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Self-assembly of acetylcholinesterase on gold nanoparticles electrodeposited on graphite

Research Article

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Abstract: The immobilisation of AChE enzyme through chemisorption on Au-modified graphite was examined with view of its prospective application in the design of membraneless electrochemical biosensors for the assay of enzyme inhibitors. The developed immobilisation protocol has been based on a two-stage procedure, comprising i) electrodeposition of gold nanostructures on spectroscopic graphite; followed by ii) chemisorption of the enzyme onto gold nanoparticles. Both the coverage of the electrode surface with Au nanostructures and the conditions for enzyme immobilisation were optimised. The proposed electrode architecture together with the specific type of enzyme immobilisation allow for a long-term retaining of the enzyme catalytic activity. The extent of inhibition of the immobilised acetylcholinesterase enzyme by the organophosphorous compound monocrotophos has been found to depend linearly on its concentration over the range from 50 to 400 nmol mL^{-1} with sensitivity 77.2% inhibition per 1 μmol mL^{-1} of monocrotophos.

Keywords: *Electrodeposited Au-nanostructures • Self-assembly • Acetylcholinesterase • Acetylthiocholine • Inhibition*

1. Introduction

Acetylcholinesterase (AChE) is a key enzyme in the nervous system of mammalian organisms, responsible for transmitting nerve impulses to the cholinergic synapses, and can be easily inhibited by a variety of toxic compounds: heavy metals, nicotine and neuroparalytic gases, medications for the treatment of Alzheimer's or Parkinson's diseases, or pesticides (organophosphorous and carbamate). The accumulation of organophosphorous and carbamate, being intensively used in modern agriculture to protect crops from insects, has to be carefully monitored [1] since they are highly toxic for humans and domestic animals and therefore pose an environmental threat, thus motivating the constantly growing scientific interest in developing electrochemical biosensors for quantitative analysis of pesticides [2].

In most AChE biosensors, the pesticide assay is based on the irreversible enzyme inhibition. Numerous immobilisation techniques have been proposed for constructing AChE biosensors: direct physical adsorption on a solid carrier [3-5], encapsulation in a polymeric film [6], immobilisation in porous membranes [7], adsorption on nanoparticle modified matrices [8-13], binding through chemisorption [14-17] *etc*. However none of them is considered universal for constructing AChE biosensors. Enzyme immobilisation on nanoparticle modified electrodes, such as gold nanoparticles (Au NPs) [8-13], carbon nanotubes (CNTs) [18-23], CdS – nanoparticles (CdS NPs) [24] *etc*. allows for a considerable improvement of both biosensor stability and sensitivity and lowers the limit of detection. Pesticides' assay with AChE biosensors commonly requires enzyme reactivation, which is not always successful, and thus makes the re-use of the biosensor awkward. Hence,

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AChE immobilisation on the single-use electrodes (*e.g.* screen- printed electrodes) has also been considered [25-27].

A simple and versatile technique for enzyme immobilisation based on gold-thiol chemistry is the spontaneous chemisorption of sulphur-containing organic compounds (cysteamine, mercaptobenzothiazole, *etc.*) on the colloid gold modified electrode surfaces thus forming a self-assembled monolayer, SAM, to which the AChE enzyme is attached through intermolecular complexes [14-17,28]. This immobilisation approach provides conformational flexibility of the enzyme and hence assures a more efficient biocatalytic process, however enabling the formation of a thicker diffusion layer that lowers electrode sensitivity. An even simpler method for AChE immobilisation is to chemisorb it directly on the surface of Au NPs without the need for additional thiolated linkers [7,8,10,13], since its protein shell contains terminal SH-groups. The obvious challenge in this case is, however, to retain the enzyme-functionalized NPs onto the electrode, which is implemented by either their incorporation in a porous membrane [7] or inclusion in a composite film cast on the surface of the working electrode [8,10] thus yielding a stable but thick catalytic layer that triggers additional diffusion limitations.

