Digest Journal of Nanomaterials and Biostructures Vol. 6, No 2, April - June 2011, p. 731- 738

CONSTRUCTION, ACTIVATION AND MODIFICATION OF PLATINUM MICROELECTRODES FOR (BIO)SENSORS DEVELOPMENT

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This paper presents the construction and surface activation of vacuum-deposed microelectrodes tested for the detection of biologically significant compounds like hydrogen peroxide and L-cysteine. It was demonstrated that the platinum working electrode surface etching by cyclic voltammetry in sulfuric acid aqueous solution lead to a significant amperometric analytical signal increase. The microelectrodes were tested as transducers for biosensor development by immobilizing acetylcholinesterase using diazonium chemistry.

(Received March 14, 2011; Accepted April 8, 2011)

Keywords: Platinum microelectrodes, Vacuum-deposition, Microelectrode surface activation, Microelectrode modification

1. Introduction

The size of microelectrodes allows the investigation *in vivo* of biochemical phenomena localized at tissue or even cellular levels and thus they are very useful in biology, medicine and environmental sciences [1]. In analytical chemistry, the microelectrodes are useful as detectors in microtechniques like capillary electrophoresis [2] or lab-on-a chip applications [3], but also allow the improvement of the detection limits due to their specific signal/noise ratio in comparison with classical electrodes [4]. Due to their small surface, the microelectrodes have low analytical signals and need special protocols for surface activation/modification or protein immobilization. In order to fulfill the specific demands of each practical application, the microelectrode must be modified with electrochemical mediators [5], enzymes [6], antibodies [7] or both mediators together with an enzyme [8]. A synergistic effect was reported for the coupling of mediators with carbon nanotubes [9].

The microelectronics technologies allow the manufacturing of thin metallic layer microelectrodes used as transducers in analytical chemistry. Glass, ceramics or semiconductors with an insulator layer of SiO_2 are used as substrate. The electrodes have a multilayer conformation. First is deposed a Cr or Ti layer for a good adherence of the second layer made of various metals like Pt, Au, or Ag/AgCl that provides the electrochemical properties required for each analytical application. The configuration of the microelectrodes is made using photolithographic techniques with micrometric resolution [10].

In this paper we investigate the possibilities to use microelectrodes for the analysis of two biological significant compounds: L-cysteine (L-Cys) and hydrogen peroxide and subsequently

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acetyl cholinesterase was immobilized on microelectrode surface. The nonessential aminoacid L-Cys is a marker for human health [11] involved in aminothiols metabolism [12] and found in nutritional supplements. There are numerous methods available for L-Cys analysis, but microelectrodes represent an attractive alternative that is adapted for localised detection in metabolism monitoring. Hydrogen peroxide is a byproduct of the reactions catalysed by FAD or FMN based oxidases [13] and it is a compound of interest in oxidant/antioxidant biological processes [14] and cell apoptosis [15]. *In vivo,* hydrogen peroxide has a short half-life and interacts with numerous biological compounds. Microelectrodes are useful for its distribution mapping within tissues and cells.

The potential of microelectrodes for biochemical investigations is unlocked by their coupling with enzymes to achieve the required selectivity in the analysis of complex biological samples [16]. This approach, even if it is more complicated than the direct microelectrodes usage, allows the expansion of the analytes to metabolites and toxins that are either enzymatic substrates or inhibitors [17]. There are numerous enzyme immobilization methods but only few of them are adaptable for microelectrodes [18]. We have chosen a diazonium based protocol that consists in electrodeposition on vacuum-deposed microelectrodes (VDME) surface of p-nitrophenyl moieties that were electroreduced to p-aminophenyl used for enzyme reticulation with glutaraldehyde [19].

Flow injection analysis is a versatile technique that allows rapid measurements with high reproducibility [20] and was used to test the microelectrodes mounted in a wall-jet flow-cell.

2. Experimental part

Reagents

Potassium ferricyanide $K_3[Fe(CN)_6]$, L-Cysteine (L-Cys), acetylthiocholine (ATCh) and hydrogen peroxide (H_2O_2) prepared daily in 0.1 M phosphate buffer (PBS) pH=7.0 supplemented with KCl 0.1 M were used for electrochemical measurements and microelectrodes characterization. A 3 mM sulfuric acid solution in purified water was used for microelectrodes were etching. Acetylcholinesterase (AChE) was immobilized on microelectrodes surface using para-nitrobenzendiazonium tetrafluoroborate (4-NBD), acetonitrile, tetrabutylammonium tetrafluoroborate (TBA) and glutaraldehyde. All the reagents were from Sigma-Aldrich. Cr, Pt, Au, Ag used for microelectrodes construction had 99.999% purity. Si substrates were from Silicon Materials-Germany, the positive photoresist type HPR 504 was from FUJI FILM (USA) and the negative photoresist type SU 8 was from Microrezist (Germany).

