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An amperometric acetylcholine biosensor based on a conducting polymer

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ABSTRACT

An amperometric acetylcholine biosensor was prepared by the generation of the conducting polymer poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine) (poly(SNS-NH_2)) on graphite electrodes. For pesticide detection, the enzymes acetylcholinesterase (AChE) and choline oxidase (ChO) were co-immobilized onto the conducting polymer poly(SNS-NH_2) films using covalent binding technique. Electrochemical polymerization was carried out using a three-electrode cell configuration via cyclic voltammetry. Characterization of resulting acetylcholine biosensor was done in terms of optimum pH, enzyme loading, range of linear response and shelf-life. Linear range was 0.12–10 mM and shelf-life 4 weeks. Sensitivity was calculated as 2.19 $\mu A\,mM^{-1}\,cm^{-2}$. The designed biosensor was tested for the determination of paraoxon-ethyl in spiked tap water samples. The results were compared with a conventional quantification method using HPLC–DAD. Linear correlation of the quantification results with both methods ($R^2 = 0.998$) was obtained.

1. Introduction

Conjugated polymers are highlighted with their distinct properties serving as multipurpose materials in many kind of application fields such as solar cells [1,2], electrochromic devices and electrochemical transistors [3,4], cell adhesion and imaging [5,6]. According to the characteristics of the application, the chemical and physical properties of these polymers can be tuned to get the most proficient and effective results in the applications. The scope of application of π-conjugated polymers (CPs) promptly augmented with their fascinating electronic and photonic properties. One of the most applicable fields for the conducting polymers is biosensors. Due to the good electrochemical and physical properties, they can be used as immobilization matrices in the preparation of enzyme-based biosensors [7–10]. Moreover, the CPs can be modified with functional groups either during the their synthesis or after the polymerization with functional structures such as polyamidoamines (PAMAM) [11,12]. By this way, more suitable and adapted polymeric film can be achieved for favorable enzyme immobilization.

Furthermore, the most challenging and critical step in biosensor preparation is the enzyme immobilization step. In order to achieve a long-life and stable biosensor, the enzyme molecules must maintain their activity after the immobilization. In addition, the immobilization must be reproducible. For this reason, the choice of enzyme immobilization method is one of the most important steps for biosensors.

On the other hand, enzyme immobilization using conjugated polymers is a verified immobilization technique for mechanically robust and stable biosensors [13,14]. CPs are considered as appropriate host for enzymes since the enzyme molecules can easily and sufficiently anchored on the electrode surface assisted by the CPs. Besides, CPs offer increased surface area, tunable morphology depending on the thickness of the film, mechanical and chemical stability and inertness. Additionally, functionalized CPs are good candidates for preparation of stable and long-life biosensors since a covalent binding can be established between CPs and enzyme molecules. Hence, a prolonged lifetime of biosensor, reliable and reproducible results can be obtained with enhanced electron transfer during the electrochemical reactions on the electrode surface.

Rising amounts of pollutants in the environment and extended use of pesticides in growing crops ask for sensitive and rapid

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analytical methods in order to monitor these substances. Beyond the wanted effects by controlled amounts of pesticide use on target organisms, environmental compartments such as soil, water and non target organisms are also contaminated. Due to these reasons, rapid, sensitive and reliable analyses techniques are always welcomed to screen toxic compounds in the environment. The widely used organophosphorous and carbamate pesticides are taken up by organisms and inhibit the activity of acetylcholinesterase (AChE) by binding to the enzyme. Hence, the hydrolysis of acetylcholine (ACh) is hindered which intervenes with the transmission of nerve impulses resulting death in high amounts. Therefore, AChE biosensors have an important place within detection techniques for pesticides without the need of many pretreatment steps of samples and offer fast and diverse application possibilities [15,16].

The aim of this work was to develop a sensitive and effective bienzymatic biosensor system with AChE and choline oxidase (ChO) for detection of organophosphate and carbamate pesticides. There are a few articles which report acetylcholine biosensors without any mediator. Hence, the use of conducting polymer as an immobilization matrix and its sufficient contribution to electron transfer in the enzymatic reactions through the working electrode will be important contributions to the literature. The biosensor working principle is based on the following equations:

$$\underset{\text{acetylcholine (ACh)}}{H_3C\text{-}COO\text{-}(CH_2)_2\text{-}N^+(CH_3)_3} + H_2O \xrightarrow{AChE} \underset{\text{choline (Ch)}}{OH\text{-}(CH_2)_2 - N^+(CH_3)_3} + CH_3 - COOH$$

$$\underset{\text{choline (Ch)}}{OH - (CH_2)_2 - N^+(CH_3)_3} + 2O_2 + H_2O \xrightarrow{ChO} \underset{\text{betaine}}{(CH_3)_3N^+ - CH_2 - COOH} + 2H_2O$$

In the electrochemical analysis, the current change generated by the consumption of the oxygen during the reactions is linearly related to choline (Ch). Since ACh is converted to Ch in enzymatic reactions via AChE, the change in the current is directly proportional to ACh in subsequent reactions. In order to overcome the two important challenges in designing pesticide biodetectors, loss of enzyme activity and low inhibition sensitivity the following strategy was pursued. The use of a conducting polymer on the basis of 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine (SNS-NH_2) as the host matrix for co-immobilization of AChE and ChO enhance the electron transfer during the substantial enzymatic reactions and accordingly, increase the sensitivity and reduce the detection limit compare to non conducting polymers. The extended surface area and superior conducting property of the CP were exploited to enhance the current responses and to prevent interferences. Concurrently, with the enhanced electron transfer and utilization of the conducting polymer, the operation potential was reduced and oxidation of interfering substances was avoided. The enzyme molecules were immobilized using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry to obtain the robust covalent binding. For the first practice test of the biosensor, the determination of paraoxon-ethyl in tap water was chosen. Paraoxon-ethyl is the oxidation product of the phosphorthionate insecticide parathion ethyl and is known to be a strong inhibitor of AChE [17]. Therefore it was chosen as model inhibitor (reference substance). In order to control the accuracy of the biosensor high pressure liquid chromatography with diode array detector (HPLC/DAD) was used as reference method.

