A NOVEL BIOSENSOR FOR ACETAMINOPHEN DETECTION WITH ROMANIAN CLAYS AND CONDUCTIVE POLYMERIC FILMS

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Abstract

The development of a biosensor based on the immobilization of horseradish peroxidase (HRP) within a Romanian clay-polyetilenimine film at glassy carbon electrodes (GCEs) surfaces for acetaminophen detection is described. HRP was immobilized on the surface of the GCE with polyethyleneimine (PEI) in clay nanoporous film, a technique that offers good entrapping and a protective environment for the biocomponent due to the hydration properties of the immobilization layer. The amperometric detection of acetaminophen was successfully achieved with a sensitivity of $6.28 \cdot 10^{-7}$ M and a linear range between $5.25 \cdot 10^{-6}$ M and $4.95 \cdot 10^{-5}$ M.

Rezumat

Prezentul studiu descrie dezvoltarea unui biosenzor prin imobilizarea peroxidazei din hrean (HRP) în interiorul unui film de argilă autohtonă şi polietilenimină la suprafaţa unor electrozi de carbon vitros (GCEs) pentru detecţia paracetamolului. Enzima a fost imobilizată la suprafaţa unui electrod de carbon vitros cu polietilenimină în filmul nanoporos de argilă, tehnică ce oferă atât o bună înglobare a biocomponetului, cât şi un mediu prielnic pentru aceasta, datorită proprietăţilor hidratante ale stratului de imobilizare. Detecţia amperometrică a paracetamolului a fost cu succes realizată cu o sensibilitate de $6.28 \cdot 10^{-7}$ M şi un domeniu liniar între $5.25 \cdot 10^{-6}$ M şi $4.95 \cdot 10^{-5}$ M.

Keywords: biosensors, acetaminophen, bentonites, montmorillonite

Introduction

Electrochemical methods are well known as very sensitive, but they lack selectivity. Electrode modification is therefore the main issue in improving selectivity. This is why the different ways of modifying the electrode surface in order to obtain better/higher electrochemical signals represents a concern for researchers all over the word.

Electrode modifications concern either the improvement of sensor selectivity by increasing the affinity for a specific analyte and rejecting, in

the same time, other interfering chemical species, or the improvement of the electroanalytical performances (higher accuracy and reproducibility, lower detection and quantification limits, the possibility to determine several electroactive species without any separation process, at different oxidation or reduction potentials).

Generally, chemical sensors contain two basic functional units: a *receptor part* which transforms the chemical information into a measurable form of energy and a *transducer part* capable to convey the energy carrying the chemical information about the sample into a useful analytical signal.

Biological sensors can be defined either as devices able to detect the presence, the movement and the number of organisms in a given environment, or as sensors which contain in their structure a biological component (bacteria, algae, tissues, cells) as receptor, this type of devices being known as biosensors [1]. In the living world, there are a lot of examples of sensors consisting in biological receptors (proteins, nucleic acids, signaling molecules) located on the cell membrane, in all the tissues, organs, or even in circulating blood stream. Enzymes were used for decades in the sensors development and this led to the apparition of a niche field of research: biosensors. More specific biosensors could be defined as sensitive and selective analytical devices which associate a biocomponent to a transducer [2]. Biosensors are applied with success in several fields (environment, food security, biomedical and pharmaceutical analysis), especially because of the stable source of the biomaterial (enzymes produced by bacteria, plants, or animal as by-products), and due to their catalytic properties and the possibility of modifying the surface of transducers in various ways.

A key step in the development and optimization of the biosensors is related to the entrapment of the enzymes at the surface of electrode, another challenge being to preserve the microenvironment of the enzyme and hence the lifetime of the biosensor. Besides the methods used before, like adsorption, cross linking, covalent binding, biological membranes, magnetic microparticles, entrapment in sol–gel etc., the immobilization into an electrochemical polymer or polymerizable matrices was successfully used in the development of the amperometric biosensors [2]. In that case, the procedure was effective and simple, and the enzyme was less affected than during other methods of entrapment.

Adsorption of proteins on clay mineral surfaces represents an important application in fields related to the agricultural and environmental sciences, but also in the pharmaceutical and biomedical analysis [3]. Organic molecules, macromolecules, and biomolecules can be easily

intercalated in solids with a 2D structural arrangement that have an open structure. Therefore, clay minerals are likely to be exploited to improve the analytical characteristics of biosensors. This type of biosensors is based on three smectite clays (laponite, montmorillonite, and nontronite) and on layered double hydroxides [4].

