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DEVELOPMENT AND EVALUATION OF SOL-GEL-BASED BIOSENSORS FOR CADMIUM IONS DETECTION

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Abstract

The enzyme-based biosensor stability and its analytical performance depend on both the immobilization process and the matrix used for immobilization of the enzyme into a stable layer. In this paper, the development and evaluation of an optimal enzymatic layer for stable encapsulation of the acetylcholinesterase enzyme (AChE) through the sol-gel method onto mediated carbon-ink screen-printed electrodes was performed, obtaining electrochemical enzyme-based biosensors. The enzyme was immobilized on the surface of the working electrode using different combinations of three sol-gel precursors, tetraethoxysilane (TEOS), tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMOS) in different ratios, without usual addition of alcohol, by comparison with the well-known cross-linking immobilization method using glutaraldehyde. Only the best suitable precursor (TEOS) was kept for further analysis. The optimized biosensors were successfully used for the amperometric detection of Cd ions; the sensor exhibits high sensitivity (1.48 ± 0.14 %/µgl^{-1}) and a low detection limit of 0.19 µg/L. Potentialities, for a short-term-use or disposable sensors, are indicated.

Key words: electrochemical biosensors; screen-printed electrodes; sol-gel layer

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1. Introduction

Electrochemical biosensors based on enzyme inhibition play an important role in the field of environmental analysis. Enzymes such as acetylcholinesterase (AChE), are inhibited by heavy metal ions, indicating their presence in natural waters and soil. These ions are well known to have pollutant effects on living organisms, increasing the need for disposable tools or systems, such as biosensors, for environmental monitoring (Rodriguez-Mozaz et al., 2006).

In present, there are several heavy metal detection methods such as atomic absorption spectrometry, inductively plasma-mass spectrometry and high performance liquid chromatography (Chen et al., 2011; He et al., 2011; Tarley et al., 2011). These methods require expensive equipment, which cannot be used for continuous measurements in the field. Moreover, some of these methods involve complicated and time-consuming sample treatment (Sartore et al., 2011). Electrochemical methods such as voltammetry or amperometry are seen as complementary to the aforementioned techniques, and are especially attractive because they allow the possibility of creating inexpensive and portable instrumentation, such as sensors and biosensors (Canh, 1993). The biosensor stability and its analytical performance depend on the matrix and the immobilization process of biological component used (Florescu et al., 2007; Pierre, 2004).

Electrochemical enzyme-based biosensors commonly rely on the enzyme that catalysis a biochemical reaction followed by the reduction or oxidation of the electrochemical product (Tutulea et al., 2012). These redox reactions can be detected at the

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electrode either directly (David et al., 2018; Tan et al., 2010; Tseng et al., 2009) or through redox mediators such as ferrocene, hexacyanoferrate or phthalocyanine compounds etc. (Florescu and David, 2017; Jayasri and Sriman Narayanan, 2007; Pauliukaite et al., 2007; Sameenoi et al., 2011; Yan et al., 2011; Wang et al., 1996) that ease the electron transfer between the electrochemical reaction and the surface of the electrode. Thus sensitive, specific and label-free analytical devices are obtained.

Biosensors can also be used for the detection of heavy metals (Chouteau et al., 2005; Frasco et al., 2007; Rodriguez et al., 2004). Enzyme inhibition may occur due to binding of the heavy metal ions to the active site of the enzyme and therefore biosensors based on enzyme inhibition can be developed. Acethylcholinesterase (AChE) is an enzyme that degrades, through its hydrolytic activity, the neurotransmitter acetylthiocholine, producing choline and an acetate group (Badea et al., 2009). Both inhibition based biosensors and those based only on the detection of the compatible substrate are very sensitive and AChE is an appropriate enzyme for inhibition detections, being able to detect inhibitors in concentrations of parts per billion (He et al., 2010; Luque de Castro and Herrera, 2003). Cadmium is harmful for the environment as well as for humans since it tends to accumulate in the liver, kidney or other tissues. Cadmium induced toxicity may occur because of its interference with antioxidant enzymes, it is altering thiol containing proteins, DNA structures and many more (Casalino et al., 2002; Koolivand et al., 2017).

