Development of a lab on a chip for nerve agent detection

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Development of a Lab on a Chip for Nerve Agent Detection

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Abstract

An escalating number of threats in terrorist activities had led to an urgent demand for innovative devices for on-site detection of chemical and biological agents as well as explosive materials. Rapid and sensitive detection of chemical and biological warfare agents (CWA) could provide an early ‘alarm’ of their release, therefore minimizing civilian casualties. Point-of-care diagnosis of individuals who are potentially exposed to chemical warfare agents will allow first-responder health providers to react quickly and efficiently. Health authority can then provide optimal, case-appropriate health care, and convince the unexposed individuals of their health safety. Such devices are required to encompass powerful analytical performance, low energy consumption and high portability. Lab-on-a-Chip (LOC) devices offer great promise for converting large and sophisticated instruments into powerful field-deployable analyzers where liquids are manipulated in a network of microchannels. Designing and fabricating miniaturized field-deployable devices whilst retaining the high sensitivity and selectivity of sophisticated laboratory-based instruments present multiple challenges. In this work, the components and systems for detection the presence of CWA or its degraded by-products in aqueous mediums were developed. The LOC device is an ‘active’ chip that not only needs to process whole human blood samples, but also to detect the presence of trace amounts of CWA from the blood samples. The report presents a literature review on possible pumping and valving solution for the chip. The design, fabrication and test of different pumping solutions are presented. Preliminary tests of the detection scheme for nerve agent Sarin are presented. The report concludes with recommendations for future works.

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List of Symbols and Abbreviations

Symbols

α	Remaining enzyme activity
Φ	Diameter
$^{\circ}\text{C}$	Temperature in degrees celsius
n	Number of same experiments conducted
W_a	Width of air chambers
W_c	Width of microchannels
f	Frequency
F^-	Fluoride ions
Q	Flow rate
ν	Kinematic fluid viscosity
A	Area
Re	Reynolds number
U	Mean fluid velocity
D_h	Hydraulic diameter
Pe	Péclet number
L	Length
D	Mass diffusivity

Abbreviations

Abs	Asorbance
AChE	Acetylcholinesterase from Electrophorus eel
APTES	3-Aminopropyltriethoxysilane
ASChI	Acetylthiocholine iodide
BSA	Bovine serum albumin
CWA	Chemical warfare agents
DI	Deionised
DTNB	5, 5'-dithio-bis-2-nitobenzoic acid
GA	Glutaradehyde
GB	Sarin
GST	Gelatine-sodium azide-trehalose coating
LOC	Lab-on-a-chip
KF	Potassium fluoride
MEMs	Microelectro-mechanical systems
PB	Phosphate buffer
PDMS	Polydimethylsiloxane
PMMA	Poly(methyl methacrylate)
SAS	Saturated ammonium sulphate

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Chapter 1

Introduction

1.1 Background

Microfluidics is a multi-disciplinary field that combines engineering, physics, chemistry, microtechnology and biotechnology. From the engineering point of view, microfluidics deals with the complexity of manipulating minute quantities of liquids. Scaling laws indicate that microfluidic technology allows the utilization of a number of microscale phenomena in analytical systems leading to better performances. Since its emergence in the 1980s, microfluidics has been widely used in the development of systems such as microchips for polymerase chain reaction (PCR) of deoxyribonucleic acid (DNA), laser inkjet print-head, sophisticated lab-on-a-chip (LOC) devices, and medical instruments.

LOC is an integrated microfluidic system on a microscale chip. LOC devices allow the integration of many microfluidic components in a single system thereby allowing laboratory-sized experiments to be conducted on a single microchip. The on-chip processes include the preparation of samples, the transportation, reactions and detection of the biological and chemical samples as well as products.

LOC devices are able to imitate conventional handling techniques of samples that are performed on the work bench of hospitals and laboratories. The advantage of miniaturization holds great potential to enable both research and healthcare to advance rapidly. Extensive interests from the research community have been attracted by such miniaturized devices, due to the above opportunities. Research in microfluidics leads to highly integrated system that is able to perform essential processing steps required for a specific application.

Research teams are often faced with challenges in transportation, metering and mixing of fluids. Other challenging tasks are valving or concentrating and separation

of molecules in a minute amount of fluid, and all on a single microchip. These challenges lead to the necessity of miniaturising ‘active’ functions and the development and integration of microfluidic components such as microvalves, micropumps, microseives, micromixers etc.^[1-3]. These devices will not only transport the fluids in the LOC but also control a precise amount of biochemical and chemical samples to be analysed. As a result, miniaturisation leads to less waste because a smaller sample volume is required for the diagnostics. Furthermore, due to the higher surface to volume ratio and shorter diffusion distances, miniaturization of chemical assays allows faster analysis and shorter response time. And in this course the cost of an diagnosis can be significantly reduced.

1.2 Objectives and scope of thesis

The main objective of this thesis is to develop a smart lab-on-a-chip (LOC) that has integrated microfluidic components such as micropumps and microvalves to perform the required steps of the detection protocol for the presence of nerve agent sarin (GB) in tainted blood. To actualise this ultimate goal, this project will focus on the design and fabrication of the microfluidic chip using polymer (poly methylmethacrylate (PMMA)). Optical fiber was the detection method used in detecting the presence of sarin in tainted blood. The results of this lab-on-a-chip (LOC) are being reported. However, this LOC can only be used in the laboratory.

To enable the microchip to function ‘actively’, that is, to be brought out of the lab into the field for use, active components, especially micropumps and microvalves, were designed and fabricated onto the desired microchip. Designs and optimization of the micropumps and microvalves, bonding technologies of different polymeric materials (PMMA and poly dimethylsiloxane (PDMS)) are investigated and reported. Immobilization of acetylcholinesterase (AChE) enzyme on different materials are also studied and reported.

1.3 Outline of the thesis

The outline of the candidature works are presented in the different chapters of the thesis.

Chapter one provides a brief introduction and description of the motivation for this project as well as its objectives and scopes. The subsequent parts of the report are comprised as follows.

Chapter two reviews and discusses the past works on LOC for detection of nerve agent sarin. This chapter will also review on the working principles of the different micropumps and microvalves reported in the past. Furthermore, different bonding technologies between different polymeric materials such as poly(dimethyl siloxane) (PDMS) and poly(methyl methacrylate) (PMMA) are also reviewed in this chapter.

In Chapter three, different fabrication techniques are discussed. The chapter also elaborates on the transition from laser micromachining of the LOC as reported earlier by the candidate to soft lithography. Since bonding a soft substrate to a hard substrate is the key technology of this project, bonding techniques for PDMS and PMMA are investigated and reported. Methods used for detecting the reaction products are also discussed in this chapter.

Chapter four discusses the design, fabrication and testing of the planned LOC. Different pumps were designed, fabricated and tested. Experimental results are presented and discussed in this report. The micropump design consists of a piezoelectric disc actuating a thin rubber gasket was build onto a PMMA chip. This design was further improved by using peristaltic concept with pneumatic actuation. The peristaltic micropump consists of a thin PDMS/adhesive membrane embedded between the PMMA actuation chamber and the PDMS microchannels. Characterization of the adhesive/PDMS membrane and the bond strength are highlighted in this chapter. Comparisons of the mixing efficiency in both PMMA and PDMS microchips are also discussed in this chapter.

Chapter five reports the investigation on the detection of nerve agent in both water samples and human blood samples in the designed LOC. Experimental results are evaluated and discussed for the suitability in the planned LOC.

Chapter six concludes the results obtained and recommends future works for this project.

Chapter 2

Literature review

In this chapter, reviews and discussions of the past work on Lab-on-a-chip (LOC) devices for detection of nerve agent, sarin, are highlighted. This chapter will also review on the working principles of the different micropumps and microvalves reported in the past. Polymeric materials such as poly(methyl methacrylate) (PMMA) and poly(dimethyl siloxane) (PDMS) are two most popular materials used in the fabrication in microchips. PDMS is a flexible material. To make it more robust, there had been an increased interest to develop techniques to bond acrylate materials to PDMS. Different bonding technologies between PDMS and PMMA are reviewed in this chapter.

2.1 Organophorous nerve agents

Organophorous compounds are chemical compounds containing carbon-phosphorus bonds. This group of compounds includes some of the commonly used pesticides and another class of very toxic organophorous chemical substances, nerve agents. Nerve agents represent one of the most important and lethal classes of chemical warfare agents. They are being classified into the ‘G’ and ‘V’ agents. (‘G’ agents were the classical agents synthesised by the Germans during the WWII, whilst the ‘V’ agents and mustard gases were synthesised by the Russians) They are hazards in both the liquid and vapor states and can cause death within minutes after exposure.

Nerve agents are highly neurotoxic, odourless, colourless, hence are capable of acting as inhibitor of acetylcholinesterase (AChE), a critical enzyme in the central nervous system and blood, which is responsible for the breakdown of the neurotransmitter acetylcholine. In the course of inhibiting the AChE, the nerve agent will cause acute, severe and rapid attacks on human and animal health. As chemical weapons, the United Nation has classified nerve agents as weapons of mass

Figure 2-1. Chemical structure of Sarin ^[4]

destruction and their production and stockpiling was outlawed in 1993 by the Chemical Weapons Convention ^[4].

Sarin ($C_4H_{10}FO_2P$, O-Isopropyl methylphosphonofluoridate) belongs to the G-type nerve agent, known by its NATO designation of GB. Sarin is a colourless, odourless and highly toxic phosphonate that acts as a cholinesterase inhibitor and disrupts neuromuscular transmission.

The lethal concentration-time dose of sarin for 50% of exposed individuals (humans) is 75 to 100 $mg \cdot min \cdot m^{-3}$. In the past, sarin had been used against the population of the Kurdish village of Birjinni in 1988, in terrorist attacks in Matsumoto city in 1994, and in the Tokyo subway in 1995. ^[5-7]

2.2 Lab-on-a-chip for nerve agent detection

In recent years, the growing concern on terrorists attacks leads to a demand for point-of-care diagnostic devices which allow fast and effective triage of casualties exposed to chemical warfare agents from the worried-well mass.

Over the past years, different analytical methods and technologies have been used to diagnose and verify exposure to these nerve agents as well as the degraded products and stimulants. These methods include: Ellman colorimetric assay for cholinesterase activity ^[8-10], identification and quantification of unbound nerve agent ^[6], microbial sensors ^[11-12], photoacoustic spectroscopy ^[13], luminescence ^[14], analysis of decomposed by products by gas chromatography-mass spectrometry (GC-MS) or

liquid chromatography-mass spectrometry (LC-MS) ^[5-7, 15]. Although mass spectrometry (MS) is the most commonly used technique as it is very sensitive and produces the most accurate data, this concept is currently only limited to laboratory analyses. A more rapid, sensitive and field-deployable method are needed to enhance our ability to response quickly to an emergency . And thus the ability to medically counteract the effects can be improved.

Fluorescence is another method that is frequently used for enzyme activity detection in the presence of a nerve agent. Zhang et. al. ^[16] developed a fluorescence-based functional group for specific ratiometric chemosensors. Viveros et. al. ^[17] developed an optical wave guide with immobilized enzyme to detect the change in fluorescence. The detection scheme was based on a competitive inhibitor, whose change in fluorescence is generated by gold nanoparticles attached to the enzyme. Bencic-Negale et. al. ^[18] reported on a nerve-agent-reactive microbead probes with fluorescence as the detector.

Another form of rapid and field deployable method of detecting nerve-agent exposure is electrochemical detection concept. Liu et. al. ^[19] used zirconia nanoparticles as selective sorbents to capture the phosphorylated AChE adduct and quantum dots were used as tags to label the monoclonal anti-AChE antibody to quantify the immunorecognition events. Wang et. al. ^[20] used carbon nanotube modified screen printed carbon electrode integrated into a microflow injection system, to detect the electroactive species that are produced from enzymatic reactions in the presence of OP pesticides. All of the above reported approaches did not use a microfluidic platform for sample analysis, and none of the papers reported, worked with whole blood as the sample.

A number of papers have reported on using LOC to detect the presence of CWA or degraded products in the presence of aqueous medium ^[12, 14, 21-26, 69-71] . Hadd et. al. ^[21] had reported using eletrokinetic means to investigate acetylcholinesterase inhibitors in microfluidic assays. Capillary electrophoresis is one of the more commonly used method for detection of organophosphorous nerve agents in microfluidic microchips. Hadd et. al. ^[21] discussed the use of computer-controlled eletrokinetics to transport the solutions of inhibitor, enzyme (acetylcholinesterase),

substrate and derivitizing agent into the LOC, where mixing took place within the microchannels. Wang et. al. ^[22] used capillary electrophoresis in a microchip for separation of organophosphate nerve agents in which amperometric detection was used. Pumera ^[23] gave a detailed review on the detection of nerve agents using capillary electrophoresis, Ding et. al. ^[24] used electrophoresis on a PDMS-based LOC with contactless conductivity to detect CWA degraded products.

Recently, LOC for diagnostics of blood samples has been an active research area. The major challenge of such LOC devices is the integration of the preparation and the analysis of the sample. In our case, the sample is whole blood. The sample must first pass through separators, cell lysis reactors, microfilters and concentrators as only some components of the whole blood are of interest for the later analysis. It is therefore necessary to separate these components from the rest.

As preliminary works for this project, the candidate had investigated the presence of trace amounts of nerve agent sarin in water samples ^[25]; and also trace amounts of sarin regenerated from spiked blood samples ^[26]. In these preliminary works, the LOC was made from PMMA using laser micromachining and reagents were introduced into the LOCs using an external syringe pump. Different reagents were pumped into the LOC and had to be manually changed. Hence, the entire investigation is limited to laboratory applications. In order to enable the entire system to be more user friendly and portable, design and fabrication of an ‘active’ LOC with miniaturized devices are necessary. In this report, the detection of sarin as the only CWA will be investigated.

2.3 Micropumps

Extensive research and a large variety of micropumps have been investigated and being realised. Micropump is one of the important microdevices that play an essential role in transporting samples and reagents through a microfluidic platform. Compactness of the micropumps and its ability to manipulate very small and precise volumes of fluids make them an ideal solution for a variety of applications. These micropumps can be stand-alone or integrated directly in the LOC.

These smart microfluidic devices have been used in many applications including controlled delivery of insulin, thermal management for microelectronics, chemical and biological analysis, cell sorting and cytometry, polymerase chain reaction (PCR), fuel cells and DNA hybridization etc. ^[27-29] Especially in bio-medical applications, it is vital that the micropumps are biocompatible, accurate, sensitive, and of light weight as well as low cost. ^[28, 30]

Micropumps reported in numerous literatures belong mainly to two major categories of mechanical or non-mechanical micropumps. This classification is based on the actuation energy required for driving the fluid within the microchannels. Mechanical micropumps are further classified as displacement pumps and dynamic pumps.

