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Short communication

Electroanalytical properties of a novel biosensor modified with zirconium alcoxide porous gels for the detection of acetaminophen

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ABSTRACT

The development of composite electrodes for biosensors construction based on HRP and zirconium alcoxide film for acetaminophen detection and finally, acetaminophen determination in pharmaceutical products is described. The enzyme immobilization is performed by retention in a polyetilenimine and zirconium alcoxide porous gel film, technique that offers a good entrapping and in the mean times a "protective" environment for the biocomponent.

The operation principle of the biosensor is based on monitoring the amperometric signal generated by reduction at the electrode surface of the enzymatically generated quinoneimine from acetaminophen. The resulting biosensor shows a linear response towards acetaminophen with a linear range of 1.96×10^{-5} M and 2.55×10^{-4} M and a limit of detection of 1.17×10^{-7} M. The proposed biosensor shows long term stability and good reproducibility.

1. Introduction

Biosensors applied in the control of drug release and concentration from pharmaceutical forms and metabolites detection represent a convenient alternative to other analytical methods. Generally biosensors are analytical devices sensitive and selective those associate a biocomponent to a transducer.

The enzymes are considered useful in the fabrication of the biosensors. Besides the problems relied to the entrapment of the enzymes at the surface of electrode, another challenge is to preserve the microenvironment of the enzyme and hence the lifetime of the biosensor. Several methods were used to immobilize the enzyme at the electrode surface like adsorption [1], cross linking [2], covalent binding [3], biological membranes [4], magnetic microparticles [5], entrapment in sol–gel [6] etc. The immobilization into an electrochemical polymer or polymerizable matrices were successfully used in the development of the amperometric biosensors, due to the fact that the procedure is effective and simple and the enzyme is less affected than during other methods of entrapment [7,8].

Lately many approaches used inorganic zirconium to immobilize enzyme during polymerization or electro-deposition [9,10]. According to the literature, nano-sized zirconium gel or thin film were used to immobilize hemoglobin [9], DNA [10], myoglobin [11] and HRP at gold electrode [12].

Zirconium oxide nanoporous gels were used to entrap the biomolecules due to their biocompatibility. The zirconium oxide nanogel was recently used for the entrapment of hemoglobin and myoglobin [13] and the protein ZrO_2 film preserve their bioactivity and show a good electrocatalytic behavior towards the reduction of H_2O_2. The analytical characteristics of the developed biosensor proved that the nanogel preserved catalytic activity and a good hydration microenvironment for the enzyme. Due to its lack of toxicity, good conductivity, affinity for groups containing oxygen, the ZrO_2 nanogels became attractive for the construction of biosensors.

A novel amperometric biosensor is described for the detection of acetaminophen (*N*-acetyl-*p*-aminophenol) as model compound. Acetaminophen is widely used as analgesic antipyretic drug having actions similar to aspirin. It is a suitable alternative for the patients who are sensitive to aspirin and safe up to therapeutic doses [14]. The large scale therapeutic use of that drug generated the need for the development of rapid and reliable methods for the determination of acetaminophen. Current methods for the analysis of acetaminophen include spectrophotometric [15], chro-

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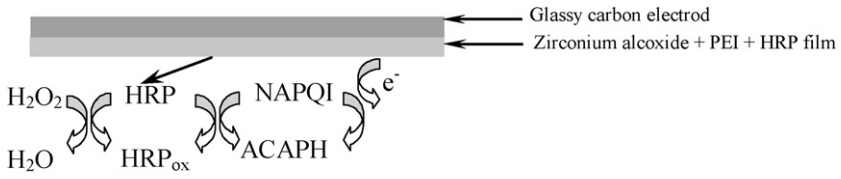

Fig. 1. The mechanism of biocatalytic peroxidation of the acetaminophen by the HRP immobilized at the surface of the electrode.

matographic [16] and electrochemical approaches [17–19]. The use of nanoporous magnetic nanoparticles for the construction of amperometric HRP immobilized biosensor was done with a good linearity range [5,20].

