FLOW-INJECTION SYSTEM WITH SITE-SPECIFIC IMMOBILIZATION OF ACETYLCHOLINESTERASE BIOSENSOR FOR AMPEROMETRIC DETECTION OF ORGANOPHOSPHATE PESTICIDES

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ABSTRACT
A flow-injection system with integrated amperometric biosensor featuring an easily replaceable immobilized acetylcholinesterase (AChE) membrane was studied. The amperometric biosensor was constructed on the basis of site-specific immobilization of AChE on a hybrid polymer membrane with integrated multi-walled carbon nanotubes. Multistage modification of the membrane and immobilization of the enzyme was proved by Fourier transform infrared spectroscopy. The optimum flow-rate of the flow-injection analysis (FIA) system was 0.5 mL/min. It gave a linear response to acetylthiocholine chloride from 2 μM to 100 μM, with an average RSD of 3.0% (n = 6). The sensitivity of the constructed biosensor was 0.093 µA/µM·cm². The Km app value of the immobilized AChE was 1.15 mM and the linear correlation coefficient R², 0.9949. The method had a low detection limit for three organophosphorus pesticides (OPs) in model pesticide solutions –paraoxon ethyl (0.9×10⁻¹² M), monocrotophos (1.8×10⁻¹² M) and dichlorvos (2.0×10⁻¹² M). This indicated that the action of multi-walled nanotubes and controlled site-specific enzyme immobilization ensured high electrocatalytic activity and selectivity of the biosensor towards pesticides. It was found that the biosensor can be reused 15 operation cycles. After storage for 30 days the enzyme membrane retained over 80% of its initial response. The FIA system was used for detection of anti-cholinesterase activity of two binary OP mixtures. The results for paraoxon + monocrotophos and paraoxon + dichlorvos showed that the total inhibition activity was not simply additive, but was lower than the sum of the individual inhibition values. Moreover, the difference between the sum of the individual inhibition values and the real results for the mixture was bigger for the binary system paraoxon and dichlorvos (7-10%) compared with that for paraoxon and monocrotophos (5-7%). The developed biosensor system is an ideal tool for monitoring of organophosphate pesticides.


Keywords: amperometric biosensor, acetylcholinesterase, site-specific immobilization, pesticides, flow-injection system

Introduction
Organophosphorous pesticides (OPs) are highly toxic compounds which are widely used in the agriculture and as chemical warfare agents (8). The OPs are neurotoxins and therefore pose serious risk to human health. These compounds may still find their way into our food and water supplies, which necessitates analytical approaches for the reliable detection of pesticides for environmental protection and food safety purposes (10). Analytical methods such as gas chromatography or high-performance liquid chromatography have been widely used for the determination of OPs (8, 22, 29). These methods are time-consuming, need specialized laboratories and do not measure the toxicological effect of the pesticides.

The acute toxicity of OPs imposed the need for fast, sensitive, selective and miniature on-site detection devices that would provide an immediate response to remedy any problem that might arise (24). Biosensors are a reliable and promising tool in this respect. As organophosphorous compounds are inhibitors of acetylcholinesterase, amperometric biosensors based on enzymatic AChE inhibition were used. The AChE biosensor response decreases in correlation with the amount of pesticides in samples and the time of incubation, as described in many publications (1, 2, 4, 14, 34, 35). In recent years, AChE has been immobilized onto various nanomaterial surfaces in order to improve the response and stability in trace pesticide detection. These nanomaterial matrices include carbon nanotubes, gold nanoparticles, etc (25, 26). Intensive efforts have been focused on the oriented and site-specific immobilization of enzymes, which has become crucial for the rational design of biosensors. A recent trend in this direction is the creation of (bio) affinity bonds between an activated support and a specific group of the protein sequence (3, 7).

The operation of a biosensor could be greatly facilitated and even automated upon integration in a flow-injection analysis (FIA) system. Various constructions of continuous flow systems have been reported (13, 17, 19, 32); all demonstrating the greater efficiency of the flow-injection system. The combination of biosensors with flow-injection operation has
gained tremendous importance in environmental and food monitoring (30).