In displacement pumps, energy is required to exert pressure on the working fluid to move through one or more boundaries, thereby creating the pumping effect.

In dynamic micropumps, energy is continuously added to the working fluid such that the momentum or pressure increases. Examples for dynamic micropumps are centrifugal micropumps and electro-osmotic pumps.

In an excellent review paper on micropumps by Laser et. al., details of the key features, characteristics and performances of various micropumps has been reported ^[27]. Figure 2-2 shows the classification of the numerous micropumps being investigated and reported in the past years.

Figure 2-2. Classification of micropumps. D. J. Laser et. al. ^[27].

2.3.1 Displacement Micropumps

For most displacement micropumps, the mode of operation is in the form of periodic actuation. There are various actuators that can be used to drive the diaphragm or membrane present in the pump chamber. When the actuator is activated, a pressure difference in the chamber is generated and induces a flow of the fluid.

Figure 2-3 shows the mechanism of a typical displacement micropump. In the next section, some of the actuation means are being discussed in this report.

Piezoelectric micropumps

Piezoelectric actuation in micropumps was the first actuation technique reported. A micropump driven by a piezoelectric actuator typically has a membrane, and a pair of check valves or valvular conduits such as diffuser/nozzle structures. When a current is applied, the piezoelectric actuator contracts the chamber causing a form of suction. This process draws the fluid into the chamber. In the subsequent step, fluid in the chamber is then ‘squeezed’ out from the chamber into the outlet. It is a very attractive form of actuation means in micropumps, as it not only provides high stroke volume and actuation force, but also a very fast mechanical response.

The first piezoelectric actuated micro diaphragm pump with passive silicon check valves to direct the fluid flow, was presented by Lintel et. al. ^[31]. In this particular design, a three-layer set-up with two glass sheets, enclose an anisotropically etched silicon wafer.

Figure 2-3. Structure and operation of a typical reciprocating displacement micropump: (a) Top view and section; (b) Discharge and suction strokes. During the discharge stroke, the driver acts to reduce the pump chamber volume, expelling working fluid through the outlet valve. During the suction stroke, the pump chamber is expanded, drawing working fluid in through the inlet valve. ^[27]

One of the commercially available micropumps that have been distributed worldwide is produced by ThinXXs (Germany). The flow rate of the micropumps is dependent on the frequency of the piezoelectric actuator. These micropumps are manufactured using injection molding; hence they can be treated as disposable devices. The micro-diaphragm with passive check valves present in the micropump, are actuated by a piezoelectric disc. ^[32]

Electrostatic micropump

In electrostatic micropumps, electrostatic forces are generated between two electrodes to actuate the pumping diaphragm. One electrode of the actuator is fixed, whilst the other is flexible. When an electric potential of the same sign is applied, the electrostatic forces cause the membrane to move outwards of the counter electrode. As a result, pressure in the pumping chamber decreases, causing an in-drawn of fluid to the chamber. Once the sign of the potential is reversed, the membrane bounces back, increasing the pressure in the pumping chamber causing the fluid to be expelled. ^[29, 33-36]

Very low power is required to operate the micropumps at several kHz with full MEMs compatibility. However, the major disadvantage is the relatively small stroke volume of the actuator, which is usually limited to about around $5\mu\text{m}$ with a corresponding actuation voltage of 200V. ^[30]

Electromagnetic micropumps

Electromagnetic actuation is not commonly used in micropumps. Permanent magnet or soft magnetic cores activated by current in energized coils are used to produce the electromagnetic force driving the diaphragm of the micropump. When an electrical current is passed through the coil, Lorentz forces are produced and cause the deflection of the diaphragm due to the interaction between the magnetic field and the electric field. Voltage required to generate the force is relatively small and on the order of 5V. ^[29]

Rotary driven micropump

There are several literatures on rotary micropumps. This section only highlights ‘rotating-gear’ and ‘viscous-force’ pumping mechanism used in rotary micropumps.

Rotary micropump reported by Chou et. al. ^[37] was able to drive the working fluid in both clockwise and anti-clockwise directions in microchannels. This pumping concept also helps to enhance the mixing of fluids in the microchannels. In this design, relatively complicated microstructures are required to achieve both pumping and mixing. Tseng et. al. ^[38] reported a new micro-rotary device that was capable of mixing and transporting samples with the same microstructures. In this design, a pump and mixer were combined together and were fabricated using PDMS. To produce the pumping effect, four layers of PDMS membrane were activated in a specific sequence. ^[38]

Displacement of fluid using viscous forces generated by a rotating component has been investigated by several researchers, each with different configurations. Blanchard et. al. ^[39] reported a new set of disk viscous micropumps. The configurations with double-disk and single-disk were capable of generating flow by the rotation of the disk. The rotational movement of the disks induces viscous stresses on the fluid that forces the fluid from the inlet channel through the pumping volume

Figure 2-4. Cross-sectional view of single-disk and double-disk pumps. ^[39]

above the single disk (or between the two disks) towards the outlet as shown in Figure 2-4.^[39]

Thermally driven micropump

In thermally driven micropumps, actuation is realized by thermal means. The actuation concept involves the volume expansion or stress induced on a material in response to the applied heat. When used in diaphragm micropumps, the thermal actuation often takes the form of thermopneumatic or shape memory alloy (SMA) actuators.

For thermopneumatic actuation, a working fluid, which can be either a gas or liquid, has to be heated and consequently expands. Expansion of the working fluid will then cause the deflection of the pump diaphragm. Such methodology of activating the micropumps relies on the diffusion of thermal energy. The thermal response is on the order of seconds. Hence, only low actuation frequencies can be achieved. However, the drawback of such micropumps is that a lot of electrical power is required to generate the large amount of thermal energy needed.^[27, 29-30, 40]

Song et. al. had developed a thermopneumatic micropump that was capable of delivering discrete amounts of liquids in the microliter range, free of pulsations in a single-stroke.^[41] This pumping concept allows users to draw in and to eject liquids from the chip without priming. Moving parts were not required to operate the device, therefore making it possible to fabricate the micropump by using low-cost polymer such as PDMS (polydimethylsiloxane). Figure 2-5 shows the microchip, designed

Figure 2-5. Principle of thermopneumatic micropump. At the right, pressure changes are induced in the chamber to draw the fluid from the inlet.^[41]

with a dead-end channel: an inlet port on one end and a sealed chamber on the other. Air trapped in the chamber will expand by thermal means. This expansion will be used to manipulate the liquid in the microchannels, similar to a pipette. Liquid flow can be electronically controlled by maintaining a constant heating or cooling rate of the heater.

Peristaltic micropump

Peristaltic micropumps incorporate the motion of actuators in series to generate pumping action. Most of the peristaltic micropumps presented in literature describe three pumping chambers with diaphragms in series. These type of micropumps may utilize many different forms of actuators such as pneumatic, piezoelectric etc. [27, 29-30, 42]

There are many advantages in utilising peristaltic micropumps in biomedical applications and integration of peristaltic micropumps with general micro-total-analysis systems. Peristaltic micropumps do not have moving parts, thus can reduce the risk of damaging particles and living cells when they are transported through the microchip. Indirectly this will help prevent the problem of channel clogging and mechanical wear inherent in microchips with moving valves. [29]

Peristaltic micropumps offer the potential to accomplish bi-directional flows via a simple adjustment of the sequence of actuation. Furthermore, it is easier to fabricate on a planar structure. High pumping forces are generated in peristaltic micropumps and they are compatible with a wide variety of working fluids. [29]

Unger et. al. reported the use of multi-layer soft lithography to fabricate active micropumps. The reported peristaltic micropump consists of three valves which are arranged on a single channel as shown in Figure 2-6. The maximum flow rate of the micropump is $2.35 \text{ nl}^{\text{s}^{-1}}$ at a pressure of 50kPa and a driving frequency of 75Hz [43].

Figure 2-6. Diagram depicting the workings of the peristaltic pump. ^[43]

Goulpeau et. al. ^[44] reported the investigation of a three-valve peristaltic micropump that was also fabricated using multi-layer soft lithography. The fabricated microvalves are in the ‘push-down’ geometry where the actuation chamber is directly above the fluidic channel. The design and pumping mechanism of these peristaltic micropumps are comparable to works reported by Unger et. al. ^[43], Studer et. al. ^[45] and Pandolfi et. al. ^[46]. High speed LEE microvalves (LEE, Connecticut, USA) bought commercially were controlled using a LABVIEW program. The valves allow a pressure switch time of below 0.5ms. A range of flow-rate measurements of 0.02-7.5 μ L/min was experimented and the relative precision was reported to be better than 4%. A single-valve model was introduced to describe the dynamics of the pump. Comparison between the theoretical models and experiments were carried out, leading to a close agreement both qualitatively and quantitatively. Through this investigation, Goulpeau et. al. ^[44] concluded that the design of the actuation system – including both internal and external micropump elements – was crucial in achieving high flow rates.

Huang et. al. ^[42, 47] reported their investigations of a pneumatic micropump that featured three membrane-enclosed chambers with differing volumes. These three enclosed chambers are serially connected and are capable of generating the pressure required to pump the fluid within the microchannels. An electromagnetic valves (EMV) switch was utilised to perform the pumping actions. Maximum pumping rate of the micropump is 3 μ l s⁻¹, at a pressure of 172.4kPa, and a driving frequency of 10 Hz, when the cross-sectional dimensions of the microchannels are 500 μ m $\times$ 100 μ m ^[47]. The design of the micropump by Huang et. al. ^[42] is shown in Figure 2-7.

Figure 2-7. Schematic illustration of the three-chamber pneumatic micropump. A constant current source is used to drive the flow sensor. Electrical signals from the flow sensor were measured by an analog-to-digital converter (ADC).^[42]

Improvement of the above design was reported by Yang et. al.^[48]. In the improved micropump design, the pneumatic micropump has a normally closed valve that is capable to prevent back flow of fluid when generating a high pumping rate and a high back pressure.

The air chambers are placed beneath the fluidic microchannels. As seen from Figure 2-8, the normally closed valve is a PDMS-based floating block that is located on the inside of the sample flow microchannel. The peristaltic motion of the PDMS membrane will create a hydraulic pressure, causing the valve to be closed. This valve helps to aid in increasing the pumping rates of the micropumps and also prevent backflow of fluids, therefore indirectly preventing back pressure. Experimental data indicated that the highest pumping rate the above-mentioned design was able to reach was 900 μ L/min, at a driving frequency of 90Hz and an applied pressure of 20 psi.^[48]

2.3.2 Dynamic Micropumps

Dynamic micropumps are capable of producing ‘continuous flow’ effects of the fluid in microchannels. Such micropumps require a direct conversion of electrical or magnetic energy into fluid motion, hence producing a steady flow in fluid. Actuation means are in the form of electroosmotic, eletrokinetic, electrochemical, electrohydrodynamic, magnetohydrodynamic concepts.

Figure 2-8. Schematic diagram showing the working principle of the normally closed microvalve. a) A cross-sectional view of the normally closed valve showing that a sample fluid can be separated by the floating block structure. b) the sample fluid can flow over the floating block when the micropump is activated. ^[48]

For electroosmotic micropumps, an electric field is applied to pull the ions along the pumping channel. The bulk fluid in turns is drag along by momentum transfer due to viscosity. In magnetokinetic micropumps, flow in the microchannel is driven by Lorentz force on the bulk fluid. ^[27-29, 33]

Electroosmotic micropump

Electroosmotic micropumps have been extensively investigated by numerous teams. This concept is used to pump electrolyte solutions. Electroosmotic pumps do not require any moving parts such as check valves or diaphragm. The pumping concept is based on a phenomenon called electroosmosis where an electrolyte solution present in the microchannels moves when an external electric field is applied. The flow direction of an electroosmotic micropump is controlled by switching the direction of external electrical field.

Lets consider a stationary solid surface in contact with an aqueous solution. When an electric field is applied, the excess counter-ions in the diffuse layer of the electrical double layer will move under the applied electric field, as illustrated in Figure 2-9. As the ions move, they drag the surrounding liquid molecules to move with them, resulting in a bulk liquid movement. This effect is called electroosmosis. The induced flow is called the electroosmotic flow. Electroosmotic pressure is a pressure required to counterbalance this electroosmotic flow. The most basic electroosmotic pumps consist of just capillaries or microchannel sections with electrodes submerged in the end reservoirs and a flow resistance in series with the channel. Flow rates produced by such pumps are very small, on the order of less than $1 \mu\text{l min}^{-1}$.

Four key parameters determining the performance of electroosmotic micropumps are:

- 1) Magnitude of the applied electric field and voltage;
- 2) Cross-sectional dimensions of the structure in which the flow is generated;
- 3) Charge density of the solid surface that is in contact with the working liquid and;
- 4) Ion density and pH of the solution.

Figure 2-9. Movement of ions under the influence of electro-osmotic flow ^[49]

Electrochemical micropump

In electrochemical micropumps, electrolysis of water occurs if a voltage is applied to electrodes immersed in the liquid. Electrolysis of water will therefore cause the formation of bubbles. These formed bubbles will be used as a means of driving force to dispense liquids, Figure 2-10.

Figure 2-10. Schematic diagram showing electrochemical actuation. ^[30]

An electrochemical micropump has its own limitations. One of the limitations is the unsteady and unreliable results due to the collapse of the generated bubbles. This is especially detrimental for applications or systems in drug administration in biomedical applications. ^[30]

Electrohydrodynamic micropump

Electrohydrodynamic micropumps are based on the electrostatic forces exerted on the charges of a dielectric fluid. There are several types of electrohydrodynamic micropumps and the difference between these electrohydrodynamic micropumps is the format of introducing the charged particles into the working fluid. ^[29]

Magnetohydrodynamic micropump

Lorentz effect can be used to drive magnetohydrodynamic micropumps. In this concept, electric current is injected into two electrodes that are located on facing side walls of a microchannel in the presence of a magnetic field, which is generated by placing permanent magnets on opposite sides of the remaining two walls of the channel. The current generates a force which is perpendicular to both the magnetic and electric fields. The Lorentz force acting onto the ionic species in the solution will

then generate a fluid flow in the microchannel. Pumping by magnetohydrodynamic means is bidirectional as it is possible to reverse the flow in the microchannel by

Figure 2-11. Magnetohydrodynamic micropump ^[29]
reversing the electric current or the magnetic field. ^[27, 29, 33]

2.4 Microvalves

A microvalve is a device that controls the fluid flow within a microfluidic chip. Besides micropumps, microvalve is another class of components that are very important and necessary for the realization of a fully integrated microfluidic system, such as a lab-on-a-chip (LOC) or micro total analysis systems.

Similar to micropumps, microvalves are also generally being classified into two main groups, namely

- i) Active microvalves whereby external systems or both mechanical and non-mechanical moving parts are used; and
- ii) Passive microvalves ^[3, 50-51].

