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Lab-on-a-chip for rapid electrochemical detection of nerve agent Sarin

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Abstract This paper reports a lab-on-a-chip for the detection of Sarin nerve agent based on rapid electrochemical detection. The chemical warfare agent Sarin ($C_4H_{10}FO_2P$, O-isopropyl methylphosphonofluoridate) is a highly toxic organophosphate that induces rapid respiratory depression, seizures and death within minutes of inhalation. As purified Sarin is colourless, odourless, water soluble and a easily disseminated nerve agent, it has been used as a weapon in terrorist or military attacks. To ascertain whether potable water supplies have been adulterated with this extremely potent poison, an inexpensive, sensitive and easy to use portable test kit would be of interest to first responders investigating such attacks. We report here an amperometric-based approach for detecting trace amounts of Sarin in water samples using a screen-printed electrode (SPE) integrated in a microfluidic chip. Enzymatic inhibition was obtained by exposing the immobilised biosensor in the microfluidic platform to Sarin in water samples. With the aid of cobalt phthalocyanine modified SPE, the device could detect Sarin at part-per-billion levels with concentration as low as 1 nM. The detection method reported here represents a significant improvement over the authors'previous optical-based detection method.

Keywords Lab-on-a-chip · Microfluidics · Electrochemical detection · Screen-printed electrode · Nerve agent · Sarin

Abbreviations

AChE	Acetylcholinestease enzyme
Ag	Silver
AgCl	Silver chloride
APTES	(3-Aminopropyl)triethoxysilane
ATChI	Acetylthiocholine iodide
ATChCl	Acetylthiocholine chloride
C	Carbon
CO_2	Carbon dioxide
CWA	Chemical warfare agent
DTNB	5,5-dithio-bis-2-nitrobenzoic acid
ECD	Electrochemical detection
GB	Sarin
OD	Optical density
OP	Organophosphorous
PDMS	Polydimethylsiloxane
PMMA	Polymethymethacrylate
ppb	Parts per billion
SPE	Screen-printed electrode
TCh	Thiocholine

1 Introduction

Nerve agents are a class of potent organophosphorus (OP) compounds that inhibit cholinesterase irreversibly and are highly toxic to humans (Delfino et al. 2009; Burnworth et al.

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2007; Hadd et al. 1999) Cholinesterase is a family of enzymes that catalyse the hydrolysis of the neurotransmitter acetylcholine (ACh) into acetate and choline. Acetylcholinesterase (AChE) and butrylcholinesterase (BuChE) are hence of interest in most clinical toxicology studies on organophosphorus chemicals. AChE is found mainly in the central nervous system and also on the membranes of circulating red blood cells. When synaptic AChE is inhibited, the hydrolytic activity of the enzyme is suppressed, resulting in the accumulation of excessive acetylcholine in neural synapses or neuromuscular junctions. Accumulated ACh exerts excessive stimulatory effects on target organs leading to respiratory arrest, status epilepticus and eventually death. In view of their extreme toxicity, relative lack of odour and colour, highly toxic OP compounds, such as the nerve agent Sarin and OP pesticide Parathion, pose viable threats to potable water sources. There is hence a need for cost-effective, portable, rapid and yet reliable OP biosensors to monitor for adulteration of potable water sources by these chemicals.

Numerous methods for detecting organophosphorus compounds have been reported in the literature, e.g. gas chromatography-mass spectroscopy (GC-MS) (Polhuijs et al. 1997; Barak et al. 1997), colorimetric (Tan et al. 2008; Viveros et al. 2006), electrochemistry (Arduini et al. 2007; Joshi et al. 2005; Du et al. 2012; Du et al. 2011; Ding et al. 2008), fluorescence (Bencic-Nagale et al. 2006) and capillary electrophoresismass spectrometry (CE-MS) (Lagarrigue et al. 2008) For field-based detection of OP compounds, the method adopted should provide good sensitivity, selectivity and portability. While GC-MS and fluorescence instruments generally provide good sensitivity for detecting trace levels of nerve agents, they unfortunately require relatively large sophisticated instrumentation that limits its portability. In contrast, electrochemical detection offers a good alternative for the development of field-based devices. The method requires relatively simple instrumentation with low power requirement while providing good sensitivity and selectivity. Electrochemical detection of nerve gas has been reported by different groups. Joshi et al. (2005) reported on the detection of nerve gas using carbon nanotube (CNT) modified thick film strip electrode. Wang et al. (2004) used chip-based detection of nerve gas using conductivity measurement. Arduini et al. (2007) applied amperometric method for the detection of nerve gas using a Prussian blue modified biosensor.

