ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2014, *59*(6-7), 421-428

Dedicated to the memory of
Professor Eugen Segal (1933-2013)

INVESTIGATIONS OF HYDROQUINONE OXIDATION INSIDE THE REACTION LAYER OF BANANA TISSUE WITH SCANNING ELECTROCHEMICAL MICROSCOPY AND DOUBLE PULSE AMPEROMETRY

Andreea LUNGU,[a] Anton Alexandru CIUCU,[b] Daniela BALA[a] and Constantin MIHAILCIUC[a,*]

[a] University of Bucharest, Faculty of Chemistry, Department of Physical Chemistry, 4-12 Regina Elisabeta, 030018 Bucharest, Roumania
[b] University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, 90-92 Panduri Av., 050663 Bucharest, Roumania

Received April 30, 2014

Scanning electrochemical microscopy (SECM) with amperometric measuring tip and double pulse amperometry (DPA) were used to investigate the biocatalytical reaction inside the enzyme layer of a banana tissue biosensor during its operation. Hydroquinone (H_2Q) has been selected as analyte for the investigations. Based on vertical SECM local maximum of H_2Q and minimum of O_2 profiles were found at approximately 70 μm far from the substrate/tissue layer boundary. Based on the obtained results, a model "banana" biosensor was constructed and used in DPA studies for H_2Q detection.

INTRODUCTION

Plant tissue-based biosensors have been used as bioreceptors in different biosensor formats. The idea of using natural tissue from plants or animals, for constructing biosensors, is based on the advantages offered by enzymes working in their own natural environments.[1] Several plant tissue based biosensors were made for measurements of phenolic compounds like catechols. Among the natural tissue used as such, one of the most important is the banana pulp slices,[2] potato,[3,4] apple,[5] mushroom,[6] avocado[4] and aubergine.[7]

The banana pulps was used as a very thin slice (membrane form) mechanically attached to the active surface of an electrode or was incorporated, by a well-known procedure, into the carbon paste obtaining the well-known "bananatrode".[2,8] Substrates catechol[9] or catechol like compounds including neurotransmitters,[10] flavonols, flavonones and flavononols[11] are oxidize by the mentioned enzyme and could be electrochemically detected at conventional electrodes surfaces. The main drawback of using mechanically attached banana slice as a membrane (biocatalytic layer) comes from the long response time (order of minutes) which depends upon the thickness of the slice influencing also the biocatalytic effect.

Hydroquinone (H_2Q) is one important isomers of phenolic compounds used in cosmetics, pesticides, flavoring agents, antioxidant, secondary coloring matters, and photography chemicals.[12] Certain phenolic compounds are highly toxic or carcinogenic which is the main reason for their determination in the environment.[13] High concentration of H_2Q can lead to fatigue, headache

* Corresponding author: cmpaul@gw-chimie.math.unibuc.ro

and tachycardia in humans[14] and can also lead to cancer such as acute myeloidleukemia.[15]

The established methods for the determination of H_2Q are commonly performed after pretreatment and separation.[16] Electrochemical biosensors appear to be an attractive and suitable option for the more sensitive and selective determinations of phenolic compounds.[17,18] To provide the necessary measuring range and sensitivity higher enzyme activity and well-tailored reaction layers would be needed.

In order to describe the function of the different enzyme based biosensors or to design efficient ones, the distribution of the different species inside the differently made, sized and formed reaction layers during their operation is necessary to be known. Scanning electrochemical microscopy (SECM) is a powerful technique to gather information about concentration profiles with high spatial resolution. Since the end of 80's, when the first SECM measurements were carried out[19,20] many experiments were done on biochemical samples. Hot spots of enzymatic activity on different surfaces have been successfully mapped with amperometric[21] or potentiometric[22] SECM tips scanned over the surface in electrolyte solution containing appropriate concentration of the substrate molecule. Investigation of concentration profiles of different species inside immobilized single and multi-enzyme layers by SECM measurements was reported.[23-25] It was hoped that knowing the concentration profiles of reactants and products of the biocatalytic process the measuring ability of the biosensors could be optimized.