In the present work, a two-step strategy for the acetylcholinesterase immobilisation on spectroscopic graphite is discussed: in the first stage gold nanoparticles were grown directly onto the graphite surface through electrochemical reduction of $HAuCl_4$ and, in the second stage, the AChE enzyme was directly chemisorbed on them, forming a self-assembled monolayer. The enzyme binds to the gold nanoparticles *via* its terminal sulfur-containing groups of the protein shell [7]. Direct deposition of Au NPs onto the graphite surface followed by the enzyme chemisorption represents a principally novel approach for designing electrochemical AChE-based biosensors, whose main advantages include:

i) enhanced enzyme load onto the electrode surface with preserved conformational flexibility of the immobilised biocatalyst that ensures retention of its catalytic activity to a big extent (over one month); and

ii) minimal diffusional limitations on the substrate access to the enzyme layer, warranting better sensitivity (~20 times higher than the literature data) and faster electrode response (at most 20 s).

The principle of detection of AChE enzyme inhibitors using acetylthiocholine as the enzyme substrate is via the following three stages:

Stage 1: AChE catalyses the hydrolysis of acetylthiocholine to thiocholine and acetic acid at room temperature:

$$\text{acethylthiocholine} + H_2O \xrightarrow{AChE} \xrightarrow{AChE} \text{thiocholine} + \text{acetic acid} \quad (1)$$

Both the non-enzymatic and the enzymatic hydrolysis of acetylthiocholine to thiocholine depend on the pH of the solution and were found to slow-down in basic media [29].

Stage 2: Electrochemical oxidation of thiocholine onto the electrode surface:

$$2\ \text{thioholine} \xrightarrow{anodic\ oxidation} \text{disulfide} + 2H^+ + 2e^- \quad (2)$$

The process takes place with the transfer of one electron from the thiocholine and subsequent dimerisation of the intermediate to disulfide [23,30]. When conventional electrodes are used [31-33], thiocholine electrooxidation proceeds at potentials higher than 0.7 V (*vs.* SCE).

Stage 3: Upon the inhibition of the AChE enzyme, the thiocholine oxidation current decreases. The extent of enzyme inhibition is determined by the equation:

$$(Inhibition)\% = \frac{I_S - I_I}{I_S} \times 100\%, \quad (3)$$

where I_S is the steady-state electrode response to the control solution (at large thiocholine concentration present); I_I is the enzyme electrode response at the same thiocholine concentration plus a given inhibitor concentration. In the present study the organophosphorus compound monocrotophos was used as a model AChE inhibitor.

2. Experimental procedure

2.1. Materials

Rods of spectroscopic graphite, type RWO (Ringsdorf Werke, Bohn, Germany) with a diameter of 0.5 cm, fitted into Teflon insulation, were used as working electrodes. Acetylcholinesterase (AChE, EC 3.1.1.7) from an electric eel with an activity of 425.94 U mg^{-1} (Fluka Biochemika); acetylthiocholine iodide with purity of over 99% (Fluka); KI (Valerus, Bulgaria) used as 2 mM aqueous solution, $HAuCl_4$ (Fisher) used as 50 mM solution in 0.1 M HCl; KH_2PO_4 and K_2HPO_4 (Sigma-Aldrich) used for preparing buffer solution were all of analytical grade and used as received.

The solution of acetylthiocholine iodide, with an initial concentration of 2×10^{-3} M was prepared just before the experiment, and during the measurements was stored in a cooling ice bath, at 1–2ºC, to avoid its spontaneous hydrolysis; the organophosphorous

compound monocrotophos $C_7H_{14}NO_5P$ (Riedel-de-Haën) used as an enzyme inhibitor was in the form of aqueous solutions with concentrations from 0.05 to 0.9 µmol mL^{-1}.

Double-distilled water was used in all water and buffer solutions.

2.2. Instrumentation

A standard, three-electrode cell with a volume of 10-20 cm^3 with working graphite electrode (with modification), reference electrode - Ag/AgCl, 3M KCl and a platinum wire as an auxiliary electrode, was used in all electrochemical experiments. All the potentials given in the paper were reported against Ag/AgCl, 3M KCl. The electrochemical measurements were performed with an electrochemical computer-controlled workstation Palm Sens, with PS Trace software (Palm Instruments, The Netherlands). During amperometric measurements, the solutions were stirred at 450 rpm with a magnetic stirrer (IkaMag RCT, Ika, Germany). The pH of the buffer solutions was controlled by a pH-meter HI 211 (Hanna Instruments, USA).