Apparatus

Platinum microelectrodes

The vacuum-deposed microelectrodes (VDME) presented in figure 1 were made in vacuum (10^{-7} torr) with a NEVA 500 equipment (Japan). The microelectrodes were made on a Si/SiO_2 substrate and the thickness of the deposed metal layer was measured *in situ* with the integrated piezoelectric quartz or by profilometry with Alphastep system Model 10-00020 (Tencor Instruments, USA). The microelectrodes adherence on the substrate was verified by the traction method. There were deposited 6 independent working microelectrodes (WE) as 6 metallic bands: 4 with 15 µm with and 2 with 30 µm width. The auxiliary microelectrode (CE) has a width of 200 µm. All WE and CE were made in a two layer configuration: Cr for adherence and Pt for electrochemical surface. The reference microelectrode (RE) has a width of 150 µm and was made from a Ag layer that was subsequently chlorinated. The length of all microelectrodes was 2.5 mm. The electric contact of the microelectrodes with the potentiostat is made with connecting traces with a bilayer configuration: Cr and Au. These traces have and expanding geometry to make the passage from the micrometric dimensions of the microelectrodes to the milimetric size 8 terminal connector with a 1.27 mm step. The connecting traces were insulated from the measuring solution with a SU 8 photoresist layer.

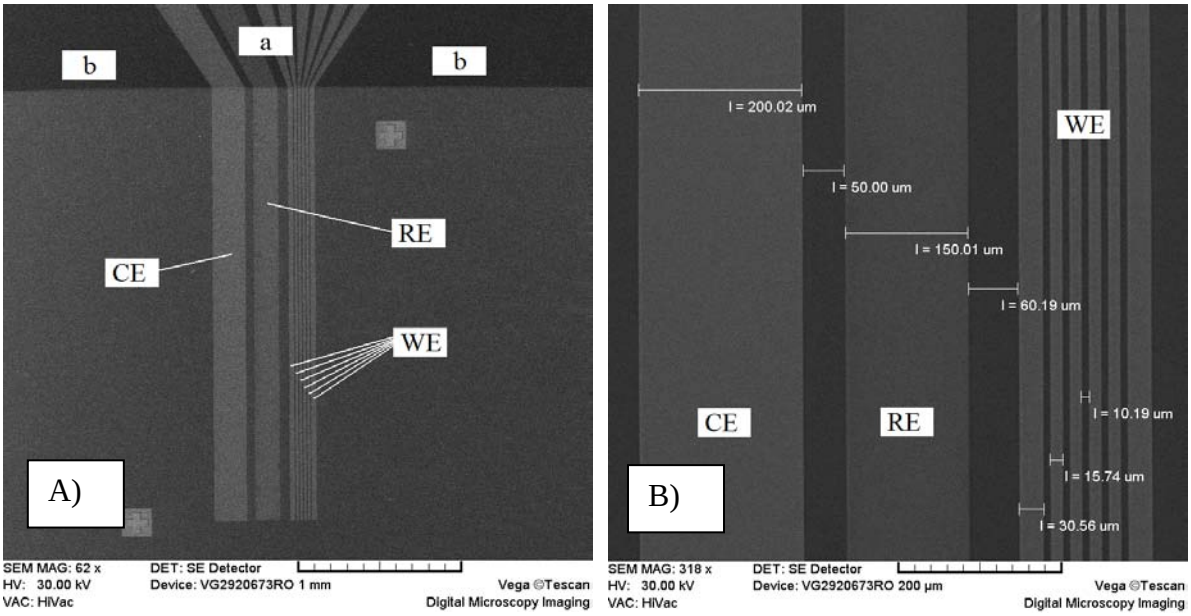

Fig. 1. The SEM images of VDME microelectrodes: A) Microelectrodes layout, B) Microelectrodes dimensions. WE – Working Electrodes; CE – Contraelectrode; RE – Reference Electrode; "a" zone – extension of the metallic strips; "b" zone – photoresist isolation.

Scanning electron microscopy (SEM)

The SEM images of VDME were obtained in secondary electrons mode with a Vega II microscope (Tescan, USA). The acceleration potential was 30 kV and the magnification was 62x for figure 1A and respectively 318x for figure 1B.