Romanian clays are cationic clays, with negatively charged alumino silicate layers. Bentonites obtained from Răzoare and Valea Chioarului deposits in operation (Maramureş County, Romania) were refined and characterized by FTIR and differential thermal analysis. The refinement was achieved by washing and decantation obtaining a more homogeneous product, rich in montmorillonite, the main component of their structure. All of the following characterization procedures and analytical experiments are based on these refined clay samples.

Separation was performed on different clay granulometric particle sizes by sedimentation, decantation, centrifugation, and ultracentrifugation after the procedures reported in the literature [5, 6], according to Stockes' law. Several fractions, below 20 μm and below 0.2 μm were separated and characterized.

The development of composite electrodes for biosensors construction based on horseradish peroxidase (HRP) and clay films for acetaminophen (*N*-acetyl-*p*-aminophenol) detection is described. 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. The enzyme immobilization was performed by retention in a polyetylenimine (PEI) and clay porous gel film, technique that offers a good entrapping and, in the meantime, a "protective" environment for the biocomponent. HRP has been a powerful tool in biomedical and pharmaceutical analysis. Many biosensors based on HRP applied in biomedical and pharmaceutical analysis are mentioned in the literature [2, 7].

Clays present some advantages, such as low price and accessibility (especially Romanian clays for us), offering in the same time a new immobilization method for biomolecules like enzymes. Hydrated clays represent a good environment for enzyme functioning and can improve the lifetime of the biosensor. On the other hand, a lot of electrochemical processes are controlled by diffusion and clays, due to their adsorbent properties, can improve or accelerate the diffusion of different molecules (pharmaceuticals in our case) at the electrode surface. In this way, electrochemical parameters can also be improved, allowing the recording of

higher currents at lower potentials, and developing thus new electroanalytical methods with enhanced performances.

Clay presents also some disadvantages, the clay deposition and the thickness of the clay film on the electrode surface being factors that can decrease the electric conductivity. In this case, the use of conductive polymers, like PEI or poypyrrrole, for the clay film immobilization at the electrode surface represents a good alternative. In spite of the water and alcohol solubility of PEI, this polymer does not involve any further polymerization process (such as the use of heat, the polymerization initiators or the potential scanning) which can damage the enzyme structure and functioning.

The purpose of this research was the exploitation of Romanian clays in electroanalysis. Therefore, clay-modified electrochemical sensors for pharmaceutical analysis based on the remarkable ion exchange and adsorbent capacity of montmorillonite could be obtained. The ion exchange capacity of clays allowed the fabrication of electrochemical sensors able to detect heavy metals in different matrices. The novel clay-modified sensor was successfully applied for the analysis of acetaminophen.

Materials and Methods

HRP (Peroxidase type II from Horseradish, EC 232-668-6), acetaminophen, hydrogen peroxide, monosodium phosphate and the disodium phosphate were provided by Sigma Aldrich; PEI (50 % in water, M_r 600000 – 1000000, density 1.08 g/cm^3 (20 ^{o}C)) was purchased from Fluka.

All reagents were of analytical grade, used as received.

Thermal behavior was also studied using differential thermal analysis performed with a MOM derivatograph, type 1500D, at 10 ^{o}C/minute temperature rate, in the range of 20 ^{o}C and 1000 ^{o}C [8].

IR analyses were recorded with a Brucker FTIR spectrometer, on Răzoare clay fraction bellow 20 μm and Valea Chioarului clay fraction bellow 0.2 μm in KBr matrix, from 4000 to 400 cm^{-1}. HRP was entrapped into the clay and PEI porous gel at the surface of GCE.

Clay water suspensions of 50 mg/mL were prepared for the fractions bellow 20 μm and 0.2 μm for Valea Chioarului clay and below 20 μm for Răzoare clay. Standard solutions of acetaminophen and hydrogen peroxide were prepared to provide a final concentration of 10^{-4} M for acetaminophen and 0.1mM for hydrogen peroxide. The stock solutions of acetaminophen were dissolved in phosphate buffer and kept in the refrigerator. All the experiments were performed in PBS (phosphate buffer saline) (pH 7.4;

0.1M) at room temperature (25ºC). The pH of the solution was measured using a ChemCadet pH-meter.