The surface morphology of the modified electrodes was examined with a scanning electron microscope (SEM), JEOL JSM-5500, operating at 10 kV for imaging. No conductive coatings or other treatment were applied to the samples prior to SEM observations.

2.3. Electrode preparation, storage and regeneration

Prior to use, the graphite electrodes were mechanically cleaned by polishing on a fine wet sand-paper, with a gradual decrease of the size of the abrasive particles (P800, P1200, P1500 and P2000) and followed by ultrasonication in bi-distilled water for 3 minutes each. Gold nanoparticles were then deposited by a brief electrolysis (1 or 10 seconds) at a constant potential of -155 mV (*vs*. Ag/AgCl, 3M KCl) from 50 mM solution of $HAuCl_4$ dissolved in 0.1M HCl. After the deposition, the surface of the electrode was washed with bi-distilled water and cleaned electrochemically in 2 M H_2SO_4 by continuous cycling over the potential range from 0 to 1.7 V for at least 20 cycles until three consecutive voltammograms overlapped.

The immobilisation of the enzyme through a self-assembly process was carried out on the gold-modified graphite electrode, which was electrochemically cleaned prior to enzyme immobilisation. The electrode surface, rinsed with double distilled water was immersed into a small-volume container with enzyme solution (1 mg mL^{-1}) and stored in a refrigerator for 24 hours. The non-adsorbed enzyme was removed by washing the electrode with bi-distilled water and buffer. For comparative experiments, Au NPs modified electrodes without the enzyme were produced.

After measurements, the enzyme electrodes were water rinsed and stored in 0.1 M phosphate buffer solution (pH = 7.6) in a refrigerator, and their activity was monitored by periodically testing the enzyme electrode response to acetylthiocholine.

The enzyme electrodes with lost AChE activity were regenerated by mechanical polishing on wet sand paper, then ultrasonicated in bi-distilled water, followed by electrodeposition of Au NPs and enzyme immobilisation.

2.4. Measurement procedure

Thiocholine electrooxidation was examined by cyclic voltammetry (CV) and constant – potential chronoamperometry. CVs were registered in 0.1 M phosphate buffer, pH= 7.6 at a scan rate of 10 mV s^{-1}. The concentration dependencies of the current were measured on Au-modified graphite with or without immobilised enzyme, as follows: the electrode was poised at a constant potential, E (typically 800 mV *vs*. Ag/AgCl, 3 M KCl) in the buffer as a background electrolyte until a steady-state current response, I_0 (µA) was established under constant stirring. Afterwards, an aliquot (20; 50; 200; 500 or 1000 µl) of the analyte stock solution (thiocholine: 2×10^{-3} M; or acetylthiocholine: 2×10^{-3} M) was added and the new value of the steady-state electrode response I_S (µA) was read. The time for reaching a steady-state value of the current under the given conditions did not exceed 0.5 minutes. All the information needed for plotting the calibration graphs was obtained from chronoamperometric records. The enzyme inhibition by the organophosphorous insecticide monocrotophos was studied by chronoamperometry using aqueous solutions with concentrations from 0.05 to 0.9 µmol mL^{-1}. Between measurements with different inhibitor concentrations the electrode was reactivated in a water solution of oxime (2- PAM, 5 mg mL^{-1}). All experimental results represent the mean value of at least three measurements (n = 3 to 6) with the regression analysis performed using MS EXCEL and Origin 7.5.