The biosensor stability and its analytical performance depend on both the immobilization process, and the matrix used for immobilization of the enzyme into a stable layer. The cross-linking method with glutaraldehyde for enzyme-based biosensors development is well known and widely used (Betancourt et al., 2006; Florescu and Brett, 2005, 2007). The sol-gel processing technique is increasingly gaining attention of world scientists due to their wide practical applications in various directions, and also has been used for biosensors development having a unique behavior regarding the encapsulation of a biological compound, especially enzymes into stable layers (Gupta and Chaudhury, 2007; Li et al., 2012; Liu et al., 2011; Sun et al., 2011; Wang et al., 2012). Using the sol-gel technique, a material layer, with properties similar to glass with a porous structure, is obtained. This material allows the encapsulation of enzyme macromolecules without using any covalent bondage. Encapsulation in sol-gel matrices is known not only to keep the enzymatic activity, but also enhances its thermal or chemical stability (Lev et al., 1997), or slowing down the unfolding of proteins (Pierre, 2004). The modification of the internal environment is possible through the mixture of different quantities of different precursors. Some are known to form larger pores than others, and hydrophobicity degree also plays an important role. The use of aqueous methods for gel preparation is wanted, in order to maintain the biological functions of the entrapped biomolecules (Brennan et al., 2002).

This paper presents the optimization of the AChE encapsulation into a stable sol-gel layer on mediated carbon-ink screen-printed electrodes for the development of enzyme-based electrochemical biosensors used for heavy metals detection. The enzymatic layer was formed on the surface of the working electrode using combinations of three sol-gel precursors, tetraethoxysilane, tetramethoxysilane and methyltrimethoxysilane in different ratios without usual addition of alcohol. The optimal biosensors parameters were obtained by studying both the effect of oxysilane precursor's combination and working conditions of inhibition based-biosensors. Analytical performance in quantification of heavy metal ions detection was also studied.

2. Material 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), tetramethoxysilane (TMOS) and methyl-trimethoxysilane (MTMOS) were purchased from Merck, Germany. Glutaraldehyde 25% (GA) and bovine serum albumin (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 6.3 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 coated with cobalt phthalocyanine (CoPC) and tetracyanoquinodimethane (TCNQ) as electrochemical mediators, and were prepared accordingly to procedures previously described (Crew et al., 2004; Valdes-Ramirez et al., 2008). 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 x 1.5 mm straight line positioned on the third side of the working electrode.

2.1. Electrochemical measurements

Measurements were made in a one-compartment cell containing modified screen-printed electrodes. Voltammetric and amperometric experiments were carried out using the electrochemical sensor interface PalmSens (Palm Instruments BV, The Netherlands) controlled with PalmScan software. The experiments were performed

in 4 different buffer solutions with a pH ranging from 5.1 to 7.0 with continuous stirring, at room temperature.

2.2. Enzyme immobilization

Enzyme immobilization method used was the encapsulation with sol-gels obtained from three oxysilane precursors mixed in different ratios. To prepare the enzymatic solution 0.7 mg AChE and 4 mg BSA were diluted with 100 µl phosphate buffer pH=7.4. For enzyme immobilization 50 µl of enzymatic solution were mixed with 100 µl sol-gel and deposited on the surface of the working electrode; 48 hours of drying at a temperature of ~ 4ºC were required. The sol-gel was obtained by mixing the sol-gel precursors in different ratios. As sol-gel preparation methods are usually aqueous as can be seen for the protocols 1, 2, 3 and 4, also non aqueous protocols were tried, such as protocols 5, 6 and 7. The mixtures obtained were intensively stirred for a few minutes. Sonication (for 15 min) was used only for aqueous protocols due to low solubility of silanes in water. Following this, the mixtures were heated in a hot air stream to evaporate the alcohol formed during hydrolysis of the oxysilanes until the solutions lost 40% of their volume (Florescu et al., 2007). Afterwards they were left for 10 min at room temperature to cool down and neutralized to neutral pH by addition of a very small quantity of 0.01 M NaOH (if needed).

The electrodes with the immobilized enzyme were kept in phosphate buffer at a temperature of ~ 4ºC when they were not used, in order to maintain their initial properties and to permit good mobility and diffusion of molecules to the enzyme and electrode surface.

3. Results and discussions

In this work the AChE enzyme immobilization was done by encapsulation with sol-gel method without usual addition of alcohol, due to enzyme degradation. AChE-based biosensors were developed and their performances compared, by immobilizing the enzyme on screen-printed electrodes (SPE) modified with two redox mediators: TCNQ (tetracyanoquinodimethane) and CoPC (cobalt phthalocyanine) (gift from Prof. J.-L. Marty, University of Perpignan Via Domitia, France). Carbon-ink used for SPE fabrication is known for its good conducting property (Wang et al., 1996). The electrodes have low production costs, easy fabrication method and good reproducibility.

3.1. Optimization of sol-gel formation

The sol-gel process is a wet-chemical technique widely used, primarily for the fabrication of materials like colloidal solutions that act as the precursor for an integrated network. Typical precursors are metal alkoxides and metal anions such as: chlorides, nitrates and acetates; which undergo various forms of hydrolysis and poly-condensation reactions, which are usually slow processes. The addition of an acid (in our case HCl) is necessary in order to catalyze the reactions (Rabinovich and Lev, 2001). The resulted gel possesses chemical inertness, physical rigidity, optical transparency, thermal stability, and experiences negligible swelling in aqueous and organic solvents. The enzyme can be immobilized by encapsulation, allowing the preservation of enzyme activity by building the porous gel network around each enzyme macromolecule (Kumar et al., 2000; Pierre, 2004). The coupling mechanism of the precursors depends on the link between the organo-functional groups and the hydrolysable groups.