In the human body the acetaminophen is metabolized to *N*-acetylbenzoquinonimine (NAPQI) [5] in a similar way as the HRP is doing in the presence of hydrogen peroxide.

The novel HRP biosensor for the detection of acetaminophen uses the poly(ethyleneimine) (PEI) to entrap the enzyme and the zirconium alcoxide. Poly(ethyleneimine) is a linear polymeric cation and kept during several weeks a good permeability. It was used by various authors as entrapment material in the development of biosensors [21] and showed a good rate of electron transfer between the biocomponent and the electrode [22].

The enzyme (horse radish peroxidase, HRP) was entrapped into a porous alcoxide gel of zirconium and poly(ethyleneimine) at the surface of a glassy carbon electrode. The obtained configuration was used to study the biocatalytic oxidation of acetaminophen in the presence of the hydrogen peroxide. The biosensor was applied to the assay of acetaminophen in drug formulation (Perdolan® and Generic drug formulation) by the standard addition method.

The mechanism of action of the HRP biosensor is presented below (Fig. 1).

2. Materials and methods

2.1. Chemicals

The horse radish peroxidase enzyme (1.11.1.7 type II, 180 U/mg) was provided by Sigma.

Acetaminophen was provided by Merck.

Composition of Perdolan® declared by the producer is 200 mg acetylsalicylic acid, 200 mg acetaminophen, 46 mg caffeine, talc, maize starch, microcrystalline cellulose, polyvidone acetate.

Composition of generic drug formulation per tablet is 200 mg acetylsalicylic acid, 200 mg acetaminophen, 46 mg caffeine, 5.6 mg talc, 45 mg maize starch, 44.9 mg microcrystalline cellulose, 22.5 mg polyvidone acetate and was made in our department.

All reagents were of analytical grade, used as received.

The zirconium alcoxide was prepared according to the literature [23] and was completely characterized.

The alcoxide nanogel was prepared by solving the ZrO_2 powder in ethanol for 2 h and mixing it for 4 h.

Two different zirconium alcoxide gels have been prepared, starting from different amounts of zirconium salt: 0.25 M and 0.4 M alcoholic solutions by refluxing for 2 h at 90 °C then allowed to cool at room temperature.

Poly(ethyleneimine) (MW 60000) from Aldrich was used without purification.

The stock solution of the acetaminophen (10^{-2} M) was dissolved in phosphate buffer and kept in the refrigerator. Fresh solutions of zirconium alcoxide and poly(ethyleneimine) were prepared before the assays in phosphate buffer pH 7.4.

2.2. Film preparation

5 mg PEI with 125 μl ethanol and 120 μl distillated water were mixed for 15 min with vortex. 6.5 μl porous gel were added and mixed another 15 min with vortex.

2.3. Enzyme immobilization

Two solutions containing 0.03 mg/ml and 0.06 mg/ml HRP in phosphate buffer 0.1 M pH 7.4 have been prepared.

Equal amounts of the enzyme solution and the above described alcoxide gel were mixed for 15 min and 20 μl of the resulting mixture was deposited on the glassy electrode surface and left over night at 4 °C for drying. For another 24 h it was left at 4 °C in 5 ml phosphate buffer for hydratation.

2.4. Electrochemical methods

Amperometry and cyclic voltammetry were performed in a conventional three electrodes setup: modified glassy carbon electrode (working electrode), platinum (auxiliary electrode), Ag/AgCl 3 M KCl (reference electrode), under stirring conditions. All the cyclic voltammetry experiments were recorded at 100 mV s^{-1}.

During amperometry, the biosensor potential was kept at 0 V. The working temperature was room temperature (25 °C).

The glassy carbon electrodes used as working electrodes were provided by BAS Inc. (West Lafayette, USA) and were carefully washed with demineralized water and polished with diamond paste (BAS Inc.).

