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Cholinesterase sensor based on glassy carbon electrode modified with Ag nanoparticles decorated with macrocyclic ligands

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ABSTRACT

New acetylcholinesterase (AChE) sensor based on Ag nanoparticles decorated with macrocyclic ligand has been developed and successfully used for highly sensitive detection of organophosphate and carbamate pesticides. AChE was immobilized by carbodiimide binding on carbon black (CB) layer deposited on a glassy carbon electrode. The addition of Ag nanoparticles decreased the working potential of the biosensor from 350 to 50 mV. The AChE sensor made it possible to detect 0.4 nM–0.2 μM of malaoxon, 0.2 nM–0.2 μM of paraoxon, 0.2 nM–2.0 μM of carbofuran and 10 nM–0.20 μM of aldicarb (limits of detection 0.1, 0.05, 0.1 and 10 nM, respectively) with 10 min incubation. The AChE sensor was tested for the detection of residual amounts of pesticides in spiked samples of peanut and grape juice. The protecting effect of new macrocyclic compounds bearing quaternary ammonia fragments was shown on the example of malaoxon inhibition.

1. Introduction

The development of instrumental tools for sensitive detection of enzyme inhibitors is of substantial interest in the environmental, food and agricultural areas [1]. Among others, organophosphate and carbamate pesticides exert irreversible inhibition of acetylcholinesterase (AChE). The pesticides or their primary metabolites form a covalent bond between the hydroxyl group of serine residue of the enzyme active site and esteric fragment of an inhibitor molecule [2,3]. The product of the reaction, i.e. phosphorylated or carbamoylated AChE does not react with a substrate. This results in a decrease of the enzyme activity quantified by appropriate transducer. The carbamoylated AChE is spontaneously re-activated in aqueous media so that maximal inhibition levels can be beyond 100%. The product of organophosphate inhibition is commonly stable and the enzyme activity decreases down to zero with an increase of the inhibitor concentration and/or incubation period.

The necessity of the detection of organophosphates and carbamates is related to the AChE biological function. This enzyme is widely present in warm-blooded living beings and is responsible for the nerve impulse transduction by hydrolysis of a natural neurotransmitter, acetylcholine.

$$\underset{\text{Acetylcholine}}{(CH_3)_3N^+CH_2CH_2O\overset{\displaystyle O}{\overset{\|}{C}}CH_3} + H_2O \xrightarrow{\text{AChE}} \underset{\text{Choline}}{(CH_3)_3N^+CH_2CH_2SH} + CH_3COOH \quad (1)$$

The inhibition of AChE in living beings increases the concentration of acetylcholine followed by abdominal cramps, muscular tremor, hypotension, breathing difficulty, slow heartbeat and death [4,5].

The residual amounts of anticholinesterase pesticides in soils, vegetables, biological tissues and food are commonly determined by gas chromatography with mass spectrometry [6,7] and flame photometry detection [7] and liquid chromatography with fluorescence/UV [8], diode-array [9] and mass spectrometry [10] detection. For sample pre-concentration, liquid–liquid [10] and solid-phase extraction techniques are often used [11]. The chromatographic detection of pesticides is summarized in recent review [12]. From other techniques, immunoassay in ELISA [13], fluorescent polarization [14] and lateral flow format [15] can be mentioned.

AChE biosensors are compact devices developed for the fast and sensitive detection of pesticides, preferably in field conditions.

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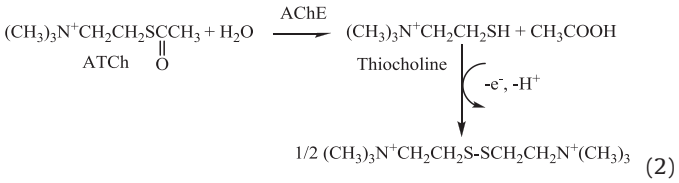
They do not compete with conventional instrumental tools but can be used for preliminary estimation of potential hazard related to anticholinesterase species. For electrochemical detection, the following strategies have been realized for the AChE inhibition quantification in past two decades: (i) the use of choline oxidase for subsequent oxidation of choline released in reaction (1) and detection of hydrogen peroxide produced [16]; (ii) the potentiometric determination of the amounts of acetic acid released [17]; and (iii) the use of artificial substrate, acetylthiocholine (ATCh), which produces electrochemically active thiocholine oxidized on the electrode (2).



(2)

The latter approach showed some advantages, i.e. simpler design of a biosensor, higher sensitivity and accuracy of the signal measurement and extended lifetime of the biosensor. Direct oxidation of thiocholine on metal and glassy carbon electrodes can be performed with rather high over voltage at 450–680 mV. The oxidation is complicated with partial poisoning of the electrode and accumulation of chemisorbed by-products affecting the reproducibility of the current recorded. Various mediators and their combinations have been proposed for AChE sensors, i.e. carbon nanotubes [18–20], ferricyanide [21], Prussian Blue [22–24], tetracyanoquinodimethane [25], and Co phthalocyanine [26–28].

In addition to them, Au nanoparticles have found application for both signal transduction and enzyme immobilization via Au–S bonds [19,23,29,30]. Silver is an alternative for gold in the assembly of biosensors. Being less hydrophobic and more reactive, Ag nanoparticles can be easily obtained in situ on the transducer surface in mild conditions compatible with those of enzyme immobilization. Meanwhile, Ag nanoparticles require additional stabilization and protection from undesired chemisorption of the reactants.