Microfluidic technologies have been widely explored to develop miniaturised devices for field-based applications allowing measurement to be done away from traditional laboratories. Such devices are referred to as lab-on-a-chip that can offer analytical results in a faster, simpler and cheaper manner as there is a reduction in the amount of samples and reagents needed for the analysis. Furthermore, microfluidic technologies can offer new platforms for the implementation of complex detection assays on a single device. (Nguyen et al. 2006) In this aspect, cheaper and faster analysis is possible as compared to traditional laboratory-based instruments.

In our previous work, absorbance detection of Sarin in blood was integrated on a lab-on-a-chip device. (Tan et al. 2008) However, absorbance measurement of colour changes has drawbacks during the implementation for field based applications. Thus, in the present work, we explore the feasibility of electrochemical detection of Sarin on a screen-printed electrode (SPE) integrated on a lab-on-a-chip device containing immobilised AChE. Such a single-use electrode is particularly attractive for field-based deployment as it can be mass produced at low cost while retaining good sensitivity and selectivity for OP compounds. Thus, incorporating electrochemical detection for Sarin onto the immobilised AChE microfluidic platform has good potential for the eventual development of a hand-held device for field-based applications. The detection concept is based on the following enzyme substrate reaction:

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Organic acid} \quad (1)$$

where thiocholine can be detected using electrochemical reaction:

$$2\text{Thiocholine} \xrightarrow{\text{Oxidation}} \overbrace{\text{dithio}-\text{bis}-\text{choline}}^{\text{Anion detected at 0.IV}} + 2e^- + 2H^+ \quad (2)$$

2 Materials and methods

2.1 Materials and fabrication of microchips

Our microfluidic device for electrochemical detection was fabricated using polymethylmethacrylate (PMMA) substrate. The top and bottom plates (Fig. 1) containing the microchannels were designed using Solidworks 2012 (Dassault Systèmes SolidWorks Corp, U.S.A.). A commercial CO_2 laser machining system (Versa Laser VLS 2.30, Universal Laser Systems, U.S.A.) transferred the designed structures on the PMMA plates. The CO_2 laser has a wavelength of 10.6 μm and a maximum power of 25 W. The maximum speed of the laser beam is 640 mm/s. The different channel heights can be adjusted by the corresponding laser power and scanning speed. Thermal bonding was conducted to bond the top and bottom plates together. A constant pressure of 2 MPa was applied for 5 min at 102 °C using a hydraulic press (Specac, Model GS15011, Ark Scientific Pte Ltd, Singapore).

The AChE immobilization process was similar to that reported previously by our group. (Tan et al. 2008) The only difference is the use of a different substrate for AChE