Flow injection analysis (FIA) system

The single-line FIA system was composed by a peristaltic pump Gilson Minipuls 3 (France), a injection valve Omnifit (USA), teflon tubes 1 mm i.d. and a flow cell from DropSens (Spain) in which the VDME were inserted. The carrier solution was PBS with a flow of 0.67 mL/min. All the electrochemical measurements were done with a potentiostat/galvanostat Autolab PGSTAT302N with ECD low-currents supplementary module from Eco Chemie (Netherlands) controlled by computer with the Nova 1.4 software.

Chronoamperometric measurements

VDME were tested by amperometry in FIA by measuring the current every second at potentials 0.4 V and 0.7 V for L-Cys detection and 0.7 V for hydrogen peroxide detection. After baseline stabilization, different concentrations of L-Cys and H_2O_2 were injected. The experiments presented in this paper were made with the 30 µm wide WE.

Cyclic voltammetry

The characterization of the VDME was made by CV by scanning the potential between -0.2 and 0.6 V at scan rate of 10 mV/s in a 1 mM potassium ferricyanide prepared in PBS. The VDME surface was etched by cyclic voltammetry: 25 cycles between -0.3 and 0.8 V with a scan rate of 50 mV/s in a 3 mM sulfuric acid solution.

Biosensor construction

The AChE immobilization protocol was adapted from a procedure already optimized using commercial platinum microelectrodes [19]. Briefly, the VDME were etched by CV in sulfuric acid then nitrophenyl was electrodeposed by potentiometry at a constant current intensity of -10 pA for 10 sec in a solution of 10 μM 4-NBD and 0.05 M TBA in acetonitrile. Then the VDME were washed with acetonitrile and water and the p-nitrophenyl was reduced to p-aminophenyl by applying a potential of -0.5 V for 10 s in PBS. Finally, the amino groups were activated by 30 min reaction with 2% (v/v) glutaraldehyde in PBS and 20 mIU AChE was deposed on the surface.

3. Results and discussion

Microelectrodes - technological realization

In a first step there were made the masks used for photolithographic configuration of the metallic traces on the substrate. The design was made with CleWin layout editor software from WieWeb Software (Netherlands).

n-type Si plates with 100 mm diameter and 500 μm thickness were used as substrate for microelectrodes deposition. They had a resistivity of 5 – 10 Ωcm and an 1,5μm insulating layer of SiO_2 was formed by thermal oxidation. Metals were vaporized by electron bombardment in vacuum.

The WE and CE consist of a 10 nm thick layer of Cr for adherence on which was deposed a layer of Pt with a thickness of 200 nm. The Cr/Pt layers were made by lift-off technique: the metals were deposited on the previous prepared substrate in a photoresist layer-the negative of the interest mask. The metal deposition in the desired geometry is obtained by dissolving the photoresist with acetone and removing thus the metal deposed on it. It was noticed that it is necessary to use a positive photoresist because the high temperature required for Pt vaporization produces cracks in the negative photoresist.

The Ag deposition for RE construction was made by lift-off using the same positive photoresist. The thickness of the deposed metallic layer was 150 nm. The AgCl was obtained by immersing the VDME in a stirred $FeCl_3$ solution for 1 minute.

The Cr/Au connectors between the electrodes in the measuring solution and the potentiostat were made by corrosion through a positive photoresist mask made by mask transfer by exposition to UV radiation. The Cr layer had a thickness of 10 nm and the gold layer had a thickness of 150 nm. The gold corrosion was made with a potassium iodide solution.

Signal amplification

One of the intrinsic disadvantages of the microelectrodes is their relatively small analytical signal due the fact that the current is directly proportional with their surface. Recent advancements in electronics led to the development of measuring equipments sensitive enough to allow the use of the microelectrodes, but in the design of a microsensor the maximization of the analytical signal is of prime importance. The initial currents measured with VDME by CV with potassium ferricyanide or amperometry for the oxidation of L-Cys or hydrogen peroxide were very small. After a surface etching by CV in sulfuric acid the recorded currents increased significantly as can be seen in figure 2 where there are presented the cyclic voltammograms before and after etching. The recorded currents for the amperometric measurements were approximately two orders of magnitude higher after the surface etching in comparison with the currents measured with the same VDME before CV treatment. All these data, together with the fact that the VDME were manufactured and stored in clean conditions, suggest that the CV in sulfuric acid does more than a simple surface cleaning and the significant increase of the measured electric currents is due to a surface etching. This simple and fast surface activation step increased the analytical signals and was used thereafter as a preliminary surface treatment.