The experiments were achieved by using an AUTOLAB PGSTAT 30 (Ecochemie, The Netherlands) equipped with GPES and FRA2 software.

All experiments were performed in phosphate buffer pH 7.4 in the presence of hydrogen peroxide 0.1 mM. The pH of the solution was controlled by a ChemCadet pH-meter.

During the amperometry studies the working potential was imposed and the background was allowed to arrive at a steady-state value. Different amounts of acetaminophen standard solution or hydrogen peroxide were added into the stirred electrochemical cell and the current was recorded as a function of time.

2.5. Microscopy and surface studies

For the surface analysis a JEOL JSM-5600LV electron microscope was used.

3. Results and discussions

3.1. Film characterization

In order to test the permeability of the film, cyclic voltammograms were recorded in the presence of the redox system model, Ferro and ferric potassium cyanide. Experiments performed at GCE–Zr alcoxide 0.25 M and 0.4 M showed similar behavior with

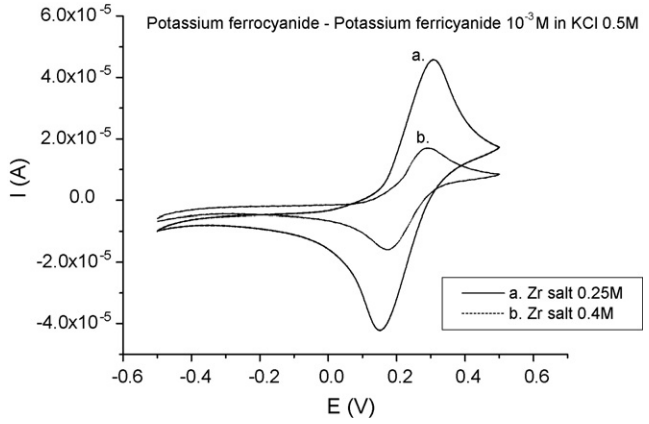

Fig. 2. Cyclic voltammetry of 10^{-3} M fero–feri potassium cyanide in 0.5 M KCl at GCE–Zr alcoxide–PEI 0.25 M (a) and GCE–Zr alcoxide–PEI 0.4 M (b).

well defined oxidation and reduction peaks (Fig. 2). The peak at 0.25 V corresponds to the oxidation of the Fe(III) while the peak at 0.196 V vs Ag/AgCl corresponds to the reduction of Fe(II). Modification of the GCE surface by the adsorption of PEI determined an increase in the charge transfer rate for the investigated redox system, illustrated by the ΔE_p decrease from 0.160 V at the GCE to 0.550 V at the PEI GCE. A similar behavior was also observed by Lojou and Bianco [22] who studied the key role of the anchoring PEI layer on the electrochemistry of redox proteins.

The zirconium alcoxide gel with the smallest amount of zirconium presented the biggest oxidation and reduction currents, due to a higher permeability and a more homogenous structure.

That is why the gel with a starting Zr salt concentration of 0.25 M was chosen for further experiments.

Differences between the film with and without zirconium alcoxide gel were investigated in the presence of 1.13×10^{-4} M acetaminophen in phosphate buffer pH 7.4 (Fig. 3). The smallest current is obtained by the PEI GCE and the ZrO_2–PEI film GCE shows an increase in signal due to the electrocatalytic response of the Zr alcoxide.

The decrease of the oxidation current until about 50% between the electrodes modified with a film of PEI and a film containing Zr alcoxide shows the improved conductive properties of the alcoxide film. Differences were observed also between a hydrated and a dry film. To explain this behavior microscopic studies were realized. Fig. 4 shows surface topography images of thin film of dry ZrO_2–PEI film (a) and hydrated ZrO_2–PEI thin film (b). As shown in Fig. 4a

