# Screen-printed bienzymatic sensor based on sol-gel immobilized *Nippostrongylus brasiliensis* acetylcholinesterase and a cytochrome P450 BM-3 (CYP102-A1) mutant

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## Abstract

Here we describe the development of a bienzymatic biosensor that simplifies the sample pretreatment steps for insecticide detection, and enables the highly sensitive detection of phosphorothionates in food. These compounds evolve their inhibitory activity towards acetylcholinesterases (AChEs) only after oxidation which is performed in vivo by P450 monooxygenases. Consequently, phosphorothionates require a suitable sample pretreatment by selective oxidation to be detectable in AChE based systems. In this study, enzymatic phosphorothionate activation and AChE inhibition were integrated in a single biosensor unit. A triple mutant of cytochrome P450 BM-3 (CYP 102-A1) and *Nippostrongylus brasiliensis* AChE (NbAChE) were immobilized using a fluoride catalyzed sol-gel process. Different sol-gel types were fabricated and characterized regarding enzyme loading capacity and enzyme activity containment. The enzyme sol-gel itself already proved to be suitable for the highly sensitive detection of paraoxon and parathion in a spectrometric assay. A method for screen-printing of this enzyme sol-gel on thick film electrodes was developed. Finally, amperometric biosensors containing coimmobilized NbAChE and the cytochrome P450 BM-3 mutant were produced and characterized with respect to signal stability, organophosphate detection, and storage stability. The detection limits achieved were 1 µg/L for paraoxon and 10 µg/L for parathion in its oxidized form, which is according to EC regulations the highest tolerable pesticide concentration in infant food.

# 1 Introduction

Organophosphates are the most common insecticides that are used in agriculture with the purpose to increase crop yields. The majority of the world-wide consumed organophosphates are phosphorothionates (appr. 80 % in Germany (CVUA Stuttgart, 2000). The disadvantages of their usage are drinking water and food contamination (Karalliedde and Senanayake, 1999). Their toxicity is based on their inhibitory effect on acetylcholinesterase (AChE), an important enzyme of the nervous system in higher organisms (Fukuto, 1990). It was demonstrated that organophosphates show chronic and acute toxicity also towards humans (Koletzko et al., 1999; Schilter and Huggett, 1998). Especially infants are supposed to have an increased susceptibility (Larsen and Pascal, 1998). In 1999, the EC has therefore lowered the threshold for pesticide concentration in infant food to 10 µg/kg (EC, 1999). Conventionally, insecticide detection is performed by gas chromatography or high-performance liquid chromatography, coupled with mass selective detectors (Anastassiades and Scherbaum, 1997; Martinez et al., 1992; Pylypiw, 1993). Drawbacks thereby are the long measurement times, the need of well-trained personal, and high apparatus expenses. No in field measuring is thus possible. As a result, a great number and variety of AChE sensors for organophosphate detection have been developed during the last decades, using mostly amperometric (Bachmann and Schmid, 1999; Kulys and D'Costa, 1990; Schulze et al., 2002; Villatte et al., 2002), potentiometric (Evtugyn et al., 1996; Ghindilis et al., 1996; Lee et al., 2001), optical (Choi et al., 2001; Danet et al., 2000) and piezoelectronic (Abad et al., 1998; Makower et al., 2003) devices. However, AChE inhibition based systems lack a sufficient sensitivity towards phosphorothionates (Jeanty et al., 2001; Navaz Diaz and Ramos Peinado, 1997; Schulze et al., 2004). These substances are weak AChE inhibitors, due to the low

reactivity of the P=S group caused by minor electronegativity of sulfur compared to oxygen (Fukuto, 1990). The toxicity of phosphorothionates is based on their *in vivo* biotransformation by microsomal P450 monooxygenases into the corresponding, strongly AChE inhibiting, oxones (Chambers and Levi, 1992; Fukuto, 1990). These processes are illustrated in figure 1. In drinking water monitoring, oxidation is commonly performed by bromine or N-bromosuccinimide (DIN38415-1, 1995, Kumaran and Tran-Minh, 1992). However, these procedures work bad in complex matrixes such as food samples. Following the example of *in vivo* biotransformation of phosphorothionates, we previously described an enzymatic activation method for these insecticides (Schulze et al., 2004). Cytochrome P450 BM-3 from *Bacillus megaterium* is a water soluble, 119 kDa natural fusion protein (Narhi and Fulco, 1986; Narhi and Fulco, 1987). It contains both monooxygenase and reductase domain on one single polypeptide chain and is therefore catalytically self-sufficient. The present study describes the combination of phosphorothionate activation and AChE inhibition by coimmobilization of the P450 BM-3 triple mutant and *Nippostrongylus brasiliensis* acetylcholinesterase (NbAChE) (Hussein et al., 2002; Hussein et al., 1999). Various AChE immobilization protocols for biosensor fabrication have been described. In a previous study we fabricated an amperometric sensor by screen-printing an AChE containing hydroxyethyl cellulose (HEC) solution on a thick film electrode, followed by crosslinking with glutaraldehyde (Bachmann and Schmid, 1999). Similar protocols use poly(vinyl alcohol) as encapsulation matrix for the enzyme or take advantage of the affinity interaction between histidine ($His_6$)-tagged AChE and nickel nitrilotriacetic acid (Andreescu et al., 2002). The sol-gel technique is another immobilization method that has become famous during the last years, and it has been successfully applied in case of AChE (Altstein et al., 1998; Andreescu et al., 2002; Anitha et al., 2004; Gill and Ballesteros, 2000; Singh et al.,

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1999). Biosensor fabrication methods that are based on sol-gel screen-printing have been published for several enzymes (Albareda-Sirvent and Hart, 2002; Wang et al., 1996). Apparently, all of these protocols use hydrochloric acid (HCl) as catalyst in order to induce the sol-gel polymerization. An alternative screen-printing protocol where the organic compound bis(2-ethylhexyl) sulfosuccinate (AOT) has the function as catalyst and also as binder in the printing process, was described by Guo and Guadeloupe (Guo and Guadalupe, 1998). Few methods for the immobilization of cytochrome P450s have been described so far (Iwuoha et al., 2000; Taylor et al., 2000). Recently, a sol-gel method using a fluoride catalyst described by Shtelzer (Shtelzer et al., 1992) was successfully applied for P450 BM-3 encapsulation (Maurer et al., 2003).

This study describes the development of a monoenzymatic and a bienzymatic biosensor for the highly sensitive detection of organophosphates, and especially for their non activated phosphorothionate forms. NbAChE and a triple mutant of P450 BM-3 were encapsulated by a sol-gel process, and a method for screen-printing the sol-gel on thick film electrodes was established. The sensitivity of the sol-gel immobilized NbAChE was demonstrated using a single enzyme set up. In a further step, enzymatic phosphorothionate activation and NbAChE inhibition were integrated by coimmobilizing both enzymes. The resulting bienzymatic sensor was evaluated by the detection of parathion as a representative of the group of phosphorothionate insecticides.