In these two major groups, the microvalves are further divided into subgroups as shown in Figure 2-12.

In our application, the design and fabrication of microvalves should be cheap, non-reactive to the chemicals used and require little power to function. Pneumatic microvalves have been identified that may be suitable for our application.

Pneumatic microvalve

Soft lithography is a technique that allows rapid prototyping of polymeric microfluidic chips and devices. Soft lithography represents an alternative for fabricating micro and nanostructures. The elastomeric stamp is usually prepared by cast moulding, where the pre-polymer of the elastomer is poured over a master having the relief structure on its surface then is cured and peeled off.

Stephen Quake's group had reported incorporating a multi-layer soft-lithography process in the microfluidic systems ^[43, 52]. Such method was used to fabricate the microchip with microchannels stacked above another. Whereby, the microchannels are separated by a very thin PDMS membrane. In which the top microchannel will act as a control channel where air is being passed through; acting as a passive microvalves.

The properties of PDMS made it suitable for the fabrication of these microvalves, as it is highly flexible, permeable to air, and compatible to most non-organic solvents as well as chemicals. The control channel receives air from an external pump or pressure source. A relatively low air pressure of about 40kPa can

Categories			
Active	Mechanical	Magnetic	External magnetic fields Integrated magnetic inductors
		Electric	Electrostatic Electrokinetic
		Piezoelectric	
		Thermal	Bimetallic Thermopneumatic Shape memory alloy
		Bistable	
	Non-mechanical	Electrochemical	
		Phase change	Hydrogel Sol-gel Paraffin
		Rheological	Electro-rheological Ferrofluids
	External	Modular	Built-in Rotary
		Pneumatic	Membrane In-line
Passive	Mechanical	Check valve	Flap Membrane Ball
			In-line mobile structure
			Diffuser
	Non-mechanical	Capillary	Abrupt
			Liquid triggered
			Burst Hydrophobic valve

Figure 2-12. Classification of microvalves. Reprinted with permission from Zhang et. al. (2007) Copyright (2007) Elsevier Science Ltd. ^[50]

cause a $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ of the PDMS membrane will buckle and squeezed into the lower microchannel that holds the fluid flow. Hence the fluid flow is blocked off. The design of this microvalve with a pneumatic control channel crossing a fluidic channel is shown in Figure 2-13;

Pandolfi et. al. ^[46] have also reported microvalves activated by low pressure. In their work, the performance of the microvalves depends on the shape of the fluid channel. Modification of the PDMS material was done by injecting silicon oil to induce localized swelling of the PDMS.

The thickness of the PDMS membrane is crucial for its application in pneumatic microvalves. PDMS membrane as thin as 3 to $10\text{ }\mu\text{m}$ are needed for many applications. Lötters et. al. reported of a controlling the thickness of PDMS by spin coating at different spin rate and spin times ^[54]. Figure 2-14 shows the relation between the spin speed and the resulting PDMS thickness.

Figure 2-13. The working principle of a pneumatic microvalve. ^[53]

Thanawng et al. reported a novel method of obtaining an ultra-thin PDMS membrane. This is possible by diluting the PDMS mixture with hexane using weight ratio. ^[55] Spin coating the diluted PDMS is necessary to create a thin PDMS film. Tensile tests were conducted by the team to illustrate the feasibility of this method.

Figure 2-14. Calibration plot of thickness of PDMS with respect to the different spin rates and duration of spinning. ^[54]

2.5 Micromixers

In microfluidics, rapid mixing is difficult to achieve because the species transport mainly relies on molecular diffusion. ^[82] A micromixer is a device that utilise mechanical microparts to mix fluids. This device represents a key technology to fields such as chemical industry, pharmaceutical industry, analytical chemistry, biochemical analysis, and high-throughput synthesis. Mixing is especially essential for biochemistry analysis, drug delivery and sequencing or synthesis of nucleic acids. Miniaturization of the fluids associated in the mixing helps to reduce the quantities of fluids involved in the chemical and/or biochemical processes, thereby reducing the cost. Reaction time is hugely enhanced by the process of mixing. Structures of micromixers are either fabricated partially or in whole using microtechnology or precision engineering. ^[75]

In recent years, numerous teams have embarked in investigating numerous designs of micromixers. Investigation of micromixers also serves as a critical understanding of transportation of substances in the microscale region. In general, micromixers can be categorised into either passive micromixers or active micromixers (Figure 2-15). Figure 2-15, shows the types of micromixers that are present.

2.5.1 Passive micromixers

Passive micromixer is the phenomenon of allowing the reagents or substances to react utilising the laws of diffusion or chaotic advection. Such forms of micromixers do not require any external energy. ^[51, 75, 83] To improve the molecular diffusion of reagents within the micromixers, increasing the contact surface between the different fluids and decreasing the diffusion path between them should be employed.

Figure 2-15. Flow chart showing the overview of different micromixers. ^[51]

Passive micromixers can be further categorised as: ^[75, 83]

- i) Parallel lamination
- ii) Serial lamination

- iii) Injection
- iv) Chaotic advection
- v) Droplet-based

Parallel lamination is the phenomenon where the stream of fluid at the inlet is being split into n substreams, then join into one stream as laminae. ^[75, 83] often the basic design is a long microchannel with two inlets ($n = 2$) to its geometry/these designs are known as the T-mixer or the Y-mixer as shown in figure 2-16 (a) and (b).

Figure 2-16. Parallel lamination micromixer: (a) the basic T-mixer and (b) Y-mixer ^[83]

Pressure is often the mean of driving the flow of fluids in parallel lamination, however, electroosmosis is another form of driving the flow as reported by Hadd et.al. ^[21, 84], and Jacobson et. al. ^[85].

Serial lamination is almost similar to parallel lamination. However, for this form of mixing, the inlet streams are first joined together as one stream before being split into numerous streams in the second stage, followed by combing into 1 stream when the fluids exit from the micromixers.

Bessoth et. al. ^[86] reported that mixing rates can be enhanced by dividing the streams into n laminae with widths $1/n$ of the original channel. This results in faster mixing by a factor of n^2 . Figure 2-17 shows the micromixers reported by Bessorth et. al.

Chaotic advection refers to the phenomenon where a simple Eulerian velocity field leads to a chaotic response in the distribution of a Lagrangian marker, such as a tracing particle. ^[75, 82, 83] Advection is another important mass transfer in flows with a

low Reynolds number. This form of mixing had been greatly investigated by numerous teams.

Figure 2-17. (a) Schematic of layer 1 of the micromixer (including points of detection). (b) Detail of layer 1 (including points of detection).^[86]

Chaotic advection can be generated by special geometries in the mixing channels or it can be induced by an external force. One of the simplest methods of inducing chaotic advection is to introduce specially design microchannels. Stroock et. al.^[87] reported introducing slanted grooves to enhanced the mixing of specimens in the microchannels. Stroock et. al.^[87], Yang et. al.^[88], Cortes-Quiroz et. al.^[89] and Tan et. al. [26], discussed on the enhanced effects of mixing when staggered herringbone structures were introduced in the micromixers. Figure 2-18 shows the design of slanted ribs and staggered herringbone microstructures reported by various teams.

Figure 2-18. Modification of mixing channels for chaotic advection. (a) slanted ribs, (b) slanted grooves, (c) staggered herringbone structures ^[83]

Liu et. al. ^[90] and Schonfeld et. al ^[91] had reported using serpentine channels to enhance mixing in the microchannels. Kim et. al. ^[92] reported designing a serpentine micromixer which was capable of achieving a high level of efficient mixing by combining two general chaotic mixing mechanisms: splitting/recombination and chaotic advection. Figure 2-19 shows the serpentine micromixer designed by Kim et. al.

Formation of droplets of mixed liquids can help to reduce the mixing path. An internal flow field is generated when the droplet moves, hence allowing mixing to take place inside the droplet. ^[83] One form of droplet micromixer design is to use flow instability between two immiscible liquids. Song et. al. ^[93] had reported using oil to form droplets in water to enhance mixing. Mao. et. al. ^[94] had reported using bubbles

Figure 2-19. Schematic diagram of the serpentine micromixer. ^[92]

as a means to enhance mixing with the microchannels.

2.5.2 Active micromixers

An external source is often used to induce disturbances in the reagents within the microchannels so as to allow mixing of the reagents to take place. Often, the external source comes in the form of pressure, temperature, electric field etc. Active micromixers, can be further categorised as the different forms of external disturbance effects:

- i) Pressure
- ii) Temperature
- iii) Electrohydrodynamics
- iv) Dielectrophoretics
- v) Electrokinetics
- vi) Magnetohydrodynamics
- vii) Acoustics

Disturbance to the pressure field had been used by as a form of active micromixers. Pulsating flows is another form that had been explored by numerous teams to generate the mixing in the active form. Pulsating flows can be generated by means of pressure generators, Sun et. al. ^[95] investigated using diverging microchannels and a custom-build dynamic pressure generator as the actuation source to produce active mixing within the micromixers. The half-angle of the diverting section and the phase difference of the two actuating pressures in mixing performances were explored.

Tsouris et. al. ^[96] reported generating an electrohydrodynamic flow generated by the motion of charge carriers in an electric field. Rapid mixing in the ‘T’ geometry of the microchannel devices achieved when a potential difference ranging from 0 to 900 V was applied to the electrodes.

Leong et. al. ^[97] reported designing a rapid microfluidic mixer that utilized a low-switching-frequency DC field to induce interactive flows for sample driving and flow dispersion generation simultaneously within the expansion chamber. Time-

pulsed driving voltages to induce electroosmotic flow velocity variations for developing a rapid mixing effect was applied to the respective inlet fluid.

2.6 Bonding technology for PMMA and PDMS

Numerous methods have been investigated to bond substrates with open microchannels, in order to obtain a properly sealed microchip. Tsao et. al. ^[56] had given a very detailed review on the different bonding methods of thermoplastic polymer in microfluidics. This section only reviews and focuses on bonding techniques for PMMA and PDMS substrates.

Bonding of microfluidic devices can be classified into either direct or indirect bonding. Whereby, indirect bonding involves the use of an adhesive layer to seal two substrates whilst direct bonding methods mate the substrates together without any additional materials added to the interface.

Indirect bonding -Adhesive bonding

Adhesive bonding is a very simple and cheap form of bonding two PMMA substrates. Adhesive in the form of glue, epoxy, sticky tapes, UV cured adhesives can be used for this purpose. However, for liquid adhesives, the challenge is to prevent clogging of the microchannels by the adhesives after the adhesives had set.

Partially cured PDMS has also been investigated as an adhesive layer for bonding of PMMA substrates. Chow et. al. ^[57] reported that a thin (10 μm to 25 μm) PDMS intermediate layer was spin-coated onto a bare PMMA substrate before placing the other PMMA substrate with channel structure onto it. These pieces with the PDMS intermediate layer were then cured in two stages. In the first stage, the layer was pre-cured at room temperature for 20 hr, followed by 90°C for 3 hours. Tensile tests were conducted, and the bonding strength was about 0.015MPa. Toh et. al. ^[67] reported a similar procedure, the only difference was that the PDMS mixture was spin coated onto an double-sided adhesive layer. This membrane was than sandwiched between two PMMA layers.

Direct bonding

Thermal fusion bonding. In thermal fusion bonding, PMMA substrates are heated to a temperature near or above the T_g of PMMA. Pressure is applied to the substrates to increase the mating contact forces. An advantage of direct thermal bonding of the substrates is the homogenous surface property when identical materials are used for both the substrate with microchannels as well as the cover layer. Thermal bonding of PMMA had been previously demonstrated by Tan et. al. ^[26], Sun et. al. ^[58], Zhu et. al. ^[59], Chen et. al. ^[60], Chen et. al. ^[72].

Solvent bonding. Generally, chlorocarbon solvents such as chloromethane, dichloromethane and chloroform are highly efficient organic solvents for allowing PMMA materials to ‘glue’ together. However, using these organic solvents often result in clogging of the microchannels as they have high PMMA solubility. Lin et. al. ^[61] reported the use of low azeotropic solvent to bond PMM microfluidic devices without causing the microchannels to clog.

Surface treatment and modification. A few papers reported modifying the PMMA surface using silanization. This method gives the modified PMMA surface a silane matrix similar to glass, allowing it to undergo plasma bonding to PDMS. Vlachopoulou et. al. ^[62] reported that they had a novel method of bonding PMMA to PDMS after doing a surface treatment to PMMA with aminopropyltriethoxysilane (APTES). Tsougeni et. al. ^[63] reported that performing a plasma treatment to the PMMA layer first, before performing surface treatment to PMMA will allow the PMMA surface to become more hydrophilic. It was discovered that when PMMA is subjected to O_2 plasma treatment (the longer the better), presence of nanostructures are observed on the surface of PMMA. These nanostructures are responsible for the increased wettability behaviour of the PMMA. Hence, after O_2 plasma treatment of the PMMA surface, APTES was able to adhere much better to the PMMA surface.

Chapter 3

Fabrication techniques and technology development

3.1 Design and fabrication

This project aims at developing a lab-on-a-chip consisting of three layers for detecting the presence of Sarin in human whole blood samples. In our preliminary work, a PMMA microchip was designed and fabricated. Figure 3-1, shows the steps to which the system that has been done previously ^[26] on a microchip.

Figure 3-1. Flow chart showing the approach of detecting the presence of nerve agent from the samples. ^[26]

The current project focuses on designing and fabricating a suitable micropump that will act as a pump and valve in the microfluidic chip for processing human blood samples and the subsequent detection of nerve agent sarin. The access ports numbered from 1 to 5, are the regions where control of reagents is necessary to allow the required chemical reactions to take place.

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, 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 entering stage (e) is almost colourless. Before testing the blood sample, a baseline 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. Discussion of the results obtained from this microchip will be further elaborated in section 5.4.

Previously, the reagents were introduced and controlled at the 5 ports (figure 3-1) using a syringe pump (CMA 100 microinjection pump Stockholm, Sweden) and manual valving. To enable the microchip to function automatically with minimum manual involvement, miniaturizing the pumping and valving systems are necessary. The micropumps and microvalves to be fabricated will be integrated on the chip. For smaller features and smaller device footprint, the new 'active' LOC will be fabricated using soft lithography process. However, the prototype will be fabricated in PMMA with laser micromachining.

Due to the requirements of the planned LOC, the micropumps to be developed should be biocompatible, precise and of low cost. The pumping mechanism of the micropumps to be investigated will be either check-valve-based or peristaltic similar to that reported by Lee et. al.^[42, 47-48].

In this project, multi-layer soft lithography approach would be used to fabricate the passive microvalves. These microvalves are parts of the micropumps. Referring to the existing microchip design^[26], and as conceptually depicted in Figure 3-1, five sets of microvalves and micropumps are required for the entire process to be carried out on the LOC. A stand-alone LOC with a multi-layer process will first be implemented before incorporating the optimised passive microvalves for the final application.

