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A capacitive field-effect sensor for the direct determination of organophosphorus pesticides

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Abstract

A novel capacitive field-effect sensor for the direct determination of organophosphorus pesticides by the enzyme organophosphorus hydrolase (OPH) is presented. The enzyme OPH hydrolyses organophosphorus compounds catalytically, thus releasing H^+ ions. By means of a silicon-based field-effect sensor, which consists of a layer sequence of Al/p-Si/SiO_2 with alternative pH-sensitive materials (Ta_2O_5, Al_2O_3 and Si_3N_4), this pH shift can be recorded directly in a potentiometric measuring arrangement. To obtain the enzyme biosensor, thus the OPH has been immobilised on top of the pH-sensitive layer structure. Typical sensor characteristics, like pH sensitivity, sensitivity towards paraoxon, reversibility of the sensor signal, response time, detection limit, long-term stability and selectivity to various pesticides have been studied.

Keywords: Field-effect sensor; Silicon technology; Organophosphorus hydrolase (OPH); Pesticide; Environmental monitoring

1. Introduction

Synthetic organophosphorus compounds—among the most toxic substances known—are used as pesticides or insecticides in agriculture and chemical warfare agents. Biosensors to detect these neurotoxins, has been an actively researched area. The majority of biosensors to date is based on the inhibition of acetylcholinesterase or butyrylcholinesterase integrated with electrochemical (potentiometric and amperometric) and optical transducers [1–3]. Although sensitive, these biosensors suffer from several limitations: poor selectivity, multi-step indirect determination, and irreversible inhibition by many compounds, to name a few. Recently, we have demonstrated the application of another enzyme, generically termed organophosphorus hydrolase (OPH), as the biological recognition element for various biosensors. This enzyme catalyses the hydrolysis of organophosphates (OPs) after releasing products that can be monitored. We have integrated this enzyme with a number of different transducers to develop biosensors that allow rapid, selective, sensitive and on-line determination of OPs [3]. Taking advantage of the fact that hydrolysis of OPs by the OPH releases proton(s), we reported a simple potentiometric biosensor in which OPH was integrated with a glass pH electrode [4]. In order to miniaturise the potentiometric biosensor, OPH was immobilised at the gate of a pH-sensitive field-effect transistor (ISFET) [5]. However, the application of miniaturised ISFET-type sensors leads to several disadvantages: instability of the required passivation layer when in permanent contact with the test sample (or electrolyte), high costs of fabrication due to photolithographical process steps, etc.

To overcome these problems, we suggest the application of a simple capacitive electrolyte–insulator–silicon (EIS) field-effect structure as transducer of an OPH-based biosensor. The layer set-up of this EIS sensor corresponds to the gate region of an ISFET, however, due to the missing photolithographic process steps, no additional passivation and encapsulation layer of the sensing area is necessary. The sensor can be easily mounted in a home-made Plexiglas cell, e.g. sealed by an O-ring. Thus, this transducer structure possesses a higher stability in the long-term than ISFET-based transducer structures and is much more cheaper in sensor preparation [6].

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In this paper, a novel EIS-based sensor for the direct determination of organophosphorus pesticides by the enzyme OPH is presented. For the transducer structure, three alternative pH-sensitive materials Ta_2O_5, Al_2O_3 and Si_3N_4 have been used. Whereas the Ta_2O_5 and Al_2O_3 layers are grown by means of pulsed laser deposition (PLD), Si_3N_4 has been deposited by low pressure chemical vapour deposition (LPCVD) onto a basic structure of Al/p-Si/SiO_2. To achieve the capacitive EIS biosensor, OPH has been immobilised by three different strategies on top of the pH-sensitive layer structure: (1) by covalent coupling via glutaraldehyde; (2) by cross-linking with nafion; and (3) by an adsorptive technique. Depending on the immobilisation procedure used and the pH-sensitive material applied, typical sensor characteristics, like pH sensitivity of the transducer material before and after the enzyme immobilisation, the biosensor performance towards paraoxon including the linear concentration range, the reversibility of the biosensor signal, the response time as well as the detection limit and the biosensor stability in the long-term have been investigated; the selectivity to various pesticides will be discussed.

