TOWARDS A NERVE GAS SENSOR FOR SECURITY PURPOSES: A SOLUTION PROVIDED BY ANALYTICAL CHEMISTRY FOR FAST DETECTION OF NERVE AGENTS USING COST EFFECTIVE, FAST FIELD INSTRUMENTS AND SENSITIVE PROBES

4.1 Introduction

Organophosphorous compounds such as Sarin (O-Isopropylmethylphosphonofluoridate), Soman (1,2,2-Trimethylpropylmethylphosphonofluoridate), Tabun (Ethyl N,N-dimethylphosphoramidocyanidate) and VX (O-ethyl-S-[2(diisopropylamino)ethyl] methylphosphonothioate) are potent nerve agents [1-4]. The extreme toxicity of nerve agents is due to their ability to bind primarily and rapidly to the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in the neuromuscular junction of the central nervous system and in blood, respectively. The high vapour pressures of these agents, their high toxicity, and their rapid effect on the central nervous system, combined with the low cost and a simple technology required for their synthesis and production of chemical weapons make them a suitable choice for terrorist attacks.

Nerve agents were employed against the Kurdish opposition in Iraq and Sarin gas was utilised in the terrorist attack by the Aum Shinrikyo sect in the Tokyo subway (1995) [5].

Also after the tragic events of September 11, 2001, there is an increased awareness of threats regarding use of chemical warfare agents. Analytical tools are therefore needed for the detection of these lethal chemicals since a rapid and reliable diagnosis is essential. Among the analytical techniques used for organophosphorous compound detection, gas chromatography and liquid chromatography have been most commonly used. These techniques have also been used to monitor the hydrolysis products of nerve agents in human plasma or urine. Minani et al [6] have demonstrated the presence of the hydrolysis product of Sarin (O-isopropyl methylphosphonic acid, IMPA) in the urine of victims of the Tokyo subway attack using gas-

chromatography with flame photometric detection. Chromatographic methods 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 specialised laboratory by skilled personnel and is not so suitable for miniaturization. The advantage of electrochemical sensors is their simple and ease preparation, the possibility to be miniaturized and cost effective. Moreover no specialized personnel is required to prepare and use them [7,8].

In literature, there are only a few papers [9-12] that report the monitoring of the nerve agents (Sarin, Soman, Tabun and VX) because, it is difficult to work with them in a non specialised laboratory, so model compounds such as paraoxon or the diisopropylfluorophosphate (DFP) are generally tested [13-16].

Mlsna et al. [9] developed a chemicapacitive sensors for chemical warfare agent analysis. The dielectric permittivity of the polymer filled chemicapacitors changed upon adsorption and desorption of the chemical vapors. 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 at 400°C.

A further approach for organophosphorous detection is based on biosensors that detect modulation of cholinesterase (ChE) activity [17-20]. White et al. [11] demonstrated the application of acetylcholinesterase based detection of Sarin using planar wave guide absorbance spectroscopy. Detection of levels of Sarin as low as 100 parts per trillion in solution and 250 pg in vapor are reported.

Lee et al. have described an assay system based on biotin-labeled ChE with streptavidin for nerve agent detection in liquid samples. LODs for Soman and Sarin were 2 and 8 pg,

respectively, for 10 min assay [12].

Our goal was the development a nerve agent detectors that can be incorporated in soldier overalls. For this purpose, the amperometric biosensor was chosen due to its high sensitivity and, also, to the possibility of being miniaturised and mass produced [21,22].

For nerve agent detection based on the amperometric measurement of ChE, two strategies can be adopted: the use of acetylcholinesterase (AChE) combined with choline oxidase together with the native substrate, acetylcholine; or the use of AChE alone with the synthetic substrate (thiocholine esters) [18,20].

The approach based on the electrochemical oxidation of thiocholine produced through the AChE catalyzed hydrolysis of the thiocholine ester, acetylthiocholine, is generally preferred because of the inherent simplicity and easiness to produce a cost effective single enzyme system. However, thiocholine detection at conventional electrodes requires high potentials with problems of the electrode surface passivation [23-25]. In this perspective, the electrochemical determination of thiocholine with cobalt phtalocyanine or TCNQ (7,7,8,8-tetracyanoquinodimethane) has been extensively studied [26-29]. Recently, the use of a Prussian Blue (PB) modified screen-printed electrode (SPE) has been demonstrated useful for the detection of thiocholine at a low applied potential (200 mV versus Ag/AgCl) due to the presence of Prussian Blue (PB) as mediator [30]. This electrode modification procedure allows an extremely easy and reproducible detection of thiocholine. In this work a biosensor based on butyrylcholinesterase (BChE) immobilized on an SPE modified with PB has been developed and tested.

