

Imperial College of Science, Technology and Medicine
Department of Bioengineering

Development of a microfluidic biosensing system for real-time monitoring of acetylcholinesterase activity

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Abstract

This thesis describes the development of a microfluidic biosensing system for the real-time monitoring of acetylcholinesterase (AChE) activity in blood samples following exposure to organophosphorus (OP) compounds.

Two methods are developed using choline oxidase (ChOx) to sense choline, the product of acetylcholine (ACh) hydrolysis, as a way of measuring AChE activity. A system where ChOx is added in flow was developed, based on the adaptation of an existing microfluidic platform comprised of LabSmith programmable components. This gave a high sensitivity of 24.68 ± 0.09 nA/mM when using optimal experimental parameters. A choline biosensor was fabricated as an alternative sensing approach, by immobilising a ChOx hydrogel layer on a combined needle microelectrode surface. The choline biosensor typically gave a sensitivity of 4 nA/mM. The choline biosensor was successfully used to monitor choline concentration in a brain microdialysate stream in a porcine model of cardiac arrest and resuscitation.

The microfluidic platform was adapted to allow addition of ACh and AChE streams to create a system where either ACh or AChE calibrations can be carried out. Using the existing microfluidic analysis system coupled with the choline biosensor, a successful AChE calibration was demonstrated.

Preliminary experiments were made to utilise microdialysis with the aim of sampling in complex media such as blood. Results demonstrate the use of microdialysis to sample different AChE activities in a stationary solution. Retrodialysis was also successfully performed to deliver ACh into AChE-containing solutions.

A microfluidic flow cell that incorporates a microdialysis probe was developed, to create a flowing system in which reactions can be tightly controlled. The flow cell coupled with a downstream choline biosensor was used to successfully probe changes in AChE activity. Sensitive AChE calibrations were performed in activity ranges up to 6 U/ml when using a standard choline biosensor, and up to 8 U/ml when using a choline biosensor with a diffusion limiting layer.

AChE solutions were exposed to OP compounds and the resulting inhibition was determined and compared with an Ellman-based assay. Dynamic inhibition experiments were performed, demonstrating the potential for the device to be used as a continuous monitoring system.

The novel microfluidic flow cell was successfully utilised to determine AChE activity in near whole blood (92%) and in 50% blood. Blood samples diluted to 50% were exposed to OP compounds and the resulting decrease in AChE activity was successfully measured, demonstrating the use of this system to monitor changes in AChE activity in biological samples.

Statement of Originality

The work presented in this thesis was carried out by myself in the Department of Bioengineering at Imperial College London under the supervision of Professor Martyn Boutelle and in collaboration with Dr Sarah Goodchild at DSTL, Porton Down. The material contained within this thesis is original and has not been submitted for a degree or diploma at any other university.

Georgia Kate Smith

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The Science Inside

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List of abbreviations

ACh acetylcholine	KCl potassium chloride
AChE acetylcholinesterase	LOx lactate oxidase
aCSF artificial cerebrospinal fluid	LUCAS Lund University Cardiopulmonary Assist System
ADC analog-to-digital converter	MS mass spectrometry
Ag AgCl silver silver chloride	MWCO molecular weight cut-off
ATCh acetylthiocholine	OPH organophosphorus hydrolase
BChE butyrylcholinesterase	PBS phosphate buffer saline
BSA bovine serum albumin	PCB printed circuit board
CE counter electrode	PEEK polyetheretherketone
ChOx choline oxidase	PEGDE poly(ethylene glycol) diglycidyl ether
CPR cardiopulmonary resuscitation	Pt platinum
CSF cerebrospinal fluid	Pt(OH)₂ platinum hydroxide
CV cyclic voltammogram	PTFE polytetrafluoroethylene
DTNB 5,5'-dithiobis(2-nitrobenzoic acid)	PU polyurethane
Fc ferrocene monocarboxylic acid	RE reference electrode
GlutOx glutamate oxidase	ROSC return of spontaneous circulation
GOx glucose oxidase	SWV square wave voltammetry
H₂O₂ hydrogen peroxide	THF tetrahydrofuran
OP organophosphorus	TNB⁻ 5-thio-2-nitrobenzoate
MD microdialysis	WE working electrode
MM Michaelis-Menten	
mPD poly(m-phenylenediamine)	

Chapter 1

Introduction

1.1 Motivation to monitor acetylcholinesterase activity

Acetylcholinesterase (AChE) is the enzyme responsible for hydrolysing the neurotransmitter acetylcholine (ACh), facilitating its removal from the synaptic cleft [1, 2]. The reaction for which is described by equation 1.1.



ACh is found at neuromuscular junctions, autonomic ganglia and in the brain [3, 4]. It has many important functions in the body, including playing a vital role in neurological processes such as attention [5], learning and memory [6]. ACh also allows contraction of skeletal muscles [4]. During a neurotransmission event, ACh is released into the synaptic cleft where it then binds to nicotinic or muscarinic ACh receptors present on the synaptic target to relay the signal from the nerve. AChE plays an incredibly important role in regulating the levels of ACh in the body; it can be found in both the central and peripheral nervous systems and in red blood cell membranes [7]. AChE is a very efficient and active enzyme, with one molecule hydrolysing approximately 25,000 molecules of ACh per second to regulate ACh levels [8].

Certain compounds act to inhibit AChE, impeding the hydrolysis and removal of ACh from the synaptic target [9]. This causes overstimulation of cholinergic targets, which has damaging effects on the body. There are two classes of AChE inhibitors: reversible and irreversible inhibitors. Reversible AChE inhibitors, beyond high level acute toxicity, are generally not harmful to the body due to their short half lives of approximately 7 - 90 hours allowing extracellular levels of ACh to still be regulated [9, 10]. Reversible AChE inhibitors can be used as treatment for symptoms of neurological diseases such as Parkinson's disease [11] and Alzheimer's disease [10, 12], by allowing build up of ACh in the brain. However, irreversible inhibitors are highly toxic as it can take weeks for the AChE activity to return to normal [13, 14].

Organophosphorus (OP) compounds, used in agriculture as pesticides and used in chemical warfare as nerve agents, are examples of these irreversible AChE inhibitors [15, 16]. OP pesticides were synthesised in large quantities following World War II [17]. Since then, their use has been restricted in the UK due to their toxicity and the environmental pollution they cause. However, they are still commonly used in countries including Brazil and China, due to availability, effectiveness and low cost, despite attempts to ban the use of several OP pesticides [18–20]. It is, therefore, a health concern for the farm workers handling these compounds and the rural community in the surrounding area. Nerve agents were first developed in the 1930s [17]. They have since been used in terrorist attacks, such as those in Japan in 1994 and 1995, where terrorists targeted civilians with sarin [16, 17]. In the 1990s, the World Health Organisation estimated that 3 million people were exposed to OP pesticides every year, with 10% of these exposures resulting in death [21]. It is, therefore, of high interest and importance to monitor exposure to these toxic compounds.

1.1.1 Toxicology of organophosphorus compounds

OP compounds are esters derived from phosphoric compounds; their general structure is shown in figure 1.1.

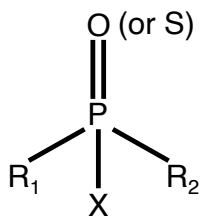


Figure 1.1: The general structure of an organophosphorus compound. The general structure consists of a phosphorus atom with a double bond to an oxygen or sulfur atom, and single bonds to two residue groups, R_1 and R_2 , and one leaving group, X .

A variety of residues, R_1 and R_2 , can be combined together with different leaving groups, X , to synthesise countless different OP compounds [22]. The OP compound inhibits AChE by entering its active site and covalently binding to the serine -OH group of AChE, while the X group, as shown in figure 1.1, is lost from the OP molecule. This process is known as phosphorylation. Following this, one of two steps can occur. Either, the enzyme undergoes reactivation, which can happen spontaneously or by oxime treatment, further discussed in section 1.1.2, or it undergoes an aging process [16]. The spontaneous reactivation consists of dephosphorylation of the inhibited AChE so that it is able to hydrolyse ACh again. The aging process consists of dealkylation of the phosphorylated AChE, this process causes irreversible damage and medical treatments will no longer work to reactivate the enzyme [16, 22]. The mechanism of inhibition of AChE following exposure to OP compounds is shown in figure 1.2.

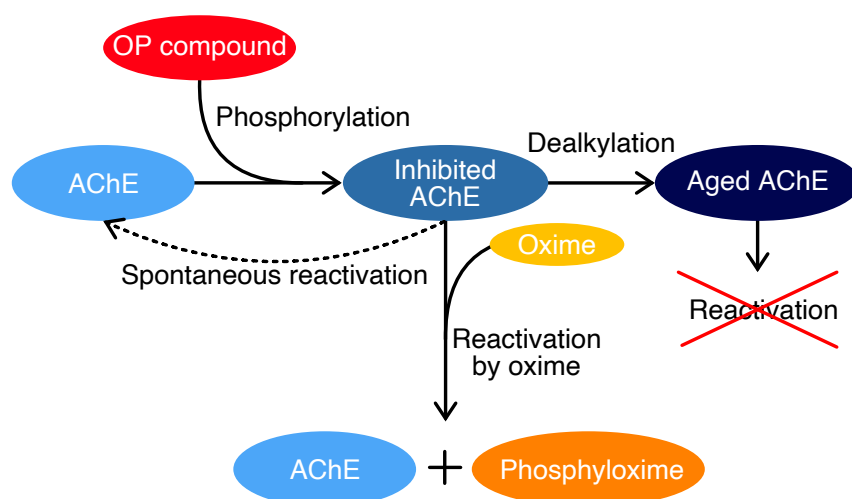


Figure 1.2: Mechanism of inhibition and reactivation of AChE. The diagram shows that following exposure to OP compounds, AChE undergoes phosphorylation and becomes inhibited by the OP compound. Following this, the AChE either undergoes an aging process by dealkylation or it experiences a reactivation process. The aged AChE cannot be reactivated. However, reactivation of the phosphorylated enzyme can occur spontaneously or following oxime treatment. The oxime acts by displacing the phosphoryl group on the inhibited AChE.

Diagnosis and treatment before the aging process occurs is critical, as the function of aged AChE cannot be restored. The aging half life of different OP-inhibited AChE varies. For example, for the nerve agent sarin, the inhibited AChE has an aging half life of approximately 3 hours, whereas for the nerve agent soman, the inhibited AChE has an aging half life of only 2 - 3 minutes [23]. AChE molecules inhibited by OP pesticides typically have a longer aging half life of 3 - 40 hours, depending on the compound [24, 25]. While pesticides pose less of an immediate threat compared to the OP compounds typically used in chemical warfare, their detection and treatment is still of interest to prevent the development of serious illness.

Exposure of OP compounds may be via inhalation, ingestion or dermal contact. OP poisoning can range from mild to severe, depending on which type and the amount of OP compound used, causing a range of neurological and physical symptoms [15], some of which are presented in figure 1.3.

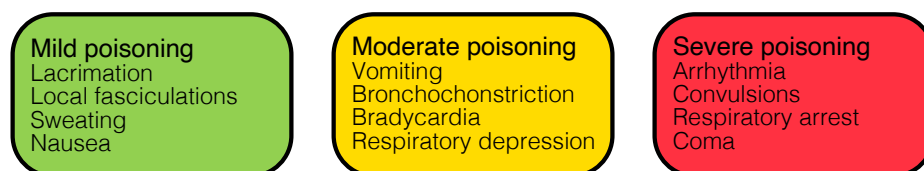


Figure 1.3: Examples of symptoms observed for different levels of OP poisoning. Mild and moderate poisoning results in more generic symptoms that may go unnoticed or lead to misdiagnosis, highlighting the importance for a detection system. Severe poisoning results in symptoms that are not specific to OP poisoning and that are life-threatening, meaning the symptoms must be treated immediately.

Severe poisoning symptoms are life-threatening. Clinical assessment is currently adequate for detecting severe OP poisoning due to the severity of the symptoms. However, mild and early-stage poisoning causes symptoms that could go unnoticed or confused with a range of other health problems. Similarly, moderate stage poisoning causes symptoms that may be misdiagnosed. It is therefore important to have a diagnostic tool that is sensitive to these mild and early-stage poisonings so that treatment can be administered as early as possible. The aging half lives of as little as 2 minutes, as previously mentioned, further validate the need for an early-stage detection system. It is also desirable to have a continuous monitoring device, which allows the tracking of AChE activity during treatment.

1.1.2 Treatment of organophosphorus poisoning

Oximes are utilised as the standard treatment following an individual's exposure to OP compounds.

The mechanism by which oximes act to reactivate phosphorylated AChE is by displacing the phosphoryl moiety from the inhibited AChE compound [16, 23]. The oxime binds to the OP compound in place of the enzyme/leaving group X, creating a phosphyloxime compound, as shown in figure 1.2. The rate of reactivation depends on a number of factors including, concentration of oximes, the source of AChE and the type of OP compound, and in turn the aging rate [26]. It is not a simple treatment as different types of oximes have varying abilities to reactivate different types of AChE inhibitors [26]. Therefore, it is of great interest to also monitor an individual's response to treatment following OP exposure.

1.1.3 Monitoring exposure to organophosphorus compounds

Detection of OP compounds can be achieved by detecting the OP compound directly, or detecting the effects of it, i.e. detecting the inhibition of AChE as a drop in the enzyme activity.

Detection of OP compounds directly

Detection of the OP compound specifically can be achieved in a couple of ways. The first method relies on the use of the enzyme organophosphorus hydrolase (OPH), which catalyses the hydrolysis of OP compounds [27]. The second method utilises an external source of AChE, which decreases in activity following exposure to OPs [27].

The OPH-based method is based on the detection of the products of OP hydrolysis. When certain OP compounds, such as paraoxon, are hydrolysed by OPH, they produce 4-nitrophenyl [28], which can be detected using a range of techniques, including electrochemical methods as 4-nitrophenyl can be oxidised [27, 29]. However, direct measurement of OP compounds in this way relies on there being free OP compounds that have not inhibited AChE, otherwise the OP compound cannot be hydrolysed. This would make it challenging to detect low-level exposure and gain an understanding of the severity of poisoning. Furthermore, detection of OP compounds alone would make it difficult to quantify how

efficiently a treatment is working, and the health of an individual; it only gives the means to say whether or not an OP compound is present and in what quantities there is a free amount of it. While this is useful and vital information in order to diagnose an individual correctly, it gives little insight into the severity of poisoning. OPH has a relatively broad specificity to different OP compounds, as it is able to cleave various phosphorus-esters, however, the OPH cleaves these bonds with varying efficiencies, making it more suitable for particular OP compounds [30]. This is a disadvantage, an effective monitoring system would ideally have the capabilities to monitor exposure regardless of the type of OP compound.

AChE-based sensors rely on the use of a fresh external source of AChE and monitoring the resulting inhibition from free OP compounds [27]. Bienzyme biosensors, combining AChE and choline oxidase (ChOx), have been developed for this purpose [31–33]. ACh is introduced into a sample where it is hydrolysed by AChE, and the resulting choline is oxidised by ChOx. The product from choline hydrolysis can be detected electrochemically or optically [33]. While this method can be applied to all OP compounds, it still relies on the detection of the free OP compound. Furthermore, it relies on a very stable sensing system to maintain high sensitivity so that the decrease in signal can be attributed to exposure to OP compounds with certainty and not to the degradation of signal.

Both of these methods have great potential for detection of an OP compound in the environment, or for initial diagnosis, however, they are not suitable for long-term or continuous monitoring of OP exposure, or monitoring the success of a treatment following OP exposure.

Detection of inhibition of AChE in blood

The activity of AChE in the blood is a key biomarker for monitoring an individual's exposure to OP compounds. Red blood cells express AChE in their membrane; the AChE is inhibited on exposure to OPs. As the AChE is inhibited, the resulting AChE activity decreases. It is possible to assess the AChE activity in whole blood by determining the rate of the formation of the hydrolysis product, choline. Methods have been developed to introduce ACh into an AChE-containing solution and detect the resultant choline [34].

The Ellman method uses the same strategy but with the alternative substrate to ACh, acetylthiocholine (ATCh) [35, 36]. The standard Ellman's assay has been widely used to monitor changes in AChE activity and is a highly optimised analytical protocol, with many research groups using their own version of the modified Ellman's assay [37, 38]. In this method ATCh is added to the sample. The ATCh is hydrolysed by the AChE in the sample, to produce thiocholine. The chromogen 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), is introduced to the system and reacts with thiocholine to produce 5-thio-2-nitrobenzoate (TNB^-), which is yellow in appearance [39]. A spectrophotometer is used to determine the absorption based on the intensity of the colour. Originally a wavelength of 412 nm was used, however, at this wavelength there is interference from haemoglobin. To overcome this, a

longer wavelength up to 470 nm has been used [37], which gives an improved TNB^- to haemoglobin absorption, however, this does result in reduced overall TNB^- absorption. The haemoglobin can be removed from samples, but this is an added preparation step. Alternatively, to detect AChE in whole blood in this way, the blood can be diluted to reduce the AChE activity such that the resulting thiocholine concentration and hence resulting yellow colour is in the range of the spectrophotometer [40, 41]. This has the added benefit of reducing the red colour from the haemoglobin, reducing interference. However, it is not ideal to have the added dilution step in sample preparation and it is hard to accurately do this outside of a lab environment. Furthermore, the Ellman method has generally been used to perform discrete measurements, which consist of one-off measurements of a sample following exposure to an OP compound at a given time point rather than continuously monitoring changes in AChE activity. Some advancements have been made to make this a continuous monitoring system, which is particularly beneficial for monitoring inhibition and reactivation kinetics [42]. However, there is still a need for a reliable, sensitive and continuous monitoring system to provide insight on changes in AChE in real-time. A device with the capabilities to sample whole blood would be highly beneficial to eliminate sample preparation from the diagnostic protocol.

Another form of cholinesterase, butyrylcholinesterase (BChE), is also present in the body and can be found in numerous locations, for example the brain, lungs and liver [43, 44]. BChE is also found in plasma, making up a proportion of whole blood cholinesterase levels [45, 46]. The physiological role of BChE in the body is unknown [44, 46] and BChE has not been found to have a direct involvement in cholinergic processes like AChE, but it may act as a substitute for ACh hydrolysis in the absence of AChE activity [46]. However, it does have a lower affinity to ACh. OPs also act by inhibiting BChE, in a similar manner that OPs inhibit AChE - a phosphorylation step occurs first, followed by a dealkylation stage [45]. BChE could also be a biomarker for OP exposure, but this often requires the use of a more specific substrate of BChE, either butyrylcholine or butyrylthiocholine, both of which are synthetic compounds [47, 48]. Furthermore, a decrease in BChE activity levels is often associated with liver malfunction [49] and may be affected by a number of other factors including chronic alcoholism, malnutrition and birth control pills, whereas AChE activity is less likely to be affected by factors other than OP exposure [50]. Because of this, monitoring OP exposure by monitoring AChE activity is more common routinely compared to monitoring BChE activity levels.

One challenge of monitoring AChE inhibition by utilising AChE levels in blood is due to inter-individual variations in AChE baseline activity levels [51, 52]. This large variation means the baseline cannot be accurately predicted, meaning it is important to have a baseline measurement to sensitively detect low-level exposure to OP compounds. Alternatively, a continuous *in vivo* monitoring system would provide valuable insight into changes in an individuals AChE levels.

1.2 Microdialysis

Sampling in complex environments is highly challenging due to large proteins, like enzymes, and cells, like red blood cells, that can interfere with the monitoring assay. A popular sampling technique that has been utilised, both *in vivo* and *in vitro*, to block out large molecules and potential interferents is microdialysis (MD) [53]. It has been employed to monitor a number of different tissues, including, the brain [54, 55], blood [56–58] and subcutaneous tissue [59].

MD is based on the diffusion of small molecules below a certain size down their concentration gradient into the dialysate stream, through a semi-permeable membrane. It is also possible to deliver molecules from the perfusate into the external environment, a technique known as retrodialysis. There are two main types of commercially-available microdialysis (MD) probes, concentric or linear probes, shown in figure 1.4A and figure 1.4B, respectively.

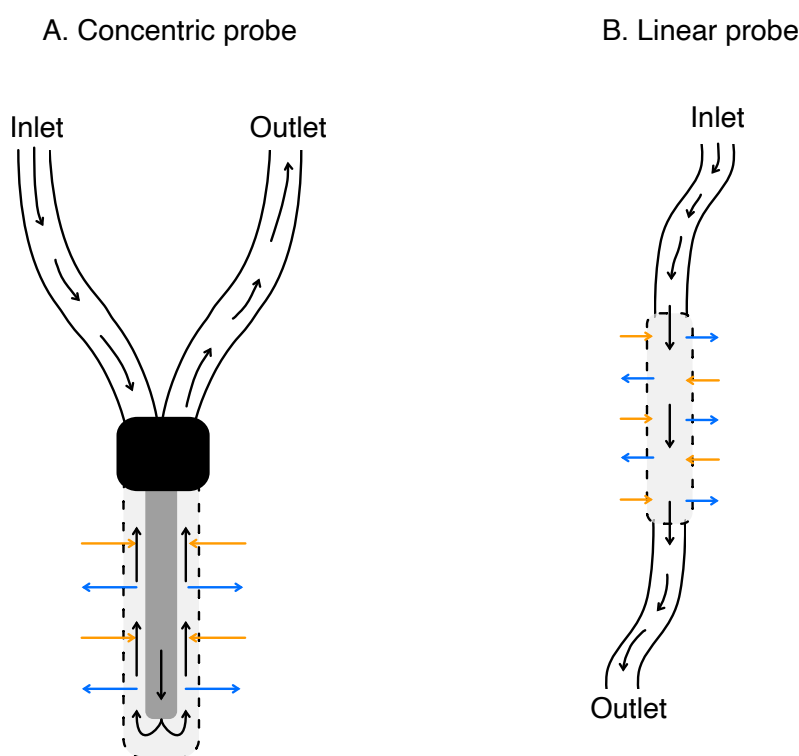


Figure 1.4: The two types of commercially available MD probes. The black arrows show the direction of the dialysate flow. The orange arrows show the direction of small molecules diffusing into the probe and the blue arrows show the direction of small molecules diffusing out of the probe. **A.** Concentric MD probes consist of the inlet tubing placed inside the outlet tubing over which the membrane is placed. **B.** Linear probes consist of the outlet tubing connected to the inlet tubing via a section of membrane.

Both probes consist of an inlet, an outlet and a semi-permeable membrane. In a concentric probe the inner inlet tubing is perfused with a physiological solution that flows out of the inlet and over the semi-permeable membrane at the tip to the outlet tubing. In a linear probe the outlet tubing is connected to the inlet tubing via a section of membrane instead.

Linear probes can be useful for sampling in homogeneous tissue, notably muscle or skin [60], to sample larger areas. They minimise tissue damage as linear probes are often thinner and offer more flexibility [60–62]. A disadvantage of linear probes is that they require two incisions to access the tissue of interest, one for the inlet tubing and one for the outlet tubing, which causes an increased risk of infection [63]. Concentric probes are useful for sampling in tissues where only one hole for insertion can be made, for example in the brain or in blood. They are useful in heterogeneous tissue, where small length probes are important to maintain spatial resolution [60].

The MD probe is placed in the tissue of interest and perfused with a solution of the same composition as the tissue in which the probe is placed. The perfusion rate varies depending on the application, which will affect the delivery or recovery of analytes. Typically, flow rates of 0.1 - 5 $\mu\text{l}/\text{min}$ are used. MD probes are characterised by their molecular weight cut-off (MWCO), typically between 20,000 and 60,000 Da [53], which defines the maximum size of the molecules that can diffuse across the membrane. They also have a particular membrane length, which can range from 1 - 20 mm.

1.2.1 Probe recovery

The probe recovery refers to the ratio of the analyte in the dialysate stream relative to the concentration of the analyte in the external environment in which the probe is placed. It is defined by the mass transport across the probe [53]. The flow rate of the perfusate will greatly affect the recovery of the analyte. Lower flow rates lead to improved recovery as there is more time for the analyte to diffuse across the probe and into the dialysate stream. A flow rate of 0.1 $\mu\text{l}/\text{min}$ has been reported to give almost 100% recovery *in vitro* [64]. However, using a lower flow rate is limiting due to an increased time delay and poor temporal resolution. Therefore, it is important to find a compromise between recovery and temporal resolution depending on the application.

As probe recovery generally is not 100%, one challenge of microdialysis sampling is determining the actual concentration in the tissue environment in which the sampling is taking place. *In vitro* recovery can be determined by adding certain known concentrations of analytes to the external environment and recording the analyte concentration in the dialysate. However, *in vitro* recovery is not representative of *in vivo* recovery as molecules have to diffuse through the tortuous extracellular environment to reach the probe *in vivo*, which is not comparable to a well stirred beaker used *in vitro*.

There are techniques to determine probe recovery *in vivo* if required, for example, no-net-flux, low flow rate or extrapolation to zero flow rate [65]. Among these, the no-net-flux technique is more commonly employed. This requires the addition of different analyte concentrations to the perfusate, and analysing the resulting concentration at the dialysate outlet [66]. The concentration in the dialysate will depend on how much of the analyte has diffused out of the probe. When the concentration of the analyte is the same in the inlet perfusate as the outlet dialysate, that is, no analyte has diffused out of the probe and no-net-flux has occurred, then this is deemed the concentration of the analyte in the external environment in which the probe is placed. This method assumes that the diffusion rates out of the probe are the same as the diffusion rates into the probe, which may not be the case [67]. Additionally, the tissue concentrations may not be stable enough to determine probe recovery with high accuracy.

Alternatively, if a baseline can be established for the analyte of interest, then relative changes from the baseline can be used to measure the changes in analyte concentrations in the external environment. This method provides valuable insight into physiological changes in analyte concentration that can be related to a pathological event. This method has been utilised for clinical monitoring and it is common among the MD community to quote dialysate concentrations [55, 59, 68–73].

1.2.2 Analysis of dialysate

Analysis of the dialysate stream can be performed online or offline. Traditionally, offline measurements are made, which involves collecting dialysate samples at particular time periods. However, these measurements are discrete and real-time monitoring is not possible with offline MD due to the time delay between collecting and analysing the sample. Useful information is lost due to very poor time resolution as samples are typically collected for 5 - 10 minutes meaning fast, dynamic changes in analyte concentrations in the tissue are missed [53].

Online MD can give continuous and real-time measurements of the analyte of interest in the dialysate stream, which is useful to see dynamic changes in the analyte concentration. Different analytical methods have been used previously including mass spectrometry [74], liquid chromatography [75, 76] and electrophoresis techniques [70, 77]. Additionally, enzymatic techniques have been employed for online detection of analytes by electrochemical or optical techniques [54, 59, 78–81]. The use of these techniques to monitor metabolites of interest is further discussed in chapter 3.

1.3 Thesis outline

Following this introduction, chapter 2 discusses the methods and common solutions used throughout this thesis.

The development of two ChOx-based sensing systems are described for the detection of the ACh hydrolysis product, choline, in chapter 3. ChOx is incorporated into the system either in-flow by modification of the microfluidic platform introduced in chapter 2 or by immobilising the enzyme onto an electrode surface to fabricate a choline biosensor. Different optimisation parameters are considered. The stability and sensitivity of both systems are investigated and the two methods are compared. In general, the choline biosensor is used in the remainder of the thesis.

Chapter 4 describes the real-time sampling of analytes in two porcine-based clinical trials. This chapter is unrelated to AChE activity detection, but describes the use of biosensing methods introduced in chapters 2 and 3 for clinical monitoring. The chapter discusses the use, adaptation and refinement of the wireless monitoring system described in chapter 2 coupled with MD. It also discusses the use of the choline biosensor *in vivo* to monitor low levels of choline in the dialysate of a pig brain.

In chapter 5, further modification of the microfluidic platform is described to perform an ACh calibration and monitor changes in AChE activity, by introducing ACh and AChE into the analysis stream. Detection of ACh hydrolysis is further investigated by the use of MD as the sampling technique. Attempts are made to refine low- and high-level AChE activity detection in a beaker, using MD coupled with the choline biosensor.

Chapter 6 discusses the development of a microfluidic flow cell, which is designed to connect directly to the microfluidic platform and incorporate MD. Two iterations of the flow cell are designed. The two designs are characterised in terms of their response times and the resulting MD probe recovery by performing choline calibrations through the flow cell. The development of the flow cell has allowed solutions to be mixed and sampled under highly-controlled conditions.

In chapter 7, the microfluidic flow cell developed in chapter 6 is used to refine the detection of AChE activity. Methods to overcome different challenges, such as sticking of AChE to the microfluidics, are discussed. This chapter investigates the use of different ACh concentrations and experimental parameters to develop an analysis system that can sensitively detect changes in AChE activity in the range found in blood.

Chapter 8 discusses the use of the developed analysis system, consisting of the choline biosensor, flow cell and microfluidic platform, coupled with MD and the previously described microfluidic chip, to sample changes in AChE activity following exposure to an OP compound. This chapter also validates the system for *in vitro* monitoring, by sampling whole blood. Finally, blood samples are exposed to OP compounds to detect changes in AChE activity in the blood.

The findings of this thesis are concluded in chapter 9. Future work is also discussed, to further validate and utilise the developed device for sensitive and continuous monitoring of AChE activity. The wide range and potential benefits of this developed device are discussed.

Chapter 2

Materials and Methods

2.1 Microelectrode fabrication

Sensors used throughout this thesis are made based on a combined needle 3-electrode system [69, 82]. They are fabricated by threading a 50 μm diameter polytetrafluoroethylene (PTFE) insulated platinum/iridium wire (90:10) (Advent Research Materials Ltd, UK) and a 50 μm diameter polyester insulated silver wire (Goodfellow Cambridge Ltd, UK) through a 27 G hypodermic needle. The insulation layer is removed from the tip by exposing the ends of each wire to a flame. The ends of the wires at the needle barrel are connected to electrical wires using conductive silver epoxy (RS Components, UK). The epoxy is left to set for at least 24 hours at room temperature or for 1 hour at 65°C. Crimp pins (Farnell, UK) are soldered to the other sides of the electrical wires to allow electrical connections. Epoxy adhesive (Farnell, UK) is used inside the needle barrel to glue the wires apart to prevent the circuit shorting and to support the wire joins inside the needle barrel. The system is supported further by injecting epoxy resin (Robnor resins, CY1301 and HY1300) into the needle shaft and barrel. The resin and hardener are combined in a 3:1 ratio by volume and sonicated to guarantee thorough mixing and remove any air bubbles. The resulting mixture is injected through the needle barrel using a hypodermic needle attached to a piece of tubing and connected to the needle electrode. A syringe is used to push the resin through the secondary needle and tubing to the internal part of the needle electrode, ensuring it is completely filled with no air bubbles. The needle electrode is topped up with resin to strengthen the wire joins and hold the wires in place. The secondary needle and tubing are placed slightly above the needle electrode and remain connected to ensure no epoxy leaks out before it is fully cured. The epoxy resin is left to cure for two days at room temperature.

When the epoxy curing process is complete, a scalpel is used to carefully remove the tubing from the needle electrode. The needle tip is sanded down using sandpaper (Buehler, UK) of various grades. Initially, P1200 sandpaper is used to create a blunt, flat edge just above the bevel of the needle. This is achieved by moving the electrode up and down the sanding paper until the desired level is reached. The needle tip is sanded further using P2500 paper, where the electrode is moved in a circle 50 times

and then rotated 90° and the process is repeated three additional times. P4000 sandpaper is then used, where the electrode is moved in a figure-of-8 motion 50 times and then rotated 90° and the process is repeated three additional times. Following the sanding process, the needle electrode is polished using three different alumina powders, $1\ \mu\text{m}$, $0.3\ \mu\text{m}$ and $0.05\ \mu\text{m}$. For each powder, water is added to create a slurry, and the electrode is moved in a figure-of-8 motion in this slurry, rotating the electrode 90° every 50 movements until the electrode is in its original position to create a flat, even surface. The electrode is then rinsed with distilled water and sonicated for 30 s to ensure nothing remained on the electrode surface, before the process is repeated with the next alumina powder. Alumina powders are used in order of their size, from largest to smallest.

The act of sanding and polishing the needle results in flat disc electrodes. The platinum (Pt) electrode is used as the working electrode (WE) and the silver electrode is made into a silver|silver chloride (Ag|AgCl) pseudo reference electrode (RE) by chloridising the silver electrode, explained in detail in section 2.2.2. An electrical wire is connected to the stainless steel needle shaft using conductive silver epoxy, to be used as the counter electrode (CE). An example of a completed microelectrode is shown in figure 2.1. A schematic of its vertical cross-section is also shown, displaying the $50\ \mu\text{m}$ Ag|AgCl RE, the $50\ \mu\text{m}$ Pt WE and the stainless steel CE. The outer diameter of the needle microelectrode is $413\ \mu\text{m}$.

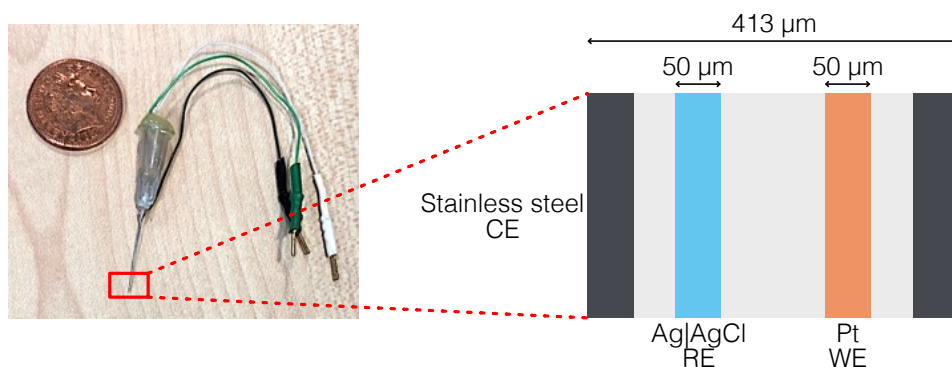


Figure 2.1: A polished microelectrode with a schematic of the vertical cross-section. The microelectrode is fabricated using a blunt needle. Its size is compared to a 1 pence coin. The cross-section shows the dimensions of the $50\ \mu\text{m}$ Pt WE and Ag|AgCl.

Following fabrication, microelectrodes are coated in a poly(*m*-phenylenediamine) (mPD) exclusion layer, described in section 2.2.2, and used as they are or further coated in a hydrogel layer containing an oxidase enzyme, described in section 2.3.2.

2.2 Electroanalytical techniques

In this thesis, two types of electroanalytical techniques are used, cyclic voltammetry and amperometry. Cyclic voltammetry is used to assess the electrode surface and amperometry is used for chloridising the silver electrode to become a reference electrode, electropolymerisation of mPD and for the detection of analytes of interest. Unless stated otherwise, these techniques were carried using in-house potentiostats and a Powerlab (ADIInstruments, New South Wales, Australia).