2. Experimental

2.1. Materials and processing

The different EIS structures of the layer sequences Al/p-Si/SiO_2/Al_2O_3, Al/p-Si/SiO_2/Ta_2O_5 or Al/p-Si/SiO_2/Si_3N_4 were fabricated from p-type Si (18–24 Ω cm, Wacker Chemitronic) with <1 0 0> orientation. A SiO_2 layer of 30 nm has been grown by means of thermal oxidation. For the preparation of the pH-sensitive materials Al_2O_3 and Ta_2O_5 (thickness: 30–50 nm), respectively, the PLD process has been employed, whereas the LPCVD technique was used to deposit the Si_3N_4 layer. The Al rear contact has been prepared by means of electron beam evaporation. All experimental details of the fabrication of the EIS structures are given elsewhere [7–9].

After preparation of the EIS structures, OPH (produced and purified according to the protocol described in [4], specific activity of 587 U/mg protein) was immobilised on the respective pH-transducing material in the following three different ways:

1. *Adsorptive immobilisation*: 60 μl of enzyme solution was dropped on the sensor surface and dried at room temperature for 12 h; followed by extensive washing with 30 mM HEPES buffer, pH 9.
2. *Coupling (cross-linking) via glutaraldehyde*: An aliquot of 25 μl from a mixture of 5 μl (10% BSA), 4 μl (2.5% glutaraldehyde) and 20 μl OPH was dropped on the sensor surface and dried for 30 min at room temperature followed by thorough washing with 30 mM HEPES buffer, pH 9.
3. *Entrapment in nafion*: An aliquot of 25 μl from a mixture of 9 μl (10% BSA), 15 μl (1% nafion) and 20 μl OPH was dropped on the sensor surface and dried for 90 min at room temperature; after that, the biosensor was extensively rinsed in 30 mM HEPES buffer, pH 9.

After immobilisation, the biosensors were mounted in a home-made measuring cell, all sensor types were, then stored in buffer solution in the refrigerator at 4 °C. Prior to measurements, the respective biosensor has been equilibrated at room temperature for at least 2 h.

2.2. Measurements

The schematic cross-section of the capacitive EIS biosensor is shown in Fig. 1. In this set-up, the pH-sensitive transducer layer can vary (Si_3N_4, Al_2O_3 or Ta_2O_5), and the immobilisation of the OPH layer is done in a different strategy (adsorptive, glutaraldehyde and nafion cross-linking). In order to study the basic sensor characteristics, the sensor chip was contacted on its front side by the electrolyte (test sample) and a reference electrode (Ag/AgCl) and on its rear side by a gold-plated pin. Capacitance/voltage (*C*/*V*) measurements were performed with an impedance analyser (Zahner electric) at a dc voltage which was swept from −3 to 0.5 V in steps of 100 mV and a superposed ac voltage with a frequency of 120 Hz and a signal amplitude of 20 mV. To obtain a dynamic sensor response, the EIS biosensor is also operated in the constant capacitance (ConCap) mode. In this measuring mode, by setting the capacitance at a fixed value (e.g. the flat band capacitance), the voltage shift that results from the generated surface potential at the interface electrolyte/sensitive layer, can be directly recorded (see also [10,11]).