Experiments concerning the biosensor optimisation were performed in our laboratory and paraoxon was used as a model compound instead of the nerve agents. Then, the developed biosensor was tested with Sarin and VX solutions in a chemical warfare specialized centre TNO Defence, Security and Safety in Rijswijk, Netherlands. The biosensor was also tested with Sarin gas.

4.2 Experimental section

4.2.1 Reagents

All chemicals from commercial sources were of analytical grade. Potassium ferricyanide from Carlo Erba (Milano, Italy) and potassium chloride from Fluka (St. Louis, USA). Butyrylcholinesterase (BChE) from horse, bovine serum albumine (BSA), S-butyrylthiocholine chloride (BTChCl), 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) and glutaraldehyde 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). Paraoxon was from Sigma Chemical Company (St. Louis, USA). Sarin and VX were provided by TNO, the Netherlands.

4.2.2 Apparatus

Amperometric measurements were carried out using a Palm Sens, Palm Instruments, Netherlands. Cyclic voltammetry (CV) was performed using an Autolab/PGSTAT-12 electrochemical system (Eco Chemie, Utrecht, The Netherlands). Screen-printed electrodes (SPEs) were produced 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 [21]. 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 cm2. Silver ink was used to print the reference electrode (see Ref. [31] for details of the electrode geometry). Before thiol measurement, the reference electrode was chlorurated by applying a potential of 0.6V between the silver ink and an external Ag/AgCl electrode for 20 s in a solution of KCl 0.1 mol l^{-1}[20].

4.2.3 Preparation of Prussian Blue (PB) modified screen printed electrodes

Prior to PB modification, SPEs were pre-treated in a 0.05 mol l^{-1} phosphate buffer + 0.1 mol l^{-1} 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 is obtained by mixing 5 μl of 0.1 mol l^{-1} potassium ferricyanide ($K_3Fe(CN)_6$) in 10 mmol l^{-1} HCl with 5 μl of 0.1 mol l^{-1} ferric chloride in 10 mmol l^{-1} HCl. The drop was carefully pipetted to be localised 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 mmol l^{-1} HCl. The electrodes were then left 90 min in the oven at 100° C to obtain a more stable and active layer of Prussian Blue [15].

The PB modified electrodes were stored dry at room temperature in the dark.

4.2.4 Preparation of a biosensor based on Prussian Blue (PB) modified screen printed electrodes

To immobilise the enzyme on the electrode surface, 2 μL of a 1% (v/v) glutaraldehyde solution were applied with a syringe exclusively on the working electrode. The solution was then left to evaporate. This, 2 μL of a mixture of BSA, enzyme and Nafion® were 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.

4.2.5 Preparation of Sarin and VX solutions

The nerve agents, Sarin and VX, were prepared in a phosphate buffer solution. The stock solutions were prepared from the pure agent each day; they were not used for more then two to five hours after preparation, to minimize the possible risk of hydrolysis of the agents.

4.2.6 Preparation of Sarin gas

An air stream contaminated with the vapor of the nerve agent Sarin was generated dynamically by evaporating the pure compound in a clean and dry nitrogen gas stream (figure. 4.1).