Electrochemical studies like cyclic voltammetry (CV) and chronoamperometry were performed in a conventional three-electrode system: new modified carbon based electrodes (working electrodes), platinum (auxiliary electrode), Ag/AgCl 3M KCl (reference electrode), under stirring conditions. All the CVs were recorded at 100 mVs^{-1}. During chronoamperometry experiments the biosensor potential was kept at 0 V vs. Ag/AgCl under continuous stirring conditions. The working potential was imposed and the background current was allowed to arrive at a steady state value. Different amounts of acetaminophen standard solution were added, every 100 seconds, into the stirred electrochemical cell and the current was recorded as a function of time. Different amounts of acetaminophen standard solution or hydrogen peroxide were added into electrochemical cell under stirring and the current was recorded as a function of time.

The obtained configuration was used to study the biocatalytic oxidation of acetaminophen in the presence of the hydrogen peroxide.

CPEs were modified by mixing different Răzoare clay concentrations (1%, 2.5%, 5%, and 10%) with "homemade" carbon paste prepared with solid paraffin [9].

Composite film electrodes (PEI/clay/GCE) were prepared as follows: PEI (5 mg) was stirred for 15 minutes in absolute ethanol (125 μL) and distilled water (120 μL), then 6.5 μL of nanoporous clay gel were added and stirred again for 15 minutes. Two different suspensions (20 μL) containing Valea Chioarului clay particles with the diameter below 20 μm and below 0.2 μm were deposited on the surface of two different GCEs and dried for 4 hours at 4°C.

GCEs were provided by BAS Inc. (West Lafayette, USA) and were carefully washed with demineralized water and polished using diamond paste (BAS Inc.). The experiments were achieved with AUTOLAB PGSTAT 30 (EcoChemie, Netherlands) equipped with GPES 4.8 software.

Results and Discussion

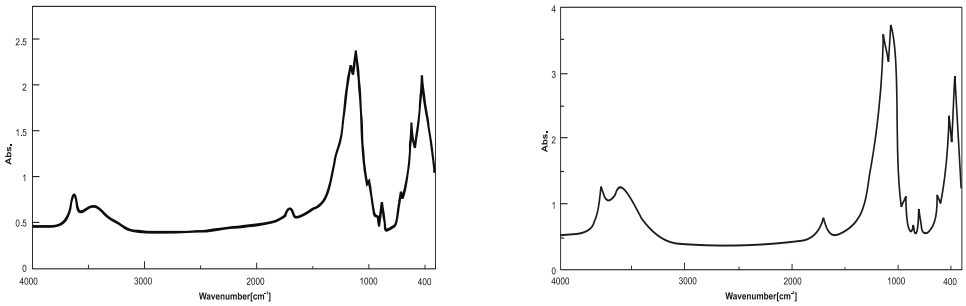
Characterization of clays

In order to complete the structural characterization of the Romanian clays several experiments were performed. The FTIR studies and the thermodifferential analysis showed the presence of a high percentage of montmorillonite.

Thus, the FTIR spectrum of Răzoare clay (Figure 1A) revealed the characteristic groups of montmorillonite. The broad band at 3447 cm^{-1}, with

its specific peak at 3620 cm^{-1}, was attributed to the stretching vibration of the hydroxyl group. The bands at 1000-1200 cm^{-1} and 466 cm^{-1} were produced by the Si-O stretching vibration. The bands at 793 cm^{-1} and 519 cm^{-1} were assigned to the Si-O-Al group [10].

The FTIR spectrum of Valea Chioarului clay (Figure 1B) presented a broad band at 3446 cm^{-1} with the specific peak at 3625 cm^{-1} attributed to the stretching vibration of the hydroxyl group [8]. The broad band at 1000-1200 cm^{-1} was assigned to the Si-O stretching vibration and the band at 520 cm^{-1} to the Si-O-Al group, all attributed to the characteristic groups of montmorillonite. In both cases the band at 1637 cm^{-1} was assigned to the bending vibration of H-O-H group [10].

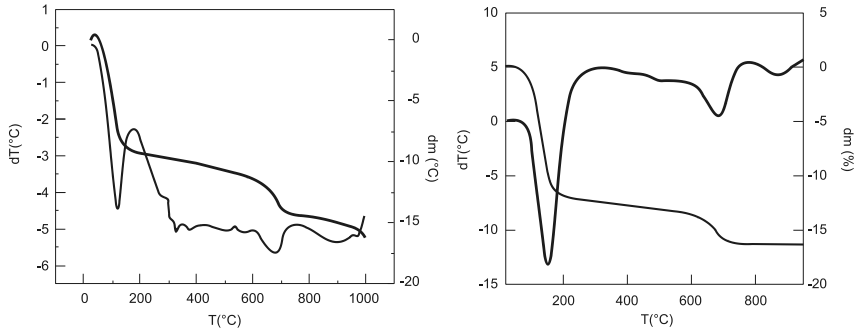


A B

Figure 1

FTIR spectra of Răzoare (A) and Valea Chioarului clays (B)

Regarding the thermodifferential analysis (Figure 2) of Răzoare and Valea Chioarului clays, a good superposition of the thermodifferential characteristic curves of montmorillonite was noticed, confirming that montmorillonite is the main constituent of both Romanian clays.