The analytical method of the developed biosensor is based on the OPH-catalysed hydrolysis reaction of organophosphate esters such as parathion [4]. A schematic of the enzyme reaction is given in Fig. 2, where G_1 represents phenoxy, thiol, cyanide or fluorine, G_2 and G_3 are standing for methyl to butyl and alkyl or phenyl, respectively, and X is an oxygen or a sulphur molecule. The catalytic hydrolysis of each molecule of these organophosphate compounds

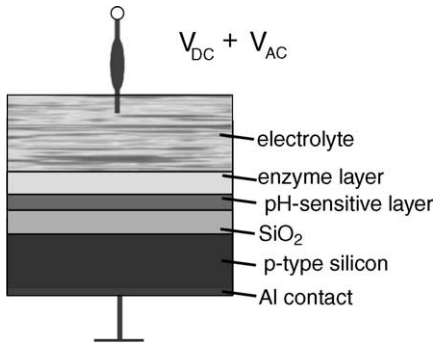

Fig. 1. Capacitive EIS biosensor with immobilised enzyme layer on a pH-sensitive transducer structure (schematically).

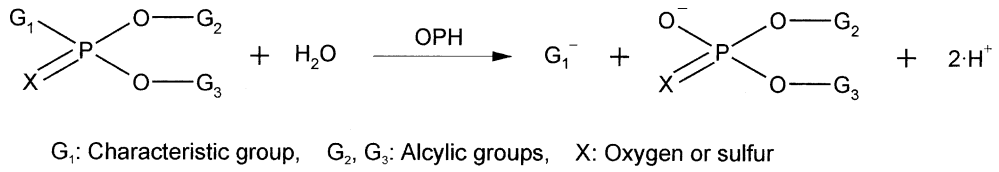

Fig. 2. Organophosphorus hydrolase (OPH)-catalysed hydrolysis reaction releasing two H^+ ions that can be detected by the pH-sensitive transducer layer.

releases two H^+ ions. As a result, the hydrogen ion concentration increases with increasing amount of the pesticide parathion. By using a weakly buffered test sample, this pH change can be detected by the underlying pH-sensitive material of the EIS biosensor. The pH sensitivity can be explained in terms of the ionisation states at the surface of the transducer layer of Si_3N_4, Al_2O_3 or Ta_2O_5 [12,13]. The resulting potentiometric response is recorded and correlated to the paraoxon concentration to be measured. The theoretical Nernstian law predicts a potential shift of 59.1 mV/pH change of one decade under standard conditions. However, the pH sensitivity of ISFETs in practical applications can be also somewhat lower [12,13].

For pH measurements, 50 mM HEPES buffer, pH 5–10, containing 50 μM $CoCl_2$ and 100 mM NaCl is used; for the paraoxon calibration measurements, 0.2 mM HEPES buffer, pH 9, containing 50 μM $CoCl_2$ and 100 mM NaCl with concentrations of 0.5–500 μM paraoxon is utilised. All measurements were carried out in a dark Faraday cage at room temperature. All chemical used are of analytical grade and are listed in the following: HEPES buffer (Fisher Scientific, Tustin, CA, USA), BSA (Sigma, St. Louis, MO, USA), glutaraldehyd (Fisher Scientific, Tustin, CA, USA), nafion (Aldrich, Madison, WI, USA), paraoxon (Sigma, St. Louis, MO, USA), pH buffer (Fisher Scientific, Tustin, CA, USA).

3. Results and discussion

Fig. 3 shows a typical *C/V* curve of a capacitive field-effect sensor with Si_3N_4 as pH-sensitive material in buffer solution, pH 7, before (full line) and after modification of the Si_3N_4 surface by OPH enzyme deposited by cross-linking (open squares). The maximum capacitance in the accumulation range is about 45 nF. Both curves indicate a nearly identical behaviour in terms of signal shape and capacitance values. The slight deviations are probably due to small variations in the additional impedance caused by the immobilised enzyme layer. The ConCap measurements allow studying the biosensor behaviour with regard to the dynamic sensor response (see also Fig. 5). The set value of the capacitance (working point, marked by arrow) was chosen from the depletion range of the *C/V* curve that is about −0.5 V. In this region, the curve has a linear shape, which guarantees that only the concentration-dependent potential shift as resulting biosensor signal is measured.

