

A lab on a chip for detection of nerve agent sarin in blood

Hsh Yin Tan^a, Weng Keong Loke^a, Yong Teng Tan^a, and Nam-Trung Nguyen^{a,b}

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Sarin (C₄H₁₀FO₂P₂O-isopropyl methylphosphonofluoridate) is a colourless, odourless and highly toxic phosphonate that acts as a cholinesterase inhibitor and disrupts neuromuscular transmission. Sarin and related phosphonates are chemical warfare agents, and there is a possibility of their application in a military or terrorist attack. This paper reports a lab-on-a-chip device for detecting a trace amount of sarin in a small volume of blood. The device should allow early detection of sarin exposure during medical triage to differentiate between those requiring medical treatment from mass psychogenic illness cases. The device is based on continuous-flow microfluidics with sequential stages for lysis of whole blood, regeneration of free nerve agent from its complex with blood cholinesterase, protein precipitation, filtration, enzyme-assisted reaction and optical detection. Whole blood was first mixed with a nerve gas regeneration agent, followed by a protein precipitation step. Subsequently, the lysed product was filtered on the chip in two steps to remove particulates and fluoride ions. The filtered blood sample was then tested for trace levels of regenerated sarin using immobilised cholinesterase on the chip. Activity of immobilised cholinesterase was monitored by the enzyme-assisted reaction of a substrate and reaction of the end-product with a chromophore. Resultant changes in chromophore-induced absorbance were recorded on the chip using a Z-shaped optical window. Loss of enzyme activity obtained prior and after passage of the treated blood sample, as shown by a decrease in recorded absorbance values, indicates the presence of either free or regenerated sarin in the blood sample. The device was fabricated in PMMA (polymethylmethacrylate) using CO₂-laser micromachining. This paper reports the testing results of the different stages, as well as the whole device with all stages in the required assay sequence. The results demonstrate the potential use of a field-deployable hand-held device for point-of-care triage of suspected nerve agent casualties.

Introduction

In recent years, the growing concern of terrorist attacks has resulted in a demand for point-of-care diagnostic devices that allow fast and effective triage of casualties exposed to chemical warfare agents from the “worried-well” masses. Sarin (C₄H₁₀FO₂P₂O-isopropyl methylphosphonofluoridate) is a phosphonate that is highly toxic to humans because it acts as an acetylcholinesterase (AChE) inhibitor to disrupt neuromuscular transmission. Sarin belongs to the G-type nerve agents and is colourless and odourless. The lethal concentration-time dose of sarin for 50% of exposed individuals (humans) is 75 to 100 mg min⁻¹ m⁻³.¹ Sarin and related phosphonates are chemical warfare agents and there is a possibility of their application in a military or terrorist attack.² Thus, there is a need for its detection in the prevention of an attack and for early treatment after exposure. Fluorescence is frequently used for enzyme activity detection in the presence of a nerve agent. The enzyme can be immobilised on packed microbeads to increase the contact surface area.³ Zhang and Swager developed fluorescence-based functional group specific ratiometric chemosensors.⁴ Simonian

et al. developed a detection scheme based on a competitive inhibitor, whose change in fluorescence is generated by gold nanoparticles attached to the enzyme.⁵ Viveros *et al.* used an optical wave guide with immobilised enzyme to detect the change in fluorescence.⁶ Bencic-Nagale *et al.* used nerve-agent-reactive microbead probes with fluorescence as the detector.⁷ All of these reported approaches did not use a microfluidic platform for sample analysis, and none of the papers reported above worked with whole blood as the sample.

Microfluidic technologies have been shown to be a promising approach for the integration of protocols for detection of nerve agents. Nerve agents are detected based on inhibition and non-inhibition concepts. In the first approach, the nerve agent works as an inhibitor of an enzyme such as AChE. The enzyme controls a hydrolysis reaction whose product can be detected electrochemically or optically. Hadd *et al.* reported a micro-fabricated device for detecting AChE inhibitors such as nerve agents.⁸ The inhibitor reduces the activity of the enzyme, which in turn affects the hydrolysis of the substrate leading to a decrease in fluorescence signals. In the non-inhibition bioassay, the nerve agents are separated by capillary electrophoresis and detected electrochemically and optically. Wang *et al.* used a microchip for capillary electrophoresis separation of organophosphate nerve agents,⁹ in which amperometric detection was employed. Pumera gave a detailed review on the detection of nerve agents using capillary electrophoresis.¹

^aDSO National Laboratories, 20 Science Park Drive, Singapore 118230

^bSchool of Mechanical and Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798.
E-mail: mntnguyen@ntu.edu.sg