An important role has the hydrophilic/hydrophobic character of the used precursors. Changing the proportions in which the hydrophobic and hydrophilic groups are found on the surface of the precursor molecules, can influence the contraction of the entire structure, resulting in contractions of the nano-cages containing the encapsulated enzyme (Azenha et al., 2005; Won et al., 2010). Khummalai and Boonamnuayvitaya (2005) showed in their work that cracking can be found in TEOS and TMOS films compared with MTMOS films that present a crack-free morphology.

In this study sol-gel precursors were used in different ratios in order to find the optimal immobilization matrix to allow maximum functionality of the encapsulated enzyme on the surface of mediator-modified electrodes. Initially, seven combinations were used: two involving TEOS in different proportions in combinations with water, three involving TEOS in combinations with TMOS and MTMOS with or without water, and two involving MTMOS in combination with TMOS in different proportions without water (Table 1) (in all situations HCl was used).

Table 1. Sol-gel protocols

No.	*Precursor Type*	*Sol-gel formation method*		*Stirring method*		*pH*	
		H_2O	*HCl*	*Sonication (min)*	*Manually (min)*	*initial*	*final*
1	TEOS	yes	yes	30	5	4-5	4-5
2	TEOS	yes	yes	120	5	4-5	4-5
3	TEOS: MTMOS	yes	yes	30	5	4-5	6
4	TEOS: TMOS	yes	yes	30	5	4-5	6
5	TEOS: MTMOS	no	yes	-	5	1	6
6	TMOS: MTMOS	no	yes	-	5	5	5
7	TMOS: MTMOS	no	yes	-	5	5	5

HCl was added as a co-solvent to ease the hydrolysis between the sol-gel precursors and water. During the gelation process of the sol, products such as alcohols often occur. Before adding the enzyme solution, the alcohol from the sol was evaporated under a beam of hot air. All sol-gel protocols were prepared at room temperature (20ºC), and after the enzyme immobilization the biosensors were kept in phosphate buffer (pH 7.4) at a temperature of 4ºC when not used in measurements.

Protocols 1-4 describe aqueous solutions, while protocols 5-7 describe non-aqueous solutions. By using protocols 1 and 2 (involving only TEOS in different ratios and water) a heterogeneous solution was obtained even after using sonication. The obtained solution was dropwise added to the surface of the working electrode, obtaining a thin, glassy, crack-free layer. TEOS and TMOS precursors are known to have an acidic pH (3.00 – 4.00) therefore the addition of very small aliquots of NaOH is necessary. We observed that the addition of NaOH lead to the precipitation of the solution containing TEOS together with TMOS, therefore protocol 4 couldn't be used for the enzyme immobilization. An explanation for these unsuccessful results might be the fact that the condensation/hydrolysis rate of the alkoxides do not match. Suratwala et al. (1998) showed that the condensation/hydrolysis rate of TEOS is much slower than that of TMOS. Protocol 3 (using TEOS:MTMOS and water) formed a transparent sol-gel solution which was dropwise added to the surface of the working electrode, but the layer cracked after drying.

The sol-gel prepared with protocol 7 by non-aqueous methods didn't turn into a gel on the surface of the electrode; no matrix could be formed to encapsulate the enzyme. Luckham and Brennan (2010) obtained a highly uniform film that did not crack by mixing a 40% (v/v) MTMOS/TMOS solution containing co-hydrolyzed components. Therefore, for the protocols 5 and 6 (mixture of TEOS/TMOS with MTMOS) the gelation was achieved and a crack-free, thin enzymatic layer was obtained. In conclusion, only some of the above protocols were kept for biosensor development. These protocols are 1 and 2: TEOS with H_2O in different ratios and the combination of MTMOS with TEOS and TMOS (protocols 5 and 6).

3.2. Electrochemical measurements

3.2.1. Amperometric measurements

Through amperometric measurements, the current given by the redox reaction of a certain species is recorded. This redox reaction undergoes on the surface of the electrode when this is kept at a fix potential (working potential) and the current depends directly on the concentration of the species. The enzymatic reaction which undergoes on the surface of the electrode involving catalytic transformation of acetylthiocholine (ACTh), in the presence of AChE (Fig. 1A).