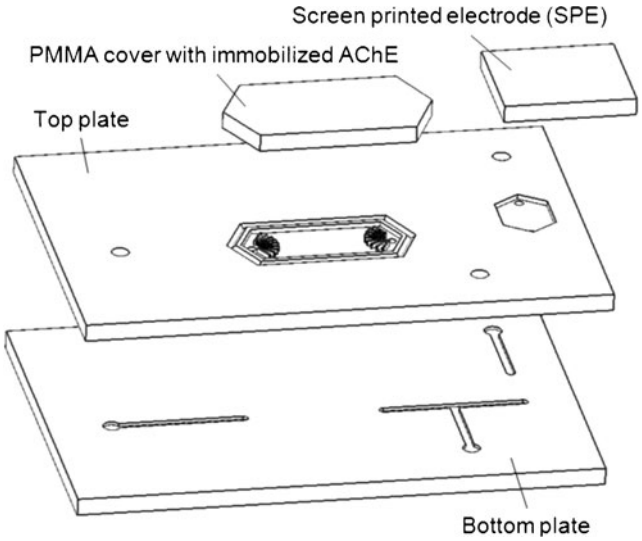


Fig. 1 Design of the microfluidic device used in the tests

immobilization. Previously, AChE was immobilized on a glass cover-slip. In the present work, we immobilized AChE on the polymer substrate PMMA. This PMMA cover, with immobilized AChE, was bonded to the main PMMA plate using ultrasonic welding (Herrmann Ultrasonics, PSDDC) to avoid heat-induced denaturation of the immobilised AChE. The bonding concept is based on focusing the ultrasonic energy to a specific protruding structure called energy director machined on the PMMA cover (Fig. 1). During the welding process, the energy director is heated up and fuses with the substrate creating a permanent bond. The PMMA cover for AChE was fabricated via hot embossing (Specac, Model GS15011, Ark Scientific Pte Ltd, Singapore). A computer-numerical-control (CNC) milled aluminium master and a PMMA wafer of 4 in. diameter were clamped together and heated up to 155 °C at a constant pressure of 3.63 MPa for 2 min. Subsequently, the temperature was reduced and the PMMA piece was separated from the mould at 70 °C. Multiple covers can be embossed on one PMMA wafer. The CO_2 laser was used to cut the PMMA wafer into individual covers.

Multiple SPEs were created on a single polycarbonate substrate. Prior to that, the designed patterns were made into a mask. Following that, the patterns were transferred onto the screen. A total of 5 patterned screens were prepared; each for a single print layer. The printing pastes Ag, C, AgCl, Mediated carbon and Dielectric (supplied by the Gwent Group, U.K.) were deposited using an industrial screen printer (Microtec MT550-TVC, Hibex Singapore Pte Ltd, Singapore). After printing each layer, the polycarbonate substrate was placed into an oven (Memmert UFE 600, Memmert GmbH + Co KG, Schwabach) for curing. Lastly, the polycarbonate substrate was cut into individual SPEs using an industrial dicing saw (DAD341, Disco Corporation, Japan). Figure 2 shows a typical SPE fabricated using the process described above.

The SPEs were bonded to the PMMA plate using a biocompatible ultra-violet (UV) curable medical adhesive (Dymax 1180-M, Blaze Technology Pte Ltd, Singapore). The adhesive was dispensed onto the surface of the SPE by a dispenser (Dymax T20010, Blaze Technology Pte Ltd, Singapore) to the region to be bonded. After dispensing, the SPE was aligned to the chamber found on the PMMA base substrate. The adhesive was then cured by exposing it to an UV spot light source with a wavelength of 365 nm for 30 s to create a permanent, leak-free bond between the SPE and the PMMA substrate. Figure 3 shows the assembled lab-on-a-chip device containing immobilised AChE and incorporated with a SPE sensor.

2.2 Reagents and chemicals

Sarin used in the present study was synthesized in-house within DSO National Laboratories, Singapore. Autoclaved ultra-pure water was used to dilute the Sarin sample. Extra precautions were taken in handling Sarin during spiking and disposal of the waste. All experiments involving Sarin were conducted within a negative suction fume hood in DSO National Laboratories and all materials involved were decontaminated after use. Phosphate buffers (PB) of pH 8.0 and pH 7.2 were prepared by mixing Na_2HPO_4 and autoclaved water. The enzyme AChE from electrophorus electricus was purchased from Fluka (Switzerland). The stock solutions of the substrates ATChI (acetylthiocholine iodide) and ATChCl (acetylthiocholine chloride) (Sigma-Aldrich, USA) were prepared in 0.1 M PB, respectively. Bovine Serum Albumin (BSA) and glutaraldehyde (GA) were purchased from Sigma Chem. Co (St. Louis, MO). Both were used in the crosslinking solution for the enzyme immobilising procedure onto the PMMA cover. Trehalose ($C_{12}H_{22}O_{11}.2H_2O$) was purchased from Fluka (UK). Gelatine (Type B from Bovine skin) was purchased from Sigma Chem Co (St. Louis, MO). A trehalose (10 % wt), gelatine (0.1 % wt) and sodium azide (0.02 wt%; Fluka) mixture was used as the protection layer for the immobilised enzyme, to permit its storage at room temperature. The protective layer is removed with a wash buffer (DSO 2007) prior to its use for Sarin detection.