A B

Figure 2

Thermodifferential analysis of Răzoare (A) and Valea Chioarului clay (B)

The thermogravimetric (TGA) and thermodifferential (TDA) analysis of Răzoare clay (Figure 2 A) showed the loss of the adsorbed water between 60° C and 200° C, accompanied by a strong endothermic effect at 115° C. The elimination of hydroxyl groups from the mineral network with an endothermic effect and decrease of mass occurs between 600° C and 750° C. The last endothermic effect, at 900° C, immediately followed by an exothermic one, showed a modification of the crystal structure to an inferior energetic state.

The first pronounced endothermic effect in the TDA curve (Figure 2 B) appeared between 60° C and 250° C due to the water loss. A substantial decrease in the clay mass occurred in the meantime according to the TG curve. Dehydration was a reversible process, at 250° C the clay being able to readsorb the water molecules, which could be then eliminated due to a new temperature exposure [11, 12].

The second endothermic effect appeared between 600° C and 750° C, followed by a loss in the clay mass due to the elimination of hydroxyl groups. A third endothermic effect occurred at 880° C, immediately followed by an exothermic effect at 900° C due to the crystal structure modifications [11, 12].

Biosensor construction

Two types of transducers were studied by using two types of clay particles (under 20 μm and below 0.2 μm): carbon paste electrode (CPE) and glassy carbon electrode (GCE). CPE has been prepared by adding various amounts of clays (1, 2.5, 5 and 10 %). The CPE being made by a mixture of graphite powder and solid paraffin are simple to make and offer a renewable surface essential for the electron transfer [9]. The use of CPE in electroanalysis is due to their simplicity, minimal cost and the possibility of facile modification by adding other compounds thus giving to the electrodes certain predetermined properties like high selectivity and sensitivity [13].

In order to realize a biosensor for the detection of acetaminophen, the electrochemical behavior of the doped CPEs was compared with the behavior of the thin PEI film GCEs. The thin PEI film deposited on the surface of a GCE exhibited a better mechanical stability in spite of its relative water solubility and an improved hydration layer, essential for the immobilization of the enzyme.

The difference between the two electrode configurations was clear. By CV recording of acetaminophen on modified CPEs, only the oxidation process could be observed (Figure 3). By comparison, the PEI film electrodes showed a reversible oxidation and reduction process (Figure 4).

The current obtained on polymeric film electrodes was, however, lower (10-15 μA) than that obtained on clay-modified CPEs (100-350 μA).

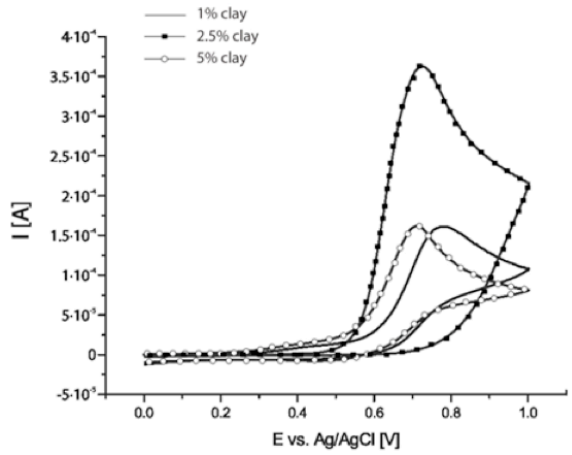


Figure 3

Cyclic voltammograms of 10-3 M acetaminophen on 1% (solid line), 2.5% (square line) and 5% (dot line) Răzoare clay-modified CPEs (KCl 0.1 M, 100 mVs-1) [14]