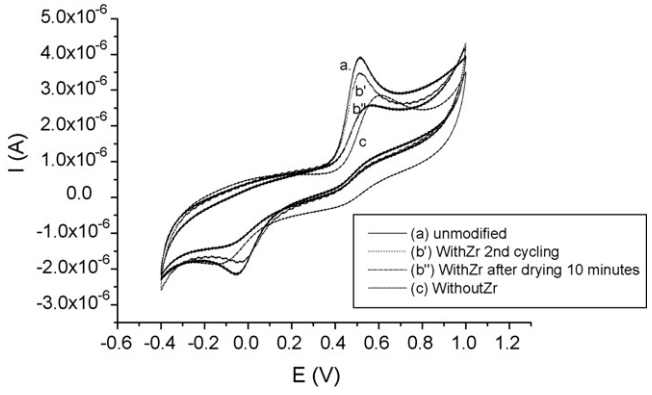

Fig. 3. Cyclic voltammograms at (a) unmodified GC electrode, (b) ZrO_2–PEI film GCE—three successive determinations and determinations after drying and (c) PEI film GCE. A solution 10^{-4} M of acetaminophen in phosphate buffer pH 7.4.

Table 1
Oxidation and reduction potentials and currents obtained with GCE, PEI GCE and thin film of ZrO_2–PEI GCE[a]

Type of electrode		Potential (V)	Current intensity (A)
GC unmodified electrode	Ox	0.517	0.388E–05
	Red	−0.047	−0.211E–05
Zirconium alcoxide–PEI GC modified electrode	Ox	0.517	0.346E–05
	Red	−0.029	−0.181E–05
PEI GC modified electrode	Ox	0.592	0.285E–05
	Red	−0.110	−0.183E–05

[a] In the presence of 1.13×10^{-4} M acetaminophen solution, $v = 100$ mV s^{-1}, reference electrode Ag/AgCl, counter electrode Pt wire.

particle size is in the ranges of micrometers. The surface topography change a lot when the ZrO_2–PEI hydrated thin film is investigated (Fig. 4b). The dry film presents cracks and pores and the hydrated film has a more homogenous structure.

This could explain the increase in stability and reproducibility of biosensor's signal. Those results were confirmed by the amperometric studies.

To completely characterize the modified electrode and for a better understanding of the acetaminophen behavior at PEI/GC and also at ZrO_2–PEI/GC modified electrodes, the cyclic voltammetric study of the hydroquinone and acetaminophen was realized.

The comparative results obtained in the presence of a 10^{-4} M acetaminophen solution at a GCE and on ZrO_2–PEI–GC modified electrode are presented in Table 1.

Electrochemical behavior of acetaminophen showed no differences between the GC unmodified electrode and ZrO_2–PEI GC electrode. Slight differences appeared after drying the electrode, in the case of the film with zirconium alcoxide that could be explained by the restructure of the film. A difference of 0.750 V between the film with and without zirconium alcoxide gel was observed by cyclic voltammetry.

To achieve the characterization of the ZrO_2–PEI film, amperometric studies were performed in phosphate buffer pH 7.4 by addition of small amounts of 1.13×10^{-4} M acetaminophen solution. Comparative successive studies made with a thin film of PEI GCE and a thin film ZrO_2–PEI GCE in days 1, 2 and 5 are presented in Fig. 5. Between measurements the electrodes were stored at 4 °C in 0.1 M phosphate buffer pH 7.4. Small amounts of a stock solution of acetaminophen 1.13×10^{-4} M were added under stirring conditions.

The reduced stability of the biosensor in the case of the film without zirconium alcoxide gel in comparison with the film containing zirconium alcoxide was observed, caused by the fast loss of the biocomponent or due to the rapid inactivation of the HRP. It can be assumed that the role of the ZrO_2 alcoxide is to preserve the enzyme hydration and the microenvironment unchanged.