2.2.1 Cyclic voltammetry

To assess the polished electrode surface, cyclic voltammetry is used. Cyclic voltammetry is a technique in which the potential at the working electrode is ramped linearly versus time to a maximum potential, and then scanned in the reverse direction to the original starting potential. The resulting current is therefore due to reactions happening at specific potentials. To assess the surface of the WE, the electrode is placed in 1.5 mM ferrocene monocarboxylic acid (Fc), details of which are given in section 2.7.2 and a cyclic voltammogram (CV) is recorded. In this work, in-house potentiostats were used, controlled by EChem software (eDAQ, UK). The potential is ramped linearly from -100 to 500 mV and then back down again at a scan rate of 10 mV/s. The scan width used is 200 ms with a sampling period of 50 ms. As the potential is increased, Fc is oxidised to Fc^+ , causing an increase in the measured current until the reaction becomes limited due to diffusion of the analyte from the bulk solution to the Pt electrode and a plateau is reached. The current at which this happens is the limiting current. When the potential is decreased, the reverse happens and Fc^+ is reduced to Fc causing a decrease in the measured current.

For an inlaid disc microelectrode, the limiting current, I_{lim} is given by equation 2.1.

$$I_{lim} = 4nrFDC \quad (2.1)$$

Where n is the number of electrons transferred per redox couple, r is the radius of the disc microelectrode, F is the Faraday constant, D is the diffusion coefficient and C is the concentration of the redox couple.

A typical scan for a microelectrode described in section 2.1 gives an oxidation wave with a limiting current of 8.29 nA, calculated using equation 2.1 with $r = 25 \times 10^{-6}$ m, $C = 1.5 \text{ molm}^{-3}$, $n = 1$, $F = 9.65 \times 10^5 \text{ Cmol}^{-1}$ and $D = 5.73 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ [83]. Sometimes, from the sanding/polishing process, silver particles can be transferred to the Pt electrode, so it is important to check that no silver artefacts appear on the CV, which would appear at ± 65 mV under these conditions. A typical CV of a clean and well-polished electrode is shown in figure 2.2.

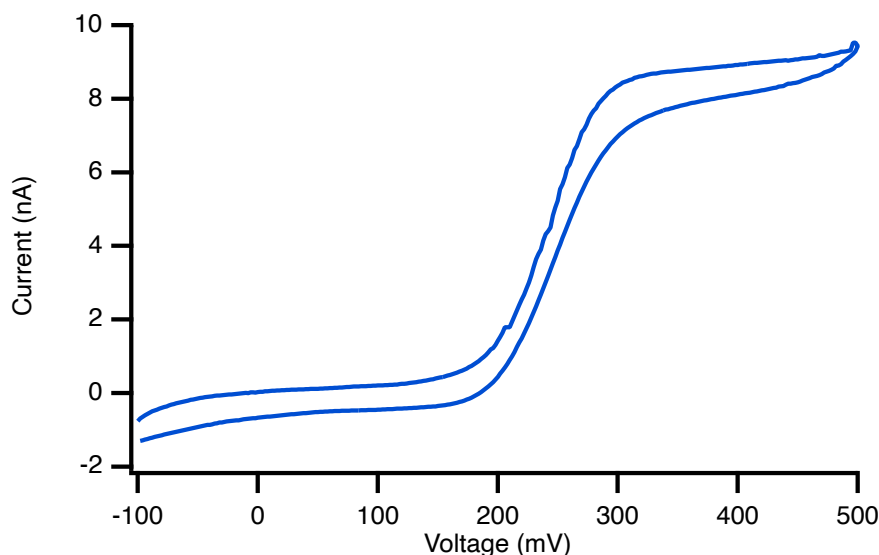


Figure 2.2: A typical CV for a polished microelectrode. The potential applied to a 50 μm Pt disc WE vs an Ag|AgCl RE is ramped linearly from -100 mV to +500 mV and then reversed, the corresponding current is recorded. The CV shown here is typical for a well-polished microelectrode, with a limiting current of approximately 8.1 nA, comparable to the theoretical value of 8.29 nA. The CV has small capacitance, which is shown by the small difference between the forward and backward scans. The CV is carried out in 1.5 mM Fc at a scan rate of 10 mV/s.

The final two polishing steps (0.3 μm and 0.05 μm alumina powders) are repeated until a satisfactory CV is achieved from the polished electrode.

2.2.2 Amperometry

Amperometry is a type of electrochemical technique where the WE is held at a constant potential vs the RE. In this thesis, this technique is used to chloridise the silver electrode, for electropolymerisation and to measure analyte concentrations. For these techniques, unless otherwise stated, the applied potential was controlled using LabChart software (ADInstruments, New South Wales, Australia).

Reference electrode

As stated in section 2.1, the RE consists of a silver wire which is chloridised to become an Ag|AgCl pseudo reference electrode. This is achieved using amperometry, where the silver electrode is treated as the WE. The microelectrode needle is placed into a 1 M potassium chloride (KCl) solution and a +0.45 V potential is applied to the silver electrode against a commercial Ag|AgCl reference electrode (Bioanalytical systems, Inc, USA) for 15 minutes. Successful chloridisation of the silver electrode can be seen under a microscope. A silver wire looks silver under the microscope, whereas a chloridised silver wire becomes darker as shown in figure 2.3.

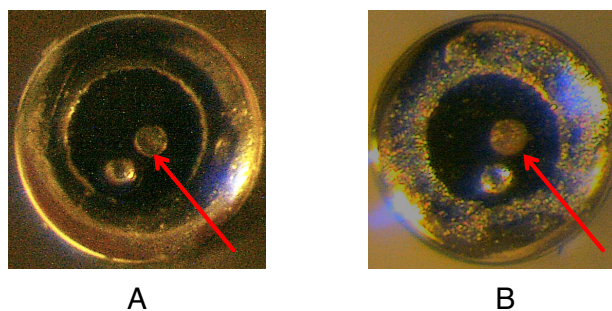


Figure 2.3: Images obtained using a microscope comparing a silver disc electrode before and after chloridising. A. A silver disc electrode before chloridising. **B.** Successful chloridisation of the silver electrode to become an Ag|AgCl pseudo reference electrode. Once the silver electrode is chloridised its appearance becomes darker.

Additionally, cyclic voltammetry can be used to assess whether the silver wire has been chloridised successfully. An unchloridised silver wire will give a CV where the backwards scan has shifted to the left and overlaps the forwards scan, as shown in figure 2.4. Otherwise a successful chloridised silver wire will produce a CV similar to that shown in figure 2.2.

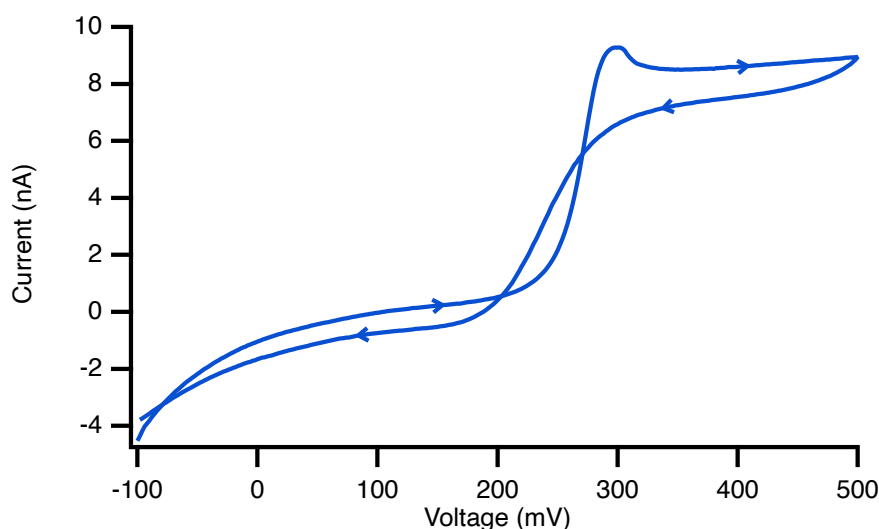


Figure 2.4: A CV for a Pt working electrode vs an unchloridised Ag|AgCl reference electrode. A CV of a 50 μm platinum disc electrode vs a failed Ag|AgCl reference electrode shows the backwards scan crossing over the forwards scan. The CV is carried out in 1.5 mM Fc at a scan rate of 10 mV/s.

mPD exclusion layer

Electropolymerisation is an amperometric technique in which the WE is held at a constant potential and placed in a solution containing a relevant monomer to initiate a polymerisation reaction to coat the working electrode. In this thesis, this technique is used to coat the sensor in an mPD layer, an exclusion layer to block out potential interferents that may oxidise at the potential applied to the WE. The structure of the monomer is shown in figure 2.5

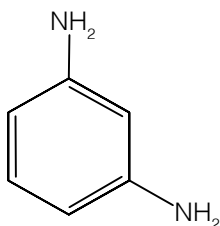


Figure 2.5: The structure of m-phenylenediamine. Electropolymerisation is used to coat the platinum WE with mPD, to block out potential interferents.

To coat the mPD on the WE surface, the electrode is placed in a 100 mM solution of mPD in 0.01 M PBS (pH 7.4). The WE is initially held at 0 V for 20 s, then held at +0.7 V for 20 minutes during which time the electropolymerisation occurs. Following this, the potential is returned back to 0 V and the electrode is left in the solution for 5 minutes to allow the mPD film to stabilise. After removal from the solution, the electrode is carefully rinsed with deionised water. Cyclic voltammetry is used to assess whether mPD has been successfully coated on the WE surface. If the mPD layer is intact and the Pt WE is well coated, the Fc molecules are unable to reach the electrode surface and therefore the oxidation and reduction peaks cannot be seen on the CV, as shown in figure 2.6.

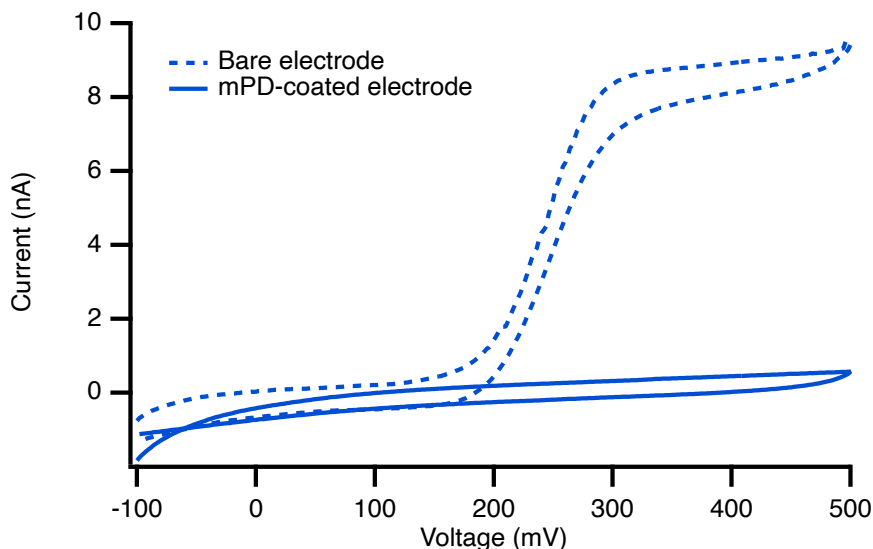
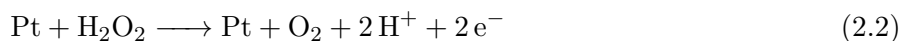


Figure 2.6: Comparison of CVs of a microelectrode with and without an mPD layer. The dotted line shows a CV for a bare, polished electrode, which has oxidation and reduction peaks, the solid line shows a CV for an electrode that has successfully been coated with mPD, showing no reduction or oxidation peaks. The CVs were carried out using a 50 μm Pt disc WE vs an Ag|AgCl RE in 1.5 mM Fc at a scan rate of 10 mV/s.

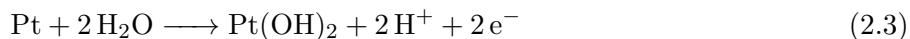
H₂O₂ detection

For the analytes of interest in this work, hydrogen peroxide (H₂O₂) is produced when the substrate is oxidised by an oxidase enzyme. To measure the H₂O₂, it is oxidised by holding the Pt WE is at +0.7 V vs the Ag|AgCl RE, and the resulting oxidation current is measured. This current is proportional to the H₂O₂ concentration, which is related to the analyte concentration.

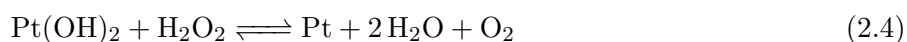
Equation 2.2 gives the overall oxidation reaction of H₂O₂ at the surface of the Pt electrode [84].



Equation 2.2 is a 2-step reaction [84]. The initial reaction that takes place when the Pt WE is held at +0.7 V vs Ag|AgCl is an electrochemical reaction described in equation 2.3.



The H₂O₂ then reacts chemically with the platinum hydroxide (Pt(OH)₂) to regenerate the Pt, as described in equation 2.4.



The regenerated Pt is reconverted to $\text{Pt}(\text{OH})_2$ as soon as it is produced because of the electrochemical reaction described in equation 2.3 that occurs due the applied potential of +0.7 V. Whilst H_2O_2 is available, Pt will be regenerated which in turn causes the reaction described in equation 2.3 to take place so 2 electrons will continuously be generated, resulting in a current proportional to the H_2O_2 concentration.

2.3 Biosensors

The microelectrodes described in section 2.1 are functionalised using enzymes to make biosensors. This section describes the theory behind enzymatic detection of analytes and the methods used to functionalise the microelectrode.

2.3.1 Enzyme kinetics

Enzymes catalyse reactions by providing a pathway with a lower activation energy for reactions to take place. The general enzymatic reaction that takes place is described in equation 2.5. Initially, the enzyme, E, and substrate, S, reversibly bind to form an enzyme-substrate, ES, complex. This ES complex irreversibly reacts to form a product and to regenerate the free enzyme.



There are three stages that take place during this reaction. The pre-steady state describes the initial period of the reaction when the substrate and enzyme first react and many ES complexes are being formed. Following this a steady-state period occurs, this is an equilibrium state where the ES concentration is constant, it is being formed at the same rate as it is being broken down. The post-steady state describes the stage when the substrate gets depleted so less ES complexes form.

Most enzyme reactions follow Michaelis-Menten (MM) kinetics, given by equation 2.6, which describes the relationship between substrate concentration, $[\text{S}]$, and the reaction rate, v during the steady-state period. V_{max} is the maximum rate of reaction that occurs when all of the enzyme active sites are saturated and K_m is the concentration of substrate at $\frac{1}{2}V_{max}$.

$$v = \frac{V_{max} \cdot [\text{S}]}{K_m + [\text{S}]} \quad (2.6)$$

This equation is displayed graphically in figure 2.7. Initially, the rate of reaction increases linearly. During this period the reaction is substrate limited; the product formation increases as the substrate concentration increases. The reaction rate then plateaus, as the enzyme active sites are fully saturated with substrate so increasing the substrate concentration has no effect on the product formation as the enzyme is functioning at maximum capacity. This stage is enzyme-limited.

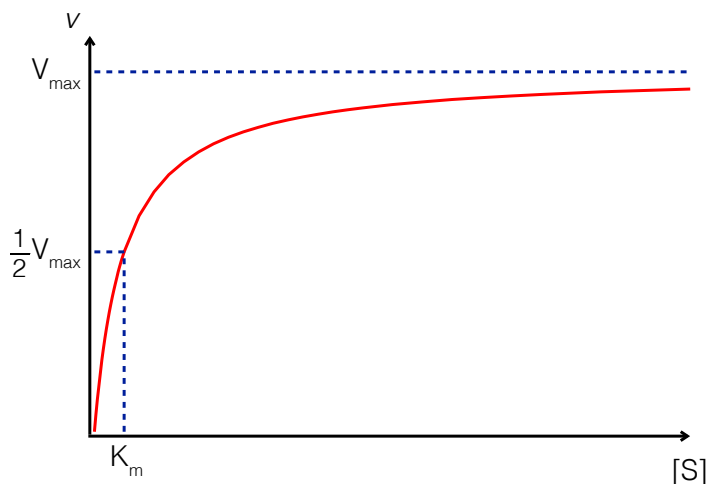


Figure 2.7: Michaelis-Menten curve. A typical curve of the reaction rate, v , versus substrate concentration, $[S]$ is plotted for an enzyme that follows MM kinetics. V_{max} describes the maximum reaction rate and K_m describes the substrate concentration at which the reaction rate is equal to half of V_{max} .

In this thesis, oxidase enzymes (SOx) are used in the biosensors. Oxidase enzymes catalyse the oxidation of their specific substrate (S) to generate a product (P) and H_2O_2 , which gets detected electrochemically as described in section 2.2.2. A schematic showing a vertical cross-section of the biosensor and this reaction is shown in figure 2.8.

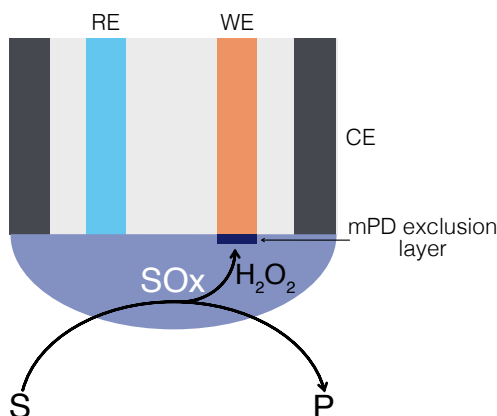


Figure 2.8: Schematic of a cross-section of a generic oxidase biosensor. The microelectrode consisting of a Pt WE, a Ag|AgCl pseudo RE and a stainless steel CE, is coated with a hydrogel containing an oxidase enzyme (SOx). When the substrate (S) for the specific enzyme diffuses into the hydrogel, it is oxidised to generate a product (P) and H_2O_2 . The H_2O_2 is oxidised at the Pt WE, held at +0.7 V vs the Ag|AgCl RE, to generate a current, which is related to the reaction rate.

The resulting current, I , that is recorded relates to the reaction rate, v , in equation 2.6, and the maximum current, I_{max} , can be related to V_{max} in equation 2.6. The recorded current is proportional to the H_2O_2 concentration which is related to substrate concentration.

2.3.2 Hydrogel layer

To functionalise the sensor, the tip of the electrode is coated in a hydrogel containing the enzyme of choice. This is achieved using a standard method developed within the Boutelle group, based on methods described by Vasylieva *et al.* [85, 86]. Within the group, lactate, glucose and glutamate biosensors have been developed [55, 69]. The hydrogel solution, prepared in 0.01 M phosphate buffer saline (PBS), consists of the relevant enzyme, lactate oxidase (LOx), glucose oxidase (GOx) or glutamate oxidase (GlutOx), glycerol, bovine serum albumin (BSA) and poly(ethylene glycol) diglycidyl ether (PEGDE), the composition of these hydrogel solutions are described in table 2.1.

	GOx hydrogel	LOx hydrogel	GlutOx hydrogel
Chemical	Concentration		
Enzyme (GOx, LOx or GlutOx)	60 mg/ml	60 mg/ml	50 mg/ml
BSA	30 mg/ml	30 mg/ml	81 mg/ml
PEGDE	14.8 mg/ml	45.6 mg/ml	78.0 mg/ml
Glycerol	10 μ l/ml	20 μ l/ml	10 μ l/ml

Table 2.1: Composition of the GOx, LOx and GlutOx hydrogel solutions.

For the glucose and the lactate biosensors, following the preparation of the relevant hydrogel mixture, 5 μ l of the solution is placed onto a microscope slide. For the glutamate biosensors, a 1 μ l of hydrogel solution is used due to the expense of glutamate oxidase. The electrode is dipped into the relevant hydrogel solution such that only the very tip of the electrode is coated. This has previously been found within the Boutelle group to make the hydrogel less fragile and susceptible to damage as the hydrogel does not go on the sides of the needle which would otherwise make it easier to accidentally wipe off. The electrode is held in place for 90 seconds before being removed from the droplet and secured with the tip facing up. The glucose and lactate biosensors are placed in the oven for 2 hours at 55°C and the glutamate biosensors are placed in the oven for 1.5 hours at 45°C. The heating process allows the PEGDE to form covalent bonds with the enzyme and BSA, creating a cross-linked matrix in which the enzyme is entrapped while still being active.

Following this process, the biosensors are stored at -20°C. Long-term storage stability has not been fully investigated but they have been used successfully after six months of storage at -20°C.

Manual biosensor calibration

A manual calibration can be performed to understand the kinetics of a given biosensor. The biosensor is initially placed in a stirred beaker containing 0.01 M PBS. The system is left so the current can stabilise following application of +0.7 V at the WE. Following this, additions of the given substrate are made to achieve a step concentration change. Following each addition of substrate, the signal increases and then stabilises. The substrate was added until there is no increase in the signal and the

plateau of the Michaelis-Menten curve has been reached.

To analyse the data, the mean and standard deviation of the plateau of each step change is taken and plotted against substrate concentration. An example of a lactate calibration curve is shown in figure 2.9. Manually calibrating biosensors in this way means the apparent V_{max} and K_m of the immobilised enzyme can be found [87].

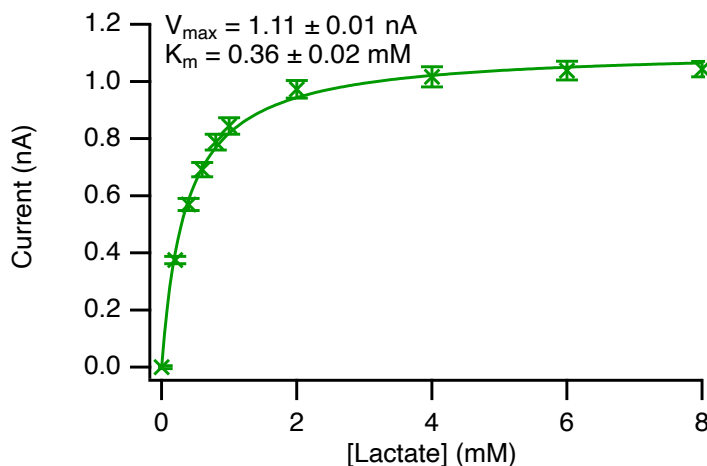


Figure 2.9: An example of a lactate biosensor calibration. The lactate biosensor was placed in a stirred beaker containing 0.01 M PBS. Lactate was sequentially added to obtain a calibration curve. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step for one calibration. The data is fitted with a Michaelis-Menten curve, the resulting apparent kinetic coefficients were found to be $V_{max} = 1.11 \pm 0.01$ nA and $K_m = 0.36 \pm 0.02$ mM. The errors represent the 95% confidence interval of the fitting parameters.

2.3.3 Polyurethane layer

In some cases, it is necessary to extend the dynamic range of the biosensors by adding a polyurethane (PU) layer. This limits the diffusion of the substrate to the enzyme, therefore decreasing the amount of substrate that is oxidised to H_2O_2 . This extends the linear relationship between the substrate concentration and reaction rate over a wider concentration range. This protocol has been developed and optimised previously in the Boutelle group [88]. A 25 mg/ml PU solution is prepared in tetrahydrofuran (THF). Polyurethane pellets are added to THF along with 0.25 μ l/ml Brij30 and left to stir in a sealed container until fully dissolved. The surfactant Brij30 is added to prevent pinhole formation during evaporation of the solvent [89]. Once the pellets have dissolved, the biosensors are held in the solution for 15 seconds and then left to dry with the tip facing up for 10 minutes. Following this initial drying period, the biosensors are held in the solution again for 15 seconds and then left to dry for 30 minutes. In between each dipping, the solution is stirred. Once dried, the biosensors are stored at -20°C .

Figure 2.10 shows a calibration curve for a lactate biosensor before and after it has been coated in PU. The dynamic range of the PU-coated biosensor has increased, as shown from the increase of the apparent K_m from 0.39 mM for the uncoated biosensor to 6.2 mM for the PU-coated biosensor.

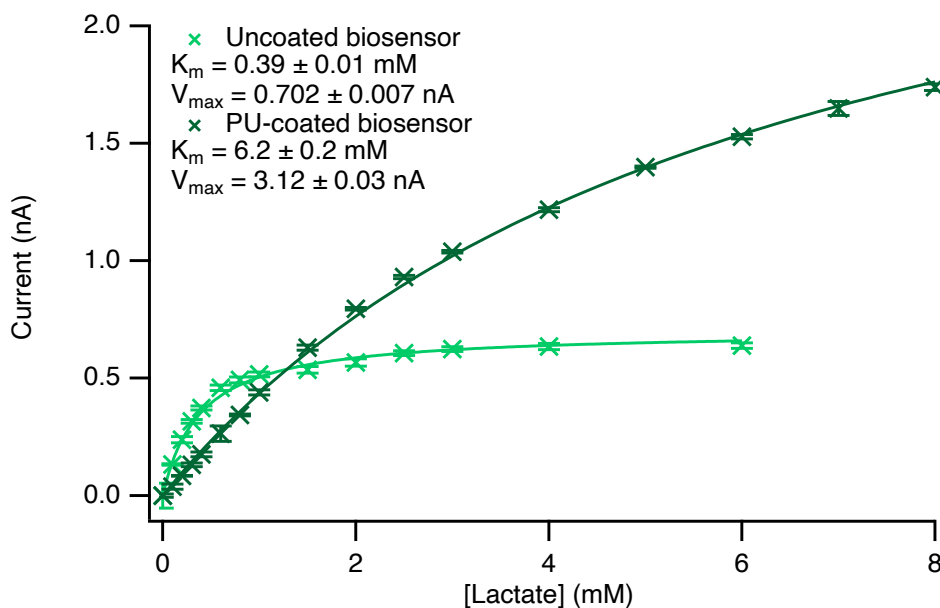


Figure 2.10: Lactate biosensor calibration before and after it has been coated in PU. The lactate biosensor, with and without the PU coating, was placed in a stirred beaker containing 0.01 M PBS. Lactate was sequentially added to obtain a calibration curve in both cases. Both calibrations are fitted with Michaelis-Menten curves. The dynamic range of the biosensor increases following coating of PU. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step. The apparent Michaelis-Menten kinetic coefficients are shown, the errors represent the 95% confidence interval of the fitting parameters.

2.4 Microfluidics

2.4.1 Microfluidic chip and sensor holders

Microfluidic chips used to house the microelectrode sensors have been developed previously within the Boutelle group [55, 78]. These chips were 3D printed using a ProJet MJP 2500 printer with VisiJet M2S-HT90 material (3D systems). The chip has an inlet, which is fitted with a C360-400 CapTite bonded-port connector for 360 μ m outer diameter (o.d.) polyetheretherketone (PEEK) tubing (Mengel Engineering, Denmark) to allow a low-volume connection to the microfluidic platform described in section 2.4.2. Running through the centre of the chip is a channel, which was designed to have a size of 350 μ m x 350 μ m (HxW). As a sample stream flows through the channel, it will flow past the electrode tip, and the H_2O_2 produced following the enzyme-mediated reaction will be oxidised. The 2-electrode microfluidic chip with electrodes inserted is shown in figure 2.11A.

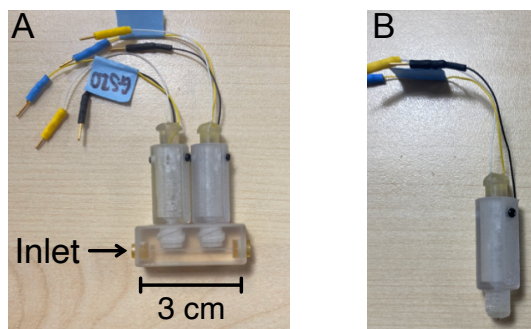


Figure 2.11: A 2-electrode microfluidic chip housing electrodes secured in 3D printed holders. A. The 2-electrode chip with electrodes inserted. There is a $350 \times 350 \mu\text{m}$ channel running through the centre, where solution flows past the electrode tips. **B.** A 3D printed holder with an electrode positioned and M2.5 grub screws securing it in place.

To fit the sensors into the microfluidic chip, the sensors are placed into 3D printed holders, shown in figure 2.11B. These holders have two pegs either side allowing them to be screwed into the slots in the microfluidic chip. This allows the electrode to be positioned in the middle of the channel and also protects it during insertion. The holders have a semi-compressible plastic (TangoPlus material) on the tip so that they create a water-tight fit meaning no leaks occur. The sensors are positioned such that the tip of the sensor sits in the middle of the channel. They are held in place with M2.5 grub screws.

2.4.2 Microfluidic platform

A microfluidic platform has been configured within the Boutelle group using LabSmith components (purchased from Mengel Engineering, Denmark) for autocalibration of the biosensors, described further in section 2.4.3, and for manipulation of low-volume sample streams [90]. Generally, push/pull syringe pumps, reservoirs and 2-way valves have been used within the Boutelle group. There are multiple different versions of the pumps, valves and reservoirs. This section will focus on the LabSmith components currently used by the Boutelle group for the development of an autocalibration microfluidic platform. Other LabSmith components are used later in this thesis and will be introduced where relevant.

The autocalibration platform consists of push/pull syringe pumps with a volume of $20 \mu\text{l}$, 2-way valves and 1 ml reservoirs. The components are connected using PEEK tubing with $150 \mu\text{m}$ inner diameter (i.d.) and $360 \mu\text{m}$ outer diameter (o.d.) and LabSmith interconnect products. These components are placed on a uProcess breadboard and controlled using uProcess software. The outlet of the board is connected to the 3D printed microfluidic analysis chip developed within the Boutelle group, described in section 2.4.1. A labelled photo of the autocalibration microfluidic platform is shown in figure 2.12.

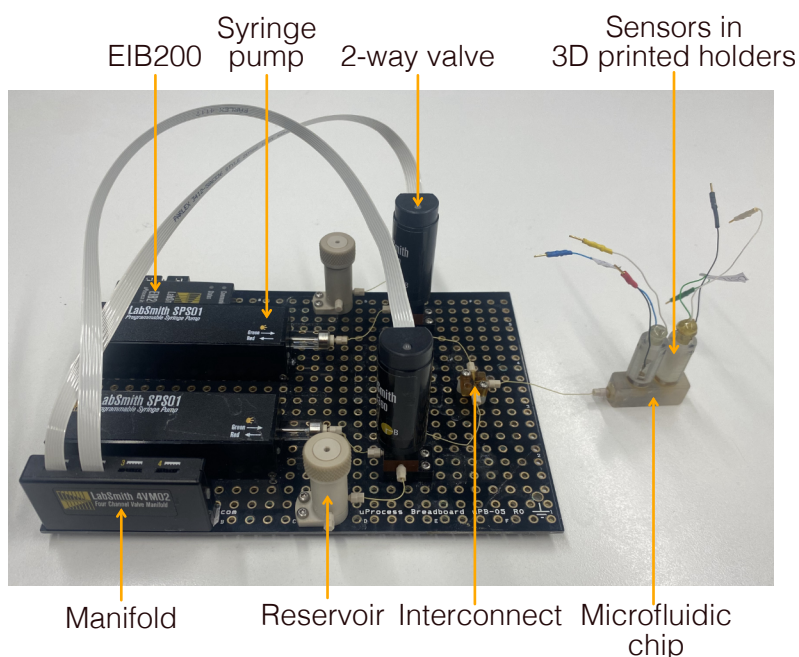


Figure 2.12: Photo of the microfluidic platform setup for a biosensor calibration. The EIB200 allows connection from the board to power and to a computer to run uProcess software to control the board. The manifold connects and allows control of the valves. The reservoirs displayed here hold a volume of 1 ml. The push/pull pumps are fitted with a glass syringe and plunger, which are available in different sizes; 20 μ l syringes are displayed here. The outlet of the board is connected to the analysis system consisting of a 3D printed microfluidic chip and the sensors fitted in 3D printed holders. The different components are connected with 360 μ m outer diameter tubing and LabSmith connectors.

A schematic of this board is shown in figure 2.13, which shows how the board is set up using the 2-way valve to have two different pathways from the pump.

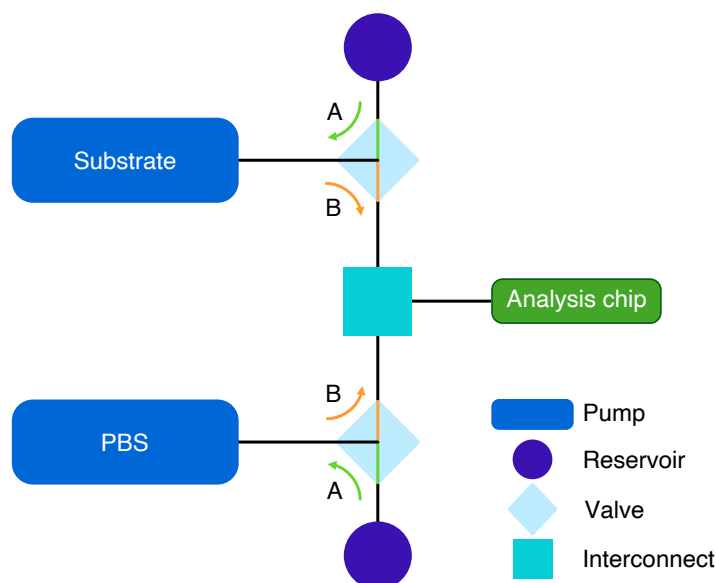


Figure 2.13: Schematic of the microfluidic platform set up for a biosensor calibration. A schematic of the microfluidic platform used for calibration of biosensors. The 2-way valves allow the flow to be controlled to either go down path A, shown in green, or down path B, shown in orange. In this schematic, when the valve is in position A the flow path is between the reservoir and pump, and the pump will fill with the liquid from the reservoir. When the valve is in position B, the flow path is between the pump and the analysis chip, and the pump will push out towards the analysis chip. When necessary, the valve is switched to configuration A to allow refilling of the pump. This can be automated by writing a uProcess (LabSmith, US) script to repeatedly run calibrations without any manual intervention.

The 2-way valves can be switched between positions A and B. In position A, shown in green in figure 2.13, the valve is directed to the reservoir and the syringe pump will fill with liquid from the reservoir. In orientation B, shown in orange in figure 2.13, the flow is directed towards the outlet of the board and the pump will push out towards the analysis chip. This process is automated by writing a script using uProcess software (LabSmith, US). The interconnects used in this thesis allow up to three flow streams to be combined with one common outlet.

This microfluidic platform was later modified and optimised to allow addition of choline oxidase (ChOx) into the analysis stream, described in chapter 3, and to allow manipulation of acetylcholinesterase and acetylcholine streams, described in chapters 5 and 6.

2.4.3 Biosensor autocalibration

The microfluidic board setup as shown in figure 2.13 is used for calibration of the biosensors. The board was previously configured in this way within the Boutelle group and used for calibration of glucose and lactate biosensors in biologically-relevant concentration ranges [90]. The flow rate of the substrate, glucose or lactate, and PBS pumps are varied while keeping the total flow rate constant, to create different concentrations of the substrate at the chip. The total flow rate is kept constant as the sensor response is determined by the mass transport of the substrate to the enzyme hydrogel and hence the sensor response is dependent on flow rate. A 5-step calibration at 2 $\mu\text{l}/\text{min}$ is typically

performed, varying the concentration of the substrate between 0 and 100% in 20% increments.