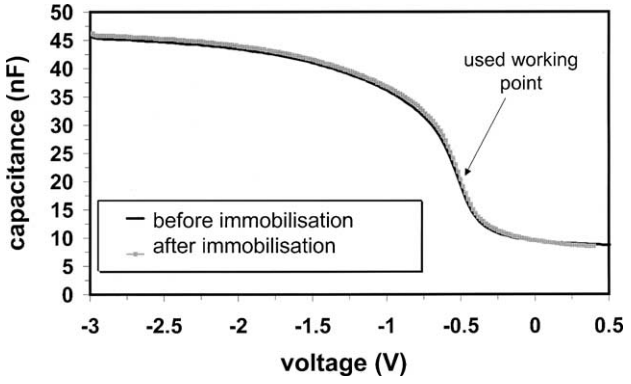

Fig. 3. Capacitance/voltage (*C/V*) curve of a capacitive field-effect sensor with Si_3N_4 as pH-sensitive material before (full line) and after enzyme immobilization (open squares) via glutaraldehyde.

To develop a capacitive EIS biosensor, first of all, the pH behaviour of the pH-transducing materials has to be checked. Therefore, in our experiments the pH sensitivity of EIS structures with Si_3N_4, Al_2O_3 and Ta_2O_5 were investigated between pH 5 and 10. For the PLD-prepared Al_2O_3 and Ta_2O_5 layers, the average pH sensitivities were close to the theoretical Nernst response, achieving sensitivities of about 52–54 mV/pH. These results also show a good agreement with measurements described in literature [7]. In contrast, for the LPCVD-deposited Si_3N_4 layers, only sub-Nernstian sensitivities of approximately 42–45 mV/pH could be achieved. It is possible to increase this sensitivity by activating the Si_3N_4 layer after preparation with hydrofluoric acid. Since the goal of this study was to primarily demonstrate the realisation of the new EIS-based biosensor with OPH, no attempt has been made to investigate the improvement of the Si_3N_4 layer's pH sensitivity.

The pH calibration plots, obtained for the different EIS biosensors after the enzyme immobilisation by glutaraldehyde cross-linking are presented in Fig. 4. In this experiment, the pH value was changed between pH 7 and 9 in intervals of less than 0.5 pH. As can be seen from the calibration graphs, the average pH sensitivities of the three transducer materials Al_2O_3, Ta_2O_5 and Si_3N_4 used are 48.3, 54.3 and 45.1 mV/pH, respectively. The curves are very linear, the regression coefficients (r^2) are varying from 0.991 to 0.998. With exception of the immobilised Al_2O_3 layer, all EIS biosensors possess pH sensitivities that are fully comparable to those achieved with sensors without OPH membrane.

Following the pH characterisation of the transducer material the performance of the biosensor for paraoxon was evaluated. Fig. 5 shows a typical ConCap measurement of

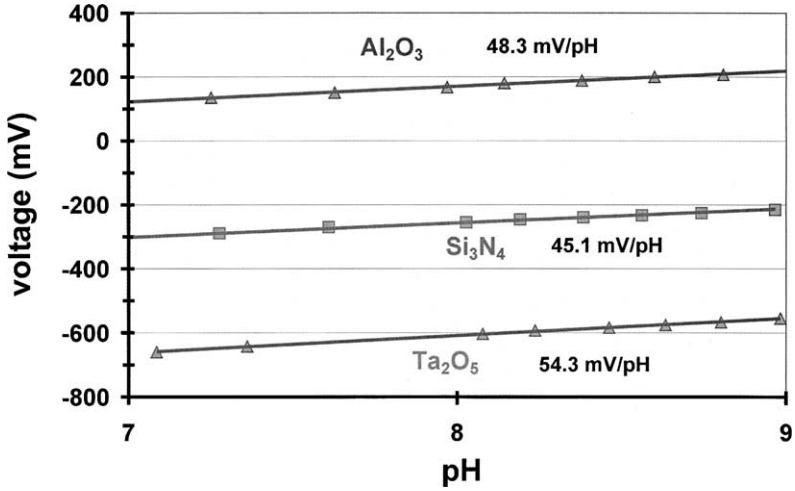

Fig. 4. The pH sensitivity of the EIS biosensor after enzyme immobilization. Al_2O_3, Ta_2O_5 and Si_3N_4 serve as transducer materials.

