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ORIGINAL PAPER

Fast, sensitive and cost-effective detection of nerve agents in the gas phase using a portable instrument and an electrochemical biosensor

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Abstract The nerve agents are chemical warfare agents known to be used during terrorist attacks. An inexpensive and portable system to be used by first responders and military personnel is of interest owing to the continuing threat of possible terrorist attacks. Amperometric biosensors based on cholinesterase inhibition show such potentialities. In this work butyrylcholinesterase was immobilized onto screen-printed electrodes modified with Prussian blue and the nerve agent detection was performed by measuring the residual activity of enzyme. The optimized biosensor was tested with sarin and VX standard solutions, showing detection limits of 12 and 14 ppb (10% of inhibition), respectively. The enzymatic inhibition was also obtained by exposing the biosensors to sarin in gas phase. Two different concentrations of sarin gas (0.1 and 0.5 mg m^{-3}) at different incubation times (from 30 s up to 10 min) were tested. It is possible to detect sarin at a concentration of 0.1 mg m^{-3} with 30-s incubation time, with a degree of inhibition of 34%, which match the legal limits (immediate danger to life and health).

Keywords Biosensor · Nerve agents · Butyrylcholinesterase · Screen printed electrode

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Introduction

Organophosphonate compounds such as sarin (*O*-isopropyl methylphosphonofluoridate), soman (1,2,2-trimethylpropyl methylphosphonofluoridate), tabun (ethyl *N*,*N*-dimethyl-phosphoramidocyanidate) and VX (*O*-ethyl-*S*-[2(diisopropylamino)ethyl] methylphosphonothioate) are potent nerve agents [1–5]. The extreme toxicity of these compounds is due to their ability to bind primarily and rapidly to acetylcholinesterase (AChE) in the neuromuscular junction of the central nervous system. The organophosphonates also have the ability to bind butyrylcholinesterase (BChE) in blood. The high vapour pressures of these agents and their rapid effect on the central nervous system, combined with the low cost and unsophisticated technology required for production, make these compounds among the preferred choices for terrorists.

Nerve agents were employed against the Kurdish opposition in Iraq and sarin gas was utilized in the terrorist attack by the Aum Shinrikyo sect in the Tokyo subway in 1995 [6].

Also the tragic events of 11 September 2001 have led to an increased awareness of threats regarding the use of chemical warfare agents; for this reason, analytical tools which combine reliability and rapidity of response for the detection of lethal chemicals are strongly required. Chromatographic methods, often used to detect nerve agents, have been demonstrated to identify the different organophosphorous compounds with high sensitivity, but they have several disadvantages for in situ monitoring, being expensive and time-consuming. Moreover, the chromatographic analysis has to be performed in a specialized laboratory by skilled personnel and is not so suitable for miniaturization. The advantage of electrochemical sensors resides in their easiness of preparation, the possibility of

their miniaturization and the low cost; moreover no specialized personnel are required [7, 8].

Despite this, only a few papers have reported [9–12] the use of this approach for the detection of nerve agents (sarin, soman, tabun and VX) because in many papers the model compounds such as paraoxon or diisopropylfluorophosphate (DFP) have been considered [13–16].

Mlsna et al. [9] developed a chemicapacitive sensor for chemical warfare agent analysis. The dielectric permittivity of the polymer-filled chemicapacitors changed upon adsorption and desorption of the chemical vapours. The detection limits obtained with sarin, soman and tabun gas were 0.4, 0.047 and 0.048 mg m^{-3}, respectively. The feasibility of a thick-film chemical sensor based on various semiconductor metal oxides to reliably detect chemical warfare agents has been studied by Tomchenko et al. [10]. Their sensors produced reliable responses to 10 ppb of sarin gas, but at 400 °C.

A further approach for organophosphonates detection is based on biosensors that detect modulation of cholinesterase (ChE) activity [17–20]. White and Harmon [11] demonstrated the application of AChE-based detection of sarin using planar wave guide absorbance spectroscopy. Detection of levels of sarin as low as 0.1 ppb in solution and 0.014 mg m^{-3} (2.6 ppb) in vapour have been reported.

Lee et al. [12] have described an assay system based on biotin-labelled ChE with streptavidin for nerve agent detection in liquid samples. The limits of detection for soman and sarin were 0.018 and 0.084 ppb, respectively, for 10-min assay. However, these methods require high temperatures or complex apparatus or the gas measurement has to be carried out in solution.

Our goal was the development a nerve agent detector that can be incorporated in soldier overalls; the papers previously cited reported low detection limits being achieved, but in both cases the systems are not suitable for incorporation in soldier overalls owing to difficulties concerning miniaturization and mass production [11, 12]. Also in both cases, specialized personnel are required. To overcome these problems, we chose the amperometric biosensor because of its high sensitivity and, also, because of the possibility of it being miniaturized, mass-produced and used by unskilled personnel [21, 22].

For nerve agent detection based on the amperometric measurement of ChE, two strategies can be adopted: the use of AChE combined with choline oxidase together with the native substrate, acetylcholine; or the use of AChE alone with the synthetic substrate (thiocholine esters) [23, 24].