3.2. Biosensor amperometric response

The electrocatalytic behavior of the enzyme electrodes towards the electrochemical reduction of acetaminophen was studied using amperometry. Fig. 6A shows the typical current–time curves of the HRP–ZrO_2–PEI GC electrode in 0.1 M phosphate buffer at pH 7.4 for successive addition of different acetaminophen concentrations under stirring conditions, at an applied potential of −0.2 V. A different behavior was observed when the HRP–ZrO_2–PEI GC electrode was stored over night in 0.1 M phosphate buffer (Fig. 6a and b), in a mixture of 1.13×10^{-4} M acetaminophen and 0.2 mM H_2O_2 (Fig. 6c and d) and in a solution of 1.13×10^{-4} M acetaminophen (Fig. 6e and f) It can be assumed that this behavior is due to the difference in the hydration of the thin film. In order to prove that is not

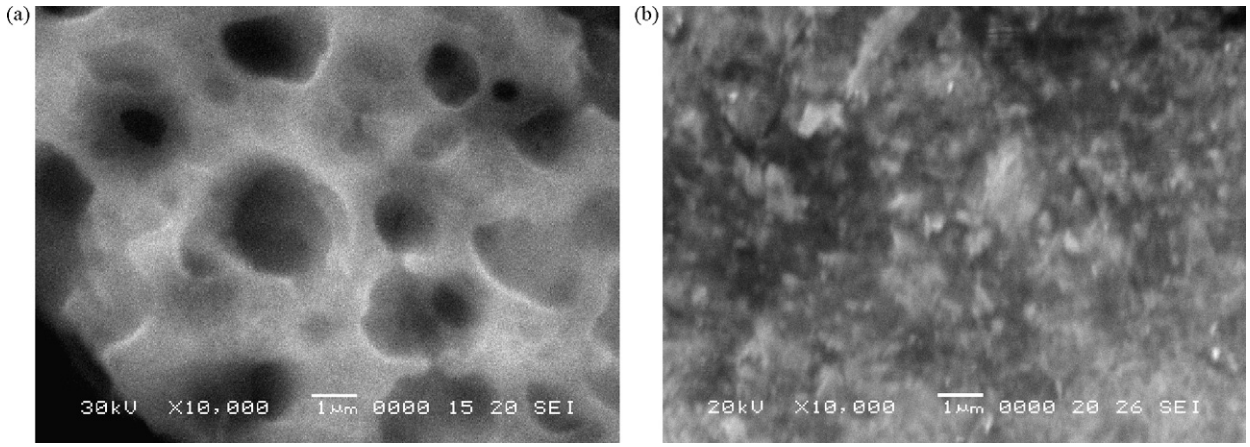

Fig. 4. Electron microscopy images of the dry (a) and hydrated film (b).

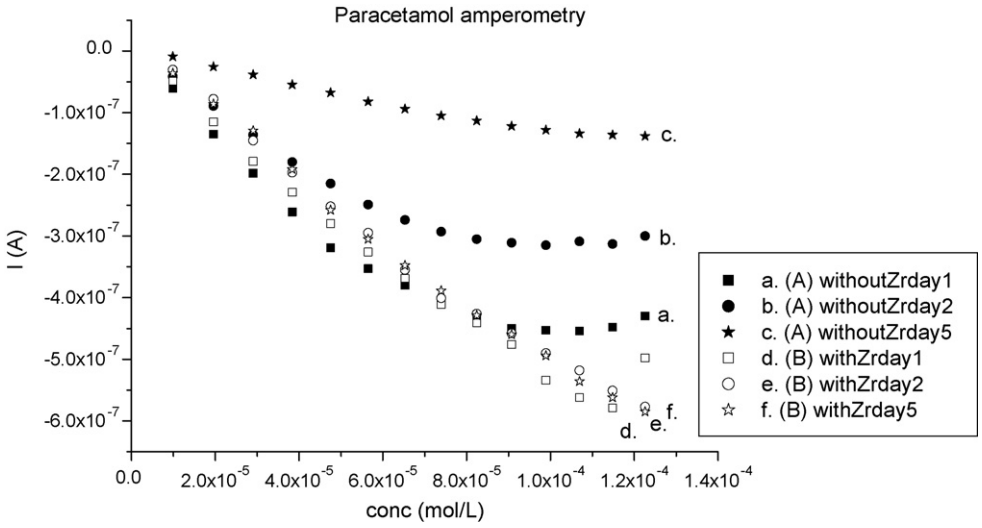

Fig. 5. Amperometric responses as a function of acetaminophen concentration along 5 days on (A) PEI GCE without Zr alcoxide, (B) ZrO_2–PEI GCE (The current was measured 1 min after addition of acetaminophen).

