicon oxynitride layer (1 μm) was grown by plasma-enhanced chemical vapour deposition (PECVD-SiO_xN_x). This layer was patterned and those areas acting as working (0.5 mm^2), counter electrode (1.36 mm^2) and a reference electrode (0.35 mm^2) were opened by dry etching (reactive ion etching). A thick-film reference electrode was fabricated by conventional screen-printing technique (Desmond et al., 1997), over the electrode defined as reference one. Ag/AgCl ink was printed through patterned stencil over the amperometric transducers at wafer level. Then, a drying cycle (80°/30 min + 120°/5 min) was applied.

After the fabrication at wafer level, the chips were separated and surface mounted on test printed circuit boards (PCB), using an UV-curable polymer as encapsulant.

Gold working electrodes were first treated by successive cycling in buffer solution between −1.2 V and −2.2 V vs. Ag/AgCl, 3 mol dm^{-3} KCl (10 cycles; scan rate, 100 mV/s) to clean and activate the electrode surface. This procedure was performed until reproducible phosphate voltammograms were recorded between −0.5 V and 0.3 V vs. Ag/AgCl, 3 mol dm^{-3} KCl. This pretreatment was employed before each electropolymerization.

2.2.1. CYP450 2B4 immobilization

A prelayer of PPy (0.1 mol dm^{-3} $LiClO_4$, 0.05 mol dm^{-3} py) was first deposited by scanning the potential between 0 V and +0.9 V vs. Ag/AgCl 3 mol dm^{-3} at a scan rate of 10 mV s^{-1}, for one complete cycle, at room temperature (Alonso-Lomillo et al., 2003, 2005). The screen-printed reference electrode was used, which allows a small volume of solution (1 ml) to be used. The oxidized form of the polymer is positively charged and requires the incorporation of doping anions, $LiClO_4$, in order to maintain its electroneutrality (Gros and Comtat, 2004). The association between polymer cations and dopant anions depends on the electrochemical conditions applied during the process of polymerization. So, in addition to the proper distribution of positive charge for anion recognition functionality, the polymers have to carry pores of suitable size, which form the host cavities for dopant anions. These steric requirements can restrict possible interference from other ions. Therefore, the sensors based on polymers, which have such additional recognition principles could also have better selectivity in comparison to other types of sensors (Sabah et al., 2006).

The PPy-CYP450 2B4 film was subsequently grown onto the gold working electrode. The electrode was immersed in a solution containing 0.1 mol dm^{-3} $LiClO_4$, 0.05 mol dm^{-3} pyrrole, and 0.133% (v/v) of the CYP450 2B4 solution at room temperature. The potential domain was scanned twice between 0 V and +0.9 V vs. Ag/AgCl 3 mol dm^{-3} at a scan rate of 10 mV s^{-1}.

After each measurement, the biosensor was regenerated by immersing it in an unstirred blank buffer solution for 30 min at 4 °C, in order to wash away the analyte from the polymer film.

3. Results and discussion

3.1. Sensor characterization

First, the stability of the developed reference electrode was tested. Its potential stability was measured in a 3 mol dm^{-3} KCl solution vs. a conventional reference electrode Ag/AgCl 3 mol dm^{-3}. A medium value of potential of 62.0 ± 0.89 mV ($n = 3$, $\alpha = 0.05$), with a drift lower than 0.23 mV h^{-1}, was recorded for at least 10 h, with an elapse time of 2 h due to the electrode hydration.

Moreover, the long-term stability was checked by measuring the potential value in a 0.1 mol dm^{-3} KCl solution. The potential measurement was constant for 20 days.

According to the Nernst equation, the electrochemical potential generated by the Ag/AgCl electrode is directly proportional to the chloride ion activity and, at 25 °C,

$$E = E_0 - 59.16 \log a_{Cl-}. \quad (1)$$

Several calibration curves were performed by ranging the chloride concentration from 0.001 mol dm^{-3} to 0.1 mol dm^{-3}. A slope of 56.8 ± 1.5 mV was obtained, which indicates a Nernstian behaviour for the developed screen-printed reference electrode.

The behaviour of the reference electrode was studied when the chloride concentration drastically changes (3 mol dm^{-3} and 0.1 mol dm^{-3}). An equilibrium potential was immediately reached, keeping a stable value of potential while chloride concentration is not changed.

Finally, the performance of the reference electrode was also checked by recording the typical cycle voltammograms of a 5 mmol dm^{-3} ferricyanide in 0.1 mol dm^{-3} phosphate solution (scan rate 100 mV s^{-1}). Voltammograms were recorded by using the developed electrode and a conventional one (3 mol dm^{-3} as inner solution), obtaining good agreement between them (Fig. 2).