Three different key fabrication techniques of the LOCs are identified as:

1. Laser micromachining of PMMA
2. Lithography and multi-layer soft lithography of PDMS
3. Bonding between PMMA and PMMS

3.1.1 Laser micromachining

Laser ablation as a form of microfabrication approach has been widely employed to fabricate poly(methyl methacrylate) (PMMA) microdevices^[56, 58, 64]. In our work, the devices were first designed using CorelDraw (Corel Co. Canada). A commercial CO₂ laser system (Universal M-300 Laser Platform, Universal Laser system Inc. Arizona, USA) engraved the designed structures on the PMMA substrate. The laser system acts as a printer, where the design can be printed directly out from the CorelDraw file. The CO₂ 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. This form of fabrication is very fast and cost effective. However, the surfaces of the fabricated microchannels are not smooth. Once the pieces of engraved substrates are obtained, they are cleaned using 70% ethanol and rinsed with de-stilled water subsequently before subjected to thermal bonding.

During thermal bonding, the PMMA pieces are subjected to a low-pressure, high temperature conditions ^[58]. Under low bonding pressure, the PMMA layers were placed between a hot plate and an upper aluminum plate. The bonding pressure on the PMMA layers could be adjusted by putting weights on top of the upper plate (the pressure was kept at 20kPa or lesser). To obtain a better flat surface of the PMMA layers, silicon wafers were placed underneath and on top of the PMMA layers during the bonding process.

The PMMA layers were first heated to 160°C in 10 min, and then it is kept at 160°C for 30 min. subsequently, the bonded PMMA layers were cooled down to 80°C in 20 min. At 80°C, the PMMA layers are 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.

3.1.2 Lithography and soft lithography technique

Lithography. The most common method of fabricating micropumps in the past was micromachining a silicon wafer followed by bonding to a cover plate made of silicon or glass. Tsao et. al. ^[56] reviewed the different types of materials used in the fabrication of microfluidic microchips. The review indicates that silicon and silica are the dominant materials for microfluidics. PDMS was the second favoured material, followed by thermoplastics then PMMA. Figure 3-2 shows the trend of materials used in microfluidics applications over the past decade reported as the number of publications.

As mentioned in Section 3.1, our project aims at an ‘active’ LOC where micropumps and microvalves are integrated on the chip to implement the protocol depicted in Figure 3-1. The fabrication may combine both micromachined PMMA and PDMS. Thus, lithography and soft lithography are the key technologies needed for the fabrication of the device.

Figure 3-2. Approximate numbers of annual publications referencing different materials for microfluidics. ^[56]

Photolithography is the process of transferring geometric patterns on a mask to the surface of a substrate material coated with photoresist. The steps involved in the photolithographic process are wafer cleaning; barrier layer formation; photoresist

application; soft baking; mask alignment; exposure and development; and hard-baking.

There are two types of photoresist: positive and negative. With positive resists, the structure to be removed is exposed to UV light. For these resists, exposure to the UV light changes its chemical structure and makes it more soluble in the developer. The exposed resist is then washed away by the developer solution, leaving windows of the bare underlying material. The mask, therefore, contains an exact copy of the pattern that remains on the wafer.

Negative resists behave in the opposite manner. Portions that are exposed to the UV light will result in polymerization of the photoresist, hence these regions are more difficult to dissolve. Therefore, when immersed in the developer solution only the unexposed portions are removed and the negative resist remains on the surface

wherever it is exposed. Masks used for negative photoresists, therefore, contain the inverse of the pattern that is to be transferred.

In our project, designs of the desired microchip were first drawn using engineering drawing software, AutoCad ® 2006, which were then printed on a polymer mask. A clean 4" wafer (Bonda, Singapore), was spin coated with the SU8-100 photoresist (Microchem, Newton, MA, USA) at 2000 rpm to yield a thickness of 150 µm. The wafer underwent soft bake at 65°C and 95°C subsequently before being subjected to exposure of Ultra Violet (UV) light at the intensity of 8.9 MW/cm² for 67 s with the polymer mask. The exposure machine, Karl Suss, MA6/BA6, was used to performed this process. After the exposure, the exposed wafer was baked at 65°C and 95°C subsequently. After baking, the wafer then underwent developing for 15 min before rinsing with DI water followed by hard baking at 150°C. Once the processes were completed, the wafer - with the microchannel structures - was checked using the SEM.

Soft lithography. Soft lithography is a technique based on photolithography developed for rapid prototyping of micro and nano-structured devices. It is called "soft" because the technique uses transparent, elastomeric material, as the "stamps", with patterned relief on the surface to generate patterns and structures. The "stamps" can be prepared by casting pre-polymers against masters patterned by conventional lithographic techniques, curing and peeling the "stamp" off. The brief procedure for soft lithography is illustrated in Figure 3-3. Soft lithography provides a convenient, effective and low-cost prototyping approach. Furthermore, the technique is easy to learn, straightforward to apply and accessible to a wide range of users. ^[71]

Poly(dimethyl siloxane) (PDMS) elastomers or also called silicone rubbers was often used in microfluidics-related works. However, there are also numerous teams using other elastomers such as polyurethanes, polyimides, and cross-linked NovolacTM resins. ^[65]

Figure 3-3. Soft lithography process ^[65].

PDMS is a widely used polymer material for prototyping devices and commercial devices. PDMS has numerous advantages over silicon and glass as it is an inexpensive and flexible material. The structured PDMS surfaces are quickly obtained and its transparency over the entire visible spectrum of light. It can also be sealed to almost any flat material to form the channels. The chemical structure of PDMS is shown in Fig 3-4:

Microvalves of dimensions of 2 mm by 100 μm by 10 μm will be designed and fabricated. These valves were investigated to determine the pressure required to close off the microchannel (2 mm in width by 0.4 mm in depth). To activate the microvalves, air from an external supply is pumped into the control channels and to cause deflection of the thin PDMS membrane. The diaphragm material of the micropump is particularly important.

Figure 3-4. Chemical structure of PDMS. R = -OH or -CH₃ ^[76]

3.1.3 Bonding between PDMS and PMMA

PDMS is a rather flexible material. To make it more robust and compatible to other technologies involving PMMA, the bonding technology of PMMA to PDMS was first to be developed. Due to the different surface and chemical properties of these two materials, bonding of PDMS to PMMA is not an easy task. It was not possible to treat both surfaces with oxygen plasma to initiate irreversible bond.

One of the methods of bonding PMMA to PDMS was modifying the mixture ratio of the PDMS elastomer and curing agent, followed by thermal bonding. Chow et. al. ^[57] reported the use of PDMS as the intermediate layer to bond 2 PMMA substrates. This was achieved by using a 3:1 mixture of elastomer and curing agents followed by a thin layer of PDMS poured onto the PMMA layers and heated at 170°C for 3 hours. Toh et. al. ^[67] had also reported a similar procedure, the only difference was that the elastomer mixture was spin coated onto an adhesive layer that consisted of double sided acrylic adhesive. The double sided adhesive layer was earlier rolled onto the PMMA substrate. Spin coating the PDMS mixture yielded a thickness of 8µm. These layers were then heated at 65°C for 3hrs followed by bonding them to another PMMA layer.

A few papers reported modifying the PMMA surface using silanization. This method gives the modified PMMA surface a silane matrix that is similar to glass, thus allowing it to undergo plasma bonding to PDMS. Vlachopoulou et. al. ^[63] reported a novel method of bonding PMMA to PDMS by modifying the surface of PMMA. However, before the chemical treatment of the PMMA surface, PMMA surface must first be made more hydrophilic to allow better adherence to the chemical. Tsougeni et. al. ^[63] reported that performing a plasma treatment to the PMMA layer before the surface treatment allows the PMMA surface to become more hydrophilic. They discovered that when PMMA is subjected to O₂ plasma treatment (the longer the better), nanostructures were observed on the surface of the PMMA. These nanostructures are responsible for the increased wettability behaviour of the PMMA. They reported that treating the plasma treated surface with aminopropyltriethoxysilane (APTES) allows the surface chemistry of PMMA to become somewhat similar to that of PDMS and glass.

The recipe reported by Valchopoulou et. al. was experimented in this project. To allow a clean PMMA surface to adhere better to APTES mixture (5% APTES + 95% autoclaved water), it first had to undergo oxygen plasma treatment. Once the APTES mixture was dropped onto the PMMA surface, the PMMA was heated at 80°C for 20 min to allow all the APTES to dry, leaving a transparent stain on the PMMA surface. After this process, this treated PMMA and a clean cured PDMS substrate undergo oxygen plasma for 25 s at a power of 70 W, a pressure of 75 kPa and an oxygen flow rate of 25 sccm. After the O₂ plasma, the treated surfaces of the two substrates were brought into intimate contact and a heavy weight of 5kg was placed onto the pieces and heated at 80°C for about 1 minutes.

It was observed that at times the surface treated PMMA was able to bond irreversibly to the PDMS, at times this method was not workable. The bonding process is not reliable. APTES treated PMMA substrate with engraved microchannels bonded to PDMS was tested for leakage. Water was pumped into the microchips at flow rates of 4.0 µl/min, 10.00 µl/min, 15.0 µl/min and 20.0 µl/min. For flow rate at 4.0 µl/min, after 90 min, the chip started to leak. As the flow rate increased, the duration after running water into the chip, at which the microchip started to leak, also became shorter. When the water still continues to be pumped into the microchip even though leaking had taken place, for 4.0 µl/min, after 105 min since the start of experiment, the PDMS was able to be peeled of completely from the PMMA substrate.

Leaking from the microchip could be due to the weakening of the attractive bonds between the PDMS and surface modified PMMA. Lee et. al. ^[98] reported that although the bond that present between the surface modified PMMA by APTES and PDMS is irreversible, any contact with water will cause the Si-O-C bond to hydrolyse. Thus, causing leaking to take place. To prevent the hydrolysis of the Si-O-C bond, they have reported by attaching sterically bulky groups to the silicon atom by treating the PMMA surface with isopropoxy modified by bis-trimethoxy-silyl-propyl-amine. This allows hydrolytically stable bonds to be form between PDMS and PMMA.

In the project reported here, a novel method of bonding PMMA and PDMS using a dry adhesive film had been developed. A hybrid membrane consisting of an

adhesive layer and a PDMS layer is able to act as the pneumatic actuator in a peristaltic micropump. This form of fabrication of the multi-layers not only allows PMMA to be bonded to PDMS, it is also easy to fabricate as well as cost effective. This hybrid membrane worked extremely well, and prevents air from the actuation chambers to leak into the microchannels. Contact to aqueous media does not cause delamination and leakage. Design, method of fabrication and results of the membrane and the resulted micropumps will be further elaborated in chapter 4 of the report.

3.2 Detection method in the microfluidic chip

Optical detection on chip was realized with optical fibres. The fabricated microchips had embedded single mode optical fibers (AFS105/125Y, Thorlabs Inc.) with a core diameter of 105 μ m, and a numerical aperture of 0.22. The fibre was inserted into the detection region to capture the optical signals. Process of the fabrication of PMMA LOC is illustrated in the flow chart shown in Figure 3-5.

One optic fiber permits light input while the other is receives the signal. Prior insertion of the optical fiber into the microfluidic chip, fiber was stripped of its cladding at the extreme ends by cutting it using a stripper and cleaver respectively (Fujikura, Japan). This process was performed to obtain a straight surface of the fiber, so that all the light waves from the laser source (Photonitech) will be captured and transmitted to the spectrometer (Ocean Optics). Insertion of the optical fiber was carried out under a microscope, Nikon, ECLI-PSE, TE-2000-S microscope and a mercury lamp (Model LH-M100CB-1, 137955, Nikon, Japan). This was then followed by fixing the positions of the optic fibers with an adhesive, epoxy glue (Araldite).

Wavelength of light at 412 nm was to be used for the measurement of the absorbance levels for the enzyme assay experiments that will be conducted with the LOC.

Figure 3-5. Flow chart of LOC fabrication using PMMA substrate. Bonding methodology is using thermal means. Optical fibers are inserted into the detection region to acquire the signals.

Figures 3-6 and 3-7, show the desktop-based set up used in the lab for measuring the absorbance levels of the enzyme assay experiments. The set up comprised of a laboratory sized laser source (Photonitech, Singapore) and a palm-sized spectrometer (Ocean Optics), lap-top (IBM) with the software for recording the

Figure 3-6. Desktop based set-up for detection of nerve agent sarin

Figure 3-7. The microfluidic device with integrated optical fiber optic sensor.

results. The absorbent measurement was carried out with a portable fibre optic spectrometer (USB 2000) and its corresponding software SpectraSuite (Ocean Optics, Florida, USA). One optical fibre was connected to the light source (wavelength 405nm – 680 nm) purchased from Photonitech, Singapore. The other optical fibre is connected to the optical input of the spectrometer by means of the HPLC connector. The absorbance signals are acquired, displayed and stored as numerical data. After recording the base-line absorbance and the absorbance of the reaction product with the enzyme assay reaction, the remaining enzyme activity within the microchip was calculated using equation (3.1).

$$\alpha = \frac{A_{\text{with sample}}}{A_{\text{without sample}}} \times 100\% \quad (3.1)$$

where α is the enzyme activity remained after inhibition, $A_{\text{with sample}}$ is the absorbance of reacted solution with GB, $A_{\text{without sample}}$ is the absorbance of reacted solution without GB.

Chapter 4

Design, fabrication and testing of microfluidic components

4.1 Micropumps

4.1.1 Investigation of ThinXXs micropumps

One of the available commercial micropumps that have been distributed worldwide is manufactured by ThinXXs (Germany). These micro diaphragm pumps are driven by piezoelectric actuation with low power consumption. Passive check valves are present in the micropumps which allow fluids to be drawn up through the inlet and exit through the outlet. These piezo-driven micropumps are capable of handling microliters of fluids. The flow rate of the micropumps is dependent on the frequency of the piezoelectric actuator. The economic manufacturing of the micropumps by injection molding allows these pumps to be treated as a disposable device. ^[32]

Figure 4-1 is a picture of the ThinXXs micropump and controller. These micropumps are small in size with a diameter of 23 mm, height of 3mm (without cover)) and weighs 3g. This micropump is able to pump water at a maximum flow rate of 7 ml/min. at 25Hz. With no flow rate, it will experience a maximum back

Figure 4-1. ThinXXs micropump ^[32]

pressure of 35kPa for water. Such capability, low cost and low power consumption, makes the ThinXXs micropump an attractive consideration for the intended LOC.