One of the reaction products, electrochemically active (thiocholine), is being transformed (dimerized) at the modified electrode surface and generates an electric current. Thus, in amperometric measurements using AChE-based biosensor the current obtained is proportional with the concentration of enzymatic substrate, ATCh. Amine (2006) and Stoytcheva (2002) showed that activity of immobilized AChE can be reversible and non-competitive inhibited by metal ions (for example cadmium). These inhibitors bind to the enzyme and the active center is deformed thus its function is impaired. In the presence of the inhibitor, a smaller quantity of the substrate (ATCh) will be transformed during the enzymatic reaction, and the amperometric biosensor will show a decrease in current intensity, proportional with the concentration of the inhibitor, for the same concentration of ATCh. Different working parameters can affect the response of the inhibition-biosensors, such as: working potential, pH of the used electrolyte and incubation time of the metal ions.

Table 2 presents the optimized protocols for the sol-gel enzymatic layers used for the deposition onto the mediated screen printed electrodes for AChE-based biosensors development. For each type of mediator-based electrode the cross-linking method with glutaraldehyde (GA) was used as comparative method for enzyme immobilization. For this purpose 100 µl of enzymatic solution were mixed with 50 µl GA 2.5% and spread on the surface of the working electrode, two hours of drying were required.

3.2.2. Working potential

The working potential of electrochemical biosensors depends both on the used material for electrode fabrication and the existence of redox mediators, such as TCNQ or CoPC. TCNQ is an organic compound showing an increased efficiency for the regeneration of a certain number of enzymatic systems. In comparison, CoPC is an organic semiconductor with a very high stability (Joseph and Menon, 2008). It was found in our previous work, that the optimal working potential for TCNQ-modified electrodes is of 0.15V and for CoPC-modified electrodes of 0.1V, confirmed also by literature (Badea et al., 2009). The following reactions describe the role of the used mediators (CoPC and TCNQ); they assure a low potential of the working electrode and facilitate the electron transfer between the substrate and the working electrode (Fig. 1.B).

3.2.3. Substrate measurements – pH studies

The amperometric measurements for enzymatic substrate detection were made in buffer solutions at different pH values (5.1, 5.5, 6.3, 7.0) for successive substrate additions, and were carried out under constant stirring. It is known that an acidic environment is needed for the existence of heavy metals in ionic form in aqueous solutions; therefore

low values of pH were also used in the view of inhibition-based biosensors development. Table 3, presents AChE-biosensors sensitivities, S, calculated as the slope of the calibration plots for all amperometric measurements of its substrate, ATCh. The calibration plots of these biosensors were done using the current (μA) obtained as the biosensor response - versus concentration of the substrate (mM) (data not shown). For sol-gel layer-based biosensors the electrodes E1, E2 and E3 have presented higher sensitivities for all used pH, as can be seen in the Fig. 2. This fact illustrates that AChE maintains its biological activity within the sol–gel matrix and the enzyme has been immobilized successfully even for lower values of pH. The electrodes E4 and E5 showed a lower enzymatic activity for neutral pH and even lower for more acidic pH. That fact implies that even though the enzyme was immobilized on the electrode surface by sol-gel matrix the enzymatic activity decreased, therefore the biosensors couldn't be used further for inhibition measurements. It is known that MTMOS forms relatively large pores-containing layers, therefore, the enzyme might leak, and small values for current are obtained (Florescu et al., 2007).

Table 2. Protocols used for the fabrication of biosensors

No.	*Mediator*	*Immobilisation method*	*Immobilisation Protocol (μl)*
E1	CoPC	Sol-Gel	TEOS:H_2O:HCL =37.5:100:6
E2	CoPC	Sol-Gel	TEOS:H_2O:HCL = 100:37.5:6
E3	TCNQ	Sol-Gel	TEOS:H_2O:HCL =100:100:6
E4	CoPC	Sol-Gel	TMOS:MTMOS:HCL =37.5:100:10
E5	TCNQ	Sol-Gel	TEOS:MTMOS:HCL =50:100:10
E6	CoPC	GA	GA = 50
E7	TCNQ	GA	GA = 50

Table 3. AChE biosensors sensitivities S (μA/mM) at different pH for ATCh detection

Electrode	*Immobilization Protocol (μl)*	*pH 7.0*	*pH 6.3*	*pH 5.5*	*pH 5.1*
E1	TEOS:H_2O:HCL =37.5:100:6	2.90±0.05	2.48±0.36	1.09±0.01	0.72±0.01
E2	TEOS:H_2O:HCL = 100:37.5:6	2.85±0.32	2.85±0.32	0.61±0.05	0.29±0.01
E3	TEOS:H_2O:HCL =100:100:6	4.46±0.05	4.03±0.09	1.87±0.02	1.12±0.03
E4	TMOS:MTMOS:HCL =37.5:100:10	0.69±0.05	-	0.18±0.01	-
E5	TEOS:MTMOS:HCL =50:100:10	0.40±0.01	-	-	-
E6	GA = 50	10.55±0.18	9.91±0.22	5.32±0.05	3.25±0.01
E7	GA = 50	1.99±0.19	2.95±0.08	0.78±0.04	0.23±0.02