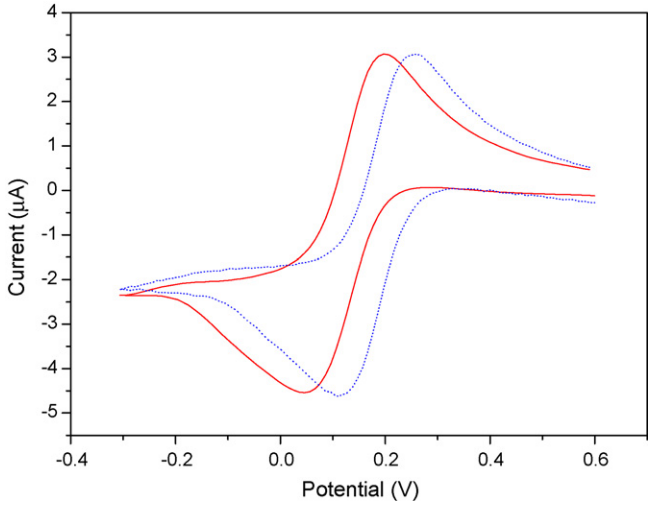

Fig. 2. Cyclic voltammograms of a 5 mmol dm^{-3} ferricyanide solution using screen-printed Ag/AgCl (solid line) and a commercial Ag/AgCl 3 mol dm^{-3} (dash line) reference electrodes. Scan rate: 100 mV s^{-1}.

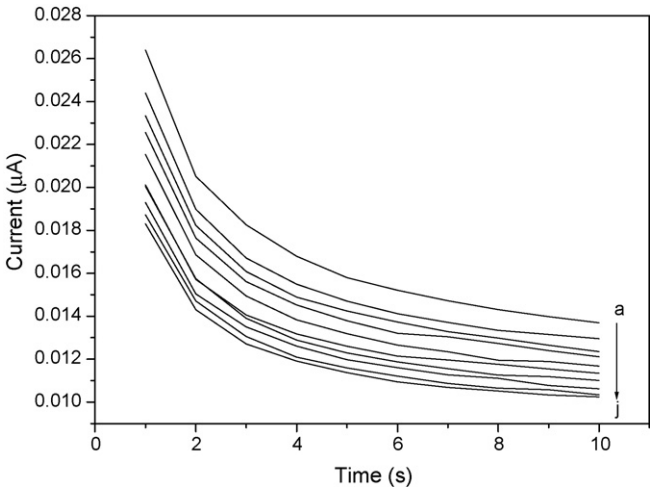

Fig. 3. Chronoamperograms of CYP450 2B4-PPy gold biosensor chip in different concentration of PB, a–j: 0.32, 0.63, 0.91, 1.18, 1.43, 1.67, 1.89, 2.11, 2.31, 2.50 μmol dm^{-3}. pH 9 and operating potential (E_{ap}) = 0.45 V.

3.2. Determination of the diffusion coefficient of PB

The diffusion coefficient of PB in solution can be estimated by using chronoamperometric experiments (Yu et al., 2007) and Cottrell's equation,

$$I = nFAD^{1/2}c\pi^{-1/2}t^{-1/2} \quad (2)$$

where D and c are the diffusion coefficient (cm^2 s^{-1}) and bulk concentration (mol cm^{-3}), respectively, and A was the electrode area (cm^2).

Fig. 3 shows the chronoamperograms at various PB concentrations for a CYP450 2B4-PPy gold biosensor chip. The plot of the current controlled by the diffusion of PB from the bulk solution to the film/solution interface vs. $t^{-1/2}$ gave straight lines. The slopes of the resulting straight lines were then plotted vs. the PB concentration, from whose slope (2.66) the diffusion coefficient D could be calculated. According to the well-accepted general mechanism of the CYP450, two electrons are transferred in the reactions (Lewis, 2001; Shumyantseva et al., 2004). Thus, the diffusion coefficient was found to be 2.42×10^{-6} cm^2 s^{-1}.

3.3. Chronoamperometric determination of PB at CYP450 2B4-PPy gold biosensor chip

The mechanism of the enzymatic reaction is based on the substitution of the biological electron delivery and transport system by the electrode (Shumyantseva et al., 2004). In order to get the maximum current response for PB, the operating potential (E_{ap}) in chronoamperometry and the pH of the solution were investigated. A 2^2 central composite design was carried out for the optimization of these parameters, taking the intensity registered at 50 s for a 3.22×10^{-5} mol dm^{-3} solution of PB as response (Alonso-Lomillo et al., 2003).

From the optimisation process based on the experimental design methodology, the following optimum values for the experimental variables in the PB determination were taken

$E_{ap} = 0.45$ V pH9.0.

The py oxidation in water solution commences around 0.65 V vs. SCE. Being less positive than this potential, the selective E_{ap} keeps the film active. A reduction in activity appears to take place gradually and has been attributed to a decrease in conductivity of the film due to oxidation reactions (Sabah et al., 2006).