Prior to designing and fabricating the micropumps for LOC, commercially available micropumps (ThinXXS, Germany) were characterized for their suitability. Several experiments were conducted using ThinXXS micropumps to pump the fluid into the microchip from our preliminary work ^[26]. ThinXXS micropumps were able to pump water into the microchip with no difficulty. However, when the medium was changed to human blood samples, it was observed that the fluid was not able to flow into the microchip. For both experiments, the micropumps were driven at a frequency of 2Hz. This could be due to the high viscosity of the human blood samples. Fluid was seen to exit via another inlet rather than the outlet.

Two sets of simple enzyme assay experiments were conducted using the ThinXXs pumps. The data obtained was compared to the data obtained from another set of experiments conducted using syringe pumps (CMA, 100 microinjection pump, Stockholm, Sweden).

Reagents used in the experiments were DTNB (0.9 mM), ASChI (1.35 mM) and AChE (1:2500). All the reagents were flowed into the microchip at the same time. The PMMA microchips used in these sets of experiments were fabricated using laser ablation followed by thermal bonding. Figure 4-2 shows the design of the microchip used in these sets of experiments.

The reaction microchannels on the 55 mm x 30 mm rectangular chip were 310

Figure 4-2. Design of microchip used in the free enzyme assay experiments.

μm (length) x $100\ \mu\text{m}$ (depth) x $80\ \mu\text{m}$ (width). A frequency of 2 Hz was chosen as the driving actuation of the ThinXXs micropumps as it is comparable to the flow rate used for the syringe pump.

Data obtained from the ThinXXs micropumps were compared against the data obtained from experiments conducted using syringe micropumps. Figure 4-3 shows the plotted data from both different sets of experiments.

As can be seen from Figure 4-3, the absorbance values obtained for experiments conducted using syringe micropump is significantly higher compared to ThinXXs micropumps. The absorbance values obtained using ThinXXs micropumps is too low upon comparing the absorbance values obtained from a spectrometer (UV-Vis, Agilent Technologies). In the experiment conducted with the spectrometer, $50\ \mu\text{l}$ of each reagent (DTNB, ASChI, AChE) were put into a cuvette and measured. The spectrometer was set to read for 45 minutes under the ‘Kinetics Assay Mode’. Path-length of the cuvette is 10mm. Figure 4-4 shows the plot of Absorbance of the kinetic

Figure 4-3. Tabulated absorbance results obtained from free enzyme assay experiments in aqueous medium using different pumping devices. ($n = 2$) assay in the cuvette measured from the UV-Vis spectrometer over time.

Figure 4-4. Plot of Absorbance against time. Absorbance values were obtained using the UV-Vis spectrometer under ‘Kinetics Assay Mode’

The results in Figure 4-4 showed that about 20 min is required for the reaction to reach saturation with an absorbance of about 1.5 Abs. The absorbance value obtained from the ThinXXs micropump of 0.25 Abs is too low (Figure 4-3). Whilst the absorbance values obtained from the syringe pump is 0.8 Abs, which is slightly lower than the expected 1.5 Abs. Therefore, ThinXXs micropumps are not suitable for the application in this project.

4.1.2 Micropumps on PMMA: Piezoelectric discs acting as micropumps

As the ThinXXs micropumps were not able to drive the fluid to the extent required by the project, hence, a micropump by means of piezoelectric discs actuation, was first designed for this purpose.

The micropump is made up of 1 piezo disc, 1 piece of rubber gasket with either ‘flaps’ or holes cut (these will act as the valves of the micropump). The regions of the micropumps were first designed and drawn using CorelDraw software. The drawings are then sent to the laser cutter (see Section 3.1.1) to be engraved either on the rubber gasket or PMMA. Two different valve designs were tried out. The designs of the pump engraved onto the PMMA sheet is illustrated in Figure 4-5. Two different valve designs are shown in Fig. 4-6.

Figure 4-5. Design of the micropump engraved onto the PMMA substrate.

Circular structures of different sizes and depths were engraved on the PMMA substrate. The different sizes and depths of the circular structures in the micropump design, were to allow the rubber gasket to be position properly in the region later in thermal bonding and also during functioning. The through holes cut into the PMMA substrate was to allow the drawing of fluid from the reservoir into the pump and exiting into the microchannels.

Figure 4-6 shows the two different valve designs that cut onto the rubber gasket and tested. For design 4-6(a), the ‘flaps’ work as check valves when the piezoelectric discs are activated. Whilst for design 4-6(b), 4 through holes were cut on the rubber gasket for the inlet and outlet regions on the PMMA. The middle region was not cut to allow the rubber gasket to act as a form of blockage to the openings on the chip when the piezo discs are activated. Due to rubber flexibility, when the piezo discs are activated, the upward and downward motions will cause the rubber gasket to deflect up and down. Thereby, creating the valving motion required to pump the fluid.

Figure 4-6. Designs of the valves cut onto the rubber gasket. (a) Design consist of 2 flaps cut on the rubber gasket. (b) 4 cut-through holes were cut on the rubber gasket to allow water to pass through.

Once the PMMA and rubber gaskets were cut using the laser micromachine, the PMMA substrates will undergo two thermal bonding processes. The first thermal bonding process was to bond the PMMA substrate with the microchannel structures with another PMMA substrate with the engraved pump designs. This will then followed by the second thermal bonding process. The rubber gaskets are placed onto the pump region on the PMMA and thermally bonded to another clean PMMA substrate. Alignment of the rubber gasket in the pump region before thermal bonding is very important for the gasket to function properly in later experiments. Once the microchips were completed, piezoelectric discs were mounted on it and secured using epoxy glue. Electric wires were soldered onto the piezoelectric discs which are connected to a power source to provide the electrical energy required to activate the piezoelectric discs. When power is activated, the piezo-electric discs will alternately move up and down. This alternating motion of the piezo-electric disc provides the pumping force for drawing up and dispensing of fluid.

Figure 4-7 shows the completed microchip with the mounted piezoelectric discs acting as the pump membrane.

This particular design with two pumps were characterized with de-ionized (DI) water as the fluid flowing into the microchannels and also the frequency of the piezoelectric discs were varied to determine the optimum working frequency of the piezoelectric discs.

Figure 4-7. PMMA microchip with mounted piezoelectric discs acting as the pump

Both valve designs were tried out with DI water. It was discovered that valve design 4-6 (b) was not able to function properly. The reason being, the valves were not able to seal pump region properly when the piezo-electric discs were activated. The leaking of water in the pump region caused the piezo-electric discs to malfunction due to short-circuitry. Therefore, design 4-6 (a) will be used to check for the performance of the designed micropump.

When two pumps were activated at the same time, there were numerous times that the fluids when entered from the inlets did not flow into the meandering microchannels. Instead fluid from the inlet will try to exit via the other inlet. To investigate the most optimum frequency at which the piezoelectric pumps could function, the 1st set of experiments conducted was to activate just one pump. Figure 4-8 shows the data obtained of the pump with varying frequency corresponding to the flow rate of the fluid within the microchannel.

Figure 4-8. Plot illustrating the relationship of 1 piezoelectric disc pump when activated and the corresponding flow rate of fluid within the microchannel. ($n = 4$)

Frequency of the pump was not increased beyond 4 Hz, reason being the ceramic portion of the piezoelectric disc is very brittle. Hence when the piezoelectric disc is subjected to long time working or high frequency, the ceramic portion cracks, hence causing the pump to stop functioning or in some experiments it was observed that the piezoelectric discs were not mounted properly to the PMMA chip and water was seen leaking out. This is dangerous as it will cause electric shock to the system and the piezoelectric discs will malfunction.

After confirming the maximum frequency that the piezoelectric discs could function, the next set of experiments to be conducted was to activate both pumps. As mentioned earlier, piezoelectric discs malfunction often, this design of micropump is not feasible for the intended application. Furthermore, the check valves present in the microchip was not able to seal properly.

Figure 4-9. Plot illustrating the relationship of 2 piezoelectric disc pumps when activated and the corresponding flow rate of fluid within the microchannel. ($n = 4$)

4.1.3 Micropumps on PDMS

The designs of the micropumps and microchannels were drawn using AutoCad 2006, which will then be printed onto a plastic mask for lithography process followed by soft lithography process using PDMS as the elastomeric substrate. Figure 4-10 shows the micropumps that had been designed and fabricated.

For both designs, the depth of the micropumps is 150 µm. Shown in Fig. 4-10 (a), the micropump designs and concept are inspired from design by Unger et. al. Whilst the design shown in Fig. 4-10 (b), was inspired by Yang et. al. ^[48].

For design 4-10 (a) shown in the figure, each vertical pump column is 8 mm in length by 0.45 mm in width for a 16 mm x 14 mm microchip. Whilst for design 4-10 (b), the dimensions are shown in Figure 4-11.

Figure 4-10. Designs of micropumps to be fabricated using soft lithography. (a) Valves are in the form of long columns. (b) Pneumatic micropumps with serially connected actuation chambers.

Figure 4-11. Schematic illustration of the micropumps shown in Figure 4-10 (b).

Once the designs of micropumps and microchannels were plotted onto a mask, lithography and soft lithography processes will be performed to yield the PDMS casts with the desired designs. After the PDMS casts were peeled away from the molds, a thin PDMS membrane (thickness $\sim 80 \mu\text{m}$) will be bonded to the microchannels and micropumps. Bonding of the PDMS layers is achieved using O_2 plasma (Power = 70W; Pressure = 75kPa, O_2 concentration = 20 sccm). Figure 4-13 shows the flow of how the microchip with micropumps were fabricated.

Figure 4-12. Design of single straight microchannel fabricated using soft lithography. Widths of microchannels experimented: 300 μm , 500 μm , 800 μm and 1000 μm .

Figure 4-13. Schematic flow chart of the fabrication process of a PDMS micropump. Step 1: PDMS mixture (10 parts resin : 1 part curing agent) was poured onto 3 wafers with microchannels, micropumps and clean wafer respectively. Step 2: after heating the wafers with PDMS mixture at 80°C, PDMS layers were peeled off. Step 3: the 3 layers will undergo O_2 plasma to be bonded together. A: Cross-sectional view of the completed microchip consisting of micropumps (1), thin membrane (2) and microchannels (3)

The thin layer of PDMS bonded to the micropump and microchannels was obtained by pouring the uncured PDMS mixture (10 parts resin: 1 part hardener) onto a wafer surface and cured at 80°C for 1 hour, before peeling it away to undergo oxygen plasma to be bonded to the other 2 layers of PDMS casts. This thin PDMS membrane will be pushed down sequentially when air filled up the micropump chambers (layer 1 in the completed microchip of Figure 4-13). With the thin PDMS membrane depressed, the liquid in the working microchannel (layer 3 of completed microchip in Figure 4-13) will be pushed in a specific direction. Hence, the liquid is then pumped forward along the channel accordingly. To prevent back-flow of the fluid, the above design can also work as an ‘active valve’. However, PDMS is permeable to air, allowing air from the actuation chambers to penetrate the thin

PDMS membrane and enter the microfluidic channels. Hence bubbles formed from the infusion of air from the actuating chambers will affect the flow rate of the fluid in the microchannels. Therefore, improvement of the actuating membrane had to be investigated.

4.2 PMMA/PDMS peristaltic micropump

Various methods were investigated to overcome the infusion of air from the air chambers into the microchannels. One of the methods was filling the air chambers with oil before applying air to the actuation chambers. A layer of oil was placed onto the membrane, in the hopes of preventing air from permeating through the membrane. Also, different ratios of PDMS resin with curing agents were also investigated. However, none of these methods yielded positive results. To overcome this challenge, the air chamber substrate was changed to PMMA and investigations were carried out to bond PMMA to PDMS. As mentioned earlier, a novel technique of bonding PMMA to PDMS had been developed. This form of bonding allows the 2 different surfaces to adhere very well and it also allows the realization of an actuating membrane for the desired peristaltic micropump design. Furthermore, this hybrid membrane prevents air fusion from the air chambers into the microchannels.

4.2.1 Bonding of PMMA to PDMS

Figure 4-14 depicts the fabrication process of a PMMA/adhesive/PDMS device. The PMMA substrate was first micromachined. The designs of the devices (test membrane and micropumps) were designed using AutoCad 2006 software followed by laser engraving of the structures onto PMMA surface. After laser machining, the PMMA pieces are washed in 70% ethanol and rinsed with de-ionised (DI) water. As depicted in Figure 4-14, the PMMA substrate was cut into the form of a circular 4-inch wafer. The air chambers are engraved into the PMMA substrate. After cleaning, the dry adhesive (Adhesive Research, Glen Rock, PA, USA) film was laminated on the PMMA wafer. The acrylic adhesive is able to adhere very well to the PMMA surface. In contact with liquids, the adhesive loses its adhering property. Hence, the adhesive film has to be in direct contact with the PMMA surface. PDMS

Figure 4-14. Fabrication process of the PMMA/adhesive/PDMS part and the peristaltic micropump; (a) PMMA wafer with engraved air chambers; (b) Dry adhesive film is laminated onto the PMMA air chambers; (c) PDMS is spin coated onto the PMMA wafer covered by dry adhesive; (d) The PMMA wafer with the adhesive/PDMS membrane and the moulded PDMS part with microchannel are treated in oxygen plasma; (e) Treated surfaces are bonded together to form the micropump with actuation chambers in PMMA and microchannel in PDMS.

was prepared, degassed and spin coated onto the PMMA wafer covered by the adhesive film, at a rate of 1500 rpm and spinning time of 30 seconds. A PDMS film of 50 μm was resulted on the adhesive layer. Thermal curing of the adhesive/PDMS layer took place at 80 $^{\circ}\text{C}$ for one hour. For the membrane tests, the fabrication process ended here. However, for the fabrication of the peristaltic micropumps, the PMMA wafer with the adhesive/PDMS membrane and the PDMS part with the microchannels underwent oxygen plasma treatment, and were subsequently bonded together. The process results in an irreversible bond. Discussion and results of the fabricated peristaltic micropump will be further elaborated later in the report.

4.2.2 Characterization of adhesive/PDMS membrane and the bond strength

Figure 4-15 shows the designs of the membranes used in the pressure test. The test chips contain different air chambers in the form of squares and circles. The edges or the diameters are 300 μm , 500 μm , 1000 μm , 1500 μm , 2000 μm , 2500 μm and 3000 μm . The depth of the air chambers in PMMA is 500 μm . All air chambers share

Figure 4-15. PDMS membrane designs for the pressure test: (a) circular air chamber, (b) rectangular air chamber.

the same pressure port.

Investigations of the adhesive/PDMS membrane and the bond strength of both circular and square air chambers were carried out by using a calibrated pressure source. Pressurised nitrogen was first connected to a pressure controller (PPC4 DH Instrument, Fluke Co., USA) which works as a precise pressure source for the tested membranes. A 45 ° mirror (Edmund Optics, Singapore) was placed next to the membrane array to allow imaging both top view and side view of the deformed membrane as shown in Figure 4-16. The deformation of the membrane was captured by a CCD camera and the corresponding software (Video Savant, IO Industries, Inc.,

Figure 4-16. Deformed membranes under applied pressure: (a) Circular membranes at 20 kPa; (b) Square membrane at 13 kPa.