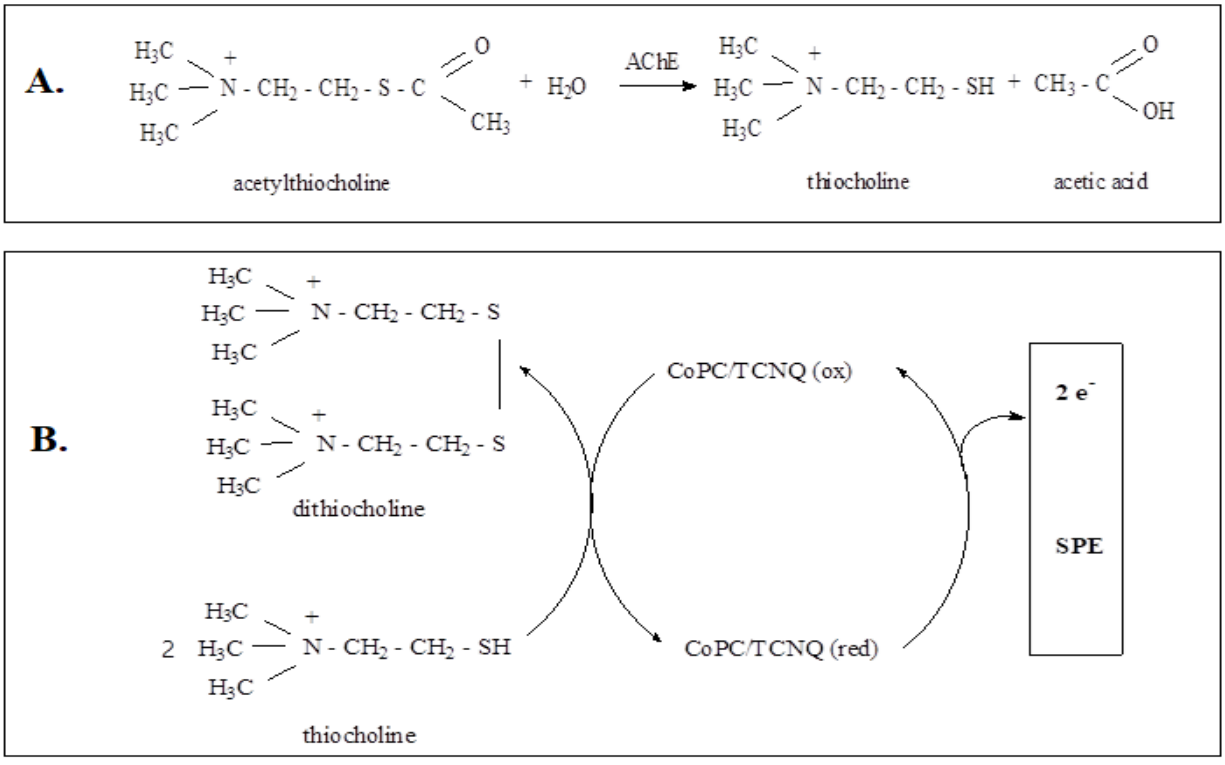

Fig. 1. A. The diagram of enzymatic reaction which undergoes on the surface of the electrode and B. The diagram of enzymatic reaction which undergoes on the surface of the mediated electrode