3. Results and discussion

3.1. Electrodeposition of Au NPs

One of the biggest challenges in developing the protocol for the AChE immobilisation on gold nanoparticles was the development of a simple, reliable and reproducible procedure for their direct deposition onto the graphite surface. The deposition method had to meet the following requirements posed by this particular practical application: uniform distribution of the deposits over the

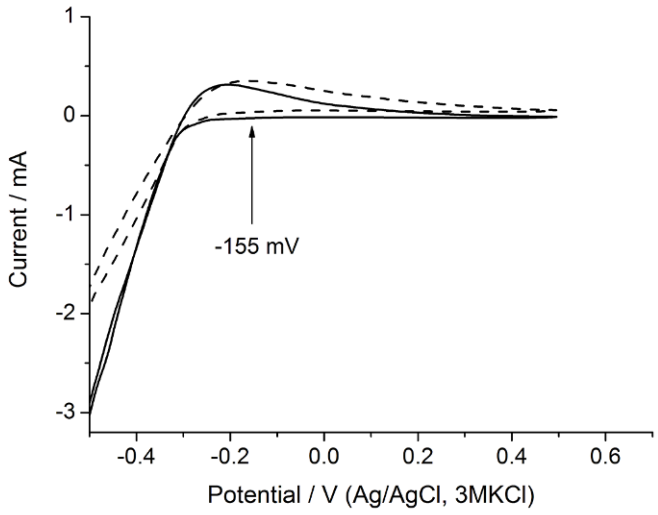

Figure 1. CVs of graphite electrode in background electrolyte 0.1 M HCl (dash) and in 5 mM $HAuCl_4$ present (solid).

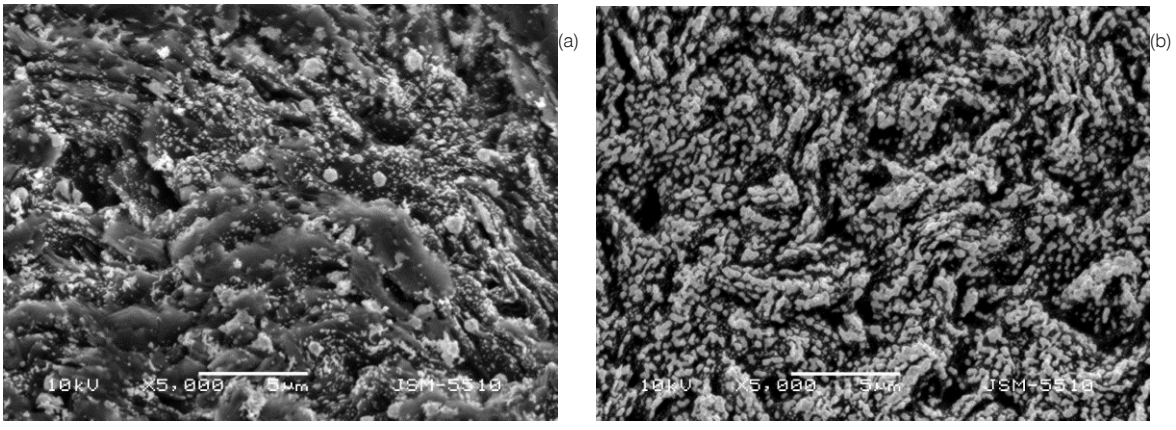

Figure 2. Scanning electron microscopy (SEM) images of Au nano-structures deposited electrochemically on spectroscopic graphite for: a) 1 s; and b) 10 s.

whole surface, small amount of deposited gold, good adhesion to the graphite and availability of sites for enzyme chemisorption.

Gold electrodeposition on graphite was carried out in an acidic solution of tetrachloroaurate at -155 mV. The applied potential was more positive than the potential at which its electrochemical reduction starts by 130-140 mV (Fig. 1), however, upon increasing the Au3+ concentration 10-fold, the coverage of the electrode surface with gold can be clearly observed by the naked eye even for deposition times as short as 1 second. The formation of uniformly distributed gold clusters that grow-up in 1 second to 3D- oblong structures with an average particle size of ca. 200 nm (Fig. 2a) have been observed. By increasing the duration of the electrolysis process to 10 seconds, larger structures were formed (Fig. 2b). A further increase in the time for deposition leads to the formation of even larger structures and multilayered formations, however does not noticeably contribute to a greater surface coverage with Au nanoparticles.

3.2. Electrochemical oxidation of thiocholine

The electrochemical oxidation of thiocholine – the product of the enzymatic hydrolysis of acetylthiocholine (stage 2 of inhibitor detection) on gold-modified graphites, was studied by both CV and amperometry.