# 2 Experimental

## 2.1 Materials and reagents

NbAChE was expressed as previously described in a *Pichia pastoris* X33 strain (Invitrogen, Karlsruhe, Germany), transformed with a pPICZ$\alpha$B vector (Invitrogen, Karlsruhe, Germany), bearing the NbAChE B gene (Hussein et al., 1999). The His$_6$-tagged triple mutant (Phe87Val, Leu188Gln, Ala74Gly) of P450 BM-3 was produced under control of the temperature-inducible P$_R$P$_L$-promotor of pCYTEXP1, using the *Escherichia coli* (*E. coli*) strain DH$\alpha$ (supE44, lacU169 [80lacZ M15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1) from Clontech (Heidelberg, Germany) (Li et al., 2000; Schwaneberg et al., 1999). The enzyme was purified by affinity chromatography on nickel sepharose, performing one washing step with PBS (50 mM, pH 7.5, 500 mM NaCl, 30 mM imidazol), and using 200 mM imidazol for elution. Paraoxon and parathion were purchased from Riedel de Haën (Seelze, Germany). Insecticide stock solutions were prepared in ethanol. NADPH tetrasodium salt was procured from Jülich Fine Chemicals (Jülich, Germany). The substrate 10-para-nitrophenoxy carbonacid (10-pNCA) was synthesized as described elsewhere (Schwaneberg et al., 1999). Nickel sepharose high performance was obtained from Amersham Biosciences (Freiburg, Germany). All other reagents were of analytical grade as supplied by Fluka (Neu-Ulm, Germany) or Sigma-Aldrich (Deisenhofen, Germany).

## 2.2 Sol-gel immobilization

In case of P450 BM-3, the solution obtained from the purification by metal affinity chromatography was used for immobilization. The molar activity of purified P450 BM-3 was measured by the pNCA-assay (Schwaneberg et al., 1999). The P450 BM-3

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concentration was determined by CO-difference spectroscopy (absorption at 450 nm minus absorption at 490nm) using an extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$ (Omura and Sato, 1964). The specific activity of the purified enzyme was calculated from the molar activity and the concentration and ranged usually around 2.0 U/mg. For NbAChE encapsulation, the supernatant resulting from the *Pichia pastoris* expression was directly used without further purification steps. Molar NbAChE activities were determined by the Ellman test (Ellman et al., 1961), and NbAChE concentrations were calculated from the molar activities and the specific activity of the free enzyme (2080 U/mg) (Hussein et al., 1999). When both enzymes were immobilized in the same sol-gel, the enzyme solutions were mixed prior to their addition to the sol suspension. All working steps with P450 BM-3 were performed at 4°C, in case of NbAChE at room temperature. Enzyme immobilization was carried out on the basis of a previously described encapsulation technique for P450 BM-3 with slight modifications (Maurer et al., 2003). Briefly, 1 mL PBS (50 mM, pH 7.5) containing 20 % (w/w) polyethylene glycol 6000 and 2.5 mL enzyme solution were mixed and then added to 5 mL of stirred tetraethoxy silane (TEOS). After 3 min, 250 μL of NaF (0.2 M aqueous solution) were added and stirring was continued for 15 min before pouring the suspension on a petri dish. After complete polymerisation, the resulting white powder was washed several times with a PBS (50 mM, pH 7.5) to remove non-immobilized enzyme. After each washing step, the enzyme activity in the obtained wash water fraction was determined and washing was continued until no activity was detectable. In case of P450 BM-3 also the concentration of the wash fractions was measured by CO difference spectroscopy. Drying of the sol-gel was performed by lyophilization over night. The enzyme loading of the sol-gel was determined by subtracting the amount of out-washed enzyme from the initial enzyme quantity that was used for immobilization.

## 2.3   Inhibition of free and sol-gel immobilized NbAChE

Free as well as sol-gel entrapped NbAChE (2.5 mg and 5 mg sol-gel) were incubated for 30 min at room temperature with different paraoxon concentrations in a cuvette as reported previously, and subsequently the remaining activity was measured by the Ellman test (Schulze et al., 2004). The inhibition was calculated as AChE inhibition [%] = $((a_0-a_i)/a_0)$ x 100. ($a_i$ = activity after incubation with insecticide, $a_0$ = activity after incubation without insecticide). In case of immobilized NbAChE, the Ellman reaction mixture was pumped in a closed loop in a flow through set-up (total volume 4 mL), consisting of a reaction tube, a syringe filter (0,4 µm diameter), a flow through cuvette (volume 100 µL) and a pump, connected to each other by tubes. The upstream filter prevented the sol-gel particles from being washed into the cuvette where the absorption change of the solution was monitored.

## 2.4   Activation of parathion by sol-gel immobilized P450 BM-3

Different amounts (5 - 50 mg) of P450 BM-3 sol-gel were added to 900 µL of a PBS (50 mM, pH 8.15) containing different parathion concentrations, and the suspension was mixed for 5 min at room temperature. Subsequently, 100 µL of an aqueous NADPH solution (5mg/mL) were added and mixing was continued for either 40 min or 80 min. The suspension was then centrifuged and the supernatant was used for NbAChE inhibition tests as described above.

## 2.5 Production of biosensors by screen-printing technique

As reported previously, glutaraldehyde AChE sensors can be made by screen-printing (DEK 249; DEK, Weymouth, UK) an AChE containing HEC solution on thick film electrodes, followed by exposure to glutaraldehyde vapor (Bachmann and Schmid, 1999). According to this procedure, the basic transducer was fabricated by consecutively screen-printing silver conducting lanes, carbon basal pads, an isolation layer, a Ag/AgCl layer, and a 7,7,8,8-tetracyanoquinodimethane (TCNQ) graphite layer on a PVC sheet. TCNQ graphite was made by evaporating a suspension of TCNQ and T-15 graphite in acetone. In the present study, different methods employing the sol-gel technique were used for the fabrication of the enzyme layer. Method 1: Based on a protocol from Guo and Guadeloupe (Guo and Guadalupe, 1998), a sol-gel paste was made by stirring thoroughly AOT : PBS (50 mM, pH 7.5) : TEOS (1:50:200, molar ratio) for 5 min. To 1 mL of the resulting sol suspension, 14 µL (2 U) of NbAChE supernatant (144 U/mL) and 600 mg TCNQ graphite were added and the suspension was homogenized. The obtained paste was immediately screen-printed on the carbon basal pads of the basic transducer. Method 2: In this case, the completely polymerized, washed and dried sol-gel was employed. The paste consisted of 26 % (w/w) sol-gel (NbAChE activity: 1.06 U/g$_{sol-gel}$), 15 % (w/w) TCNQ graphite and 59 % HEC solution (3 % (w/w) aqueous solution). These components were mixed, stirred for 1 h and the resulting paste was screen-printed. Method 3: A paste consisting of 15 % (w/w) TCNQ graphite and 85 % HEC solution (3 % (w/w) aqueous solution) was made by stirring the components for 1 h. The paste was printed on the carbon basal pads of the transducer and the obtained electrode was dried for 1 h at 90 °C. Different sol-gel pastes were made by stirring different ratios of polymerized, washed and dried sol-gel (NbAChE activity: 1.06

U/$g_{sol-gel}$), (13 - 26 % (w/w)) in HEC solution (3 %, (w/w) aqueous solution ) for 45 min, and the pastes were screen-printed on the TCNQ graphite layer. The optimal sol-gel ratio in the HEC solution was found to be 26 % (w/w). Exceeding this value made the mixture too viscous to be printed. Method 4: The sensor was constructed corresponding to method 3 with the following modifications: The mesh aperture of the screen that was used for sol-gel printing was 249 μm (45 μm in case of methods 1-3). The sol-gel ratio suspended in the HEC solution was varied from 13 - 36 % (w/w) and the HEC ratio of the HEC solution itself was varied between 1 - 3 % (w/w). A paste consisting of 20 % sol-gel in 1 % HEC was found to be the optimum, giving a homogenous sol-gel layer.