Recently, lab-on-a-chip (LOC) for diagnostics of blood samples has been an active research topic.¹⁰ The challenge of such devices is the integration of the preparation and the analysis of a sample. Sample preparation usually starts with the whole blood. Before entering the analysis stage, the sample should pass through separators, cell lysis reactors, microfilters, and concentrators. Since only some components of the whole blood are of interest for the later analysis, it is necessary to separate these components from the rest. Cell separation is usually the first step in sample preparation. The next step is breaking down the cell membrane in a cell lysis reactor. The sample is subsequently filtered to reject particulates and other components that may affect the later analysis. Before entering the analysis stage, the analyte can be preconcentrated to improve detectability. Readers may refer to a recent review by Toner and Irimia on preparation of blood samples.¹⁰ Liu *et al.*¹¹ reported a LOC for sample preparation, polymerase chain reaction amplification, and DNA microarray detection. The polymeric LOC device for blood typing reported by Kim *et al.*¹² uses whole blood as the input sample and consists of sequential stages such as micromixer, microreactor and microfilter. There were very few reports on complete integration of all processes from sample preparation to detection on a single LOC device.

Previously, a prototyping technology for LOC devices made of PMMA was established by our research group. LOC devices for polymerase chain reaction of deoxyribonucleic acids¹³ and for detection of sarin in water samples¹⁴ were developed and tested. In this paper, we report the design, fabrication and test of a polymeric LOC for detection of sarin in blood samples. The following sections report the detailed implementation of the detection approach based on absorption measurements of the inhibition reaction. The design and fabrication of different components of the LOC are also discussed.

Detection approach of nerve agent, sarin

The LOC device reported in this paper uses diagnostic technology called “Scentmate”,¹⁵ developed and patented by DSO National Laboratories, Singapore. Its implementation in a microfluidic platform can reduce the amount of blood sample required and possibly also the analysis time. Fig. 1 gives a scheme of the processes used in our LOC device. At stage (a), nerve agent is regenerated from whole blood. An aliquot of whole blood enters the device at inlet 1 and is mixed with a nerve gas regeneration agent entering at inlet 2. The regeneration reaction was realised in a micromixer. With the introduction of a protein precipitation chemical into the sample, process stage (b) serves as both reaction and filter for precipitated blood proteins. Stage (c) is realized as a chamber with packed beads to further trap the particulates and to remove the fluoride ions F⁻ from the sample. At stage (d), the sample is mixed with a substrate for the enzyme-based hydrolysis reaction and a chromophore to indicate the activity of the reaction of the enzyme. In stage (d), the enzyme is immobilised in the reaction chamber. For a longer shelf-life, the enzyme is protected by a coating layer. In this case, the coating layer is removed with an extra washing step before the measurement. If nerve agent exists in the blood sample, the enzyme is inhibited, hence hydrolysis of substrate is prevented and chromophore remained unconverted. As a result, the sample

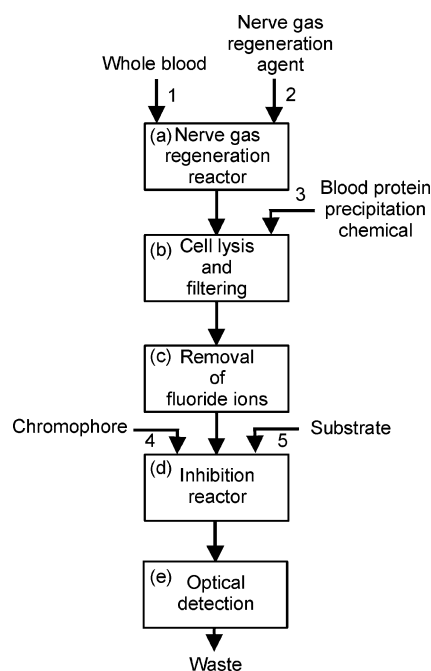


Fig. 1 Schematic approach for detection of nerve agent sarin in blood.

entering stage (e) is almost colourless. Before testing the blood sample, a base line measurement is carried out with the substrate, the chromophore and the enzyme only. The colour can be determined by an absorbance measurement at the wavelength of 412 nm. Fig. 2 depicts the schematic diagram of sarin inhibition of acetylcholinesterase and consequential inability to produce a colorimetric outcome.

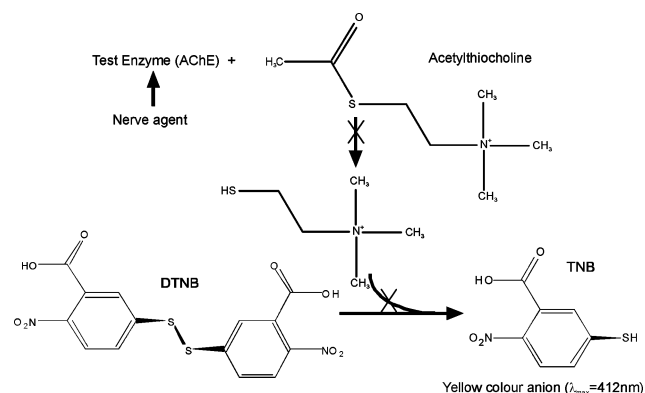


Fig. 2 Schematic diagram of sarin intoxication on acetylcholinesterase.