In many studies, the performance of the biosensors has been tested for each analyte separately. The detection of more than one analyte simultaneously, however, should also be studied since the AChE sensors are not specific to one pesticide and give total anti-cholinesterase activity in real samples. Some more complicated systems use different enzymes, different types of the same enzyme or engineered variants of AChE (5, 6, 9, 33) simultaneously in order to differentiate between the pesticides. These methods necessitate the use of multivariate methods or data analysis by feed-forward artificial neural networks for evaluation of the results. Kok and Hasirci (20) investigated binary carbamate mixtures by a simple method with an acetylcholinesterase–choline oxidase biosensor. However, there is still little information about the evaluation of different pesticide mixtures.

In our previous work (15) we described the construction of an amperometric biosensor on the basis of site-specifically immobilized AChE on a hybrid polymer membrane with integrated multi-walled carbon nanotubes (MWCN). The combination of the highly conductive and electrocatalytic behavior of MWCNs with the controlled site-specific enzyme immobilization resulted in a stable and sensitive sensor towards ATCh.

Here we describe a flow-injection system with integrated amperometric biosensor featuring an easily replaceable AChE-immobilized membrane described above. This is very important for the detection of irreversible enzyme inhibitors because of the easier replacement of the enzyme membrane and utilization of a single working electrode. The complicated composition of the enzyme membrane was proved by FT-IR analysis. The objective of this work was to optimize the conditions of analysis of the AChE–FIA system and to employ it in the detection of three organophosphorus pesticides – paraoxon ethyl, monocrotophos and dichlorvos in model pesticide solutions and binary mixtures. The response of our biosensor to the different pesticides and their mixtures was analyzed in order to evaluate its performance.

Materials and Methods
Materials
Acrylonitrile - methylmethacrylate - sodium vinylsulfonate membranes (PAN) were prepared without support as previously described by us (15). The ternary copolymer (acrylonitrile—91.3%; methylmethacrylate—7.3%, sodium vinylsulfonate—1.4%) was a product of Lukoil Neftochim, Burgas. Ultrafiltration membranes of acrylonitrile copolymer were measured to be 4 µm thick and could retain substances with molecular weight higher than 60,000 Da. MWCNs (2–6 nm in diameter; 0.1–10 µm in length, >90% purity) and pyridine-2-aldoxime methochloride (PAM) were purchased from Sigma–Aldrich (St. Louis, USA). Acetylthiocholine chloride (ATCh) and AChE (Type C3389, 500 U·mg−1 from electric eel) were purchased from Sigma–Aldrich and used as received. Bovine serum albumin (BSA), glutaraldehyde (GA) and concanavalin A (Con A) were also purchased from Sigma–Aldrich. Phosphate buffer solution (PBS, pH 7.6) and other reagents were of analytical reagent grade. All solutions were prepared with double distilled water.

Preparation of acetylcholinesterase biosensor
The chemical modification of MWCNs and chemical modification of PAN membranes was described in detail in our previous paper (15). The immobilization of enzyme was carried out in seven steps. The first procedure included the activation of the amino groups of modified membrane with glutaraldehyde (10%, PBS, for 1 h at room temperature). The next step was to immerse the activated membrane in a mixture of modified MWCN and BSA (from 11 mg MWCN-NH₂ in 1 mL 10% BSA solution). Then the membrane was immersed in a glutaraldehyde solution (10%, PBS) once more. After being carefully washed with bidistilled water the activated membrane was immersed in Con A solution – 1.5 mg/mL for 1 h at room temperature, then the non-reacted aldehyde groups and the adsorption sites were blocked by incubating the membrane in PBS (pH 7.6) (70 U/mL) in PBS (pH 7.6)
for 24 h at 4 °C. Finally the enzyme membrane was washed with bidistilled water.

**Flow-injection analysis system**

The flow-injection system was comprised of four major elements: three electrodes – a platinum working electrode, a calomel reference electrode and an auxiliary platinum wire electrode; a flow-cell with a working volume of 2 mL; a multichannel peristaltic pump (Ismatec, model IPC), four-way injection valve (Supelco Rheodyne Model 5020) with 100 µL sample injection loop and an amperometric detector (Palm Instruments BV, The Netherlands). The analyte solutions were introduced into the sample loop by means of a plastic syringe. The configuration of the flow-injection system is presented in Fig. 1a. The electrochemical cell used in flow-injection analysis is shown in Fig. 1b.