the EIS biosensor (with Si_3N_4 as pH-sensitive material) in the concentration range between 0.5 and 500 μM paraoxon. The sensor output signal was recorded for each concentration over a 3-min period and was changed at the times marked by arrows. Between each measurement, the biosensor was operated in HEPES buffer, pH 9, to firstly wash the sensor before detecting the next pesticide concentration, and to secondly validate the reproducibility of the biosensor signal. As evidenced by the signal recorded for the buffer in between measurements of the paraoxon, the baseline signal of the biosensor was fairly stable (represented by the horizontal dotted line) during the course of the experiment (Fig. 5). With paraoxon concentrations increasing from 3 to 500 μM, the concentration of the H^+ ions resulting from the catalytical reaction is also raised. As a consequence, the sensor output signal decreases in order to adjust the constant capacitance value, required in the ConCap mode.

Fig. 6 depicts the response curves for paraoxon of the capacitive EIS biosensor with Si_3N_4 as pH-sensitive transducer material. The three curves reflect the different methods of OPH immobilisation. The plots were prepared from the steady-state response of the sensor after 3 min. As can be seen from the graph, only the biosensors with the glutaraldehyde-immobilised OPH membrane (type I) and nafion-immobilised OPH membrane (type II) demonstrate a distinct dependence of the biosensor signal (delta voltage) on the paraoxon concentration. As expected the curve follows an exponential profile according to the Nernst equation. In contrast to the results of the first two types of biosensor, there is almost no dependence of the sensor signal on the paraoxon concentration for the adsorptively immobilised OPH biosensor (type III). A slight increase of the output voltage to a maximum of 30 mV was observed which is about three times smaller than for cross-linked enzyme membranes.

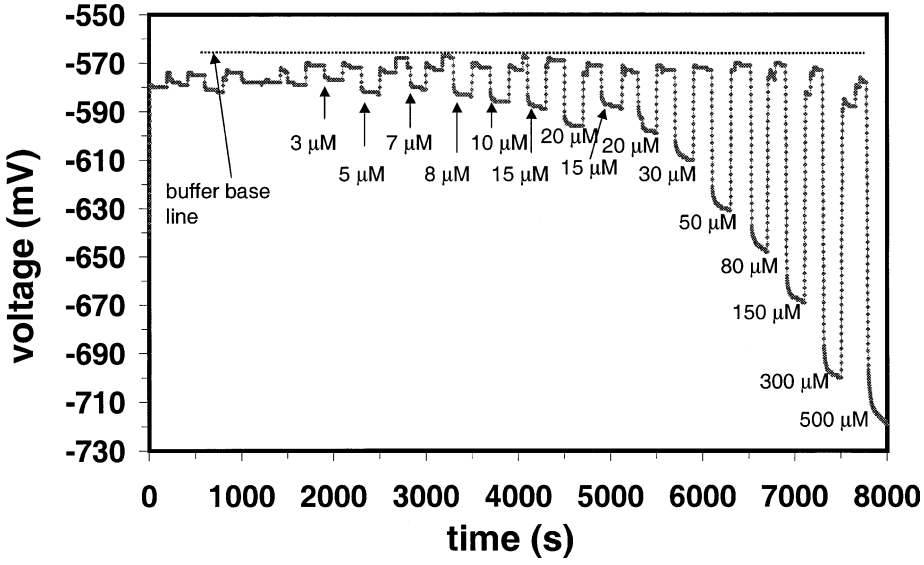

Fig. 5. Typical constant capacitance (ConCap) plot vs. time of the EIS biosensor. The dotted line corresponds to the buffer concentration that is alternatingly measured with the increasing paraoxon concentration.

