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Performance Evaluation of Acetylcholinesterase-Based Biosensors for Detection of Heavy Metals

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Abstract: In this work, mediated carbon-ink screen-printed electrodes are used to develop enzyme-based biosensors for detection of selected heavy metals. Two immobilisation methods were used for acetylcholinesterase (AChE) immobilisation on electrode surface together with two redox mediators. The methods used for the encapsulation of the enzyme were: cross-linking with bi-functional reagents like glutar-aldehyde (GA) and encapsulation with sol-gel method.The used mediators were TCNQ (tetracianoquinodimethan) and CoPC (cobalt phthalocyanine). Comparison between biosensors performance was made function of electrolyte pH, especially for more acidic values were heavy metals are present in ionic form.

Keywords: Biosensors; Screen-printed electrodes; Acetylcholinesterase; Heavy metals.

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Introduction

Electrochemical enzyme-based biosensors commonly rely on the enzyme that catalyzes the reduction or oxidation of the analyte. These redox reactions can be detected at the electrode either directly, or through redox mediators such as ferrocene- and osmium-based redox polymers [1]. Biosensor configurations are also occasionally used for the detection of heavy metals in order to monitor their concentrations, due to the potential health and ecological hazard they present [2-4].

Acetylcholinesterase (AChE) is an enzyme that degrades, through its hydrolytic activity, the neurotransmitter acetylcholine, producing choline and an acetate group. It is mainly found at neuromuscular junctions and cholinergic nervous system, where its activity serves to terminate synaptic transmission [5]. The inhibition of this enzyme helps us to determine the toxicity levels in our surroundings. Inhibition based biosensors are more sensitive than those based only on the detection of the compatible substrate and AChE is an appropriate enzyme for inhibition detections, being able to detect inhibitors in concentrations of at the low ppb level [6,7].

In this work, we have used mediated carbon-ink screen-printed electrodes because of low production costs, easy fabrication method and good reproducibility. Carbon-ink is known for its good conducting property [8] and the mediators ease the electron transfer from the surface of the electrode. The used mediators were TCNQ (tetracianoquinodimethan) [9] and CoPC (cobalt phthalocyanine) [10]. Two methods were used for the encapsulation of the enzyme solution on the surface of the working electrode: cross-linking with bi-functional reagents like glutar-aldehyde (GA) and encapsulation with sol-gel [11]. Regarding their use for cadmium inhibition measurements, these electrodes were mostly used for single time detections.

Experimental

Materials and Methods

The enzyme acetylcholinesterase (AChE) from Electrophorus electricus 906 U/mg and compatible acetylthiocoline substrate (ATCh) were purchased from Sigma Chemicals Co., UK. The sol-gel precursors tetraethoxysilane (TEOS), trimethoxysilane (TMOS) and methyl-trimethoxysilane (MTMOS) were purchased from Merck, Germany. Glutaraldehyde 25% (GA) and bovine serum albumine (BSA) were also purchased from Sigma Chemicals Co., UK.

The supporting electrolyte was 0.1 M acetate buffer (ph = 5.1 and 5.5) and 0.1 M phosphate buffer (pH = 6.0 and 7.0). All chemicals used for the preparation of stock and standard solutions of heavy metals, as well as sodium phosphate, disodium hydrogen phosphate, sodium chloride, acetic acid, sodium acetate and sodium hydroxide and all other chemicals were of analytical reagent grade and purchased from Sigma-Aldrich or Merck. All solutions were prepared with bi-distilled water.

The screen-printed electrodes used in this study are containing cobalt phthalocyanine (CoPC) and tetracianoquinodimethan (TCNQ) as mediators, and were prepared accordingly to procedures previously described [12,13].

The diameter of the surface area of the working electrode is 0.4 mm, the auxiliary electrode is a 16 x1.5 mm curve line surrounding on two sides the working electrode and the Ag/AgCl pseudo-reference electrode is a 5 x1.5 mm straight line positioned on the third side of the working electrode.

Electrochemical Measurements

Measurements were made in a one-compartment cell containing modified screen-printed electrodes. Voltammetric and amperometric experiments were carried out using the electro-chemical sensor interface PalmSens (Palm Instruments BV; The Netherlands) controlled with PalmScan software. The experiments were performed in four different buffer solutions with a pH ranging from 5.1 to 7.0; always, with continuous stirring.