Recently, two Ag electrodes have been successfully used for the detection of thiocholine as a product of enzymatic reaction. A flow injection system with a silver electrode on-line was described for the amperometric detection of thiocholine liberated up-front in a flow-through detector [31]. AChE was immobilized on a golden support. The response was generated by oxidation of silver in the presence of thiocholine at about 80 mV. The comparison of thiocholine oxidation on Ag, Pt, glassy carbon and Au showed the lowest potential of 0.08 V on silver electrode used then for paraoxon detection [32].

In this work we have described a simple and reliable protocol for the synthesis of Ag nanoparticles in the reaction of thiacalix[4]arene bearing catechol fragments in the substituents at the lower rim. The macrocycle applied as a reducing agent shields the surface of nanoparticles from aggregation and dissolution and also provides reversible and fast electron exchange on the electrode interface. The electrodes modified with Ag nanoparticles decorated with macrocyclic ligands were successfully applied for dopamine detection [33] and as a transducer in the aptasensor for ochratoxin A determination [34]. In this work, we recommend the AChE sensor with glassy carbon electrode modified with Ag nanoparticles for the detection of anticholinesterase pesticides and protective agents testing.

2. Experimental

2.1. Reagents

AChE from electric eel (687 U/mg prot.), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide chloride (EDC), 2-(*N*-morpholino) ethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS), 2-pyridine aldoxime (2-PAM), ATCh chloride were purchased from Sigma (St Louis, MO). Malaoxon (Pestanal, stock solution 1 mg/mL in ethanol), malathion (Pestanal, 97.2% solid), paraoxon (96% solid), aldicarb (Pestanal, 99.9% solid) and carbofuran (Pestanal, 99.9% solid) were purchased from Fluka (Germany). Carbon black (CB, N220) of industrial standard grade was obtained from Cabot Corporation (Ravenna, Italy). All other reagents were of analytical grade.

5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetrakis-[1-(2′-hydroxyethyl)-*N*-(3″,4″-dihydroxyphenyl)amidocarbonyl)-methoxy)-2,8,14,20-tetrathiacalix[4]arene in *1,3-alternate* conformation (**TC-0**) was synthesized as described elsewhere [33]. For the synthesis of Ag nanoparticles, 0.01 M stock solution of the thiacalix[4]arene in acetone was prepared and then diluted with 50% (v/v) aqueous acetone prior to use.

The following thiacalix[4]arenes bearing quaternary ammonia groups at the substituents at the lower rim have been tested (Fig. 1): 5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetrakis-[(*N*-(30,30,30-trimethylammoniumpropyl)carbamoylmethoxy)]-2,8,14,20-thiacalix[4]arene tetranitrate (**TC-1**). 5,11,17,23-tetra-tert-butyl-25,26,27,28-tetrakis-[(*N*-phthalimideammoniumpropyl) carbamoylmethoxy]-2,8,14,20-thiacalix[4]arene tetranitrate (**TC-2**), 5,11,17,23-tetra-tert-butyl-25,26,27,28-tetra-[(*N*-(3′,3′-dimethyl-3′-ethylammoniumpropyl) carbamoylmethoxy]-2,8,14 20-thiacalix[4]arene tetranitrate (**TC-3**), 5,11,17,23-tetra-tert-butyl-25,26,27,28-tetrakis-[(*N*-(30,30-dimethyl-30-benzylammoniumpropyl)carbamoylmethoxy)]-2,8,14,20-thiacalix[4]arene tetranitrate (**TC-4**). All the thiacalix[4]arenes were synthesized at the Organic Chemistry Department of Kazan Federal University in accordance with [35] as iodide salts. Their structure and purity were confirmed by NMR ^{1}H and ^{13}C, IR-spectroscopy, MALDI-TOF and elemental analysis. Prior to use, all the thiacalix[4]arenes were treated with $AgNO_3$ to get nitrate salt forms. The anion exchange was monitored by Dionex ICS 5000 (Thermo Fischer Scientific Inc., USA).

Electrochemical measurements were performed in PBS (0.05 M disodium phosphate + 0.1 M NaCl) adjusted to pH 7.8. For electrode washing, 1.0 M NaCl solution containing 0.05 M phosphate buffer and 0.1 M ethanolamine was used. The substrate and inhibitor solutions were prepared directly prior to their use in measurements by aliquot dilution of stock solutions by Millipore Q® water. The ATCh stock solution was stored at 4 °C for not more than one week.

2.2. Apparatus

Electrochemical measurements were performed with AUTOLAB PGSTAT 302N potentiostat with FRA module for electrochemical impedance (EIS) measurements (Metrohm Autolab B.V., The Netherlands). Three-electrode cell with glassy carbon electrode (working area 1.67 mm^2), Pt auxiliary electrode and double junction Ag/AgCl reference electrode (AUTOLAB Cat. no. 6.0726.100) were used in all the electrochemical measurements.

EIS spectra were recorded using NOVA software (Metrohm Autolab B.V., The Netherlands) in the presence of 0.01 M $K_3[Fe(CN)_6]$ and 0.01 M $K_4[Fe(CN)_6]$. The amplitude of the applied sine potential was 5 mV. The direct current potential was calculated as half-sum of the peak potentials of the redox couple $[Fe(CN)_6]^{3-/4-}$. The EIS spectra were recorded within the frequency from 100 kHz to 0.04 Hz with a sampling rate of 12 points per decade.