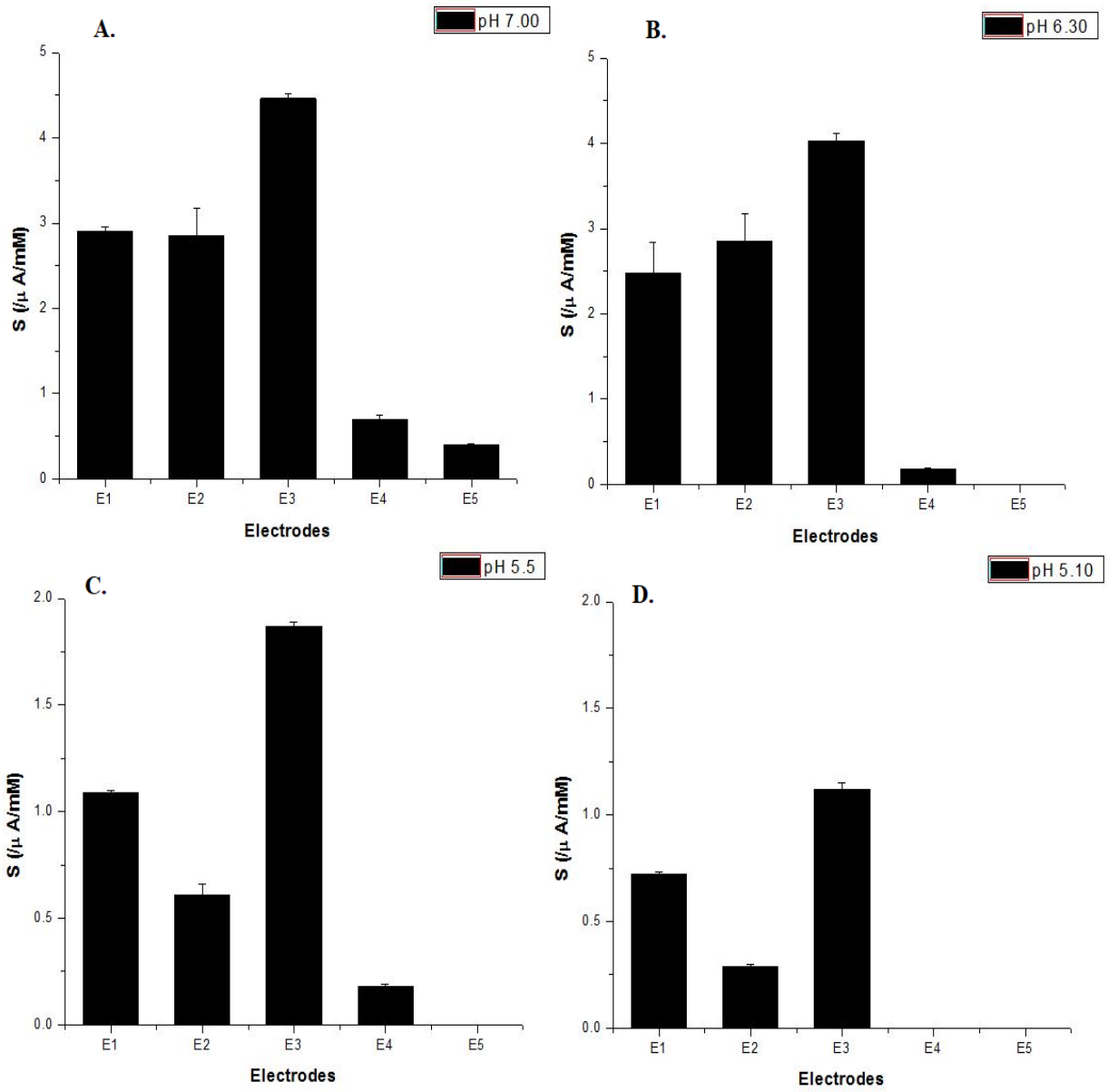

Fig. 2. Sensitivity values of sol-gel layer based biosensors at different pH values: A. pH = 7.0, B. pH = 6.3, C. pH = 5.5, D. pH = 5.1 (details in the text)

If we compare the sensitivities for enzymatic substrate detection of biosensors based on different mediators and the same enzyme immobilization method, we conclude that E3 using the sol-gel immobilization method, has a better performance than E7 with GA. This shows how important the mediator is regarding the electron transfer, and its compatibility to a certain immobilization method.

We can also conclude that that sol–gel matrix doesn't modify the catalytic behavior of AChE and the sol-gel pores or nano-cages containing the encapsulated enzyme allow easy access of the substrate or of enzymatic products in the buffered solution.

3.2.4. Inhibition measurements

For a good enzymatic activity and also to ensure the presence of heavy metals in ionic form we have chosen a pH value of 5.5 for working buffer solutions. In all inhibition experiments the AChE biosensors were first incubated in the buffer solution containing metal ions for a specific time (incubation time) followed by the amperometric detection after substrate addition. The substrate concentration was kept at a constant value of 0.6 mM and the concentration of heavy metal ions was gradually increased.

The best value for the incubation time was determined to be 10 minutes. This value was determined for a concentration of 25 μg/L Cd^{2+} and a constant value of 0.6 mM for the substrate. Trying different incubation times of 10, 15 and 20 minutes, as shown in Fig. 3, the optimal value has been chosen 10 minutes. Increasing the incubation time from 15 to 20 minutes didn't show any significant change to justify increasing of the measuring time.