2. Experimental

2.1. Materials

Choline oxidase (ChO, EC 1.1.3.17, 14 U mg^{-1} from *Alcaligenes* sp), acetycholinesterase (AChE, EC 3.1.1.7, 518 U mg^{-1} from *Electrophorus electricus* (electric eel)), acetylcholine chloride, paraoxon-ethyl, n-hydroxysuccinimide (NHS), n-(3-dimethylaminopropyl)-n′-ethylcarbodiimide hydrochloride (EDC), and all chemicals used for the synthesis of the monomer and electropolymerization was purchased from Sigma–Aldrich Co. LCC. (St. Louis, USA). Acetonitrile, hydrochloric acid, sodium hydroxide were purchased from Merck (Darmstadt, Germany) and tetrahydrofuran (THF) from Acros (Geel, Belgium). All chemicals were analytical grade.

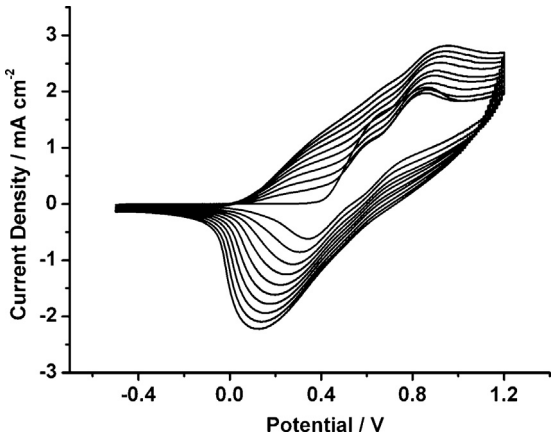


Fig. 1. Repeated potential-scan electropolymerization of SNS-NH_2 in 0.1 M $NaClO_4$/$LiClO_4$/ACN electrolyte–solvent system at a scan rate of 100 mV s^{-1} on graphite (up to 10 cycles).

2.2. Instrumentation

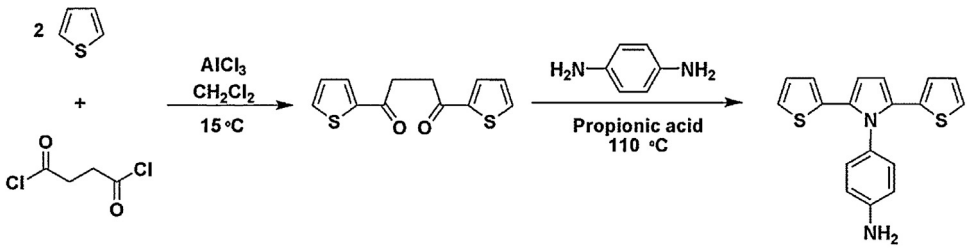
All electrochemical measurements were performed with the potentiostat CompactStat (Ivium Technologies B.V., Eindhoven, Netherlands) in a three-electrode cell configuration consisting of a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode (Fig. 1). A platinum wire as the counter electrode and a silver wire as the pseudo reference electrode were used. Amperometric measurements were performed in a three-electrode system. In amperometric analyses, the data were given as the average of five measurements and standard derivations were recorded as ±SD. All measurements were performed at ambient conditions (25 °C).

For investigation of surface characteristics, scanning electron microscopy (SEM) (JEOL JSM-6400 model, Japan) and X-ray photoelectron spectroscopy (XPS) (PHI 5000 Versa Probe (F ULVACPHI, Inc., Japan/USA) with monochromatized Al Kα radiation (1486.6 eV) 10 as an X-ray anode at 24.9 W were used.

HPLC (Agilent 1100 Series, Karlsruhe, Germany) equipped with a diode array detector (DAD) and a LiChrospher 100 RP-18 (250 mm × 4.6 mm, 5 μm) column (Merck, Darmstadt, Germany) was used for the chromatographic analyses of paraoxon ethyl. The mobile phase of ammonium acetate (50 mM, pH 6) and acetonitrile (1:9) was run isocratic at a flow rate of 0.8 mL min^{-1} for 8 min. The injection volume of the samples was 10 μL. The detection was performed at 270 nm. For the calibration a stock solution of paraoxon ethyl (1 μg μL^{-1}) was prepared in methanol. Calibration standards in methanol of 0.5, 1.0, 5.0, 10, 25 and 50 ng μL^{-1} were prepared from the stock solution.

2.3. Synthesis of 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine (SNS-NH_2)

SNS-NH_2 was preferably utilized as functional monomer due to the free amino groups bearing on its backbone. SNS-NH_2 was synthesized according to the literature [18]. First,



Scheme 1. Synthetic route to SNS-NH_2.