The approach based on the electrochemical oxidation of thiocholine produced through the AChE-catalysed hydrolysis of the thiocholine ester, acetylthiocholine, is generally preferred because of the inherent simplicity and easiness to produce an economic single-enzyme system. However, thiocholine detection at a conventional electrode requires high potentials with problems of passivation at the electrode surface [25]. In this perspective, the electrochemical determination of thiocholine with cobalt phtalocyanine or 7,7,8,8-tetracyanoquinodimethane (TCNQ) has been extensively studied [26–29]. Recently, the use of a Prussian blue (PB) modified screen-printed electrode (SPE) has been demonstrated to allow the detection of thiocholine at low applied potential (+200 mV vs Ag/AgCl) owing to the presence of PB as a mediator [30]. This modification procedure allows an extremely easy and reproducible means for detection, also suitable for mass production of modified electrodes. Experiments concerning biosensor optimization were carried out in our laboratory and paraoxon was used as a model organophosphorous compounds instead of nerve agents. Then, the biosensor developed was tested with sarin and VX solutions in a chemical warfare specialized centre in Rijswijk, the Netherlands. The biosensor was also tested with sarin gas.

Experimental

Reagents

All chemicals from commercial sources were of analytical grade. Potassium ferricyanide was from Carlo Erba (Milan, Italy) and potassium chloride was from Fluka (St. Louis, USA). BChE from horse serum, bovine serum albumin (BSA), *S*-butyrylthiocholine chloride (BTChCl), 5,5′-dithio-bis(2-nitrobenzoic acid), glutaraldehyde and paraoxon were purchased from Sigma Chemical Company (St. Louis, USA). Nafion (perfluorinated ion-exchange resin, 5% v/v solution in lower alcohols/water) was obtained from Aldrich (Steinheim, Germany). Sarin and VX were provided by TNO defence, security and safety centre in Rijswijk, the Netherlands.

Apparatus

Amperometric measurements were carried out using a portable PalmSens instrument (Palm Instruments, the Netherlands). Cyclic voltammetry was performed using an Autolab/PGSTAT-12 electrochemical system (Eco Chemie, Utrecht, the Netherlands).

The sarin gas was analysed using an HP6890 gas chromatograph with a 5973N MSD instrument (Agilent). The gas chromatograph was equipped with a Factourfour VF-5MS column (Varian, 50 m×0.32-mm inner diameter, film thickness 0.25 μm). Helium was used as the carrier gas with a flow-rate of 1 ml min^{-1}. The temperature programme used was 40 °C (1 min), 5 °C min^{-1} to 160 °C (5 min).

Electrodes

SPEs were produced in our laboratory with a 245 DEK (Weymouth, UK) screen-printing machine. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrode [20]. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were home produced in foils of 20. The diameter of the working electrode was 0.2 cm, resulting in a geometric area of 0.031 cm^2. Silver ink was used to print the reference electrode (see [30] for details of the electrode geometry). Before thiol measurement, the reference electrode was chlorinated by applying a potential of +0.6 V between the silver ink and an external Ag/AgCl electrode for 20 sss in a solution of 0.1 M KCl [31].

Preparation of PB-modified SPEs

Prior to PB modification, SPEs were pretreated in a 0.05 M phosphate buffer plus 0.1 M KCl, pH 7.4 by applying a positive potential of 1.7 V for 3 min. PB modification of SPEs was then accomplished by placing a drop (10 μl total volume) of "precursor solution" onto the working electrode area. This solution was obtained by mixing 5 μl of 0.1 M potassium ferricyanide in 10 mM HCl with 5 μl of 0.1 M ferric chloride in 10 mM HCl directly on the surface of the working electrode. The drop was carefully pipetted to be localized exclusively on the working electrode area. The solution was left on the electrode for 10 min and then rinsed with a few millilitres of 10 mM HCl. The electrodes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of PB [32]. The PB-modified electrodes were stored dry at room temperature in the dark.

Preparation of a biosensor based on PB-modified SPEs

To immobilize the enzyme (BChE or AChE) on the electrode surface, 2 μl of a 1% (v/v) glutaraldehyde solution was applied with a syringe exclusively on the PB-modified working electrode. The solution was then left to evaporate. Then, 2 μl of a mixture of BSA, enzyme and Nafion was applied on the working electrode. The mixture was obtained by mixing 25 μl of BSA (5% w/v prepared in water), 25 μl of Nafion (0.1% v/v diluted in water) and 25 μl of a stock enzyme solution with a concentration ranging from 1.5 to 150 U ml^{-1}.

Preparation of paraoxon, sarin and VX solutions

The organophosphate compound paraoxon and the organophosphonate compounds sarin and VX were prepared in 0.05 M phosphate buffer solution at pH 7.4. Stock solutions of sarin and VX were prepared daily from the pure agent and they were not used for more than 5 h after preparation to minimize the possible risk of hydrolysis. The real concentration of nerve agent was checked every time before measurement.