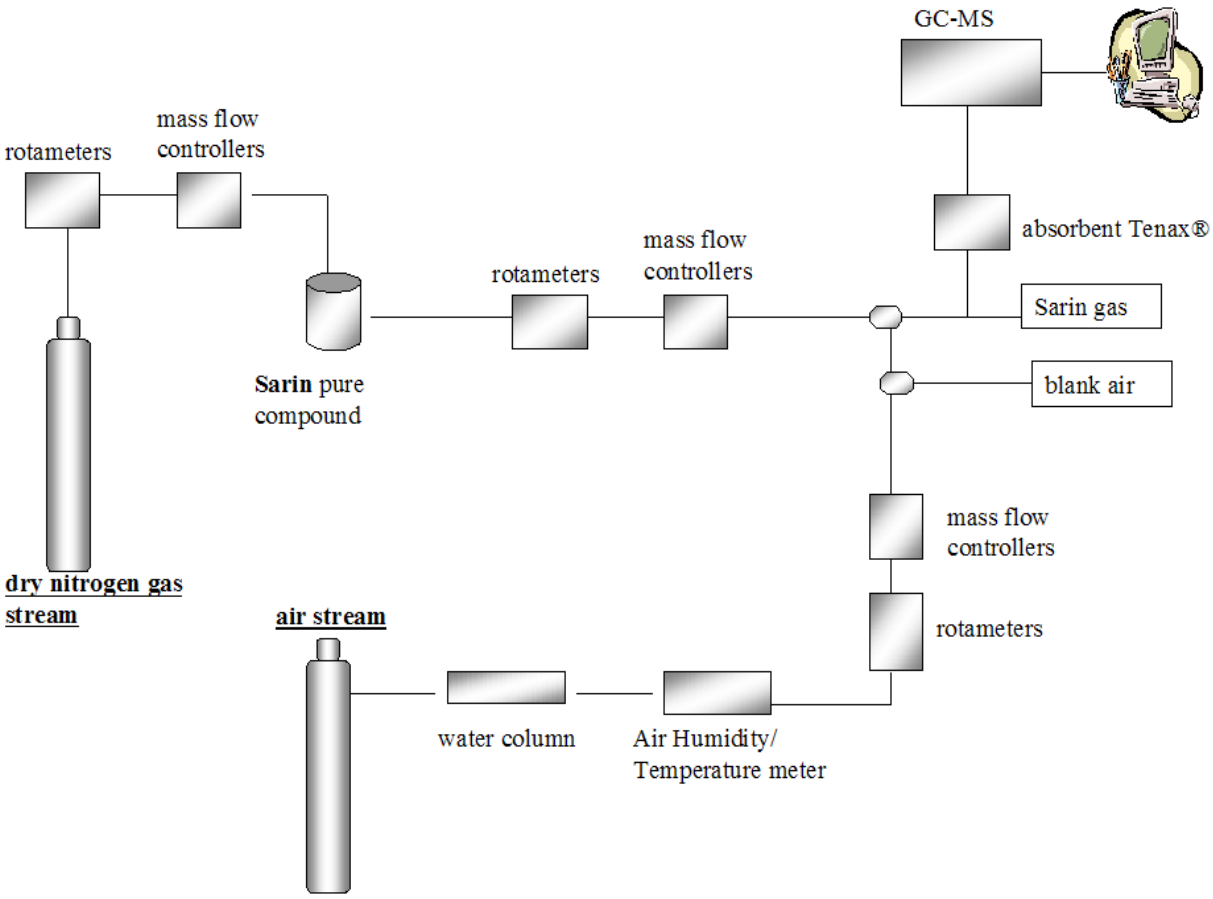


Figure 4.1 Experimental production of Sarin gas

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

The various gas flows have been set and controlled by Bronkhorst Hi-Tec type F-201 mass flow controllers and Rota-Wehr L63 type 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 being 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 (rH).

The target concentration of the generated Sarin vapor has been 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 have been used for the sampling of the generated sarin contaminated air stream.

The air stream (770 l/hr) served as the source for exposure of the sensors. A custom build glass bell jar device has been used to provide a stable and even flow across the sensors.

4.2.7 Biosensor enzymatic activity measurement

Butyrylthiocholine measurements were performed using a chronoamperometric “drop” procedure in phosphate buffer solution (0.05 M + 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 amount of butyrylthiocholine was placed onto the BChE biosensor, in such a way that the counter and reference electrodes were also covered. After applying the potential, the signal was recorded continuously and the current value at the steady state was measured. The time needed for the stabilisation of the current was 5 min.

4.2.8 Stability of BChE immobilized onto PB modified screen printed electrode

BChE immobilized screen-printed electrodes were divided into groups of two electrodes. Each group was used to measure one time point. The catalytic activity of the BChE immobilized screen-printed electrode was measured as describe above. The biosensors were dried at room temperature. Catalytic activity of each biosensor was measured.

4.2.9 Paraoxon standard solution 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 cholinesterase 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 toward the substrate was measured as described above and the degree of inhibition was calculated as a relative decay of the biosensor response (Equation 4.1).

$$I\% = [(i_0 - i_i)/i_0] \times 100 \qquad \text{Eq. 4.1}$$

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

4.2.10 Sarin and VX solution measurements

The inhibitory effect of Sarin and VX on the BChE biosensors was evaluated using the procedure adopted for paraoxon standard solution measurements.

4.2.11 Sarin gas measurements

The inhibitory effect of Sarin gas on the BChE biosensor was evaluated using procedures described in section 4.2.6. In this case, during the incubation time, the surface of the working electrode was covered with 5 μL of phosphate buffer 0.05 M +KCl 0,1, pH=7.4 and, then, the

biosensor was exposed to the blank air stream. After, the response toward the substrate was measured obtaining i_0 (see equation 4.1). 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 sensors were fixed one next to the other on the surface of the fume cupboard where the vapor stream was generated.

The system for Sarin gas production is characterized by a glass switching valve and, so, it was possible to switch very quickly between the blank air and the air stream containing the Sarin gas.

4.1.12 Safety considerations

The stock solutions of Sarin and VX and the stream of Sarin gas were prepared using of appropriate safety conditions created in TNO laboratory.