## 2.6  Biosensor measurement

All sensor experiments to determine the NbAChE activity were carried out in 5 mL of a stirred PBS (10 mM potassium phosphate, 50 mM NaCl, pH 7.5) at room temperature. The NbAChE activity was represented by the current output caused by monitoring thiocholine formed by enzymatic hydrolysis of acetylthiocholine chloride (final concentration: 1 mM). Thiocholine was detected by oxidation at +100 mV versus Ag/AgCl. In order to test the operational stability of the sensors, the NbAChE activity was measured in five consecutive measurements. For inhibition experiments with paraoxon, biosensors were incubated 30 min without stirring at room temperature in 5 mL of a PBS (50 mM, pH 7.5), containing different insecticide concentrations. In case of parathion, the PBS contained NADPH (1mg/mL) besides the insecticide and the incubation time was varied from 30 min to 90 min. The percentage of inhibition was calculated as AChE inhibition [%] = (($a_0$-$a_i$)/$a_0$) x 100. ($a_0$ = initial AChE activity, $a_i$ = AChE activity after incubation with sample).

The fabricated sensors were stored at 4°C in petri dishes without the addition of drying agents. In order to test the storage stability, inhibition experiments were performed once a week after the fabrication, each time with another sensor. The used parathion concentration was 20 µg/L and the incubation time was 1 h at room temperature.

# 3   Results and discussion

## 3.1   *Sol-gel immobilization*

Different sol-gels containing just one enzyme as well as coimmobilisates, entrapping both enzymes in the same matrix, were prepared. Since the sol-gel particles disturbed the absorption measurement in the spectrometric activity tests, the activity of the sol-gel encapsulated enzymes was measured using a flow through system. The total activity in the enzyme solution that was used for the encapsulation experiment will be called initial activity in the following. Three different NbAChE sol-gels were prepared using an initial activity of 5 U, 50 U and 250 U. Best results were obtained with 50 U, yielding an activity of 1.06 ± 0.03 $U/g_{sol-gel}$. A 10-fold decrease of the initial activity to 5 U resulted in an approximately proportionally lowered sol-gel activity of 0.16 ± 0.01 $U/g_{sol-gel}$. In contrast, starting with 250 U did not lead to a significant improvement. The examination of the wash water in this case showed that nearly all enzyme was washed out. Two different enzyme concentrations were used in the production of P450 BM-3 sol-gels. An increase of the initial activity from 6 U to

9 U resulted in a proportional rise of the sol-gel activity from 0.24 ± 0.01 U/g$_{sol\text{-}gel}$ to 0.39 ± 0.02 U/g$_{sol\text{-}gel}$.

Two types of coimmobilisates were fabricated using the same initial NbAChE activity of 50 U for both, but different P450 BM-3 amounts. Coimmobilisate type 1 was prepared with an initial P450 BM-3 activity of 9 U. The NbAChE activity in the resulting gel was found to be 0.82 ± 0.06 U/g$_{sol\text{-}gel}$ and the P450 BM-3 activity was 0.16 ± 0.02 U/g$_{sol\text{-}gel}$ (ratio 5 :1). The activity of both enzymes was thus slightly lower than in the single enzyme sol-gels. This may be due to saturation effects, since the wash water examination showed increased enzyme loss. The encapsulated enzyme amounts were determined to be 5.6 pmol/g$_{sol\text{-}gel}$ NbAChE and 667 pmol/g$_{sol\text{-}gel}$ P450 BM-3 (ratio 1 : 119). In order to increase the P450 BM-3 to NbAChE activity ratio in the sol-gel, an initial P450 BM-3 activity of 90 U was used for the fabrication of coimmobilisate type 2. The resulting NbAChE activity in the sol-gel was determined to 0.32 ± 0.04 U/g$_{sol\text{-}gel}$ and the P450 BM-3 activity to 0.70 ± 0.05 U/g$_{sol\text{-}gel}$ (ratio 1:2.2). The gel contained 2.2 pmol/g$_{sol\text{-}gel}$ NbAChE and 2920 pmol/g$_{sol\text{-}gel}$ P450 BM-3 (ratio 1 : 1327). Corresponding to the large P450 BM-3 amount that was used for the production of coimmobilisate type 2, a high enzyme ratio was removed in the washing steps. Since no significant decrease of the specific activity was observed, out-washed P450 BM-3 was recycled by filtration and purification. The enzyme solution was then concentrated to the desired value and again used for immobilization. In this manner, three cycles could be performed before a loss of the specific P450 BM-3 activity was observed.

## 3.2 Paraoxon detection by sol-gel immobilized NbAChE in a spectroscopic assay

Inhibition tests were performed with suspensions of two different NbAChE sol-gels (NbAChE activity 1,06 $U/g_{sol-gel}$ and 0,16 $U/g_{sol-gel}$) and compared to the inhibition of free NbAChE. As can be seen in figure 2, entrapped NbAChE showed a similar sensitivity towards paraoxon as the free enzyme. The mean inhibition ratio (inhibition of immobilized / free enzyme) using the sol-gel of higher activity was 66 %. In case of the other sol-gel, 90 % average inhibition was achieved. This gel showed also the higher sensitivity towards paraoxon at lower concentrations (between 0 µg/L and 10 µg/L). The detection limit, defined as signal to noise ratio greater 3, was 1 µg/L in this case, thus the same as obtained with the free enzyme. Using the sol-gel with the higher NbAChE content, the detection limit was determined to be 5 µg/L. Altstein et al. described an insecticide detection system based on an AChE sol-gel located in a microtiter plate (Altstein et al., 1998). Inhibition tests revealed detection limits (defined as 20 % inhibition) laid between 8 µg/L (methidathion, 2h incubation) and 440 µg/L (omethoate, 20h incubation). These detection limits, achieved after longer incubation times, were higher by a factor of 100 to 4000 than in our study. Altstein reported a 3 to 5 times lower sensitivity of the encapsulated enzyme towards organophosphates when compared to the free enzyme (comparison of the 50 % inhibition values ($I_{50}$)). As can be estimated in the study presented here from figure 2, the $I_{50}$ values of the entrapped enzymes are close to the free NbAChE in the range of 8-10 µg/L. Navaz Diaz and Peinado reached a detection limit of 360 µg/L for the organophosphate naled, thus ranging on the same level as Altstein (Navaz Diaz and Ramos Peinado, 1997).