Canada). Images of the deformed membranes captured are then analysed by a program written in Matlab (MathWorks, MA, USA). The centre of deflection and the

radius of curvature of the deformed membrane were measured at different applied pressure. The pressure used for the circular air chambers varies from 2 kPa to 20 kPa, whilst for the square air chambers it varies from 2 kPa to 13 kPa. Figure 4-16 shows the typical images of deformed membranes of both circular and rectangular cases.

Figure 4-17 shows the effect of increasing pressure on the centre of deflection and the radius of curvature of circular membranes with different diameters. The results show that the bond quality between PDMS and PMMA is good; no delamination occurs at the maximum pressure of 20 kPa. At higher pressures, the membrane would burst while the bond was still intact. The results also indicate that the larger the applied pressure the higher is the centre of deflection of the membrane. At the same applied pressure, a larger membrane experiences a larger deflection. The relatively large deflection would allow the adhesive/PDMS membrane to be used as a pneumatic actuator for microvalves and micropumps. With increasing pressure, the radius of curvature of the deformed membrane decreases as shown in Figure 4-17(b). For the largest membrane with a radius of 3 mm, beyond a critical pressure of about 18 kPa, the radius of curvature increases with increasing pressure. This behaviour may be caused by the tear-off effect of the more rigid adhesive layer, making the overall membrane becomes softer. The change in the behaviour of deformation versus pressure can also be observed in the deflection data, Fig. 4-17 (a).

The square membranes were tested up to 13 kPa. The pressure is limited by the deflection of the membrane exceeding the image frame that can be captured by the CCD camera. Similar to the circular membranes, an increasing pressure causes an increase in the centre of deflection of the membrane and a decrease in radius of curvature. Due to the larger membrane area ($A = A$), the deflection for the square membrane ($A = a^2$) was significantly larger than that of a circular membrane ($A = \pi a^2/4$). As shown in the inserts of Figures 4-17 and 4-18, where a is the diameter of the circular membrane and the length of the square membrane. At 20 kPa, a 3-mm circular membrane reaches a centre deflection of approximately 2.20 μm . At 13kPa, a 3-mm square membrane reaches a centre deflection of approximately 3.5 mm. Although the membrane was deformed and took the shape of a balloon, the membrane did not detach from the PMMA substrate. For the same design footprint, a square membrane would allow a larger deflection. The square membrane was therefore used for designing the peristaltic pump described in the next section.

Figure 4-17. Geometry of circular membranes at different applied pressures: (a) centre deflection; (b) radius of curvature.

Figure 4-18. Geometry of square membranes at different applied pressures: (a) centre deflection; (b) radius of curvature

4.2.3 Operation and characterization of peristaltic micropump

A peristaltic micropump (fabrication process shown in fig. 4-14) was fabricated by bonding PMMA wafer with the adhesive/PDMS membrane on a PDMS part containing the microchannel of the pump. When the rigid PMMA air chambers are filled with compressed air, the membrane deflects to the bottom of the microchannel, which has a height of only 150 μm . Fluid flow inside the microchannel is sealed off and displaced by combining the peristaltic motion of the three actuating membrane as depicted in Fig. 4-19. The air chambers in PMMA are placed 1 mm apart. The overlapping area between the air chamber and the microchannel defines the actuating membrane which is depicted as the grey areas in the schematics of Fig. 4-19. Fig. 4-20 shows the assembled microchip.

Figure 4-19. Control circuit and the experimental setup for characterizing the peristaltic micropump. The schematic of the micropump is shown on top. The active areas of the pump chambers are shaded grey. The size of the pump chamber is determined by the width of the pump channel W_c and the width of the actuation chamber W_a .

Figure 4-20. The fabricated microchip comprising of the micropumps and the microchannels

The area of the actuating membrane is $A = W_a \times W_c$ where W_a and W_c are the widths of the air chamber and the microchannels, respectively. Two different widths of air chamber $W_a = 1$ mm and 2 mm and three different channel widths of $W_c = 0.5$ mm, 0.8 mm and 1.0 mm were tested. DI water mixed with red food dye was used as the test liquid. The micropumps were activated by three two-way solenoid valves. The valves are switched when the direction of the applied current is changed. A customized switching circuit using relays was built for this purpose, Fig. 4-19.

As the circuit requirement is similar to that used by Nguyen et. al. ^[99], a slight modification was carried out for driving the peristaltic micropump. With the two-way solenoid valve, air from a pressure source is diverted between the air chamber in PMMA and the environment. The control circuit is connected to a data acquisition (DAQ) module (National Instruments, Texas, USA). Figure 4-19 illustrates the hardware setup for testing the micropumps.

LabVIEW 8.5 and NI-DAQmx 8.8 (National Instruments, Texas, USA) were used as the platforms to run the control program of the micropump. Transistor-transistor-logics (TTL) signals are sent from the DAQ module to the relay circuit. In order for the fluid to be pumped in one direction, the three actuating membranes are controlled in a peristaltic manner. Table 1 shows the actuating sequences of the peristaltic micropump. “1” and “0” indicate the "ON" and "OFF" states respectively. The six states listed in Table 4-1 form one pumping cycle. Therefore, if the pump is operating at a frequency of f , each state should last $1/(6f)$ seconds.

In the characterization experiments, the pumping frequency was varied. Compressed air at 138 kPa works as the pressure source. The average pumping velocity $v = x/t$ was determined by measuring the displacement of the front meniscus x in the tubing connected to the pump and the corresponding time t . With the known cross-sectional area A of the tubing, the flow rate can be determined as $Q = v \times A$. In all the experiments, the measurement time was fixed at $t = 120$ sec. The uncertainties in measuring the time and the displacement are $\Delta t = 0.1$ sec and $\Delta x = 0.5$ mm. The error propagation for the average pumping velocity can be determined as:

$$\Delta v = \frac{\Delta x}{t} + \frac{x}{t^2} \Delta t = \frac{\Delta x}{t} = \frac{v}{t} \Delta x \quad (4.1)$$

Hence, the error in the measure velocity consists of two componenets: $\Delta v = \Delta x/t$ from length measurement and the relative error $\Delta v/v = \Delta t/t$ from time measurement. Applying this error analysis to the experiments, the systematic error contributed by the length measurement is 0.5 μ l/min and the relative systematic error contributed by the time measurement is 0.8%.

Table 4-1. Actuating sequence of the three membranes in the peristaltic micropump.

Sequence	Membrane 1	Membrane 2	Membrane 3
1	0	0	0
2	1	0	0
3	1	1	0
4	1	1	1
5	0	1	1
6	0	0	1

To investigate the long-term stability of the micropumps, the pump with width $W_a = 2$ mm and channel width $W_c = 0.5$ mm was tested at pumping frequencies of 10Hz and 30Hz for 3 days each. The flow rates were sampled during this time span and depicted in Figure 4-21.

Figure 4-21. Flow rates at 10Hz and 30 Hz sampled from pump operation over three days ($W_a = 2$ mm and $W_c = 0.5$ mm)

Figure 4-22(b) shows the performance of micropumps with a larger air chamber width of $W_a = 2$ mm. For smaller channel width of $W_c = 0.5$ mm and 0.8 mm, the twice as large actuating membranes double the flow rate as compared to the performance of pumps with $W_c = 2$ mm. For the larger microchannel with $W_c = 1$ mm, the improvement in flow rate is not that apparent.

The results show that at same pumping frequency, the larger the channel width, the higher the flow rate. At frequencies beyond 20 Hz, the flow rate remains constant or even drops slightly in the case of channel width = 1 mm. This phenomenon is caused by the limited dynamics of the switching relays. A solid-state

Figure 4-22. Performance of peristaltic micropumps with different channel widths W_c ; (a) Actuation chamber with a width of $W_a = 1$ mm; (b) Actuation chamber with a width of $W_a = 2$ mm.

relays with a higher dynamics would allow a higher pumping flow rate at frequency beyond 20 Hz.

4.3 Micromixers

Micromixers are another very important component in a LOC, where the bulk of the reactions take place. Especially for biological or chemical experiments where time and the mixing of reagents are crucial for reactions to take place.

4.3.1 Micromixers in PMMA

Previously, achieving effective mixing of reagents in the microchannels was one of the numerous challenges faced while the experiments were conducted. With poor mixing of reagents, long duration was required to obtain the results. Tan et. al. ^[25] reported setting the flow rate of 4.0 $\mu\text{l}/\text{min}$ at which the reagents (DTNB, ASChI & AChE) pumped into a very long micromixer (dimensions: 309.85 mm (length) x 100 μm (depth) x 80 μm (width) Figure 4-2). Such a slow flow rate was necessary for the reagents to have sufficient time to react by diffusion. The fluid dynamics of the reagents' flow within the microchannels was laminar. Diffusion time is long as compared to the residence time in the microchannel and this is not ideal. Therefore, efficiency mixing of reagents in the microchannels had to be further enhanced to reduce the experimental duration.

Turbulence and chaotic advection are the two forms of flows that are being introduced to fluids to enhance mixing ^[51, 75]. Chaotic advection is not turbulence and it can be created in a simple two-dimensional flow using time dependent disturbance or in a three-dimensional flow without time dependent disturbance. ^[75]

In turbulent flows, the motions of the fluids are random and irregular. Fluids in the microchannels can disperse quickly to enhance mixing of fluids. Chaotic advection, is generated by stirring the flow of the fluids and this form of mixing is very effective for fluids flowing with very low Reynolds number. Equation 4.1, is the formula for calculating the Reynolds number for fluids.

$$\text{Re} = UD_h/\nu \quad (4.2)$$

Where U is the mean fluid velocity in m/s ; D_h is the hydraulic diameter in m and lastly ν is the kinematic fluid viscosity in m^2/s .

In most microfluidic cases, flow of the fluids are generally laminar, therefore a low Reynolds number can be expected. Péclet number (Pe), is a measure of the relative importance of advection to diffusion, has to be considered. The higher the Pe number, the more important is the advection.

$$Pe = UL/D \quad (4.3)$$

Where U is velocity in m/s; L is the characteristic length in m; and D is the mass diffusivity in m^2/s .

The flow of fluids in microfluidics is generally laminar. To enhance the mixing of fluids in the microchannels, chaotic advection is being induced by manipulating the laminar flow in the microchannels ^[26, 51, 75]. This phenomenon is an attractive engineering solution for achieving efficient stirring in biofluids. Total strain affected on the macromolecules in the biofluid is limited. One form of creating chaotic advection in the fluidic flow is to include the presence of ‘surface pattern’ etched into the microchannels. With the inclusion of ‘surface patterning’, both length of the micromixer and time for complete reaction of reagents to take place are greatly reduced. This concept also allows an increased in surface contact between the different fluids.

Decreasing the diffusion path between the fluids is another way to improve mixing of fluids in the microchannels. To reduce the diffusion path, the widths of the microchannels are made smaller. However, smaller channel widths will require higher pressure to pump the blood into the microchannel. In our investigations, a couple of the surface pattern designs were tried out:

- i) Diagonal pattern
- ii) Herringbone pattern

Figure 4-23 show the designs of the micropatterns present in the micromixers that were being investigated.

Figure 4-23. Design of micromixers. (a) Microchip design with herringbone structures. Number of microchannels are indicated in ‘red’ on the diagram; (b) Enlarged view of herringbone structures; (c) Enlarged view of diagonal patterning.

Both micromixer designs were tested using fluorescent dye and water. However, it was observed that microchip with the diagonal patterning, Figure 4-23 (c), did not yield good mixing within the microchannels. Microchips with design 4-23 (c) were tested in the earlier report and the results yielded were almost equivalent to microchips with no microstructures etched in the microchannels.

Designs with herringbone structures were tested out by conducting 2 sets of experiments using fluorescence solution and enzyme, substrate and chromophore reagents to determine the efficiency of mixing in the microchannels:

- Set A: Water and fluorescence dye .
- Set B: DTNB, ASChI and free AChE

The micromixer was tested using a fluorescent dye ($C_{20}H_{10}Na_2O_5$). The 10x 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 liter, autoclaved for 20 min] was added to the dye solution. 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) was used to record the concentration field of the fluorescent dye. Figure 4-24 shows the images of the fluorescent dye at different places along the mixing channels at a flow rate of 140 $\mu\text{l}/\text{min}$. At the entrance, the fluorescent and non-fluorescent liquids were clearly separated by an interface. With the help of the herringbone structures and the turns, stretching and folding were observed. As the fluids flow along the microchannels, the fluorescence colour of the mixture became more uniform (from channel 3 onwards). The results showed that the herringbone structures were able to induce chaotic advection in the mixing channel.

Figure 4-24. Concentration field in the micromixer at a flow rate of 140 $\mu\text{l}/\text{min}$

Once the design has been confirmed, several enzyme assay tests were carried out with the microchip using DTNB (0.9 mM), ASChI (1.35 mM) and AChE (1:2500). Comparisons were made with another set of tests conducted on a microchip without microstructures etched in the microchannels. Flow rates at which the enzyme, substrate and chromophore were introduced into the microchips were also varied. The flow rate was varied so as to enable us to investigate the optimum flow rate to be used for subsequent experiments.

The results show that the presence of herringbone structures in the microchannels, helped to enhance the efficiency of mixing of the reagents. Replicating the similar herringbone structures in a PDMS microchip is challenging, as it will require a two

Figure 4-25. Comparison of experimental results between chip with herringbone structures and chip without herringbone structures. step lithography process.

4.3.2 Micromixers in PDMS

The micromixers on the microchip were designed according to the previous microchannel design from Tan et. al.^[26]. Microchannels designs fabricated to test for mixing efficiency are shown in Figure 4-12 (a) and Figure 4-12 (b). The microchips

were fabricated using soft lithography process, and the PDMS casts with the microchannels then undergo O_2 plasma. The whole process of lithography and soft lithography process has been discussed in section 3.1.2.

The PDMS microchips measuring 17 mm x 18 mm, contain microchannels of dimensions: $600\text{ }\mu\text{m}$ x $100\text{ }\mu\text{m}$. These microchannels have micro-patterns which will enhance mixing of the reagents in the microchannels. Similar to the PMMA microchips containing the micromixer designs, the micromixers in PDMS has:

- 1) Rectangular columns: These rectangular patterns measure: $100\text{ }\mu\text{m}$ (width) x $250\text{ }\mu\text{m}$ (length) x $150\text{ }\mu\text{m}$ depth. The distance between 2 rectangular patterns is $150\text{ }\mu\text{m}$.
- 2) Herringbone structures: The herringbone designs in the PDMS microchannels are slightly different from that in the PMMA microchannels. The structure is being split up so as to allow fluid to flow through. The reason being, the microstructures are of the same depth as the microchannel depth. Hence if there is no gap in between the herringbone structure, fluid will not be able to flow through. Figure 4-26 shows the structure of the herringbone structures in the PDMS microchips.