An example of a current trace recorded during a 5-point calibration is shown in figure 2.14.

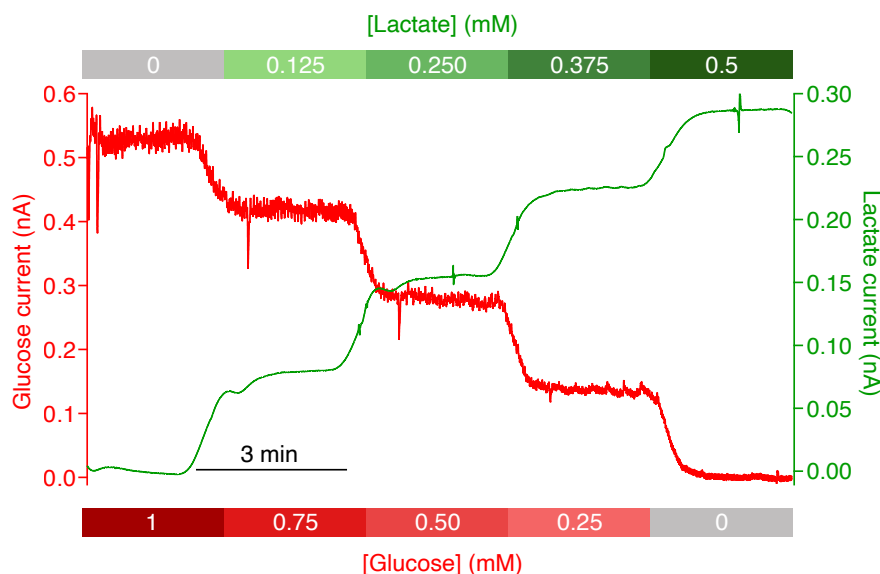


Figure 2.14: The current trace recorded during a 5-point autocalibration for a glucose and a lactate biosensor. The total flow rate for this calibration was held constant at 2 $\mu\text{l}/\text{min}$. The lactate concentration was varied from 0 to 0.5 mM and simultaneously the glucose concentration was varied from 1 to 0 mM. Each concentration step lasted 3 minutes. The glucose current response is shown in red and the lactate current response shown in green. The current was recorded at 200 Hz and smoothed using a 201-point Savitzky-Golay filter.

For each concentration step, the mean and standard deviation of the current is taken and is plotted against the substrate concentration as shown in figure 2.15, giving a calibration curve.

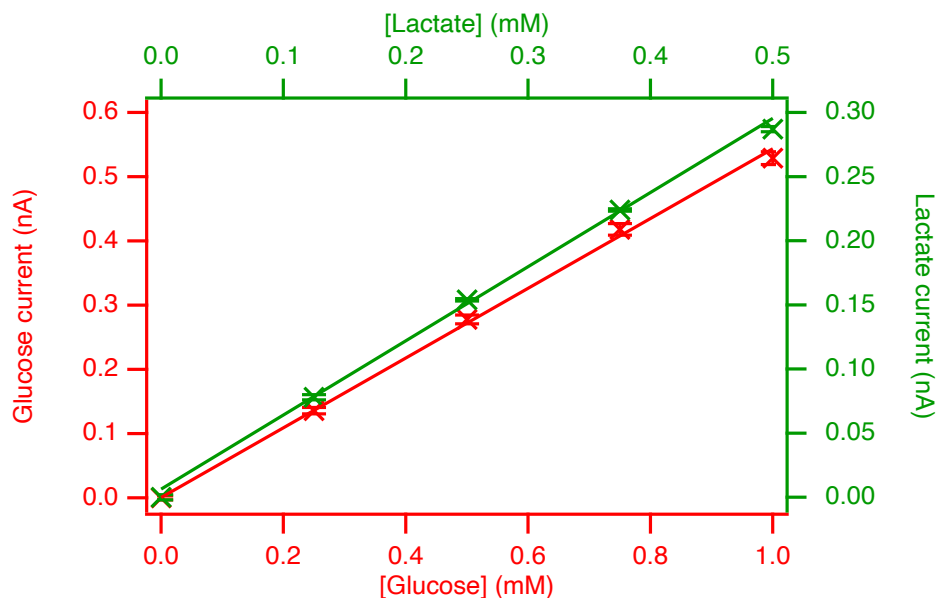


Figure 2.15: Glucose and lactate autocalibration curves. The glucose calibration curve is shown in red and the lactate calibration curve shown in green. The biosensors used here were not coated in polyurethane and the substrate concentrations were chosen to be in the linear range. The markers and error bars show the mean $\pm$ standard deviation of the current for each concentration step shown in figure 2.14. The points have been fitted using linear regression, the gradient of which corresponds to the sensitivity of the biosensor.

When in the linear range of the biosensor, a linear regression is used to fit the data and the resulting gradient of the graph gives the sensitivity of the sensor. Typically, the sensitivity of a glucose or lactate biosensor coated without PU is approximately 1 nA/mM and with PU is 0.2 nA/mM. If the calibrations are performed with substrate concentrations beyond the linear range of the biosensor, the curve is fitted using the MM equation described by equation 2.6 in section 2.3.1.

2.5 Wireless monitoring

In this thesis, some of the monitoring of analytes, either on the bench-top, or monitoring *in vivo*, has been done wirelessly when taking the equipment to external sites. In this scenario, sensors are controlled using an in-house wireless potentiostat, powered by a 3.7 V battery. The potentiostat transmits data via Bluetooth to an Android app that has been developed within the Boutelle group [78]. This app is installed on a Samsung tablet to display and store the data. A box has been designed and 3D printed to house the electronics and hold the microfluidic chip, described in section 2.4.1, in place. The printed circuit board (PCB) is shown in figure 2.16A, and the bluetooth module shown in figure 2.16B.

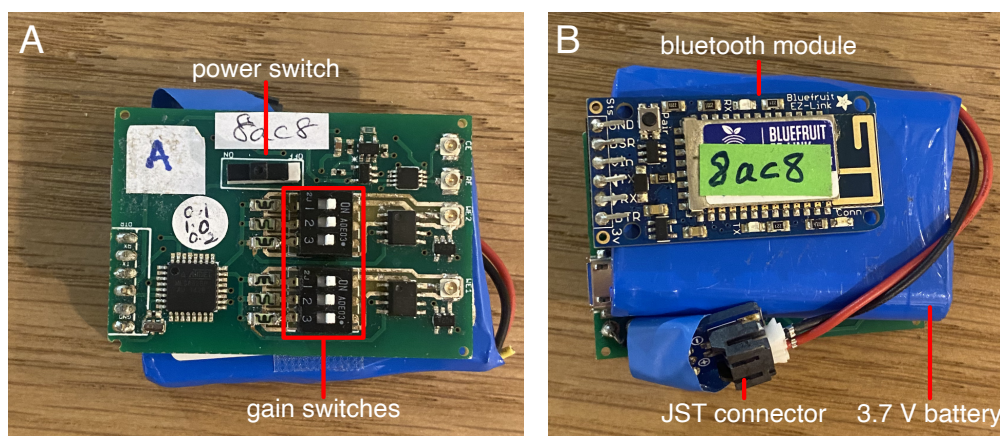


Figure 2.16: The wireless potentiostat. A. A labelled photo of the top-side of the potentiostat showing the power and gain switches. **B.** The Bluetooth module connected to the potentiostat is shown, with the 3.7 V battery connected using a JST connector.

Different views of the housing for the wireless electronics, with a 2-electrode analysis chip, are shown in figure 2.17.

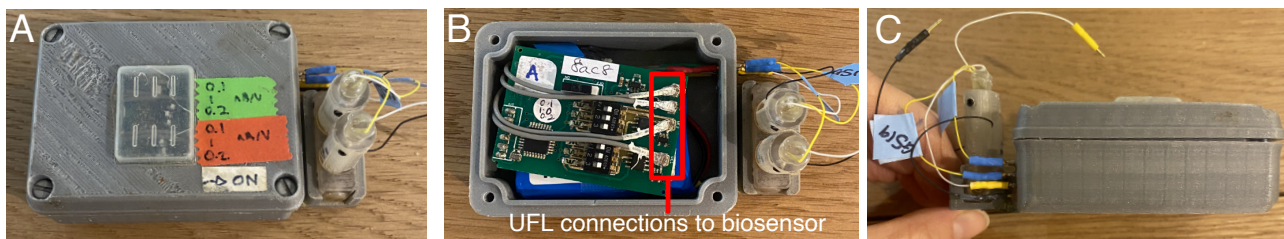


Figure 2.17: The housing for the wireless electronics. **A.** The closed box is shown with a 2-electrode analysis chip inserted into the slot. **B.** The wireless electronics inside the box. UFL cables are used to connect the biosensors to the potentiostat. **C.** A side view of the housing for the wireless electronics, showing the electrical connections between the biosensors and UFL cables.

The potentiostat has two channels, with each channel having three different gains; 1 nA/V, 0.1 nA/V and 0.2 nA/V or 0.4 nA/V depending on the chosen potentiostat and the application. The gain selected is controlled by the switches labelled in figure 2.16A. The gain options allow the current range of the potentiostat to be chosen based on the sensor response. The current is converted to a voltage using an operational amplifier. The voltages are digitised by the internal 10-bit analog-to-digital converter (ADC) of the microcontroller (ATMega328). The number of bits of the ADC is therefore $2^{10} = 1024$ bits. The voltage range is determined by the analog reference which has been set to 3.3 V. This gives a voltage resolution of $3.3/1024 = 3.2$ mV/bits. Based on this, the theoretical current measured at the working electrode can be calculated using equation 2.7, where I is the current in nA, S is the recorded data in bits and G is the selected gain on the potentiostat in nA/V.

$$I = (3.2 * 10^{-3}) * S * G \quad (2.7)$$

The app display is shown in figure 2.18. The top graph shows a 5-point glucose calibration and the bottom graph shows a 5-point lactate calibration.

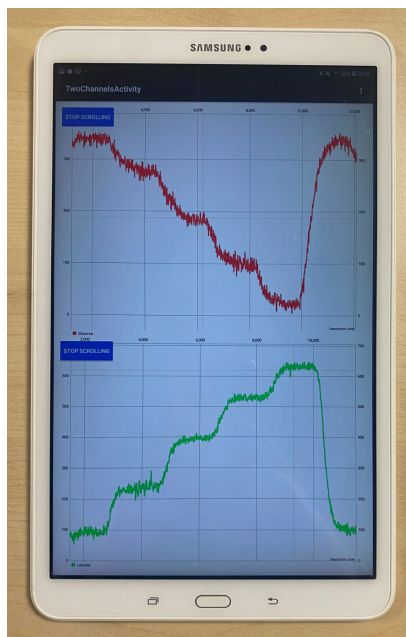


Figure 2.18: The tablet display during a glucose and lactate calibration. The top graph displays a 5-point glucose calibration, shown by the red trace, and the bottom graph displays a 5-point lactate calibration, shown by the green trace. Each point displayed is an average of 10 samples. The recorded data is sampled at 10 Hz.

In this thesis, the wireless potentiostats are used to run various experiments in chapters 4 and 8. The 3D printed box has been adapted to allow different size microfluidic chips to be used and to facilitate easier electrical connections between the sensors and potentiostat, also discussed in chapters 4 and 8.

Work was also undertaken by myself and Dr Sally Gowers to develop a new version of the potentiostat with a different ADC to provide better resolution and the app was improved to allow monitoring from additional channels. Full delivery of this work goes beyond the time-scale of this thesis.

2.6 Microdialysis probes

Commercially available microdialysis (MD) probes were used within this thesis. Two different types of probes were commonly used, shown in figure 2.19.



Figure 2.19: Microdialysis probes used in this thesis. A. CMA 70 brain MD probe. **B.** CMA 12 animal MD probe.

Flexible CMA 70 brain microdialysis probes were purchased from Mdiaalysis (Sweden). These have a molecular weight cut-off (MWCO) of 20 kDa, a membrane length of 10 mm and a membrane diameter of 600 μm . These probes were used for preliminary MD experiments discussed in chapter 5. CMA 12 animal microdialysis probes were purchased from Linton Instrumentation (UK). These probes have a MWCO of 20 kDa, a membrane length of 4 mm and a membrane diameter of 500 μm . They were used due to their rigidity making them suitable for incorporation into a flow cell, the development of which is described in chapter 6.

2.7 Common reagents and solutions

Choline oxidase from *Arthrobacter globiformis* was purchased from Sekisui diagnostics. All other chemicals, including acetylcholinesterase from *Electrophorus electricus*, were purchased from Sigma Aldrich.

2.7.1 Phosphate buffered saline

One phosphate buffered saline (PBS) tablet (Sigma Aldrich) was dissolved in 200 ml of deionised water to make 0.01 M PBS solution.

2.7.2 Ferrocene monocarboxylic acid solution

A 1.5 mM Fc solution was used as the redox couple in cyclic voltammetry to assess the electrode surface as described in section 2.2.1. The composition of the 1.5 mM Fc is shown in table 2.2.

Chemical	Concentration (mM)
Ferrocene monocarboxylic acid (Fc)	1.5
Ethylenediaminetetraacetic acid (EDTA)	1
Sodium citrate	100
Sodium chloride	150

Table 2.2: Composition of 1.5 mM Fc solution

2.7.3 Substrate standards and enzyme solutions

To prepare choline and acetylcholine (ACh) standards, choline chloride and acetylcholine chloride were dissolved in 0.01 M PBS respectively. Initially, the solutions were made from a bottle that had been opened and, therefore, exposed to the air previously. However, due to the hygroscopic nature of choline chloride and acetylcholine chloride variable results were achieved. Therefore, it was important to dissolve the batch of either chemical immediately after opening the container and further dilute this down to obtain the required concentration for the standards. To prepare choline oxidase solutions, the enzyme was weighed and dissolved in the relevant volume of 0.01 M PBS.

Two different grades of acetylcholinesterase (AChE) were used, type V-S and type V-IS (Sigma Aldrich), which had > 1000 U/mg protein and 200 - 1000 U/mg protein, respectively. Initially, to prepare AChE solutions, a volume of 0.01 M PBS was added to the bottle containing the enzyme to make a 1 mg/ml solution. To improve the stability of the AChE solution, BSA was added to the solution to make a 1 mg/ml BSA solution.

Chapter 3

Choline detection

3.1 Introduction

Choline is a nutrient that plays many important roles in the body, including in acetylcholine synthesis, cell membrane synthesis, and liver and muscle function [91]. Choline has also been studied as a potential biomarker for Alzheimer's disease [92]. Abnormal choline metabolism, as well as changes in an individual's choline level, has been seen in multiple cancers [93, 94], hence choline could be used as a biomarker for cancer detection. Furthermore, detection of choline has been utilised for monitoring acetylcholinesterase activity [95]. Described in detail in chapter 1 and 5, acetylcholinesterase (AChE) is the enzyme that catalyses the hydrolysis of acetylcholine (ACh) into choline and acetic acid and by monitoring the hydrolysis product choline we are able to detect changes in AChE activity.

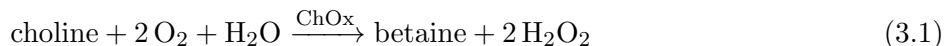
In this thesis, choline detection will be used to characterise AChE activity. In this chapter I describe the development of a choline sensing system.

3.1.1 Current methods for choline detection

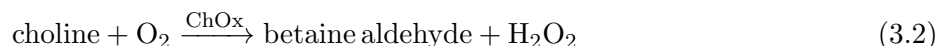
Methods to monitor choline include liquid chromatography coupled with mass spectrometry (MS) [96], ion chromatography [97], gas chromatography coupled with MS [98] and electrophoresis coupled with MS [92]. These methods have been shown to be highly sensitive and selective, however they are labour intensive, expensive and time consuming. They also do not offer a continuous measurement. Enzyme-based sensors are a popular alternative; these include electrochemical, colorimetric, chemiluminiscent and fluorescence types. These sensors are simple and low-cost while still retaining good sensitivity. Amongst the types of enzyme-based sensors, electrochemical methods have been found to give the highest sensitivity for choline response [99]. An extensive review of the different enzymatic methods has been conducted by Rahimi *et al* with a good summary of the different parameters achieved, i.e. linear range and sensitivity [99].

3.1.2 Enzyme-based choline detection mechanism

Enzyme-based sensors use the enzyme choline oxidase (ChOx), which catalyses the oxidation of choline to produce betaine and two hydrogen peroxide (H_2O_2) molecules, this reaction is shown in equation 3.1.



This is a 2-step reaction. The first step is the oxidation of choline to betaine aldehyde and one H_2O_2 molecule, shown in equation 3.2.



The second step is the oxidation of betaine aldehyde to betaine and one H_2O_2 , shown in equation 3.3.



Enzyme-based sensors rely on the detection of the oxidation product, H_2O_2 , the concentration of which will be relatable to the choline concentration. A range of optical methods can be used to do this, such as colorimetric, fluorescent and chemiluminescent methods.

Colorimetric biosensing of choline relies on a bienzymatic system, ChOx and a peroxidase or peroxidase-mimic, and a chromogenic peroxidase substrate. The H_2O_2 reacts with the chromogenic peroxidase substrate in the presence of a peroxidase or peroxidase-mimic enzyme to produce a change in colour [100, 101]. This multi-stage method to detect choline is more complex in the fabrication process and does not offer improved sensitivity compared to other methods, however, once the biosensor is fabricated and reagents are prepared, it is a simple method and offers fast detection of changes in choline levels that can be detected visually by the naked eye. However, it is hard to detect small changes in choline levels using this method, as it is limited by what can be detected by the naked eye. In order to achieve greater sensitivity, it has to be coupled with a spectrometer or a form of image capture and post-processing is required [102–104].

Fluorescent choline biosensors rely on the use of fluorophores or fluorescently-labelled molecules which absorb electromagnetic radiation and produce a change in fluorescent signal. Commonly, nano-materials have been used for this purpose. For example, carbon dots [105] and quantum dots [106] have been successfully used for choline detection as the H_2O_2 produced by choline oxidation has been found to quench the fluorescence of both carbon and quantum dots. While fluorescence-based assays often offer a wide response range and can be sensitive, they require a spectrometer to detect the resulting fluorescence, and often the fabrication process is complex.

Chemiluminiscent methods rely on the use of a luminophore, which reacts with H_2O_2 to produce light. The reaction can be initiated by using electrochemical methods or catalysed by using a peroxidase enzyme [107, 108]. While these sensors have been developed to offer low detection limits and a wide linear range, they do require another device to read the chemiluminescence output, and, again, is a multi-stage detection mechanism.

3.1.3 Method to monitor choline in this thesis

In this thesis, an electrochemical enzyme-based approach was developed. This method was chosen as only one enzyme, ChOx, is required, making it a simple approach and excellent sensitivity can be achieved [99]. H_2O_2 can be detected electrochemically using a range of techniques. Commonly, the dynamic electrochemistry methods square wave voltammetry (SWV) [109] and cyclic voltammetry are used [110]. Although SWV has been shown to offer improved sensitivity, these methods do not offer a continuous measurement and require significant post-processing [111]. In this thesis, amperometry is used as previously described in section 2.2.2 to offer a continuous measurement and minimal post-processing of the signal. A constant +0.7 V is applied to a platinum (Pt) working electrode (WE) vs the Ag|AgCl pseudo reference electrode (RE) to oxidise H_2O_2 , this generates a continuous current which is related to the choline concentration. This method offers the advantage of an easy-to-fabricate enzyme-based sensor that can be coupled with small electronics for continuous monitoring of choline.

In this thesis, two approaches were developed to create a method to detect choline using ChOx: (i) an in-flow ChOx system and (ii) a choline biosensor, shown in figure 3.1.

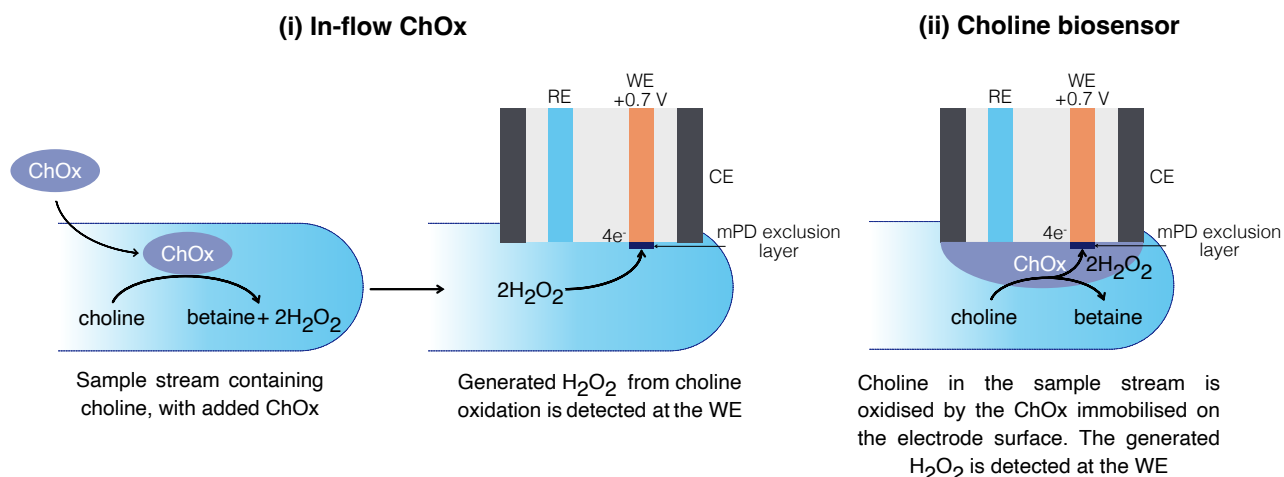


Figure 3.1: Schematics to describe the two enzymatic methods to detect choline in a sample. In both methods, ChOx is used in the system to oxidise choline to produce $2\text{H}_2\text{O}_2$. The generated H_2O_2 is detected using amperometry by holding the WE at +0.7 V to oxidise the H_2O_2 and produce 2e^- for every H_2O_2 molecule, described in more detail in section 2.2.2. In method (i), ChOx is introduced into the sample in-flow, allowing the enzyme to mix with choline present in the sample and oxidise it to produce $2\text{H}_2\text{O}_2$ in solution. This itself is then oxidised as it flows past the electrode surface. In method (ii) ChOx is immobilised on the electrode surface to create a choline biosensor using a method based on that described in section 2.3. As the choline in the sample flows past the biosensor surface, it is oxidised to produce $2\text{H}_2\text{O}_2$ molecules, which are oxidised at the electrode surface. In each schematic, the case is shown for one choline molecule.

The in-flow ChOx system introduces the enzyme into the solution to oxidise the choline, producing $2\text{H}_2\text{O}_2$ molecules, which are detected using a mPD-coated sensor. By having the free enzyme in solution, it allows the reaction time between the choline and ChOx to be controlled. In theory, the in-flow system can provide a maximum signal for a given flow rate, as with perfect mixing all the choline can be converted to H_2O_2 . However, the in-flow ChOx system requires the continuous addition of reagent, which becomes costly and is less practical. The choline biosensor was fabricated based on the method described in section 2.3 to fabricate the glucose and lactate biosensors. The amount of choline converted will always be less as there is limited reaction time between the choline and ChOx. Both approaches are explored in this chapter and the optimisation of different parameters are considered. The sensors were optimised up to a point where the sensitivity and stability were sufficient for the application of monitoring in plasma and whole blood, in the μM range [112].

3.2 In-flow ChOx system

To monitor choline using the in-flow ChOx system, mPD-coated sensors were fabricated. The microfluidic platform introduced in section 2.4.2 was modified to include an additional pump to allow the addition of the enzyme ChOx into our system, as shown in figure 3.2.

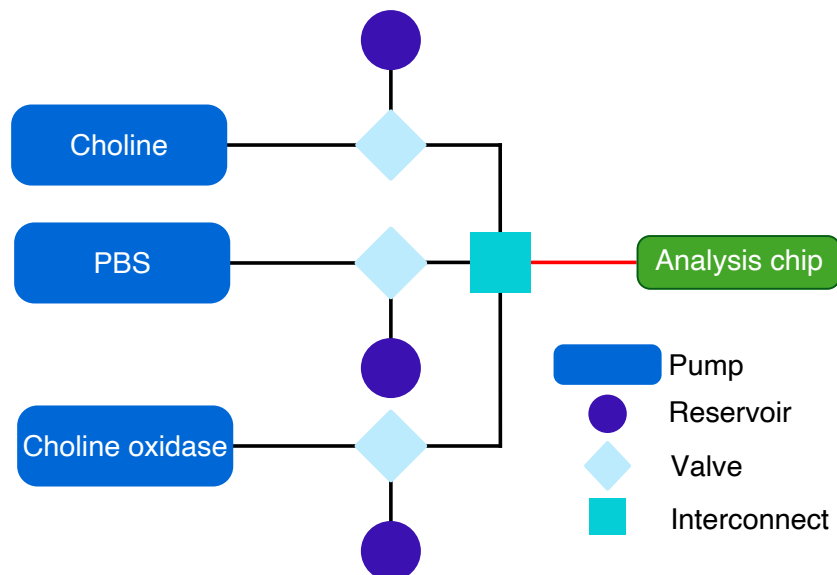


Figure 3.2: Schematic of the adapted microfluidic board to allow addition of ChOx in-flow. An additional pump, reservoir and valve was added to the microfluidic board described in section 2.4.3 to allow the addition of ChOx in flow. The choline and ChOx meet at the interconnect and mix together in the reaction tubing highlighted in red, where choline is oxidised to generate H_2O_2 . The length of the reaction tubing and flow rate determines how long the ChOx and choline mix together and therefore determines how much choline will be oxidised to H_2O_2 before the liquid stream comes into contact with the sensors in the analysis chip.

The flow rates of each pump were chosen to control the concentration of ChOx and choline at the chip. The length of tubing highlighted in red, the reaction tubing, determines the interaction time between the choline and ChOx in solution for a set flow rate. This determines the amount of choline oxidised and the signal achieved; the longer the tubing, the more interaction time and therefore the more choline converted to H_2O_2 until there has been 100% choline conversion and there is no benefit of increasing the reaction time. However, increasing the length of tubing decreases the temporal resolution due to Taylor dispersion and also increases the time delay in measuring the signal [113]. Therefore, it was important to find a balance between interaction time, temporal resolution and time delay. Two different lengths of reaction tubing, 5 cm and 30 cm, were used to determine the effect of the length of tubing on the signal obtained from oxidation of choline. The corresponding reaction times were 21 s and 2 min 7 s, respectively. The choline concentration was varied from 0 to 80 μM in 20 μM steps. The ChOx concentration remained constant at 0.2 mg/ml, corresponding to an activity of 2.72 U/ml. The total flow rate of the system was 2.5 $\mu\text{l}/\text{min}$. The flow rate of the ChOx pump was held constant at 0.5 $\mu\text{l}/\text{min}$, and the PBS and choline pumps were varied to have a total flow rate of 2 $\mu\text{l}/\text{min}$. The calibration curves are shown in figure 3.3.

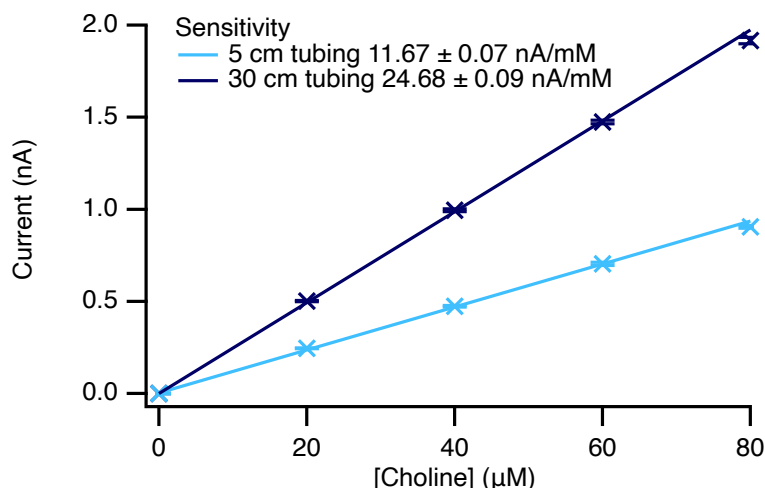


Figure 3.3: Calibration curves for the in-flow ChOx system using 5 cm and 30 cm lengths of reaction tubing. Calibration curves obtained using the in-flow ChOx system (0.2 mg/ml ChOx), with 5 cm and 30 cm lengths of reaction tubing, shown in light and dark blue, respectively. The choline concentration was varied from 0 to 80 μM in 20 μM steps. The total combined flow rate was held constant at 2.5 $\mu\text{l}/\text{min}$. The sensitivity, found by linear regression, of the system when using the 30 cm length of reaction tubing was $24.68 \pm 0.09 \text{ nA}/\text{mM}$, approximately twice the sensitivity of the system with the 5 cm length of reaction tubing, $11.67 \pm 0.07 \text{ nA}/\text{mM}$, suggesting not all of the choline was converted when using the 5 cm reaction tubing. The errors on the sensitivities represent the 95% confidence interval of the fitting coefficient. The markers and error bars show the mean $\pm$ standard deviation respectively for a 1 minute sample of each concentration step.

The sensitivity of the 30 cm reaction tubing setup is approximately twice the sensitivity of the 5 cm reaction tubing setup. It was expected that the ratio of the sensitivities obtained for each length of reaction tubing would be the same as the ratio of the lengths of each reaction tubing. However, as this is not the case, the results suggest that maximal signal could have been obtained with a piece of tubing less than 30 cm in length, but longer than $2 \times 5 \text{ cm} = 10 \text{ cm}$.

As a further step this experiment was repeated with the 30 cm tubing coiled, achieved by wrapping it around a 2 cm diameter pen. This was to investigate if coiling the tubing would further improve the signal by improving the mixing of the enzyme and substrate as poor mixing may have been the limiting factor for the current obtained. The experimental protocol is shown in figure 3.4 on the left, which shows that a 5 cm tubing calibration was performed before and after the 30 cm tubing calibration (LT) and the 30 cm coiled tubing calibration (CT). The purpose of this was to check the degradation of the system sensitivity over time. The calibration before the LT calibration is referred to as T1, the calibration between the LT and CT calibrations is referred to as T2 and the calibration performed after the CT calibration is referred to as T3. The calibration curves in figure 3.4 are those obtained during the short tubing calibrations (T1, T2 and T3), with the sensitivities shown.

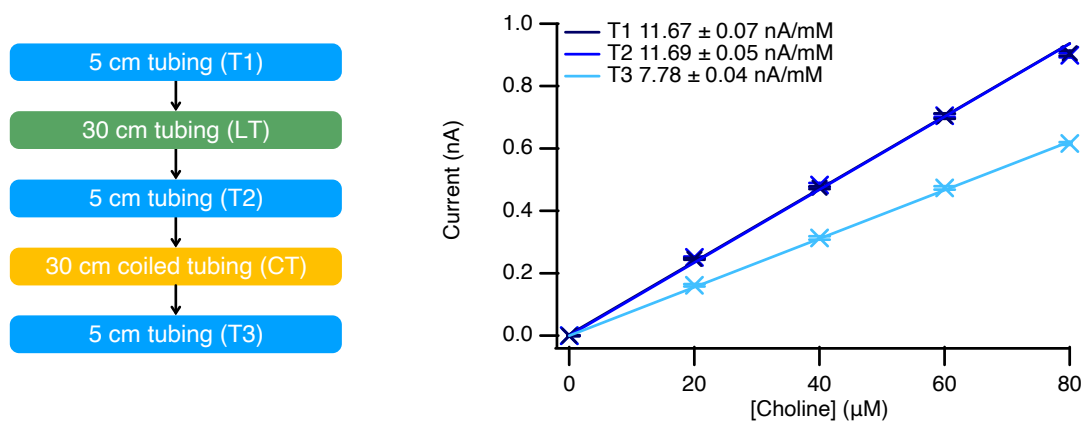


Figure 3.4: A summary of the experimental protocol and the resulting calibration curves for the different 5 cm tubing calibrations performed for the in-flow ChOx system. The calibration curves T1, T2 and T3 refer to the position in the experimental protocol of these calibrations as shown by the flow diagram on the left. The calibration curves for the 5 cm length of reaction tubing show that there is a large drop in sensitivity between calibrations T2 and T3, between which an experiment was performed with the 30 cm length of reaction tubing coiled. This data can be used by taking the ratio of the sensitivities for $\frac{T1}{T2}$ and $\frac{T1}{T3}$ to normalise the LT and CT calibration data.

The graph shows that initially the calibrations are quite stable, shown by T1 and T2, with T2 mostly overlaying T1. However, a noticeable decrease in sensitivity is observed between the T2 and T3 calibrations, between which the CT calibration was performed, as referenced in the experimental protocol on the left of figure 3.4. To properly assess the differences between the two 30 cm tubing types, the data was normalised using the 5 cm tubing calibrations. The ratio $\frac{T1}{T2}$ and $\frac{T1}{T3}$ were taken and used as a multiplication factor. There are two ways the multiplication factors could be used: (i) the $\frac{T1}{T2}$ ratio could be used to normalise the LT calibration and the $\frac{T1}{T3}$ ratio could be used to normalise the CT calibration or (ii) the LT calibration could be kept the same, and the $\frac{T1}{T2}$ ratio could be used to normalise the CT calibration. However, given that the ratio $\frac{T1}{T2}$ is 0.9983 (approximately equal to 1), only method (i) was used as method (ii) has little effect on sensitivities. Therefore, only the ratio $\frac{T1}{T3}$ was used to normalise the CT data and the LT data was left unchanged. The results were compared to the original values.

The calibration curves are shown in figure 3.5 which demonstrate that the normalised CT calibration curve is very different to the original calibration curve, also shown.