The ratio between the measured absorbance with a blood sample and the measured baseline absorbance is defined as the remaining enzyme activity:

$$\alpha = \frac{A_{\text{with sample}}}{A_{\text{without sample}}} \times 100\% \quad (1)$$

A drop in absorbance or remaining enzyme activity indicates the existence of sarin. The lower the remaining activity, the higher is the concentration of sarin in the sample. These five process stages make a single on-line analysis of nerve agent in whole blood possible.

Experimental

Samples and reagents

The nerve agent sarin (GB) used in our studies was synthesized in-house within DSO National Laboratories. Whole blood was drawn from an adult male and spiked with sarin and used as the sample for our experiments. The synthesized sarin was first diluted using autoclaved water. Next, the solution was pipetted into a blood sample to obtain the required concentration for the experiments. Extra precautions had to be taken while 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.

Potassium fluoride (KF) works as the nerve gas regeneration chemical, which reacts with the spiked blood and regenerates sarin. A saturated salt solution (SAS) works as the blood protein precipitation chemical.

Phosphate buffers (PB) of pH 8.0 and pH 7.2 were prepared by mixing Na_2HPO_4 , NaH_2PO_4 and autoclaved water. The chromophore DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) (Sigma, USA) solution was prepared by dissolving DTNB in 0.1 M PB. The enzyme AChE from *Electrophorus electricus* was purchased from Fluka (Switzerland). The substrate ASChI (acetylthiocholine iodide) (Sigma–Aldrich, USA) was prepared by dissolving ASChI into 0.1 M PB. Bovine serum albumin (BSA) and glutaraldehyde (GA) were purchased from Sigma Chem Co (St. Louis, MO, USA). Both were used in cross-linking solution for the immobilised enzyme procedure. Trehalose ($\text{C}_{12}\text{H}_{22}\text{O}_{11}\cdot 2\text{H}_2\text{O}$) was purchased from Fluka (UK). Gelatine (Type B from bovine skin) was purchased from Sigma Chem Co (St. Louis, MO, USA). Trehalose (10% wt) and gelatine (0.1% wt) were mixed with sodium azide (0.02 wt %) (Fluka, Switzerland) to form a solution (GST solution) which was used to protect the immobilised enzyme on the glass piece, allowing storage of the immobilised enzyme at room temperature. The surfactant 50% Tween 20 (Polysorbate 20, 1.05–1.07 kg l^{-1}) and approx. 50% lauric acid were purchased from DUCHEFA (Haarlem, Netherlands). These two reagents were used for preparing the wash buffer that removes the protecting GST layer above the immobilised enzyme.

Chromic acid was prepared by dissolving 5 g of potassium dichromate (Alfa Aesar, USA) in 5 ml of autoclaved water before adding 100 ml of concentrated sulfuric acid (Merck, Darmstadt, Germany).

Design and fabrication of the lab-on-a-chip device

The LOC device is implemented on a 79 mm × 37.5 mm chip. Fig. 3 shows the overall design with the five stages from (a) to (e). The reactor for nerve gas regeneration is designed as a micromixer based on chaotic advection with herring-bone structures. The channel width and depth are 2 mm and 0.4 mm, respectively. The total length of the mixing channel is 100 mm. The herring-bone structures are designed as ridges with a width of 0.5 mm and a height of 0.2 mm, Fig. 3(a). Unlike the traditional design¹⁶ with two secondary flows and alternate width changes, our new design reverses the direction of the secondary flow in each period to improve the folding effect on each half of the channel. Since this paper focuses on the

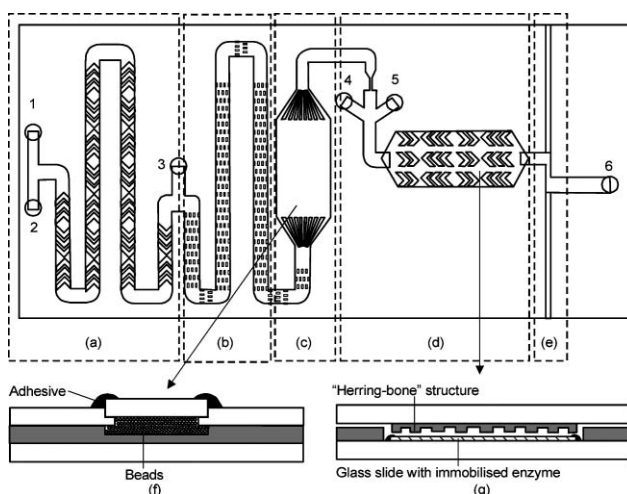


Fig. 3 Design of the lab-on-a-chip for detection of nerve agent in blood: (a) nerve gas regeneration reactor; (b) cell lysis and filtering; (c) removal of fluoride ions; (d) inhibition reactor; (e) optical detection.