4.3 RESULTS AND DISCUSSION

4.3.1 Biosensor based on Prussian Blue modified SPE

The choice of electrochemical mediator for amperometric thiocholine detection has previously been evaluated. The electrochemical determination of thiocholine with cobalt phtalocyanine or 7,7,8,8-tetracyanoquinodimethane (TCNQ) have been extensively studied in literature. Recently our group has investigated the performance of Prussian Blue (PB) as a mediator of thiocholine oxidation [30]. The major advantage associated with the use of the Prussian Blue modified sensor is the very high stability even under strong conditions [32], thus making it very useful for practical applications. This mediator shows a good stability (when stored several months dry at room temperature in the dark) and good reproducibility (RSD=7%) [30], and, also, the modification of the electrode surface is easy to perform as well as being amenable to mass production techniques. Moreover, the Prussian Blue modified sensors allow the measurement of thiocholine at μM levels (LOD=5μM), with good

sensitivity (143 mA $M^{-1}cm^{-2}$) and a broad linear range ($5 \bullet 10^{-6}$-$5 \bullet 10^{-3}$ M) [30]. For these reasons, and given our long experience in the use of Prussian Blue 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 a rapid response, the choice of enzyme is a crucial point. In our case the choice of the ChE enzyme is based on the ability of nerve agents to irreversible inhibit these enzymes and also the high turnover number of ChE permits an activity measurement in a short time. In the literature, the development of ChE's engineered to have very high affinity towards organophosphorous compounds such as dicholrvos, malaoxon and paraoxon have been reported [33,34]. Among the various ChE enzymes available we had chosen AChE from *electric eel* and BChE from *horse serum* because these are commercial available. In fact, given a perspective to develop a biosensor for mass production, it is important to use reagents commercial available. In our 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 the AChE from *electric eel*. The BChE showed a higher inhibition than the AChE by both paraoxon and chlorpyriphos-methyl oxo belonging to the class of organophosphates. Because our goal is to develop a biosensor for nerve agents (organophosphorous compounds), the BChE from *horse serum* was chosen as enzyme to immobilize on Prussian Blue SPEs to develop a biosensor for nerve agent detection.

4.3.2 The BChE biosensor

The approach to detection nerve agents in this work is based on the biosensor developed for pesticide detection in our previous work [20]. In this work, the enzyme immobilization to create a nerve agent biosensor was optimized to obtain a faster and simpler measurement procedure whereas for the pesticide biosensor the immobilization was carried out using ChE

in the presence of glutaraldhehyde 1% v/v, BSA 5% w/v and Nafion 1% v/v, this formulation required a washing step between the measurements. 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 nerve agent biosensor, the membrane was altered by using Nafion® 0.1% (for details see experimental section). Using this type of membrane we have observed that is possible make repetitive measurements without an intervening washing step.

In addition, the concentration of enzyme was optimized. Due to the fact that nerve agents detection involves an irreversible inhibition of the enzyme, the lowest feasible concentration of enzyme was necessary to reach a low detection limit. The dependence of the response on the amount of the enzyme immobilized on the electrode surface is from 0.001U until to 0.100 U was investigated. A value of 0.025 U was chosen as the best compromise between a low enzyme loading, sufficiently high substrate signal and good storage stability, in order to have a biosensor that would be stable at RT and dry conditions. The reproducibility of the biosensors obtained under this condition was good: RSD% of 3% was observed for five replicates using the same biosensor (RSD% intra-electrode). Five different biosensors were also tested with the same concentration of BTChCl resulting in a RSD% value of 10% (inter-electrodes RSD%). Figure 4.2 shows the calibration curve obtained for different concentrations of substrate (i.e. BTChCl). 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 condition at RT with a residual percentage of enzymatic activity equal to (89±4)% .