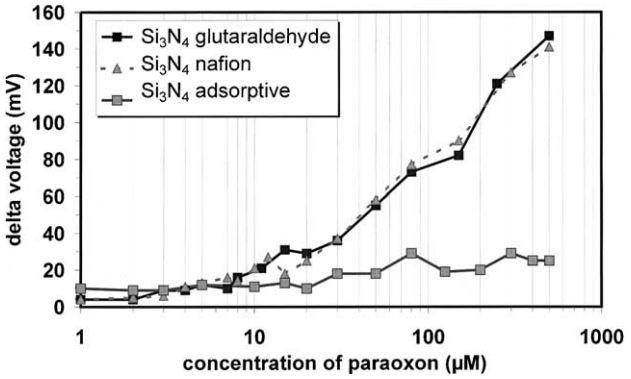

Fig. 6. Calibration curve of the enzyme biosensor for paraoxon with Si_3N_4 as pH-sensitive transducer. The enzyme OPH was immobilised by adsorptive-, nafion- and glutaraldehyde coupling.

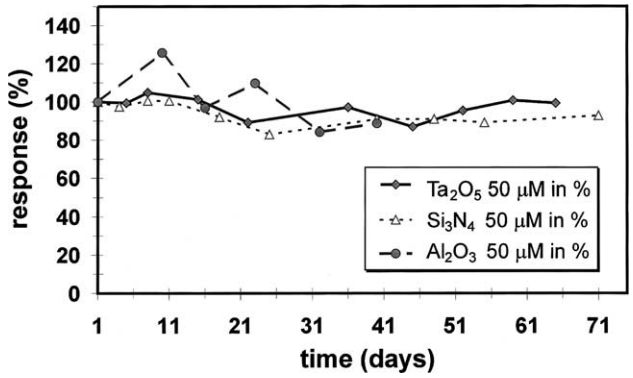

Fig. 8. Comparison of the long-term stability of the three different enzyme biosensors with the transducer materials Si_3N_4, Al_2O_3 and Ta_2O_5. For each sensor, the first measurement was defined as 100% response.

This behaviour can be attributed to the low enzyme loading when immobilised by adsorption and leaching from the pH-sensitive transducer surface. A possible explanation might be that in the case of the adsorptive immobilisation only a weak binding to the transducer surface results. In contact with solution, then the enzyme activity can be decreased due to this weak binding by simply washing it away from the transducer surface. Similar biosensor performance, i.e. the poor adhesion of the adsorptively immobilised enzyme layer on the pH transducer, was also observed with the other transducer materials, Al_2O_3 and Ta_2O_5. Therefore, for further investigations, EIS biosensors of types I and II immobilisation were selected.

Fig. 7 summarises the performances of biosensors prepared by immobilising OPH by cross-linking with glutaraldehyde on the three different pH-transducing materials—Al_2O_3, Ta_2O_5 and Si_3N_4. Similar curves are also obtained for type II biosensors. The calibration curves can be divided into two linear regions, one spanning the concentration range of 2–10 µM, and the other one, from 10 to 50 µM paraoxon.