In this work we are reporting the results obtained by SECM measurements inside a reaction layer existing in a close vicinity of a cellulose acetate (dialysis) membrane which separates the banana tissue, kept in a glass tube, from an electrolyte solution containing H_2Q as electroactive species. A model biosensor based on a reaction layer containing banana pulp as native enzyme source was constructed. Hydroquinone (H_2Q) has been selected as analyte for the investigations. Local maximum of H_2Q and minimum of O_2 profiles were found at approximately 70 μm far from the substrate/tissue layer boundary (dialysis membrane) in SECM experiment. It became possible so to establish the thickness of the banana pulp layer itself that was utilized in the construction of the model biosensor which was used in the detection of the H_2Q in double pulse amperometry (DPA) experiments.

RESULTS AND DISCUSSION

The function of the "bananatrode" is based on the tyrosinase (Tyr-ase) alyzed reaction of hydroquinone with dissolved oxygen.[26-28] The H_2Q from the sample solution diffuse through the dialysis membrane, interact with Tyr-ase present in the banana pulp and being transformed into p-benzoquinone (Q), the enzyme being transformed in its reduced form. The p-benzoquinone formed, diffuse into two directions: one into sample solution and the other toward the electrode surface where it can be reduced back to hydroquinone by means of an electrode reaction if an appropriate electrode potential is applied. In our case the last electrochemical reaction was possible by maintaining the electrode potential at a very reducing value, -0.2 V, which is more cathodic comparing to the potential of the cathodic peak resulted in previous CV experiments (results not shown here). The reduced form of the enzyme is oxidized back to its natural form by the dissolved oxygen that exists inside the banana pulp. The generated current depends on the analyte concentration.

$$H_2Q + 1/2O_2 \xrightarrow{\text{Tyr-ase}} Q + H_2O$$

The O_2 can be supplied either across the dialysis membrane from the sample solution where the oxygen comes from air, the upper side of the solution being open, or from the air directly in the banana pulp volume, its upper side being also open. The thickness of the enzyme containing layer has basic effect on the working condition of the electrode. It influences the diffusion process of electrochemical active species inside the layer, response time as well as the signal.

SECM studies

The progress of the chemical oxidation reaction of the H_2Q by the Tyr-ase can be followed amperometricaly with platinum microdisc electrode by detecting the Q reaction product. Using a Pt UME (ultramicroelectrode) disk-shaped, which moves from the upper side of the banana pulp volume toward the dialysis membrane through the banana pulp, at the steady state conditions in the bulk of the solution the following equation describes the limiting current obtained at the Pt UME:

$$i_{l,\infty} = 4nFDc^*a$$

where n is the number of electrons involved in the electrode reaction, F is the Faraday constant, D is

the diffusion coefficient of the electroactive species, c* is the bulk concentration of the electroactive species and a is the UME radius.

During the movement of the Pt UME, at a very slow speed (not disturbing the concentration profiles installed inside the biochemical reaction layer) through the banana pulp the current has a constant value, given by the above equation, but approaching the dialysis membrane, in the biochemical reaction layer, where the H_2Q is consumed, the current will increase as the distance decrease due to the production of Q in the immediate vicinity of the membrane. The current as a function of distance is recorded at an applied electrode potential (E_a) at which either only one electrode reaction, that of Q, is occurring (E_a = -0.2 V) or two electrode reactions, those of Q and oxygen, are occurring (E_a = -0.6 V). In this way, information on the concentration profiles inside the chemical reaction layer could be obtained and an evaluation of the optimal thickness of the biocatalytic layer of the banana pulp could be realized.

The current-distance curves for the three mentioned concentrations of H_2Q (see electrochemical measurements in experimental section) in the sample solution are shown in Fig. 1; these curves were obtained at +0.4 V working electrode potential where the H_2Q could be oxidized. As can be seen in Fig. 1, the current has a steady-state appearance at large distance but it increases in an exponential manner for distances lower than almost 70 μm. In this domain, the Pt UME explores the chemical reaction layer where the H_2Q is oxidized.