The enzyme electrode was inserted in the flow-cell along with the reference and auxiliary electrodes. The inlet and outlet for carrying solutions were just at the two opposite ends of the cell to minimize the dead volume. PBS flow-rate was set at the optimum value and the working potential was set at 630 mV. The substrate solution was fed to the flow-cell in two ways: by spiking a 100 µL of 100 µM substrate solution into the PBS carrier stream and by a continuous flow of 100 µM substrate solution.

**Measurement procedures for detection of OPs**

OP determination was carried out as follows: the initial amperometric response (A₀; peak area of the oxidation current) to 100 µM solution of acetylthiocholine chloride (ATCh) was measured in the working buffer (pH 7.6, 0.1 M PBS) stream; then 100 µL OP sample was injected into the flow cell and stopped in order to incubate the biosensor with the OPs for 7 min. Then the amperometric response to 100 µM ATCh (Aₗ) was measured again. Thus the inhibition percent (I%) was calculated by the following equation:

\[ I\% = \left( \frac{A_0 - A_l}{A_0} \right) \times 100 \]

Three kinds of OPs were tested – dichlorvos, paraoxon and monocrotophos. All measurements were performed at room temperature (about 25 ± 2 °C).

**Reactivation of the immobilized AChE**

After each inhibition measurement the immobilized enzyme was reactivated by feeding a 5 mM solution of PAM to the cell for 7 minutes. This was followed by a thorough washing of the membranes with PBS solution (pH 7.6, 0.1 M PBS).

**FTIR measurements**

The surface chemical composition of the membranes was characterized by attenuated total reflectance Fourier transform infrared spectroscopy (FTIR/ATR). FTIR/ATR measurements were carried out using a Perkin Elmer Spectrum One FT-IR spectrometer equipped with a horizontal ATR (HATR) unit. The HATR utilizing a zinc selenide (ZnSe) crystal, a parallel-sided plate of about 5 cm by 1 cm, with the upper surface exposed to the sample and with a 45° nominal incident angle. All spectra were collected using 4 scans in the range from 4,000 to 650 cm⁻¹ with a 4 cm⁻¹ spectral resolution. The background spectrum was collected through the air exposed HATR unit.

**Results and Discussion**

**Preparation of enzyme membrane. FTIR analysis of chemically modified membranes and enzyme membrane**

The chemical modification of the polymer membranes and immobilization technique of enzyme involved seven steps (Table 1). The amount of bound proteins and the enzyme activity were determined, respectively 0.878 mg/cm² and 26.52 mol/min1 cm². The higher activity of enzyme was ensured by blocking the residual free aldehyde groups, which significantly reduced the random immobilization. This emphasized the specific bonding between Con A and AChE and yielded a substantially higher activity. The addition of preliminarily aminated MWCNs, before Con A, resulted in an enhanced AChE activity, higher enzyme loading capacity and improved electrical conductivity of the membrane. The multi-stage modification of the membrane and immobilization of enzyme required to be proven. This was achieved by FTIR analysis.

**Fig. 2.** FTIR spectra of initial (sample 0), chemically modified (samples 1 - 5) membranes and enzyme membrane (sample 7) in the range 2800 - 3800 cm⁻¹ (a) and 1800 – 1100 cm⁻¹ (b).

First, the changes of the membrane which occurred during the chemical treatment were analyzed. To highlight this Fig. 2 shows the spectra obtained after the various steps of
Steps in the chemical modification of the polymer membranes and enzyme immobilization

<table>
<thead>
<tr>
<th>Number</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>untreated membranes</td>
</tr>
<tr>
<td>1</td>
<td>NaOH + EDA</td>
</tr>
<tr>
<td>2</td>
<td>NaOH + EDA + GA</td>
</tr>
<tr>
<td>3</td>
<td>NaOH + EDA + GA + (MWCNT+BSA)</td>
</tr>
<tr>
<td>4</td>
<td>NaOH + EDA + GA + (MWCNT+BSA) + GA</td>
</tr>
<tr>
<td>5</td>
<td>NaOH + EDA + GA + (MWCNT+BSA) + GA + Con A</td>
</tr>
<tr>
<td>6</td>
<td>NaOH + EDA + GA + (MWCNT+BSA) + GA + Con A + BSA</td>
</tr>
<tr>
<td>7</td>
<td>NaOH + EDA + GA + (MWCNT+BSA) + GA + Con A + BSA + E</td>
</tr>
</tbody>
</table>