Enzyme Immobilisation

Two enzyme immobilization methods were used: cross-linking with GA and bioencapsulation with different sol-gel recipes. For cross-linking method 10 µl of enzymatic solution were mixed with 5 µl GA 2.5% and spread on the surface of the working electrode, two hours of drying were required. For sol-gel method 5 µl of enzymatic solution were mixed with 10 µl sol-gel and spread on the surface of the working electrode, 48 hours of drying at a temperature of ~ 4ºC were required. To prepare the enzymatic solution 0.7 mg AChE and 4 mg BSA were diluted with 100 µl phosphate buffer pH=7.4. The electrodes with the immobilized enzyme were kept in a solution of phosphate buffer at a temperature of ~ 4ºC when they were not in use.

Results and Discussions

Biosensor attributes which permanently need to be optimized are response time, initial and long term activity, analytical range, sensitivity, selectivity, detection limit and reproducibility [14]. The biosensor stability and analytical performance depends on the immobilisation process, comparing both cross-linking and sol-gel method, and the matrix used [15].

The cross-linking method with GA is well known and widely used [16]. The sol-gel processing technique has a unique behaviour regarding bio-encapsulation. Encapsulation in sol-gel matrices, not only improves the enzymatic activity, but also enhances thermal or chemical stability. Sol-gels are known to slow down the unfolding of proteins [14]. In order to obtain biologically compatible doped sol-gels, some requirements must be taken into account. An important requirement is the use of aqueous methods for gel preparation, in order to maintain the biological functions of the entrapped biomolecule [7].

Also, the properties of the sol-gel precursors should be easily changeable to allow the modification of the internal environment. This is possible through the mixture of different quantities of different precursors: some are known to form larger capsules than others, and hidrophobicity also plays an important role here. There are also different types of polymers such as PEG (polyethylene glycol) or PVA (polyvinyl alcohol) added to the sol-gel mixture in order to model the capsule around the enzyme [17,18].

Several sol-gel recipes which combine the three precursors (TEOS, MTMOS and TMOS) were used in order to find the best immobilization matrix to allow a maximum functionality of the enzyme (results not shown). All recipes were prepared at room temperature at 20ºC, and after the enzyme immobilization the electrodes were kept in phosphate buffer (pH 7.4) at a temperature of 4 ºC when not used in measurements. Table I shows these recipes, regardless the encapsulation method, which encapsulated the enzyme successfully, kept his catalytic activity and which formed biosensors which showed a good performance.

Table I: *Recipes used for the fabrication of biosensors*

No.	Mediator	Immobilisation method	Recipe / Composition of sensing system
1	TCNQ	GA	GA + enzyme solution
2	TCNQ	Sol-Gel	$TEOS:H_2O:HCl = 37.5:100:6$ + enzyme solution
3	CoPC	Sol-Gel	$TEOS:H_2O:HCl = 37.5:100:6$ + enzyme solution
4	CoPC	Sol-Gel	$TEOS:H_2O:HCl = 100:37.5:6$ + enzyme solution
5	CoPC	GA	GA + enzyme solution

Cyclic Voltammetry Measurements

This type of measurements offers qualitative information about the electrochemical reactions which undergo on the surface of the working electrode. The redox peaks are different for each type of mediator. The working potential needed for the amperometric measurements can be determined after the position of the redox peaks, therefore the working potential for CoPC electrodes is at 0.1 V and for TCNQ electrodes at 0.15 V (results not shown).

Amperometric Measurements of the Substrate

Through amperometic measurements, we can determine the current given by the electro-chemical reaction of a certain species, at certain concentrations, which undergoes on the surface of the working electrode. The current intensity depends directly on the concentration of the analyte. The pH of the working electrolyte influences the biosensors response. Normally the enzyme pH is closer to the physiological one. In our experiments we want to detect heavy metals that are present in ionised form usually at acid pH. For this reason, measurements at different pH values (5.1, 5.5, 6.3, 7.0; see Fig. 1) are done for different enzymatic substrate (ATChE) concentrations (0.2mM, 0.4 mM, 0.6 mM).

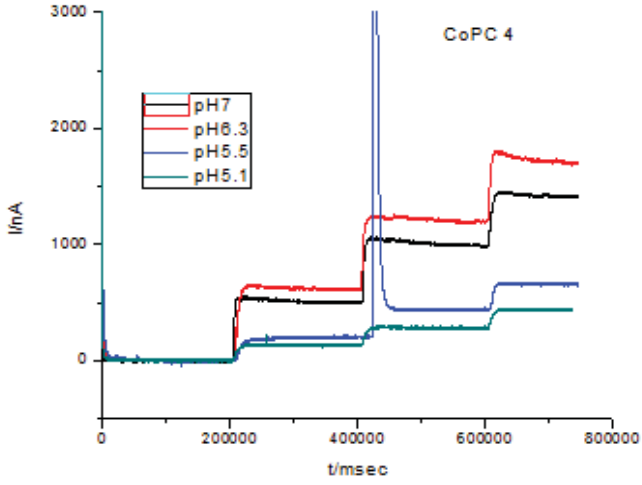

Fig. 1. *The response of biosensor 4, mediator CoPC, sol-gel immobilization method at pH 5.1, 5.5, 6.3 and 7.0 for substrate (ATChE) concentrations of 0.2mM, 0.4 mM and 0.6 mM.*