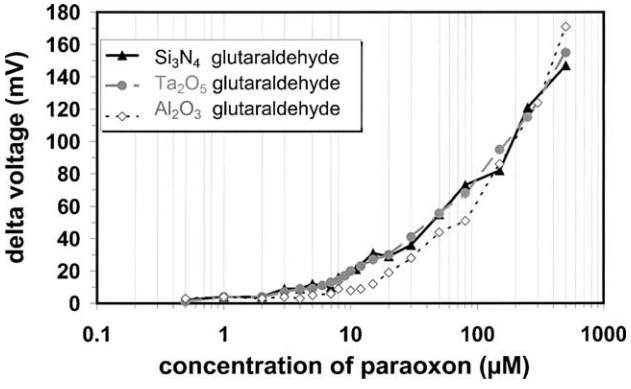

Fig. 7. Influence of the calibration plot towards paraoxon of the enzyme biosensor on the pH-transducing layer used (Si_3N_4, Al_2O_3 and Ta_2O_5). Each data point represents the mean value of three measuring points for paraoxon concentrations above 15 µM, and the mean value of five measuring points of concentrations below 15 µM. The error bars represent ±1 standard deviation.

From the practical view point, the lower concentration range is of more interest. Here (2–10 µM), the sensitivities of all three biosensors are quite similar, reaching slopes of about 1 mV/µM. The lower detection limit of the three biosensors for paraoxon, evaluated as three times the standard deviation of the signal for the HEPES buffer, was determined to be about 2 µM. However, further extensive analytical measurements are required to evaluate the exact quantities. These analytical characteristics are similar to the OPH-modified glass pH electrode [4]. Although these biosensors do not provide the nanomolar detection limits exhibited by acetylcholinesterase-based potentiometric biosensors, the OPH-modified sensors presented in this work have much faster response time, can be reused, can be employed for on-line monitoring, are selective for organophosphates and do not require consumable supply of enzyme substrate.

The time-dependent behaviour of the biosensor signal towards paraoxon over a measuring period of 70 days is presented in Fig. 8. The curves correspond to the EIS biosensors with the three different transducer materials Al_2O_3, Ta_2O_5 and Si_3N_4. The respective sensor signal was evaluated at a concentration of 50 µM paraoxon and normalised to 100% response for the first day of measurement. Within the investigation period, all biosensors show a reproducible output signal. Even after more than 2 months in operation, the biosensor response still has at least 90% of the original sensor signal. This long-term stability was achieved for both types I and II biosensors. In comparison to recently published results of a disposable potentiometric enzyme sensor for the direct determination of organophosphorus pesticides [14], the presented silicon-based biosensor has a high long-term stability and also a somewhat better detection limit.

Like paraoxon, many other organophosphates such as parathion, methyl parathion, diazinon, etc. can also be hydrolysed by OPH to yield H^+ ions. Therefore, it is expected that the developed biosensor should also be able to detect and determine other organophosphates. In contrast, only a negligible sensitivity towards non-organophosphorus compounds, like atrazine, simazine, sarin and sutan might be

expected because these compounds are not substrates for OPH. First investigations could qualitatively demonstrate these presumptions, however, further detailed studies are necessary to also quantitatively validate these results.

4. Conclusions

For future research studies, the developed biosensor should be integrated in a flow-injection analysis system to demonstrate its availability for real sample monitoring. The combination of a potentiometric with an amperometric sensor, for example, will improve the sensitivity and selectivity characteristics. Both sensors can be integrated as hybrid on a single chip. In such a dual biosensor chip, the amperometric detection principle is relied on the amperometric determination of *para*-nitrophenol, whereas the potentiometric detection principle takes benefit on the catalytic hydrolysis of H^+ ions. Due to the catalytic reaction, only some of the pesticides generate *para*-nitrophenol in the case of the amperometric transducer. In contrast, all pesticides lead to a pH change (but with different intensities) for the potentiometric transducer. As a result, when simultaneously measuring several different pesticides by both electrochemical methods, one can achieve a signal pattern with different signal changes of the respective sensor signal. In this way, the increased quantity of information should offer the possibility to distinguish between different organophosphorus compounds. Moreover, even the combination of two different enzymes, the first one allowing the direct substrate determination (e.g. OPH) and the second one detecting the inhibition reaction (e.g. AChE) can further enhance the information content of this type of biosensor.

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