Voltammetric curves recorded in the presence of thiocholine on the gold modified graphite electrodes for 1 and 10 seconds are illustrated in Fig. 3. A pair of clearly expressed peaks is observable on the CVs with peak-to-peak separations of 120 mV and 90 mV, respectively, indicating quasi-reversible electrochemistry of the process. These peaks, however, are not associated with the electrochemical oxidation of thiocholine, since the constant potential amperometric measurements

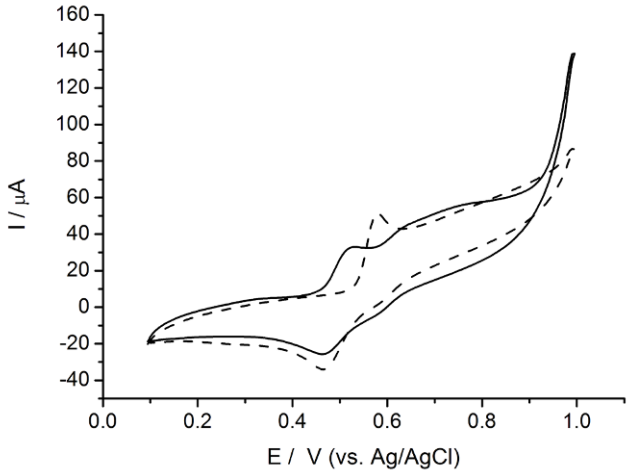

Figure 3. CVs of Au-modified graphite electrodes in 1 mM thiocholine present; graphite with Au electrodeposited for 1 s (dash) and 10 s (solid); 0.1 M phosphate buffer, pH = 7.6; scan rate 10 mVs^{-1}; room temperature.

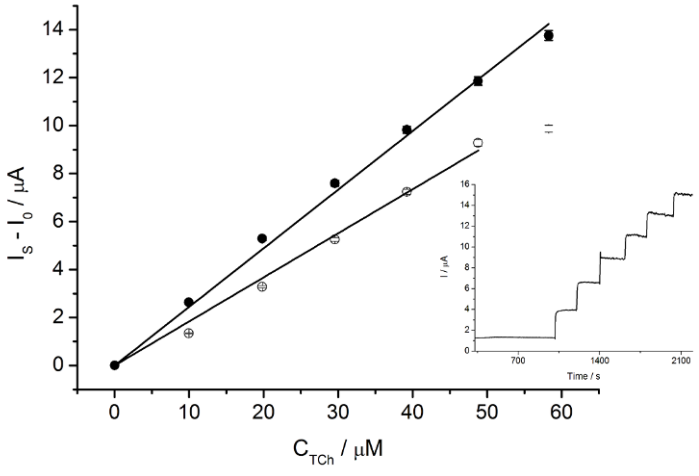

Figure 4. Electrode response as a function of thiocholine concentration at potential 0.8 V (vs. Ag/AgCl); open circles: Au electrodeposited for 1 s (regression equation Y = 0.184 X; R^2 = 0.992); closed circles: Au electrodeposited for 10 s (regression equation Y = 0.245 X; R^2 = 0.995); electrolyte: 0.1 M phosphate buffer, pH = 7.6; room temperature. Inset: Chronoamperometric record upon addition of thiocholine; electrode: graphite with electrodeposited Au for 10 s; experimental conditions as stated above.

indicated very weak electrode response at applied potentials below 0.8 V. Although much cheaper than acetylthiocholine chloride, the use of acetylthiocholine iodide as the substrate for AChE causes undesired electrooxidation of the iodide ions at the working potential, as it was shown earlier by Stoytcheva *et al.* [34] and the quasi-reversible peaks observed on the CVs are most probably due to this process.

The dependence of the electrode response on thiocholine concentration (Fig. 4) shows that the increased duration of the Au electrodeposition, up to 10 seconds, results in a 33% improvement in electrode sensitivity: from 0.184 μA $μM^{-1}$ (open circles) to 0.245 μA $μM^{-1}$ (closed circles), keeping in the same time strict linearity of the calibration plot and low noise level. However, the electrooxidation of thiocholine on graphite modified with Au through electrodeposition for a duration twice as long (20 seconds) does not lead to an improved electrode sensitivity, despite the amount of gold (determined from coulometric data) being increased by 60% as compared to the 10 second modification. Obviously, as discussed above, the increased load of the modifier does not substantially contribute to the enhancement of the electrochemically active electrode surface. Therefore, all further discussions will concern mainly the performance of the Au-electrodeposited on graphite for 10 seconds.