EDA: ethylenediamine; GA: glutaraldehyde; MWCNT: multi-walled carbon nanotubes; BSA: bovine serum albumin; Con A: concanavalin A; E: enzyme

Main peaks and assignments for FTIR spectra

<table>
<thead>
<tr>
<th>Wave number (cm⁻¹)</th>
<th>Assignment SAMPLE 0</th>
<th>Assignment SAMPLE 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>3288</td>
<td>OH and NH stretching</td>
<td></td>
</tr>
<tr>
<td>3310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2934</td>
<td>C–H asymmetric stretching for CH₂</td>
<td>C–H asymmetric stretching for CH₂</td>
</tr>
<tr>
<td>2874</td>
<td>C–H symmetric stretching for CH₂</td>
<td>C–H symmetric stretching for CH₂</td>
</tr>
<tr>
<td>2243</td>
<td>C≡N stretching</td>
<td>C≡N stretching</td>
</tr>
<tr>
<td>1726</td>
<td>C=O for aldehyde or carboxylic acid or ketone</td>
<td>C=O for aldehyde or carboxylic acid or ketone</td>
</tr>
<tr>
<td>1639</td>
<td>C=C stretching for vinyl</td>
<td>C=O and C–N stretching AMIDE I</td>
</tr>
<tr>
<td>1652</td>
<td></td>
<td>C–N stretching and N–H bend</td>
</tr>
<tr>
<td>1536</td>
<td></td>
<td>C–N stretching and N–H bend AMIDE II</td>
</tr>
<tr>
<td>1453</td>
<td>C–H band</td>
<td>C–H band</td>
</tr>
<tr>
<td>1395</td>
<td>SO₂ stretching for sulfonate</td>
<td>SO₂ stretching for sulfonate</td>
</tr>
<tr>
<td>1362</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1220</td>
<td>C–N stretching</td>
<td>C–N stretching</td>
</tr>
</tbody>
</table>

Modification. Also in this case the most important differences are around 3330 cm⁻¹ and in the range of 1700-1520 cm⁻¹ (Fig. 2a, b). The spectrum of sample 1 (after treatment with NaOH and ethylenediamine) indicated, with respect to the spectrum of sample 0, an increase for the peak at 3300 cm⁻¹ due to the presence of N-H and a high peak at 1655 cm⁻¹ due to C=O stretching for AMIDE and another peak at 1560 cm⁻¹ due to N-H bend. The sample 2 spectrum showed a decreased peak due to NH group (at 3300 and 1560 cm⁻¹) and a decrease of the peak around 1655 cm⁻¹, as well as an increased peak at 1726 cm⁻¹ also due to C=O stretching of aldehyde group.

Finally, in spectra 5 and 7 there were high peaks due to protein AMIDE I and II and a decrease of the aldehyde peak at 1726 cm⁻¹ because this group is involved in the immobilization of proteins. The AMIDE I and II peaks were higher, indicating an increase in the amount of immobilized protein. In fact, we found that the area under the two peaks increased from sample 5 to 7 with about 30%. The spectrum of sample 6 is not shown because it is the almost the same as that of spectrum 5.

Fig. 3 and Fig. 4 show the spectra obtained by analyzing the membrane before the chemical treatment (Fig. 3) (sample 0) and after enzyme immobilization (Fig. 4) (sample 7). All the spectra were normalized to the peak at 1453 cm⁻¹, which is due to C–H stretching for CH₃ attached to oxygen (from the methylmethacrylate in the initial membrane), because this peak does not seem to be modified during the different modification and immobilization steps since the CH₃-O group is probably not involved in the chemical modification of membranes. The main differences between the two spectra were in the region around 3300 cm⁻¹, where the contribution of the N-H stretch due to the presence of proteins introduces a significant increase in the peak of the OH and in the region between 1750-1520 cm⁻¹. In fact, from the comparison of the figures, it is evident that the peak due to C=O stretching (at 1724 cm⁻¹) was reduced, while two peaks of high intensity, due to AMIDE I and II of proteins, were present in spectrum 7. The AMIDE I band (at 1652 cm⁻¹) is the most intense absorption band in protein. It results from the C=O stretching vibration of the amide group coupled to the stretching of the C-N bond. These vibrational modes are sensitive to hydrogen bonding and...
coupling between transition dipole of adjacent peptide bonds and hence are sensitive to secondary structure. The AMIDE II band (at 1536 cm$^{-1}$) involves both in-plane N-H bending and C-N stretching. The assignments of peaks are listed in Table 2.