## 3.3 Parathion activation by sol-gel immobilized P450 BM-3 in suspension and subsequent insecticide detection in a spectroscopic assay

The ability of encapsulated P450 BM-3 to convert parathion to paraoxon was tested by subsequent AChE inhibition experiments. The P450 BM-3 sol-gel, prepared with 9 U initial P450 BM-3 activity, was therefore incubated with parathion and NADPH. After incubation, a defined amount of the supernatant was used for NbAChE inhibition experiments as described for paraoxon. An incubation time of 40 min and a sol-gel amount of 50 mg/mL reaction volume were found to be the optimum for the conversion. Figure 3 shows the resulting calibration curve compared to the curve that was obtained when paraoxon was directly used for NbAChE inhibition. The transformation efficiency for the parathion conversion (average ratio of inhibition caused by paraoxon and pretreated parathion) was calculated to be 77 %. The detection limit was in both cases 1 µg/L of insecticide, indicating a quasi quantitative oxidation of parathion at low concentrations. To our knowledge, this is the first protocol demonstrating parathion activation by sol-gel immobilized cytochrome P450. Navaz Diaz and Peinado achieved detection limits ranging from 13.9 to 57.6 mg/L for several non-activated phosphorothionates using an AChE sol-gel (Navaz Diaz and Ramos Peinado, 1997). This means the sensitivity is 3 to 4 orders of magnitude lower than in the work presented here and can be explained by the lower reactivity of the P=S group of phosphorothionates (Fukuto, 1990). The results thus demonstrate the requirement of phosphorothionate activation for a highly sensitive detection as it was achieved in this study by using P450 BM-3.

*3.4   Biosensor fabrication*

To achieve mild conditions during P450 BM-3 immobilization we investigated the usage of sol-gel procedures that employ non-acidic catalysts. Enzyme containing sol-gel pastes were screen-printed on thick film electrodes (Bachmann and Schmid, 1999), following two different strategies. On the one hand, a paste containing a freshly prepared sol solution was printed, according to a procedure from Guo and Guadaloupe (Guo and Guadalupe, 1998) (method 1). In order to test the operational stability of the produced electrodes, 5 consecutive measurements were performed. The decrease of the current signal (figure 4) was in accordance with the observation that the sol-gel layer was washed away from the electrode. The second strategy was to screen-print a paste containing completely polymerized sol-gel powder. In a one step procedure, a mixture of sol-gel, TCNQ graphite and HEC solution was printed (method 2). However, the electrodes showed the same operational instability as the sensors fabricated according to method 1. In a two step protocol (method 3), the TCNQ graphite layer was made first and a suspension of the sol-gel in HEC solution was printed in a second step. The sensor showed a stable current signal ranging at about 50 nA in case of full AChE activity (U) and 1mM acetylthiocholine. This is in the range of biosensors with glutaraldehyde crosslinked AChE, which reached current outputs of about 100 nA (Bachmann and Schmid, 1999). In an alternative protocol (method 4), the amount of sol-gel that was deposited on the electrode was increased by employing a screen with a larger mesh aperture, yielding a stable current signal ranging at about 160 nA (sol-gel used: coimmobilisate 2).

P450 BM-3 sol-gel screen-printing protocols have not been reported so far. Usually, acidic conditions are used for the immobilization of biomaterials that are known for their robustness (Albareda-Sirvent and Hart, 2002; Wang et al., 1996). In our study,

encapsulation of the enzymes was performed under mild pH conditions. Another difference to reported methods is that they are usually one step procedures. According to these, the biocomponent is mixed into a sol and the viscous suspension is printed, formation of the polymer matrix then mainly occurs on the electrode surface. The herein described technique is a two step procedure where encapsulation and printing are separated. The major disadvantage of this method is the time and work intensity. On the other hand, the presented mode offers two big advantages compared to one step protocols and it is, to our knowledge, unique in literature. First, the properties of the entrapped biocomponent can be completely characterized prior to printing, as this study exemplifies. The technique can therefore easily be extended to other biomaterials. Second, screen-printing P450 BM-3 in a HEC solution led to deactivation of the enzyme, due to the shearing in the process. Employing method 4 gave electrodes that were useful for parathion detection (see below). This suggests that the completely polymerized sol-gel matrix has an strongly protecting effect.

## 3.5  Paraoxon detection by the NbAChE biosensor

Inhibition tests with paraoxon were performed using electrodes fabricated by method 3 and the results were compared to those obtained with the sensor that was produced by crosslinking AChE with glutaraldehyde (Bachmann and Schmid, 1999) (see figure 5). In both cases the detection limit, defined as signal to noise ratio greater than 3, was 1 µg/L paraoxon (3,6 nmol/L). Various sol-gel AChE sensors have been reported using different sensor systems and experimental conditions. Andreescu et al. produced an amperometric sensor by dropping an AChE containing sol suspension on a screen-printed thick film electrode (Andreescu et al., 2002).

They described a detection limit, defined as 20 % AChE inhibition, of 7 µg/L (24 nmol/L) for paraoxon. Doong observed 30 % inhibition at a paraoxon concentration of 149 µg/L (0,54 µmol/L) using a fiber optic AChE sol-gel sensor (Doong and Tsai, 2001).

### 3.6 Parathion detection by the bienzyme biosensor

When a mixture of NbAChE sol-gel (1.06 U/$g_{sol-gel}$) and P450 BM-3 sol-gel (0.39 U/$_{sol-gel}$) was used to produce sensors following method 3, no inhibition was observed with a parathion concentration of 1000 µg/L. By contrast, a sensor that was made with coimmobilisate type 1 yielded an 20 % inhibition at a concentration of 1000 µg/L and an incubation time of 60 min. Using the same parameters for coimmobilisate type 2 resulted in 60 % inhibition with 1000 µg/L. Biosensors that were constructed with coimmobilisate type 2 according to method 4 yielded an inhibition of 20 ± 2 % after 30 min incubating with 20 µg/L parathion. When incubated for 1 h, the inhibition value increased to 37 ± 3 %. Contrary to these findings, incubating 90 min did not lead to further improvement, probably due to inactivation of P450 BM-3. Adjusting an incubation time of 1 h, the detection limit was determined to be 10 µg/L (figure 6). In theory, the less NbAChE is entrapped, the less paraoxon has to be converted by P450 BM-3 to cause significant inhibition. It was therefore expected that the ratio of the two encapsulated enzymes would have the biggest influence on the inhibition. However, an increase of the ratio of the P450 BM-3 to NbAChE activity by a factor 10 just yielded a three times higher inhibition value. On the other hand, the most important factor turned out to be the total sol-gel amount, meaning the total enzyme quantity on the electrodes. Yet, we assume that only a locally enhanced paraoxon concentration near the electrode surface is produced. Consequently, the less

diffusion barriers between the two enzymes exist, the higher is the possibility that a generated paraoxon molecule can hit the NbAChE before diffusing away from the electrode surface. This assumption can explain the better results obtained with the coimmobilisate, since the enzymes are located in the same matrix and so less diffusion barriers are between them.

To our knowledge this paper describes the first AChE inhibition based biosensor that integrates phosphorothionate activation on the biosensor. It has been demonstrated that these insecticides have a low inhibitory effect on AChE and are thus difficult to detect by AChE based biosensors (Barber et al., 1999; Jeanty et al., 2001; Jokanovic, 2001; Navaz Diaz and Ramos Peinado, 1997; Schulze et al., 2004). Jeanty et al. observed a 1000-fold lower toxicity of phosphorohionates towards AChE compared to their oxidized homologues (Jeanty et al., 2001). No detection was thus possible below concentrations of 10 µmol/L (2910 µg/L). Schulze et al. reported an AChE inhibition of 19 % with 2000 µg/L parathion (Schulze et al., 2004). Barber et al. used 2910 µg/L at 37 °C and an incubation time of 60 min, resulting in an inhibition of 10 % (Barber et al., 1999). The sensitivity that was obtained in the present work thus means a 100-fold improvement. The detection limit achieved for parathion lies in the range of thresholds obtained for paraoxon by AChE systems reported elsewhere (Andreescu et al., 2002; Doong and Tsai, 2001).