1,4-di(2-thienyl)-1,4-butanedione was synthesized via double Friedel-Crafts reaction. Secondly, the resulting product was reacted with benzene-1,4-diamine in the presence of catalytic amount of propionic acid in toluene. At the end of 24 h, the desired molecule was obtained after column chromatography as pale yellow solid (Scheme 1).

2.4. Electrochemical polymerization of the monomer

Before use, graphite rods were polished on emery paper and rinsed several times with deionized water. SNS-NH_2 was electrochemically polymerized on the graphite electrode in 0.1 M $NaClO_4$/$LiClO_4$/ACN electrolyte/solvent system at room temperature via scanning the potential between −0.5 V and +1.2 V with cyclic voltammetry. The polymer thickness was controlled by adjusting scan number during electropolymerization. According to the previous works and experiments, 100 cycles of electropolymerization was chosen at a scan rate of 100 mV s^{-1} [19,20]. Charge involved in the film formation was calculated as 7.05×10^{-3} C and the thickness of the polymer film was estimated as 0.16 μm (Fig. 1). When electropolymerization was completed, the polymer coated surface of the electrode was rinsed with deionized water to get rid of the organic impurities.

2.5. Preparation of the poly(SNS-NH_2)/AChE–ChO biosensor

ChO and AChE were co-immobilized onto the amine functional conducting polymer using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS). 9.0 μL of ChO solution (50 mM in sodium phosphate buffer pH 7.5 containing 1392 U/cm^2 ChO, 0.4 M EDC and 0.1 M NHS) was spread over the graphite electrode coated with the conduction polymer. Immediately after that, 3.0 μL AChE solution (50 mM in sodium phosphate buffer pH 7.5 containing 536 U/cm^2 AChE) was immobilized onto the electrode surface and left to dry at room temperature. Then, the enzyme electrode was stored at 4 °C for overnight. Before use, it was rinsed with deionized water to get rid of unbound enzyme molecules and reagents.

Amperometric measurements were performed in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.5) under gently stirring. During the measurements, the current changes due to the enzymatic activity were followed. When the current reached a steady state, the substrate (ACh) was added to the buffer solution in the reaction cell and the current response as a result of the decrease of the oxygen level by the enzymatic reactions was monitored at −0.7 V. This change in the current as the response of the biosensor was recorded in each measurement. The buffer solution was refreshed after each measurement. Substrate (ACh) stock solutions were prepared in phosphate buffer solution daily and stored in dark. For the characterization, of the biosensor the response on ACh was measured under optimized conditions.

2.6. Application test with paraoxon-ethyl

Poly(SNS-NH_2)/AChE-ChO biosensor was used for the determination of paraoxon-ethyl in spiked tap water samples. In order to measure the inhibition effect of paraoxon-ethyl on the biosensor, the current responses were recorded for the same amount of acetylcholine (ACh) before and after exposure of the biosensor to the paraoxon-ethyl solution. The paraoxon-ethyl solutions were prepared from the stock solution (cf. Section 2.2) by dilution with phosphate buffer (50 mM pH 7.5). For inhibition, the biosensor was kept in buffer solutions with different concentrations of paraoxon-ethyl for 5 min. After these incubations, the biosensor was rinsed with phosphate buffer solution and deionized water many times. The percentage of the inhibition was calculated as

$$\%\,\text{Inhibition} = \left[\frac{I_0 - I_i}{I_0}\right] \times 100.$$

With this data a calibration curve was calculated. Moreover, the biosensor was recovered by washing with 50 mM phosphate buffer solution (pH 7.5) for 30 min after incubations in concentrated pesticide solutions. Furthermore, tap water samples from Braunschweig, Germany were spiked with the stock solution of paraoxon-ethyl (cf. Section 2.2) to obtain concentrations of 0.5–50 mg L^{-1}. The concentrations were determined with the biosensor. The spiked tap water samples were also analyzed with HPLC/DAD.

During inhibition applications, after each inhibition study, the biosensor was washed with deionized water for several times and reactivation was achieved. After 89% inhibition, the biosensor retained 79% its initial activity.

3. Results and discussion

3.1. Characterization of the polymeric film

The factors affecting the biosensor performance must be optimized in order to yield the best biosensor responses and sensitivity. In order to maintain the activity and three dimensional structures of the enzyme molecules after immobilization, all the parameters affecting the enzyme microenvironment must be optimized.

Immobilization of the AChE and ChO molecules in conducting polymer layer was attempted to block the release of the enzyme molecules from the active layer during the operations and to assure it from the inhibitor effects. CPs with their organized molecular structure serve as three dimensional host materials for enzymes and sustain the activity of enzymes. Via crosslinking, simple and one-step biosensors can be achieved with functional CPs with a high operational and shelf-life stability and reproducibility [21].

Poly(SNS-NH_2) bears free amino groups in its structure assisting the covalent linkage with the enzyme molecules for a robust immobilization. This polymer as support was recently offered for the immobilization of enzymes concerning its high stability and functional groups available for the immobilization [19,20]. After that, immobilization was conducted and biosensor responses

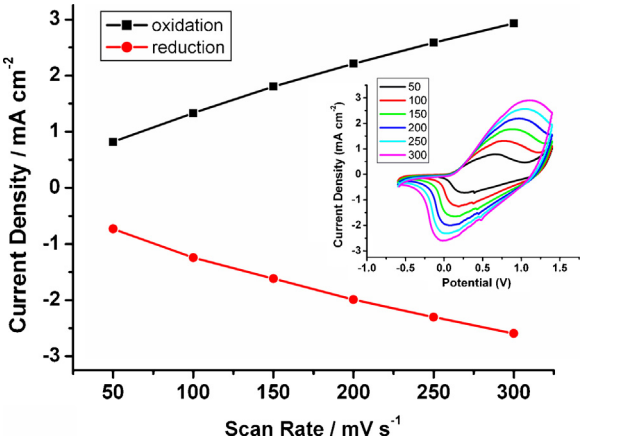


Fig. 2. Scan rate dependence of poly(SNS–NH_2) film in 0.1 M $NaClO_4$/$LiClO_4$/ACN solvent electrolyte/system at 50, 100, 150, 200, 250 and 300 mV s^{-1}.