Preparation of sarin gas

An air stream contaminated with the vapour of the nerve agent sarin was generated dynamically by evaporating the pure compound in a clean and dry nitrogen gas stream in the TNO laboratory (Fig. 1).

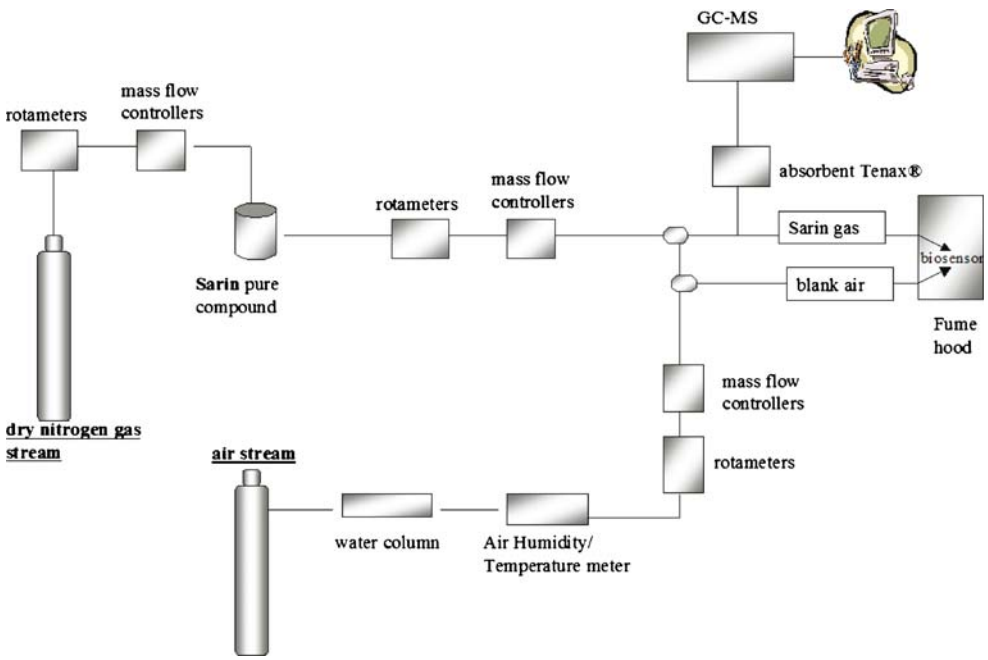

Fig. 1 Experimental production of sarin gas

Subsequently, the gas stream was diluted with clean, pressurized (and humidified) air using a TNO-built dynamic vapour generation system. The exhaust of the vapour generation system is open-ended; thus, the system delivers the sarin vapour to the sensors almost at atmospheric pressure.

The various gas flows were set and controlled by Bronkhorst Hi-Tec F-201 mass-flow controllers and Rota-Wehr L63 rotameters. Humid air was generated by bubbling a clean, dry and pressurized air stream through a water column and mixing the resulting gas stream with the contaminated air stream to obtain the required humidity. The primary humidified air flow was split in two equal parts, one containing blank air and the other one was used for the dilution of the concentrated sarin gas. The air humidity was monitored with a Novasina type MIK 3000-C combined air humidity/temperature meter. The experiments were carried out at ambient temperature and at fixed air relative humidity.

The target concentration of the sarin vapour generated was verified by sampling and by off-line analysis using a combination of gas chromatography and mass spectrometry (GC-MS). Glass sampling tubes filled with the absorbent Tenax® TA were used for the sampling of the sarin-contaminated air stream.

The air stream (770 l h^{-1}) served as the source for exposure of the sensors. A custom-built glass bell jar device was used to provide a stable and even flow across the sensors.

Biosensor enzymatic activity measurement

Butyrylthiocholine (or acetylthiocholine) measurements were performed using an chronoamperometric "drop" procedure in phosphate buffer solution (0.05 M plus 0.1 M KCl, pH 7.4) with an applied potential of +200 mV vs Ag/AgCl [20]. The drop (50 μl) of buffer containing different amounts of butyrylthiocholine was placed onto the BChE biosensor (or acetylthiocholine in the case of the AChE biosensor) in such a way that the counter and reference electrodes were also covered. After applying the potential, we recorded the signal continuously and measured the current at the steady state. The time needed for the stabilization of the current was 5 min.

Paraoxon, sarin and VX standard solutions measurements

The inhibitory effect of paraoxon on the BChE biosensor was evaluated by determining the decrease in the current obtained for the oxidation of thiocholine that was produced by the enzyme. The ChE biosensor was first incubated by dropping paraoxon solution onto the working electrode, for a certain period (incubation time), and then rinsing three times with distilled water. After that, the response towards the substrate was measured as described before and the degree of inhibition was calculated as the relative decay of the biosensor response (Eq. (1)):

$$I(\%) = [(i_0 - i_i)/i_0] \times 100 \quad (1)$$

where i_0 and i_i represent the biosensor response before and after the incubation procedure, respectively.