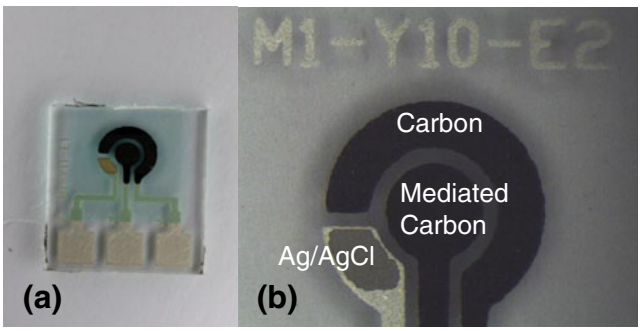


Fig. 2 Screen-printed electrdode **a** Co-Phthalocyanine mediated SPE; **b** The electrode region

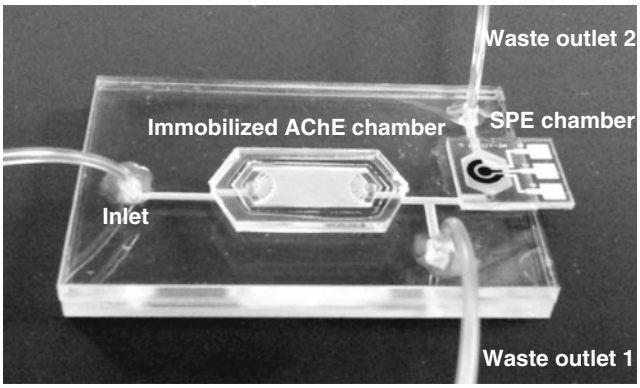
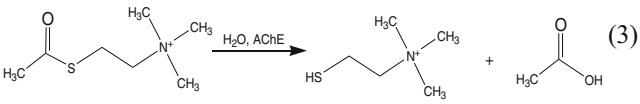


Fig. 3 Immobilized AChE and SPE chip used in experiments

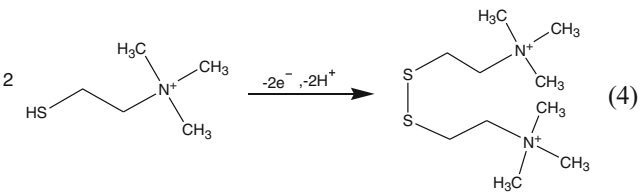
2.3 Experimental

The samples and reagents were introduced into the microchips with SPEs by a syringe pump (CMA 100 microinjection pump, Stockholm, Sweden). The microchips with SPEs (Fig. 2) were fabricated by Singapore Institute of Manufacturing Technology (SIMTech) and DSO National Laboratories. When AChE reacts with acetylthiocholine, acetate acid and thiocholine are formed as the products:



(3)

Thiocholine produced by the above enzymatic hydrolysis of acetylthiocholine is oxidised at a suitable applied potential:



(4)

The resulting current output correlates to the concentration of thiocholine ions present in the solution. This end-point assay approach, through the measurement of amount of end-products formed within a fixed substrate reaction time, is used to estimate AChE activity. As a high working potential of the above detection scheme is more prone to chemical interference and subsequent electrode fouling, we employed cobalt-phthalocyanine modified SPE (Hart and Hartley 1994; Pchelintsev and Millner 2008; Pritchard et al. 2004) for the present work. This permitted the application of a low working potential of approximately 100 mV, applied against the Ag/AgCl reference electrode, resulting in a much lower background current.