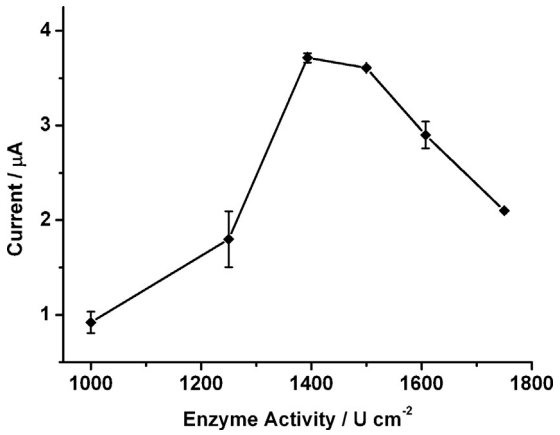


Fig. 3. Effect of increasing amounts of ChO (AChE (1.5 U)) on the biosensor response (in 50 mM phosphate buffer, pH 7.5, 6.0 mM acetylcholine, 25 °C, −0.7 V). Error bars show standard deviation (SD) of five measurements.

were recorded. In order to see the effect of conducting polymer film, a biosensor lacking of poly(SNS-NH_2) was prepared. Without the polymer layer, it was even impossible to keep the enzyme molecules on the graphite surface; hence, no response could be recorded.

For the further characterization of the polymeric film, poly(SNS-NH_2) was subjected to scan rate variation analyses. With the help of these experiments, diffusion capability of the prepared polymeric film was investigated. Since the electron transfer is important and sensitive in acetylcholine biosensors, the host materials must assist electron transfer profoundly. In scan rate studies, a monomer free system was used. Cyclic voltammograms for the polymeric film at different scan rates showed that the polymer redox process is quasi-reversible. Current versus scan rate graph showed the linear relationship between scan rates and current for both oxidation and reduction (Fig. 2). The increase in the peak current intensity and the linearity indicate the processes are non-diffusion controlled and the polymer is well-adhered to the graphite electrode surface. Thus efficient electron transfer in the catalytic reactions on the electrode surface is assured, enhancing the electrochemical properties and the performance of poly(SNS-NH_2)/AChE–ChO biosensor.

3.2. Optimization of the biosensor response

In order to optimize the amount of immobilized ChO in the biosensor, the currents of six different biosensors with increasing amounts of ChO from 1000 U/cm^2 to 1750 U/cm^2 (equal to 0.2–0.35 mg protein) a constant thickness of the conducting polymer, a constant amount of crosslinker and of the AChE (536 U/cm^2) were compared. The maximum response of the biosensor was obtained with a ChO activity of 1392 U/cm^2 (Fig. 3). At higher amounts (1607 and 1750 U/cm^2) it was observed that the enzyme molecules of ChO leached from the electrode surface due to excess loading. Moreover, it can be considered that with the increase of the enzyme concentration, the enzyme layer gets thicker which may constrain the diffusion of the substrate and slow down the electron transfer on the electrode.

Furthermore, the AChE activity was optimized. Keeping ChO amount (1392 U/cm^2 per electrode which equals to 0.28 mg protein) and other parameters constant, electrodes with 89, 179, 536 and 893 U/cm^2 of AChE were compared. The maximum response was obtained with the 536 U/cm^2 AChE containing electrode. Thus for further studies, enzyme amounts of 1392 U/cm^2 ChO and 536 U/cm^2 of AChE were used in the biosensor construction.

Since the protonation of the enzyme molecules differs with varying pH in their microenvironment, pH of the working buffer is notably capable to affect the activity, response and sensitivity of the biosensor [22]. Thus, the effect of the pH on the amperometric biosensor response was investigated using 50 mM phosphate buffer solution between pH 6.0 and 8.0 in the presence of acetylcholine (6.0 mM). Fig. 4 shows that at pH < 7 and pH ≥ 8 the enzyme activity is reduced. The optimum enzyme activity was found at pH 7.5 and thus used for the further studies.

3.3. Surface characterization of poly(SNS-NH_2)/AChE–ChO biosensor

The surface morphologies of the poly(SNS-NH_2) coated graphite electrode before and after AChE/ChO immobilization were investigated by scanning electron microscopy (SEM). In Fig. 5(A), the image corresponding to 100-cycle polymeric film is given. It shows a porous and cauliflower 3D structure of the CP. Thus, the polymer served as an appropriate host for the enzyme molecules. In Fig. 5(B), after covalent immobilization of AChE/ChO, the cauliflower structure of the conducting polymer alters to a more regular structure due to the formation of covalent bonds between AChE/ChO and poly(SNS-NH_2). The adequate and satisfactory immobilization can be concluded from the SEM images.