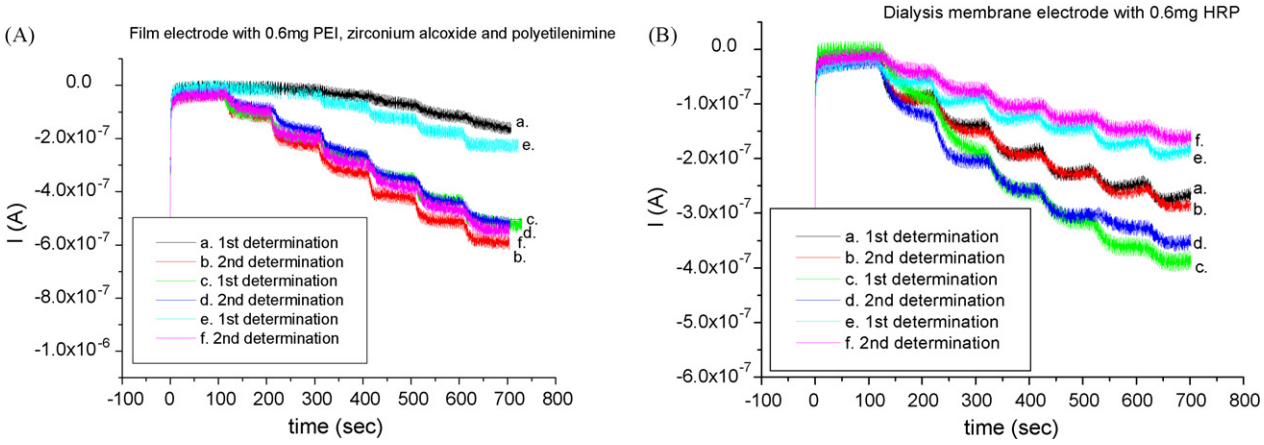

Fig. 6. (A) Amperometric study on the modified electrode film: 5 mg PEI, 125 μl ethanol, 120 μl distillated water, 6.5 μl zirconium alcoxide gel, 0.6 mg HRP and (B) amperometry: dialysis membrane electrode: 5 mg PEI, 125 μl ethanol, 120 μl distillated water, 6.5 μl zirconium alcoxide gel, 0.6 mg HRP: (a and b—two consecutive determinations) left over night in PB 0.1 M, (c and d—two consecutive determinations) left over night in acetaminophen 1.13×10^{-4} M and 0.2 mM H_2O_2 PB 0.1 M, (e and f—two consecutive determinations) left over night in acetaminophen 1.13×10^{-4} M PB 0.1 M.