Figure 4-26. Herringbone structures in the PDMS microchannels.

These herringbone structures measure: 250 μm (length) x 60 μm (width) x 150 μm (depth). The horizontal gap in between each structure is 90 μm whilst the vertical gap between each structure is 250 μm .

As the method of fabricating the microchip has been translated from laser micromachining to soft lithography, optimization of the PDMS microchips has to be carried out. Varying flow rates were being tried out: 100 $\mu\text{l/hr}$, 500 $\mu\text{l/hr}$, 900 $\mu\text{l/hr}$ and 120 $\mu\text{l/hr}$. These experiments were conducted to investigate the mixing efficiency of the reagents within the microchannels. Two different fluids were pumped into the microchannels and images were captured with a digital video camera. The two fluids were DI water and water mixed with fluorescein dye. It was observed that the microchannel design shown in Figure 4-12 (b) was not able to allow fluid to be pumped through. Perhaps the gaps between the herringbone structures in the microchannels were too small for the fluid to pass through. Therefore, the mixing efficiency test on PDMS microchip will be based on the design shown in Fig. 4-27. The circled regions indicated on Figure 4-27, are regions where mixing efficiency of the fluorescence with water are recorded.

Figure 4-27. Microchannels to be fabricated with PDMS. 1, 2, 3 indicate 1st channel, 2nd channel, 3rd channel. Circled regions are also regions where readings were taken.

The mixing efficiency of the fluorescence and water was observed under microscope. It was seen that the lower the flow rate, the region where the fluids have uniform mixing is nearer to the inlet. As discussed earlier in Section 4.2.1, chaotic advection is generated by stirring the flow of the fluids and this form of mixing is very effective for fluids flowing with very low Reynolds number. Furthermore, in most microfluidic cases, the flow of the fluids is generally laminar. With laminar flow, a low Reynolds number can be expected. Hence, it is vital to consider the Péclet number (eq 4.3). The higher the Pe number, the more important is the advection. In the experiments conducted, at lower flow rates, the fluids were able to mix better due to a longer mixing time and diffusion. However, with a higher flow rate, the duration for the fluids to mix properly is reduced. For example, the experiment conducted at a flow rate of 1200 $\mu\text{l}/\text{min}$, mixing in the microchannels did not take place even until the outlet. Hence, as the flow rate increases, the region where the mixing has taken place will be further away from the inlet.

4.4 Immobilization of enzyme on PMMA and PDMS

Acetylcholinesterase (AChE) is the enzyme that is vital in the procedure to detect the presence of GB, by indication of its activity through the colour change of the chromophore and substrate. In order to detect the presence of GB regenerated from the blood samples spiked with sarin, it is necessary to have post-inhibition results compared against pre-inhibition result. In order to obtain pre- and post-inhibition data, it is necessary to immobilize the AChE. Furthermore, it is not possible to use flow AChE together with spiked blood into the microchip at the same time. The red colour of the haemoglobin in blood prevents the optical fiber from picking up light signals at 412 nm.

Previously, the AChE was immobilized on a thin glass cover slips (Fisher Scientific, Pittsburg, PA, thickness of 0.1mm) cut into pieces measuring 15mm x 7mm. 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 APTES, the chemical necessary for cross-linking of AChE. After this process, this glass slide was then positioned by an adhesive on the bottom of PMMA layer in the enzyme chamber.

Next, thermal bonding of the PMMA pieces with the glass slide was carried out, before enzyme immobilization process is carried out for the next 3 days. For the first day of enzyme immobilization, cross-linked enzyme solution (10 units/ml AChE, 2.38µg/ml BSA, 0.119% wt/vol GA) was pumped into the inhibition chamber. The whole device was kept at 4°C for 12 hours. The following day, the enzyme solution was washed by pumping in wash buffer (1 part 50% tween 20 and 1000 parts 0.1M PB pH 7.2) into the inhibition chamber. Next the GST solution (10% wt/vol. trehalose, 0.1% gelatine and 0.02% sodium azide) was pumped into the inhibition chamber. The chip was kept at 4°C for another 4 hours. After the 4 hours of incubation, the GST solution was then pumped out from the inhibition chamber. The chip was lyophilized at -40°C overnight and subsequently placed in a vacuum desiccator for 2 hours. This step is to ensure the long term storage of the device with immobilized enzyme.

In the previous method, much preparation had to be done on the glass side. A much simpler method of immobilizing AChE directly onto the microchip material had been investigated and being used in the experiments. Data and observations had been recorded and this method of immobilizing the AChE onto either PMMA or PDMS has proved to be reliable and stable.

It was discovered that treating the surface of PMMA or PDMS with aminopropyltriethoxysilane (APTES) allows AChE to be immobilized on PMMA or PDMS. APTES is a cross-linking solution in enzyme immobilization that had been used widely by numerous teams ^[26, 77-81]. The APTES solution was freshly prepared using 5% (Vol./Vol.) APTES stock diluted in autoclaved water.

To enable the surface of PMMA or PDMS to become more hydrophilic, PMMA or PDMS specimen first had to undergo oxygen plasma (Power of 100W; pressure of 100 kPa; oxygen flow rate of 50 sccm) for 60 s. The recipe was similar to that reported by Valchopoulou et. al. ^[62]

The PMMA or PDMS specimen were then removed from the RIE chamber and the prepared APES solution was dropped onto the surface of the PMMA or PDMS and left on the hotplate at 80°C until the APTES dried and left a transparent

film on the PMMA or PDMS. Thermal bonding or plasma-assisted bonding was conducted to ensure the complete microchip fabrication.

Once the microchip has been completed, 3 days of enzyme immobilization followed. This enzyme immobilization process is almost similar to that reported earlier in this section and by Tan et. al. [26]. For the first day of enzyme immobilization, cross-linked enzyme solution (10 units/ml AChE, 2.38 $\mu\text{g/ml}$ BSA, 0.119% wt/vol GA) was pumped into the inhibition chamber. The whole device was kept at 4°C for 12 hours. The following day, the enzyme solution was washed by pumping in wash 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% gelatine and 0.02 % sodium azide) was pumped into the inhibition chamber. The chip was kept at 4°C for another 4 hours. After the 4 hours of incubation, the GST solution was then pumped out from the inhibition chamber. The chip was lyophilized at -40°C overnight and subsequently placed in a vacuum desiccator for 2 hours. This step is to ensure the long term storage of the device with immobilized enzyme.

Once the microchip has been immobilized with enzyme, a simple enzyme assay test using DTNB (5, 5'-dithio-bis-2-nitobenzoic acid) and ASChI (acetylthiocholine iodide) was conducted. When there is a distinct change in colour of the substrate/ chromophore mixture from colourless to yellow, this indicates that AChE is present. The results of the enzyme assay experiments conducted in these microchips will be further discussed in Sections 5.2 and 5.3.

4.5 Fabrication of PMMA microchip for nerve agent detection

The LOC device is implemented on a 79 mm $\times$ 37.5 mm chip. Fig. 4-28 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 herringbone structures. The channel width and depth are 2 mm and 0.4 mm, respectively. The total length of the mixing channel is 100 mm.

Figure 4-28. 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

The herring-bone structures are designed as ridges with a width of 0.5 mm and a height of 0.2 mm, Figure 4-28 (a) (as discussed in section 4.2.1). Unlike the traditional design^[87] with two secondary flows and alternate width changes, this design reverses the direction of the secondary flow in each period to improve the folding effect on each half of the channel. Experimental results of the micromixers have been discussed in section 4.2.1.

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 Figure 4-28 (b). The filter chamber, Figure 4-28(c), 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. Just

before inlets 4 and 5, the width of the microchannel is narrowed to prevent backflow of reagents when the chemicals are introduced into the inhibition reactor. In the inhibition reactor, a glass piece was coated with immobilized enzyme and a protective coating. The reaction chamber measures in length, width and depth, 15 mm, 7 mm and 0.6 mm, respectively. Herring-bone patterns are present on the top PMMA layer over the reaction chamber. These herring-bone patterns are present to improve the transport of reagents to the glass surface with immobilized enzyme, Figure 4-28 (d). The reaction products enter the detection path which is 4 mm long and 0.6 mm wide. Grooves are machined into the chip for aligning optical fibres.

The device was fabricated on PMMA using laser micromachining. The device was first designed using CorelDraw (Corel Co., Canada). Information was then sent to a commercial CO₂ laser system to engrave the designed structures on the PMMA substrate. The different channel heights can be adjusted by the corresponding laser power and scanning speed.

The device depicted in Figure 4-28 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 Figure 4-28 (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 Figure 4-28 (c) and 4-28 (f) were machined on the cover layer. The bottom layer holds the glass piece for immobilized enzyme. The three PMMA layers were bonded using the low-pressure, high-temperature thermal bonding process. Under low bonding pressure, the PMMA stack was first heated to 165 °C in 10 min, and then kept at 165 °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 (Figure 4-28 (c)) through the opening in the cover layer. The filter chamber

was subsequently sealed by an adhesive (Araldite, Vantico Ltd, Duxford, UK) with a PMMA sheet, Figure 4-28 (f).

AChE was immobilized on a small glass piece. Glass cover-slips (Fisher Scientific, Pittsburg, PA, USA) with a thickness of 0.1 mm were cut into pieces measuring 15 mm $\times$ 7 mm. The glass piece was first cleaned using chromic acid and rinsed in ethanol and dried in an oven at 100 °C. The glass piece was subsequently coated with APTES (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, immobilization process of AChE is carried out. The immobilization process of AChE on glass slide had been discussed in section 4.4.

After the immobilization of AChE on the APTES–coated glass piece and it was protected with a GST layer, optical fibres were inserted and fixed with adhesive, Figure 4-28 (e). For this purpose, we used single-mode optical fibres (AFS105/125Y, Thorlabs Inc., Newton, NJ, USA) with a core diameter of 105 μ m, a clad diameter of 125 μ m, 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. 4-29.

Figure 4-29. Fabricated PMMA microchip for detecting nerve agent.

Chapter 5

Detection of nerve agent Sarin in aqueous and blood media

Experimental data collected and reported in this section are based on enzyme AChE immobilized directly onto the microchips. Details on the immobilization are reported in Section 4.3. For the experiments, two different types of microchips were fabricated using PMMA and PDMS. The nerve agent, Sarin (GB), used in the studies was synthesized in-house within DSO National Laboratories. The synthesized sarin was first diluted using autoclaved water. Extra precaution had to be taken in handling sarin during dilution and disposal of the waste. All experiments involving sarin were conducted within a negative suction fume hood in DSO National Laboratories.

5.1 Chemical reaction of GB on AChE

Sarin (GB), GB reacts rapidly with the serine hydroxyl group in the active site of AChE, thereby forming a phosphonate ester. The phosphorylated enzyme regenerates extremely slowly therefore making the enzyme inaccessible for its parent substrate, acetylcholine.

However, as mentioned earlier the enzyme used in the assay tests is Acetylcholinesterase (AChE). The substrate used is ASChI (acetylthiocholine iodide), which produces a yellow colour solution, when catalysed and reacts with the chromophore, DTNB (5, 5'-dithio-bis-2-nitobenzoic acid), Fig. 5-1.

Equation 3.1 (section 3.5) was used to calculate the percentage of enzyme activity remained in the LOC after GB was transported through. Calculation of the percentage of remaining enzyme activity was done using the absorbance data collected by the spectrometer (Ocean Optic, USA). The colour from the resulting

Figure 5-1. Sarin intoxication on acetylcholinesterase.

anion after reaction, can be determined by absorbance measurement at a wavelength of 412 nm .

5.2 Enzyme assay tests conducted using the PMMA LOC with immobilized AChE in aqueous medium

After AChE was immobilized onto the PMMA microchips, one batch of microchips was equipped with embedded optical fibers, whilst the another batch of microchips did not have optical fibers.

The PMMA microchips measuring 51 mm (length) x 20 mm (width), contain microchannels of dimensions: 2mm (width) x 0.4 mm (depth); enzyme immobilization chamber of dimensions: 7 mm (width) x 15 mm (length). Figure 5-2, shows the design of the PMMA microchips.

Figure 5-2. Microchip designs with enzyme chamber to house immobilized enzyme. (a) Microchip to be experimented using observation. (b) Microchip design with optical fibers to be embedded in the detection region.

5.2.1 Experiment 1 - Visual observation

Microchips without embedded optical fibers were used in this set of experiment. Mixture of DTNB and ASChI was first pumped into the microchip for 5 minutes at a flow rate of 10 $\mu\text{l}/\text{min}$. A very distinct yellow colour could be observed at the outlet. GB (Sarin) of concentration of 200 nM was then pumped into the microchip at a flow rate of 10 $\mu\text{l}/\text{min}$ for 10 minutes. The relatively long time was necessary to allow the complete inhibition of the enzyme by GB to take place. After the inhibition process, DTNB and ASChI mixture was pumped into the chip again. The reacted solution was observed to remain colourless indicating that there was no reaction between the AChE and the substrate with the chromophore mixture. The colorless reaction product means that GB has inhibited the immobilized enzyme, resulting in no obvious reaction.

The above experiment showed that a novel yet simple method has been derived to immobilized AChE onto the PMMA. Hence, proving a means to fabricate a

portable yet simple and low-cost microchip for the detection of GB in water samples, using visual observation.

(a)

(b)

Figure 5-3. (a) A drop of yellow reacted solution (DTNB +ASChI) was observed at the outlet of the microchip with immobilized AChE. (b) A drop of near to colourless solution (DTNB + ASChI) was observed at the outlet of the microchip with immobilized AChE after GB had been flowed into the microchip

As it was difficult to capture the yellow colour on camera, strips of filter paper were cut and placed at the outlet of the microchip to absorb the reacted solution

Figure 5-4. Stripes of filter paper with the various solution absorbed

(Figure 5-4). The colour of the used filter paper was then compared to the mixed DTNB/ASChI solution and wash buffer.

5.2.2 Experiment 2 - Detection with optical fiber

The same microchip was fabricated with immobilized AChE in the enzyme chamber. However, for this set of microchips, two optical fibers were inserted in the viewing chamber. One fibre guides light from the laser source into the detection region, whilst the other optical fibre receives the light and guides it to the optical sensor as well as the processing software on the computer. Figure 5-5 shows the data obtained for the pre-GB test and the post-GB test.