X-ray photoelectron spectroscopy (XPS) was utilized in order to investigate the covalent binding in the modified electrode surfaces. XPS spectra of poly(SNS-NH_2) and poly(SNS-NH_2)/AChE–ChO were resolved by a fitting program as shown in Fig. 6. The peaks at 283.8 and 284.3 eV can be attributed to aromatic carbons of thiophene

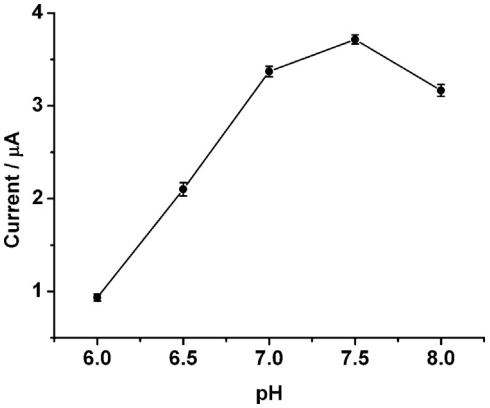


Fig. 4. Effect of pH (50 mM sodium phosphate buffer at pH 6.0, 6.5, 7.0, 7.5, 8.0; 6.0 mM acetylcholine, 25 °C, −0.7 V, ChO 3.9 U, AChE 1.5 U) on the biosensor response. Error bars show standard deviation (SD) of five measurements.

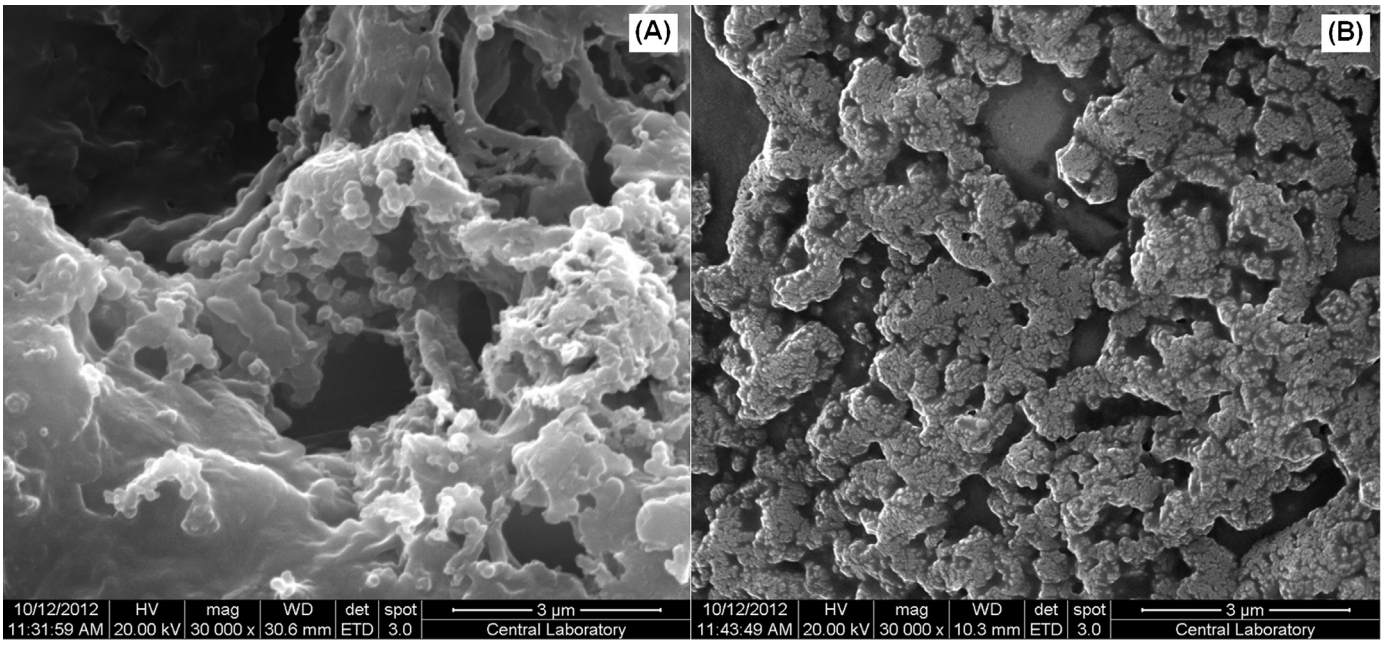


Fig. 5. Surface characteristics of (A) conducting polymer poly(SNS-NH_2); (B) enzyme immobilized conducting polymer, poly(SNS-NH_2)/AChE-ChO biosensor via SEM images.

and pyrrole units (C_α, C_β of pyrrole ring and C–S, respectively) [23] (Fig. 6(A) and (B)). The peaks at higher energies (286.2 eV) can be assigned to the carbons atoms of C–N in the polymeric structure due to the presence of pyrrole and benzenamine units. The successful immobilization of enzymes was proved with the new three peaks formed by the ejected photoelectrons from the carbon atoms of enzyme and amide bond formation in the second spectrum (Fig. 6(B)). The enzyme immobilized surface illustrated two signals at 286.6 eV (C═O) and 287.3 eV ((O═C)—N) representing the amide bond formation between free amino groups of poly(SNS-NH_2) and enzyme molecules. The generation of these peaks and the increase in the intensity of C—N bonds in the spectrum confirm the efficacious immobilization and binding of AChE and ChO molecules. The additional peak at 288.9 eV was generated by the carbon atoms in carboxylic acids in the protein structure of the enzymes [24,25]. Furthermore, it is noted that the nitrogen spectrum for polymeric and enzyme conjugated polymeric structures also supported the successful attachment. In Fig. 6(D), the peak at 400.4 eV can be attributed to the amide nitrogens [23,25] which occurred after the immobilization affirming the immobilization. In Fig. 6(C), the peak in nitrogen spectrum for polymer at 399.6 eV appears due to the presence of free amine groups in the structure of poly(SNS-NH_2) [26,27]. The binding energy at 401.3 eV is assigned to protonated amine groups in the structure of free amino containing polymeric film [26,28]. The emission band in the spectra at 398.4 eV can be assigned to a tertiary benzenamine substituted amine in pyrrole units [26,29]. After immobilization of the enzymes, the disappearance of this band due to the utilization of amino groups of the polymer in immobilization and new appeared emission band at 400.4 eV belonging to amide bond shows the successful covalent bonding.