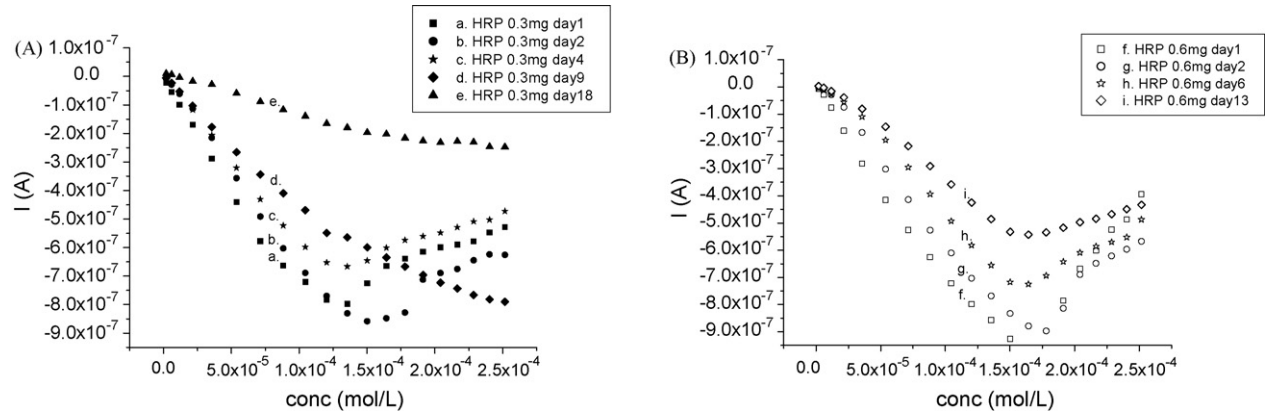

Fig. 7. Typical current–time response of HRP–ZrO_2–PEI GCE for successive addition of 0.2 mM H_2O_2 to 5 ml stirring 0.1 phosphate buffer, pH 7.4 (applied potential −0.2 V vs Ag/AgCl)—calibration curve (A 0.3 mg HRP and B 0.6 mg HRP).

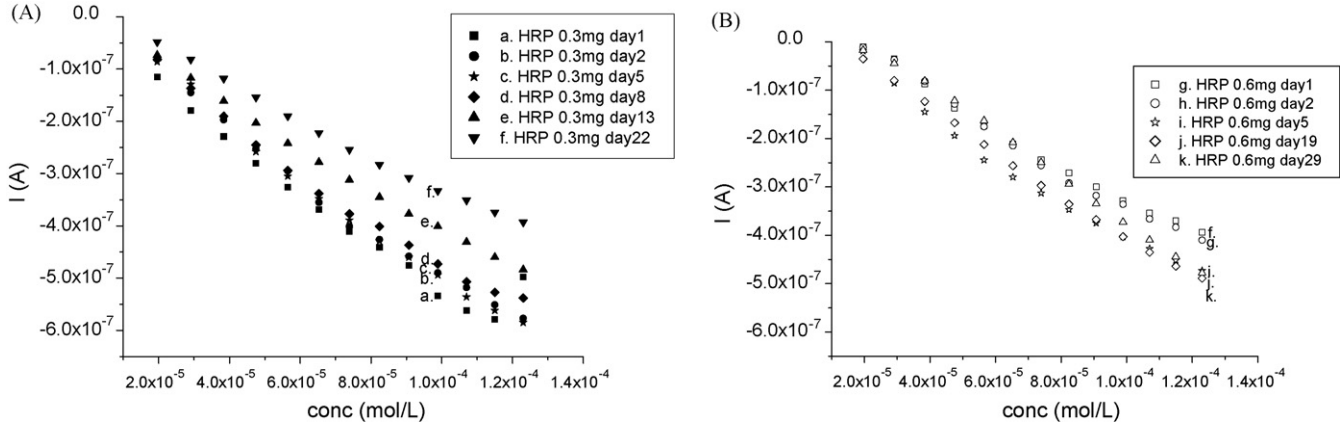

Fig. 8. Typical current–time response of HRP–ZrO_2–PEI GCE for successive addition of 0.2 mM H_2O_2 to 5 ml stirring 0.1 phosphate buffer, pH 7.4 (applied potential −0.2 V vs Ag/AgCl)—linearity domain (A 0.3 mg HRP and B 0.6 mg HRP).

only an accumulation effect due to the presence of the thin film a similar experiment was made with a cellulose membrane (Sigma dialysis tubing) used to immobilize the thin film at the surface of the electrode.

When the biosensor was left over night in acetaminophen and hydrogen peroxide (the reaction still going) no difference between the first and the second determination was observed. In the case of the membrane sensor (Fig. 6B) containing just HRP still no difference between the first and the second determination occurred, in all the three situations: over night in acetaminophen and hydrogen peroxide, in acetaminophen and in phosphate buffer.