## 3.7 Storage stability

The robustness of NbAChE was previously observed and the storage stabilities of biosensors range from days up to more than a year depending on the origin of the AChE [data not shown]. Regarding AChE sol-gel sensors, a lifetime of more than 6 months at -20°C under vacuum is reported (Andreescu et al., 2002). However, the

more fragile P450 BM-3 is the limiting factor in case of the bienzymatic sensor described here. The half life of P450 BM-3 in solution was described to be 26 days at 4 °C and just 2 days at room temperature (Maurer et al., 2003). The storage stability of the presented sensor at 4 °C was examined by determination of the AChE inhibition value caused by 20 µg/L parathion, in order to monitor both enzymes. The results show only a slight decrease of the inhibition during 4 weeks (figure 7). The starting value was an AChE inhibition of 37 %, while at the end still 31 % (84 % of the starting value) were achieved. These results are comparable to the storage stability determined by the pNCA assay that is reported for sol-gel entrapped P450 BM-3 (Maurer et al., 2003).

## 4  Conclusion

This publication describes an AChE inhibition based biosensor that integrates enzymatic phosphorothionate activation, enabling the direct and sensitive detection of this type of insecticides. Parathion, a prevalent food contaminating phosphorothionate, could be detected at a concentration of 10 µg/L, which is the pesticide threshold in infant food set up by EC regulations and about 100 times more sensitive than commonly described for AChE sensors. In addition, the developed test with an incubation time of 1 h and an overall test time of about 2 h is faster than conventional phosphorothionate detection using chromatographic methods that require about one day. The presented sensor can be produced in a high throughput mode, since all steps of the fabrication including enzyme deposition are performed by the semi-automated screen-printing technique. The mild encapsulation conditions and the protecting role of the polymer matrix in the printing process make this fabrication procedure particularly attractive for fragile biomaterials. Finally, the

elimination of sample pretreatment, combined with the good storage stability seem to us as major properties towards biosensor commercialization and application for field measurements of hazardous food contaminants.

## Acknowledgements

## Legends to Figures

Figure 1:

Illustration of the reaction sequence, exploited in this study, used for the detection phosphorothionate type insecticides by AChE inhibition. The toxicity of these compounds *in vivo* is based on P450 monooxygenase action following analogous mechanisms (Chambers and Levi, 1992; Fukuto, 1990).

Figure 2:

Calibration curve for the inhibition of free AChE and immobilized AChE in sol-gels with different enzyme activity. The inhibition was caused by incubating the enzyme for 30 min with different paraoxon concentrations (n=3). The volume activity of free enzyme was $7.20*10^{-2}$ U/mL. In case of the sol-gel with the higher enzyme activity (initial activity of 50 U applied for immobilization), 2.5 mg ($2.65*10^{-3}$ U) per mL test volume were used. The amount of the other sol-gel (initial activity of 5 U applied for immobilization) was 5 mg ($8*10^{-4}$ U).

Figure 3:

Calibration curve for the inhibition of free AChE ($7.20*10^{-2}$ U/mL), caused by incubation for 30 min with paraoxon and pretreated parathion (n=3). The parathion pretreatment comprised of incubating the insecticide for 40 min with 50 mg of P450 BM-3 sol-gel (initial activity of 9 U applied for immobilization) and NADPH (0.5 mg) in 1 mL test volume.

Figure 4:

Operational stability of AChE sol-gel biosensors fabricated by three different methods (initial activity of 50 U applied for sol-gel immobilization in case of methods 2 and 3). Five consecutive measurements were performed with each electrode (n=2).


Figure 5:

Calibration curve for paraoxon using the AChE sol-gel biosensor (initial activity of 50 U applied for immobilization, incubation time 30 min, n=3), compared to an AChE biosensor with AChE immobilized by crosslinking with glutaraldehyde.


Figure 6:

Calibration curve for parathion using the sol-gel bienzyme biosensor (incubation time 1 h, n=3). The sensor was produced by method 4 using coimmobilisate type 2.


Figure 7:

Storage stability of the bienzyme biosensor. AChE inhibition caused by incubation with 20 µg/L parathion for 1 h at different times after sensor fabrication (n=3). The sensor was produced according to method 4 using coimmobilisate type 2.
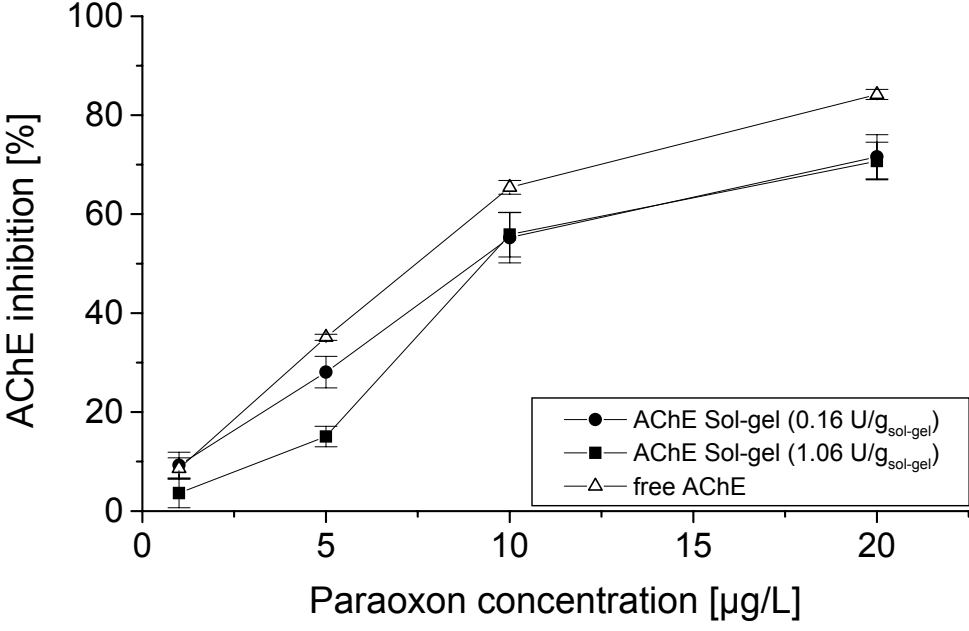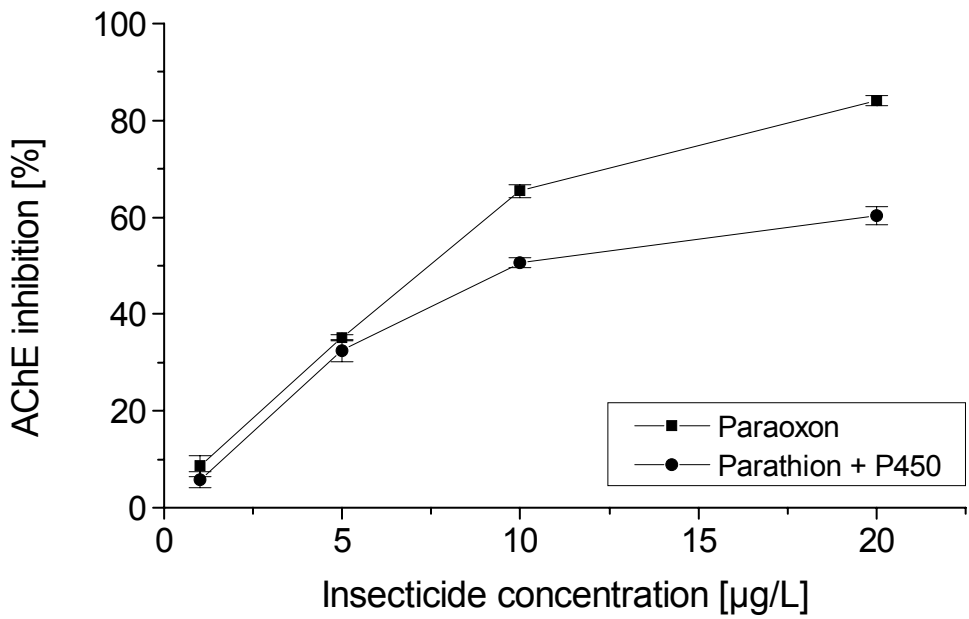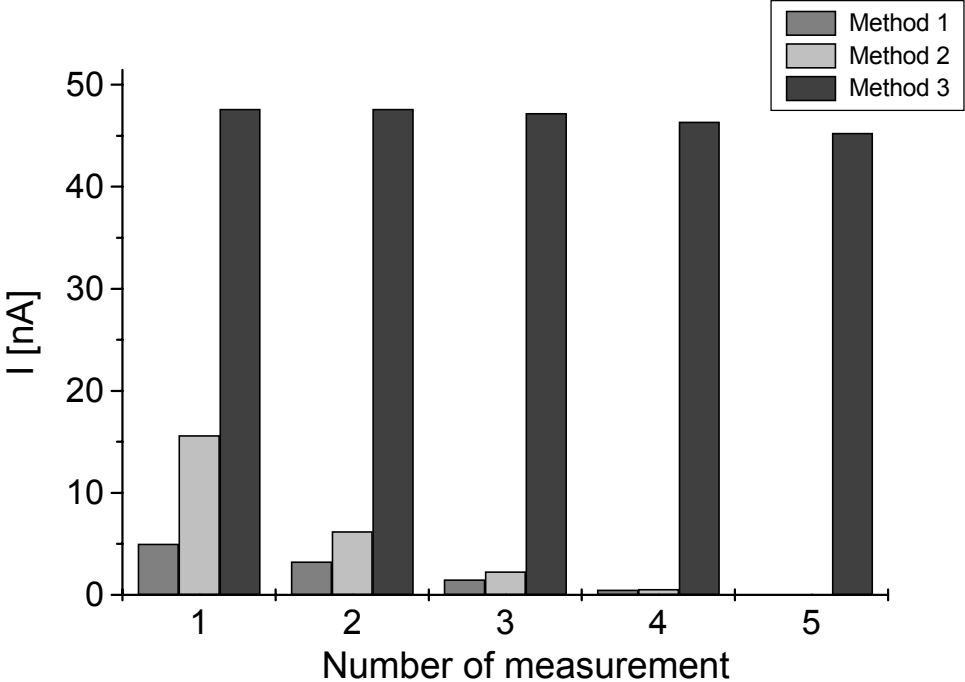
Figure 1
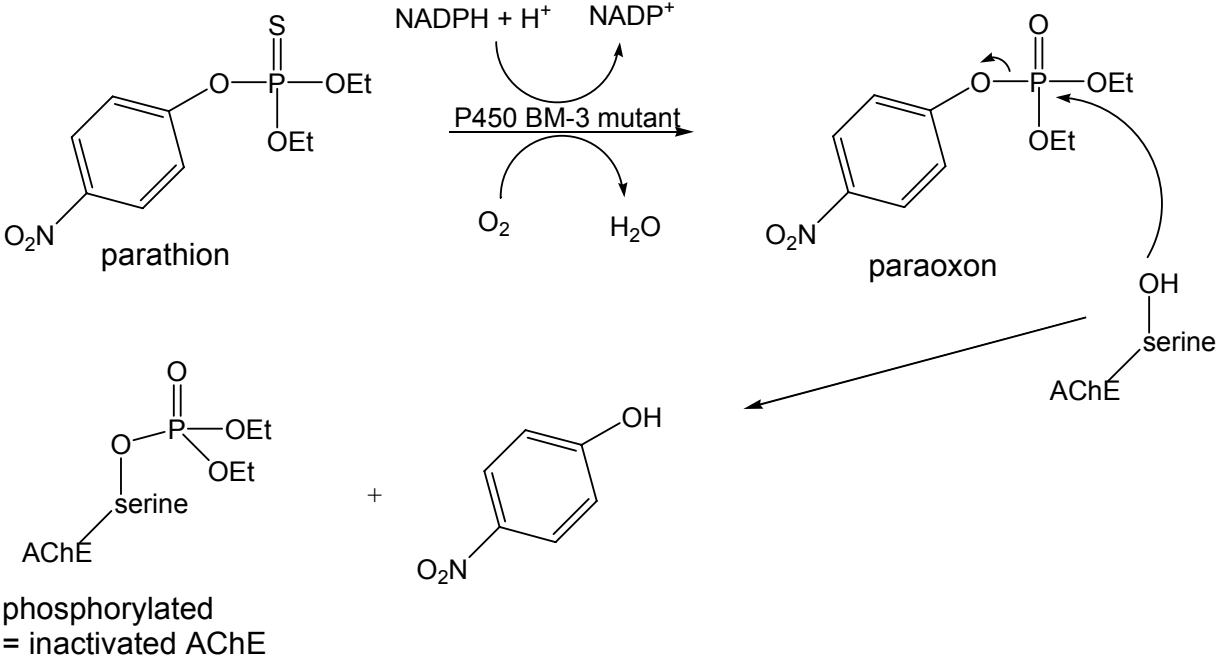
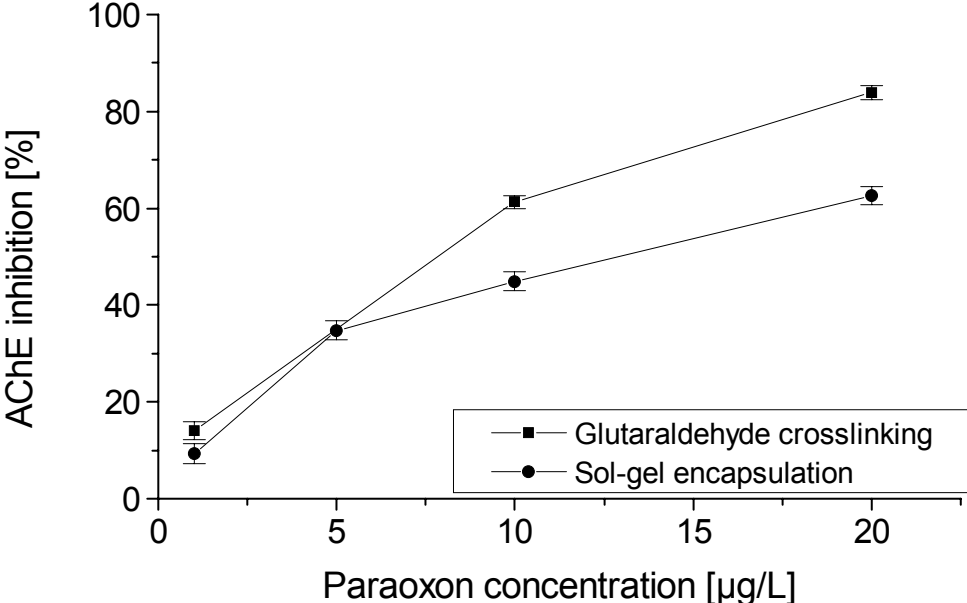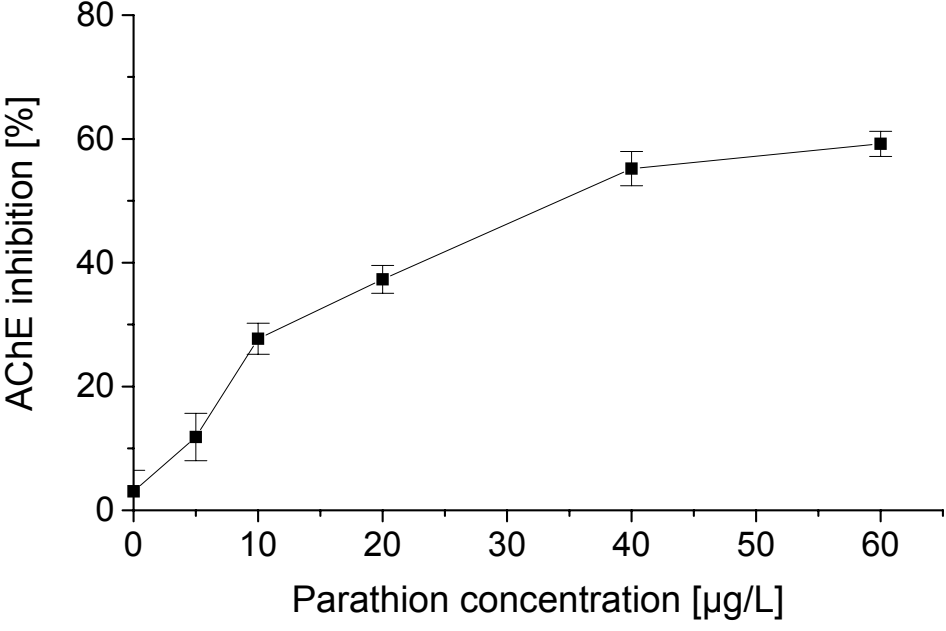Figure 2