Sarin gas measurements

The inhibitory effect of sarin gas on the BChE biosensor was also evaluated. In this case, during the incubation, the surface of the working electrode was covered with 5 μl of 0.05 M phosphate buffer plus 0.1 M KCl, pH 7.4, then the biosensor was exposed to the blank air stream. Afterwards, the response towards the substrate was measured and i_0 was obtained (Eq. (1)) using the procedure described above. To measure the i_i the same procedure was adopted but, in this case, the biosensor was exposed to the sarin gas stream. In each case, three biosensors were used simultaneously and the air flow was nearly perpendicular to the surface of each biosensor. The biosensors were fixed one next to the other in front of the entrance of the gas stream in the fume hood.

The system for sarin gas production is characterized by a glass switching valve; this made it possible to switch very quickly between the blank air and the air stream containing the sarin gas.

Safety conditions

The stock solutions of sarin and VX (highly toxin compounds) were prepared under appropriate safety conditions. In fact in order to avoid contact with powder or inhalation of the vapour of nerve agents, the operators wore laboratory coats, gloves, masks and glasses. Also a dedicated fume hood was used during sample preparation and analysis.

Results and discussion

The choice of electrochemical mediator for amperometric thiocholine detection has previously been evaluated. Recently our group investigated the performance of PB as a mediator of thiocholine oxidation [30]. The major advantage associated with the use of the PB-modified sensor is the very high stability even under strong conditions [32], thus making it very useful for practical applications. This mediator shows good analytical performances towards thiocoline [30] together with good stability and reproducibility (relative standard deviation, RSD, of 7%) [30]. Also, the modification of the electrode surface is easy to perform

as well as being amenable to mass-production techniques. For these reasons, and given our long experience in the use of PB-modified SPEs, these sensors were selected as probes for thiocholine detection in order to develop a biosensor for nerve agent detection.

To develop a biosensor with high sensitivity and rapid response, the choice of the enzyme is critical. In our case the choice of the ChE enzyme was based on the ability of nerve agents to irreversibly inhibit this enzyme, and the high turnover number of ChE permits the activity measurement to be made in a short time. Among the various ChE enzymes available we chose AChE from electric eel and BChE from horse serum because of their commercial availability. In fact, given the perspective to develop a biosensor for mass production, it is important to use reagents that are commercially available. In previous work [20] we analysed the different ability of some carbamates (carbaryl, aldicarb) and organophosphates (chlorpyriphos-methyl oxo, paraoxon) to inhibit BChE from horse serum as well as AChE from electric eel. BChE was inhibited more than AChE by both paraoxon and chlorpyriphos-methyl oxo. The inhibition constants for paraoxon, a pesticide frequently used as a nerve agent model compound (as widely reported in the literature) [15, 33], were calculated using AChE and BChE biosensors. Inhibition of ChE by organophosphate (or organophosphonate) follows pseudo-first-order kinetics when the concentration of the inhibitor is much higher than the concentration of the enzyme and when the concentration of the inhibitor does not vary during the experiment, as in our case. In this work, the results were investigated using the kinetic approach described by the following equation:

$$\ln(E/E_0) = -k_i I_0 t \quad (2)$$

where E_0 and E are the enzymatic activity before and after inhibition, I_0 is the concentration of the inhibitor and t is the incubation time. The activity of the biosensor was plotted against incubation time at a fixed concentration of paraxon (200 ppb) and values of 2.9×10^{-4} and 8.4×10^{-4} ppb^{-1} min^{-1} were obtained as inhibition constants (k_i) using AChE and BChE biosensors, respectively (Fig. 2). Because our goal was to develop a sensitive biosensor for nerve agents (organophosphonate compounds), BChE from horse serum was chosen as the enzyme to be immobilized on PB-modified SPEs for this purpose.