Table 2
Correlations coefficients and limits of detection for the HRP–ZrO_2–PEI GCE for different amounts of HRP during 29 days of essays

Modified electrode	Correlations coefficients, R^2
HRP 0.3 mg day 1	0.9411
HRP 0.3 mg day 2	0.9863
HRP 0.3 mg day 5	0.9902
HRP 0.3 mg day 8	0.9853
HRP 0.3 mg day 13	0.9961
HRP 0.3 mg day 22	0.9938
HRP 0.6 mg day 1	0.9881
HRP 0.6 mg day 2	0.9818
HRP 0.6 mg day 5	0.9808
HRP 0.6 mg day 19	0.9949
HRP 0.6 mg day 29	0.9983

To improve the response of the HRP–ZrO_2–PEI GC electrode, different quantities of enzyme were immobilized at the surface. An amperometric study made during 18 days shown in Figs. 7 and 8 proved that the fact of increasing the quantity of the enzyme at the surface of the electrode (from 0.3 mg to 0.6 mg) does not improve the limit of detection of detection of 1.17×10^{-7} M and the linear domain between 1.96×10^{-5} M and 2.55×10^{-4} M of the modified electrode.

The loss of biosensor's response in time could be due either to the inactivation or to the loss of the enzyme from biosensor's surface.

Fig. 7 exhibits typical current–time curves of HRP–ZrO_2–PEI GCE to the reduction of H_2O_2 for two different quantities of HRP entrapped at the surface of the electrode and the inset of Fig. 7 shows the calibration plot linearity range of the biosensor (the correlation coefficients are presented in Table 2).

A linear trend, not passing through the origin, was found between 1.96×10^{-5} and 2.55×10^{-4} M with an RSD of ±7% in the 1st day, ±5% in the 2nd and 5th day and of ±2% in the 8th, 13th and 22nd day for the electrode having 0.3 mg HRP. Better residuals with the increase of time could be explained by film hydration. Better repeatability in time was also observed.

3.3. Acetaminophen assay

The biosensor was applied to the acetaminophen assay in drug formulation, Perdolan® and a generic solid dosage form made in our

department. The standard addition method was used. The addition of same amount of 2×10^{-3} M acetaminophen standard solution and Perdolan® recovered pharmaceutical form (acetaminophen 2×10^{-3} M) in 5 ml 0.1 M phosphate buffer pH 7.4 showed a perfect superposition of the corresponding responses. (The current was measured after 1 min of acetaminophen addition).

A linear trend of current vs acetaminophen concentration was found for concentrations between 2.0×10^{-5} M and 1.6×10^{-4} M ($R^2 = 0.9996$, RSD of slope = 20%, $n = 5$).

The optimization of the parameters related to the modification procedure was performed with the aim of obtaining the maximum reproducibility of the measurements.

The biosensor was tested after 60 days of storage in phosphate buffer and the HRP–ZrO_2–PEI GCE signal decreased with 30%.

4. Conclusion

A HRP Zr alcoxide porous gel biosensor for acetaminophen determination has been realized. The enzymatically generated reactive oxidized species of acetaminophen were electrochemically reduced and the amperometrical signal was recorded.

The LOD of this method for acetaminophen is 1.17×10^{-7} M and linear range is between 1.96×10^{-5} M and 1.22×10^{-4} M.

The investigated zirconium alcoxide porous gel represents an interesting way for biocomponent immobilization onto the glassy carbon electrode, conferring in the mean time a hydrated environment for the enzyme. The cyclic voltammetry assays showed that the zirconium alcoxide porous gel was a biocompatible material, capable to preserve the enzyme bioactivity. The ZrO_2–PEI thin film exhibited good electrocatalytical and electroanalytical response towards acetaminophen and H_2O_2.

The HRP Zr alcoxide porous gel biosensor may represent an interesting analytical device for investigation of other compounds that can be peroxidated.

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