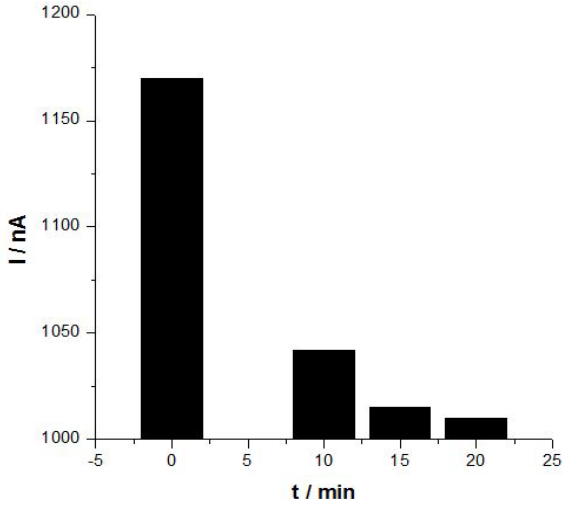

Fig. 3. Optimization of the incubation time for $Cd2^+$ ions detection

The calibration plots of inhibition-based biosensors is done using the degree of inhibition (I(%)) determined with the following expression (Eq. 3):

$$I(\%) = 100\frac{(I_0 - I)}{I_0} = 100\frac{\Delta I}{I_0} \quad (3)$$

where: *I(%)* stands for the degree of inhibition, I_0 is the response given by the biosensor for the detection of the substrate in the absence of the inhibitor, and I is the answer given by the biosensor for the detection of the substrate in the presence of the inhibitor.

The inhibition effect on the immobilized enzyme was studied by recording the amperometric signal at a constant potential of 0.15 V (TCNQ electrodes) and 0.1 V (CoPC electrodes) during a two steps typical inhibition experiment as illustrated in Fig. 4. The first arrow corresponds to the addition of inhibitor (first step). The second arrow refers to the addition of the substrate in a buffer solution in the absence and presence of the inhibitor (second step). As it can be seen, the value of current intensity decreases when the substrate was added after the addition of inhibitor (10 min as incubation time). The Cd ions reduce the activity of AChE by interacting with its active sites, limiting the possibility of the substrate to bind to the enzyme.

Table 4 summarizes the parameters of the used inhibition-based biosensors using the two steps method as described into the above paragraphs. These parameters are: sensitivity S_i (for inhibition), limit of detection and linear detection range for Cd^{2+}. Biosensors E1, E2, E6 and E7 have been utilized as inhibition-based biosensors and calibration curves for cadmium ions as inhibitors were plotted. The concentration of the ATCh substrate was kept at 0.6 mM.

The detection limit representing the minimum concentration value of the $Cd2^+$ which can be detected was calculated with the formula: $LOD = 3\sigma_D/S$, where σ_D is the standard deviation. S_i is the sensitivity of the inhibition-based biosensor, calculated from the slope obtained after fitting the calibration curve done using the degree of inhibition, I(%), versus concentration of the inhibitor (µg/L). The linear range represents the domain of inhibitor concentrations for which the calibration curve has linear shape. Analyzing the data from the Table 4, we can conclude that the most performing biosensor is E2 (immobilization method with sol-gel) (Fig. 5).

Table 4. The values for sensitivity, limit of detection and linear range for the analyzed biosensors for $Cd2^+$ ions detection

Biosensor	***Mediator***	***S_i (%/µgl^{-1})***	***LOD (µg/L)***	***Linear range (µg/L)***
E1	CoPC	0.69 ± 0.26	1.12	2.50 – 25.00
E2	CoPC	1.48 ± 0.14	0.19	2.50 – 25.00
E6	CoPC	0.40 ± 0.07	0.52	2.50 – 25.00
E7	TCNQ	0.53 ±0.088	0.49	2.50 – 25.00