The current-distance curves, obtained at -0.2 V working electrode potential and at -0.6 V working electrode potential for the three above mentioned concentrations of H_2Q are shown in Fig. 2A and 2B respectively.

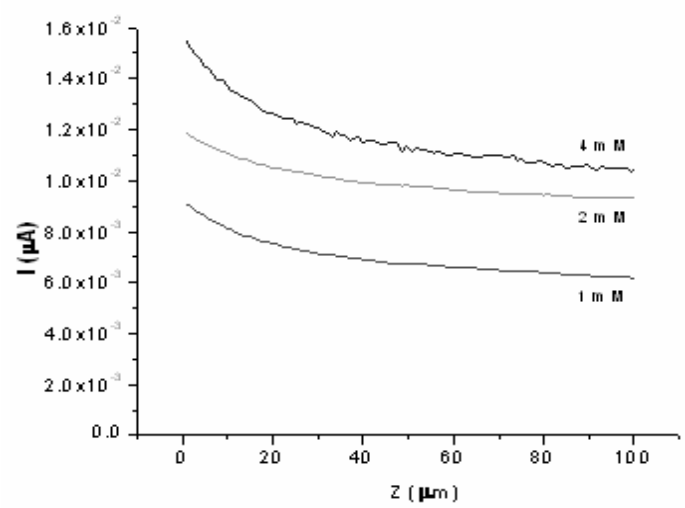

Fig. 1 – H_2Q concentration profiles for different initial H_2Q concentrations.
Applied electrode potential for Pt UME was + 400 mV vs. Ag quasi-reference electrode.

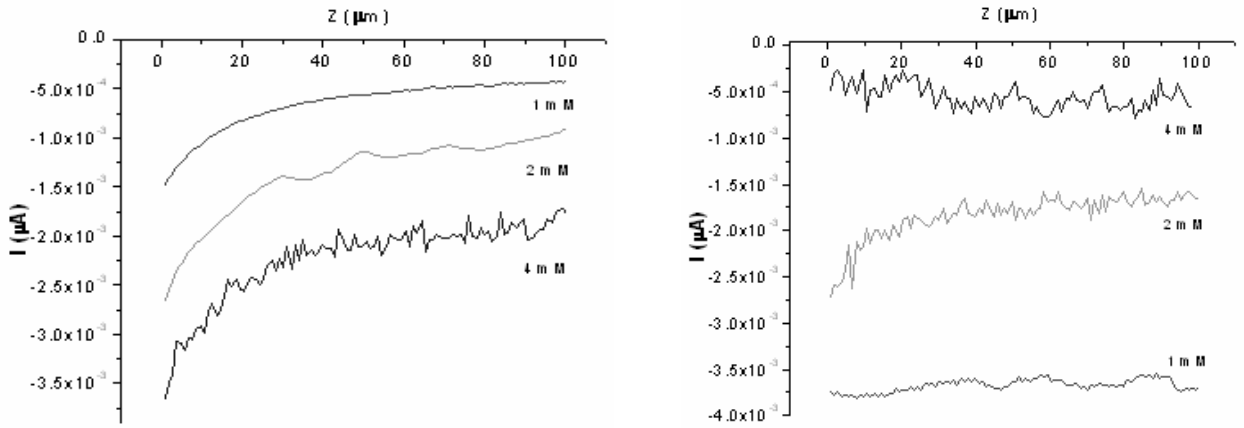

Fig. 2 – The current-distance (approaching) curves for the three different H_2Q concentrations in PBS of pH 7.33 at -0.2 V (A) and -0.6V (B) working electrode potential.