Autolab station and General Purpose Electrochemical Systems software (GPES, Version 4.9.004) were used to perform amperometric experiments on the SPE. The SPE was bonded to the PMMA microfluidic device. The device consists of a chamber with immobilized AChE and another chamber to house the SPE. The following formula was used to calculate the percentage of AChE activity remained on microchip after inhibition by Sarin in water samples. The remaining AChE activity will correspond to the amount of enzyme inhibition by Sarin.

$$\alpha = \frac{I_{post}}{I_{pre}} \times 100\% \quad (5)$$

3 Results and discussion

3.1 Investigation of optimum voltage for amperometric experiments

An initial experiment was performed with a SPE chip (Fig. 4) to determine the optimal voltage for amperometric detection of thiocholine. AChE and ATChCl were mixed at room temperature for at least 1 h to ensure the complete reaction of the substrate ATChCl with AChE. PB (pH 8.0, 0.1 M) was then pumped into the chip at a flow rate of 100 μl/min. Current values were taken for a duration of 120 s. Three sets of PB background current value I_{PB} were taken. These values were averaged at the plateau region and recorded as the baseline for the electrode. Next, the reacted AChE and ATChCl solution was pumped into the electrode well at the same flow rate of 100 μl/min. Similarly, three sets of current values I_{TChCl} were obtained. The change in amperometric readings in the presence of thiocholine ions was evaluated:

$$\Delta I = I_{TChCl} - I_{PB} \quad (6)$$

The potential investigated ranged from 0.1 V to 0.5 V. Figure 5 showed that at V_{in}=0.1 V, the amperometric readings are the highest as compared to other V_{in}. Henceforth, V_{in}=0.1 V was the optimal voltage applied for detection of thiocholine with the cobalt-phthalocyanine modified SPEs.

3.2 Detecting trace concentrations of Sarin in water samples with immobilized AChE and SPE

The assembled lab-on-a-chip device used for indirect amperometric detection of Sarin is shown in Fig. 3. As mentioned above, the detection of Sarin is based on the irreversible inhibition of AChE immobilised within the device. Within a fixed inhibition period, the amount of Sarin present could be correlated to the level of AChE inhibition achieved. However, as the amount of thiocholine formed by the remaining uninhibited AChE is a function of substrate reaction time, the latter could influence the assessment of percentage AChE inhibition when an end-point assay is used here to determine

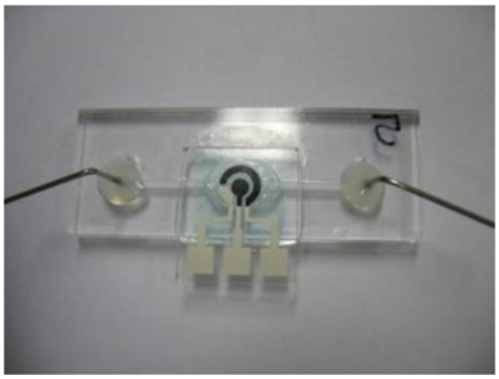


Fig. 4 SPE chip used for amperometric analysis of thiocholine at different applied potentials

remaining AChE activity as opposed to a kinetic AChE assay approach. The influence of substrate reaction time on device's ability to detect trace levels (10 nM) Sarin is investigated as follows.

The device was first flushed with the proprietary wash buffer (WB) described in the "Scentmate" patent. (DSO 2007) The enzyme chamber was soaked with the WB for about 10 min prior to using the enzyme chamber for the experiments. As the composition of WB has a detrimental effect on the performance of the SPE, WB is prevented from entering the electrode chamber by blocking the waste outlet 2 with a syringe. At the cross-junction, the WB was seen exiting from the waste outlet 1 preventing WB entering the electrode chamber. After soaking the enzyme chamber for approximately 5 min, WB was flushed out with the syringe pump. PB was then delivered into the microchips at a flow rate of Q=100 μl/min and amperometric readings were taken. These data were recorded as the baseline reading of the electrode. Next, ATChCl was pumped into the chambers at Q=100 μl/min. Once both chambers were filled up, the flow was then stopped. It is important that no air bubbles were trapped in either chamber.