whole chip design, details of the effectiveness of this micromixer will be investigated and reported in the future. Experimental results of the micromixers are reported in the subsequent sections. The reactor for precipitation of blood proteins is designed as a mixing channel with rectangular pillars for filtering the particulates resulting from the blood protein precipitation process. The rectangular pillars measure 0.2 mm × 0.5 mm and are positioned in an array as depicted in Fig. 3(b). The filter chamber is designed as a chamber with packed filtration beads. The chamber is 15 mm long, 7 mm wide and 0.6 mm high. The inlet and outlet of the chamber are designed with smaller microchannels to prevent the microbeads escaping from the channel, Fig. 3(c). Before the inlets 4 and 5 for the substrate and the chromophore, the width of the channel is narrowed to prevent backflow while introducing these chemicals into the inhibition reactor. The inhibition reactor contains a glass piece coated with immobilised enzyme and a protective coating. The reaction chamber measures in length, width and depth, 15 mm, 7 mm and 0.6 mm, respectively. The reaction chamber is located on the other side of the main device layer, with herring-bone patterns to improve the transport of reagents to the glass surface with immobilised enzyme, Fig. 3(d). The reaction products enter the detection path which is 4 mm long and 0.6 mm wide. Groves are machined into the chip for aligning optical fibres.

The device was fabricated in polymethylmethacrylate (PMMA) using laser micromachining. The device was first designed using CorelDraw (Corel Co., Canada). A commercial CO_2 laser system (Universal M-300 Laser Platform, Universal Laser Systems Inc., Arizona, USA) engraved the designed structures on the PMMA substrate. 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^{-1} . The different channel heights can be adjusted by the corresponding laser power and scanning speed.

The device depicted in Fig. 3 was implemented in three basic PMMA layers. The main layer contains on one side, the channel structures, the herring-bone patterns, the rectangular pillars and the guides for the optical fibres. The herring-bone structures in the inhibition reactor as shown in Fig. 3(d) are machined on the

other side of the main layer. The access holes and the opening for packing the filtration beads as depicted in Fig. 3(c) and 3(f) were machined in the cover layer. The bottom layer holds the glass piece for immobilised enzyme. The three PMMA layers were bonded using the low-pressure, high-temperature thermal bonding process previously developed by our group.¹⁷ Under low bonding pressure, the PMMA stack was first heated to 160 °C in 10 min, and then kept at 160 °C for 30 min. Next, the bonded stack was cooled down to 80 °C in 20 min and annealed at this temperature for another 30 min to relieve the stress. Finally, the bonded device was cooled to room temperature and removed from the hot plates.

After bonding the PMMA layers, filtration beads (Merck, Darmstadt, Germany) with diameters ranging from 63 µm to 200 µm were packed into the filter chamber (Fig. 3(c)) through the opening in the cover layer. The filter chamber was subsequently sealed by an adhesive (Araldite, Huntsman Advanced Materials, Utah, USA) with a PMMA sheet, Fig. 3(f).

The enzyme was immobilised on a small glass piece. Glass cover-slips (Fisher Scientific, Pittsburg, PA, USA) thickness 0.1 mm were cut into pieces measuring 15 mm × 7 mm. The glass piece was cleaned using chromic acid and rinsed in ethanol and dried in an oven at 100 °C. The glass piece was subsequently coated with APES (3-aminopropyl)triethoxysilane (Sigma–Aldrich, USA), a chemical used for cross-linking AChE. Before the thermal bonding process described above, the glass piece was positioned using an adhesive on the bottom PMMA layer. After the thermal bonding process, cross-linked enzyme solution (10 units ml⁻¹ AChE, 2.38 µg ml⁻¹ BSA, 0.119% wt/vol GA) was pumped into the inhibition chamber. The whole device was kept at 4 °C for 12 h. Subsequently, the enzyme solution was washed by pumping the washing buffer (1 part 50% Tween 20 and 1000 parts 0.1 M PB pH 7.2) into the inhibition chamber. Next, the GST solution (10% wt/vol trehalose, 0.1% wt/vol gelatine and 0.02% wt/vol sodium azide) was pumped into the reaction chamber. The LOC device was then kept at 4 °C for 4 h. After 4 h incubation, the GST solution was pumped out from the inhibition chamber. The LOC device was lyophilised at -40 °C overnight and subsequently placed in a vacuum desiccator for 2 h. This step ensures the long-term storage of the device with immobilised enzyme.