The results shown in Figure 5-5 indicated a definite drop in absorbance in the post-test. This drop in absorbance proves that AChE was immobilized on the PMMA. The method of immobilizing enzyme onto PMMA was therefore successful. However, further improvement will be carried out to allow the yellow colour of the reacted solution to be more observable. A similar experiment on PDMS fabricated microchips with immobilized AChE will be conducted to examine the suitability of PDMS for enzyme immobilization.

Figure 5-5. Plot showing the data obtained for Pre-GB and Post-GB tests. Optical fibers and a spectrometer (Ocean Optics) were used to capture and analyzed the data.

After the initial test, enzyme assay experiments with PMMA microchips were carried out to check for consistency of data obtained. Three sets of enzyme assay tests were conducted in the PMMA microchips with immobilized AChE on different days and different devices. Each test used a new PMMA microchip. In these three sets of experiments, GB of a concentration of 10 nM was used for the inhibition. The protocol of this test is similar to that described above. The microchip was first flushed with wash buffer so as to remove the protecting GST coating, followed by pumping of DTNB (0.6 mM) and ASChI (0.9 mM), to obtain the pre-inhibition absorbance. After the pre-inhibition test, GB was pumped into the microchip at a flow rate of 10.0 $\mu\text{l}/\text{min}$ for 10 min to allow inhibition of the immobilized AChE to take place. After the inhibition process, wash buffer was once again pumped into the microchip to remove the remnant GB prior to pumping in DTNB and ASChI to obtain the post-inhibition absorbance values. Flow rate of all the reagents were set to flow at 10.0 $\mu\text{l}/\text{min}$.

Figure 5-6. The percentage immobilized AChE activity remained in microchip after 10 minutes of inhibition with 10 nM GB on PMMA microchips for different dates.

Calculation of the percentage immobilized AChE remained in the microchip after a 10 min incubation of 10 nM GB, was done using equation (3.1). Figure 5-6 shows that the data obtained from the inhibition tests with immobilized AChE on PMMA microchips were consistent.

5.3 Compatibility of PDMS for the use with CWA

In the past, we have reported that using PMMA as the substrate material for microchannel fabrication allowed the detection of trace amounts of GB.^[17] However, PMMA as a rigid material and not ideal as the material for fabricating micropumps or microvalves. Hence, softer and flexible materials are to be investigated for possible use in the microvalves of the LOC. PDMS was chosen as the material to be used in the fabrication of the micropumps and microvalves on the microchip. However, numerous papers reported the high absorbing ability of PDMS causing the material to swell. Swelling of PDMS therefore affects its properties. In the experiments, where PDMS is used as the substrate for microchips, the change in material properties will have some adverse effects on the results of assays conducted on the microchip.^[68] Till

date, there have not been papers reporting on using PDMS as the material for detection of by products of CWA in microfluidics. Most of these papers reported on using PDMS microchips for detecting the degradation products using electrophoresis. Hence, investigations need to be carried out to check whether PDMS is a compatible material for CWA usage. PDMS microchips fabricated and to be immobilized with AChE, were done using the protocol as explained in Section 4.5.

5.3.1 Experimentation of PDMS microchips with immobilized AChE in aqueous medium

Since the dimensions of the microchannels in the PDMS-based LOC differ from the dimensions of the microchannels in the PMMA-based LOC, optimization of the flow rate had to be carried out. A simple enzyme assay test using DTNB and ASChI was conducted with the PDMS-based LOC with immobilized AChE. Figure 5-7 shows the microchip design that will be fabricated and experimented.

Soft lithography is used to fabricate this microchip. The soft lithography process used for this microchip fabrication had been discussed in section 3.1.2. The microchip measures: 51 mm (length) x 15.3 mm (width). The microchannels have a depth of 150 μm .

The experiments started with the washing step, where wash buffer (1 part 50% Tween 20 and 1000 parts 0.1 M PB pH 7.2) was pumped into the microchip for about 10min to remove the GST cover. Next both DTNB (0.6 mM) and ASChI (0.9 mM) are pumped into the LOC to obtain the pre-inhibition readings. The solution of GB (10 nM) was then pumped into the LOC for 10 min to allow inhibition of the

Figure 5-7. PDMS microchip design to be fabricated using lithography and soft lithography. Optical fibers will be inserted into the detection region to acquire the signals during the experiments.

immobilized AChE to take place. Thereafter, wash buffer was once again pumped into the LOC to remove all the GB present in the LOC. In the last step, DTNB and ASChI were pumped into the chip to obtain the post-inhibition readings. Once the post-inhibition readings are obtained, calculations are carried out to obtain the percentage AChE activity remained in the microchip. Equation 3.1 is used to calculate the percentage enzyme activity remained in the microchip.

Since the dimensions of the microchannels are much smaller as compared to the microchips fabricated in PMMA, the former flow rate of 20 $\mu\text{l}/\text{min}$ cannot be used in the new experiments. The flow rate of 20 $\mu\text{l}/\text{min}$ was previously optimised for the preliminary experiments^[26] In the optimization experiments, flow rates were varied to allow sufficient time for the substrate and enzyme to react. An optimisation test was conducted to investigate the most suitable flow rate to be used for the PDMS microchips. Different flow rates were tried out and the resulted absorbance values of the enzyme assay experiments obtained were recorded. Figure 5-8 shows the absorbance values obtained at different flow rates.

Figure 5-8. Plot illustrating the absorbance values corresponding to the flow rate at which the reagents were pumped into the PDMS microchips with immobilized AChE.

As observed from Figure 5-8, flow rate of 4.0 $\mu\text{l}/\text{min}$ yielded an almost similar reaction time and absorbance value as compared to flow rate set at 10 $\mu\text{l}/\text{min}$ for experiments conducted in a PMMA microchip with immobilized AChE. Therefore, flow rate of 4.0 $\mu\text{l}/\text{min}$ will be utilised to pump the reagents into the PDMS microchips to check for PDMS compatibility to GB.

Three sets of enzyme assay experiments were conducted, to investigate the consistency of the data obtained. Figure 5-9 shows the results obtained with the PDMS-based microchips are somewhat consistent, with some slight variance. The presence of GB could be detected in the PDMS microchips with immobilized AChE.

5.3.2 Comparing the data obtained with the data obtained for PMMA microchips

The data obtained for the PDMS-based microchips were compared with the data obtained with PMMA-based microchips with immobilized AChE. Figure 5-10 shows that the data obtained for PDMS microchips are almost comparable to those

Figure 5-9. Chart showing the percentage immobilized AChE activity remained in microchip after 10min of inhibition with $[\text{GB}] = 10\text{nM}$ for PDMS microchips

Figure 5-10. Comparison of percentage of immobilized AChE remained in the different type of microchips after 10min of inhibition with 10 nM GB.

obtained from the PMMA microchips. Thu, we can conclude that PDMS can be used as the substrate material for fabricating the microchips.

5.4 Enzyme assay tests conducted using the PMMA-based LOC with immobilized AChE in whole blood medium

A PMMA microchip was designed and fabricated and was capable of detecting trace amounts of sarin present in a small volume of human blood samples.

The design of the LOC device as shown in Figure 4-28 was used to conduct the enzyme assay tests. For this particular PMMA microchip design, three different sets of experiments were designed to assess the effectiveness of the different stages of the LOC device.

In the first experiment, the inhibition reactor (Figure 4-26) was tested with blood sample prepared outside the LOC device, bypassing the nerve gas regeneration reactor, lysis reactor and filter chamber. In the second experiment, the filter chamber and the inhibition reactor were then tested, bypassing the nerve agent 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.

5.4.1 Testing of immobilised enzyme reaction chamber and absorbance detection unit

A volume of 200 μ l of whole blood was spiked with 4 μ l of sarin leading to a concentration of 200 nM. Nerve agent regeneration chemical KF (pottasium fluoride) was added to spiked blood and let to react for 5 minutes. (KF acts as the nerve agent regeneration chemical) Next, protein precipitation chemical SAS (saturated ammonium sulphate) 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 with a flow rate of 60 μ l/min each through inlets 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 each pumped at a flow rate of 20 μ l/min into the LOC device. The cold blood filtrate was then pumped into the device at inlet 4 at a flow rate of 60 μ l/min. Once immobilised enzyme chamber was filled with the filtrate, the syringe pump was stopped and inhibition was allowed to take place inside the enzyme chamber for 15 minutes. Wash buffer was then pumped into the device through inlets 4 and 5 to flush away the blood filtrate. Finally DTNB and ASChI are introduced into the device through inlets 4 and 5 at a flow rate of 20 μ l/min each. The absorbance was then recorded accordingly.

5.4.2 Test of microfilters, immobilised enzyme reaction chamber and absorbance detection

In this test, the blood sample was prepared in the same way as previously 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 is the same as in the previous experiment.

An amount of 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 $\mu\text{l}/\text{min}$ via inlet 3. The pumping process continued until all the blood mixture had gone through the device. The entire experiment took about 23 to 24 minutes. After the inhibition reaction, the measurement of the absorbance follows the same protocol of experiment 1.

5.4.3 Test of the complete PMMA LOC device

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

The test started with pumping 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 kept at approximately 2:9:14, we used syringes of different volumes 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 based on the 5-ml syringe as reference. For checking the effect of the residence time and the corresponding inhibition time the flow rates at inlets 1, 2, and 3 were set at 48 $\mu\text{l}/\text{min}$ (3 $\mu\text{l}/\text{min}$ spiked blood, 15 $\mu\text{l}/\text{min}$ KF and 30 $\mu\text{l}/\text{min}$ SAS) and 22.4 $\mu\text{l}/\text{min}$ (1.4 $\mu\text{l}/\text{min}$ spiked blood, 7 $\mu\text{l}/\text{min}$ KF, and 14 $\mu\text{l}/\text{min}$ SAS). Three sets of experiments were conducted to assure the repeatability of the test. The residence times were approximately 25 minutes and 65 minutes, respectively. After the inhibition reaction, the measurement of the absorbance follows the same protocol of the first experiment.

The test of the whole LOC device in experiment 3 shows that approximately only 5% of the enzyme was inhibited at a total sample flow rate of 48 $\mu\text{l}/\text{min}$ and a corresponding total residence time of 25 minutes. At this flow rate, the residence time in the regeneration reactor is about 1.5 minutes, while the protocol for the preparation outside the LOC required 3 minutes 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. Measurement with a slower total sample flow rate of 22.4 $\mu\text{l}/\text{min}$ and a corresponding total residence time of 65 minutes supports this hypothesis. The inhibition rate of the enzyme was increased to about 43 %.

5.4.4 Discussion on nerve agent detection on PMMA-based LOC with immobilized AChE on glass cover slip

Figure 5-11 shows the remaining enzyme activities measured in all three experiments. Despite the different levels of inhibition, all experiments show the

Figure 5-11. Comparison of the % enzyme remained on chip for regeneration process taken place outside microchip (by passing the mixture through manifold and microchip) & regeneration process taking place within chip.

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 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 the third experiment shows that approximately only 5% of the enzyme was inhibited at a total sample flow rate of 48 $\mu\text{l}/\text{min}$ and a corresponding total residence time of 25 minutes. At this flow rate, the residence time in the regeneration reactor is about 1.5 minutes, while the protocol for the preparation outside the LOC required 3 minutes 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 ^[74]. Measurement with a slower total sample flow rate of 22.4 $\mu\text{l}/\text{min}$ and a corresponding total residence time of 65 minutes supports this hypothesis. The inhibition rate of the enzyme was increased to about 43 %.

Chapter 6

Conclusion and future work

6.1 Conclusion

The results presented in the report show that PMMA-based LOC was able to detect the trace amounts of sarin spiked into human whole blood samples. However, the current PMMA-based LOC relies on syringe pump and manual handling of the reagents in order to allow the entire procedure to take place within the microchip. Therefore, micropumps and microvalves will be designed and integrated onto the LOC to do away with the manual handling of reagents,

The work thus far allowed us to demonstrate that it is possible to detect the trace amount of sarin in aqueous medium in both PMMA and PDMS substrates. Through this investigation, PDMS shows its compatibility for CWA usage. Hence, PDMS can be used for fabricating the micropumps and microcvalves, which will have intimate contact with the CWA and all the reagents used in the device.

The possibility of immobilizing AChE directly onto both PDMS and PMMA was also demonstrated. These promising results allow the simplification of immobilization process. However, a suitable micropumps design that is able to handle the reagents in the microchip has to be developed and tested. This challenge will be the future works of this project.

During the course of the project, different methods of bonding PMMA and PDMS have been investigated. And in the course of it, a novel method of bonding PMMA to PDMS had been developed. Through this technique, a thin membrane comprising of laminating a thin layer of double-sided dry adhesive on the PMMA substrate followed by spin coating a layer of pre-cured PDMS mixture. These layers will then undergo thermal curing followed by oxygen plasma to bond irreversibly to another PDMS part. This fabrication technique is suitable for batch processes and the

thickness of the membrane is controllable. Different air chamber sizes and different air pressure was shown to affect the deflection of the membrane. For the same design foot print, square membranes were able to deflect more than the circular membranes. The square membranes were used in peristaltic micropumps as pneumatic actuators. Pumping of fluid by the designed micropumps was successfully achieved. The highest flow rate by the largest air chamber and channel was 96 $\mu\text{l}/\text{min}$ at a pumping frequency of 10 Hz. This micropump was capable of functioning at a high driving pressure of 138 kPa. This novel fabrication technique of the micropumps prevents bubble formation in the microchannel from the infusion of air from the actuation chambers. The layer of adhesive is impermeable to air and adhere well to the PMMA surface. The bond between PMMA to PDMS is strong and reliable as the different layers did not delaminate in different tests. The micropumps based on the combination of PMMA, dry adhesive and PDMS is powerful, and suitable to work with other components in a more complex LOC platform.

6.2 Future work

Although the chemical reaction of all nerve agents on AChE is similar, the device developed in this project, will also be tried with other nerve agents, to investigate for this viability.

The device presented in this report can also be used for the detection of organophosphorous insecticides. As the workings of insecticides on AChE is similar to that of GB. Thus, the device is suitable for other applications in occupational hygiene in agriculture. However, this have yet to be investigated in future works. With such technology, this can serve as one step nearer to reaching a point-of-care device for monitoring the levels of AChE in humans.

On the technology front, novel methods for bonding of PDMS to PMMA had been explored and investigated, hence enabling a more robust LOC made of both PDMS and PMMA. Various designs of PDMS micropumps was designed and fabricated. These micropumps have yet to be tested and characterized. In future work, the fabricated micropumps will first be tested with simple enzyme assay solutions

before embarking on GB solution. Only when the design of the micropumps are safe and reliable, it will be tested with human whole blood samples.

However, the current LabView program and switching board used for controlling the external solenoid valves do not allow the EMVs to switch faster. Also the set-up comprised of a number of equipment which will not be applicable for bringing them out-field. For future work, a microprocessor will be used in place of all the switching board.

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