Next, enzyme-substrate reaction was allowed to take place. Twenty seconds before the stipulated reaction time, the

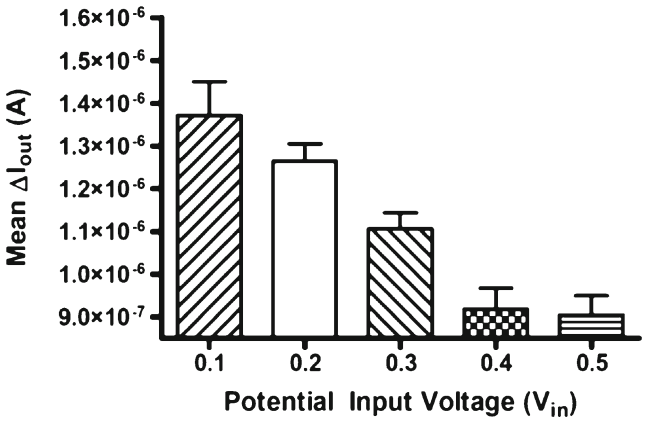


Fig. 5 Effect of applied potential on the amperometric response of oxidation of thiocholine on cobalt phthalocyanine modified SPE integrated in a chip (n=3)

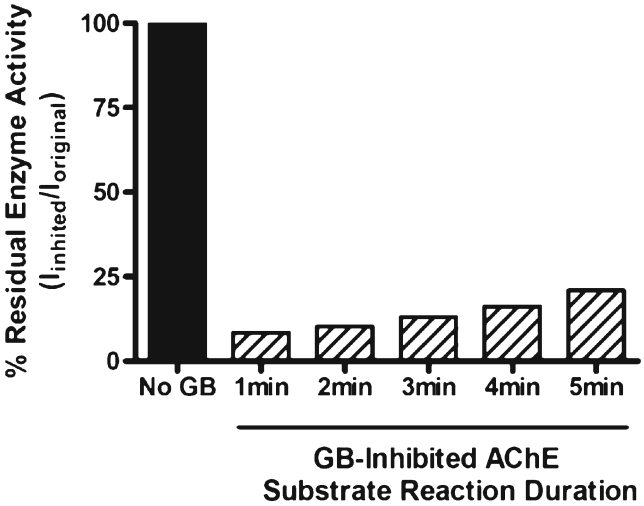


Fig. 6 Investigations on effect of varying AChE assay durations on ratio of I_{TChC} derived from pre- and post- inhibition of immobilized AChE enzyme by 10 nM of GB; V_{in}=0.1 V

electrochemical station was activated. The syringe pump was turned on 20 s later. The data collected was subsequently plotted. The same procedure was repeated for 2, 3, 4 and 5 min of substrate reaction time. All data collected were tabulated and compared. After getting the pre-inhibition readings, a dilute aqueous solution of Sarin (10 nM) was pumped into the enzyme chamber until the chamber was filled up. The syringe pump was stopped and inhibition was allowed to take place for 15 min. The dilute Sarin solution was removed and ATChCl was pumped back into the enzyme chamber. The previous protocol of varying substrate reaction duration was repeated and amperometric readings recorded for each reaction time instance.