After the immobilisation of the enzyme on the APES-coated glass piece and it was protected with a GST layer, the optical fibres were inserted and fixed with adhesive, Fig. 3(e). For this purpose, we used single-mode optical fibres (AFS105/125Y, Thorslab Inc., Newton, NJ, USA) with a core diameter of 105 µm, a clad diameter of 125 µm, and a numerical aperture of 0.22. The buffer layer was removed before the fibres were inserted into the groves machined previously by the CO₂ laser. The completed device without the optical fibres is shown in Fig. 4.

Detection method

The absorbance measurement using the two optical fibres depicted in Fig. 3(e) was carried out with a portable fibre optic spectrometer USB2000 and its corresponding software SpectraSuite (Ocean Optics, Florida, USA). One optical fibre is connected to the light source (wave length 405–680 nm) purchased from Photonitech, Singapore. The other optical

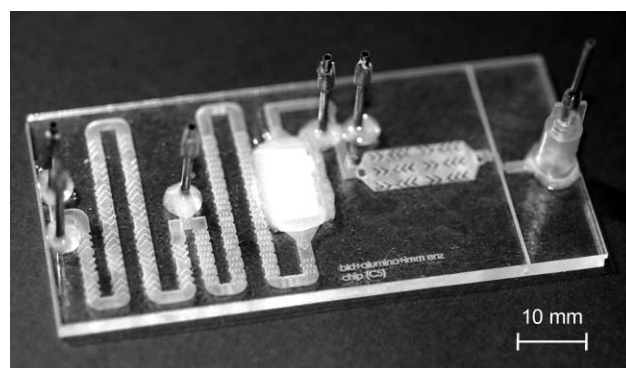


Fig. 4 The fabricated LOC device.

fibre is connected to the optical input of the spectrometer. The absorbance signal is acquired, displayed and stored as numerical data. After recording the base-line absorbance and the absorbance of the reaction product with the blood sample, the remaining enzyme activity was calculated according to eqn (1).

Results

Test of the micromixer

In the following experiments, the samples and chemicals were delivered into the LOC device using a programmable syringe pump (CMA 100 microinjection pump, Stockholm, Sweden).

The micromixer was tested using a fluorescent dye (C₂₀H₁₀Na₂O₅). The 10× TBE buffer [108 g Tris-base, tris-(hydroxymethyl)aminomethane, 55 g boric acid, and 40 ml 0.5 M ethylene diamine tetraacetic acid (pH 8.0) for 1 l, autoclaved for 20 min] was added to the dye solution to enhance the emitted fluorescence. The dye has an excitation wavelength and an emission wavelength of 490 nm and 520 nm, respectively. A CCD camera attached to an inverted epi-fluorescence microscope (Eclipse TE200-S, Nikon, Japan) was used to record the concentration field of the fluorescent dye. Fig. 5 shows the images of the fluorescent dye at different places along the mixing

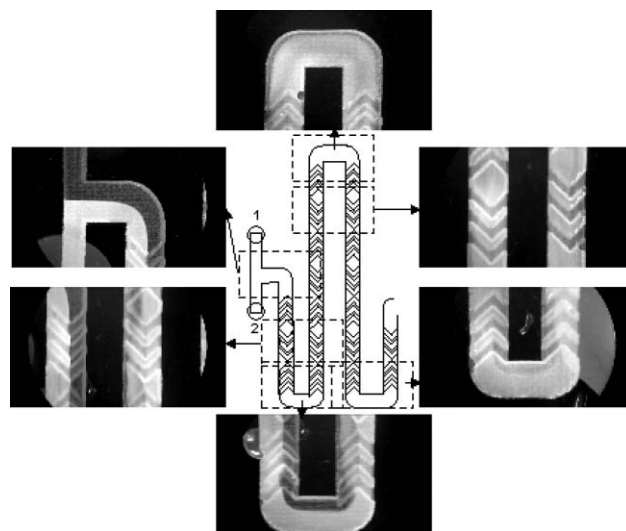


Fig. 5 Concentration field in the micromixer at a flow rate of 140 µl min⁻¹.

channels at a flow rate of $140 \mu\text{l min}^{-1}$. At the entrance, the fluorescent and non-fluorescent liquids were clearly separated by an interface. With the help of the herring-bone structures and the turns, stretching and folding were observed. The results showed that the herring-bone structures were able to induce chaotic advection in the mixing channel.

To evaluate the advantage of the herring-bone structures, an AChE–ASChI (enzyme–substrate) assay test was conducted on a micromixer with herring-bone structures and another micromixer without these structures. The measured absorbance data was plotted and compared in Fig. 6. The micromixer with herring-bone structures showed a clear improvement in detected absorbance.