The results shown, that the AChE-based biosensors are working also at lower pH but with lower sensibility. The stability of the biosensors was evaluated by repeated measurements in different days, over a timeframe of 40 days, at the same substrate (ATChE) concentration of 0.6 mM. The results show that the biosensors remained active even after 40 days. Here, it should be taken into account, that the electrodes were used for inhibition measurements and in such cases, the electrodes are of single-use. In order to determine if our biosensors are reproducible, we analysed three electrodes, using the most stable sol-gel recipe. The pH value (5.5) and the substrate concentration value (0.6 mM) were kept constant. Two of the electrodes had very similar response values.

Regarding the substrate measurements, a comparison between all electrodes was made at a pH=5.5 and the results are shown in Fig. 2.

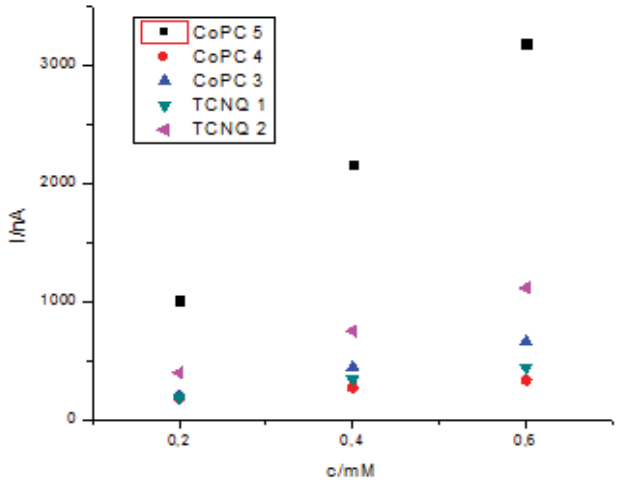

Fig. 2. *Comparison between all biosensor responses at pH 5.5 for three successive additions of 0.2 mM ATChE substrate.*

From the above figure we can conclude that the CoPC 5 electrode, with the enzyme immobilized by cross-linking with GA has the highest sensitivity. Several authors described a better enzymatic activity by using GA. If we compare the sensitivity of the two different mediators using the same immobilization method, we conclude that the TCNQ 2 electrode using the sol-gel immobilization method, has a better performance than the TCNQ 1 electrode (GA). This shows how important the mediator is regarding the electron transfer, and its compatibility to a certain immobilization method.

Inhibition Measurements

In order to keep a balance between a good enzymatic activity and the presence of heavy metals in ionic structure we choose a pH value of 5.5. Heavy metals need an acid pH to be found as ions, and to be able to bind to the active sites of the enzyme.

The best value for the incubation time was determined to be 10 min. This value was determined for a concentration of 25 ppb Cd, after trying different incubation times of 10, 15, and 20 min. (not shown).

The degree of inhibition was determined after the following expression:

$$I(\%) = 100\,(I_1 - I_2)/I_1 = \Delta I/I_1$$

where I stands for the degree of inhibition, I_1 is the answer given by the biosensor for the detection of the substrate, and I_2 is the answer given by the biosensor for the detection of the substrate in the presence of the inhibitor.

Calibration curves (see Figs 3 & 4) play an important role in the characterization of a biosensor. Cadmium ions in different concentrations were used while the concentration of the substrate was kept at 0.6 mM. The enzyme inhibition depends on the concentration of the cadmium ions. For the TCNQ 2 electrode, the calibration curve could not be constructed because the enzymatic activity had become very weak in the course of time.

From the calibration curves, it was also possible to calculate the detection limit of each biosensor. The detection limit represents the minimum concentration value of the analyte which can be detected with the help of a biosensor. In order to calculate the detection limit we used the formula: LOD = $3\sigma_D$ / S, where σ_D is the standard deviation, and S the sensibility of the biosensor. The higher the sensibility is, the lower is the detection limit, which means that the biosensor is very sensitive.