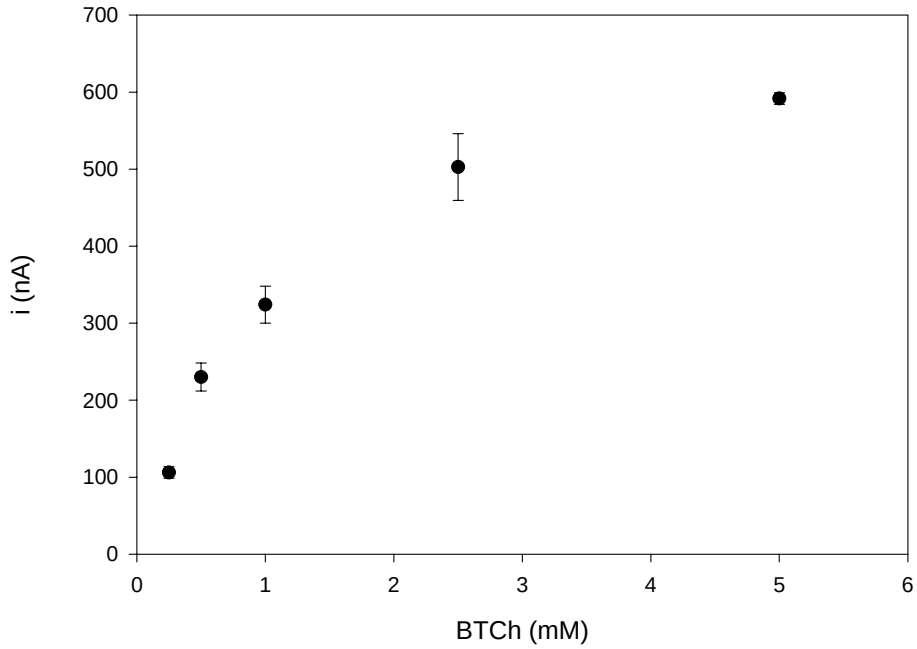


Figure 4.2 Calibration plot of butyrylthiocholine using an BChE biosensor. Applied potential:+200 mV vs Ag/AgCl. AChE= 0.01 U, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4.

4.3.3 Preliminary studies: paraoxon determination using BChE biosensors

Before the Sarin detection, the biosensor was optimized using as a model the organophosphorous compound paraoxon. Its irreversible inhibition is characterized by a higher degree of inhibition at longer incubation times (reaction time between inhibitor and enzyme), thus the study of incubation time was carried out (table 4.1). 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.

Incubation time (min)	Degree of inhibition (I) %			
	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

Table 4.1 Study of incubation time using BChE biosensors and different organophosphates (Paraoxon or Sarin or VX). BTChCl (5 mM) were used a substrate. Applied potential: +200 mV vs Ag/AgCl, phosphate buffer 0.05 M+ KCl 0.1 M, pH 7.4. Measurement carried out in "drop condition

Following the incubation with inhibitors, the biosensor was rinsed three times with distilled water and the residual enzymatic activity was measured using a new solution of phosphate buffer containing substrate (medium exchange method [35,36]). For paraoxon inhibition, all the measurements were carried out using a "drop system". A linear range from 25 until to 100 ppb of paraoxon was obtained (table 4.2). Measurements were replicated three times and an average RSD of 7% was obtained.

Organo-phosphate	Linear range (ppb)	Detection limits (ppb) (10% of inhibition)	50% of inhibition (ppb)	R^2
Paraoxon	25-100	17	85	0,97
Sarin	10-20	10	20	0,88
VX	20-150	18	180	0,89

Table 4.2 Analytical parameters BChE biosensors relative to different organophosphates. BTChCl (5 mM) were used a substrate. Applied potential: +200 mV vs Ag/AgCl, phosphate buffer 0.05 M+ KCl 0.1 M, pH 7.4. Incubation time=10 min. Measurement carried out in "drop condition".

4.3.4 Sarin and VX determination using the BChE biosensor

The biosensor optimised using paraoxon as model organophosphorous compound was then used to test standard solutions of Sarin at the TNO laboratory. The standard solutions were prepared in phosphate buffer solution at pH=7.4 and were used only for 8 hours, because its known the hydrolysis of nerve agents in solution [4]. The study of incubation time was carried out using 200 ppb of Sarin solution ranging from 1 min until to 5 min of incubation or 20 ppb at incubation times equal to 5 and 10 min (table 4.1). An incubation time equal to 10 min was chosen for the calibration curve. Under these conditions, the linear range proceeds up to a limiting value of 20 ppb (table 4.2). The detection limit defined as 10% of inhibition was equal to 10 ppb. The results obtained showed that Sarin inhibition was slightly higher than paraoxon inhibition towards the enzyme on biosensor. To better understand the results obtained, a study of BChE inhibition by VX was carried out, knowing that VX inhibits more strongly ChE than Sarin (the value of LD_{50} for VX was found to be equal to 10 mg/person while the LD_{50} of Sarin is equal to 1700 mg/person [37]). For this study, incubation time using a VX solution of 200 ppb was carried out from 1 min until to 10 min (table 4.1). Also in this case, an incubation time of 10 min in phosphate buffer was ultimately selected as a good compromise between the requirement for a rapid assay and an achievement of high degree of inhibition. A linear range from 20 ppb up to a limiting value of 150 ppb was obtained (table 4.2). The detection limit defined as 10% of inhibition was equal to 18 ppb.