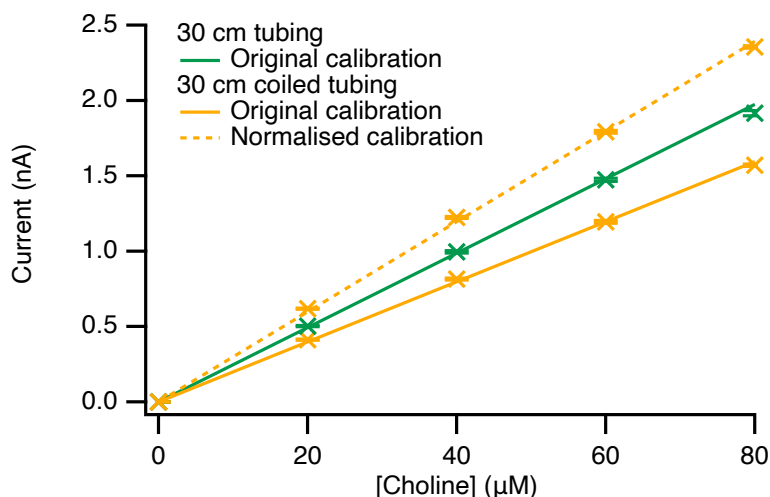


Figure 3.5: Calibration curves for the in-flow ChOx system using a straight and a coiled 30 cm length of mixing tubing. The results for the two tubing types are shown, with normalised result for the coiled tubing calibration also shown. The ratio of the short tubing calibrations T1 and T3, $\frac{T_1}{T_3}$, was used to normalise the coiled tubing calibration data. Results for the normalised data show that the coiled tubing provides better sensitivity than the straight tubing. However, the original calibrations suggest that the LT setup gives a better sensitivity than the CT setup.

The calibration curves show there is a discrepancy in which experimental setup gives better sensitivity and more data is needed to conclude whether coiled tubing does improve mixing. Linear regression has been used to fit the curves and the gradients give the resulting sensitivities. The sensitivities for each experimental setup and the normalised data is shown in table 3.1.

Calibration	Sensitivity (pA/μM)
30 cm tubing	24.7 ± 0.9
30 cm coiled tubing	19.94 ± 0.07
Normalised 30 cm coiled tubing	29.90 ± 0.07

Table 3.1: Sensitivities for each experimental protocol used for the in-flow ChOx method

Taking an average of the two normalised coiled tubing results gives a sensitivity of approximately 24.9, which is similar to the straight 30 cm tubing sensitivity. This is a plausible result; if the drop in sensitivity of the system was linear, a true normalisation factor would be an average of the two ratios, $\frac{T_1}{T_2}$ and $\frac{T_1}{T_3}$, which would give approximately the average of the two coiled tubing calibrations. Furthermore, it is likely that, based on the 30 cm tubing and 5 cm tubing results discussed previously, the length of reaction tubing to achieve maximal signal should be 10 cm, hence improving the mixing should have no effect as the ChOx already has excess time to oxidise all of the available choline. This is theoretical, therefore further experimental work is needed to understand the system fully. These results show that there are options to improve the sensitivity of the system if it is needed by changing the length of the reaction tubing.

3.2.1 Stability

The mPD-coated sensor gets fouled, from enzyme sticking and blocking the electrode surface, over time and decreases in sensitivity [90]. Similarly, the ChOx enzyme loses sensitivity over time when at room temperature as the enzyme denatures and becomes less active. As these sensors are to be used continuously over several hours, it is important to understand how quickly the in-flow system drops in sensitivity. This will provide insight into how often the sensors need calibrating, or how frequently the ChOx solution needs to be changed.

Initially, an experiment was carried out using the microfluidic platform configured as shown in figure 3.2, with a reaction tubing length of 5 cm. The flow rates of the pumps were chosen such that the total flow rate was 2 $\mu\text{l}/\text{min}$, to make it comparable to the biosensor experiments which were conducted at this flow rate. The ChOx flow rate was held constant at 0.4 $\mu\text{l}/\text{min}$. The flow rates of the PBS and choline pumps were varied to create changing choline concentrations at the analysis chip, with the their combined flow rates remaining constant at 1.6 $\mu\text{l}/\text{min}$. The ChOx concentration was 1 mg/ml, corresponding to 0.2 mg/ml at the chip, and the maximum choline concentration at the chip was 8 μM . This concentration was chosen to be comparable to the biosensor experiments, as 10 μM is well within the biosensor linear range, discussed further in section 3.3, and it is within the range of plasma choline levels. It was also important to check the experimental system was sensitive enough to monitor these low levels of choline. A 5-point calibration was left to run for multiple calibrations over a 65-hour time period. After the sensitivity of the system had dropped to <10% of its original value, at approximately 49 hours, the solution in the ChOx reservoir was replaced with a fresh 1 mg/ml solution to see whether the drop in sensitivity was due to the ChOx solution degrading or the sensor fouling. The 5-point calibration curves were plotted and a line fitted to obtain the sensitivity of the experimental setup at a given time. The resulting sensitivities have been plotted against the elapsed experimental time, shown in figure 3.6.

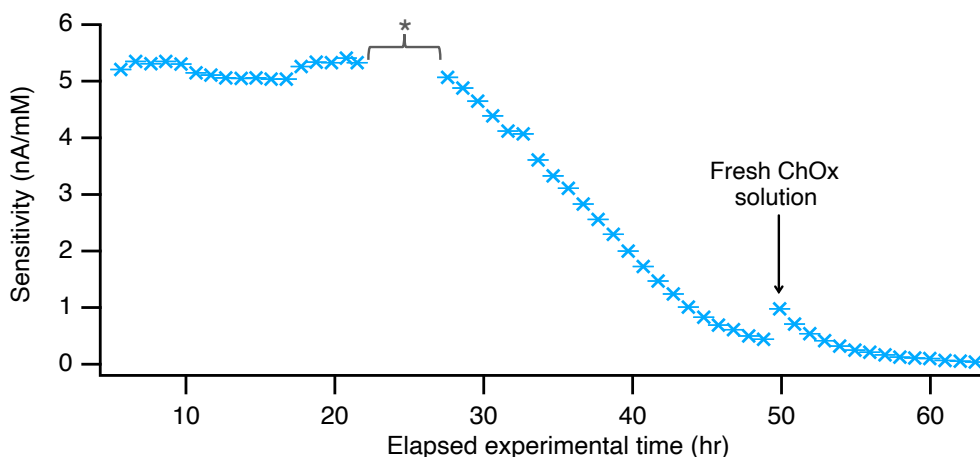


Figure 3.6: The sensitivity decay of the in-flow ChOx system when using a bulk solution of 1 mg/ml ChOx. 5-point choline calibrations up to 8 μM were performed continuously over 62 hours to investigate the stability of the in-flow ChOx system. The calibration curves were fitted using linear regression, the gradient gives the sensitivity of the sensor. The resulting sensitivities have been plotted against the elapsed experimental time. The asterisk shows a period where the signal was unstable and therefore the sensitivities have not been plotted. The markers and error bars show the gradient $\pm$ 95% confidence interval of the gradient obtained from the linear regression of each calibration.

The results show that at approximately 30 hours the sensitivity starts to decline. The asterisk shown in figure 3.6 annotates a period during the experiment where the signal was unstable. The increase in sensitivity following the addition of fresh ChOx solution suggests that the ChOx had degraded, however, it is only a small increase, suggesting that the more dominant factor causing loss of signal was the sensor degrading due to the sensor fouling.

This experiment was repeated with a bulk enzyme concentration of 0.5 mg/ml to determine the effect of a lower enzyme concentration on stability and sensitivity. Using a lower enzyme concentration is desirable because it costs less, however, it may impact the stability of the enzyme solution. To further investigate the degradation of the ChOx enzyme specifically, a 4-pump microfluidic platform was set up as shown in figure 3.7, and designed to give a 5-step calibration alternating between fresh ChOx solution and aging ChOx enzyme solution to determine the degradation of the aging ChOx.

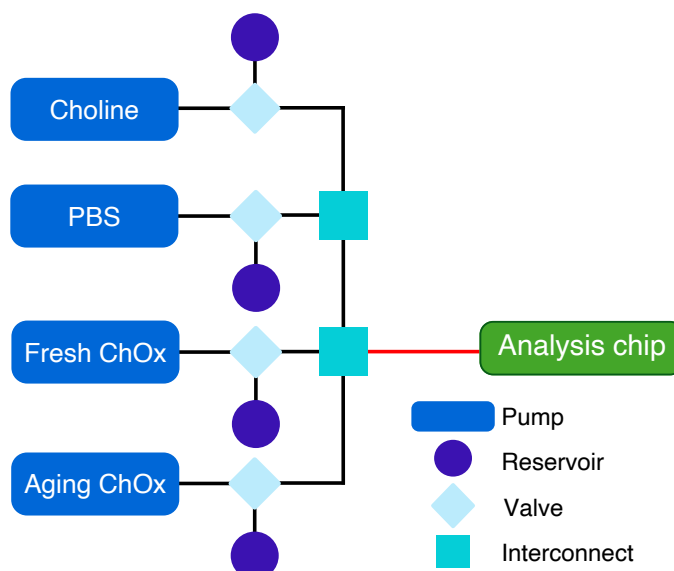


Figure 3.7: A schematic of the microfluidic platform configured to alternate between performing a calibration with an aging ChOx solution and a fresh ChOx solution. A uProcess script was written to alternate between performing a 5-point calibration using the pump containing the ‘fresh’ ChOx solution and running a 5-point calibration using the pump containing the ‘aging’ ChOx solution.

The total flow rate of the system was constant 2 $\mu\text{l}/\text{min}$. As before, the flow rate of the ChOx pump was constant at 0.4 $\mu\text{l}/\text{min}$ and the combined flow rate of the PBS and choline pumps was 1.5 $\mu\text{l}/\text{min}$. Due to the lower enzyme concentration of 0.5 mg/ml, corresponding to 0.1 mg/ml in the reaction tubing, the experiment was carried out with a higher choline concentration of 80 μM choline to ensure the calibration steps could be seen as it was not known how large an effect the different enzyme concentration would have on the sensitivity. Initially, 0.5 mg/ml of ChOx was made in 0.01 M PBS and placed in each reservoir. The ChOx solution in the designated ‘fresh’ pump was replaced approximately every 2 hours. There was a length of time overnight where there was no access to the experiment so the ‘fresh’ ChOx was replaced the following morning. Results of this are shown in figure 3.8.

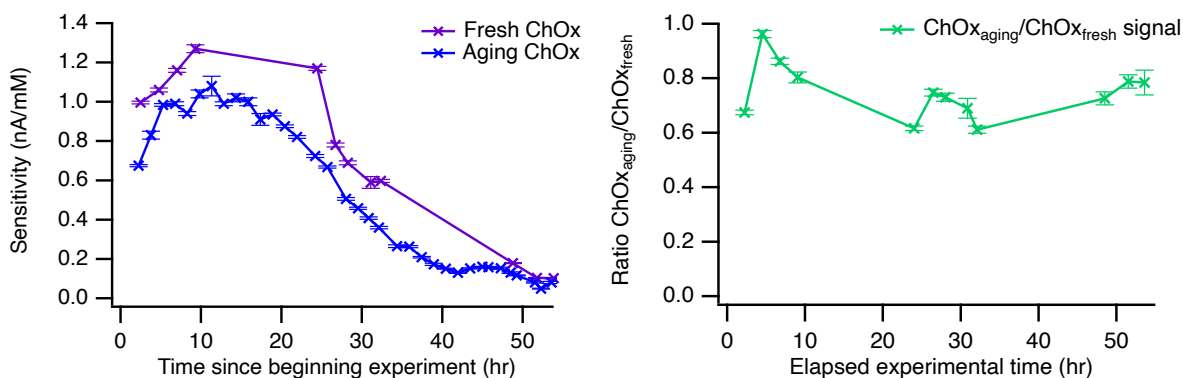


Figure 3.8: Sensitivity decay of the in-flow ChOx system when using fresh and aging enzyme solution. Left: Sensitivities obtained during a 5-point calibration up to 80 μM when using fresh ChOx solution and aging ChOx solution. The sensitivities obtained during the ChOx_{fresh} calibration are relatively stable until 24 hours, following that the sensitivity reduces at a similar rate to the ChOx_{aging} sensitivities. The ChOx_{aging} results are also relatively stable initially, but start to degrade at around 15 hours. Right: The ratio of the sensitivity obtained from the ChOx_{aging} sensitivity/ ChOx_{fresh} sensitivity. The ratio shows that following an initial decline of the ChOx_{aging} sensitivities compared to the ChOx_{fresh} sensitivities, the ratio is relatively stable, suggesting that the dominating factor of loss of signal in this system comes from the electrode fouling. The markers and error bars show the ratio $\pm$ the propagated error of the ratio using the error of the sensitivities.

The left graph shows that for the aging 0.5 mg/ml ChOx solution, ChOx_{aging}, the sensitivity of the system starts to degrade at approximately 15 hours of elapsed experimental time. Comparing this to figure 3.6 suggests that the ChOx solution is less stable at lower concentrations. However, it is important to consider that this experiment used a different mPD-coated sensor, and the mPD coating may vary causing changes in how quickly the sensor degrades over time. It is also important to note that the ChOx_{fresh} calibrations do not show a drop in sensitivity until approximately 24 hours, which would suggest the initial degradation of the ChOx_{aging} sensitivities is due to the lower enzyme concentration degrading at a faster rate.

The ratio of the fresh enzyme to the aging enzyme was taken, as shown by the right graph in figure 3.8. The graph shows that the ratio of ChOx_{aging}/ChOx_{fresh} shows the ratio initially increased, possibly indicating flow problems with the ChOx_{aging} pump that later stabilised. The ratio remains quite stable following an initial decline, from approximately 24 hours of elapsed experimental time. This suggests that at this point the sensor is becoming fouled by enzyme sticking and preventing H₂O₂ reaching the electrode surface. Taking this and the aging ChOx trace into account, it is likely that this system, with this enzyme concentration, can only be used for approximately 15 hours before the sensors should be replaced as the steep drop in sensitivity could lead to inaccurate results.

Further work could be done to investigate if the electrode fouls as quickly with a polyurethane (PU) layer. Previously, as described in section 2.3.3, the purpose of the PU layer has been to extend the dynamic range of the biosensors. In this case, the dynamic range of the system will be unaffected, however, it could provide an extra protection layer for the sensor. Therefore, it could be an option to investigate this to improve stability during use, as changing the sensor and/or solution every 15 hours

would mean needing to change the sensor in the morning and evening, which is not desirable. Using a higher concentration of ChOx solution could be beneficial as these preliminary results suggest it could extend the time-frame over which the system can be used.

Check to see if ChOx is sticking to the analysis chip

Enzymes are large proteins, therefore they have a tendency to stick to soft plastics. If the ChOx was to stick, the increase in ChOx in the system may cause an increase in the amount of choline converted to H_2O_2 , which would mean that frequent calibrations would be needed to ensure they are accurate for the amount of enzyme in the system. There are ways to stop enzyme sticking, which include pretreating a plastic surface, either with a surfactant or a protein, or mixing the enzyme with a surfactant and/or protein containing solution. This is discussed in more detail in section 7.

To determine if the ChOx was sticking, the microfluidic board was set up as shown in figure 3.2. A uProcess script was written that alternated between two calibration cycles - one cycle had ChOx present and the other one did not have ChOx present. If the ChOx was sticking, then the calibration with no ChOx being run through the board would still show a change in signal as the choline would be oxidised due to the presence of leftover ChOx. If no ChOx was sticking, then no change in signal would be seen when running the calibration cycle without ChOx.

The results of this experiment are shown in figure 3.9, which shows the filtered trace.

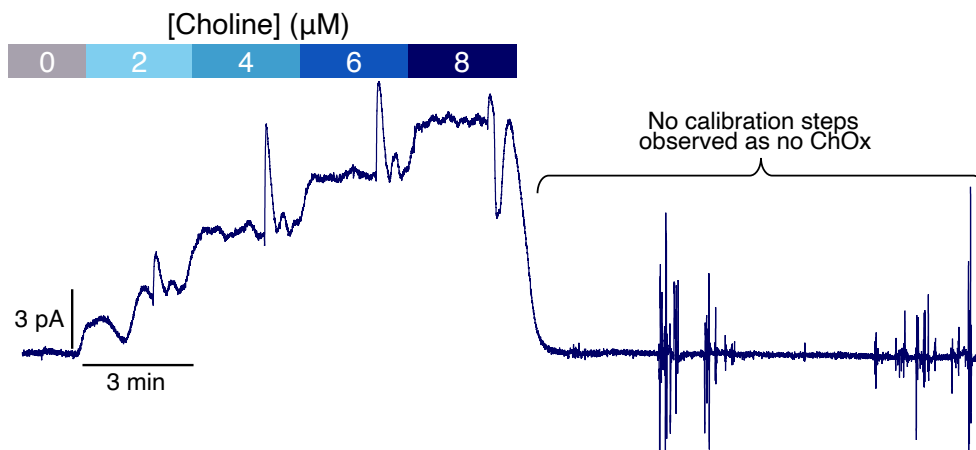


Figure 3.9: An example of the trace obtained during tests to see if ChOx was sticking to the microfluidic platform. The microfluidic platform was set up to alternate between a 5-point choline calibration up to 8 μM with ChOx present and one with no ChOx present to check if the ChOx was sticking to the microfluidic platform. The trace shows that with ChOx present, the choline calibration can clearly be seen, and with no ChOx present, there is no change in signal. This suggests no ChOx is sticking to the microfluidic platform. The current is sampled at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

When the ChOx is present in the system, a 5-point calibration can clearly be seen. When no ChOx is present in the system, there is no change in signal, showing that no ChOx is sticking in the tubing of the microfluidic platform or inside the analysis chip.

3.3 Choline biosensor

For the choline biosensor, ChOx is immobilised on the electrode surface. In this section, I present the development, fabrication and optimisation of the choline biosensor. Different parameters, including the sensitivity and the stability of the biosensor were determined.

3.3.1 ChOx hydrogel layer

To fabricate the choline biosensor, a ChOx hydrogel was made and immobilised on the tip of an mPD-coated electrode. Prior to this step, the electrodes were polished, the silver wire was chloridised to create the Ag|AgCl pseudo reference electrode, and the mPD exclusion layer applied to the electrode surface by electropolymerisation as discussed in sections 2.1 and 2.2.2, respectively. A cyclic voltammogram (CV) was recorded to assess the electrode surface at each stage of the fabrication process as previously described in section 2.2.1. Only electrodes that exhibited a limiting current between 7.5 and 11 nA were used beyond the referencing stage of the fabrication process.

Initially, to make the ChOx hydrogel solution, the protocols for the lactate oxidase (LOx) hydrogel and glucose oxidase (GOx) hydrogel were copied with ChOx replacing the respective enzyme. As described in section 2.3.2, the hydrogel solution is prepared in 0.01 M PBS and consists of the enzyme, glycerol, bovine serum albumin (BSA) and poly(ethylene glycol) diglycidyl ether (PEGDE). The ChOx hydrogel solution was more viscous when following the GOx protocol compared to the LOx protocol, which made it easier to coat the electrode tip, therefore the hydrogel made following the GOx protocol was used going forward. Table 3.2 shows the composition of this ChOx hydrogel using this method.

Chemical	Concentration
ChOx	60 mg/ml
Bovine Serum Albumin (BSA)	30 mg/ml
Poly(ethylene glycol) diglycidyl ether (PEGDE)	14.8 mg/ml
Glycerol	10 μ l/ml

Table 3.2: Composition of the ChOx hydrogel solution

To fabricate the choline biosensors, the mPD-coated electrodes were dipped in a 5 μ l droplet of the hydrogel for 90 s. Following this, the biosensors needed to be held at a specific temperature to initiate the cross-linking reaction between the PEGDE, BSA and ChOx. For the glucose and lactate biosensors, the hydrogels are cured for 2 hours at 55°C. To investigate the use of different temperatures for the different enzyme, ChOx, the duration and temperature at which the hydrogel was cured at was varied and compared. According to the data sheets provided by the supplier of the enzymes, Sekisui, ChOx has been found to be stable at 40°C and below (10 minutes, pH 7.5), whereas GOx is stable at 50°C and below (15 minutes, pH 7.0) and LOx is also stable at 50°C (10 minutes, pH 7.0). Due to the activity of ChOx dropping off significantly at temperatures above 40°C, the sensors were placed in the

oven at lower temperatures but for longer periods of time to allow the chemical reaction between the PEGDE, BSA and the enzyme ChOx to take place, which will take longer at lower temperatures. To investigate this, the sensors were fabricated by curing the hydrogel at temperatures of 35°C or 40°C for 3, 4 or 5 hours.

Microfluidic setup

To test the sensors, the microfluidic platform was configured in the standard calibration format as described in section 2.4.2, figure 2.13. The sensors were made in batches of 2 or 3 and placed in a 3-electrode microfluidic chip. To perform a calibration up to 10 μM , 0.01 M PBS and a 10 μM solution were placed in each reservoir on the microfluidic platform. A stock 1 mM choline solution had previously been prepared and frozen in aliquots; this was diluted to make the 10 μM choline solution. A uProcess script was written to automatically, continuously perform a 5-step calibration, the corresponding choline concentrations at the microfluidic chip were 0, 2.5, 5, 7.5 and 10 μM , with each concentration step lasting 3 minutes. The concentration range was chosen so that the calibration was well within the dynamic range of the enzyme, the K_m of the free enzyme is 1.2 mM for choline and 8.7 mM for betaine aldehyde. The apparent K_m of the immobilised enzyme will be lower than this, as previously seen for the glucose and lactate biosensors. Being within the dynamic range made it possible to compare sensitivities of the biosensors by using linear regression to fit a straight line to the calibration. An example of the trace and calibration curve obtained during a 5-point 10 μM choline calibration is shown in figure 3.10.

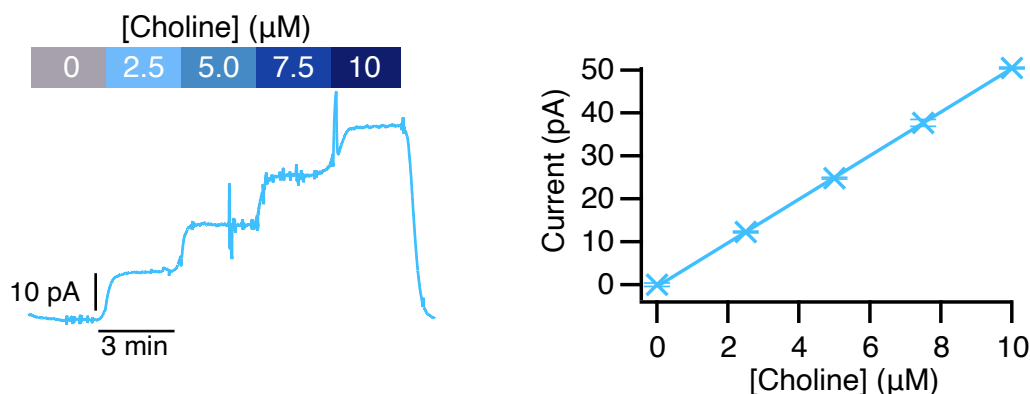


Figure 3.10: An example of the current trace recorded during a choline biosensor calibration and the resulting calibration curve. Left: The current trace recorded during a 5-point choline biosensor calibration up to 10 μM . The current was recorded at 200 Hz and the trace was smoothed using a 201-point Savitzky-Golay filter. Right: The resulting calibration curve for the choline biosensor. The markers and error bars show the mean $\pm$ standard deviation for each concentration step of one calibration sample. This choline biosensor was fabricated using the GOx hydrogel protocol, the composition of which is shown in table 3.2, and placed in the oven for 5 hours at 35°C.

3.3.2 Comparison of hydrogel curing conditions

The sensitivities of various biosensors obtained using the different fabrication conditions are shown in figure 3.11 using a box and whisker plot.

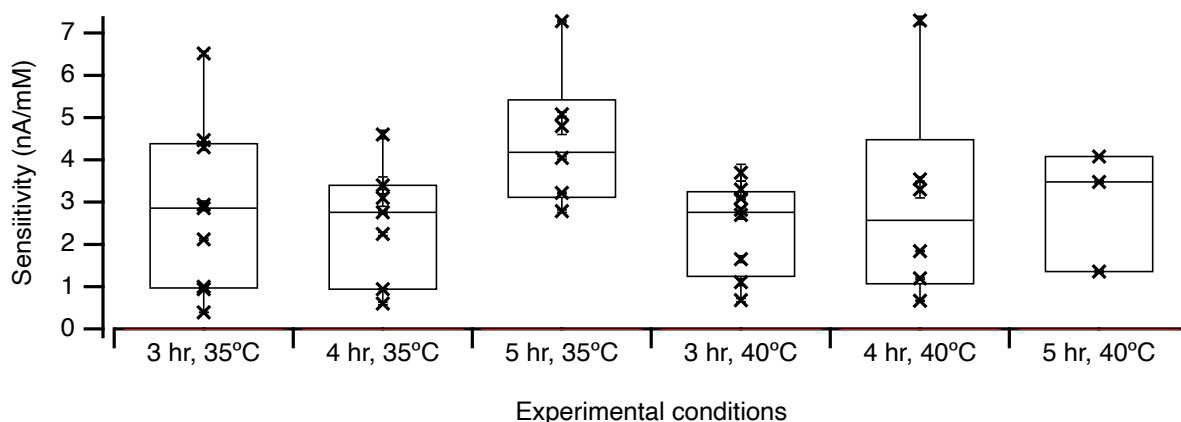


Figure 3.11: A box and whisker plot comparing the sensitivities obtained for choline biosensors fabricated using the GOx hydrogel protocol and placed in the oven at 35°C or 40°C for different lengths of time. The different sensitivities are also plotted as individual points, which were obtained by performing a linear regression on the calibration curve and taking the gradient as the sensitivity. The error bars represent the 95% confidence interval of the linear regression.

Very high sensitivities were achieved for all of the choline biosensors regardless of the protocol. There was a spread in responses for each protocol, with some outliers in each case. However, based on the median and interquartile range, the protocol of 5 hours at 35°C gave the best overall sensitivity.

For these choline biosensors, sensitivities of up to 7 nA/mM were achieved. For comparison purposes, the glucose and lactate biosensors described in section 2.3 without an outer diffusion limiting layer, the sensitivities achieved are typically 1 nA/mM. One of the reasons for the comparatively high sensitivity will likely be due to the two-step reaction shown in equations 3.2 and 3.3, with the overall reaction producing two H_2O_2 molecules that can in turn be oxidised at the electrode. It is important to note again that the ChOx used here has a specificity to betaine aldehyde of 46.2%, which will likely affect the relative reaction velocities. Based on this, depending on the solution, it is possible that two H_2O_2 molecules will not be produced for every choline molecule, or it will not necessarily be detected by the sensor. However, it is still likely that more H_2O_2 molecules will be oxidised on average for each choline molecule, contributing to the overall current compared to just one H_2O_2 molecule for every glucose and lactate molecule. Another reason is that the glucose and lactate biosensors are placed in the oven at a higher temperature of 55°C, which may be causing some of the enzyme to denature and lose activity. The temperature profile shows that both GOx and LOx are stable at higher temperatures than choline oxidase, but the activity of GOx does decrease to 80% when held at 55°C for 15 minutes, and the activity of LOx decreases to 80% when at 60°C for 10 minutes (no data on 55°C). As these sensors are in the oven for 2 hours, it is likely that some of the activity is lost, which could be a contributing factor to the lower sensitivity.

Experimental limitations

This experiment to compare different experimental conditions used during the choline biosensor fabrication process had some limitations. As previously stated, the sensors were fabricated and placed in the oven in batches of 2 or 3 sensors. On analysing the data, I found that biosensors further along the microfluidic chip, in position 2 or 3, would sometimes give a better sensitivity than a biosensor in position 1. This could have been a result of cross-talk; some H_2O_2 produced at the biosensor in position 1 may not have been oxidised and could have travelled downstream to the biosensors in position 2 or 3 and contribute to the recorded current at these electrodes. A test was performed to determine if any H_2O_2 was travelling downstream. The microfluidic platform was configured in the same standard calibration format, shown in figure 2.13. An mPD-coated sensor was placed downstream of the choline biosensor. A 5-step choline calibration was performed in the same concentration range, 0-10 μM , as for the biosensor comparison tests. The resulting current trace and calibration curves of this experiment are shown in figure 3.12.

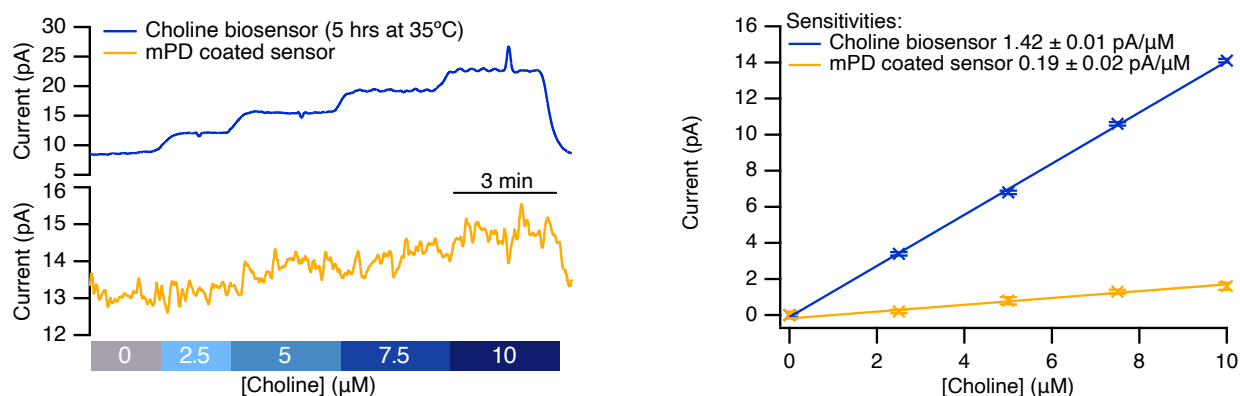


Figure 3.12: An example of a calibration trace and curve obtained to determine if H_2O_2 travels downstream after oxidation of choline at a biosensor. An mPD-coated sensor was placed downstream of a choline biosensor to determine if any H_2O_2 produced by the choline biosensor travelled downstream. Left: The current trace showing a clear 5-point choline calibration recorded by the choline biosensor, and a small change in signal for the mPD-coated electrode. It should be noted that the y-axis ranges are different for the two traces. The traces were recorded at a frequency of 200 Hz and smoothed using a 201-point Savitzky-Golay filter. Right: The corresponding calibration curves, fitted using a linear regression. The sensitivities of each calibration is given, the error is given by the 95% confidence interval. The signal measured at the mPD-coated sensor was 13.4% of the signal measured at the choline biosensor, suggesting that some H_2O_2 produced at the choline biosensor travels downstream. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step for one calibration cycle.

The aim of this experiment was to determine if any H_2O_2 travelled downstream to the mPD-coated sensor as a result of choline oxidation at the choline biosensor upstream. The results show that there is a small amount of H_2O_2 travelling downstream, shown by the calibration at the mPD-coated sensor. This suggests cross-talk was contributing to the signal of the choline biosensors that were in positions 2 or 3 of the chip. However, the relative signal measured at the mPD-coated sensor was 13.4% of the choline biosensor signal, which does not account for the differences seen between the sensitivities of

the biosensors. Therefore it is reasonable to compare the biosensors regardless of which position the biosensor was in.

To perform the multiple biosensor calibrations, two different microfluidic platforms were used. The platforms had the same overall layout, however the configuration of the interconnect was different for the two boards. One board had the choline and PBS mixing in an L shape and the other board had it mixing in a T shape, as shown in figure 3.13.

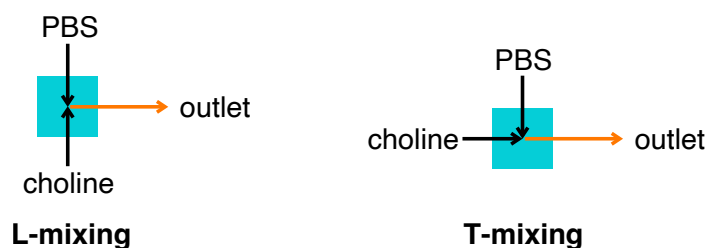


Figure 3.13: The schematics the two mixing orientations: L-mixing or T-mixing. The two mixing orientations were used to calibrate the choline biosensors.

One concern in microfluidics is how well two solutions mix when they meet in laminar flow. If the PBS and choline streams are not fully mixed, the way in which the two input streams meet may determine the degree of mixing. In the case where the final solution is not well mixed, the position of the sensor in the stream will affect the ‘true’ choline concentration that it samples. Therefore, it was important to check if the orientation of the input flows did affect the overall mixing of the solutions. This was tested by performing a 5-point calibration up to 10 μM with the microfluidic platform in the two different orientations. The resulting calibration curves are shown figure 3.14.

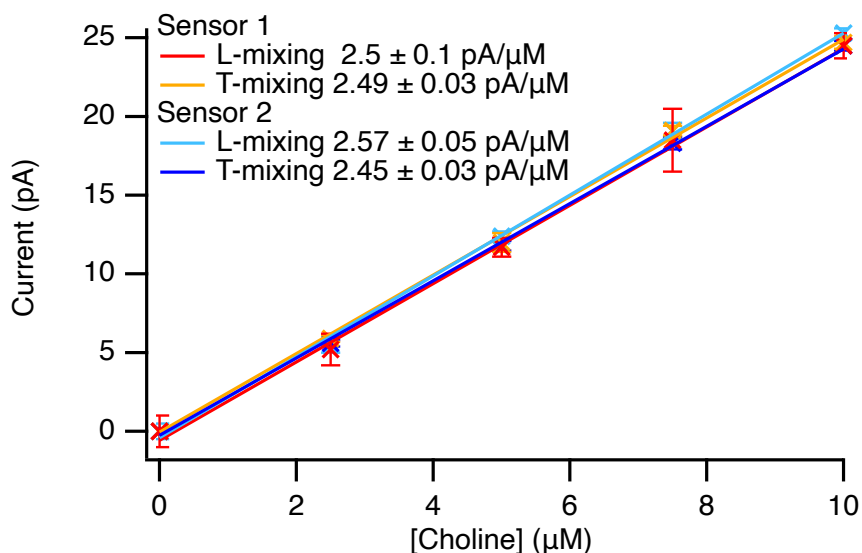


Figure 3.14: Calibration curves obtained when comparing two different mixing configurations on the microfluidic platform. The microfluidic board was configured so that the choline and PBS streams either met in an L-shape or a T-shape, as shown in figure 3.13. A 5-point choline calibration up to 10 μM was performed using two biosensors to compare the two methods of mixing, to determine if there was a difference in the resulting sensitivities. The sensitivity of each sensor and configuration of mixing is given. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step during one calibration. The calibration curves have been fitted using linear regression. The sensitivity is given by the gradient and the error is given by the 95% confidence interval.

For sensor 1, based on the errors of the fitting coefficients of the linear regression, there is no difference in sensitivity of the two mixing configurations. Sensor 2 shows a slight variation, however, the difference is negligible compared to the differences seen in the biosensor sensitivities as shown in the box and whisker plot in figure 3.11. Therefore, the biosensor sensitivities can be compared regardless of which board orientation was used.

The final limitation of this experiment is that only a small sample of biosensors were tested for each protocol, particularly for the group of sensors which were placed in the oven for 5 hours at 40°C, which only had three biosensors in the sample. The sensitivity achieved for all of the protocols was very good, and it is possible that any protocol could have been used, however 5 hours at 35°C showed slightly improved sensitivity and lower variability and was therefore used going forward. The high sensitivity means the biosensors are capable of detecting very low concentration ranges. If necessary, further work could investigate the best biosensor protocol in terms of sensitivity and stability.