The strong absorption bands of amide I and amide II bands, as well as NH and NH$_2$ bands, are characteristic of enzyme, indicating the AChE was successfully immobilized on the modified membrane surface. All the differences in the spectra confirm that the procedures for modification of the membrane and immobilization of enzyme had been successful.

### Table 3

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Interval of linearity, M</th>
<th>$R^2$</th>
<th>Equation</th>
<th>Detection limit, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon ethyl</td>
<td>$1 \times 10^{-11}$ - $1 \times 10^{-8}$</td>
<td>0.9957</td>
<td>$y = 26.1 \ln(x) + 293.7$</td>
<td>$0.9 \times 10^{-12}$</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>$5 \times 10^{-11}$ - $1 \times 10^{-9}$</td>
<td>0.9954</td>
<td>$y = 16.3 \ln(x) + 182.3$</td>
<td>$1.8 \times 10^{-12}$</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>$1 \times 10^{-11}$ - $1 \times 10^{-7}$</td>
<td>0.9952</td>
<td>$y = 10.0 \ln(x) + 112.8$</td>
<td>$2.0 \times 10^{-12}$</td>
</tr>
</tbody>
</table>

### Table 4

Comparison of the AChE biosensor performance in flow-injection system with such reported by other authors

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Flow rate, mL/min$^{-1}$</th>
<th>Linear interval, M</th>
<th>Detection limit, M</th>
<th>Incubation time, min</th>
<th>Storage time, days</th>
<th>Residual activity, %</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon ethyl</td>
<td>0.25</td>
<td>$1 \times 10^{-12}$ - $1 \times 10^{-8}$</td>
<td>$4 \times 10^{-13}$</td>
<td>6</td>
<td>21</td>
<td>85</td>
<td>(23)</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>1</td>
<td>$5 \times 10^{-11}$ - $1 \times 10^{-9}$</td>
<td>$4 \times 10^{-11}$</td>
<td>20</td>
<td>76</td>
<td>90</td>
<td>(28)</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>0.5</td>
<td>$1 \times 10^{-8}$ - $4 \times 10^{-7}$</td>
<td>$1 \times 10^{-9}$</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>(27)</td>
</tr>
<tr>
<td>Paraoxon ethyl</td>
<td>1.5</td>
<td>$1 \times 10^{11}$ - $1 \times 10^{-7}$</td>
<td>$1 \times 10^{-9}$</td>
<td>10</td>
<td>21</td>
<td>70</td>
<td>(16)</td>
</tr>
<tr>
<td>Paraoxon methyl</td>
<td>1.0</td>
<td>$1 \times 10^{-9}$ - $2.5 \times 10^{-7}$</td>
<td>$1 \times 10^{-9}$</td>
<td>15</td>
<td>single use SPE</td>
<td>-</td>
<td>(33)</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>1.0</td>
<td>$1 \times 10^{-10}$ - $1 \times 10^{-7}$</td>
<td>$1 \times 10^{-10}$</td>
<td>15</td>
<td>single use SPE</td>
<td>-</td>
<td>(33)</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>1.0</td>
<td>$1 \times 10^{-18}$ - $1 \times 10^{-6}$</td>
<td>$1 \times 10^{-17}$</td>
<td>20</td>
<td>92</td>
<td>65</td>
<td>(21)</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>1.0</td>
<td>$2 \times 10^{-9}$ - $5 \times 10^{-7}$</td>
<td>$7 \times 10^{-11}$</td>
<td>20</td>
<td>76</td>
<td>90</td>
<td>(28)</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>0.35</td>
<td>$1 \times 10^{-7}$ - $8 \times 10^{-5}$</td>
<td>$8 \times 10^{-8}$</td>
<td>15</td>
<td>150</td>
<td>90</td>
<td>(31)</td>
</tr>
<tr>
<td>Paraoxon ethyl</td>
<td>0.5</td>
<td>$1 \times 10^{-11}$ - $1 \times 10^{-8}$</td>
<td>$0.9 \times 10^{-12}$</td>
<td>7</td>
<td>120</td>
<td>50</td>
<td>here</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>0.5</td>
<td>$1 \times 10^{-11}$ - $1 \times 10^{-7}$</td>
<td>$1.8 \times 10^{-12}$</td>
<td>7</td>
<td>120</td>
<td>50</td>
<td>here</td>
</tr>
<tr>
<td>Monocrotophos</td>
<td>0.5</td>
<td>$1 \times 10^{-11}$ - $1 \times 10^{-7}$</td>
<td>$2.0 \times 10^{-12}$</td>
<td>7</td>
<td>120</td>
<td>50</td>
<td>here</td>
</tr>
</tbody>
</table>