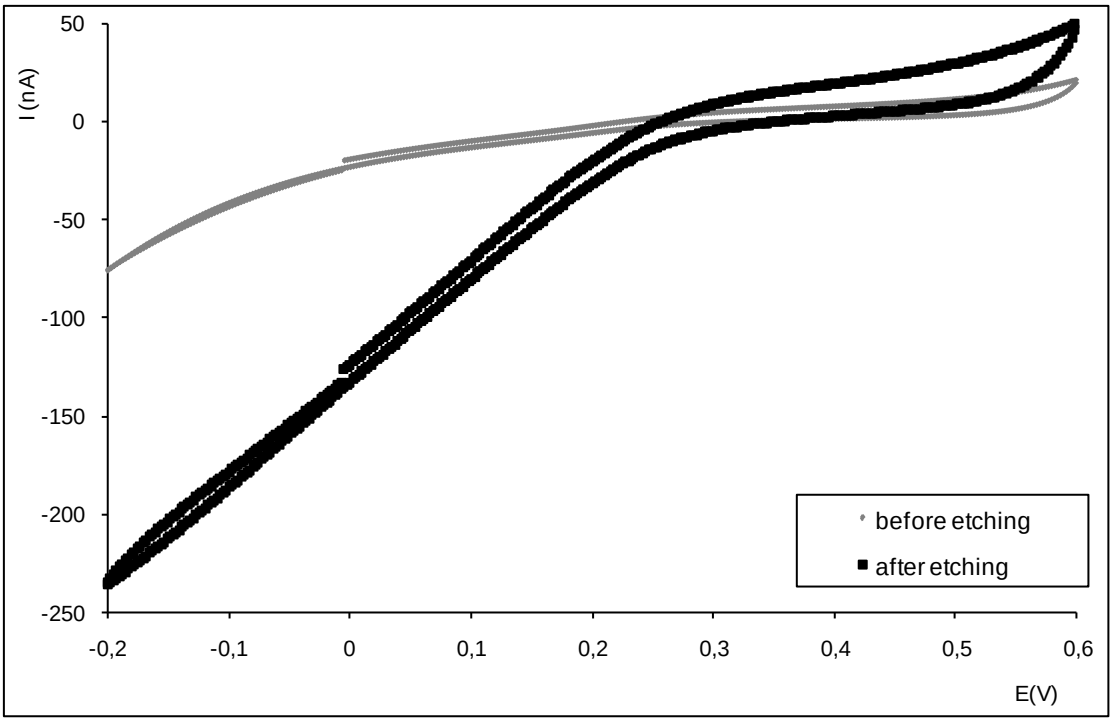

Fig. 2. Cyclic voltammograms in 1 mM potassium ferricyanide of a VDME before and after etching by CV in 3 mM sulfuric acid.

L-Cys detection

The L-Cys detection is based on the oxidation of the thiol moiety from the amino-acid structure to dithio groups and thus the L-Cys is transformed in cystine. This reaction takes place on WE made from platinum without the need of an electrochemical mediator. The L-Cys detection was made at two potentials: 0.4 V and 0.7 V. At the lower overpotential the magnitude of the recorded currents is smaller, but in some complex samples this disadvantage may be compensate by minimizing some interferences from the matrix. The baseline measured at 0.4 V varies between dozens and hundreds of pA for different VDME, while for a potential of 0.7 V the baseline value is 1-5 nA. The increase of the baseline value does not impede on the analytical signal measurements as the measured currents are much higher and the noise (baseline variation) and not its absolute value is important for a low of the limit of detection as can be seen in figure 3.