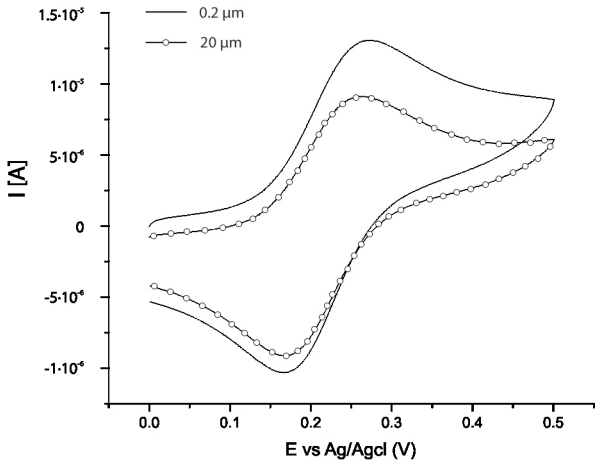


Figure 4

Cyclic voltammograms of 10^{-4} M acetaminophen solution using 20 μm (dot line) and 0.2 μm (solid line) Valea Chioarului clay immobilized in a 1 mg/mL PEI film (0.1 M phosphate buffer pH 7.4, 50 mVs^{-1})

An increase of the current was noticed on different particle sizes of Valea Chioarului clay, the best response for acetaminophen being recorded for the 0.2 μm, due to the greater active surface (Figure 4).

In the human body, acetaminophen is metabolized to *N*-acetylbenzoquinonimine (NAPQI) [15]. The same conversion can be achieved *in vitro* by HRP in the presence of hydrogen peroxide.

The amperometic studies were made by recording the electrochemical reduction of the enzymatically generated electroactive oxidized species of acetaminophen (NAPQI) in the presence of hydrogen peroxide after stepwise addition of small amounts of 10^{-4} M acetaminophen solution [15].

$$\text{Acetaminophen} + H_2O_2 + 2H^+ + \text{HRP} \rightarrow \text{NAPQI} + 2H_2O$$
$$\text{NAPQI} + 2e^- + 2H^+ \rightarrow \text{Acetaminophen}$$

The linear range was calculated as the ratio of the standard deviation of the blank baseline (0.1 M phosphate buffer, pH 7.4), the noise and the biosensor's response to acetaminophen [2]. The amperometric assays were realized at -0.2V, which represents the reduction potential of NAPQI. The HRP-clay-PEI film on the glassy carbon biosensor had a sensitivity of $6.28 \cdot 10^{-7}$ M and a linear range between $5.25 \cdot 10^{-6}$ M and $4.95 \cdot 10^{-5}$ M.

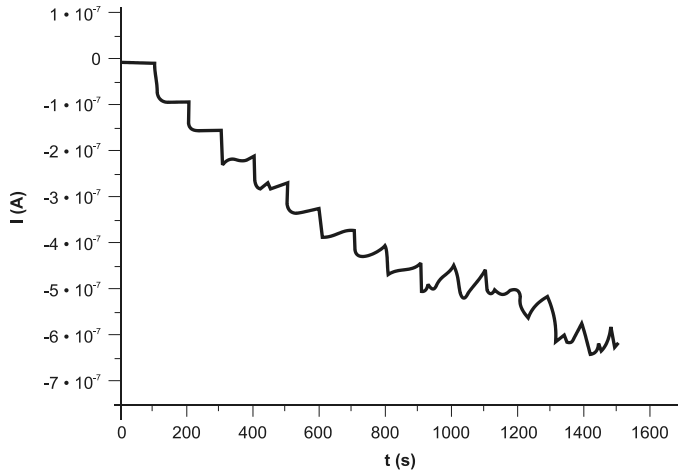


Figure 5

Amperometric response of the clay-modified electrode (0.2 µm Valea Chioarului clay in 1 mg/mL PEI /HRP/GCE) after successive additions of 50 µL of 10^{-4} M acetaminophen in phosphate buffer pH 7.4 and 0.2 mM H_2O_2

The calibration curve equation of the amperometric biosensor was $y = 0.0139x + 3 \cdot 10^{-8}$ ($R^2 = 0.996$) and presented a linear range between $5 \cdot 10^{-6}$ - $4.5 \cdot 10^{-5}$ M. The reproducibility was also tested on the same electrode after 10 successive analyses in three different days. The RSDs of the slopes of the

linear responses calculated by Line weaver-Burk method were less than 15%.

Conclusions

A novel biosensor based on HRP immobilization in a PEI and clay porous gel film for acetaminophen was developed. The amperometric detection of acetaminophen was successfully achieved with a sensitivity of $6.28 \cdot 10^{-7}$ M and a linear range between $5.25 \cdot 10^{-6}$ M and $4.95 \cdot 10^{-5}$ M. The clay offered both a good entrapping and a "protective" environment for the biocomponent. This immobilization strategy could be exploited in the development of other biomolecules for the detection of pharmaceuticals.

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