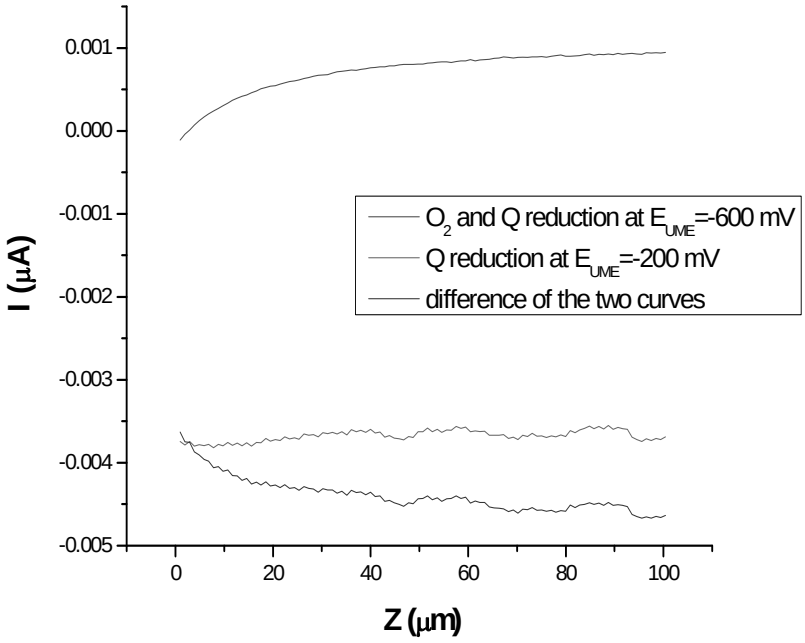

Fig. 3 – The current-distance (approaching) curves for 1 mM H_2Q in PBS of pH 7.33 at two different working electrode potentials: -0.6 V (simultaneous reduction of O_2 and H_2Q) and at -0.2 V (reduction of O_2 only) and their difference.

If the current obtained at -0.2 V is extracted from the current resulted at -0.6 V, as can be seen in Fig. 3 for 1 mM H_2Q concentration in the sample solution, one has the current due mainly to the reduction of oxygen. This current of oxygen reduction seems to be more or less constant with the distance due to the fact that the oxygen diffusion is not a limiting process; its supply in the system is constant because of the free access of the oxygen through the open surfaces of the banana layer and sample solution to the atmosphere. But in the very narrow layer in vicinity of the membrane, there is a very small lack of oxygen and this could be assigned to its involvement in the regenerating reaction of the oxidized form of Tyr-ase.

DPA studies

The established conditions in SECM studies (the thickness of the banana pulp layer) were used in construction of a "bananatrode" model biosensor which was utilized for H_2Q detection in DPA experiments.

The obtained results in DPA experiments are presented in Fig. 4 for seven additions of 20 μL H_2Q of 50 mM concentration. The response time itself is not as short as in the Tyr-ase based sensors; it takes almost 1-2 minutes for response to appear due to a much more complicated diffusion process which occurs inside a more complicated heterogeneous space which can produce much more complicated spatial and time dependences of concentrations of enzyme as well as of reaction product (Q).

Plotting the sum of cumulated current jumps ($\sum_{k=1}^{n} I_{salt,k}$) vs. the existing concentration in the solution (c_k) after the addition number k, *i.e.*, $\sum_{k=1}^{n} I_{salt,k}$ vs. c_k, linear behavior is obtained on the entire concentration domain explored for each series of additions accomplished. For example, in the case of seven additions of 20 μL H_2Q 50 mM the line equation is $I_{salt} = 5.692 \cdot 10^{-8} - 4.626 \cdot 10^{-6} c$. The main parameters of linear fittings are given in Table 1; the slope of the plot $\sum_{k=1}^{n} I_{salt,k}$ vs. c_k is the sensitivity of the method in $nA/\mu M$ (for all four series of additions, see Table 1). The mean sensitivity is -4.83 $nA/\mu M$.

In the Tables 2 and 3 the level of concentrations in the bulk solution after the addition of H_2Q and the cumulated value of current jumps at the addition number k are presented for each of the four different series of additions.