The effect of I^- oxidation at 0.8 V and pH = 7.6 on Au NPs modified graphite for 10 seconds was examined using KI solution with the same concentrations as

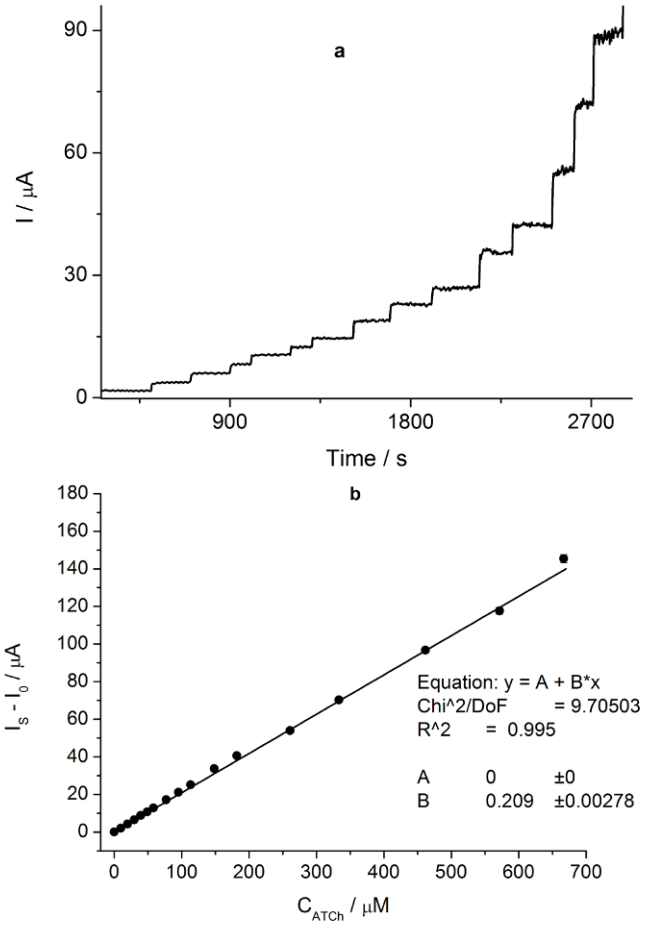

Figure 5. a) Chronoamperometric record of the AChE-electrode responce upon addition of acetylthiocholine; b) Calibration plots of the same enzyme electrode (AChE self-assembled on Au NPs modified graphite for 10 s); applied potential 0.8 V (vs. Ag/AgCl); 0.1 M phosphate buffer, pH = 7.6; applied potential 0.8 V (vs. Ag/AgCl); room temperature. Y-error bars correspond to the 2.43 % standard deviation.

thiocholine iodide. Under our experimental conditions it has been found that the iodide electrooxidation contributes to the electrode response with 44.8% of the signal, *i.e.*, the partial rates of electrochemical oxidation of thiocholine and I^- under the given conditions are almost the same.

3.3. Immobilisation of AChE through self-assembly

The choice of electrode material and the immobilisation method [35] are of key importance for the design of a reliable biosensing device, since these affect its operational characteristics: sensitivity, response time, stability, reproducibility, *etc*.

A key element in this procedure was the electrochemical cleaning of the modified surface, preceding the chemisorption of the enzyme as it was found that the enzyme scarcely attaches to non-pre-treated electrodes. During the electrochemical treatment two clearly expressed peaks of oxygen desorption were observed on the CVs, suggesting that the surface oxides formed on Au NPs would allow the enzyme adsorption on them only to an insignificant extent, or would even block it. Alternatively, an enzyme layer was found to form on the electrochemically cleaned electrodes, which reaches maximal surface coverage within ~20 hours. The degree to which this process took place was probed by catalytic activity during acetylthiocholine hydrolysis, *i.e.*, by the response of the enzyme electrode when aliquots of the substrate were added.