3.4. Analytical characterization

With the optimum conditions, biosensor responses for different amounts of acetylcholine were recorded to generate a calibration curve (Fig. 7). Linear correlation was found to be 0.12–10 mM (Fig. 7, inset). Moreover, limit of detection (LOD) and sensitivity were calculated as 0.11 mM for acetylcholine and 2.19 mA mM^{-1} cm^{-2}, respectively. These values for the performance of the designed biosensor are in the range of the values that are reported in the literature (Table 1). The response time was only 7 s and thus even shorter than the reported once. Hence, the effectiveness of the immobilization and well-applicability of poly(SNS-NH_2) to biosensor construction were proven. Furthermore, due to the presence of covalent binding between enzyme molecules and matrix, a stable and long-life biosensor with high sensitivity was achieved. The shelf-life was found as 30 days conserving the same biosensor configuration and stability. The biosensor can be utilized for weeks without loss of activity in many applications.

Moreover, high operational stability is one of the most required properties of a biosensor. For repeatability of the acetylcholine biosensor response, ten successive measurements were performed at the same day with 6.0 mM acetylcholine. Standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.119 and 3.2%, respectively. For reproducibility of the biosensor, 5 different biosensors were prepared in the same manner and current responses for 6.0 mM acetylcholine were recorded as 3.94 ± 0.05 μA.

In order to be capable to perform accurate analyses with various kinds of samples, measurements must be done interference free and biosensor must be specific to the target substrate. Therefore, various possibly interfering substances such as ascorbic acid, urea, cholesterol and glucose were investigated for their effect on the poly(SNS-NH_2)/AChE–ChO biosensor response since these products can be easily oxidized on the electrode surface. Ascorbic acid, urea, cholesterol and glucose solutions were prepared in a range of concentrations from 1.0 mM to 0.1 M and during the amperometric measurements, these materials were injected to the reaction medium instead of the substrate. No interference effect was observed during all experiments. Moreover, the same content of pesticide solutions used in pesticide analyses without paraoxonethyl were prepared and measured. No interference was recorded. However, when ACh was injected to the reaction cell, the signal response can be easily obtained in 7 s as given in Fig. 8. This duration is much lower than the ones in literature (Table 1). This fast response was an indication of no diffusional drawbacks of the catalytic reactions on the biosensor surface.

Moreover, since the potential −0.7 V versus Ag reference electrode was used in all amperometric measurements, the possibility

Table 1
Characteristics of AChE-biosensors from the literature compare to the biosensor prepared in this study.

Biosensor	Immobilization technique	Linear range	LOD	Response time	Shelf life	Reference
P(HEMA)/AChE-ChO	Covalent	0.1–10 mM	NR	NR	60 days	[30]
PVA/AChE–ChO	Adsorption	5.0–100 μM	2.0 μM	2 min	30 days	[31]
ZnO sol gel/AChE–ChO	Entrapment	1.0 μM to 1.5 mM	0.6 μM	10 s	10 days	[22]
Silica membrane/AChE–ChO	Adsorption	6.0 μM to 8.0 mM	5.2 μM	3 min	80 days	[32]
$PVFClO_4$/AChE–ChO	Adsorption	0–12 mM	1.0 μM	50 s	25 days	[33]
PAMAM/AChE–ChO	Crosslinking	0.2–1.3 mM	40 μM	1.2 min	14 days	[34]
Poly(SNS-NH_2)/AChE–ChO	Covalent	120 μM to 10 mM	111 μM	7 s	30 days	This work

NR: not reported.

to oxidize various electroactive species in the reaction medium was blocked. To lower the working potential is desirable in amperometric analyses to eliminate the background current changes and interference effects. The oxygen consumption can be readily followed with this structure where no higher voltages needed. Hence, this is also an advantage for this design and it can be comfortably applied for real sample measurements.

The designed biosensor effectively works in a great wide linear range. As a consequence of this wideness, the LOD value is calculated as 111 μM which is slightly higher than the reported ones.