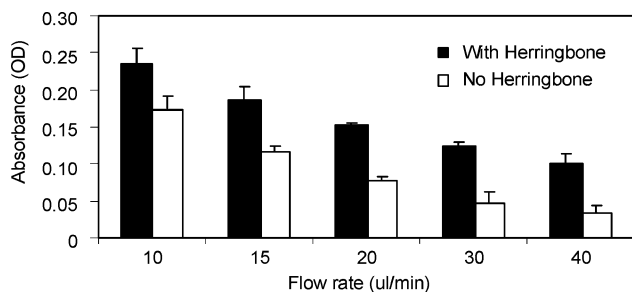


Fig. 6 Comparison of the effectiveness of mixing in a micromixer with herring-bone structures and a micromixer without the herring-bone structures.

Optimization of flow rates

The flow rates of the sample and the reagents are directly related to the residence time and the time needed for enzyme-assisted reaction between the substrate and the chromophore, as well as the inhibition reaction between the nerve agent and the enzyme. A slow flow rate increases the residence time and the corresponding absorbance signal but also the total analysis time. Therefore, a compromise between signal strength and analysis time needs to be achieved.

The reaction kinetics between substrate and chromophore was measured using a spectrometer (8453 UV-Visible spectrophotometer, Agilent Technologies, USA). The reagents for this test have the same concentration and ratio as used later in the LOC device. $50 \mu\text{l}$ of each reagent (0.9 mM DTNB , 1.35 mM ASChI and $0.1084 \text{ units ml}^{-1} \text{ AChE}$) were mixed and placed in the measurement cuvette. The detection path length of the cuvette is 10 mm . Fig. 7(a) depicts the time function of the measured absorbance. The results show that the absorbance needs about 20 min to reach saturation. Upon comparing these results with the data depicted in Fig. 6, a flow rate of $20 \mu\text{l min}^{-1}$ was chosen for future experiments.

In the test with the LOC device, the residence time was estimated based on the flow rate, the channel cross sections, the channel length and the optical path length of the measurement. The projected absorbance (the solid line in Fig. 7(b)) was taken from the time function shown in Fig. 7(a). The dashed line depicted in Fig. 7(b) is the absorbance signal collected from the LOC device without immobilised enzyme. Chromophore DTNB, substrate ASChI and enzyme AChE were pumped into the reactor with the same flow rate. Braun–luer-locked 5 ml

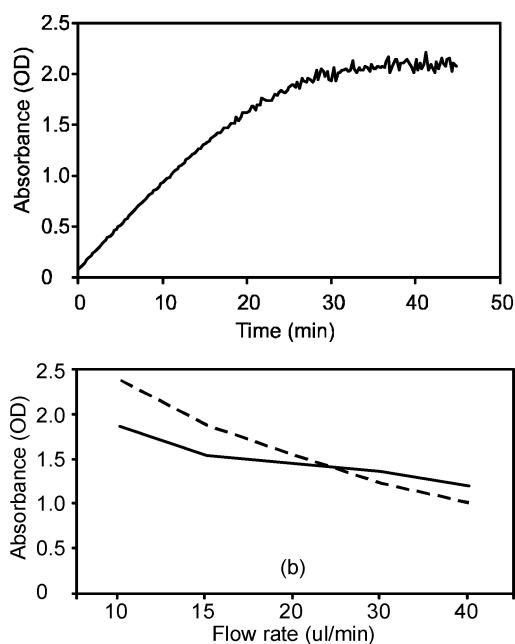


Fig. 7 Reaction kinetics and dependence on flow rate: (a) time function of absorbance measured with a commercial UV-Vis spectrometer; (b) projected and actual absorbance as function of flow rate and residence time.

syringes were used in the test. As shown in Fig. 7(b), at a flow rate of about $25 \mu\text{l min}^{-1}$, the absorbance measured in the LOC device reached the same level as that of a commercial cuvette. Thus, a flow rate of $20 \mu\text{l min}^{-1}$ was selected for each syringe in the subsequent test to ensure the same signal strength as in a commercial detection system, and at the same time to keep the analysis time at its minimum.

Optimization of inhibition time

In the test above, the absorbance was measured with free flowing enzyme. The situation may differ when the enzyme is immobilised on a glass piece packed in the inhibition reaction chamber, Fig. 3(c). To estimate the required inhibition time, remaining enzyme activity was measured in a two-step test. Before the test, the protective GST layer was removed by a washing step using the wash buffer to expose the immobilised enzyme AChE. The base line signal was collected, in the first step, by introducing a 0.6 mM DTNB solution and a 0.9 mM ASChI into inlets 4 and 5, respectively. In the second step, 50 nM of sarin solution was introduced into inlet 3. The pumping time was recorded and taken as the inhibition time.