As shown in table 4.1, using 5 min as incubation time and 200 ppb of VX, a degree of inhibition equal to (31±4)% was obtained whereas in the same conditions (5 min of incubation time) but using 200 ppb of Sarin as nerve agents, the degree of inhibition was (70±2)%. The results obtained seem to suggest that the immobilization adopted for SPE can change degree of inhibition by nerve agents, due probably to the diffusion through the membrane. In fact, as can be seen in figure. 4.3, the VX have a major steric hindrance than Sarin, probably give more difficult diffusion into the membrane. To better understand these

results the inhibition was carried out in batch mode under stirring condition using 200 ppb of VX. Under these conditions, and using an incubation time of 5 min, a degree of inhibition equal to 43% (with lower RSD equal to 1%) was obtained, that is an increase of 10%, approximately. These results seem to confirm that the lower degree of inhibition is due to the lower diffusion of VX through the membrane relative to Sarin.

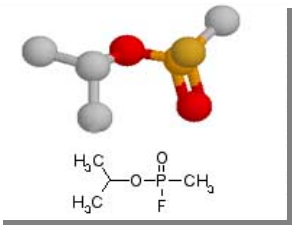


Figure. 4.3 a. Structural formula of Sarin

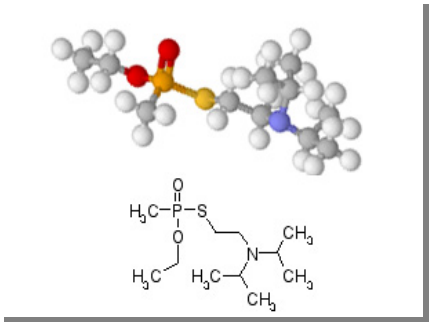


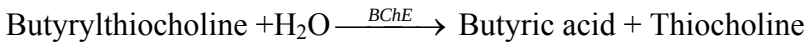
Figure. 4.3 b. Structural formula of VX

4.3.5 Sarin gas measurements

Our purpose is to develop a biosensor for Sarin gas measurement, given that in terrorist attack the Sarin in gas phase has been utilised, the terrorist choice of Sarin gas being due to the possibility to contaminate a bigger space in a few minutes. Sarin gas in the TNO laboratory was produced with a sophisticated instrument realised by them (figure. 4.1). We tested only two different concentrations of Sarin gas equal to 0.1 mg/m^3 and 0.5 mg/m^3 at different incubation times (from to 30 s until to 10 min) because the preparation of Sarin gas is difficult to achieve. These concentrations were chosen knowing that the level Immediately Dangerous to Life or Health (IDLH) is equal to 0,2 mg/m^3 for 5 min [38].

Firstly, the exposure of the biosensor to Sarin gas at 0.5 mg/m^3 in dry condition and in wet condition was evaluated. The incubation step was carried out in different ways. For the measurement made in the wet condition, was added 5 µL of phosphate buffer at pH=7.4 on

the biosensor over the working electrode, while in dry condition no buffer was added. As expected, only under wet conditions the inhibition (equal to 97±3%) was observed, in fact during the reaction between Sarin (inhibitor) and ChE a molecule of water is required, as it can be seen in scheme 4.1.



Scheme 4.1 Enzymatic reaction

Moreover, as a control the effect on the biosensor of the air stream in absence of nerve agent in wet condition was evaluated. After 10 min of exposure no change in biosensor activity was observed, showing that the air stream itself had no effect on enzyme activity. A plot of the relative inhibition of BChE activity as a function of incubation time using Sarin gas at 0,5 mg/m^3 is reported in figure. 4.5a and a typical response of butyrylthiocholine using the BChE biosensor before and after the exposure are reported in figure. 4.4.