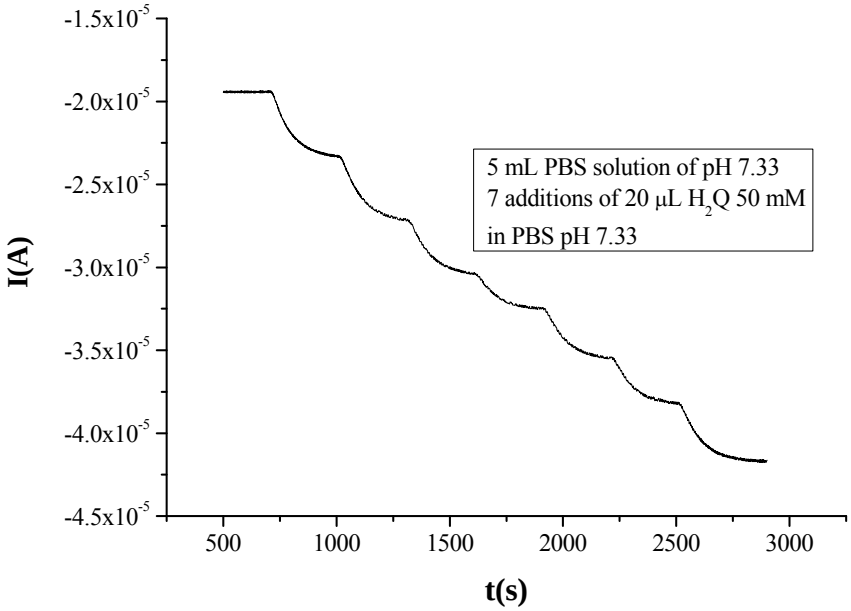

Fig. 4 – The current-time responses for one (20 μL H_2Q 50 mM in PBS of pH 7.33) of the four series of seven constant-volume additions.

Table 1

Analytical parameters for the linear dependence of the cumulated current jumps (in A) on H_2Q concentration (in mM) for each addition series

Adding volume series (μL)	Correlation coefficient (R^2)	SD	Slope
10	0,9962	7,92 E-8	-5,41E-6
15	0,9990	5,32 E-8	-4, 95E-6
20	0,9992	5,85 E-8	-4, 63E-6
25	0,9980	1,19 E-7	4, 53E-6

Table 2

Cumulated values of H_2Q concentration in the bulk solution for each series of additions

	c_{H_2Q} (mM)			
Adding volume (μL)	10	15	20	25
No. of adding volume				
0	0	0	0	0
1	0.0995	0.1496	0.1992	0.2487
2	0.199	0.2982	0.3968	0.495
3	0.298	0.446	0.5929	0.7389
4	0.3965	0.5929	0.7874	0.9804
5	0.495	0.7389	0.9804	1.2195
6	0.593	0.8841	1.1719	1.4563

Table 3

Cumulated values of currents after each series of addition of H_2Q

	I(A)			
Adding volume (μL)	10	15	20	25
No. of adding volume				
0	0	0	0	0
1	-7E-7	-7E-7	-8E-7	-9E-7
2	-1.3E-6	-1.5E-6	-1.8E-6	-2.1E-6
3	-1.8E-6	-2.2E-6	-2.7E-6	-3.2E-6
4	-2.3E-6	-3E-6	-3.5E-6	-4.3E-6
5	-2.8E-6	-3.7E-6	-4.5E-6	-5.6E-6
6	-3.3E-6	-4.3E-6	-5.4E-6	-6.4E-6

The level of reproducibility of the model biosensor is dictated by the renewal of "bananatrode" thin reaction layer; this layer could be rapidly and reproducibly renewed if desired. The stability of the model biosensor depends on the stability of the tyrosinase in banana tissue which is greater compared with that of extracted soluble enzyme.

EXPERIMENTAL

Chemicals and materials

Hydroquinone, NaH_2PO_4, and Na_2HPO_4 were analytical reagents grade and purchased from Sigma. Phosphate buffer solution (PBS) of pH 7.33 was prepared in deionized water as well as 1.0 M hydroquinone solution used as stock solution. When not in use, the hydroquinone stock solution was kept in dark in refrigerator. Cellulose acetate (dialysis) membrane with 20 μm thickness (from Autoanalyzer accessories) was used to separate the reaction layer and the sample solution. Banana fruits were purchased from the local supermarket. Pt wires obtained from Goodfellow Metals, Cambridge Science Park, England, together with a borosilicate glass capillary 1 mm inner diameter taken from World Precision Instruments, were used in ultra-microelectrode (UME) construction for SECM studies.