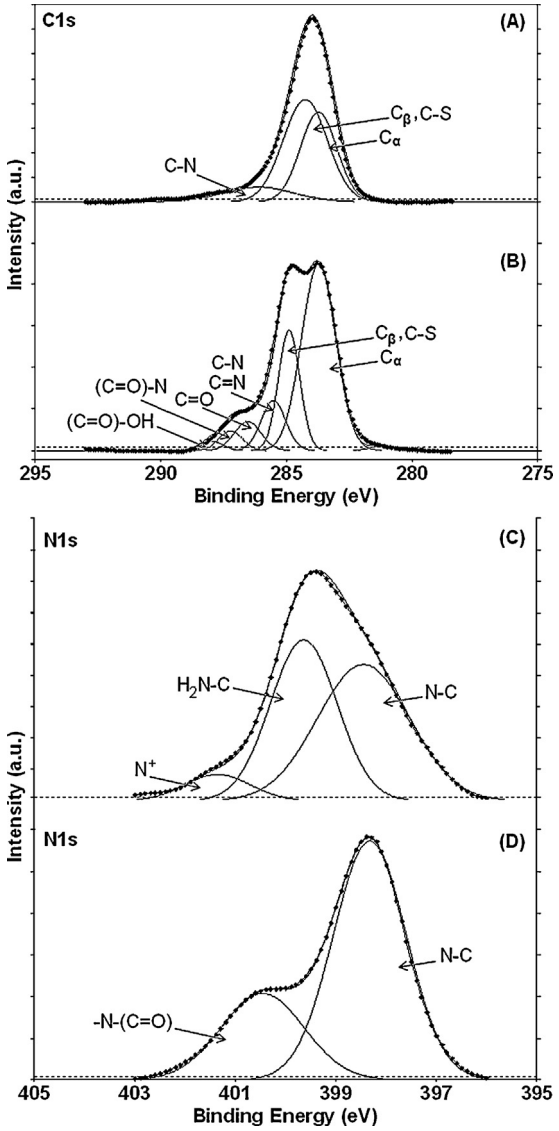


Fig. 6. XPS C 1s spectra for (A) poly(SNS-NH_2) and (B) AChE–ChO immobilized polymer, N 1s spectra for (C) poly(SNS-NH_2) and (D) AChE–ChO immobilized polymer.

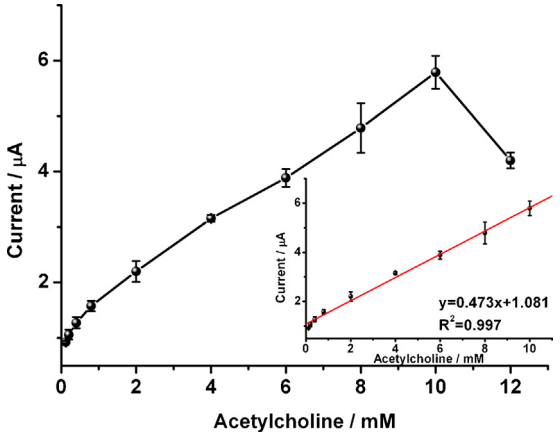


Fig. 7. Calibration curve for acetylcholine (in 50 mM phosphate buffer, pH 7.5, 25 °C, −0.7 V). Error bars show standard deviation (SD) of three measurements.

However, this value is sufficient for the further applications and did not affect the pesticide detection since in these applications indirect detection of pesticide is done. Moreover, LOD was determined at a signal to noise ratio of 3. Without conducting polymer, it was impossible to have a linear range. It is clear that the higher current signals was due to the accelerated electron transfer in the presence of conducting polymer toward electrode surface as stated before and supported by the data obtained from the scan rate experiments without using diffusional redox mediators. Without such an addition of redox mediators, this LOD value is quite sufficient for further applications. Furthermore, with these lower amounts of enzymes, it is great to have such a low LOD value.

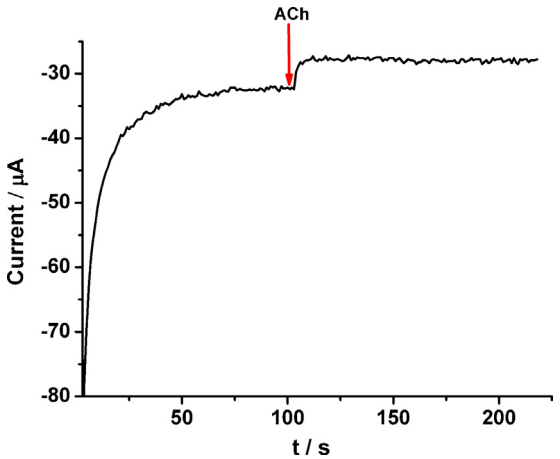


Fig. 8. Typical amperometric signal response of the biosensor to 8 mM acetylcholine (in 50 mM phosphate buffer, pH 7.5, 25 °C, −0.7 V).

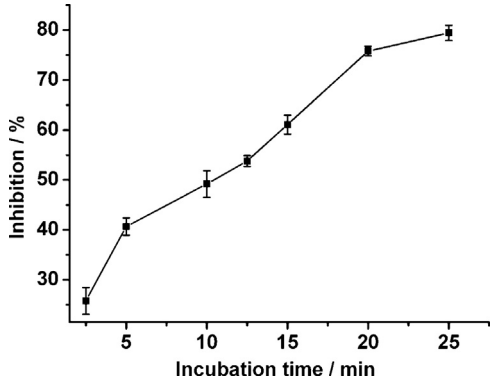


Fig. 9. The dependence of the inhibition (%) by paraoxon-ethyl (0.25 μg L^{-1}) from the incubation time. Error bars show standard deviation (SD) of three measurements.