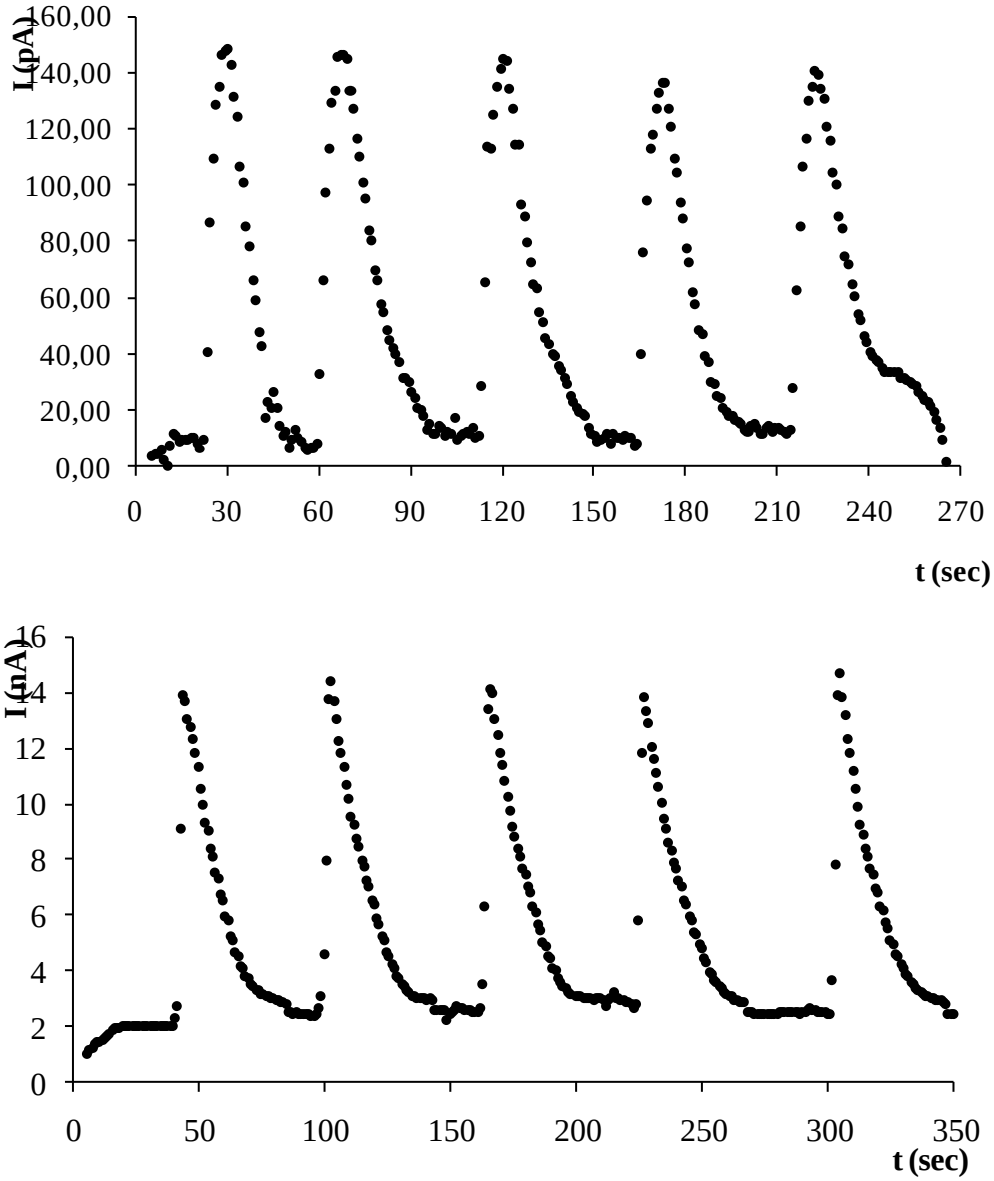

Fig. 3. FIA signals obtained using VDME: A) 0.1 mM L-Cys at potential of 0.4 V; B) 1 mM L-Cys at a potential of 0.7 V.

The relative standard deviation (RSD) of the measurements made at 0.4 V is 3.5% (I=137±4.9 pA; n=5) for a 0.1 mM L-Cys sample. The limit of detection (LOD) obtained for a potential of 0.4 V is 10 μM and the calibration graph is linear between 10 μM and 5 mM with the equation: I(pA)=800.54xL-Cys (mM)+103.52; R^2=0.9925. The RSD for the measurements at 0.7 V is slightly higher: 4.5 %, the analytical signals for a 1 mM L-Cys sample is I=12.2±0.55 nA; n=5. The use of this higher overpotential allows the improvement of the LOD to 3 μM and the calibration graph is linear up to 3 mM with the equation: I(nA)=8.604xL-Cys (mM)+0.9535; R^2=0.9808. The low noise and baseline values coupled with the reproducibility of the L-Cys detection and the calibration curves at the two tested potentials suggests that the VDME are suitable for (bio)sensors applications.