Electrodes

A homemade ultra-microelectrode (UME) was prepared from the Pt wire (25 μm diameter) and was used in amperometric SECM measurements. For electrode construction the Pt wire was sealed into a soft glass capillary under vacuum using a home-made apparatus and finally a disk-shaped Pt electrode at the end of the capillary was obtained. The detailed electrode preparation procedure was published elsewhere.[23,29] Fig. 5 shows the experimental setup used in SECM experiments.

In DPA studies a model "bananatrode" biosensor was constructed and used in amperometric measurements. For its construction a Pt disk-shaped electrode was covered with a nylon net, and after that a carefully cut slice layer of banana pulp was deposited on the electrode surface; finally a dialysis membrane (20 μm thickness) was fixed on top of the electrode by using a suitable "O" ring. The schematic drawing of the home-made "bananatrode" electrode is shown in Fig. 6. The experimentally setup was similarly to that used in SECM studies.

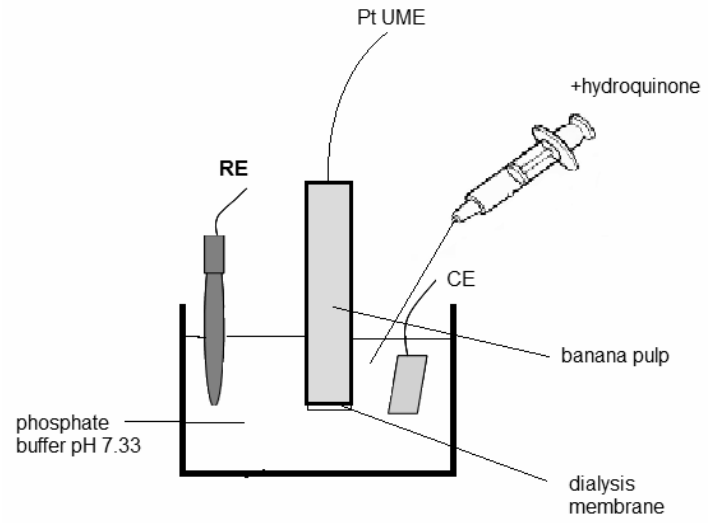

Fig. 5 – The experimental setup used in SECM experiments.

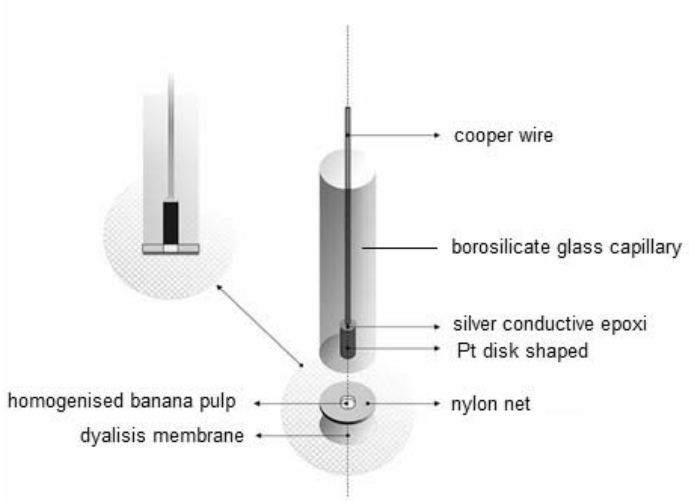

Fig. 6 – The sketch of the construction of the "bananotrode".

Three electrodes in one-compartment cell were used in all experiments. All potentials were measured and given referred to Ag used as quasi-reference electrode in the case of SECM experiments and to Ag|AgCl|KCl (3 M) (Metrohm) used as reference electrode in the case of DPA experiments. A Pt wire of sufficient area was used as a counter electrode in SECM experiments and a Pt of 3 mm in diameter from Methrom in DPA experiments. All measurements were carried out at room temperature.