3.5. Application tests of the poly(SNS-NH_2)/AChE–ChO biosensor

3.5.1. Effect of incubation time on response of poly(SNS-NH_2)/AChE–ChO biosensor

The designed biosensor was tested for the determination of paraoxon-ethyl. Preincubation method was chosen in order to eliminate the competition between substrate (ACh) and inhibitor (paraoxon-ethyl) and to get the maximum inhibition [30]. The biosensor was immersed in 0.25 μg L^{-1} (0.001 μM) paraoxon-ethyl solution for a given time. Then, after rinsing the biosensors with phosphate buffer and distilled water, the biosensors response was studied for 6.0 mM acetylcholine. The inhibition extent gradually increases with the increase in the incubation time of the paraoxon-ethyl solution (Fig. 9). Due to the drastic incubation effect for low amount of pesticide and more pronounced incubation activity, incubation time was selected as 5 min.

3.5.2. Calibration of the biosensor for the determination of paraoxon-ethyl

The decline of the current response (inhibition %) of the biosensor was proportional to the pesticide concentration (Fig. 10). Two ranges of linear correlation with different sensitivity were observed. The first linear range was between 0.0083 μg L^{-1} and 0.67 μg L^{-1} with a high sensitivity (slope of linear equation: 77). Above 75% inhibition the sensitivity of the biosensor decreased obviously and a second linear range from 0.67 μg L^{-1} to 16.7 μg L^{-1} with a low sensitivity (slope of linear equation: 0.83) was recorded. The maximum inhibition was not 100% due to the equilibrium established between pesticide and active sites in the enzyme [35].

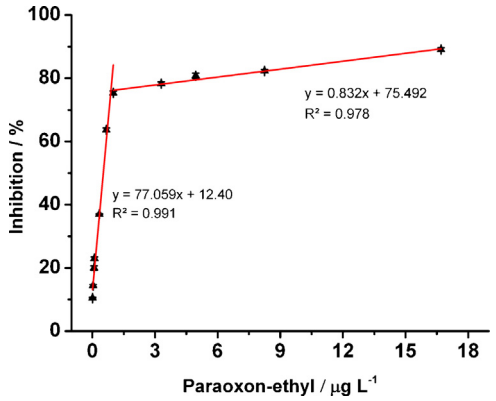


Fig. 10. Calibration curve for paraoxon-ethyl detection (in phosphate buffer, 50 mM, pH 7.5; 25 °C, −0.7 V, 6.0 mM acetylcholine, 5 min incubation time). Error bars show standard deviation (SD) of three measurements.

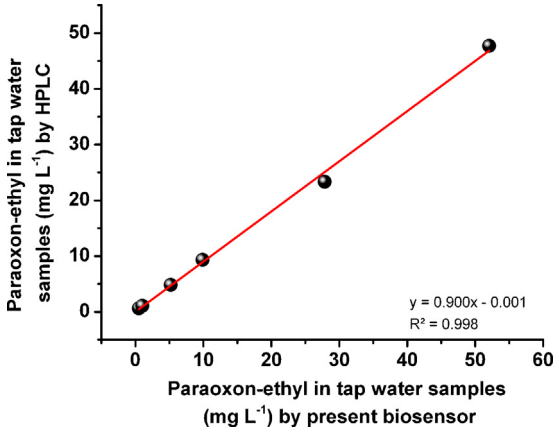


Fig. 11. Correlation of the quantification results for paraoxon-ethyl in tap water with the new designed biosensor (*x*-axis) and HPLC/DAD analysis (*y*-axis).

The LOD for paraoxon-ethyl was found as 0.0079 μg L^{-1} for an inhibition degree of 10% criterion. The detection limit value is lower than those reported earlier; for instance, SWCNTs/AChE biosensor with a LOD of 2.0 μg L^{-1} [36], CPE/AChE biosensor with LOD of 0.86 μg L^{-1} [37], and PB/GNP/AChE–ChO biosensor with LOD 10 μg L^{-1} [38].

3.5.3. Determination of paraoxon-ethyl in spiked tap water

For a first test of the applicability of the designed poly(SNS-NH_2)/AChE–ChO biosensor to determine AChE-inhibitors in real water samples, tap water was spiked with paraoxon-ethyl. The water samples were prepared by spiking the tap water with paraoxon-ethyl at final concentrations between 0.5 and 50 mg L^{-1}. No pretreatment of filtration were done prior to use the water samples. Their inhibition was measured as described above. Paraoxon-ethyl concentrations of the spiked water samples were quantified in parallel with the biosensor and with HPLC/DAD. The HPLC method with a practical calibration range of 0.5–50 mg L^{-1} was much less sensitive than the biosensor. The results of both methods were well correlated with each other with a regression coefficient of R^2 = 0.998 (Fig. 11). Considering this result, reliability of the designed biosensor was proven.

4. Conclusion

Combining conducting polymer and the enzymes AChE and ChO a biosensor was developed that was proven to work rapidly, reliable and accurately for the detection and quantification of paraoxon-ethyl, which is a model compound for AChE inhibition by organophosphates. After generation of conducting polymer film on the electrode surface, the quality of the immobilization of the enzymes and sensitivity of the biosensor were enhanced. Moreover, the working potential for amperometric measurements was reduced to −0.7 V blocking the interference effects of various electroactive species. The detection limit is lowered enabling the ability to work on real samples in the field. The LOD level is exceedingly sufficient for detection of hazardous amounts of pesticides in water samples. The prepared poly(SNS-NH_2)/AChE–ChO biosensor with high sensitivity, wide linear range and lower detection limit was a result of robust covalent binding of the enzymes onto the functional polymeric film on the transducer surface. Hence, the biosensor was successfully applied for the detection of paraoxon-ethyl with inhibition technique without requiring any pretreatment or regeneration. Present study reveals that this biosensor can practically be used as an electrochemical device to replace HPLC detector.

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