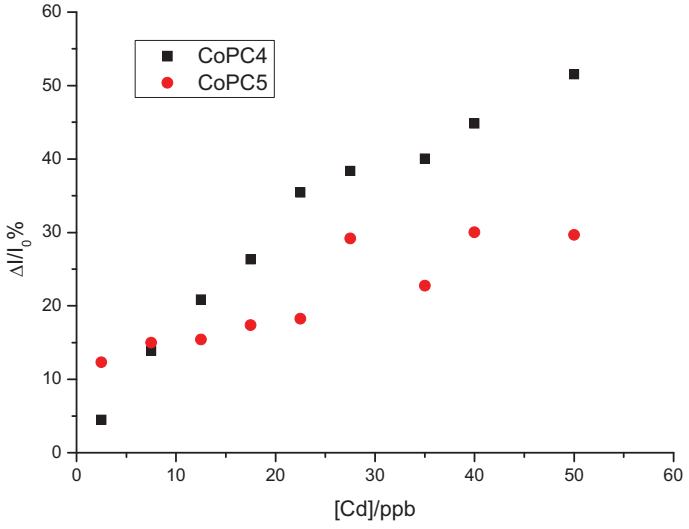

Fig. 3. *Comparison between the calibration curves of CoPC electrodes (different enzyme immobilization methods).*

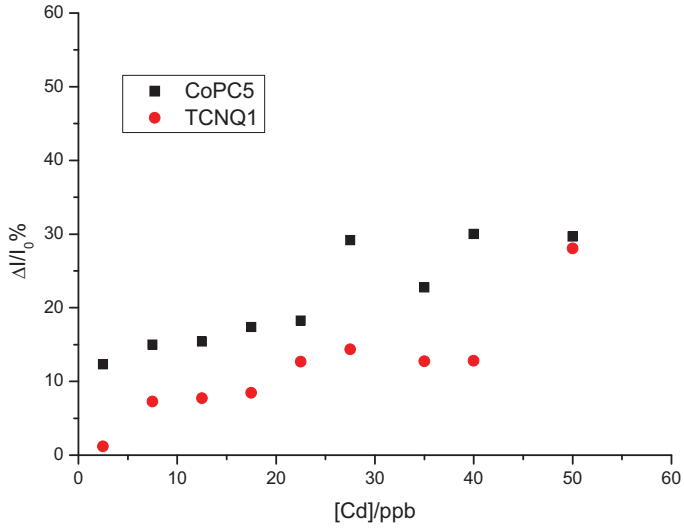

Fig. 4. *Comparison between the calibration curves of CoPC and TCNQ electrodes with the same enzyme immobilization method (GA).*

Table II below summarizes some characteristics of the biosensors like: sensitivity, limit of detection and linear detection range:

Table II: *The values for sensitivity, limit of detection and linear detection range for the analyzed biosensors.*

Electrode	S (%ppb)	LOD (ppb)	Linear detection range (ppb)
CoPC 5	0.285 ± 0.04	0.389	2.5 - 40
CoPC 4	1.122 ± 0.14	0.366	2.5 - 40
CoPC 3	0.695 ± 0.26	1.12	2.5 - 40
TCNQ 1	0.528 ±0.088	0.494	2.5 - 30

By analyzing the data from the table, it can be concluded that the most performing electrode is CoPC-4 (immobilization method with sol-gel). It has the highest sensitivity and the lowest LOD. The linear detection range changes only for a different type of mediator.

Conclusions

In this work, we have used mediated carbon-ink screen-printed electrodes for enzyme-based biosensors development used as detection tool for heavy metal detection. Two immobilisation methods were used for acetylcholinesterase (AChE) immobilisation on electrode surface together with two redox mediators. We obtained different characteristics for each type of biosensors. After performing the substrate measurements, it can be stated the sol-gel method keeps the enzyme in a stable conformation and the enzymatic activity is preserved as well as in the immobilization method with glutaraldehyde, independent of the mediator type.

Regarding the inhibition measurements, we found the the biosensor using sol-gel for enzyme immobilisation and CoPC as electrochemical mediator (CoPC-4) to be the most sensitive ($S = 1.122 \pm 0.14$), with LOD of 0.37 ppb and a linear detection range up to 40 ppb. The biosensors obtained with the immobilization method with sol-gel, are more sensitive than those obtained with the cross-linking immobilization method. The sol-gel precursors play here a very important role, influencing the enzymatic activity through pore size and acidity. Taking into account the value of the detection limit, we have obtained both very sensitive electrodes (CoPC 4 and TCNQ1); but, also less sensitive electrodes. Finally, all the electrodes have had short response times (up to 10 seconds), even in the presence of an inhibitor.

Acknowledgements

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