Data obtained was used to compare between the pre- and post-currents, denoted as I_{pre} and I_{post} respectively. The ratio of these 2 currents was determined per substrate reaction duration. This ratio is then used as an estimation of residual AChE activity as illustrated in Fig. 6. The results show that the introduction of Sarin into the enzyme chip leads to a drop of

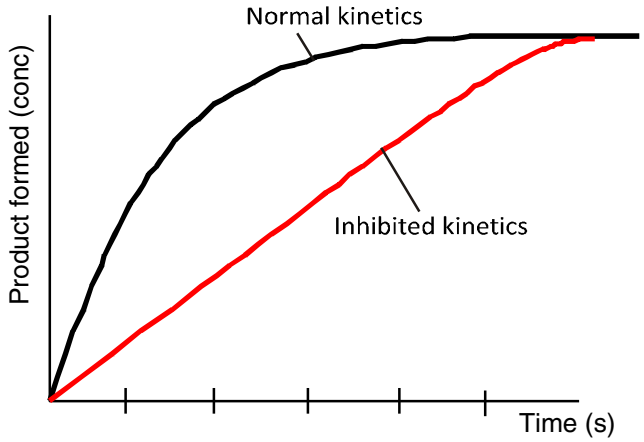


Fig. 7 Schematic of enzyme reaction kinetics: normal and inhibited plot

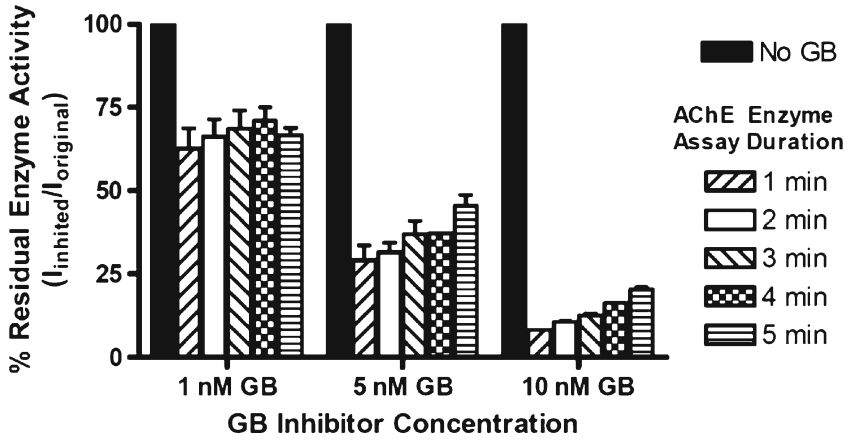


Fig. 8 Percentage of AChE remained on microchip after inhibition by different GB concentration, $n=2$

active AChE and a corresponding drop in thiocholine level, which is detectable as a much lower amperometric current. I_{post}. In addition, at the same Sarin inhibitor concentration, the ratio of pre- and post-current was observed to increase with increasing substrate reaction times (Fig. 6), which could be interpreted erroneously as a reduced inhibition of enzyme activity arising from presence of lower concentration of Sarin. Although this artificial increment in I_{post} is small, it is nonetheless important to fix the duration of reaction between the enzyme and substrate to ensure consistent I_{post} readings.

To understand the influence of substrate reaction duration on the perceived level of residual enzyme, one needs to compare the typical enzyme kinetics of a normal uninhibited enzyme reaction with that of an inhibited enzyme (Fig. 7). While the uninhibited enzyme displays initial rapid substrate hydrolysis rate that flattens out early, the inhibited enzyme has a much lower substrate hydrolysis rate. Hence, it is obvious from Fig. 7 that the ratio of end-product formation by the uninhibited and inhibited AChE would change during substrate hydrolysis, thus accounting for the changing ratio of pre- and post-current observed with the GB-inhibited AChE in Fig. 6.

Three different GB concentrations were subsequently investigated with the above mentioned procedure with a substrate hydrolysis duration similarly varied from 1 to 5 min. The results, illustrated in Fig. 8, indicate that the percentage of AChE remaining on chip depends on both substrate reaction time and the concentration of Sarin.