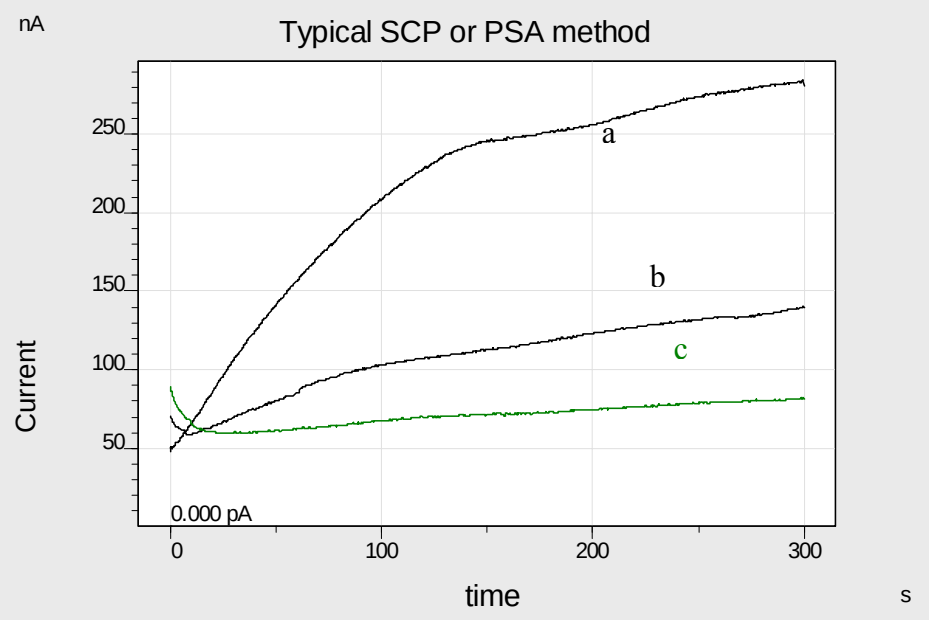


Figure. 4.4 Original recording obtaining using biosensor in phosphate buffer solution 0,05 M +KCl 0,1 M , pH 7.4. Applied potential: + 200 mV vs Ag/AgCl.

a) signal recorded using a solution of butyrylthiocholine 5 mM in phosphate buffer before the exposure of biosensor at Sarin gas;
b) signal recorded using a solution of butyrylthiocholine 5 mM in phosphate buffer after the exposure of biosensor at 0.1 mg/m^3 of Sarin gas;
c) signal recorded using a solution of butyrylthiocholine 5 mM in phosphate buffer after the exposure of biosensor at 0.5 mg/m^3 of Sarin gas.

As shown in figure 4.5 a, 30 s of incubation time is necessary to obtain a degree of inhibition equal to (45±4)% using 0.5 mg/m^3 of Sarin gas. This showed that the procedure exposing the biosensor (with few μL of phosphate buffer on the working electrode) to the nerve agent gas is an effective approach and also quite simple as it is only important that the biosensor be in wet condition with buffer (phosphate buffer at pH=7.4 for the ChE activity optimal conditions).

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

After the good results obtained with 0.5mg/m^3, a lower concentration of Sarin gas was tested (0.1 mg/m^3). The results obtained are shown in figure 4.5b and the response of butyrylthiocholine using BChE biosensor before and after the exposure is reported in figure 4.4.

Also in this case it is possible to detect Sarin gas using an incubation time of 30 s with a degree of inhibition equal to (34±1)%. However, with a incubation time of 1 min, twice inhibition was obtained (56±1)% (figure 4.5b), showing a linear correlation between incubation time and degree of inhibition. In fact the linearity of inhibition degree is usually

observed in a range from 20% until to 60% degree of inhibition [39]. As we can see in the figure 4.4, the time of measurement of enzymatic activity was chosen equal to 5 min. However, to make a fast measurement of nerve agents a shorter measurement time measuring would be possible measuring the rate of enzymatic reaction in the first minute instead 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 measurement time of 2 min, a limit lower both for time and concentration than Immediately Dangerous to Life or Health (0,2 mg/m^3 for 5 min).