3.3.3 Investigating the dynamic range of the biosensor

The dynamic range of the choline biosensor will determine over what concentration range it can measure. To investigate this, manual calibrations were performed, as described in section 2.3.2, to determine the apparent K_m and V_{max} of the choline biosensors by fitting the graph using a Michaelis-Menten curve. Ten choline biosensors fabricated at the same time, which had been cured for 5 hours at 35°C, were placed in a stirred beaker and additions of choline were made, and the resulting current measured. Measurements were made over a choline concentration range of 0 - 4 mM. The results for these choline biosensors are shown in figure 3.15.

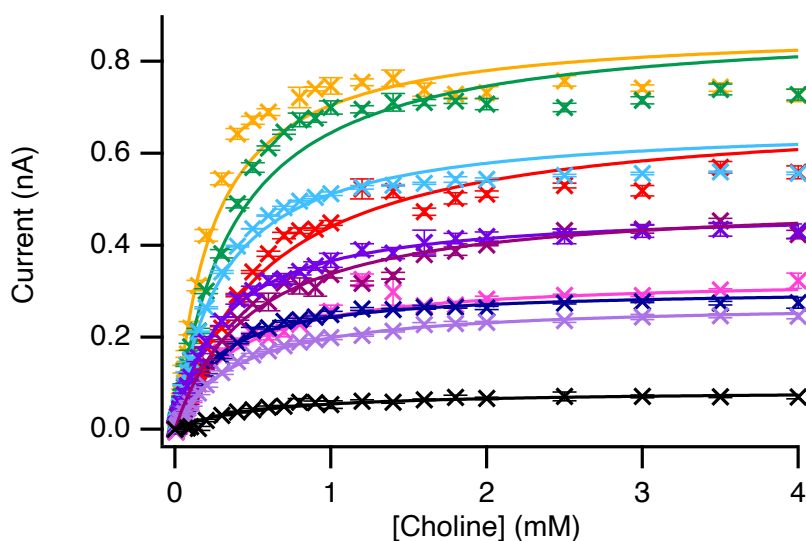


Figure 3.15: Calibration curves for 10 biosensors obtained from manual calibrations. The choline biosensors were placed in stirred beakers containing 0.01 M PBS. The different colours represent the different biosensors. Two choline biosensors were calibrated per beaker. Aliquots of choline were sequentially added to obtain a calibration curve. The mean $\pm$ standard deviation of each concentration step is shown. The curves are fitted using the Michaelis-Menten equation shown in 2.6.

The average apparent V_{max} and K_m of the ten biosensors was 0.5 ± 0.3 nA and 0.4 ± 0.1 mM, respectively. This means the dynamic range of the biosensors is, on average, 0 - 400 μ M. Differences between the biosensors response may result from differences in the electrode surfaces or from the fabrication process which will have some human error associated with it.

3.3.4 Extending the dynamic range of the choline biosensor

For choline levels in the blood, quoted to be approximately 66 μ M in whole blood and 11 μ M in plasma [112], it is not necessary to extend the dynamic range of the choline biosensor, however, there may be other scenarios where detecting higher levels of choline is necessary. For example, in AChE monitoring where the activity of AChE is high, large amounts of choline will be produced following hydrolysis of acetylcholine (ACh). In this case, it may be necessary to have a diffusion limiting layer on the biosensor to extend its dynamic range. This would allow the biosensor to sensitively detect

differences in choline levels at higher concentrations.

Adding a polyurethane (PU) layer to the biosensor is a technique previously developed within the Boutelle group by Dr Sally Gowers to increase the dynamic range of a given biosensor, as described in section 2.3.3 [88]. The PU layer acts to reduce diffusion of the substrate to the biosensor, thus decreasing the recorded current and increasing the recorded value of the apparent K_m for the biosensor.

A PU layer was added to six of the biosensors to investigate the effect this would have on the dynamic range of the sensors. The biosensors with the PU coating were placed in a stirred beaker containing 0.01 M PBS, and a manual calibration was performed. The choline concentration range measured was 0 - 7 mM. Three of the biosensors showed a noticeable increase in their dynamic range, the results of these manual calibrations are shown in figure 3.16.

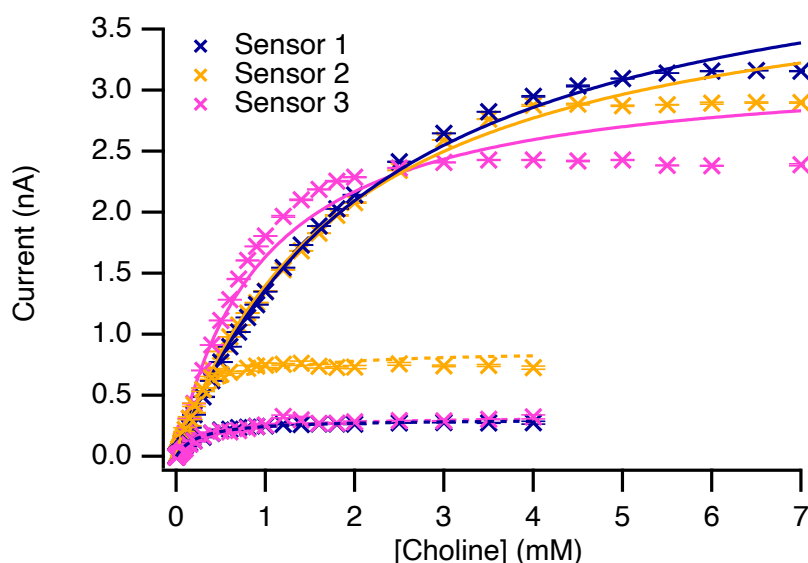


Figure 3.16: Resulting calibration curves obtained for three choline biosensors with and without a PU coating. The biosensors were placed in a stirred beaker containing 0.01 M PBS. Aliquots of choline were sequentially added to obtain these calibration curves. The dotted lines show the calibration curves obtained before the coating the biosensors in PU and the solid lines show the calibration curves after the coating the biosensors in PU. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step. The calibrations are fitted using Michaelis-Menten kinetics.

For these three biosensors, the average apparent V_{max} and K_m increased from 0.5 ± 0.3 nA to 3.9 ± 0.7 nA and 0.28 ± 0.06 mM to 1.7 ± 0.7 mM, respectively. Of the three other biosensors that had a PU coating, one was knocked and the PU layer was potentially damaged so the results are not shown and the other two biosensors showed no change in the apparent K_m . The results before and after the PU coating for the two biosensors that did not show a significant change are presented in figure 3.17.

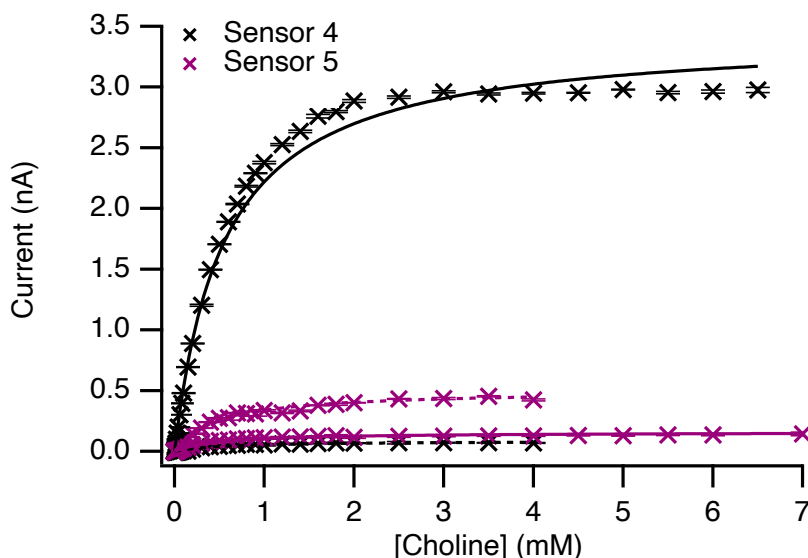


Figure 3.17: Calibration curves obtained for an unsuccessful PU coating of two choline biosensors. The choline biosensors were placed in a stirred beaker containing 0.01 mM PBS. Aliquots of choline was sequentially added to the beaker. The resulting calibration curves are plotted. The dotted line represents the calibrations obtained before the PU-coating attempt and the solid lines show the calibrations obtained after the PU-coating attempt. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step. The calibrations are fitted using Michaelis-Menten kinetics. While the V_{max} has changed for both biosensors, no change in the value of K_m was observed.

Neither sensor showed a change in their apparent K_m value. The V_{max} has significantly increased for sensor 4, which suggests that the PU coating is not covering the electrode, but it could be the case that the tetrahydrofuran (THF) used in the solution has changed the structure of the hydrogel, causing a change in the apparent V_{max} . This is also an observation that was made previously by Dr Gowers [88]. For sensor 5, it is possible that the PU layer is too thick and almost completely blocking the surface, resulting in a low signal and reduced V_{max} .

As described further in Dr Gower's thesis, the PU coating method is known to be inconsistent, partly due to rapid evaporation of the THF used in the protocol, which could explain why not all biosensors were successfully coated in the PU [88]. The V_{max} does consistently increase, a trend also seen with the lactate biosensor in figure 2.10, which is an unusual and unexpected result.

The value of main interest is the K_m as this determines the dynamic range of the biosensors. The results presented in figure 3.16 for the three biosensors showing a noticeable increase in the apparent K_m show that having the PU coating can improve the dynamic range of the biosensor, making it more suitable for particular applications. One such application is to monitor high AChE activities in blood. An example of a PU-coated biosensor being used for this purpose can be found in chapter 8.

3.3.5 Stability of the choline biosensor during continuous use

As for the in-flow ChOx method, biosensors can also drop in sensitivity and therefore it is important to understand the stability of the choline biosensor. This will provide insight into how often the sensor should be calibrated whilst in continuous use and how long it can be used for before it needs to be replaced.

The stability of the biosensor also played a key role when choosing the experimental conditions for fabricating the biosensors. One concern with using a lower temperature to cure the hydrogel was that the cross-linking reaction may not have gone to completion, even after extending the time over which the enzyme is held at a lower temperature, making the hydrogel layer less stable. As it is impractical to hold the sensors at a low, specific temperature for a long period of time, it was important to find a balance between the time in the oven, practicality and the stability of the choline biosensor. This was a key reason why the longer oven time of 5 hours was chosen to use going forward, however, to ensure that 5 hours at 35°C was sufficient for this application, the stability of the biosensor using these fabrication conditions was further investigated.

The microfluidic platform was configured as shown in figure 2.12 for a biosensor calibration. A 5-point choline calibration up to 10 μM was performed continuously for 24 hours, and the resulting sensitivity of the biosensors were investigated. Two choline biosensors were used to perform this test that had been fabricated using the GOx hydrogel protocol (see section 3.3.2) and held at 35°C for 5 hours. The calibration curves were plotted and fitted using a linear regression. The gradient of the linear regression gave the sensitivity and was plotted against elapsed experimental time as shown in figure 3.18.

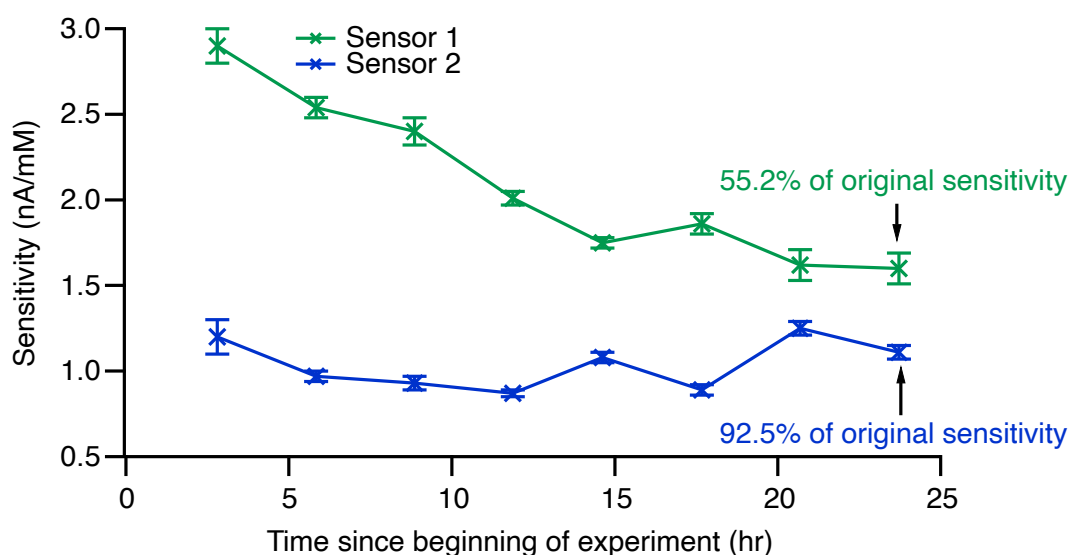


Figure 3.18: The sensitivity decay of the two choline biosensors over time. 5-point choline calibrations up to 10 μM were performed over a long period of time to investigate the degradation of the choline biosensors. These biosensors were fabricated by cross-linking the hydrogel at 35°C for 5 hours. The markers the sensitivity obtained from the linear regression of the calibration curves and the error bars represent the 95% confidence interval of the fit.

The results show that the biosensors are usable over a 24 hour period. After 24 hours, sensor 1 showed a 44.8% drop in sensitivity and sensor 2 showed a 7.5% drop in sensitivity. As the sensitivity of the biosensors did fluctuate, it would be best to run a biosensor calibration every 4 hours to ensure the sensitivity used to convert a recorded current to concentration is accurate. Further work could also be performed to investigate whether it is possible to fit and predict the degradation of biosensor sensitivity and the results could be used to model the sensitivity over time.

3.4 Interference tests

The aim of this thesis is to determine AChE activity in whole blood using the choline sensing system. Therefore, it is important to consider that there are electroactive species present in the blood that may interfere with the analysis system. In order to address this, an mPD-exclusion layer is used on both the biosensors and on the sensors used for the in-flow ChOx method to exclude large and charged molecules from reaching the electrode surface.

The response of two biosensors to interferents was investigated to confirm their selectivity. Common interferents include ascorbic acid, uric acid and acetaminophen. For these tests, ascorbic acid, which is known to be the most problematic interferent, was used. Ascorbic acid can be found in plasma and erythrocytes in low concentrations of 40 - 80 μM and 40 - 60 μM , respectively [114], and in the brain in higher levels, 100 - 500 μM [115]. While the main application is not brain monitoring, the choline biosensor was used *in vivo* in brain microdialysis, described further in chapter 4, therefore it was of interest to ensure no interference could be seen from ascorbic acid in this higher range.

Two choline biosensors, one with and one without a PU-coating, were used to perform these interference tests. The reason for this was to see if there was any additional protection provided with the PU-coated sensor. The calibration board was set up in the standard calibration format, shown in figure 2.13, a 5-point choline calibration up to 10 μM was performed, followed by a 5-point ascorbic acid calibration up to 500 μM . This low concentration of choline was used as studies have found the choline concentration in cerebral spinal fluid (CSF) is $2.72 \pm 0.44 \mu\text{M}$ ($n = 145$) [116]. The resulting calibration curves for the two choline biosensors are shown in figure 3.19.

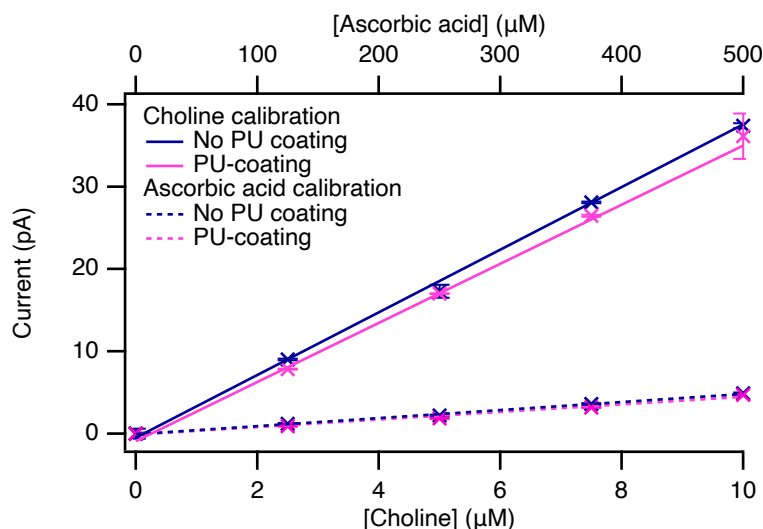


Figure 3.19: Calibration curves obtained for two choline biosensors, one with and one without PU, to determine the interference from ascorbic acid. A 5-point choline calibration up to 10 μM and ascorbic acid calibration up to 500 μM were performed to the interference from ascorbic acid, a known electroactive molecule that is in the blood and in the brain. The calibration curves show that the response to 500 μM ascorbic acid is much less compared to the response to 10 μM choline for both sensors, demonstrating that the the biosensor fabricated with the mPD and hydrogel layer is successful. The markers and error bars represent the mean $\pm$ standard deviation of each current step during one calibration. The sensitivities of the different responses were found by linear regression. The sensitivities to choline for the biosensor with the PU coating and without the PU coating were 3.59 ± 0.02 nA/mM and 3.81 ± 0.03 nA/mM, respectively. The sensitivities to ascorbic acid for the biosensor with the PU coating and the biosensor without the PU coating were 0.0092 ± 0.0001 nA/mM and 0.0098 ± 0.0003 nA/mM, respectively.

The calibration curves demonstrate that for both biosensors, with or without the PU coating, the response to 10 μM choline is much higher than the response to 500 μM ascorbic acid. The sensitivity to ascorbic acid was just 0.26% of the sensitivity to choline for both the PU-coated biosensor and the biosensor with no PU coating. No difference was observed in effectiveness at blocking out interferents depending on the PU-coating, suggesting that this layer does not enhance or impair the effectiveness of the mPD exclusion layer. This is a very small sample size, but initial results are promising.

These results successfully show the effectiveness of the biosensor with the mPD exclusion layer at blocking out electroactive interferents. This demonstrates the potential for the biosensor to be used for *in vivo* monitoring to selectively detect low choline concentrations. Further experiments could be performed to investigate the effectiveness of the mPD layer alone, for example by comparing the response of a biosensor with and without an mPD layer.

3.5 Conclusion

In this chapter I have demonstrated that choline can be sensitively detected using two different enzymatic methods; by introducing ChOx in-flow or by immobilising ChOx on the electrode surface. Different experimental conditions were trialled to determine the best protocol to use in either case moving forward.

The in-flow ChOx system showed excellent sensitivity of 24.68 ± 0.09 nA/mM when using a flow rate of 2.5 μ l/min, a bulk ChOx solution of 1 mg/ml in the reservoir, corresponding to 0.2 mg/ml at the microfluidic chip, and a 30 cm piece of reaction tubing. However, when reducing the reaction tubing length to 5 cm, the flow rate to 2 μ l/min, and the ChOx concentration to 0.1 mg/ml at the microfluidic chip, the sensitivity is reduced to 5 nA/mM. This is affected further when the ChOx concentration is reduced. Therefore, the sensitivity of the in-flow ChOx system largely depends on the experimental parameters chosen, and they need to be controlled in order to get repeatable results.

The protocol used to fabricate the choline biosensors was based on that used by the Boutelle group for glucose and lactate biosensors. As ChOx loses a large proportion of activity at high temperatures, the choline biosensors were placed in the oven at lower temperatures for longer durations to cure the hydrogel compared with the protocol for glucose and lactate biosensors. Different experimental conditions were tried and high sensitivities were obtained for all protocols. The final temperature and duration chosen was 35°C and 5 hours in the oven. The mean sensitivity obtained using this protocol was 4.5 ± 1.6 nA/mM. The sensitivity achieved using this protocol was on average slightly higher than for the other protocols and it also gave the least amount of variability between sensors. The longer oven time allows longer for the cross-linking reaction to take place while still being a practical as the biosensors are able to be fabricated from a polished electrode within a day if required.

Stability experiments showed promising results for both methods. The in-flow ChOx method showed good stability for up to 30 hours depending on the experimental conditions used. The system is initially very stable before dropping at a fast rate. The choline biosensors were usable for at least 24 hours, however, it would be recommended to do fairly frequent calibrations, approximately every 4 hours, to ensure that any fluctuations in sensitivity can be accounted for.

Adding a PU coating to the choline biosensor was also investigated to extend the linear range of the biosensor. On average, with no PU coating, the apparent K_m of the biosensor was found to be 0.4 ± 0.1 mM. The apparent K_m of the biosensor increased to 1.7 ± 0.7 mM with the PU coating. This gives two options for the choline biosensor going forward, to detect lower levels of choline it is better to use a biosensor without the PU coating, whereas to detect higher levels, as needed in chapter 8, it is necessary to have the PU coating so that the biosensor is in the correct dynamic range.

Interference tests were performed using two choline biosensors, one with and one without a PU coating. This was to ensure that there was minimal interference from electroactive molecules. To test

for potential interferents, ascorbic acid was used as it is present in blood and high levels are present in the brain. Both choline biosensors, regardless of the PU coating, had a sensitivity to ascorbic acid that was 0.26% of the sensitivity to choline.

To summarise, the choline biosensor offers a simple and sensitive choline detection mechanism. It is advantageous over the in-flow ChOx method as it does not require the addition of the enzyme into the sample stream. Furthermore, it is a simpler detection method and as the choline is oxidised as it comes into contact with the biosensor, it does not require such precise design of the microfluidics. The choline biosensor has excellent sensitivity. Therefore, for the *in vivo* measurements and monitoring in real samples, the choline biosensor was chosen as the monitoring method.

Chapter 4

Online *in vivo* monitoring

4.1 Introduction

As discussed in chapter 1, microdialysis (MD) is a very useful technique to sample complex media. Within the Boutelle group, MD has been utilised to sample metabolites and ions in the brain and in interstitial fluid [59, 69]. As part of this research, I was a member of a team who collaborated with a group in Copenhagen to monitor glucose and lactate in a porcine brain during cardiac interventions [117]. Doing this work allowed me to use the wireless monitoring system and MD, providing insight into how it can be utilised for this thesis. Following on from this work, I was a part of a team who extended our existing *in vivo* analysis system to improve its robustness and the monitoring capabilities by sampling a number of analytes. In another collaboration with researchers in Innsbruck, we were able to use this improved analysis system to continuously monitor choline for the first time *in vivo* along with glucose, lactate and glutamate in the porcine brain during cardiac interventions. The project provided an opportunity to test the capability of the choline biosensor for monitoring *in vivo*.

A simplified model of the setup used in both projects is shown in figure 4.1. The MD probe is perfused with artificial cerebrospinal fluid (aCSF). The outlet of the probe is connected to our analysis system consisting of the biosensors, microfluidic chip, and the wireless electronics as introduced in sections 2.3, 2.4 and 2.5, respectively.

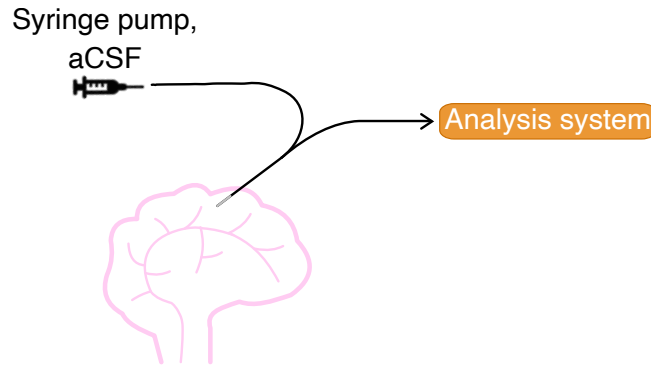


Figure 4.1: A simplified model of the experimental setup used during monitoring in a porcine brain. An MD probe is inserted into the brain of a sedated pig. The probe is perfused with aCSF using a CMA 107 pump (MDialysis, Sweden). The outlet of the probe is connected to our analysis system, consisting of the microfluidic chip, biosensors and the wireless electronics.

4.1.1 Motivation of this research

Survival rates of victims that suffer from cardiac arrest following resuscitation attempts are just 10% [118]. Neurological damage may occur due to compromised blood flow to the brain during cardiac arrest and resuscitation events, or from reperfusion injury following return of spontaneous circulation (ROSC) [119–121]. Studies have found that cardiopulmonary resuscitation (CPR) provides just 25% of normal cerebral blood flow which is below the 40-50% needed to maintain cellular integrity [119, 120]. The main motivation of both research cases discussed in this chapter is to monitor key analytes in the brain to gain an understanding of brain health during cardiac arrest and resuscitation attempts.

4.2 First attempt at monitoring glucose and lactate in a porcine model

This experimental animal work was performed in collaboration with medical professionals and researchers from Righospitalet, Copenhagen. All procedures were approved by the Danish Animal Experiments Inspectorate (Protocol No.: 2017-15-0201-01371) and by the Department of Experimental Medicine, University of Copenhagen.

Using the microfluidic and wireless systems, described in sections 2.4 and 2.5, respectively, coupled with MD allowed the real-time monitoring of glucose and lactate levels in the pig brain. Glucose and lactate biosensors were fabricated as described in section 2.3 in the Boutelle lab in London, including an mPD coating to prevent interference from electrochemically active species and a polyurethane (PU) coating to extend the dynamic range of the biosensors. My role in this research was to fabricate glucose and lactate biosensors and to travel to Copenhagen, where I helped set up our analytical monitoring system and to perform the measurements.

The microfluidic platform, lab laptop and wireless monitoring system, including the wireless electronics, tablet and biosensors were flown to Copenhagen. The biosensors were calibrated prior to each experiment, using the autocalibration system described in section 2.4.3. The MD probe used for these experiments was a CMA 70 bolt catheter (MDialysis), with a MWCO of 20 kDa and a membrane length of 10 mm. For each experiment, the MD probe was inserted by vets through a burr hole into the cortex via an intracranial bolt, and left to allow tissue stabilisation. It has previously been reported that it takes 12 - 15 minutes for the tissue to stabilise following probe insertion [122]. The MD probe was perfused with aCSF and the flow rate of the MD pump was set to 1 $\mu\text{l}/\text{min}$, chosen as a compromise between good recovery of analytes and still providing good temporal resolution. After the stabilisation period of 15 minutes, the biosensors were connected to the probe outlet via a 3 cm length of fluorinated ethylene propylene (FEP) tubing and the baseline dialysate was recorded for at least 20 minutes. Following this, a cardiac arrest was induced in the pig using a defibrillator to pace the heart between 200 and 400 bpm, and there was no intervention for 3 minutes. Resuscitation attempts were then made in line with the council advanced life support guidelines, which included use of a Lund University Cardiopulmonary Assist System (LUCAS) to supply mechanical chest compressions for 2 minutes, defibrillation every 2 minutes if there was a shockable rhythm, and administration of adrenaline every 3 - 5 minutes until ROSC occurred and when necessary thereafter. Glucose and lactate were monitored during this period to investigate whether there were signs of ischemia in the brain due to poor blood flow.

An example of the results recorded for one pig are shown in figure 4.2.

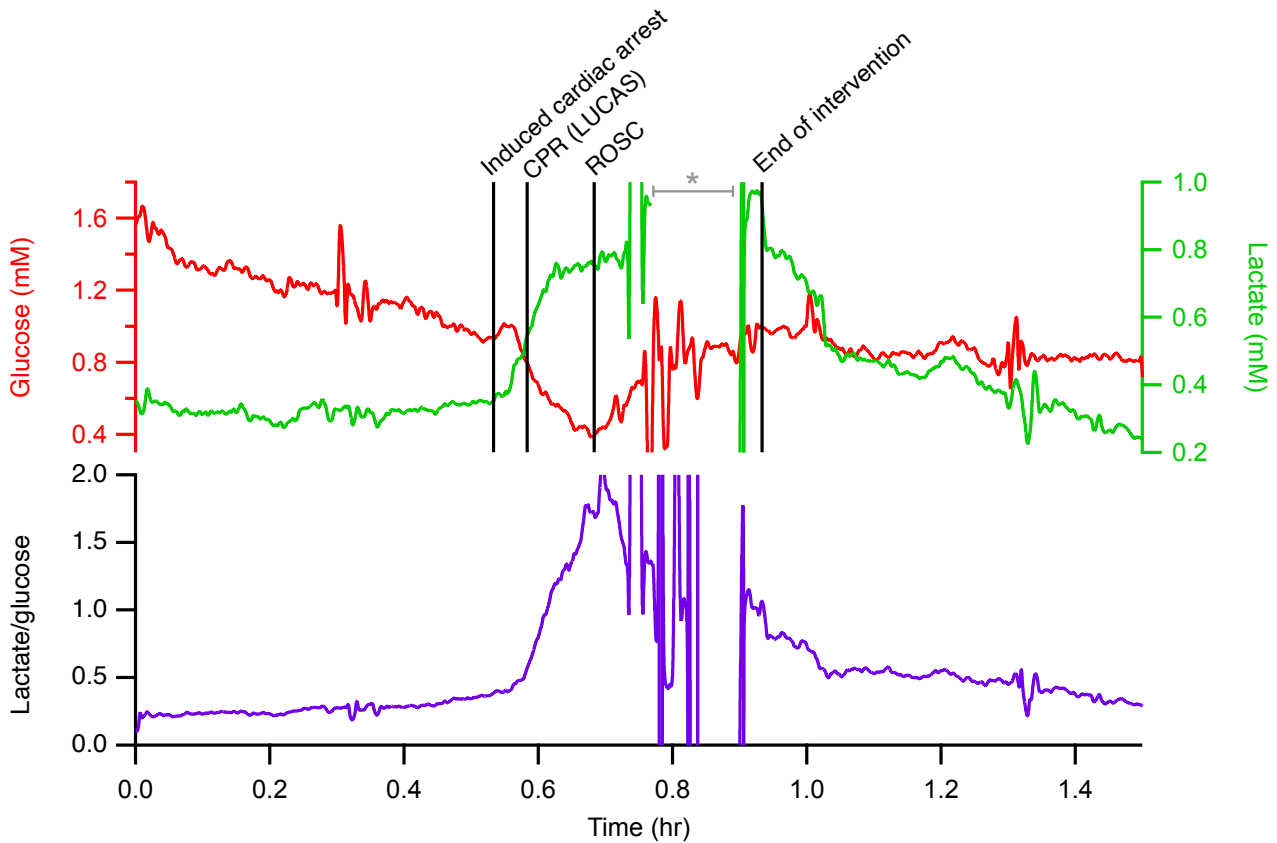


Figure 4.2: An example of glucose and lactate levels recorded in the dialysate of a pig brain during different cardiac interventions. Top: The recorded trace shows a decrease in glucose following cardiac arrest and a simultaneous increase in lactate levels. This is typical of ischemia suggesting poor blood flow to the brain. Following ROSC, glucose levels increase and lactate levels begin to decrease, suggesting a return of blood flow to the brain. Bottom: The lactate/glucose ratio is shown. Taking the ratio is useful to omit any flow artefacts. The ratio shows very clearly a sharp increase following cardiac arrest, followed by a slow decrease which coincides with ROSC. Note the asterisk shows a time period where the lactate biosensor goes out of the range which also impacts the lactate/glucose ratio. The data was collected at 10 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

Following cardiac arrest, the glucose levels decrease and the lactate levels increase, which is typical of ischemia. This suggests poor perfusion of the brain even during resuscitation. Following ROSC, the glucose levels begin to increase and lactate levels start to decrease, demonstrating good recirculation of the blood, with both analytes returning to baseline levels. By taking the ratio of lactate to glucose, any flow artefacts or changes in probe recovery are removed from the trace. The ratio shows there is a sharp increase in the lactate to glucose ratio following cardiac arrest, followed by a slow decrease once ROSC occurs. This is consistent with the compromised blood flow during cardiac arrest and demonstrates that CPR is insufficient in maintaining sufficient perfusion pressure in the brain. Once ROSC has occurred, the blood flow begins to return to normal, demonstrated by the return of analyte concentrations to baseline.

During this research, a measurement in peripheral tissue was also performed, by placing an MD probe in the pig leg. The purpose of this was to see if ischemia also occurred in peripheral tissue, which is less metabolically active. The MD probe used was, again, a CMA 70 bolt catheter and perfused with aCSF at a flow rate of 1 $\mu\text{l}/\text{min}$. The results of this are shown in figure 4.3. As before, a cardiac arrest was induced and resuscitation was attempted by performing CPR.

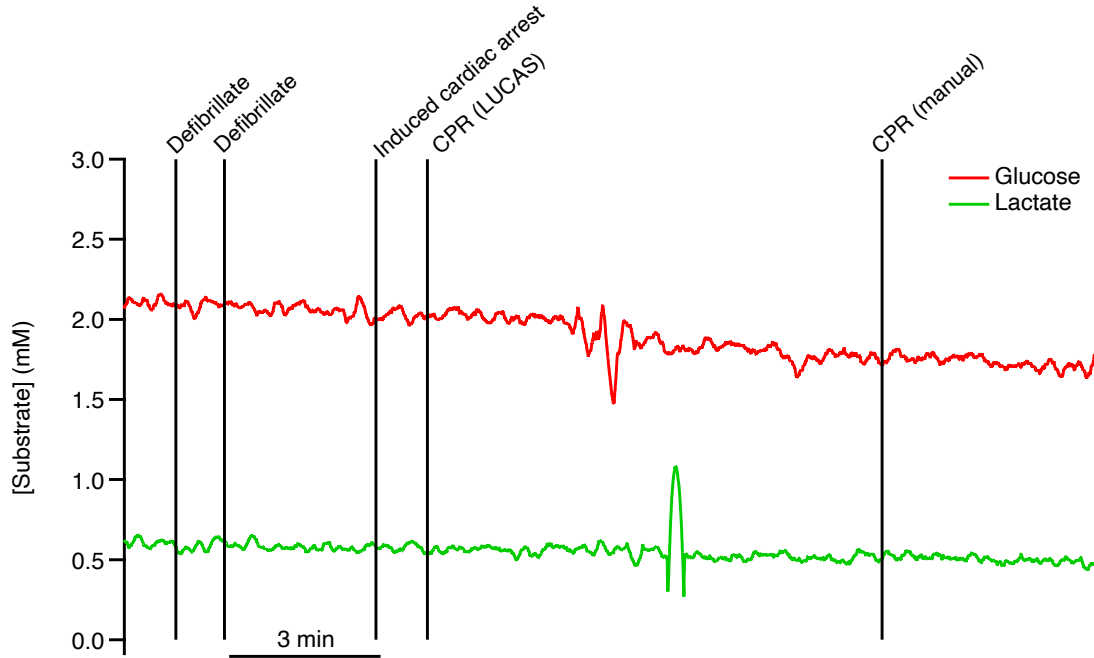


Figure 4.3: Dialysate glucose and lactate levels in the leg during cardiac interventions and resuscitation attempts. The trace demonstrates that the glucose and lactate levels in the leg dialysate remain relatively stable during different cardiac interventions and resuscitation attempts. The trace has been sampled at 10 Hz and smoothed using a 201-point Savitzky-Golay filter.