With an increase in Sarin concentration, the percentage of AChE activity levels remaining on the microchip becomes smaller. When the degree of AChE inhibition was lower, as observed with 1 nM Sarin, the influence of substrate reaction duration on the estimated percentage of residual enzyme activity was lesser. The results clearly show that the combination of SPEs and the immobilized AChE in the microchip is able to detect Sarin in water samples in a concentration-dependent manner with the lower detection limit of 1 nM Sarin. This performance of the lab-on-chip device for detection of Sarin in aqueous solution is superior to conventional gas chromatography based analytical methods in terms of detection speed, detection limit and amount of sample required.

3.3 Comparison of optical method with electrochemistry for detection of GB

We compared the performance of the current method with a previously reported lab-on-chip device using optical detection of AChE activity. (Tan et al. 2008) The results, illustrated in Fig. 9, indicated that the natural logarithm of (Inhibited vs Original Current ratio) is correlated linearly against the concentration of Sarin inhibitor. It has been established in prior studies (Loke et al. 1998; Wandhammer et al. 2011) that under a pseudo-first order reaction condition, the inhibitor concentration could be related in linear fashion to the residual enzyme activity through this equation [Inhibitor]$=b-a\times$ ln(% enzyme activity). Henceforth, Fig. 9 suggests that end-point enzyme assay by electrochemical detection provides a good estimate of enzyme activity that is typically derived from kinetic assays tracked continuously by optical means. Assessment of residual

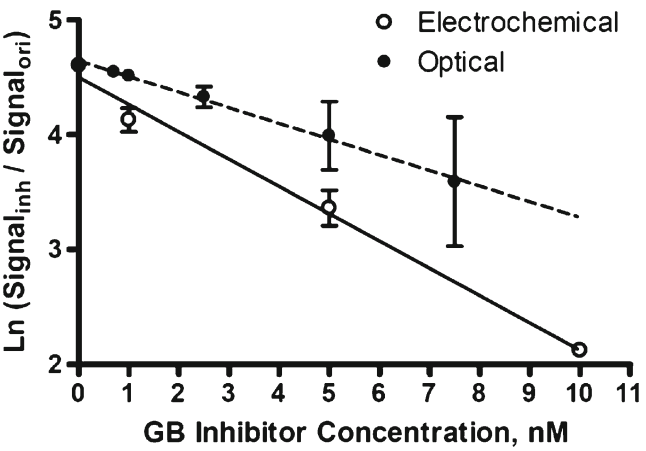


Fig. 9 Comparison of percentage of AChE remained after inhibition by different GB concentrations in water samples

enzyme activity by electrochemical detection resulted in a steeper drop in (post/pre) signal ratio with increasing Sarin concentration as compared with optical assay. At the lowest Sarin concentration (1 nM), a significant drop of 37.3 % was observed with (post/pre) signal ratio. In contrast, the optical detection method recorded a smaller reduction of 10 % in (post/pre) signal ratio at a similar concentration of 1 nM Sarin. Moreover, optical detection displayed a higher variance in enzyme assay reading as Sarin concentration increases. The comparison shown here indicated that electrochemical detection is more sensitive and reliable than optical detection for monitoring enzyme inhibition by OP agents. Electrochemical detection is therefore recommended over optical detection for implementation within a handheld device in view of its lower power requirement, simpler instrumentation and superior portability.

4 Conclusions

We reported the design, fabrication and test of a microchip with cobalt-phthaolocyanine modified SPEs that was able to detect trace amounts of Sarin in aqueous medium. The results demonstrated that the microchip was able to successfully detect the presence of Sarin at a concentration down to 1 nM. The enzyme activity was tested by obtaining the amperometric reading of the resulting thiocholine product ions. Experiments were carried out for the determination of the optimum applied potential for amperometric studies of the thiocholine. We also reported the kinetics of the different substrate reaction time with the inhibited enzyme. The performance of the amperometric detection was then compared to the optical detection. The data obtained indicated that the electrochemical detection is more sensitive. For field deployable applications and devices, it is necessary for the device to be sensitive, portable, rugged and consume minimum power. With the reported investigations, we had shown that electrochemical method is more sensitive than the optical method and has a good potential for field applications.

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