Equipment

The SECM apparatus used in this work was a home-made one controlled by a software interface. A detailed report about construction and performance has been published earlier.[23,29]

Amperometric studies (DPA) were carried out using a potentiostat-galvanostat system AutoLab PGStat 12, controlled by General Purpose Electrochemical System (GPES) electrochemical interface for Windows (version 4.9.007).

Electrochemical measurements

In SECM studies a 10 mL Erlenmeyer flask filled with 5 mL of blank solution (PBS, pH = 7.33), and the tube containing the banana pulp layer having at its end the diffusion membrane of cellulose acetate immersed into the blank solution, was firstly used to obtain the i vs. z trace representing the current evolution with the distance z as the Pt UME moves toward the membrane through the banana layer as shown in Fig. 6. An equilibration time of 5 min was used to allow the PBS to diffuse into the banana pulp layer and then the SECM experiment was started. The current was almost constant on the entire explored distance. In the blank solution, an exact amount of H_2Q was added in order to obtain final concentrations of 1.0, 2.0 or 4.0 mM. The small molecules of H_2Q and oxygen can easily diffuse through the dialysis membrane. In each case, the SECM experiment was started by moving the Pt UME from the upper side to the bottom side, the approaching curve being recorded.

In DPA the home-made "bananatrode" was used to investigate the biocatalytic redox reaction between Tyr-ase enzyme inside the banana pulp and added H_2Q in the bulk of the electrolyte solution used in the electrochemical cell; the buffer solution diffuse inside the banana pulp volume serving both as "solvent" and supporting electrolyte. Initially the "bananatrode" was immersed inside the measuring cell (10 mL) containing 5 mL of PBS pH=7.33 and the DPA was started for 1800 s. When the current reaches its stationary level, at about constant time interval, constant-volume additions of H_2Q were made. Four different experiments were done, using the "bananatrode" and volumes of 10 or 15 or 20 or 25 μL 50 mM H_2Q concentration were added in the bulk solution. In DPA technique the following operational parameters were chosen: initial electrode potential $E_{in} = 0.1\,V$ (no electrode reaction occurs), electrode potential for the first step $E_1 = 0.2\,V$, applied for $t_1 = 1\,s$ (no electrode reaction is driven to the "bananatrode"), $E_2 = -0.2\,V$ for reducing Q or $E_2 = -0.6\,V$ for reducing simultaneously both Q and oxygen, applied for $t_2 = 0.03\,s$.

DPA studies were carried out with a probe solution intensively stirred with a magnetic stirrer in order to assure a very homogeneous probe solution.

CONCLUSIONS

Two kinds of studies concerning the redox activity of enzyme Tyr-ase belonging to its natural environment (i.e. banana pulp) were performed. First experiment, based on a Pt UME disk-shaped, was dedicated to SECM investigation of the biocatalytic effect of Tyr-ase on hydroquinone contained in the bulk solution. The local changes of the concentrations of hydroquinone and oxygen within a very thin reaction layer in vicinity of the dialysis membrane were recorded by SECM technique using the approaching curve at two working electrode potentials -0.2 V and -0.6 V, respectively. From the data measured and interpreted one can advance that a reaction layer thickness is approximately 70 μm.

Based on SECM results, in the second experiment, a model "bananatrode" biosensor was used to detect H_2Q by means of DPA technique. The response time of the tissue biosensor was

estimated to be in between 1-2 min. The banana pulp reaction layer of the model biosensor could be rapidly and reproducibly renewed. Cost efficient way of preparation and relatively long life time are important advantages of the elaborated model biosensor. Further experimental improvements and analytical evaluation including selectivity studies of this type of biosensor are currently in progress with particular emphasis on rapid H_2Q measurements. The present sensor might be useful for measurements in tap water, where H_2Q is found in micromolar levels.

Acknowledgements: The financial support of Roumanian Grant no 251/2011 funded by UEFSCDI and of Bilateral Project no. 667/2013 between Roumania and Hungary, financed by UEFSCDI, is highly appreciated.

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