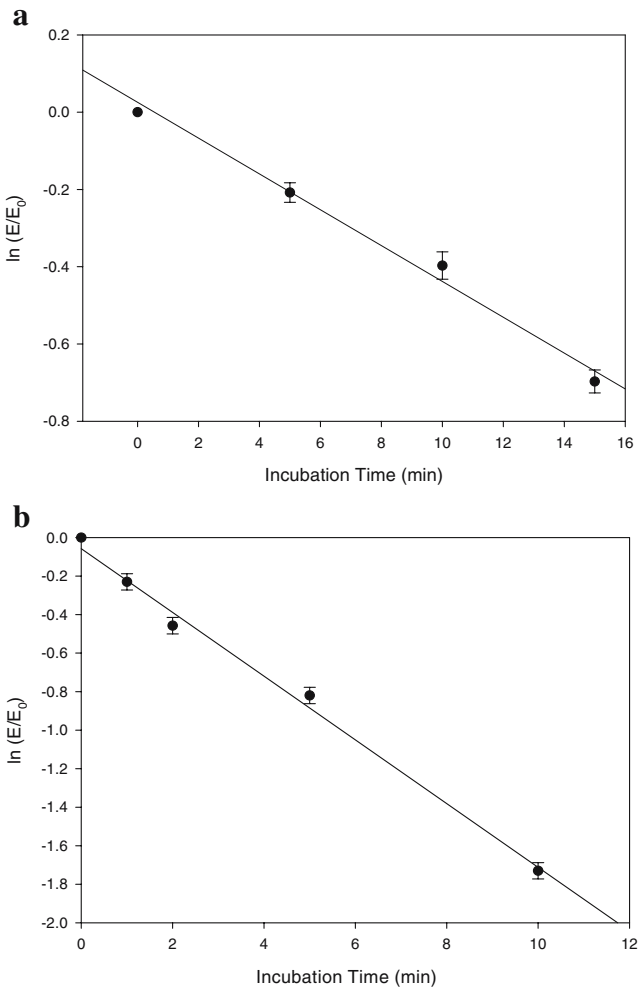

Fig. 2 Study of $\ln(E/E_0)$ vs incubation time using **a** acetylcholinesterase (AChE) or **b** butyrylcholinesterase (BChE) biosensors. Paraoxon concentration 200 ppb. Applied potential +200 mV vs Ag/AgCl; 0.01 U AChE, 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4. In the case of the AChE biosensor 1 mM acetylthiocholine chloride (ATChCl) was used, while for the BChE biosensor 5 mM butyrylthiocholine chloride (BTChCl) was used. All the values are the average of triplicate measurements

The BChE biosensor

On the basis of the results previously obtained, the enzyme immobilization to create a nerve agent biosensor was optimized in this work to obtain a faster and simpler measurement procedure. In the former experiments, the immobilization was carried out using ChE in the presence of 1% v/v glutaraldhehyde, 5% w/v BSA and 1% v/v Nafion. With this formulation, a washing step between two successive thiocholine measurements was required. We had supposed that thiocholine is likely to be entrapped between the electrode surface and the enzymatic layer and would thus affect successive measurement [20]. To avoid this problem, for the nerve agent biosensor, the concentration of Nafion was decreased to 0.1% (for details see "Experimental"). Using this type of membrane we have observed that is possible to make repetitive measurements without any intervening washing step.

In addition, the concentration of enzyme was optimized. Owing to the fact that nerve agent detection involves an irreversible inhibition of the enzyme, the lowest feasible concentration of enzyme was necessary to achieve a low

detection limit. The dependence of the response on the amount of the enzyme immobilized on the electrode surface from 0.00 to 0.100 U was investigated. A value of 0.025 U was chosen as the best compromise between a low enzyme loading, a sufficiently high substrate signal and good storage stability. The reproducibility of the biosensors obtained under this condition was good: RSD of 3% was observed for five replicates using the same biosensor (intraelectrode percentage RSD). Five different biosensors were also tested with the same concentration of BTChCl, resulting in a RSD value of 10% (interelectrode percentage RSD). Figure 3 shows the calibration curve obtained for different concentrations of substrate (i.e. BTChCl) that could be described by the Michaelis–Menten equation. Using Michaelis–Menten theory it is possible to calculate the maximum rate (V_{max}=740 nA) and the apparent Michaelis–Menten constant (K_M^{app}=1.2 mM). A substrate concentration of 5.0 mM was then chosen for the inhibition measurements. The optimized biosensor showed a storage stability of 1 month in dry conditions at room temperature with a residual percentage of enzymatic activity of 89±4% .

Preliminary studies: paraoxon determination using BChE biosensors

Owing to security reasons and to the fact that the use of sarin and powerful nerve agents require specialized structures, the biosensor was first optimized using an organophosphorous pesticide (paraoxon) as a model compound. Its irreversible inhibition is characterized by a higher degree of inhibition at long incubation times (reaction time between inhibitor and enzyme); thus, a study of incubation time was carried out (see the second column of Table 1). On the basis of these results, an incubation time of 10 min in phosphate buffer was selected as a good compromise between the requirements for a rapid assay and the achievement of the highest degree of inhibition.

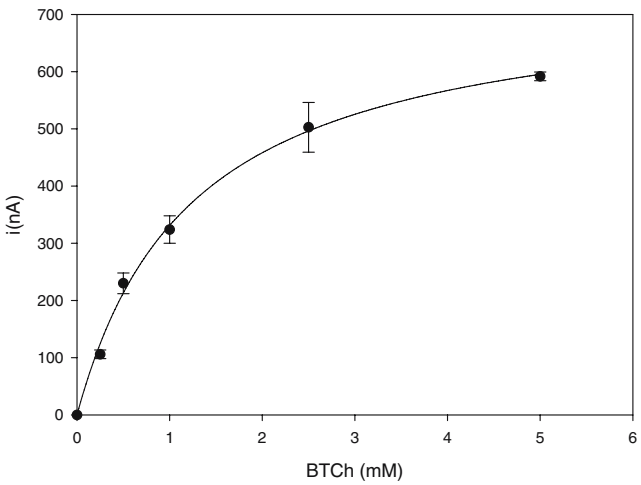

Fig. 3 Calibration plot of butyrylthiocholine using the BChE biosensor. Applied potential +200 mV vs Ag/AgCl; 0.01 U BChE, 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4. All the values are the average of triplicate measurements. *BTCh* butyrylthiocholine

A linear range from 25 to 100 ppb paraoxon was obtained (Table 2). A detection limit (calculated as the concentration giving 10% inhibition) of 5 ppb was obtained by plotting the values of ln (E/E_0) vs inhibitor concentration and by using the equation y=0.0545-8.61×10^{-3} x; by using the same plot, 74 ppb resulted as the concentration giving 50% inhibition.