The chronoamperometric record, presented in Fig. 5a demonstrates that the enzyme electrode current increased stepwise upon the addition of acetylthiocholine aliquots, the enzymatic hydrolysis of which generates thiocholine and acetic acid (Reaction 1) followed by the electrochemical oxidation of thiocholine (Reaction 2). The higher the substrate concentration, the higher the noise level observed. The increased

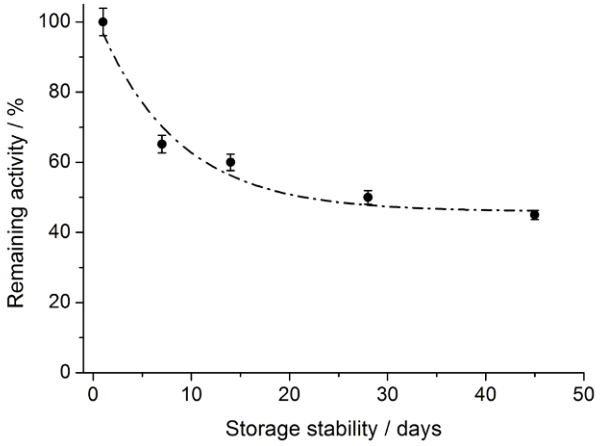

Figure 6. Remaining activity of the enzyme electrode as a function of the storage time. The Y-error bars correspond to the 2.43% standard deviation.

noise is most probably due to the electrochemical oxidation of iodide ions, but could also result from other side reactions (see *e.g.* Reaction 2).

The calibration graph (Fig.5b) of the produced AChE –electrode, shows a linear trend over the studied range of substrate concentrations – above 600 μM, with a regression correlation coefficient of 0.995 and a slope of 0.209 μA μM^{-1} (0.135 μA μM^{-1} – for 1 second) representing the electrode sensitivity. A slight decrease of the electrode sensitivities after enzyme chemisorption is most probably associated with the coverage of the electrode surface with a protein monolayer that hampers the penetration of the enzymatically produced thiocholine to its surface. The detection limit towards acetylthiocholine was estimated to be 2.5 μM at a signal to noise ratio of 5. The enzyme electrode response reached it steady-state value in at most 20 seconds.

The determined electrode sensitivity of 0.209 μA μM^{-1} towards acetylthiocholine is considerably improved compared to the one reported in the previous studies of the group (implemented with a biosensor with AChE immobilised on a modified PAN membrane with incorporated gold nanoparticles [7]), and much greater than that reported in the literature for biosensors with a similar method of enzyme immobilisation (AChE chemisorbed on gold nanoparticles), *viz.*: about 18-24 times as high as the fabricated by Du and co-workers [11] and approximately 48-64 times as high as the reported by Shulga and co-authors [13] biosensor. This finding is obviously due to the much greater number of chemisorbed enzyme molecules resulting from both the extended electrode surface area and the enlarged number of available centres for enzyme chemisorption owing to the electrochemical cleaning of the gold surface. Indeed, upon exchanging the enzyme substrate to acetylthiocholine chloride it is expected that the electrode sensitivity will drop to ca. 0.115 μA μM^{-1} since, as mentioned above, the contribution of the electrooxidadation of iodide is considerable. Yet, the anticipated electrode sensitivity will significantly exceed the literature data reported so far.

3.4. Reproducibility and stability of AChE enzyme electrode

The inter-assay precision, or fabrication reproducibility, was evaluated by determining the response to 50 μL 2 mM solution of ATCh of six different electrodes and the relative standard deviation was found to be 2.43%. The intra-assay precision of the sensors was assessed by testing one enzyme electrode for six replicate determinations and the RSD was calculated to be 1.12% for an ATCh concentration of 50 μM. These results were indicative of an acceptable reproducibility regarding ATCh determination.

The stability of the enzyme electrode during long-term storage was examined for over a month by testing its activity with respect to its substrate ATCh. Fig. 6 shows the residual activity of the enzyme electrode as a function of its storage time. The residual activity was determined as the percentage of the measured signal at a constant concentration of the substrate, to the current at the same concentration, measured with a freshly prepared AChE-electrode. As it is evident from Fig. 6, the catalytic activity of the immobilized enzyme decreases by half over the first 10 days, and over the next 40 days it gradually goes down to 40% of the initially measured signal with the same electrode.