Hydrogen peroxide detection

Hydrogen peroxide is another (bio)chemical compound that may be amperometrically detected using WE from platinum without the need of electrochemical mediators. For this analyte it is necessary to use a 0.7 V potential and the LOD was 30 μM. The RSD determined by the successive analysis of a 0.1 M H_2O_2 solution was 5.1 % (I=27.7±1.4 nA; n=5). The calibration curve is linear from LOD up to 3 mM H_2O_2 with the following equation of calibration graph: I(nA)=397.25x H_2O_2+33.144; R^2 = 0,9903. The possibility to detect the hydrogen peroxide allows the use of WMDE for the development of microbiosensors based on FAD-class enzymes or its direct use for the investigation of numerous redox processes that involve this compound.

Biosensor construction

The VDME were used as transducers for the development of microbiosensors. As model enzyme it was used AChE because it is capable of hydrolyzing a thiolic artificial substrate (acetylthiocholine) to acetic acid and thiocholine. As it was presented previously, the VDME are capable to detect compounds with a thiol moiety. Thus, the electrodes will not respond to the substrate that has the thiol group acetylated, but will detect small quantities of the enzymatic reaction product. The VDME are also capable to detect hydrogen proxides and thus they may be used for the development of microbiosensors based on FAD containing enzymes.

Unlike thiols on gold surface, the diazonium electrodeposition involves radical additions and multilayers may be formed. If a too thick organic stratum is formed on the electrode surface the electrons transfer to and from solution will be hindered. The immobilization process was characterized by CV and it was noticed that after p-nitrophenyl electrodeposition and reduction to p-aminophenyl the measured currents were slightly reduced in comparison with etched VDME. The binding of AChE leads to a decrease of the CV current (see figure 3), but this is due to the high dimension of the protein that alters the diffusion between the bulk of the solution and the electrode surface and not to the surface fouling and thus the thiols generated on the electrode surface are still detected [19].

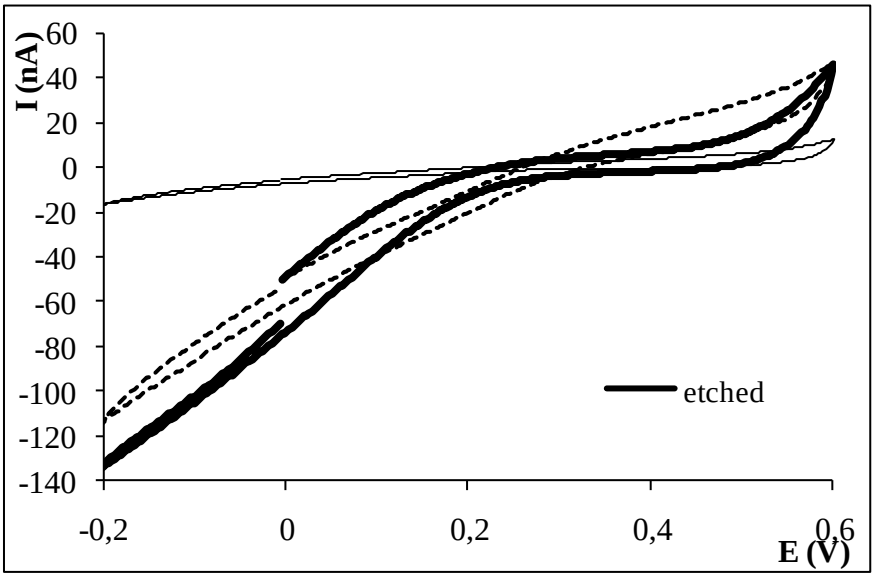

Fig. 4. Cyclic voltammograms in 1 mM potassium ferricyanide of a VDME during AChE immobilization process.

4. Conclusions

VDME with Pt WE were produced and used in a FIA system for the direct detection of the hydrogen peroxide at 0.7 V and L-Cys at 0.4 V by amperometric measurements. It was observed the surface etching by CV in a 3 mM sulfuric acid solution lead to an important increase of the analytical signal. The VDME had low background current and good reproducibility of the measurements. A model enzyme was immobilized on the WE proving that the VDME may be

used as transducers for enzymatic biosensors development. On the same chip there were deposited 6 independent WE with different widths. This allows to increase the complexity of the performed analysis by making differential measurements: e.g. one eWE with immobilized enzyme for analyte and electroactive interferents detection and another WE without enzyme for interferents quantification. From the diference between the two signals one can mitigate the negative influence of interferents on the analytical signal.

Acknowledgements

This work was supported by Romanian Ministry of Education and Research UEFISCDI (projects PN II-RU TE-100/2010, MICROSEN 11049/2007 and BLUEBOXSENS 82102/2008). Ovidiu Ilie Covaci is a Ph.D. student with a scholarship funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/6/1.5/S/19.

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