The results show relatively stable levels of both glucose and lactate, especially when compared to the dynamic changes seen in the brain dialysate glucose and lactate levels. The peripheral tissue is less metabolically active and therefore seems to be less sensitive to changes in perfusion compared with the more metabolically active brain.

The benefit of using the wireless monitoring system for this clinical monitoring was that it could be placed near the monitored area, meaning a shorter outlet connection tubing was needed than often required in a clinical setting [54], improving temporal resolution. Our monitoring system was compact and discrete, meaning it was ideal for a busy and crowded clinical environment as it did not get in the way or interfere with the rest of the experiment. For future experiments, adaptations to the monitoring equipment were needed to allow the monitoring of more metabolites and to create a more robust system, these modifications are described in the next section.

4.3 Modifications made due to limitations of the equipment

Whilst the system used previously in Copenhagen had worked well for the online monitoring of metabolites, there were a couple of problems with the housing for the wireless electronics. To connect the electrical wires of the biosensors to the potentiostat, the wires had to be positioned awkwardly to meet the electrical connections. This led to issues with the electrical wires not being long enough which caused wires to break or meant extension cables were required. The wireless monitoring app was also prone to crashing when the gains were changed on the potentiostats, or when the box was handled vigorously; in both cases this caused the potentiostat to move and momentarily lose power meaning the connection between the wireless board and app was lost.

To overcome these issues, we made improvements to the system before carrying out the *in vivo* monitoring experiments in Innsbruck. Additionally, for the experiments performed in Innsbruck, it was of interest to monitor more metabolites, such as glutamate and choline, therefore a potentiostat was required that allowed connection to four sensors. This potentiostat was designed by Dr. Gowers based on the previous 2-electrode PCB discussed in section 2.5. I modified the wireless monitoring app and the arduino code for the microcontroller to allow for data collection from four channels. Although preliminary tests were promising, further optimisation of the PCB was required to handle the extra channels, but this is beyond the scope of this thesis and is currently being improved. In addition, it was also challenging to test the issues with the PCB as the wireless monitoring app also needed further optimisation to handle the increase in data collection. Due to these problems with the 4-electrode PCB and given the short time-frame before the experiments, it was easier to use two 2-electrode wireless potentiostats and two tablets that had the original wireless monitoring app installed. Therefore, the wireless housing needed to be redesigned to allow two potentiostats to fit into one box. This meant the potentiostats needed to be separated with a divider so that they would not come into contact with each other. Based on the issues listed previously, further requirements for the potentiostat housing were to secure the potentiostats in place so that they did not become displaced during movement of the system or when changing the gains to avoid losing bluetooth connection, and to have improved connections between the PCB and the electrical wires of the sensors.

A larger box was designed using SolidWorks (Dassault Systèmes). A removable divider was included to separate the two potentiostats. Additionally, extruded plastic walls were added to the design to secure the potentiostats firmly in place. Finally, the lid was changed so that it was cut into 2 parts. One end of the lid was designed to have the electrical connections, which allowed the sensor wires to go over the top of the lid. This meant that the wires were not held in awkward positions and also meant that it was easier to connect the wires initially. The second part of the lid was built to cover the potentiostats. There were two ‘windows’ inserted into this part of the lid so that gain and power switches could be accessed as before, without having to remove the lid. The lid was made in two

parts so that it was possible to access inside the main box without having to disconnect the sensors. Drawings of this modified electronic housing are shown in figure 4.4.

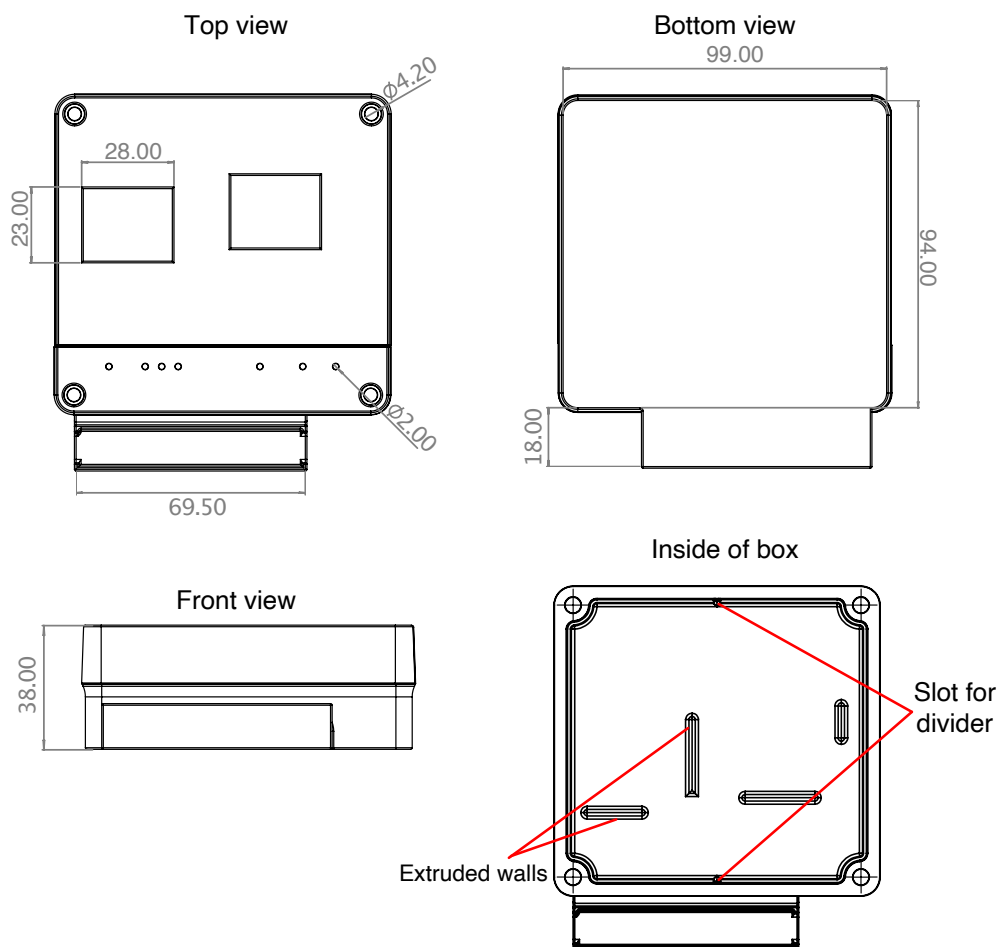


Figure 4.4: Drawings of different orientations the electronic housing designed for Innsbruck. The dimensions are given in mm. The top view shows the view of the box with the lid attached, with two ‘windows’ cut out to access the potentiostats and change the gain, additionally there are small holes to allow electrical connections to be made between the electrical wires of the biosensors and the UFL leads. For this monitoring, there were two different PCBs used. The PCBs had the same electrical design, but the footprints of them varied slightly making their shape and, therefore, fit in the box slightly different. This is why the position of the ‘windows’ also differ slightly. The bottom view shows the general shape of the box and useful dimensions. The front view shows the height of the box, which was made taller to allow the UFL leads to bend upwards without breaking within the box. The view of the inside of the box shows the extruded walls to hold the potentiostats in place and the slots for the plastic divider (not shown) to separate the two potentiostats.

The box was 3D printed using an original Prusa i3 printer (Prusa Research, Czech Republic) with PLA material. Photos of the box with the potentiostats enclosed and the biosensors connected are shown in figure 4.5.

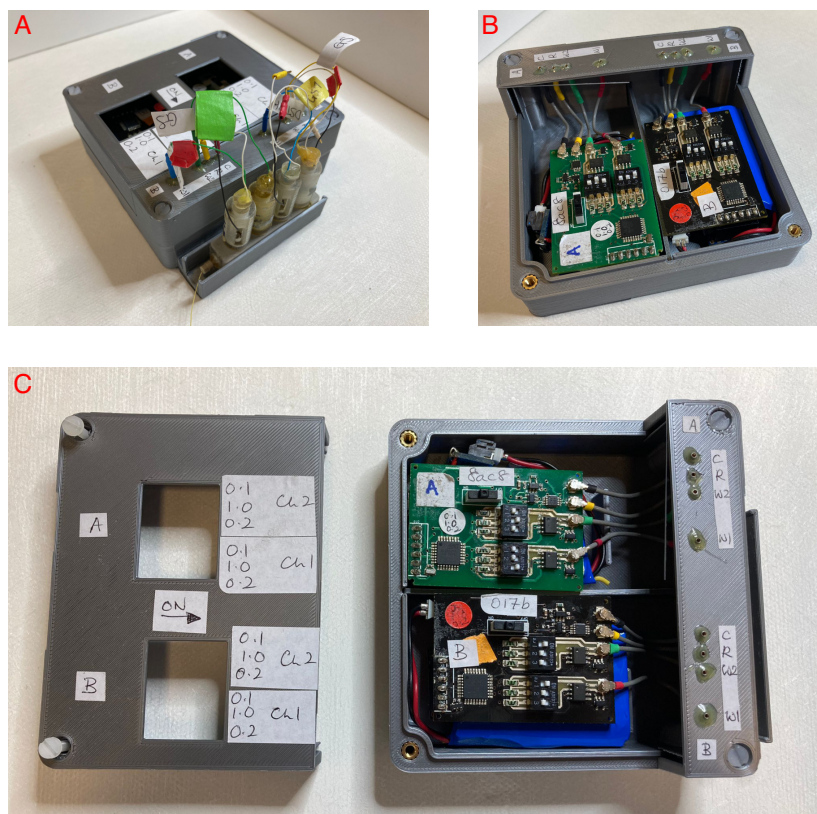


Figure 4.5: Photos of the 3D printed box used to house the wireless electronics during *in vivo* monitoring. **A.** A 4-electrode chip has been inserted into the chip holder and the biosensors are connected to the potentiostat through adapters in the lid as shown. **B.** The inside of the box is shown, demonstrating the UFL connection and why the additional height is needed. **C.** A clear image showing the potentiostats side by side, separated by the divider. Also shown is the brass nuts in the corners of the box, where M3 screws are inserted through the lid to hold the box together.

The chip holder was made large enough to hold another type of chip, not shown, to allow addition of cofactors to detect pyruvate. This is beyond the scope of this thesis. The chip shown here was fabricated to hold four sensors. The chip holder width was designed to hold the chip firmly so it did not fall out, but not so tight that the chip was hard to move. To hold the box together, M3 knurled brass nuts were inserted into the corners of the box, and M3 plastic screws were used.

This new box was successfully used in future experiments, as described in section 4.4, and the adaptations made the system more robust, and there were fewer issues encountered regarding the bluetooth and electrical connections.

4.4 Monitoring multiple metabolites in a porcine model

The experiments performed in Innsbruck were a part of a collaboration with medical professionals and researchers at the Medical University Innsbruck. This experimental animal study was approved by the Institutional Animal Care and Use Committee of the Medical University Innsbruck and the Austrian Ministry of Science, Research and Economy (Protocol No.: BMFWF-2021-0.895.386). The study was conducted at the Experimental Research Unit of the Department of Anaesthesiology and Intensive Care Medicine of the Medical University Innsbruck. Experiments were done in compliance with EU regulations for animal experimentation (Directive 2010/63/EU of the European Parliament and the European Council).

As with the previous collaboration, the aim of this research was also to investigate brain health during cardiac arrest and resuscitation. The protocol was very similar to that used in Copenhagen. However, in this case longer monitoring was possible as, if ROSC occurred, the pig was monitored for 2 hours. The medical research is beyond the scope of this thesis, however, it gave our group the opportunity to measure more analytes that are of interest in brain health including glutamate, which had not been monitored in this context before. We also took the opportunity to monitor choline; it was not known what to expect for choline levels during ischemic events, but it was of interest to test the biosensor *in vivo* to see how it performed. Ions were also monitored, however, this will not be discussed as a different type of technology is used for ion detection and it is beyond the scope of this thesis.

MAB 7.80.10 animal MD probes (Microbiotech, Sweden) were used in these experiments. The MD probes had a MWCO of 15 kDa and a 10 mm membrane length. These probes were chosen as they are cheaper than the CMA 70 brain probes. The probes were inserted by a neurosurgeon and perfused at a flow rate of 1 μ l/min with cerebral MD perfusion fluid (MDialysis, Sweden) using a CMA 107 MD pump (MDialysis, Sweden). The flow rate was chosen again to give good recovery of analytes while maintaining good temporal resolution. The MD probe was inserted for at least an hour before any measurements were made, meaning tissue stabilisation would have occurred. Prior to connection to the MD probe outlet, the biosensors were calibrated using a larger version of the autocalibration microfluidic platform previously described in section 2.4.3. This larger version had additional pumps and reservoirs to allow more analytes to be calibrated, including four metabolites and two ions.

A 4-electrode microfluidic chip was used to accommodate the four biosensors for metabolite monitoring: glutamate (glut), choline (ch), glucose (glu) and lactate (lac), positioned as shown in figure 4.6.



Figure 4.6: The position of the different biosensors in the microfluidic chip. The glutamate (glut) biosensor is first in the sample stream, followed by the choline (ch), glucose (glu) and lactate (lac) biosensors.

This chip was connected to the outlet of the MD probe after the sensors had been calibrated, via LabSmith interconnect products and 360 μm tubing. It was expected that the glucose and lactate levels would be in the mM range [117], which is higher than the levels in the μM range expected for glutamate and choline typically seen in human cerebrospinal fluid (CSF) [116, 123, 124]. This difference in expected concentrations means the glucose and lactate biosensors will likely generate high levels of H_2O_2 relative to the glutamate and choline biosensors. This can cause cross-talk issues if the glutamate and choline biosensors are placed downstream of the glucose and lactate biosensors, as any H_2O_2 not oxidised at the glucose or lactate biosensor will travel downstream to the glutamate and choline biosensors and be oxidised there, contributing to the signal seen at these sensors. This is why the glutamate and choline biosensors are positioned upstream of the glucose and lactate biosensors. Cross-talk is not normally an issue when monitoring glucose and lactate alone as the analytes are expected to be in similar concentration ranges.

The protocol here was to induce a cardiac arrest by applying a 60 V, 50 Hz alternating current via two subcutaneous needle electrodes, followed by a 5-minute period of no intervention. After the 5-minute period, chest compressions using a LUCAS device were initiated. After 1 minute of LUCAS chest compressions, adrenaline was administered. After a further minute, it was paused and a defibrillator was used to shock the heart. After a further minute, LUCAS was paused to check for ROSC. If there was ROSC, the interventions were stopped and the pig was monitored for 2 hours, after which a high concentration of potassium chloride (KCl) (100 mg per 100 g body weight) was administered intravenously to euthanise the pig. If no ROSC occurred, the 3 minute cycle was repeated up to four more times (a maximum of 15 minutes) depending on whether ROSC was achieved. Following the death of the pig either from no ROSC or the KCl administration, there was a no touch period of 20-30 minutes, meaning we were also able to see the change in analytes as a result of the death. The time delay due to the length of outlet tubing was approximately 16 minutes. The time delay here was slightly longer than can normally be achieved using the wireless monitoring system. As ions were being monitored too, the combined monitoring system was bigger which meant the system had to be placed further away from the pig head.

An example of glucose, lactate and glutamate brain dialysate concentrations during cardiac arrest, resuscitation attempts and ROSC is shown in figure 4.7.

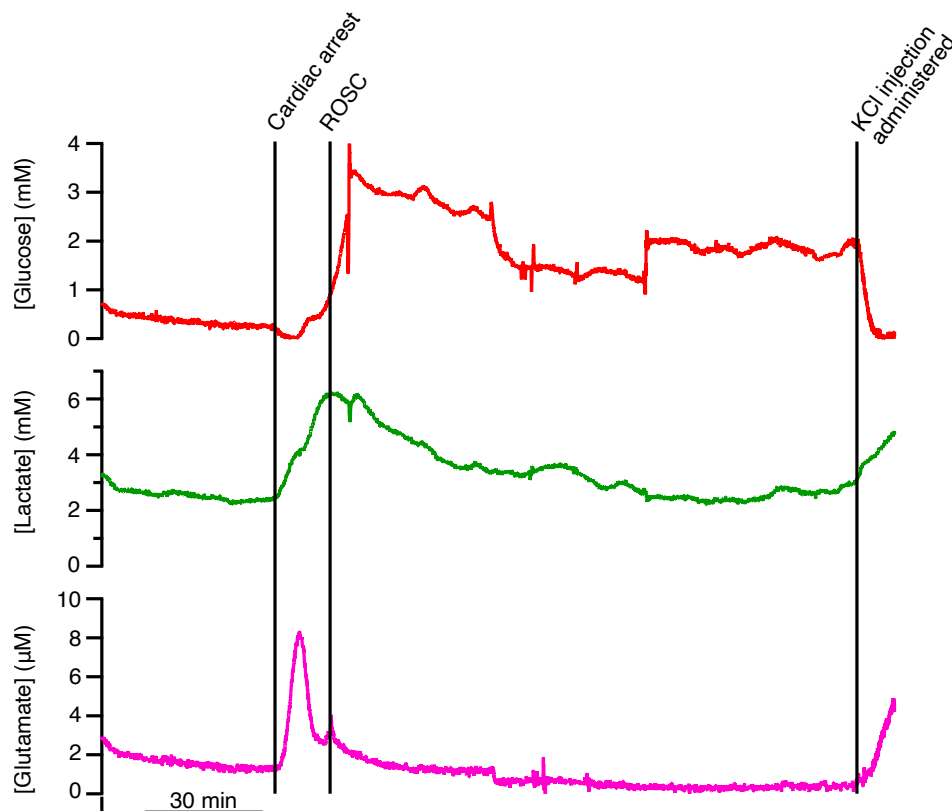


Figure 4.7: Glucose, lactate and glutamate brain dialysate concentrations during cardiac arrest, resuscitation, ROSC and following a KCl infusion. The results show a small decrease in glucose levels following cardiac arrest, along with an increase in lactate levels. Glucose levels recover to above the baseline value following ROSC, and lactate levels decrease to baseline values. These observations are in agreement with previous the previous results presented in figure 4.2. A spike in glutamate levels is recorded following cardiac arrest. The data has been shifted to account for the time delay as a result of the length of tubing between the MD probe membrane and the analysis system. The trace has been sampled at 10 Hz and smoothed using a 201-point Savitzky-Golay filter.

Following cardiac arrest the dialysate glucose levels decrease by approximately 0.5 mM and the lactate levels increase by approximately 3.5 mM. This trend is in agreement with the results obtained in Copenhagen. There is also a peak in glutamate concentration following cardiac arrest. This pig had ROSC soon after resuscitation attempts started (approximately 9 minutes). Following ROSC, the glucose concentration increased to above the baseline level, and the lactate concentration slowly decreased back down to the baseline level. The glutamate concentration also returned to baseline levels. After 2 hours of monitoring post-ROSC, a KCl injection was administered to euthanise the pig. Following this, glucose levels dropped to zero, whereas glutamate and lactate levels increased. The trace ends at the point that the analysis system was removed from the probe. Choline was not monitored here.

These results were interesting and exciting in that glutamate, an analyte previously monitored in traumatic brain injury [55], was monitored during cardiac interventions.

During another experiment, choline was monitored along with glutamate, glucose and lactate. However, following the experiment, it was clear that the choline signal was following a very similar

trend to the glutamate signal, suggesting there may have been cross-talk in the choline signal from the glutamate biosensor. In the standard calibration, choline and glutamate standards were placed in the same pump, therefore, as these levels changed together, the cross-talk could not be seen. Hence, to check for any cross-talk, additional calibrations were needed and these were performed after the experiment. Specifically, 3-point calibration was performed with the glutamate and choline solution combined (up to 20 μM glutamate, 10 μM choline), followed by a 3-point calibration with just glutamate (up to 20 μM), to determine if there was any cross-talk from the glutamate biosensor at the choline biosensor. The choline biosensor response to these two calibrations is shown in figure 4.8.

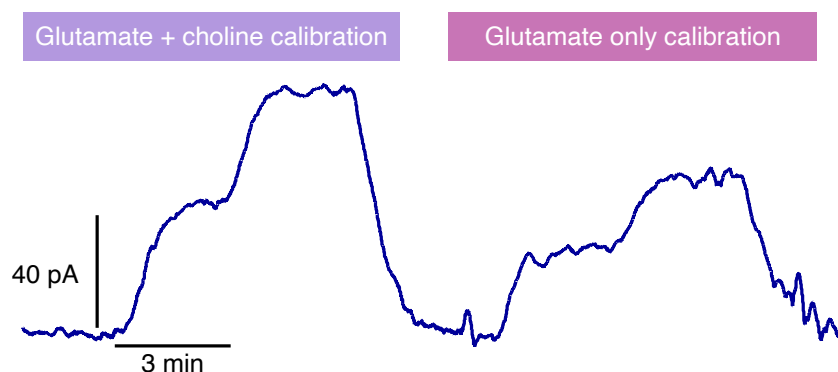


Figure 4.8: The choline biosensor response to a combined glutamate and choline calibration, and a glutamate only calibration. The current trace for the choline biosensor, placed downstream of a glutamate biosensor. The first calibration shown is the current response to a glutamate and choline calibration, where the glutamate and choline standards have been placed in the same reservoir. The second calibration shown is the choline biosensor response to a glutamate only calibration, suggesting there is cross-talk from H_2O_2 travelling downstream from the glutamate biosensor to the choline biosensor. The current was recorded at 10 Hz. This trace has been smoothed using a 201-point Savitzky-Golay filter.

The current trace in figure 4.8 shows that there was cross-talk from the glutamate biosensor, as there is a clear response from the choline biosensor when there is only glutamate in the calibration solution. This was large relative to the signal obtained for the glutamate and choline combined calibration and therefore needs to be taken into account. Performing the calibrations separately in this way made it possible to subtract the portion of the signal due to the glutamate cross-talk from the choline signal, leaving the signal due to choline concentration only. This was achieved by calculating the glutamate concentration from the glutamate biosensor calibration and signal. The glutamate concentration was then converted to current using the calibration obtained for the choline biosensor response to the glutamate only calibration. This current, which was the current resulting from cross-talk, was subtracted from the total choline current.

The results for the four analytes monitored in the pig brain dialysate are shown in figure 4.9.

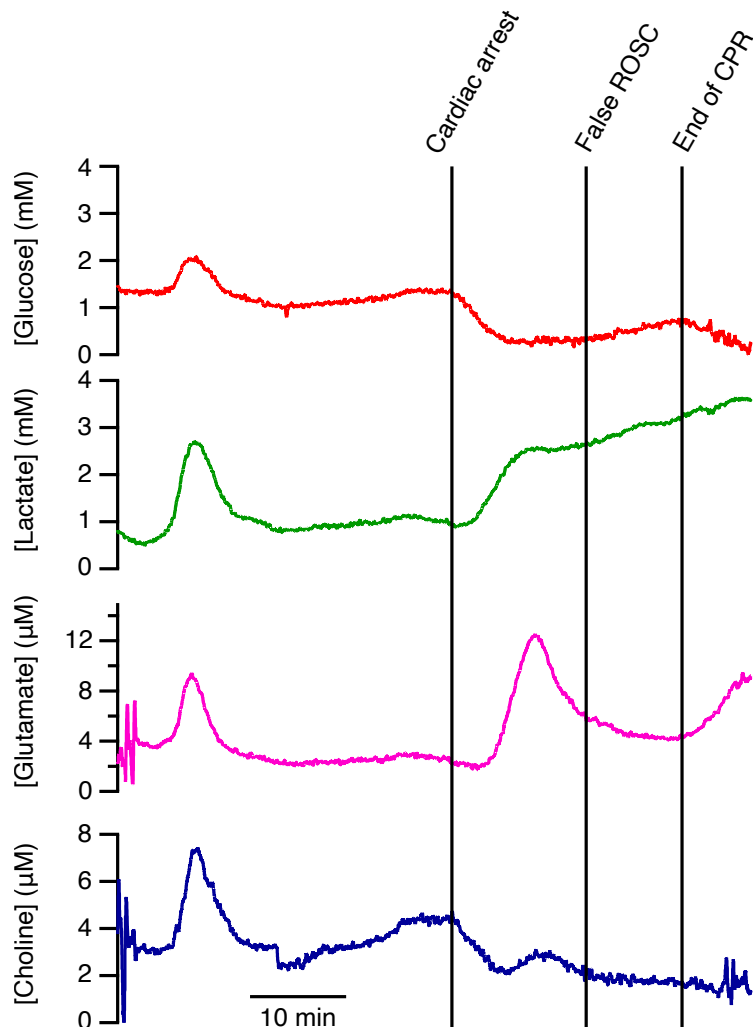


Figure 4.9: The glucose, lactate, glutamate and choline brain dialysate concentrations recorded during cardiac arrest and after the pig death. The traces were sampled at 10 Hz and have been smoothed using a 201-point Savitzky-Golay filter.

The choline level shown is with the cross-talk from the glutamate signal removed. After attaching the analysis system to the MD probe outlet, which corresponds to the beginning of the trace, there is a large peak in all four analyte signals. There were flow issues at the beginning of the experiment so it is likely that this peak is a flow artefact. The flow issues were resolved following this peak. For this pig, there was no ROSC but there was a brief moment where it was thought ROSC had occurred, labelled ‘false ROSC’. The experiment shows that following cardiac arrest, the glucose levels decrease and lactate levels increase, in agreement with the experiments carried out in Copenhagen. Following this, the glucose levels remained relatively stable at low levels, with a small increase occurring around the time of the false ROSC, which may suggest perfusion pressure was momentarily restored. The glucose signal decreased to zero following the end of CPR with the LUCAS device. The lactate levels continued to increase throughout the experiment, but at a slower rate compared to the sharp increase immediately following cardiac arrest. As expected, baseline glutamate levels are very low. Following

cardiac arrest, glutamate levels also increased, however there was a delay to see the increase in signal compared to the more immediate changes seen in glucose and lactate. Following the initial increase in glutamate, it then stabilised at a level approximately twice the baseline level. Following the end of CPR with the LUCAS device, glutamate levels increased again. Choline levels decreased following cardiac arrest, this is possibly related to acetylcholine (ACh) levels in the brain, however, further investigations are required. The levels momentarily increase, and then slowly decrease. No change in trend was observed after CPR by LUCAS compressions was stopped. The levels recorded are very low, between 2 and 4 μM , demonstrating excellent sensitivity of the biosensor to detect and resolve changes in low concentration ranges.

4.5 Conclusion

In this chapter, the use of MD to sample different analytes in the brain has been demonstrated by using the biosensors coupled with the wireless monitoring system.

Initial experiments monitored glucose and lactate levels in brain dialysate. Following cardiac arrest, lactate levels increased and glucose levels decreased, which is typically seen in ischemia. These results suggest that the brain is being poorly perfused. These trends continued throughout resuscitation attempts, suggesting that these attempts are insufficient to maintain blood flow to the brain. If ROSC occurred, the glucose levels would increase again, and lactate levels would slowly decrease, demonstrating reperfusion of the brain.

An MD probe was placed in the leg to determine if the same trends were observed in peripheral tissue. The results showed relatively stable levels of both glucose and lactate in the dialysate during the same interventions. This suggests that peripheral tissue is not as affected by the poor perfusion due to its lower energy demand.

The electronic housing and analysis system were adapted to allow monitoring of multiple analytes and improve robustness of the system. Results showed, again, that monitoring glucose and lactate in the brain provides valuable insight into brain health. Similar trends were seen, with dialysate glucose levels decreasing and lactate levels increasing following cardiac arrest. Following ROSC, the opposite trends were observed, suggesting reperfusion of the brain. Glutamate proved to be a useful biomarker in detection of poor perfusion, as a large peak in glutamate brain dialysate levels was observed following cardiac arrest. After the death of the pig, glutamate levels increased again.

The choline biosensor was successfully used *in vivo*, demonstrating its capabilities for monitoring extremely low choline levels (μM ranges) in complex media using MD. Interestingly, choline levels decreased at the onset of cardiac arrest, and continued to decrease. More investigation is required to understand the trends observed in choline levels, which are likely linked to ACh levels in the brain. The use of the choline biosensor coupled with microdialysis for sampling in the brain demonstrates

the potential of the system to monitor AChE activity in complex medias, such as blood, which is the overall aim of this project. This is discussed further in chapter 5.

Chapter 5

Acetylcholinesterase monitoring

5.1 Introduction

As discussed in chapter 1, the overall aim of this work is to sensitively detect changes in acetylcholinesterase (AChE) activity following an individual's exposure to organophosphorus (OP) compounds. AChE is the enzyme responsible for regulating levels of the neurotransmitter acetylcholine (ACh), by hydrolysis. The reaction is described by equation 5.1.



Following the discussion in chapter 1, it is clear that an advantageous way to sensitively detect exposure to all OP compounds is by detecting the change in an individual's AChE activity, as opposed to detecting OP compounds directly.

The discussion also highlighted the importance of a continuous monitoring system, providing many advantages, including determining the kinetics of different OP inhibitors, the kinetics of OP treatments and by continuously monitoring changes in an individual's AChE activity.

5.1.1 Proposed monitoring system

The aim of this thesis is to develop a device that has the capability to continuously monitor AChE activity. The proposed system will introduce ACh into the sample where it will be hydrolysed by AChE to produce choline; this product can be detected using either of the two methods described in chapter 3. The amount of ACh hydrolysed, and therefore the amount of choline produced, will depend on the activity of AChE in the sample, therefore using the proposed method, we are able to probe changes in the AChE activity. As the aim is to monitor in whole blood, it is important to consider the background levels of choline already present, and whether this is a constant level. A study carried out by Hirsch *et al.* investigated the effects of dietary choline intake on serum choline levels [125]. The average fasting serum choline level was found to be $8.2 \pm 0.6 \mu\text{M}$, which rose to $10.9 \pm 1.2 \mu\text{M}$ just

30 minutes after consuming a low-choline meal supplemented with 100 g lecithin (containing 2-3% choline). In this study, serum choline levels continued to rise over a 12-hour period to 30.0 ± 2.5 μM . This demonstrates how choline levels can vary depending on what an individual may consume during the day, therefore it is necessary in continuous *in vivo* monitoring to consider measuring the background levels of choline regularly. Another study conducted by Awwad *et al.* investigated the plasma and whole blood choline levels of 61 individuals [112]. The range of plasma and whole blood choline levels were found to be 6.7-15.4 μM and 43.2-154.8 μM , respectively. The large range also suggests that there is relatively large variations in choline levels between people.

To take account of this, my approach is to perform a baseline choline measurement. This also means that the signal takes into account any interferences from the blood. Taking the differential measurement between the baseline choline signal and the signal after adding ACh into the system results in a value that is wholly dependent on AChE activity. An outline of the detection mechanism is given by the flow diagram in figure 5.1.



Figure 5.1: A flow diagram to detailing the order of measurements for the detection system. First, a basal choline measurement is made. ACh is introduced into the sample, and the resulting choline is measured due to ACh hydrolysis. The amount of ACh hydrolysed will depend on the activity of AChE in the sample. The differential measurement will determine the signal due to AChE activity.

The capability of the monitoring system to perform measurement 1 has been demonstrated in chapter 3. This chapter will investigate how AChE activity can be monitored by performing measurement 2, measurement of choline following ACh hydrolysis.

5.2 Detecting AChE activity using the microfluidic platform

This section explores introducing AChE into a sample stream using the microfluidic platform, adapted for this use. As a proof of principle, I first investigate the use of AChE to detect changes in ACh, before investigating detection of changes in AChE activity while holding the ACh concentration constant.

5.2.1 Detecting changes in ACh concentration

To initially explore the feasibility of adding AChE to react with ACh in-flow using the microfluidic platform and analysis chip, the microfluidic board was modified to perform an ACh calibration. This established whether choline could be monitored following ACh hydrolysis. The microfluidic board setup is shown in figure 5.2, an AChE pump and an ACh pump were added to the basic layout shown

in figure 2.13 to allow their addition to the sample stream. In this experiment, choline was detected by addition of ChOx in flow.

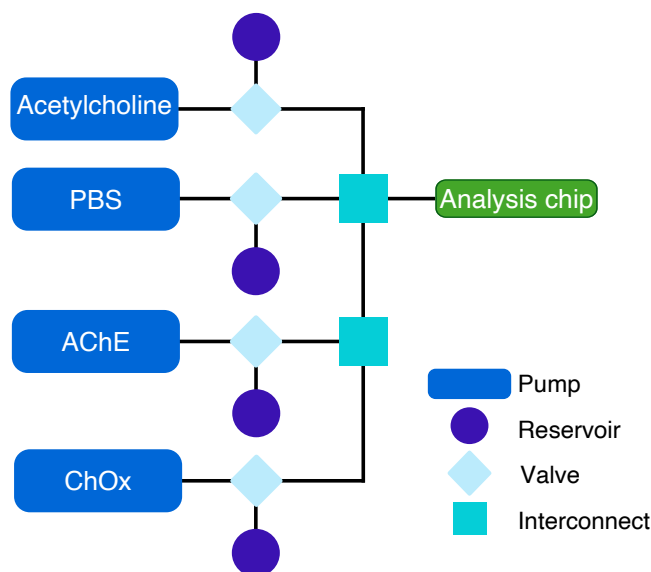


Figure 5.2: Schematic of the adapted microfluidic board to perform an ACh calibration. Two additional pumps, reservoirs and valves have been added to the microfluidic board described in section 3.2 to allow manipulation of ACh and AChE streams.

The flow rates of the AChE and ChOx pumps were held constant at 0.25 $\mu\text{l}/\text{min}$ and the flow rates of the ACh and PBS pumps were varied to give a changing ACh concentration. The combined flow rate of the ACh and PBS pumps were held constant at 2 $\mu\text{l}/\text{min}$. Initially, a 5-point ACh calibration up to 80 μM was performed. These concentration ranges were chosen to ensure that the step change in ACh was large enough to see as it was unknown how sensitive the system would be, and the same concentrations of choline had been detected using this method in chapter 3. The activity of AChE used for this experiment was 4.5 U/ml in the reaction tubing. The ChOx concentration was 1 mg/ml (13.6 U/ml), corresponding to a final concentration of 0.1 mg/ml in the reaction tubing. The bulk concentration of 1 mg/ml was used as this was found to be more stable based on experiments described in chapter 3.

The calibration trace and ACh calibration curve is shown in figure 5.3.