Sarin and VX determination using the BChE biosensor

The biosensor optimized using paraoxon as a model organophosphorous compound was then used to test standard solutions of sarin. The standard solutions were prepared in phosphate buffer solution at pH 7.4 and were used only for 5 h, because the hydrolysis of nerve agents in solution has been reported [4]. The study of incubation time was carried out using a 200 ppb sarin solution ranging from 1 to 5 min of incubation or a 20 ppb solution at incubation times of 5 and 10 min (Table 1). An incubation time of 10 min was chosen for the calibration curve. Under these conditions, the linear range extends up to a limiting value of 20 ppb (Table 2). A detection limit defined as 10% inhibition equal to 12 ppb was obtained from the equation y=0.7478-0.07412x, derived by plotting ln(E/E_0) vs inhibitor concentration (Fig. 4a). The value of the inhibition constant for sarin (k_i=1.1×10^{-3} ppb^{-1} min^{-1} using 200 ppb as the inhibitor concentration) was evaluated using the plot of ln(E/E_0) vs incubation time (y=-0.122-0.223x). The results obtained showed that the sarin inhibition was slightly higher than paraoxon inhibition towards the enzyme immobilized on the biosensor. VX as a nerve agent was also tested. For this study, the incubation time using a VX solution of 200 ppb was varied from 1 to 10 min (Table 1). Also in this case, an incubation time of 10 min in phosphate buffer was ultimately selected as a good compromise between the requirement of a rapid assay and the achievement of a high degree of inhibition. A linear range from 20 ppb up to a limiting value of 150 ppb was obtained (Table 2). The detection limit defined as 10% inhibition was equal to 14 ppb; 50% inhibition equal to 176 ppb was evaluated using the equation y=-0.05497-3.623×10^{-3}x, obtained by plotting the enzymatic activity vs VX concentration (Fig. 4b).

As shown in Table 1, using 5 min as the incubation time and 200 ppb VX, we obtained a degree of inhibition of 31±4%, whereas in the same conditions (5 min of incubation time) but using 200 ppb sarin as the nerve agent, we obtained a degree of inhibition of 70±2%.

Under our experimental conditions the order in which organophosphonate compounds inhibited the BChE from

Table 1 Study of incubation time using different organophosphates
Butyrylthiocholine chloride (5 mM) was used as a substrate. Applied potential +200 mV vs Ag/AgCl; 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4. All the values are the average of triplicate measurements

Incubation time (min)	Degree of inhibition (%)			
	Paraoxon (200 ppb)	VX (200 ppb)	Sarin (200 ppb)	Sarin (20 ppb)
1	–	–	25±4	–
2	37±2	–	51±4	–
5	52±4	31±4	70±2	31±8
10	82±2	52±9	–	49±7

horse serum was sarin>paraoxon>VX, with an inhibition constant for sarin of 1.1×10^{-3} ppb^{-1} min^{-1} (9.8×10^{5} M^{-1} min^{-1}). The results obtained are slightly different form those reported by Ashani et al. [34], in which the order was sarin>VX>paraoxon using nonimmobilized BChE from human plasma, with an inhibition constant for sarin of 13.3×10^{6} M^{-1} min^{-1}. In that case the inhibition constant is higher than the k_i obtained in our work, probably owing to the use of different sources of ChE, BChE from horse serum, and also to the immobilization adopted in this study.

Sarin gas measurements

Our purpose was to develop a biosensor for sarin gas measurement, given that in terrorist attacks sarin in gas phase has been utilized. Sarin gas in the TNO laboratory was produced with a sophisticated instrument realized by that laboratory (Fig. 1).

Firstly, the exposure of the biosensor to sarin gas at 0.5 mg m^{-3} (100 ppb) in dry conditions and in wet conditions was evaluated. The incubation step was carried out in different ways. For the measurement made in the wet conditions, 5 μl of phosphate buffer at pH 7.4 was added over the working electrode, while in dry conditions no buffer was added. As expected, only under wet conditions was the inhibition (97±3%) observed.

Moreover, as a control, the effect on the biosensor of the air stream in the absence of nerve agent in wet conditions was evaluated. After 10 min of exposure, no change in biosensor activity was observed, showing that the air stream itself had no effect on enzymatic activity.