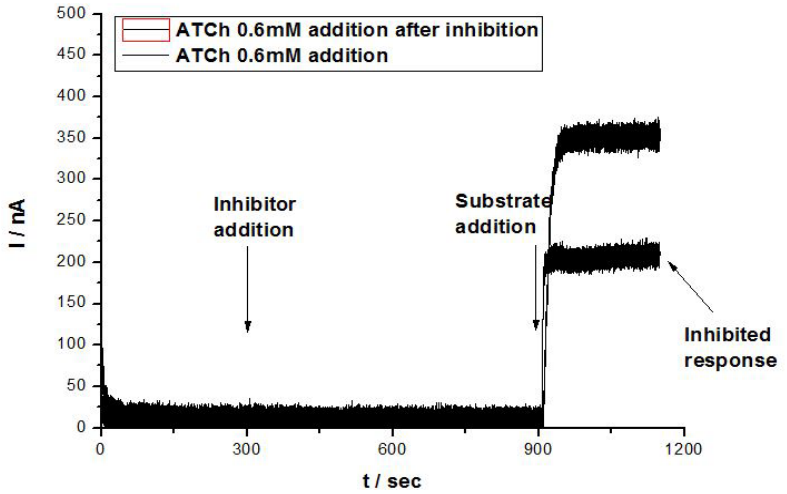

Fig. 4. The effect of inhibition on AChE-based biosensor response

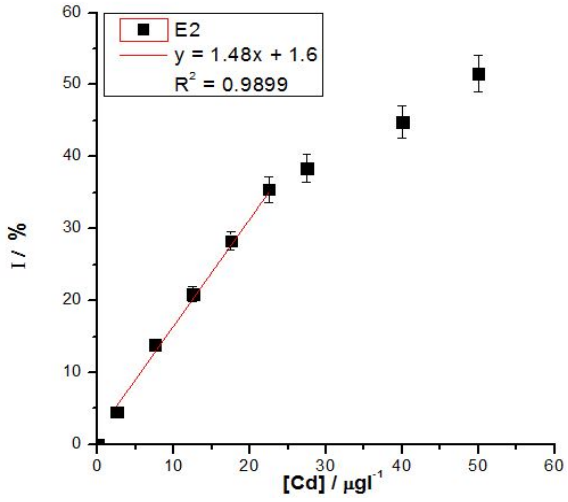

Fig. 5. Calibration plot for Cd^{2+} using inhibition-based biosensor E2

3.2.5. Stability of AChE-based biosensors at substrate measurements

The biosensors with optimal sol-gel layer configuration, (TEOS:H2O:HCL = 100:37.5:6), was also employed to test the reproducibility at substrate measurements using three different biosensors, and a RSD of 4.7% was obtained. The stability of the AChE-biosensors was evaluated by repeated measurements in different days, with the same electrode, over a timeframe of 40 days, for the same substrate concentration of 0.6 mM. Results of testing newly prepared biosensors with ATCh after different time intervals showed that the response of the biosensors increased slightly during 10 days after the first testing, which can be due to reorganization of the sol – gel network and has been noted previously (Pauliukaite et al., 2006), and then began to decrease, until 33% of the initial value, as seen in Fig. 6.

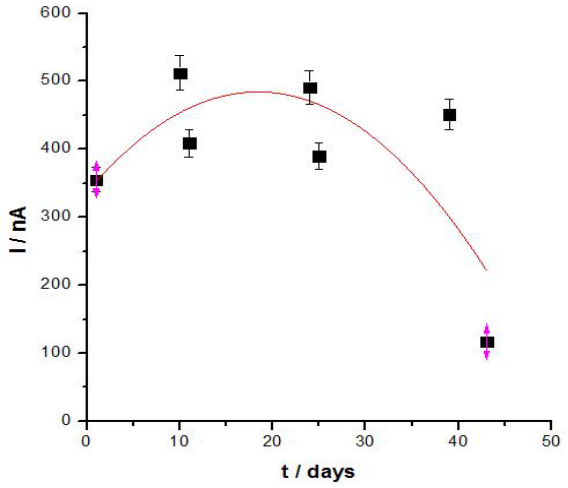

Fig. 6. The stability in time of E2 at pH=5.5 for enzymatic substrate measurements

Nevertheless, this loss of sensitivity is not a problem for short-term use or disposable sensors and was also found not to be a drawback in the measurement of substrate or inhibitor using the standard addition method.

No reactivation substances for enzyme activity were used after inhibition measurements were performed, but we kept the electrodes in a phosphate buffer solution all the time when not used. We observed that the optimum reactivation time for the enzyme was around 3 days, depending on the immobilization method.

3.2.6. Preliminary analysis of Cd ions on real samples using AChE-based biosensors

River water samples were analyzed using AChE-based biosensors with biosensor E2 configuration (TEOS:H_2O:HCL = 100:37.5:6), using the standard addition method. Water samples were collected at various locations in Brasov County, Romania and used after they were acidified at pH 5.5. Analysis led to acceptable values in agreement with independent values from the atomic absorption spectroscopy, the standard method (5.51 ± 1.26 and 5.79 ± 0.24 µg/L, respectively).

These results demonstrate the potentiality of this type of cheap and disposable biosensor for heavy metal ions detection in water. Other approaches based on analysis of different real samples, such as biological ones are currently under investigation.

4. Conclusions

This work has been concerned with the development of electrochemical enzyme biosensors based on mediated carbon-ink screen-printed electrodes for use as cheap, short-term or disposable biosensors for heavy metal ions detection. Evaluation of the system was carried out using determination of Cd^{2+} with acethylcholinesterase enzyme immobilized by sol-gel method and the influence of sol-gel precursors in different ratios without usual addition of alcohol shown.

Measurements with CoPC modified biosensor and the optimized combination (TEOS:H_2O:HCL = 100:37.5:6) present better sensitivity with LOD = 0.19 µg/L. Application to analysis of river water was demonstrated. These results demonstrate the potentiality of this type of enzyme-based biosensor for future applications in environmental monitoring. They are shown to be a viable alternative to expensive standard method, as atomic absorption spectroscopy.

Future work concerns the incorporation of other enzymes for the measurement of a variety of enzyme inhibitors and analysis of different real samples, such as biological ones.

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