3.5. Inhibition studies of AChE enzyme electrode

In order to prove that the immobilised AChE enzyme retains its enzymatic activity upon immobilisation to a large extent and the registered electrode response

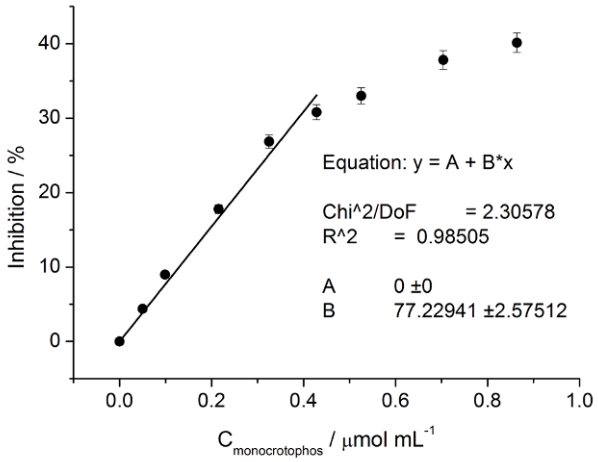

Figure 7. Linear dynamic range of the dependence of the extent of inhibition of AChE on monocrotophos concentration, at room temperature. The error bars correspond to the standard deviation.

is due to the electrochemical oxidation of the thiocholine produced by the enzymatic hydrolysis of acetylthiocholine, the purposeful irreversible inhibition of the immobilised AChE with the organophosphorous insecticide monocrotophos was performed. The response of AChE-enzyme electrode to the enzyme inhibitor was studied in an acetylthiocholine-rich buffer solution (pH =7.6) at a working potential of 0.8 V (*vs*. Ag/AgCl) and room temperature.

The effect of monocrotophos concentration on the degree of AChE inhibition is presented in Fig. 7. As one can see, the linear part of the "calibration plot" of the inhibition degree versus the monocrotophos concentration, *i.e*., the range where the inhibition is controlled by a first-order reaction, spans over the range from 0.05 µmol mL^{-1} to 0.4 µmol mL^{-1}. Immobilised AChE exhibited good initial sensitivity towards the pesticide, up to a percentage inhibition of 30% at the upper limit of the linearity range. The overall results outline the good potential of the discussed immobilisation system for the construction of a biosensor for pesticide detection, since the inhibition pattern shows analogy to a great extent with other biosensing systems reported so far [7]. Further work is needed, however, in order to optimise the performance of the reported electrode to design a biosensor for assaying the AChE inhibitor monocrotophos.

4. Conclusions

A membrane-less amperometric enzyme electrode was developed with the AChE enzyme immobilised through chemisorption onto Au NPs modified graphite. The electrode was characterized with respect to its basic operational characteristics at a working potential of 800 mV (*vs*. Ag/AgCl), as follows:

- the detection limit of 2.5 µM towards the enzyme substrate acetylthiocholine;
- electrode sensitivity towards ATCh of 0.209 µA μM^{-1};
- response time of 20-30 seconds;
- storage time up to 50 days within which time the enzyme retains up to 40% of its initial activity;
- degree of inhibition by the insecticide monocrotophos that depends linearly on its concentration over the range from 0.05 to 0.4 µmol mL^{-1} with a 'sensitivity' of the inhibitor determination - 77.23% inhibition per 1 µmol mL^{-1} monocrotophos (R^2 = 0.985).

The results presented herein demonstrate the principle opportunity to create a cost-effective electrochemical biosensor for the determination of the enzyme inhibitor monocrotophos. Future work concerning the increase of biosensor sensitivity towards the inhibitor, decrease of the noise level and improvement of the detection limit, by *e.g*. replacing the enzyme substrate used in this work with acetylthiocholine chloride, is planned. Another promising improvement to be done is to decrease the operating potential so that to minimize the contribution of the side electrooxidative reactions.

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