Then the system was challenged against the sarin gas at two different concentrations 0.1 (40 ppb) and 0.5 mg m^{-3} at different incubation times (from 30 s to 10 min) and the concentration of sarin gas was confirmed using GC-MS on-line with the system. We tested only two different

Table 2 Analytical parameters relative to different organophosphates

Organophosphate	Linear range (ppb)	Detection limits (ppb) (10% inhibition)	50% inhibition (ppb)	R^2
Paraoxon	25–100	5	74	0.97
Sarin	10–20	12	20	0.88
VX	20–150	14	176	0.89

Butyrylthiocholine chloride (5 mM) was used a substrate. Applied potential +200 mV vs Ag/AgCl; phosphate buffer 0.05 M+ 0.1 M KCl, pH 7.4; incubation time 10 min. All the values are the average of triplicate measurements

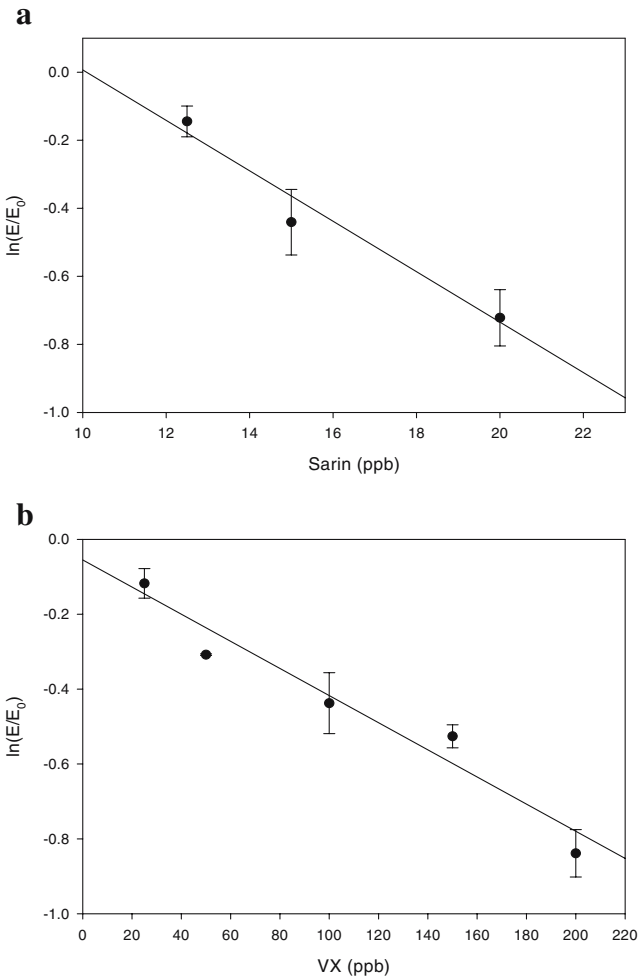

Fig. 4 Study of $\ln(E/E_0)$ vs inhibitor concentration. **a** Sarin and **b** VX using the BChE biosensor. Incubation time 10 min; applied potential +200 mV vs Ag/AgCl; 0.01 U BChE, 5 mM BTChCl, 0.05 M phosphate buffer + 0.1 M KCl, pH7.4. All the values are the average of triplicate measurements

concentrations of sarin gas because sarin gas is difficult to prepare. These concentrations were chosen knowing that the level immediately dangerous to life or health (IDLH) is 0.1 mg m^{-3} [35].

The degree of inhibition obtained by exposing the biosensor to sarin gas with different incubation times is reported in Fig. 5. As shown in Fig. 5a, 30 s of incubation time is sufficient to obtain a degree of inhibition of 45±4% using 0.5 mg m^{-3} sarin gas. This demonstrated that the procedure of exposing the biosensor (with a few microlitres of phosphate buffer on the working electrode) to the gas flow of the nerve agents is an effective approach and also is quite simple.

It is likely that an equilibrium between sarin in the gas phase and in the aqueous phase (a drop on the working electrode) was achieved. This equilibrium is a dynamic equilibrium because the molecules of sarin irreversibly bonded to BChE are removed from the aqueous phase, but are restored from the gas phase. This dynamic equilibrium is probably the reason for the higher degree of inhibition observed in this procedure than for the sarin tested using a drop of sarin aqueous solution on the biosensor.

After the good results obtained with 0.5 mg m^{-3}, a lower concentration of sarin gas was tested (0.1 mg m^{-3}) (Fig. 5b).

Also in this case it is possible to detect sarin gas using an incubation time of 30 s with a degree of inhibition of 34± 1%. However, with a incubation time of 1 min, double the inhibition was obtained 56±1% (Fig. 5b), showing a linear correlation between incubation time and degree of inhibition. In fact, the linearity of the degree of inhibition is usually observed in a range from 20 to 60% inhibition [36]. The plots obtained permit us to evaluate the behaviour of the degree of inhibition as a function of incubation time using two different concentrations of sarin gas.

A typical response to butyrylthiocholine using the BChE biosensor before and after the exposure to sarin gas is reported in Fig. 6. As we can see in the figure, the time of measurement of enzymatic activity was chosen as 5 min. However, to make a fast measurement of nerve agents, a shorter measurement time would be possible by measuring the rate of enzymatic reaction in the first minute, instead of the value at steady state. Then, using the biosensor developed it is possible to detect 0.1 mg m^{-3} with an incubation time of 30 s and a measurement time less than 5 min.