**Optimal conditions of the flow-injection system and calibration curve**

A flow rate which would give a large amperometric response and fast response time but also be economical with flow buffer was desired for the flow-injection system. To this end a range of flow speeds was evaluated for the amperometric response profile of the electrodes (Fig. 5). The registered current went through a peak value (at 0.5 mL/min) with the increase of the flow-rate, after which the current slightly decreased and was insignificantly influenced by the flow-rate increment. The stronger the current, the better the signal-to-noise ratio, that is why 0.5 mL/min was selected as the optimum flow-rate to carry out further experiments.

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**Fig. 3.** FTIR spectrum of the initial membrane, before the chemical treatment (sample 0).

**Fig. 4.** FTIR spectrum of the enzyme membrane (sample 7).
Fig. 5. Biosensor response to 100 µL of 100 µM ATCh solution as a function of carrier flow-rate.

This flow rate was found to give a linear response to acetylthiocholine chloride from 2 µM to 100 µM (Fig. 6), with an average RSD of 3.0% (n = 6). Then the amperometric response leveled out to a plateau, showing a typical Michaelis-Menten process. The substrate concentration of 100 µM was chosen for further experiments. The linear regression equation was

\[ I (\mu A) = 0.0724(x) - 0.3568 \]

with a correlation coefficient of 0.9974 (n = 10), Fig. 7a. The detection limit was 0.2 µM at a signal-to-noise ratio of 3, and the sensitivity, 0.093 µA/µM cm². The results for determination of substrate with the flow-injection system were better than the results obtained using the AChE biosensor in the batch mode (15), where the sensitivity of analysis was 0.064 µA/µM cm² and the detection limit, 0.34 µM.

The \( K_m^{app} \) value of the immobilized AChE was calculated by fitting the experimental data to the Michaelis-Menten equation using the Lineweaver-Burk electrochemical representation of 1/[current] versus 1/[ATCh] (Fig. 7b). At pH 7.6 and at room temperature (about 25 ± 2 °C), the \( K_m^{app} \) of the immobilized AChE was 1.15 mM and the linear correlation coefficient \( R^2 \) was 0.9949 (n = 6). This value was lower than that of 15.56 mM for AChE immobilized in Al₂O₃ sol gel (31); 1.75 mM for PDDA/AChE/PDDA/CNT/GC (23); 1.5 mM for AChE adsorbed on polyethyleneimine modified electrode (18) and higher than 0.309 mM for AChE-Chitosan-AuNPs/Au (12); 0.45 mM for AChE/AuNPs–CS–SiSG/GCE (11), indicating that AChE site-specifically immobilized on PAN membrane containing MWNT had a greater affinity to ATCh. It demonstrates that the immobilized AChE is suitable for the Michaelis-Menten kinetic equation.

**Pesticide detection**

**Inhibition detection in model pesticide solutions**

Each measurement cycle consists of calibration (in order to determine the initial enzyme membrane activity), incubation of the enzyme membrane in the investigated pesticide sample, measurement of the residual activity of the immobilized enzyme, reactivation of the inhibited sensor and calibration again. The duration of one measurement cycle could be greatly reduced when using a flow-injection system mainly by reducing the incubation and the reactivation time. In our previous work (15) we measured in batch mode only the inhibition degree of paraoxon. The reported duration of the incubation and reactivation steps was 50 minutes which was mainly due to the fact that the latter were performed under batch mode (the enzyme membrane was immersed in the pesticide sample as well as in the solution of PAM). The employed flow-injection system was expected to intensify the mass transfer of inhibitor/reactivator molecules to the immobilized enzyme, which should result in reducing the time for each step of the measurement cycle.