Figure 3

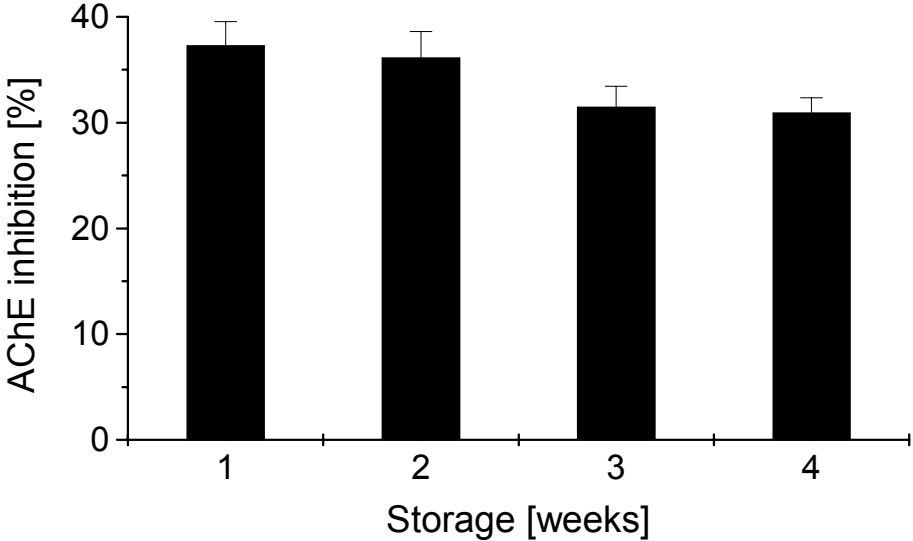Figure 4

Figure 5

Figure 6

Figure 7

# References

Abad, J.M., Pariente, F., Hernandez, L., Abruna, H.D., Lorenzo, E., 1998. Determination of organophosphorus and carbamate pesticides using piezoelectric biosensors. Anal. Chem. 70, 2848-2855.

Albareda-Sirvent, M. and Hart, A.L., 2002. Preliminary estimates of lactic and malic acid in wine using electrodes printed from inks containing sol-gel precursors. Sens. Actuators B Chem. 87, 73-81.

Altstein, M., Segev, G., Aharonson, N., Ben Aziz, O., Turniansky, A., Avnir, D., 1998. Sol-gel-entrapped cholinesterases: A microtiter plate method for monitoring anti-cholinesterase compounds. J. Agric. Food Chem. 46, 3318-3324.

Anastassiades, M. and Scherbaum, E., 1997. Multiresidue method for determination of pesticide residues in citrus fruits by GC-MSD. Dtsch. Lebensmitt. Rundsch. 93, 316-327.

Andreescu, S., Barthelmebs, L., Marty, J.L., 2002. Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides. Anal. Chim. Acta 464, 171-180.

Anitha, K., Mohan, S.V., Reddy, S.J., 2004. Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides. Biosens. Bioelectron. 20, 848-856.

Bachmann, T.T. and Schmid, R.D., 1999. A disposable multielectrode biosensor for rapid
simultaneous detection of the insecticides paraoxon and carbofuran at high
resolution. Anal. Chim. Acta 401, 95-103.

Barber, D., Correll, L., Ehrich, M., 1999. Comparison of two in vitro activation systems for
protoxicant organophosphorous esterase inhibitors. Toxicol. Sci. 47, 16-22.

Chambers, J.E., Levi, P.E., 1992. Organophosphates - Chemistry, Fate, and Effects.
Academic Press, San Diego, California.

Chemical and Veterinary Official Laboratory (CVUA) Stuttgart, 2000. Jahresbericht des
Chemischen und Veterinäruntersuchungsamtes Stuttgart. CVUA Stuttgart, Fellbach,
Germany.

Choi, J.W., Kim, Y.K., Lee, I.H., Min, J., Lee, W.H., 2001. Optical organophosphorus
biosensor consisting of acetylcholinesterase/viologen hetero Langmuir-Blodgett film.
Biosens. Bioelectron. 16, 937-943.

Danet, A.F., Badea, M., Marty, J.L., Aboul-Enein, H.Y., 2000. Flow analysis for determination
of paraoxon with use of immobilized acetylcholinesterase reactor and new type of
chemiluminescent reaction. Biopolymers 57, 37-42.

DIN38415-1, 1995. Suborganische Testverfahren (Gruppe T), Teil 1: Bestimmung von
Cholinesterase-hemmenden Organophosphat- und Carbamat-Pestiziden. Dtsch.
Norm.

Doong, R.A. and Tsai, H.C., 2001. Immobilization and characterization of sol-gel-
encapsulated acetylcholinesterase fiber-optic biosensor. Anal. Chim. Acta 434, 239-
246.

EC, 1999. Commission Directive 1999/50/EC of 25 May 1999 amending Directive 91/321/EEC on infant formulae and follow-on formulae. Official J. Eur. Communities L 139, 29-31.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-92.

Evtugyn, G.A., Budnikov, H.C., Nikolskaya, E.B., 1996. Influence of surface-active compounds on the response and sensitivity of cholinesterase biosensors for inhibitor determination. Analyst 121, 1911-1915.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245-254.