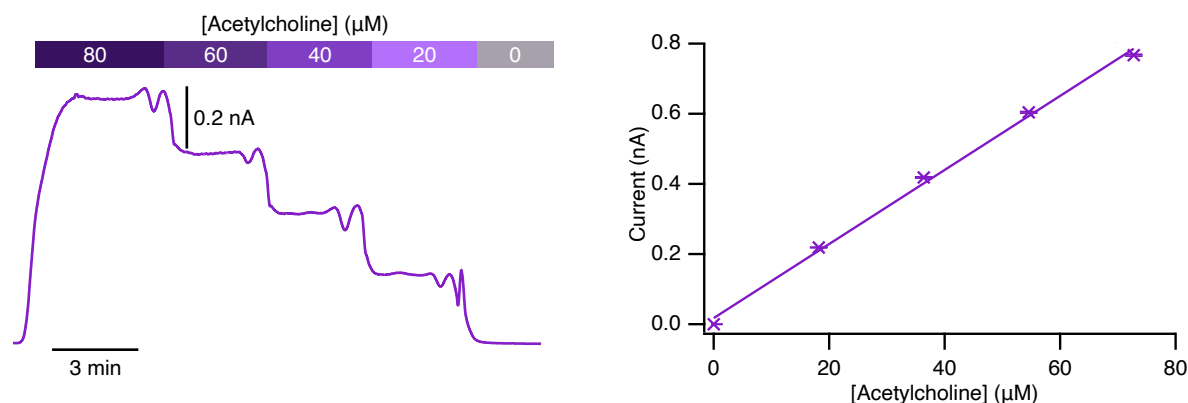


Figure 5.3: Current recorded during a 5-point ACh calibration up to 80 µM and the corresponding calibration curve. The calibration was performed at 2.5 µl/min, taking into account the addition of ChOx and AChE in-flow. The flow rates of the ChOx and AChE pumps were held constant at 0.25 µl/min. Left: The recorded current trace is shown. The current was recorded at 200 Hz and smoothed using a 201-point Savitzky-Golay filter. Right: The resulting ACh calibration curve is shown. The markers and error bars represent the mean $\pm$ standard deviation of each calibration step. The data is fitted using linear regression. With these experimental parameters, the analysis system has a sensitivity to ACh of 10.6 ± 0.3 nA/mM. The error is given by the 95 % confidence interval.

The calibration script was adapted to start at 100% and step downwards, as shown in the trace of figure 5.3, to see the biggest step change first and therefore check if the calibration was working as expected. This experiment showed clear concentration steps because the system was in the substrate-limited region for each concentration step; there was an excess of enzyme relative to the substrate. The AChE activity used was 4.5 U/ml, which corresponds to $4.5 \mu\text{mol}/\text{min}/\text{ml} = 4.5 \text{ mM}/\text{min}$ of ACh being hydrolysed. The reaction length used in this experiment was 5 cm, corresponding to a reaction time of approximately 20 seconds for the flow rate of 2.5 µl/min, so in theory it was expected that the ACh would be fully hydrolysed. This clear ACh calibration demonstrates that AChE and ACh can be introduced together in-flow to produce choline, which can be detected using the choline analysis system.

5.2.2 Checking for a background interference signal from ACh

To check that the system is in fact detecting choline as a result of ACh hydrolysis, an ACh calibration was run with no AChE added to the sample stream. This experiment was performed with a choline biosensor and the board was configured as shown in figure 2.13. A 5-point choline calibration up to 10 µM was performed followed by a 5-point ACh calibration up to 10 µM. A low choline concentration calibration was performed to ensure it was well within the linear range of the biosensor so the data can be fitted using linear regression and the corresponding sensitivity can be calculated. The results of two choline biosensors are shown in figure 5.4.

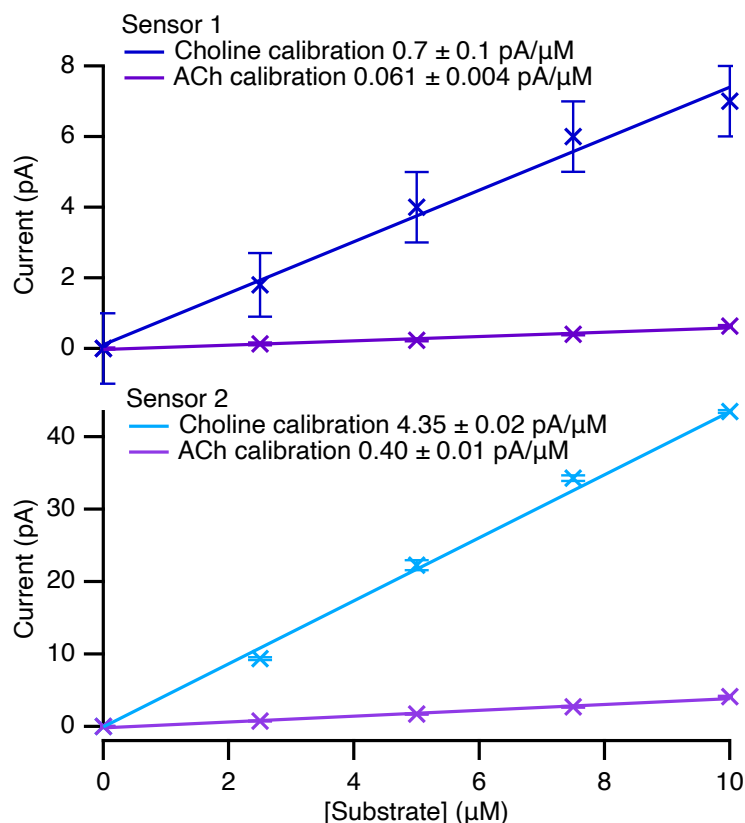


Figure 5.4: Calibration curves obtained for a choline calibration and an ACh calibration with no added AChE. The calibrations were performed with two choline biosensors placed in a 3D printed chip. Initially, a choline calibration was performed up to 10 μ M, followed by an ACh calibration up to 10 μ M, with no AChE added. In theory, there should be no background current from ACh. The corresponding calibration curves are shown for two biosensors, blue shows the biosensor response to choline and purple shows the biosensor response to acetylcholine. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step. The data has been fitted using linear regression. The gradient of the line gives the sensitivity, which has been quoted. The error on the sensitivity is given by the 95% confidence interval of the regression. Note sensor 1 has an unusually high signal-to-noise ratio. The sensitivity of the sensor 1 to choline is also uncharacteristically low.

For sensor 1, the sensitivity of the choline biosensor to ACh was 8.7% of the sensitivity to choline. For sensor 2, the sensitivity of the choline biosensor to ACh was 9.2% of the sensitivity to choline. Relatively, these are tiny signals so it can be deduced that there is little ACh background signal compared to the choline signal obtained due to ACh hydrolysis. As the microfluidic platform and analysis chip has ‘seen’ AChE before, it is possible that some of this is sticking and causing the hydrolysis of ACh.

To investigate this further, the microfluidic platform was configured as shown in figure 5.2 and different calibration cycles were performed. The tubing and microfluidic chip were changed so the microfluidic platform had not ‘seen’ AChE before. Initially, an ACh calibration up to 100 μ M was performed with ChOx (1 mg/ml) but no AChE present, at a flow rate of 2 μ l/min. The resulting calibration is shown by the red line in figure 5.5.

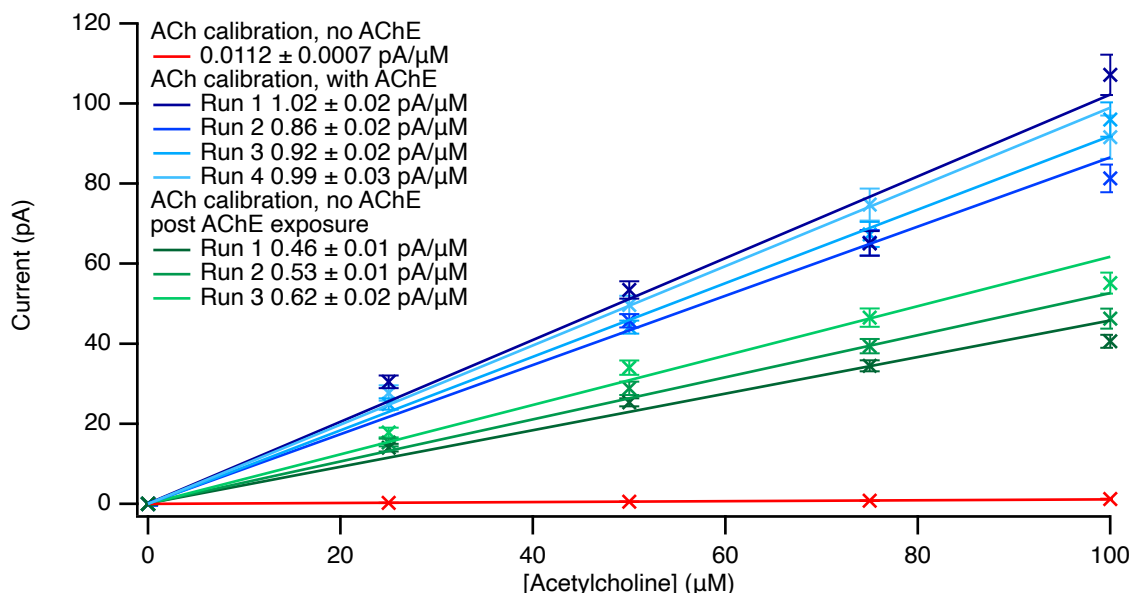


Figure 5.5: Calibration curves obtained during different ACh calibration cycles, with and without AChE, to check for interference from ACh. Calibrations were performed with the in-flow ChOx (1 mg/ml) at a total flow rate of 2 μ l/min. Initially, an ACh calibration up to 100 μ M was performed with no AChE present, shown by the red line. Following this, alternating calibrations were performed, one with 10 U/ml AChE present (blue) followed by one with no AChE present (green), and calibrations were repeated in this way. The green lines represent the ACh calibrations with no AChE present, but after the microfluidic platform and analysis chip had been ‘exposed’ to AChE. The markers and error bars represent the mean $\pm$ standard deviation of each concentration step for one calibration. Each calibration is fitted using linear regression, the sensitivities shown are given by the gradient and the error represents the 95% confidence interval of the fit.

The results show that when no AChE is present, and the microfluidic platform has not ‘seen’ the enzyme, a very small signal is recorded. Following this calibration, an ACh calibration was performed with 10 U/ml AChE present. This is a high AChE activity, but chosen to fully investigate if there is any effect from the AChE sticking to the board. This is shown by the dark blue line in figure 5.5. The results demonstrate a much larger signal is obtained from ACh when AChE is present. This is expected as the AChE present will hydrolyse the ACh to give choline, which is oxidised by the ChOx also present in the solution. The generated H_2O_2 will be detected at the electrode surface.

The ACh calibration was repeated with no AChE present, shown by the dark green line in figure 5.5. The signal recorded during this calibration, after the microfluidic platform has ‘seen’ AChE, is much higher compared to when the platform had not ‘seen’ AChE, shown by the red line. This clearly shows that some of the enzyme has indeed remained in the microfluidic platform and/or analysis chip.

Repeated ACh calibrations were run, alternating between a calibration containing AChE (blue) and a calibration with no AChE (green). The results show that the signal seen from ACh with no AChE present increases as the microfluidic platform and analysis chip are exposed to more AChE, again, suggesting that more enzyme is sticking and this is the reason for a background signal. The repeated ACh with AChE calibrations do not show such a clear trend, suggesting that the ACh is being fully hydrolysed, thus any additional enzyme sticking to the microfluidic platform/chip is not

having a significant effect.

Given that a very high AChE activity of 10 U/ml was used, it is unknown how big an effect lower enzyme concentrations will have on the system, and it is possible that the problems are arising from the enzyme sticking to the microfluidic chip. Sampling using microdialysis (MD) will likely improve this problem as AChE will be blocked out of the MD probe and hence the analysis stream. This is investigated further in section 5.3.

5.2.3 Detecting changes in AChE activity

The successful detection of choline following ACh hydrolysis demonstrated in section 5.2.1 showed that the addition of an AChE stream to the microfluidic platform led to the hydrolysis of ACh. The next stage was to determine if it is possible to detect changes in AChE activity based on ACh hydrolysis. For this experiment, the microfluidic platform was set up as shown in figure 5.6, where the AChE and PBS pump flow rates were varied to create step changes in the AChE activity, and the ACh pump flow rate was held constant, resulting in an AChE calibration as the amount of ACh hydrolysed was dependent on the AChE activity. For this experiment, a choline biosensor was used and the ChOx pump was removed from the board to reduce the number of time-dependent reactions occurring in the reaction tubing, highlighted in red, and analysis chip.

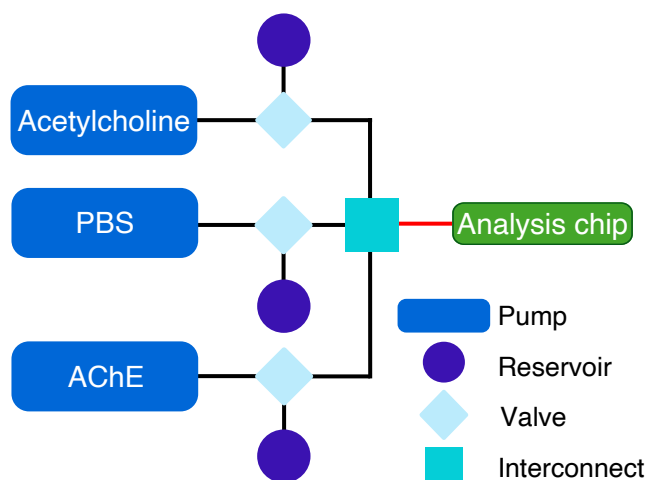


Figure 5.6: Schematic of the microfluidic board configured to perform an AChE calibration. To perform an AChE calibration, an ACh pump and an AChE pump is needed. Detection of choline produced by hydrolysis of ACh was performed with a choline biosensor, therefore the ChOx pump was removed from the microfluidic platform shown in figure 5.2. The red tubing highlights the reaction tubing, this refers to the tubing in which the AChE hydrolyses the ACh.

As the results presented in figure 5.5 suggest that the AChE was sticking, this experiment was performed with a lower enzyme activity to ensure step changes could be seen. The AChE activity was varied between 0 and 1 U/ml in 25% increments, and the ACh concentration was held constant at 1.25 mM to ensure that there was an excess of substrate, required to achieve a difference in signal for the different AChE activities. The trace and corresponding calibration curve is shown in figure 5.7.

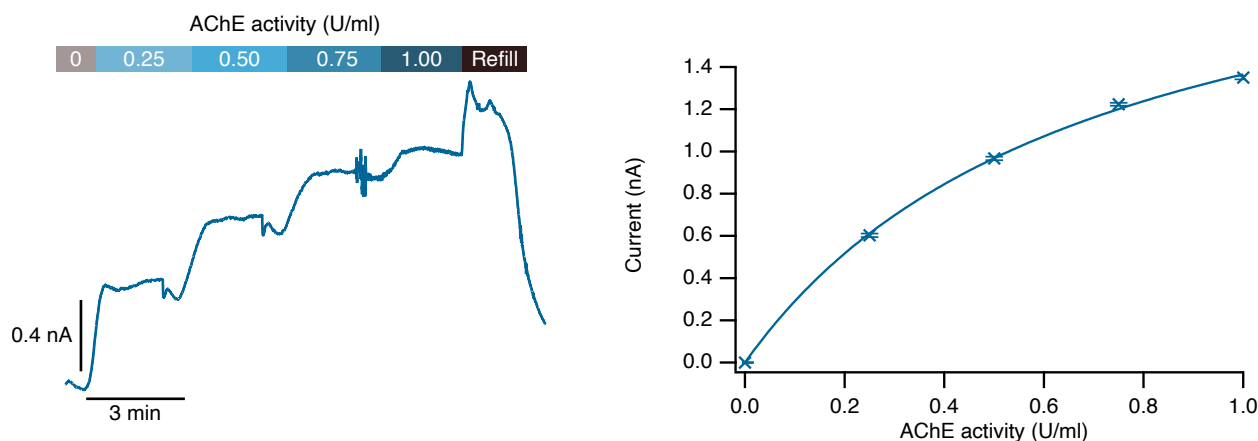


Figure 5.7: Current recorded during a 5-point AChE board calibration with the corresponding calibration curve. The AChE activity was varied from 0 to 1 U/ml in 0.25 U/ml steps. To determine the signal due to AChE activity, 1.25 mM ACh was added to the sample stream, and hydrolysed to produce choline by the AChE present. Different AChE activities result in different amounts of ACh being hydrolysed, and therefore different amounts of choline being produced. The current trace due to the choline is shown on the left. The current was recorded at 200 Hz and the trace was smoothed using a 201-point Savitzky-Golay filter. The calibration curve is shown on the right. Markers and error bars show the mean $\pm$ standard deviation. The calibration was fitted with a Michaelis-Menten curve as the result is non-linear, likely due to the ChOx biosensor saturating as there is a high turnover of ACh.

On the 1 U/ml AChE calibration step, there is a large artifact due to the refilling of the pumps. During refilling, the flow to the analysis system stops, which also means the flow in the mixing tubing stops. Refilling takes approximately 12 s, during which more ACh will be hydrolysed than previously seen as there is an additional 12 s of interaction time between the ACh and AChE in the reaction tubing. For 1 U/ml activity of AChE which corresponds to 1 μ mol of ACh converted per minute per ml, this activity therefore converts 1 mM of ACh per minute, so an additional 12 s of reaction time results in an additional 0.2 mM of ACh being hydrolysed, hence a spike in the signal.

The reaction tubing length used was approximately 5 cm with an inner diameter of 150 μ m, which corresponds to a volume of 0.88 μ l. The total flow rate is held constant at 2 μ l/min, therefore there is a reaction time between the AChE and ACh of approximately 27 s. The highest enzyme activity used here was 1 U/ml, corresponding 1 mM of ACh being converted per minute. In 27 s approximately 0.45 mM of ACh will be converted to choline. The reason that the calibration is not linear is likely to be because a high level of choline is being produced, meaning the levels are beyond the linear range of the choline biosensor (apparent K_m is typically 0.4 μ M). However, these are still promising results and in the future a PU membrane can be added to the outside of the biosensor to extend its dynamic range if necessary, or the reaction tubing can be shortened so less ACh is converted to choline.

5.3 Microdialysis

In this thesis, MD was chosen as the sampling technique to allow sampling in complex media, such as blood. The methodology of MD is described in chapter 1. By using MD, large molecules, such as enzymes, and cells, such as red blood cells, are prevented from entering and disturbing the analysis system. MD was also chosen as it allows delivery of molecules below the molecular weight cut-off (MWCO), a technique known as retrodialysis. This has potential to be useful to deliver ACh for monitoring changes in AChE activity *in vivo*.

Two approaches are proposed for sampling using MD: (i) add ACh to the sample externally to the probe and (ii) add ACh to the sample through retrodialysis. The two approaches are tested in this section. For all experiments, the MD probes are perfused at a flow rate of 2 $\mu\text{l}/\text{min}$ using a CMA 107 pump (MDialysis, Sweden).

5.3.1 Detecting choline through microdialysis directly and following acetylcholine hydrolysis

As previously discussed, the level of choline is approximately 66 μM in whole blood and 11 μM in plasma [112], however, it varies throughout the day and depending on an individual's diet [125].

As described in section 5.1.1, before initiating the AChE measurement by adding ACh to the sample, the background choline level will be determined so that it can be subtracted from the AChE signal. Initial MD experiments therefore investigated the detection of choline directly, before exploring the detection of choline following ACh hydrolysis.

To detect choline through MD, an MD probe was placed in a stirred beaker containing 40 ml of 0.01 M PBS. For these experiments, CMA 70 brain microdialysis probes were used, with a MWCO of 20 kDa, purchased from MDialysis (Sweden), due to their availability in the lab. Choline was added to the beaker in 2.5 μM increments. A choline biosensor was used in the analysis chip, connected to the outlet of the MD probe, for detection of choline in the dialysate stream. The experimental setup is shown by the left schematic in figure 5.8.

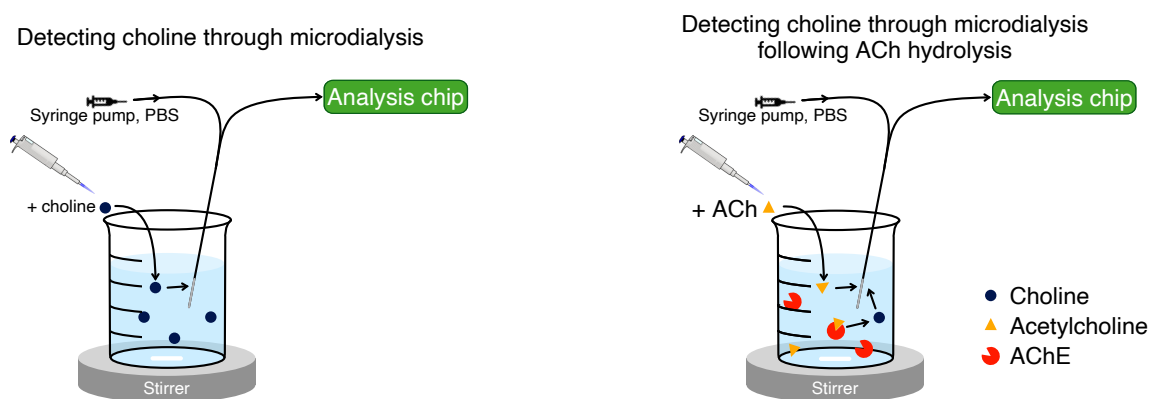


Figure 5.8: Experimental setup for microdialysis experiments performed in the beaker. In both experiments, a CMA 70 brain MD probe (MWCO 20 kDa) was perfused with 0.01 M PBS at 2 $\mu\text{l}/\text{min}$ and placed in a stirred beaker. Left: Schematic showing the setup for an experiment to detect choline additions into the beaker through MD. Right: Schematic showing the experimental setup designed to detect choline following ACh hydrolysis. ACh additions were made into a beaker containing AChE. The ACh was hydrolysed by the AChE to produced choline, which diffused into the MD probe and is detected downstream with the choline biosensor.

After each addition of choline, the signal was left to stabilise before the next addition. The mean and standard deviation of each concentration step was taken and plotted against the corresponding concentration. The resultant calibration curve is shown in green in figure 5.9.

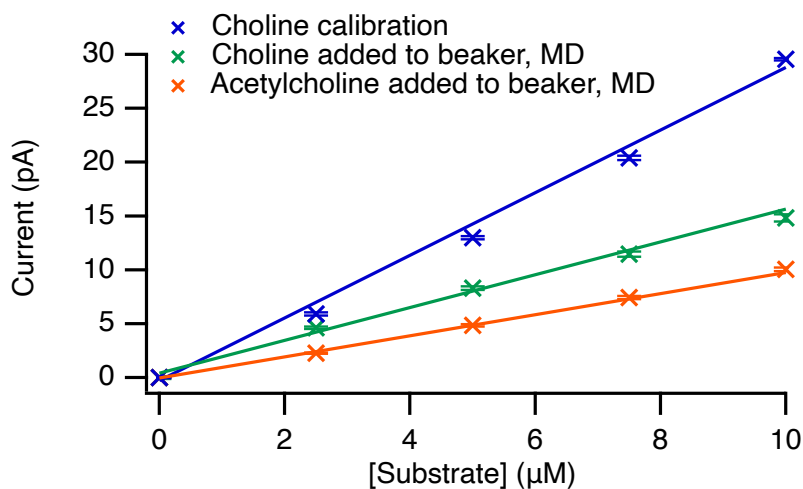


Figure 5.9: Calibration curves recorded for a standard choline calibration and microdialysis calibrations. An autocalibration was performed using the microfluidic platform to obtain the choline calibration curve, shown in blue. A microdialysis probe was placed in a stirred beaker containing 40 ml of 0.01 M PBS, and 2.5 μM choline additions were made to obtain the green calibration curve. The microdialysis probe was placed in a beaker containing 0.025 U/ml AChE in 0.01 M PBS. 2.5 μM additions of ACh were made and the resulting choline from ACh hydrolysis is detected downstream using a choline biosensor, shown by the orange calibration curve. The markers and error bars show the mean $\pm$ standard deviation of each concentration step. The calibrations have been fitted using linear regression.

To gain an understanding of how the final system will work, the next step in these MD experiments was to detect the recovery of choline following the hydrolysis of ACh. The experimental setup is shown by the right schematic in figure 5.8. The MD probe was placed in a beaker containing 40 ml of 0.025 U/ml AChE solution made in 0.01 M PBS. ACh was then added to the beaker in 2.5 μ M increments. The calibration curve for this experiment is shown in orange in figure 5.9.

The successful signal obtained from the addition of ACh into the beaker demonstrates that the AChE hydrolysed the ACh and the resulting choline diffused into the MD probe for detection at the analysis system. The resulting current is related to the level of choline, which is proportional to the amount of ACh hydrolysed. This is essentially an ACh calibration through microdialysis. The activity of AChE in the beaker was 0.025 U/ml, corresponding to 0.025 μ mol of ACh hydrolysed per minute per ml, which is equivalent to 25 μ M of ACh hydrolysed per minute. This AChE activity was chosen to demonstrate that small AChE activities could be detected.

5.3.2 Detecting changes in AChE activity through microdialysis

Following successful choline and ACh calibrations using MD, the next step was to try to detect different AChE activities. This requires a constant concentration of ACh to be added to the system. To detect changes in AChE in this way, it was important to be in an enzyme-limited region, so that a different amount of ACh is converted depending on the enzyme activity.

An overview of the experimental protocol is shown in figure 5.10. A CMA 12 animal MD probe was placed in a stirred beaker containing 6 ml of an AChE solution in PBS. An addition of ACh was made to the beaker, so that the final ACh concentration was 10 mM. The choline produced by hydrolysis of ACh diffused into the MD probe for detection using the analysis system connected to the outlet of the MD probe. This was repeated with different activities of AChE, ranging from 0 - 3 U/ml. This experiment used a different probe compared with the previous experiment due to the availability in the lab at the time of the experiment.

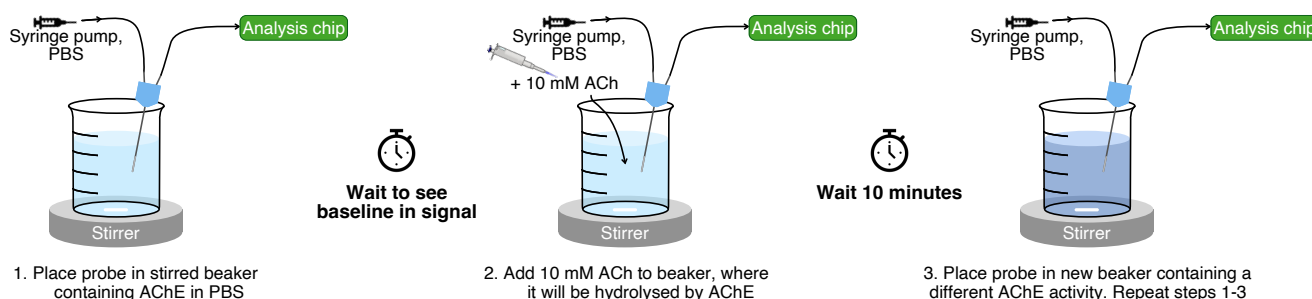


Figure 5.10: The experimental protocol to determine different AChE activities. A CMA 12 animal MD probe, perfused at 2 $\mu\text{l}/\text{min}$ with 0.01 M PBS, is initially placed in a stirred beaker that contains a certain AChE activity in 0.01 M PBS (step 1). The system is left to reach a stable baseline. 10 mM ACh is then added to the beaker (step 2). The ACh will be hydrolysed to choline at a rate that depends on the AChE activity and diffuse into the MD probe. The dialysate will then travel downstream to the analysis chip containing a choline biosensor. Ten minutes after the addition of ACh to the beaker, the probe is placed into a new beaker containing a different AChE activity in 0.01 M PBS (step 3), and steps 1-3 are repeated again.

An extract of the current trace recorded during this experiment is shown in figure 5.11.

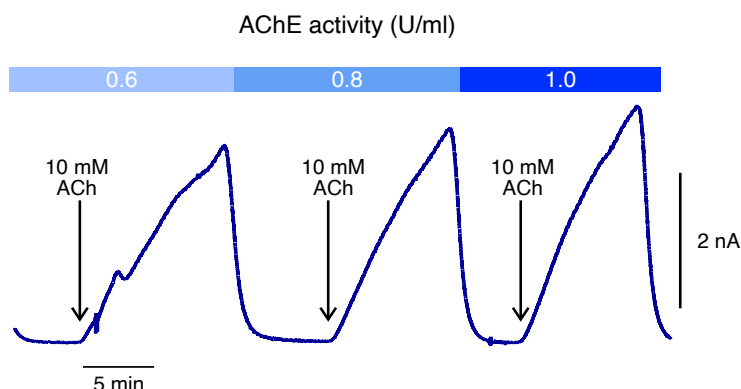


Figure 5.11: A section of the current trace recorded during the sampling of solutions containing different AChE activities. 10 mM ACh additions were made, and the increase in current was recorded at the choline biosensor placed downstream. The arrows show the point at which 10 mM additions were made, after the time delay to record the change in signal has been taken into account. The current was recorded at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

The change in the resulting current corresponded to the change in choline level, which was due to the change in enzyme activity leading to a change in the amount of ACh hydrolysed. As the AChE generates more choline, more choline diffuses into the probe which travels downstream where it is detected at the choline biosensor, causing an increasing signal. The signal increase stops when the probe is moved to a fresh solution without ACh, causing the signal to return to baseline.

A plateau could have been reached by waiting for the enzyme to fully hydrolyse the ACh, however the plateau would be the same regardless of the enzyme activity, as the same amount of ACh would be hydrolysed, it would just take a different amount of time to reach the plateau. Therefore, it was not necessary to wait for the signal to stabilise.

To analyse this data, a 10 s average of the signal was taken 2 minutes after the addition of ACh was made. The resulting currents are plotted against AChE activity in figure 5.12.

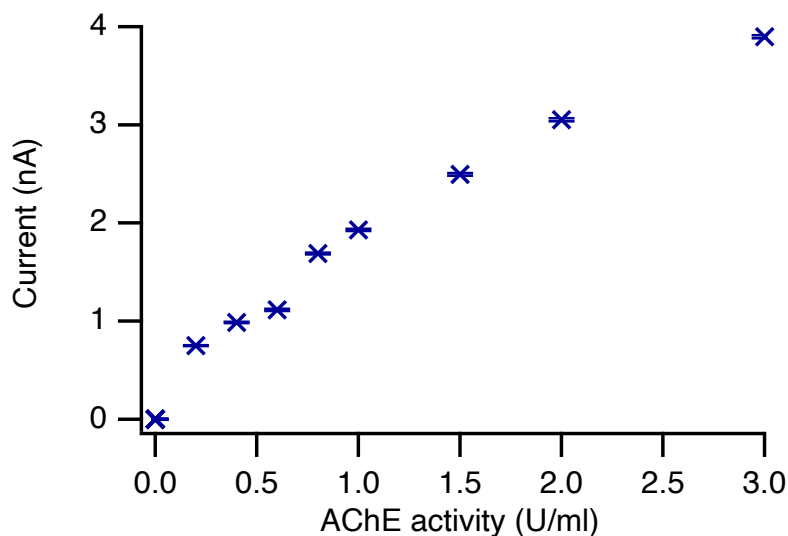


Figure 5.12: The average current obtained due to ACh hydrolysis for different AChE activities. A 10 s average of the current is taken 2 minutes after the addition of ACh. The rate of ACh hydrolysis depends on the enzyme activity, therefore a given time after ACh is added to the AChE containing solution, there should be a different amount of ACh hydrolysed depending on the AChE activity. The markers and error bars represent the mean $\pm$ standard deviation of the 10 s sample.

The curve clearly demonstrates that increasing the AChE activity results in an increase in the recorded signal. A higher AChE activity causes a higher rate of hydrolysis ACh. Therefore, for a given time point, there is a greater amount of choline that has been produced, which has been detected by the analysis system.

5.3.3 Delivery of ACh through retrodialysis

A further step of monitoring AChE activity continuously is moving towards monitoring *in vivo*. This could potentially be achieved by introducing ACh through the microdialysis stream, which would be beneficial as it would avoid manually adding the substrate into the sample. Delivering ACh in this way is known as retrodialysis.

Experimental protocol

The experimental protocol is shown in figure 5.13. For these experiments, the probe was perfused with a certain concentration of ACh at a flow rate of 2 $\mu\text{l}/\text{min}$. The probe was initially placed in a 1 ml solution of 0.01 M PBS. The signal was left to stabilise giving the background signal of the experiment. Following signal stabilisation, the MD probe was placed in a solution containing a known AChE activity. The probe was left in the solution for at least 10 minutes, which allowed a change in signal to be observed before switching back to a fresh PBS solution.

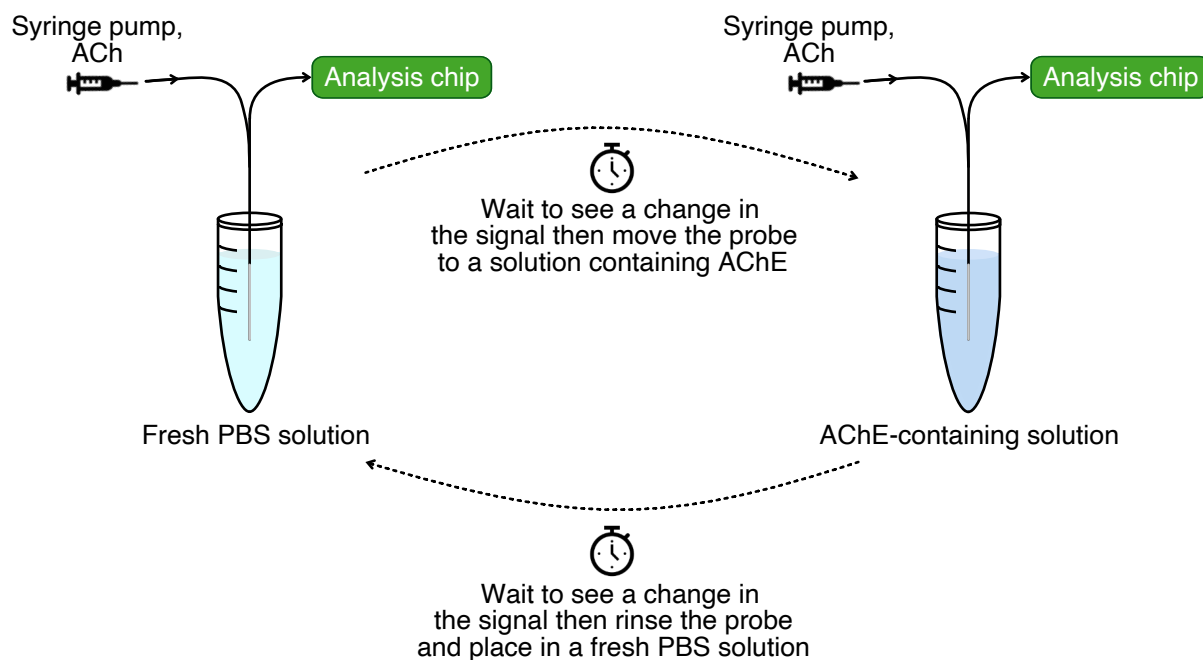


Figure 5.13: Experimental protocol to add ACh to a sample via retrodialysis and probe different AChE activities. The MD probe is perfused with a certain concentration of ACh. The probe is placed in PBS solution and the baseline signal is recorded downstream at a choline biosensor. After the signal has stabilised, the probe is moved to a solution containing a known activity of AChE. After at least 6 minutes, the probe is placed in a fresh 0.01 M PBS solution. All solutions are 1 ml volume.