The residual activity of the enzyme was influenced by the exposure time of AChE to the pesticide. The exposure time was controlled by the time of stop-flow. For these experiments we used paraoxon – the pesticide with high inhibitory potential. It was found that the higher the paraoxon concentration was (1×10⁻⁷ M, 1×10⁻⁸ M), the faster and greater the degree of inhibition was. Even a low concentration of paraoxon (1×10⁻¹¹
M) could cause an apparent inhibition after a 10-min exposure. Considering the whole analytical time and sensitivity of the amperometric measurements, a 7-min exposure time was chosen as the best compromise between signal and exposure time.

As regeneration is one of the key points in the development of inhibition enzyme biosensors, we tried to regenerate the AChE biosensor after a complete measurement (50% paraoxon inhibition) with incubation of 5 mM PAM. The results showed that only 30% of the responses were recovered after a short regeneration time (2 min). We found that the whole response of the biosensor was recovered by feeding a 5 mM solution of PAM to the cell for 7 min.

The FIA system was employed in the detection of three organophosphorus pesticides – paraoxon ethyl, monocrotophos and dichlorvos in model pesticide solutions. The relations between the degree of inhibition (I %) and the corresponding pesticide concentration (ranging from $10^{-14}$ to $10^{-4}$ M) are presented in Fig. 8.

The detection limit was calculated on the basis of 10 amperometric measurements of the biosensor response to a blank sample (without pesticide) and can be regarded as the inhibitor concentration that reduces the mean biosensor response with the tripled value of the standard deviation of the measured biosensor responses.

Table 3 contains summarized data for the linear intervals with the corresponding equations, correlation coefficients and the respective detection limits for each pesticide. The enzyme system was found to be effective for the detection of all the three pesticides. The slope of the equation of paraoxon was the highest, meaning that the sensitivity of the biosensor to this pesticide is the highest. It was also determined that monocrotophos and dichlorvos have a wider linear working range compared to paraoxon.

As seen from Table 3, paraoxon featured the lowest detection limit, followed by monocrotophos and dichlorvos and this could be explained with the higher inhibitory potential of paraoxon compared to those of monocrotophos and dichlorvos. Although monocrotophos was a more potent inhibitor than dichlorvos, the similarity of the detection limits could be explained with the similar diffusion rates of these pesticides (similar molecular weight, respectively 223.2 g/mol, 220.98 g/mol). These results showed that the biosensor has a higher biological affinity to pesticides. The action of multi-walled nanotubes (MWNTs) and controlled site-specific enzyme immobilization ensured high electrocatalytic activity and sensitivity of the biosensor towards pesticides (as inhibitors of AChE).

The reproducibility of the AChE biosensor for paraoxon detection was examined. Based on the percent inhibition observed for the concentrations of paraoxon in the range of $1\times10^{-11}$-$1\times10^{-8}$ M, a relative standard deviation of 3.4% ($n = 6$) was obtained. These results were indicative of an acceptable reproducibility regarding pesticide determination. The operation stability was measured with $1\times10^{-7}$ M paraoxon concentration. It was found that the biosensor could be reused for 15 operation cycles. When the flow-injection system was not in use, the enzyme membrane was stored at 4°C in 0.1 M PBS, pH 7.6, containing 0.02% NaN$_3$. The half-life storage...
time of the enzyme membrane (50% residual activity) was studied. After storage for 30 days the enzyme membrane retained over 80% of its initial response, which is in coherence with other authors’ reports (Table 4) and after 120 days, 50%.

The obtained sensitivity for paraoxon (slope 26.1) was significantly better than previous results with the AChE biosensor in batch mode (15) where paraoxon could be measured with lower sensitivity (slope 5.917). The reason for this difference is due to the small volume of the electrochemical flow cell (2 mL) and its special construction, which provides very good hydrodynamic conditions for washing and regenerating the enzyme membrane. The FIA system is advantageous over that using electrodes in the batch mode, whereby an average response to acetylthiocholine is measured from one batch of electrodes, then the enzyme membrane is separated and is exposed to pesticide in another batch, after that in PAM for reactivation and then the enzyme membrane is again assembled to the electrode to measure the average response to acetylthiocholine from another batch. All these operations take up a lot of time – 50 min. Using a flow rate of 0.5 mL/min ATCh ensured that the current returned rapidly to the baseline so that injections can be repeated every minute and the pesticide incubation and reactivation takes 7 min. This means that for one sample the time was 15 min, 3.5 times faster than experiments under batch mode (15).