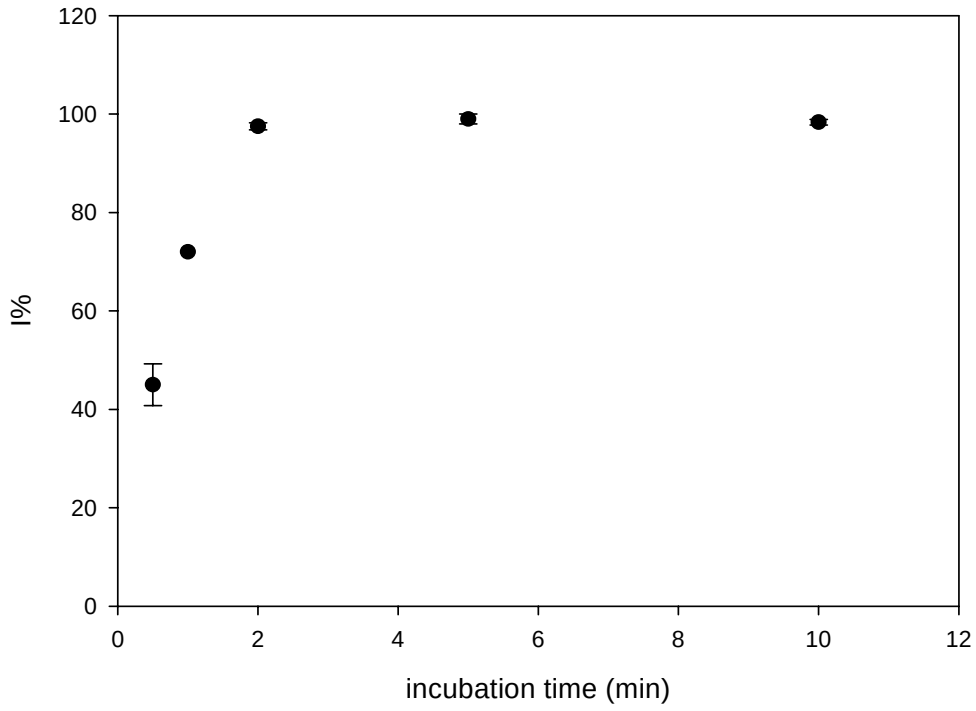


Figure. 4.5a Plot for the relative inhibition of BChE activity as a function of incubation time using Sarin gas at 0,5 mg/m^3 . Measurement conditions: 0.05M phosphate buffer +KCl 0.1 M, pH=7,4; applied potential +200 mV (vs Ag/AgCl), 5 mM BTChCl..

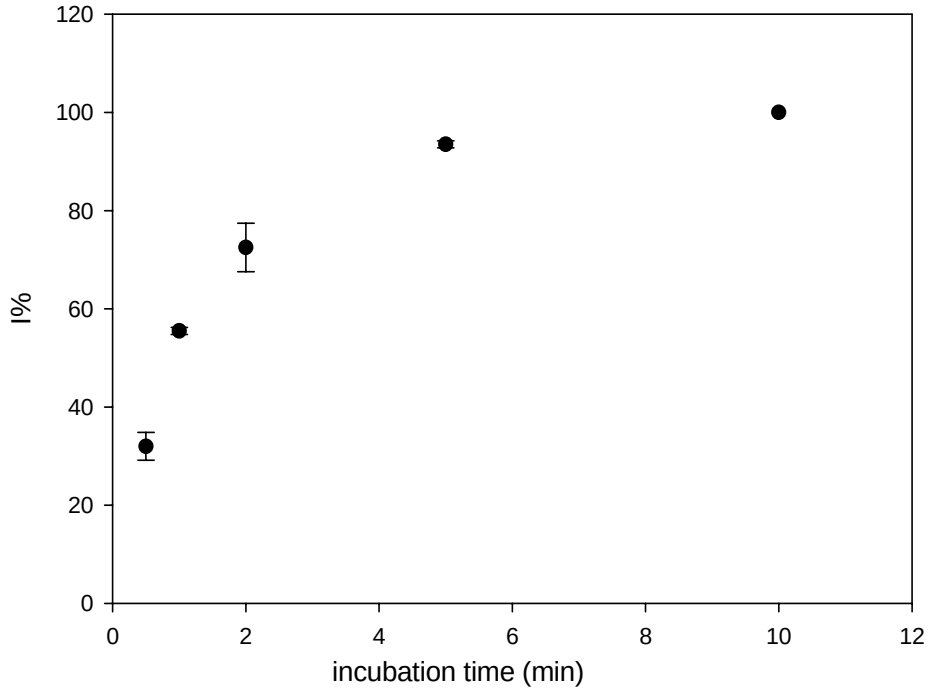


Figure. 4.5b Plot for the relative inhibition of BChE activity as a function of incubation time using Sarin gas at 0,1 mg/m^3. Measurement conditions: 0,05M phosphate buffer +KCl 0,1 M, pH=7,4; applied potential +200 mV (vs Ag/AgCl), 5 mM BTChCl.

4.4 Conclusions

In this work the biosensor for nerve agents detection was developed. The system was project in order to be incorporated in soldier's overalls. For this purpose, the amperometric technique was chosen due its high sensitivity and also because of the possibility to miniaturise and mass produce. The butyrylcholinesterase (BChE) has been immobilised onto screen printed electrodes modified with Prussian Blue (PB-SPE) and the nerve agents detection was evaluated measuring the residual activity of enzyme. The optimised biosensor was tested with

Sarin and VX standard solutions showing detection limits of 10 ppb and 18 ppb (10% of inhibition), respectively. Given that, in terrorist attacks, the Sarin in gas phase has been utilised, thus we have challenged our biosensor against the Sarin gas. Two different concentrations of Sarin gas (0.1 mg/m^3 and 0.05 mg/m^3), at different incubation times (from to 30 s until to 10 min), were tested. It is possible to detect the Sarin gas in 30 s as incubation time, with a degree of inhibition of 43% and 34%, respectively.

The results obtained showed that using the developed system it is possible to detect low concentration of Sarin in a few minutes according to the level Immediately Dangerous to Life or Health (IDLH) is equal to 0,2 mg/m^3 for 5 min [38].

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