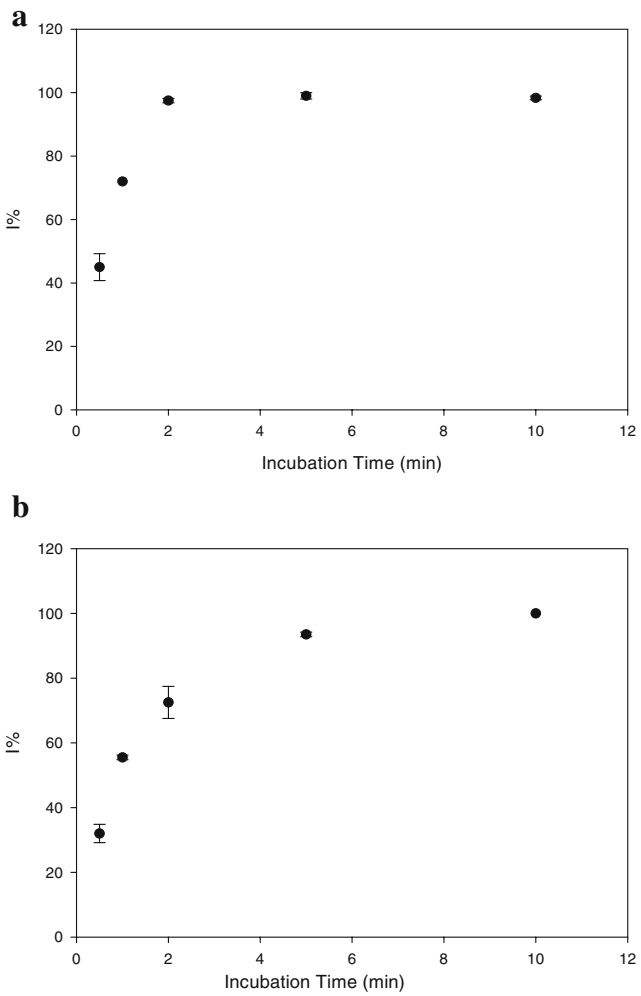

Fig. 5 The relative inhibition of BChE activity as a function of incubation time using aarin gas at 0.5 mg m^{-3} (**a**) and 0.1 mg m^{-3} (**b**). Measurement conditions 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4; applied potential +200 mV vs Ag/AgCl; 5 mM BTChCl

Conclusions

In this work a biosensor for detection of nerve agents was developed. BChE was immobilized onto SPEs modified with PB and the detection of nerve agents was evaluated by

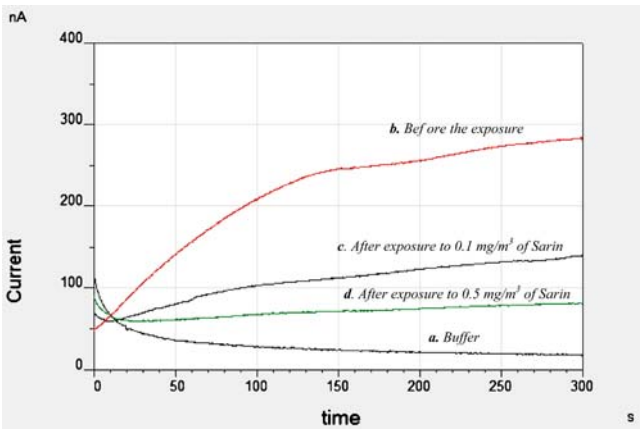

Fig. 6 Original recording obtained using a biosensor in 0.05 M phosphate buffer solution + 0.1 M KCl, pH 7.4. Applied potential + 200 mV vs Ag/AgCl. Signal recorded in phosphate buffer (*a*) and in a solution of butyrylthiocholine (5 mM) before the exposure of the biosensor to sarin gas (*b*) and after 1-min exposure to 0.1 mg m^{-3} (*c*) and to 0.5 mg m^{-3} (*d*) of sarin gas

measuring the residual activity of the enzyme. The optimized biosensor was tested with sarin and VX standard solutions, showing detection limits of 12 and 14 ppb (10% inhibition), respectively. Thus, we have challenged our biosensor against sarin gas. Two different concentrations of sarin gas (0.1 and 0.5 mg m^{-3}), at different incubation times (from to 30 s to 10 min), were tested. The results show that it is possible to detect sarin gas at 0.1 and 0.5 mg m^{-3} in 30-s incubation time, with a degree of inhibition of 43 and 34%, respectively.

The system developed allows us to detect low concentrations of sarin in a few minutes, according to the level IDLH, which is 0.1 mg m^{-3} [35].

To our knowledge the work presented here presents a high novelty feature; in fact there are no examples of amperometric devices based on cholinesterase inhibition tested for nerve agents in the literature owing to the difficultly in working with nerve agents. This kind of test is usually performed with mimic compounds, which have much easier handling characteristics.

Moreover, the procedure developed of exposing the biosensor to the gas flow of the nerve agents is an effective and simple approach in order to automate and miniaturize the system.

The analytical study with the real analytes (in this case nerve agents) has also highlighted some issues such as the lower inhibition of VX than sarin on BChE which could not be taken into account when using a model compound.

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