Ghindilis, A.L., Morzunova, H.C., Barmin, A.V., Kurochkin, I.N., 1996. Potentiometric biosensors for cholinesterase inhibitor analysis based on mediatorless bioelctrocatalysis. Biosens. Bioelectron. 11, 837-880.

Gill, I. and Ballesteros, A., 2000. Bioencapsulation within synthetic polymers (Part 1): sol-gel encapsulated biologicals. Trends Biotechnol. 18, 282-296.

Guo, Y.Z. and Guadalupe, A.R., 1998. Screen-printable surfactant-induced sol-gel graphite composites for electrochemical sensors. Sens. Actuators B Chem. 46, 213-219.

Hussein, A., Harel, M., Selkirk, M., 2002. A distinct family of acetylcholinesterases is secreted by Nippostrongylus brasiliensis. Mol. Biochem. Parasitol. 123, 125-134.

Hussein, A.S., Chacon, M.R., Smith, A.M., Tosado-Acevedo,R., Selkirk,M.E., 1999. Cloning, Expression, and Properties of a Nonneuronal Secreted Acetylcholinesterase from the Parasitic Nematode Nippostrongylus brasiliensis. J. Biol. Chem. 274, 9312-9319.

Iwuoha, E.I., Kane, S., Ania, C.O., Smyth, M.R., de Montellano, P.R.O., Fuhr, U., 2000. Reactivities of organic phase biosensors 3: Electrochemical study of cytochrome

P450(cam) immobilized in a methyltriethoxysilane sol-gel. Electroanalysis 12, 980-986.

Jeanty, G., Ghommidh, C., Marty, J.L., 2001. Automated detection of chlorpyrifos and its metabolites by a continuous flow system-based enzyme sensor. Anal. Chim. Acta 436, 119-128.

Jokanovic, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139-160.

Karalliedde, L. and Senanayake, N., 1999. Organophosphorus insecticide poisoning. eJIFCC [online computer file] 11.

Koletzko, B., Aggett, P.J., Agostoni, C., Baerlocher, K., Bresson, J.L., Cooke, R.J., Decsi, T., Deutsch, J., Janda, J., Manz, F., Moya, M., Rigo, J., Socha, J., 1999. Pesticides in dietary foods for infants and young children. Report of the Working Group on Pesticides in Baby Foods of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). Arch. Dis. Child. 80, 91-92.

Kulys, J. and D'Costa, E.J., 1990. Printed Amperometric Sensor Based on TCNQ and Cholinesterase. Biosens. Bioelectron. 6, 109-115.

Kumaran, S. and Tran-Minh, C., 1992. Determination of Organophosphorous and Carbamate Insecticides by Flow Injection Analysis. Anal. Biochem. 200, 187-194.

Larsen, J.C. and Pascal, G., 1998. Workshop on the applicability of the ADI to infants and children: consensus summary. Food Addit. Contam. 15 , 1-9.

Lee, H.S., Kim, Y.A., Chung, D.H., Lee, Y.T., 2001. Determination of carbamate pesticides by a cholinesterase-based flow injection biosensor. Int. J. Food Sci. & Tech. 36, 263-269.

Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450 BM-3 into an indole-hydroxylating catalyst. Chem.-Eur. J. 6, 1531-1536.

Makower, A., Halamek, J., Skladal, P., Kernchen, F., Scheller, F.W., 2003. New principle of direct real-time monitoring of the interaction of cholinesterase and its inhibitors by piezolectric biosensor. Biosens. Bioelectron. 18, 1329-1337.

Martinez, C.R., Gonzales, R.E., Moran, A.M.J., Mendez, H.J., 1992. Sensitive method for the determination of organophosphorus pesticides in fruits and surface waters by high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. 607, 37-45.

Maurer, S.C., Schulze, H., Schmid, R.D., Urlacher, V., 2003. Immobilisation of P450BM-3 and an NADP(+) cofactor recycling system: Towards a technical application of heme-containing monooxygenases in fine chemical synthesis. Adv. Synth. Catal. 345, 802-810.

Narhi, L.O. and Fulco, A.J., 1986. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in Bacillus megaterium. J. Biol. Chem. 261, 7160-7169.

Narhi, L.O. and Fulco, A.J., 1987. Identification and characterization of two functional domains in cytochrome P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in Bacillus megaterium. J. Biol. Chem. 262, 6683-6690.

Navaz Diaz, A. and Ramos Peinado, M.C., 1997. Sol-gel cholinesterase biosensor for organophosphorus pesticide fluorimetric analysis. Sens. Actuators. B Chem. 38-39, 426-431.

Omura, T.I. and Sato, R.J., 1964. The carbon monooxide-binding pigment of liver microsomes. J. Biol. Chem. 94, 2370-2378.

Pylypiw, H.M., 1993. Rapid Gas-Chromatographic Method for the Multiresidue Screening of Fruits and Vegetables for Organochlorine and Organophosphate Pesticides. J. AOAC Int. 76, 1369-1373.

Schilter, B. and Huggett, A.C., 1998. The ADI as a basis to establish standards for pesticide residues in food products for infants and young children. Food Addit. Contam. 15, 83-89.

Schulze, H., Schmid, R.D., Bachmann, T.T., 2002. Rapid detection of neurotoxic insecticides in food using disposable acetyicholinesterase-biosensors and simple solvent extraction. Anal. Bioanal. Chem. 372, 268-272.

Schulze, H., Schmid, R.D., Bachmann, T.T., 2004. Activation of phosphorothionate pesticides based on a cytochrome P450 BM-3 (CYP102 A1) mutant for expanded neurotoxin detection in food using acetylcholinesterase biosensors. Anal. Chem. 76, 1720-1725.

Schwaneberg, U., Schmidt-Dannert, C., Schmitt ,J., Schmid, R.D., 1999. A continuous spectrophotometric assay for P450 BM-3, a fatty acid hydroxylating enzyme, and its mutant F87A. Anal. Biochem. 269, 359-366.

Shtelzer, S., Rappoport, S., Avnir, D., Ottolenghi, M., Braun, S., 1992. Properties of Trypsin and of Acid-Phosphatase Immobilized in Sol-Gel Glass Matrices. Biotechnol. Appl. Biochem. 15, 227-235.

Singh, A.K., Flounders, A.W., Volponi, J.V., Ashley, C.S., Wally, K., Schoeninger, J.S., 1999. Development of sensors for direct detection of organophosphates. Part I: immobilization, characterization and stabilization of acetylcholinesterase and organophosphate hydrolase on silica supports. Biosens. Bioelectron. 14, 703-713.

Taylor, M., Lamb, D.C., Cannell, R.J., Dawson, M.J., Kelly, S.L., 2000. Cofactor recycling with immobilized heterologous cytochrome P450 105D1 (CYP105D1). Biochem. Biophys. Res. Commun. 279, 708-711.

Villatte, F., Schulze, H., Schmid, R.D., Bachmann, T.T., 2002. A disposable acetylcholinesterase-based electrode biosensor to detect anatoxin-a(s) in water. Anal. Bioanal. Chem. 372, 322-326.

Wang, J., Pamidi, P.V.A., Park, D.S., 1996. Screen-printable sol-gel enzyme-containing carbon inks. Anal. Chem. 68, 2705-2708.