After that, DTNB and ASChI were pumped into the reaction chamber again to measure the inhibition results. The remaining enzyme activities were measured (see eqn (1)) at two different flow rates and the different inhibition times are depicted in Fig. 8. The results show clearly that a short inhibition time such as 5 min results in considerable remaining enzyme activity. An inhibition time of 5 min is insufficient for free flowing sarin to inhibit the immobilised enzyme AChE. The results also show that for a longer inhibition time, the flow rate of $20 \mu\text{l min}^{-1}$ for each reagent results in only a slightly higher remaining enzyme activity. Thus in the subsequent tests, a flow rate of $20 \mu\text{l min}^{-1}$ for each reagent was set and an inhibition time of 15 min was selected.

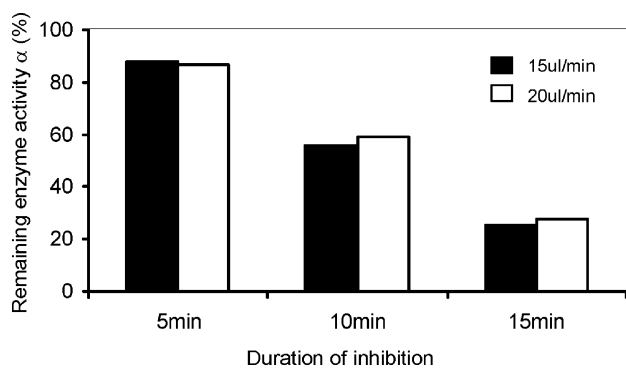


Fig. 8 Remaining enzyme activity at different flow rates and inhibition times.

Subsequently, the LOC device was tested with whole blood samples spiked with sarin. The experiments were designed to assess the effectiveness of the different stages of the LOC device. In the first experiment, the inhibition reactor (Fig. 3(d)) was tested with a blood sample prepared outside the LOC device, by-passing the nerve gas regeneration reactor, lysis reactor and filter chamber. In the second experiment, the filter chamber and the inhibition reactor were tested, by-passing the nerve gas regeneration reactor and the lysis reactor. In the final experiment, the whole system was tested with whole blood spiked with sarin as the input sample.

Experiment 1: test of immobilised enzyme reaction chamber and absorbance detection

To start, 200 μl of whole blood was spiked with 4 μl of sarin leading to a concentration of 200 nM. Nerve gas regeneration chemical KF was added to spiked blood and left to react for 5 min. Next, the protein precipitation chemical SAS was added to the KF and spiked blood mixture. The mixture was then transferred into a microplate containing filtration beads and filtered using the Whatman vacuum manifold. The filtrate was subsequently collected and transferred into a container that was kept on ice.

The test started with the washing step, where the wash buffer was pumped at a flow rate of 60 $\mu\text{l min}^{-1}$ through each inlet, 4 and 5, into the inhibition reaction chamber to remove the protective GST layer and to expose the immobilised enzyme. Next, the base line absorbance was measured, where both substrate ASChI (0.9 mM) and chromophore DTNB (0.6 mM) were pumped at a flow rate of 20 $\mu\text{l min}^{-1}$ into the LOC device. The cold blood filtrate was then pumped into the device at inlet 4 at a flow rate of 60 $\mu\text{l min}^{-1}$. Once immobilised the enzyme chamber was filled with filtrate, the syringe pump was stopped and inhibition was allowed to take place inside the enzyme chamber for 15 min. Wash buffer was then pumped into the device through inlets 4 and 5 to flush away the blood filtrate. Finally, DTNB and ASChI were introduced into the device through inlets 4 and 5 at a flow rate of 20 $\mu\text{l min}^{-1}$ each. The absorbance was then recorded accordingly.

Experiment 2: test of microfilters, immobilised enzyme reaction chamber and absorbance detection

In this test, the blood sample was prepared in the same way as previously described and has a sarin concentration of 200 nM. However, after mixing with KF and SAS, the sample

was not filtered. The washing step and the measurement of the base line absorbance was the same as in experiment 1.

2.5 ml of the unfiltered blood mixture (spiked blood, KF, and SAS) was pumped into the LOC device at a flow rate of 60 $\mu\text{l min}^{-1}$ via inlet 3. The pumping process continued until all the blood mixture had gone through the device. The entire experiment took about 23–24 min. After the inhibition reaction, the measurement of the absorbance followed the same protocol as experiment 1.

Experiment 3: test of the complete LOC device

The washing step for removing the GST layer protecting the immobilised enzyme followed the same protocol as in experiments 1 and 2. The whole blood sample spiked with 200 nM sarin was prepared outside of the LOC device.