**Inhibition detection in binary pesticide mixtures**

The above observation was confirmed by the results of an investigation of the inhibition exerted by binary mixtures of the OPs. Two sets of samples were prepared (paraoxon + monocrotophosphos and paraoxon + dichlorvos), each sample having a final volume of 10 mL. The pesticide concentrations were selected to be within the linear intervals, respectively 0.1 ppb, 0.5 ppb and 0.7 ppb. The enzyme system cannot differentiate between pesticides in a mixture and can detect total anti-cholinesterase activity instead. The purpose of these experiments was to determine whether the total inhibition is the sum of the inhibition of two pesticides, whether the theoretical sum is different from the experimental sum and whether the pesticide with a greater inhibitory potential would discriminate the amount of a weaker inhibitor in the mixture.

The inhibition curves for binary pesticide mixtures of paraoxon + monocrotophosphos (Fig. 9) and paraoxon + dichlorvos (Fig. 10) showed that the total inhibition activity was not simply additive but was lower than the sum of the individual inhibition values (Fig. 8; Fig. 9 and Fig. 10, where the concentration of one pesticide in the mixture is 0). A similar behavior was observed by Kok and Hasirci (20) for binary mixtures of aldicarb - carbofuran and aldicarb - carbaryl. Bachmann et al. (6) used a multisensor for simultaneous detection of binary organophosphate mixture (malaoxon and paraoxon) and found that the concentrations of malaoxon and paraoxon were discriminated with predication errors of 0.9 and 1.6 mg/L, respectively. Moreover the difference between the sum of the individual inhibition values and the real results for the mixture...
was bigger for the binary system paraoxon and dichlorvos (7-10%) compared with paraoxon and monocrotophos (5-7%). This could be explained by the fact that dichlorvos is a much weaker inhibitor than paraoxon and paraoxon significantly discriminated the interaction of dichlorvos with the enzyme. This masked effect was weaker for monocrotophos. These results are easily explained with the higher inhibitory potential of paraoxon than those of monocrotophos and dichlorvos and by the competition for the enzyme active site. Moreover, monocrotophos is a more potent inhibitor than dichlorvos and has a similar diffusion coefficient, and therefore would inhibit the immobilized enzyme to a greater extent under equal conditions. A theoretical justification of the possibility of such pesticide discrimination could be found in the mathematical model proposed by Zhang et al. (36). This model reveals that the degree of inhibition exerted by a certain pesticide depends on its inhibitory potential, but also on its diffusion coefficient and concentration at equal other conditions. Such a competitive behavior could be utilized for the discrimination of multiple pesticides present in analyzed samples based on the distinctive diffusion rates (i.e. molecular geometry) and inhibitory potencies of different OPs.

Conclusions
The present work was focused on the integration of an AChE biosensor in a flow-injection system in order to optimize the working conditions and create continuum analysis for rapid and sensitive measurements of OPs. The construction of the amperometric biosensor was on the basis of site-specific immobilization of AChE on a hybrid polymer membrane with integrated multi-walled carbon nanotubes. The AChE-immobilized membrane was easily replaceable with fresh one. This is very important for the detection of irreversible enzyme inhibitors. The prepared biosensor showed good precision, reproducibility and stability. It was found that the biosensor based on site-specific immobilization AChE on a hybrid polymer membrane with integrated multi-walled carbon nanotubes is an excellent platform for the detection of paraoxon, monocrotophos and dichlorvos. The AChE-FIA system presented a low detection limit for paraoxon – 0.9×10^{-12}; monocrotophos – 1.8×10^{-12} and dichlorvos – 2.0×10^{-12} M. The system was employed in the detection of binary OP mixtures. The total anti-cholinesterase activity of binary pesticide mixtures were found to be lower than the sum of the individual inhibition values. It was confirmed that the observed competitive behavior of the organophosphorous pesticides in the binary mixtures could be utilized for their discrimination on the basis of molecular geometry and inhibition potency with the use of only one acetylcholinesterase. The information obtained about the response of our biosensor to different pesticides and their mixtures was used as a means to evaluate the performance of the biosensor.

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