Fig. 4 shows the chronoamperogram of the modified electrode during the successive addition of 100 μl of a 10^{-5} mol dm^{-3} PB solution, registered under the optimum conditions. Measurements were taken under stirring conditions.

Control experiments were done in which no CYP450 was deposited. A poor chronoamperometric response for PB was obtained in comparison to the developed biosensor.

The chronoamperograms recorded at 1.43 μmol dm^{-3} of a PB solution were used to characterise the biosensor reproducibility and repeatability. Residual standard deviation (RSD) values of 13.68% and 5.51% ($n = 3$, $\alpha = 0.05$) were obtained, respectively.

The limit of detection (LOD) was also assessed. Calibration curves in the concentration range 0.32–2.50 μmol dm^{-3} of PB were obtained from those chronoamperometric responses at 50 s. The parameters of these calibrations were optimally evaluated. In order to avoid incorrect adjustments due to the existence of anomalous points, least median squares regression (LMS) was used (Massart et al., 1986; Rousseeuw and Leroy, 1989). Then, the detection limit was calculated using the DETARCHI program, with evaluation of the probability of false positive (α) and negative (β) (Alonso-Lomillo et al., 2003; Blanco et al., 1994; ISO11843-2, 2000). For $\alpha = \beta = 0.05$, the LOD medium obtained was 0.289 μmol dm^{-3}.

Antiepileptic drugs such as primidone, ethosuximide and phenytoin did not hinder the analysis of PB, since at a concentration of 1.64×10^{-5} mol dm^{-3} these compounds gave no response under the optimised conditions.

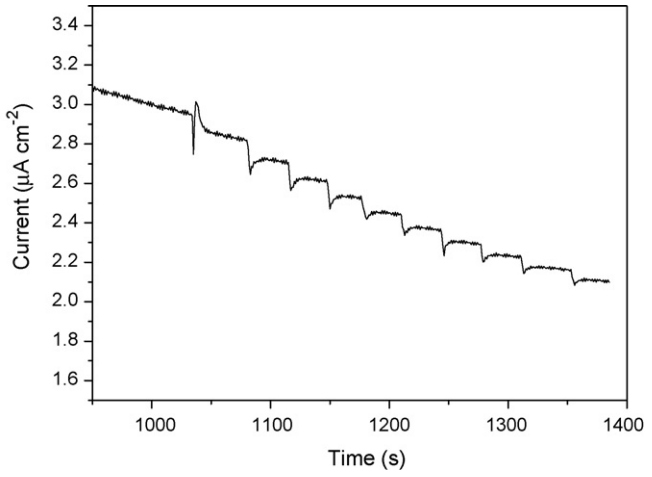

Fig. 4. Chronoamperogram of CYP450 2B4-PPy gold biosensor during the successive addition of 100 μl of a 10^{-5} mol dm^{-3} PB solution, registered under the optimum conditions.

3.4. Determination of PB in pharmaceutical drugs

The CYP450 2B4-PPy gold biosensor chip was applied to the determination of PB in LUMINALETAS® by Kern Pharma. A tablet was dissolved into ethanol-water (10:90, v/v), sonicated until complete dissolution, centrifuged and finally, filtered.

Next, the PB concentration was evaluated by comparison with a standard addition of identical volume (50 μl) of a 10^{-3} mol dm^{-3} PB solution to a sample of the dissolved drug. The concentration found was 3.93 mmol dm^{-3} ± 0.80 ($n = 3$ and $\alpha = 0.05$).

This value was checked by HPLC determination. A calibration curve in peak area ratios was obtained over the concentration range 0.004–0.0032 mg mL^{-1} for PB. The PB peak area found in the LUMINALETAS® solution was [0.564 ± 0.028] ($n = 5$, $\alpha = 0.05$), which corresponds to 4.049 mmol dm^{-3} of a PB in the stock solution.

4. Conclusions

Since PB is one of the preferred substrates of CYP450, this enzyme has been used as the biological component in the development of biosensors for the selective determination of this antiepileptic drug. In order to use a small cell volume and, therefore, small amount of reagents, three-electrode configurations chips have been fabricated by photolithographic and screen-printing techniques. Screen-printed Ag/AgCl reference electrodes have been characterized. The viability of these electrodes for further applications has been showed. Thus, CYP450 was immobilized by Py electropolymerization onto the gold working electrode.

Chronoamperometric experiments enabled determination of PB diffusion coefficient, as well as the concentration of PB in different kind of samples. Once experimental variables have been optimized through the experimental design methodology, reproducibility, repeatability and LOD have been calculated. Finally, the performance of the biosensor has been checked by applying it to the determination of PB in pharmaceutical drugs. PB concentration has been analyzed in LUMINALETAS® by the standard addition method. HPLC, which has been used as reference technique, showed good agreement with the proposed method.

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