The test started with the pumping of the spiked whole blood sample, KF and SAS through inlets 1, 2 and 3, respectively. As the volume ratio between spiked blood, KF and SAS was to be kept at approx. 2 : 9 : 14, we used different volume syringes for the respective reagents; *i.e.* 1 ml syringe for spiked blood, 5 ml syringe for KF and 10 ml syringe for SAS. The flow rate of the pump was set with the 5 ml syringe as the reference. To check the effect of the residence time and the corresponding inhibition time, the flow rates at inlets 1, 2, and 3 were set at 48 $\mu\text{l min}^{-1}$ (3 $\mu\text{l min}^{-1}$ spiked blood, 15 $\mu\text{l min}^{-1}$ KF, and 30 $\mu\text{l min}^{-1}$ SAS) and 22.4 $\mu\text{l min}^{-1}$ (1.4 $\mu\text{l min}^{-1}$ spiked blood, 7 $\mu\text{l min}^{-1}$ KF, and 14 $\mu\text{l min}^{-1}$ SAS). Three sets of experiments were conducted to ensure the repeatability of the test. The residence times were approx. 25 min and 65 min, respectively. After the inhibition reaction, the measurement of the absorbance followed the same protocol as experiment 1.

Discussion

For all experiments, the required time for the washing step was 1 to 2 h to entirely remove the GST layer. This time could be shortened using a higher flow rate and thus a higher shear stress or a thinner layer of GST to coat the immobilised enzyme.

Fig. 9 shows the remaining enzyme activities measured in all three experiments. Despite the different levels of inhibition, all experiments show the successful detection of sarin with respect to a drop in absorbance. Experiment 2 shows clearly that the sample cleanup absorbent packed in the device was effective in removing the fluoride ions, and more than 50% of

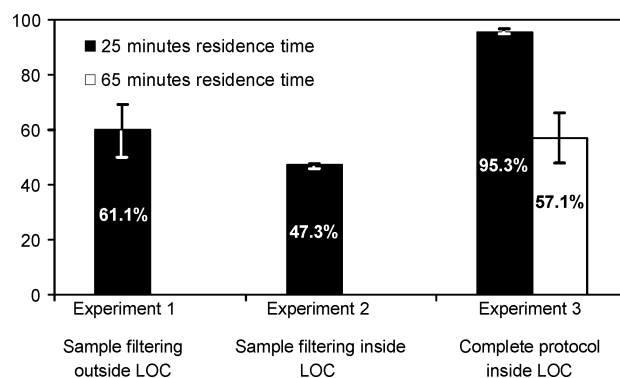


Fig. 9 Remaining enzyme activity in the three testing experiments.

the immobilised enzyme was inhibited. The results show that on-chip sample preparation was even better than the conventional process outside the LOC device.

The test of the whole LOC device in experiment 3 shows that only approx. 5% of the enzyme was inhibited at a total sample flow rate of $48 \mu\text{l min}^{-1}$ and a corresponding total residence time of 25 min. At this flow rate, the residence time in the regeneration reactor was about 1.5 min, while the protocol for preparation outside the LOC required 3 min for the regeneration. Since the conditions for the filtering stage and the immobilisation stage were the same as in experiments 1 and 2, the reason may be due to inefficient mixing in the first two stages or the insufficient residence time for the regeneration reaction.¹⁵ Measurement with a slower total sample flow rate of $22.4 \mu\text{l min}^{-1}$ and a corresponding total residence time of 65 min supports this hypothesis. The inhibition rate of the enzyme was increased to approx. 43%.

Conclusions

We report the design, fabrication and test of a LOC device for the detection of regenerated nerve agent in human blood samples. The detection concept is based on the inhibition reaction between the nerve agent and an enzyme. The enzyme activity was tested by hydrolysis of a substrate. The extent of the hydrolysis reaction was measured with a chromophore. Detection of sarin in whole blood spiked with a low level sarin concentration, 200 nM, was successfully achieved, shown as a significant drop in absorbance. Experiments were carried out for the determination of the kinetics of the inhibition reaction to estimate the required sample flow rate and inhibition time. The effectiveness of each stage of the LOC device was tested in three separate experiments. The results showed that the immobilization and protection of the enzyme on the chip was successful. The clear drop in absorbance showed that the inhibition reactions were successful in all three experiments. The lower remaining enzyme activity in experiment 2 showed that filtering and clean up of fluoride ions on the chip was more effective than the on-the-bench protocol. Compared to experiment 2, the results of experiment 3 showed that improvement is needed to address the problem of effective mixing and required residence time. The current relatively long test time can be improved by optimising the

thickness of the GST layer and the washing protocol. Effective removal of the protecting GST layer would shorten the analysis time significantly. Currently, the pumping and valving processes were carried out outside the LOC. In the future, we aim to incorporate these processes into the chip to make it suitable for easy use in portable applications. The device presented in this paper can also be used for detection of organophosphorus insecticides. Thus, the device is suitable for other applications in occupational hygiene in agriculture.

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