Experimental results

To initially trial this technique, a CMA 70 brain probe was perfused with 5 mM ACh. The probe was placed in an unstirred 1 ml solution of 0.01 M PBS and the resulting baseline current was recorded. This was higher than expected as shown in figure 5.14. This suggested that a large background signal was being observed due to the ACh in the dialysate stream. This is likely to be from previously used AChE being stuck inside the analysis chip, or possibly sticking to the MD probe.

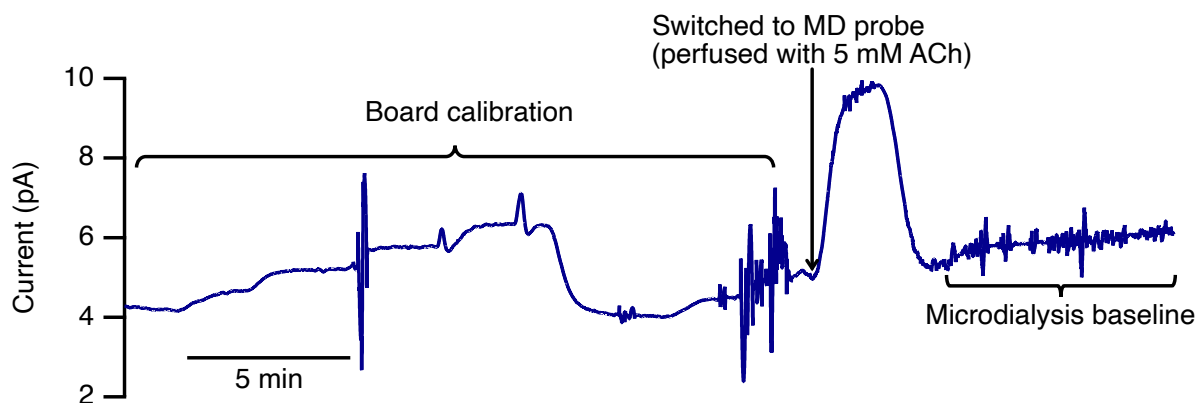


Figure 5.14: The current trace recorded when switching from a board choline calibration up to 10 μ M to dilaysate containing 5 mM ACh. The baseline was expected to return to a similar current seen for a PBS baseline during the board calibration, however there was a large background current due to the 5 mM ACh. The current has been recorded at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

Eppendorf tubes were prepared containing 1 ml volumes of different AChE activity, 1, 2, 3 and 4 U/ml, corresponding to 1, 2, 3 and 4 mM of ACh hydrolysed per minute. The probe was placed in these volumes, as described previously by the experimental protocol, and the resulting signal from ACh hydrolysis was detected downstream at the choline biosensor. The resulting current trace is shown in figure 5.15.

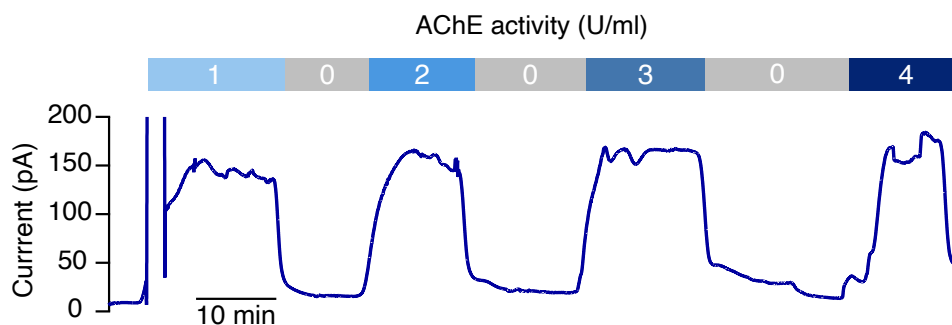


Figure 5.15: Current trace recorded for different AChE activities when ACh was delivered using retrodialysis. The MD probe was perfused with 5 mM ACh at 2 μ l/min. The probe was placed into solutions containing different AChE activities in 0.01 M PBS. The resulting choline from ACh hydrolysis was detected at the choline biosensor placed downstream. The current was recorded at 200 Hz. The trace has been smoothed with a 201-point Savitzky-Golay filter.

The trace shows that no clear difference can be seen between the signals obtained when the probe is placed in different enzyme activities. This suggests that the experiment was in a substrate-limited region. There was also a slight increase in the baseline level in the PBS vial (no AChE added, but with ACh still perfused through the MD probe), suggesting that there may be some AChE sticking to the probe to cause this increase in baseline. The mean of the baseline steps and the AChE current steps were taken. The differential measurement between response in AChE solution and that in the previous PBS solution was taken and plotted against the corresponding AChE activity to get a calibration curve, as shown in figure 5.16.

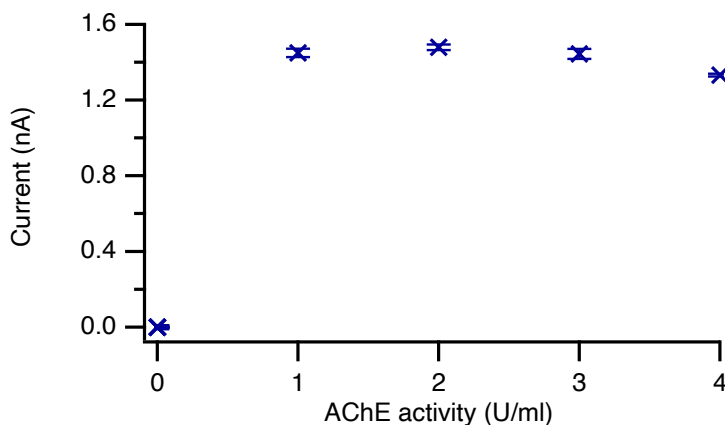


Figure 5.16: Average current obtained for different AChE activities when ACh was delivered through retrodialysis. The markers and error bars represent the mean $\pm$ standard deviation of each current step.

This reiterates that no difference in the amount of ACh hydrolysis can be seen between the different AChE activities, suggesting there is an excess of enzyme.

As the pump was being perfused at 2 $\mu\text{l}/\text{min}$, the maximum amount of ACh deliverable can be calculated. In 2 μl of 5 mM ACh, there is 0.01 μmol of ACh. If this all diffused out of the probe, this would correspond to 0.01 μmol of ACh being delivered per minute. In 1 ml of solution, 0.01 μmol of substrate corresponds to a concentration increase of approximately 10 μM ACh in the solution every minute. While the solution was not stirred in the hope that the ACh concentration surrounding the probe would be higher, it's clear that little ACh is being delivered. This is an oversimplified model of the microdialysis system, but it provides an idea as to whether the parameters are in the correct range. As the lowest activity of AChE used in this experiment was 1 U/ml, corresponding to 1 mM of ACh being hydrolysed per minute, it is unsurprising that the average signal for each AChE activity is similar.

To further test the use of retrodialysis to deliver ACh, the experimental parameters were modified. The probe was perfused with 100 mM ACh, much higher than the 5 mM previously used. At a flow rate of 2 $\mu\text{l}/\text{min}$, this corresponds to a maximum 0.2 μmol of ACh being delivered to the solution every minute. Smaller AChE activities were sampled to see if a difference could be seen in the signal. The probe was placed in different eppendorfs containing 1 ml of solution with AChE activities of 0.02, 0.04, 0.08 and 0.12 U/ml, as described by the experimental protocol in figure 5.13. The current trace recorded during this experiment is shown in figure 5.17.

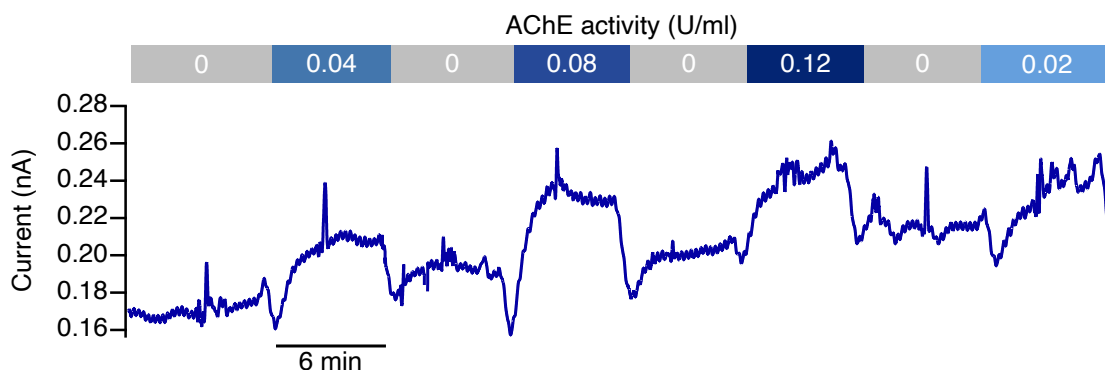


Figure 5.17: Current trace recorded for different AChE activities when sampling with an MD probe perfused with 100 mM ACh. An MD probe perfused at 2 $\mu\text{l}/\text{min}$ with 100 mM ACh was placed in AChE-containing solutions ranging from 0 - 0.12 U/ml. In between each measurement, the probe was placed in a solution containing fresh 0.01 M PBS. These 0 U/ml AChE baseline measurements show an increase in the background signal. The current was recorded at 200 Hz. The trace has been smoothed with a 201-point Savitzky-Golay filter.

The trace clearly shows that the baseline current is increasing following the AChE activity measurements, suggesting that there is some AChE sticking to the MD probe. It can also be seen that the current response is not very stable. This meant that the timings had to be well monitored, and to analyse this data, a 1 minute average was taken 3 minutes after the probe had been placed in the respective solution. Again, a differential measurement was taken between the mean current recorded for an AChE solution and the mean baseline current recorded in a PBS solution before it. The differential measurement was plotted against the AChE activity, as shown in figure 5.18.

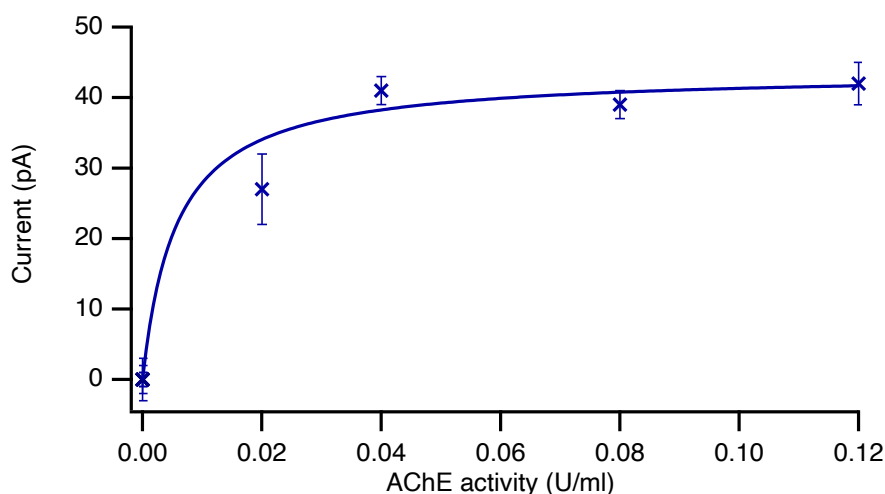


Figure 5.18: The average current obtained for different AChE activities. Retrodialysis was used to deliver ACh into an AChE-containing solution by perfusing an MD probe at 2 $\mu\text{l}/\text{min}$ with 100 mM ACh. Markers and error bars represent the mean $\pm$ standard deviation of each current step. The data has been fitted using MM kinetics.

The data points were initially fitted using MM kinetics, as the choline biosensor could be saturating causing the plateau in signal. However, it is hard to tell if it is a good fit. A better fit may be a linear fit between the 0 and 0.02 U/ml AChE activity and a horizontal fit between the 0.04 - 0.12 U/ml activities, as shown in figure 5.19. The horizontal fit was based on the average of the three currents obtained for 0.04, 0.08 and 0.12 U/ml AChE activity. Where the two lines meet is the approximate AChE activity at which the system becomes substrate limited.

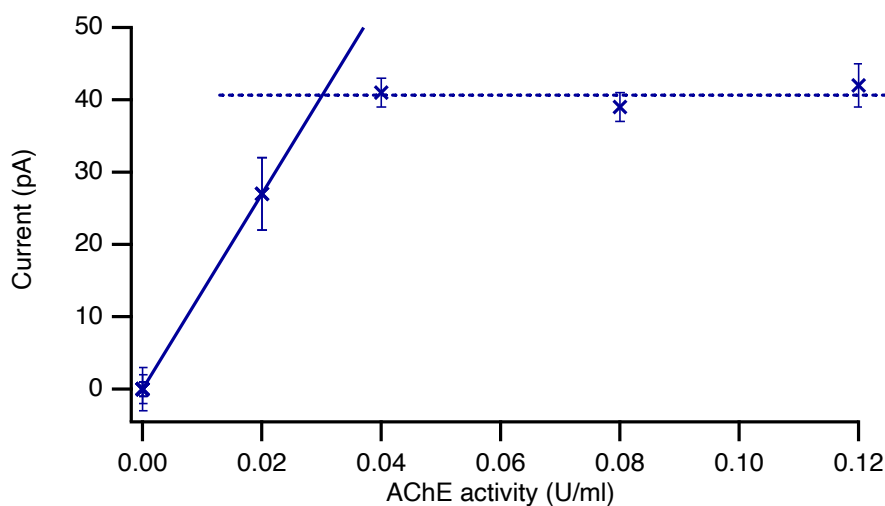


Figure 5.19: The average current obtained for different AChE activities. Retrodialysis was used to deliver ACh into an AChE-containing solution by perfusing an MD probe at 2 $\mu\text{l}/\text{min}$ with 100 mM ACh. Markers and error bars represent the mean $\pm$ standard deviation of each current step. The data has been fitted using a linear fit for the low enzyme activities. The dotted line shows a horizontal fit for activity ranges that have become substrate limited.

The results suggest that the experiential parameters are still not quite correct, and there is still not enough ACh being delivered to see a difference in hydrolysis rates for the chosen enzyme activities. The large background current is also a problem, as this makes the system less sensitive as an increasing amount of AChE is appearing to stick to the MD probe, so the actual amount of AChE in a given solution is greater than it appears.

To reduce the effect of the background current resulting from ACh, two probes could be used; one probe can be used to deliver ACh, whilst the other is used to detect the resulting choline. The probe used to pick up the resulting choline would be perfused with PBS, reducing the background signal from ACh.

5.3.4 Two-probe system

Having the ACh in the dialysate led to a large background current, which would vary depending on how much ACh was diffusing out of the probe, determined by the concentration gradient. Therefore, a new approach was trialled, to deliver ACh using one MD probe and pick up the resulting choline with another MD probe. This method has its drawbacks; the sensitivity of the system is likely to be reduced as the choline will diffuse into both probes. However, it allows greater control over the system, for example the probes can have different flow rates to optimise the delivery of ACh and also increase pick up of the resulting choline (low flow).

Experimental setup

In this section, different ways to secure the probes are trialled. For these experiments, the probes used were CMA 70 brain probes. Three methods were used: (A) probes were taped together, (B) probes were placed in solution unsecured and (C) probes were secured in place by glueing them in an eppendorf lid. The three methods are shown in figure 5.20.

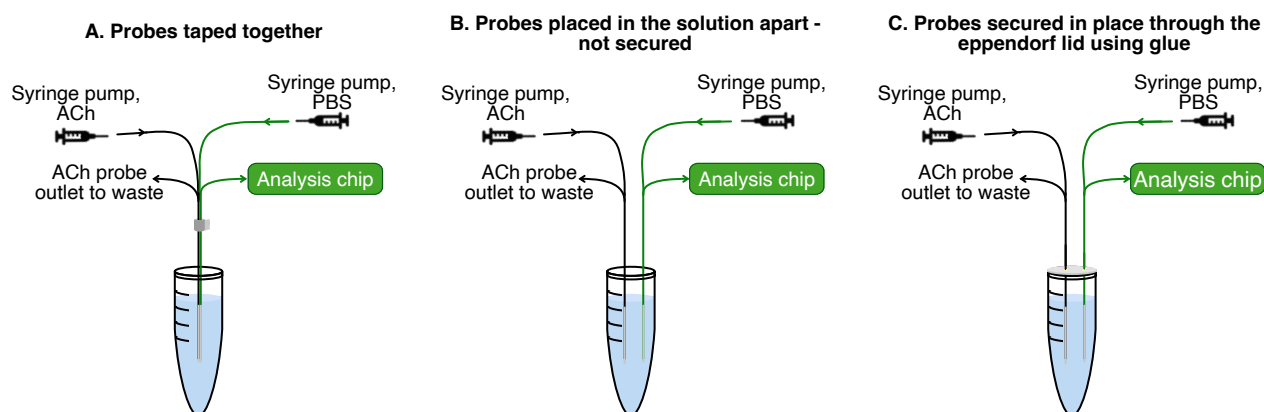


Figure 5.20: Different experimental setups when sampling AChE activity using two probes. For each experimental setup, two probes are used. The probe used for choline recovery is perfused with PBS and connected to the analysis chip housing the choline biosensor. The probe used for retrodialysis of ACh is perfused with 100 mM ACh, and the outlet goes to waste. (A) shows the two probes taped together. (B) shows the 2 tapes placed in solution, with no method of securing them in place. (C) shows the probes secured in place by glueing them in an eppendorf lid.

To trial the use of one probe for retrodialysis and the other probe for choline recovery, initially the two probes were placed in a 1 ml solution containing AChE and the resultant choline was detected. The solution was not stirred to try to prevent dilution of ACh into the bulk solution. Two of the different experimental setups for the probe were initially trialled: (i) the probes were taped together and placed in the solution, shown by (A) in figure 5.20 and (ii) the probes were placed in the solution and held in place, shown by (B) in figure 5.20. The experimental protocol presented in figure 5.13 was then followed, but with the 2 probes used instead of the singular probe. The probes were placed in 1 ml solutions containing 0.02, 0.04, 0.16 and 0.32 U/ml AChE. The results for the two experimental setups are shown in figure 5.21.

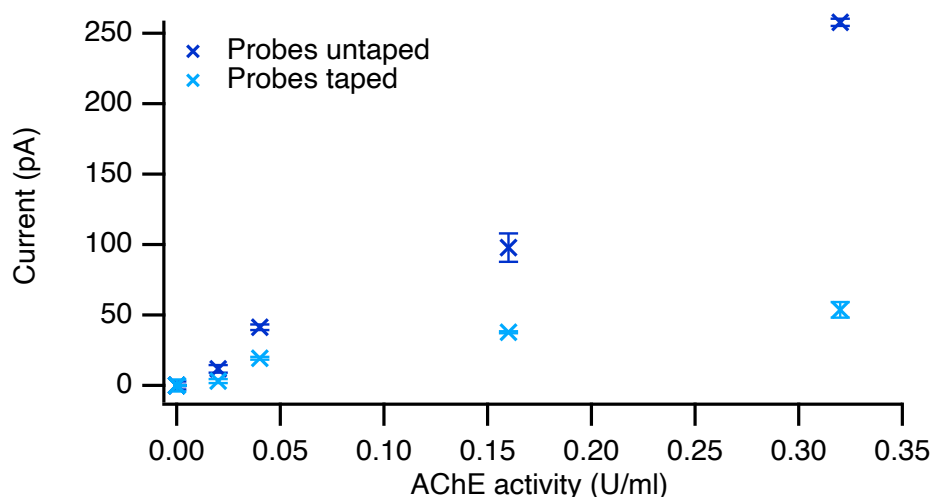


Figure 5.21: The currents recorded when sampling AChE activities using two different two probe systems. The resulting choline from ACh hydrolysis was detected at the outlet of the MD probe using a choline biosensor. The marker and error bars represent the mean $\pm$ standard deviation of the current step.

There is a large difference in the signal obtained with the probes untaped and with the probes taped. As the solution was not stirred, the separation of the probes affected the experiment in terms of how much choline would diffuse into the second probe and at what rate. When the probes are taped together, they tend to stick together, so it is possible that the ACh diffuses straight into the second probe or does not diffuse out of the probe in that orientation because of it. When the probes were not taped together, they could still be held quite close together. However, overall it was easier to control the spacing between the two probes when they were taped together. To investigate this further, the taped probes were placed in 1 ml solutions containing AChE activities over a larger range, from 0.02 U/ml to 0.40 U/ml. The trace of these results is shown in figure 5.22.

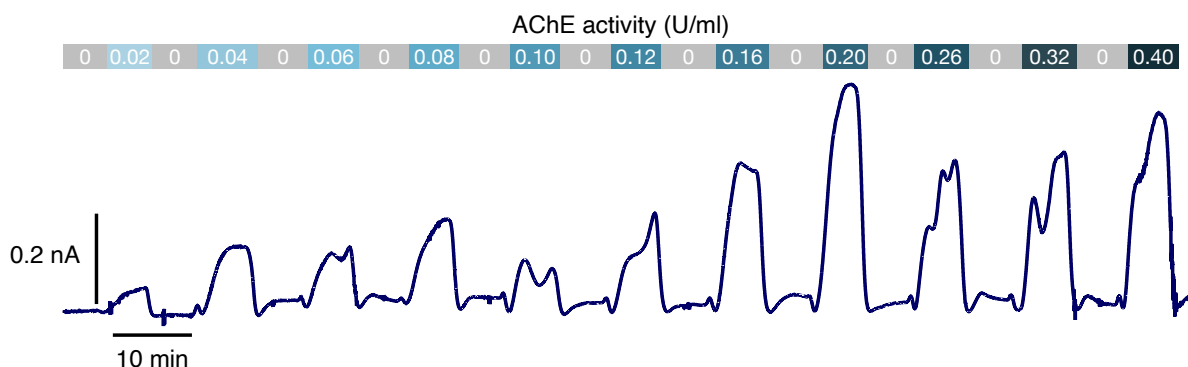


Figure 5.22: Current trace recorded when sampling different AChE activities using the two probe system. The two probes were taped together, as shown by method A in figure 5.20 and placed in solutions containing different AChE activities. The current was recorded at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

The results show that the baseline remains very low relative to the signal due to the hydrolysis of ACh. Comparing this to the experiment conducted with one probe using similar AChE activity ranges, as shown in figure 5.17, there seems to be a much less noticeable change in baseline, suggesting that having a separate probe to deliver ACh is beneficial. To quantify this, the average of the baseline step was taken and plotted against the elapsed experimental time, presented in figure 5.23.

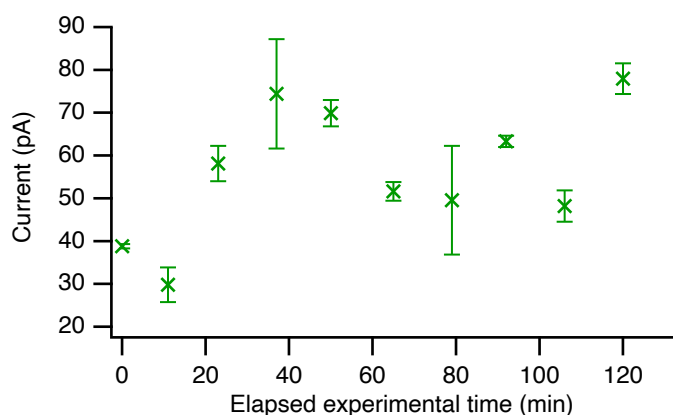


Figure 5.23: Average ACh baseline recorded during the experiment to detect different AChE activities. The results show that the baseline ACh level fluctuates between measurements. The markers and error bars represent the mean $\pm$ standard deviation of each baseline step.

While there is some fluctuation in baseline, it is on a small scale (10s of pA) relative to the signal (100s of pA), suggesting that the two probe system is beneficial in improving noise from the ACh baseline.

A differential measurement was taken between the mean of the current resulting from the AChE hydrolysis reaction and the mean of the baseline prior to that measurement. The results were plotted against AChE activity, which are shown in figure 5.24.

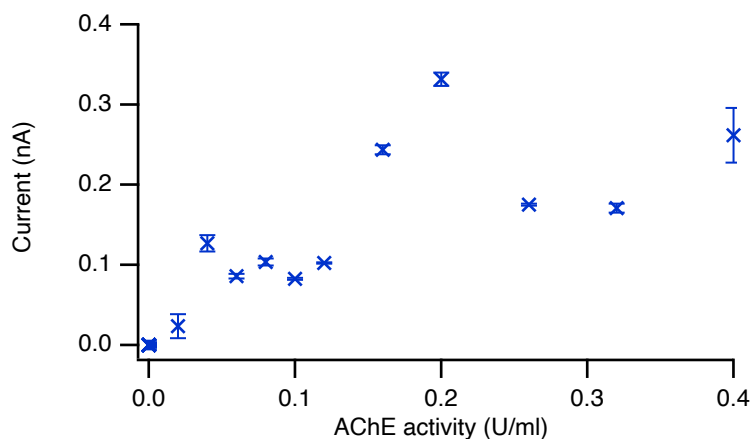


Figure 5.24: The average current obtained for different AChE activities using the two probe system. One probe was perfused with 100 mM ACh at 2 μ l/min, with the outlet going to waste. The other probe was perfused at 2 μ l/min, with the outlet connected to the analysis chip, for detection of resulting choline from ACh hydrolysis. The probe was placed in solutions containing different AChE activities. Markers and error bars represent the mean $\pm$ standard deviation of each current step.

There is some indication of an upwards trend, however, there is also some scatter in the data. Studying different parts individually does show a promising trend, for example, from 0 - 0.08 U/ml, there is a linear change in current. From 0.10 - 0.20 U/ml, there is also an increasing trend. The results at higher activities were also promising but the trend is less definitive. Even taped together, the probes flare out or stick together, which is hard to control as the membranes themselves cannot be held in place due to their fragility.

As an attempt to better control the separation of the probes and make this separation more consistent, the probes were placed through an eppendorf lid and glued in the lid, as shown by schematic C in figure 5.20. The probes taped together and the probes secured apart were then placed repeatedly in 0.20 U/ml AChE to see if the results were reproducible for either experimental setup. The results of this are shown in figure 5.25.

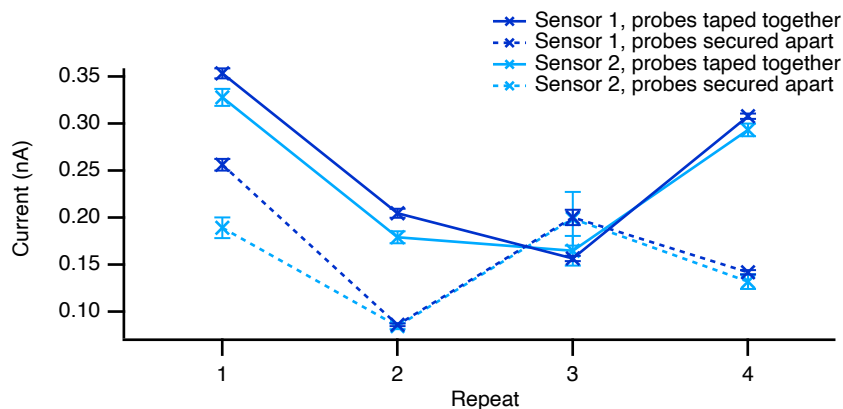


Figure 5.25: The average current obtained in when two-probe system is placed in identical solutions containing 0.20 U/ml AChE activity. There are two variations of the two-probe system used: the probes are taped together or they are secured apart. Two choline biosensors in a 3D printed chip at the outlet of the MD probe were used to record the recovered choline concentration. The dotted line shows the results when the probes are secured apart, the solid line shows the results when the probes are taped together. Dark and light blue lines represent sensor 1 and 2, respectively. The markers and error bars represent the mean $\pm$ standard deviation of each current step.

Two biosensors were used for this test, to check that the signal was consistent across the two sensors. The results show that both methods gave a large amount of variation. The mean and standard deviation were calculated for the different set ups. For the ‘glued apart’ setup, the mean current was calculated to be 0.17 ± 0.07 nA and 0.15 ± 0.05 nA for sensor 1 and 2 respectively. For the ‘taped together’ setup, the mean current was calculated to be 0.26 ± 0.09 nA and 0.24 ± 0.08 nA for sensors 1 and 2, respectively.

The experimental setup was flawed as the separation between the probes was inconsistent. Due to the probes being flexible, they could flare out or stick together, this happened in both the untaped and taped experimental setup. It was tricky to keep the same separation between the membranes even when the probes were glued apart, meaning the experiment was variable despite attempts to control parameters.

Based on these experiments, it was determined that to sample a solution in this way, the parameters need to be highly controlled. The time-dependent nature of the reactions taking place, paired with the requirement to be in an enzyme-limited region meant that creating reproducible results was tricky.

5.4 Conclusion

The aim of this chapter was to demonstrate that measurement two of the proposed detection protocol, as shown in figure 5.1, could be achieved. This measurement is detecting AChE activity by adding ACh to a sample and detecting the resulting hydrolysis product choline.

In section 5.2, I have successfully shown that by introducing AChE into a sample stream, an ACh calibration can be achieved, demonstrating that the detection of choline following ACh hydrolysis was

possible with the modified analysis system. This was further refined to perform an AChE activity calibration by holding the ACh concentration constant and measuring different AChE activities due to different amounts of ACh being hydrolysed.

These were very promising preliminary experiments. However, the goal for this device is to sample in individuals potentially exposed to OP compounds by sampling their whole blood AChE activity. MD is proposed as the sampling technique in blood to block out large, potential interferents. Initial experiments discussed in chapter 5.3.1 successfully demonstrated the ability to sample choline through MD, both directly and following ACh hydrolysis.

Following this, different AChE activities were detected via MD. Initially, ACh was dosed into a AChE-containing sample and the resulting choline was recovered by the MD probe and detected, as discussed in section 5.3.2. Different AChE activities led to different rates of ACh hydrolysis. However, as the AChE in solution continued to generate more choline, there was an ever increasing signal, meaning the differences in AChE activity could not be seen in the raw data, and it had to be analysed by looking at specific time points. This experiment did show potential as, for a given time point, there was an increasing current seen as the enzyme activity was increased. As this was achieved through MD, it would be suitable to sample blood in this way.

To further refine the experimental protocol and not have to manually dose in ACh, retrodialysis was trialled as a potential technique to deliver ACh into a solution. Using one probe to deliver ACh and pick up resulting choline showed promising results at low AChE activities, however, there was a large, interfering background signal from ACh, likely due to increasing enzyme sticking to the MD probe. As more enzyme sticks, the true enzyme activity in a solution changes, leading to inaccurate results. It also makes the system less sensitive, as it is important to be in an enzyme-limited region, but as more enzyme sticks the system moves towards a substrate-limited region.

The use of two probes was trialled in an attempt to remove the interference from background ACh; one probe was used to deliver ACh and the other probe was used to recover the resulting choline. This improved the background signal from ACh, however, results were not repeatable and the system was highly dependent on factors including the separation of the probes.

One challenge with delivery of ACh through retrodialysis was that it was difficult to deliver enough ACh into the solution to see a difference in enzyme hydrolysis rates. Because of this, the solutions were not stirred to avoid dilution of the ACh. The rates of diffusion of molecules in and out of the probes are highly dependent on the concentration gradients, and as the solution was not stirred, this became highly dependent on how far the probes were from one another and led to inconsistent results. It is possible that repeatable results could be achieved by placing the two probes in a stirred beaker and changing the flow rate of the delivery probe to deliver more ACh in solution. However, it was important to create a more robust method and resolve stable signals so I decided to move away from this method.

It is clear that these experiments need to be performed under highly controlled conditions due to the time-dependent nature of the reactions taking place. The goal of this device is for use in continuous and real-time monitoring. The experiments presented in this chapter have not yet achieved this. The single probe experiment, presented in section 5.3.2, in which ACh was delivered externally presented the most potential for probing changes in AChE activity. However, the difference in hydrolysis rates produced by different AChE activities could not be seen in the raw data due to ever-increasing signals. A situation in which there is a limited reaction time, as shown in section 5.2.3, that also incorporates MD for sampling in complex media is needed.

Chapter 6

Development of a microfluidic flow cell

6.1 Introduction

Following the microdialysis (MD) experiments in a beaker described in chapter 5, it became apparent that due to the time-dependent nature of the reactions taking place, it is important that experiments are done in a highly controlled environment. Furthermore, acetylcholinesterase (AChE) is a very active enzyme which means it will turn over a large number of acetylcholine (ACh) molecules, therefore it is desirable to have a system where it is possible to control how long the AChE and the ACh have to interact. This will avoid the issues seen in section 5.3.2 of having to look at specific time points or at the rate of reactions, which is not ideal as it adds to the post-processing.

The proposed solution is to have a flowing device to control the final AChE activity that replicates the AChE calibration performed using the microfluidic platform, as presented in section 5.2.3. An additional requirement was to incorporate MD into the analysis system to allow sampling of complex media, such as blood, therefore the device needs to be developed to enable this.

The proposed solution is a flow cell, where the solution flows through a channel in which a MD probe is placed. As the solution flows past the MD probe, any molecule small enough to diffuse across the semi-permeable membrane, below the molecular weight cut-off (MWCO), will do so. The outlet of the MD probe is connected to the analysis system containing the choline biosensor, as described previously for the microdialysis experiments in chapter 5.

In this chapter I discuss two iterations of the flow cell design and characterising the flow cell. The flow cell was used for a series of experiments designed to understand the dynamics between AChE and ACh and to determine different AChE concentrations, the results of which are presented in chapter 7.

6.2 Flow cell design

The flow cell design consists of two components, the main body and the MD probe holder. I designed the two parts of the flow cell in SolidWorks (Dassault Systèmes, France). The printing was carried out by PhD student Chiara Cicatiello; further details of the printing are given in the relevant sections.

The main body consists of the channel through which the solution will flow. The MD probe holder is inserted into the flow cell body so the MD probe is held securely in place and remains in the same position throughout the experiment. The flow cell was designed for a CMA 12 MD animal probe, with a MWCO of 20 kDa and membrane length of 4 mm. This probe was chosen due to its rigidity and small size. The rigidity meant that it would not move in the channel and the small size enabled the flow cell to be compact and the channel diameter to be minimised. The probe is shown in figure 6.1 with the important dimensions highlighted.

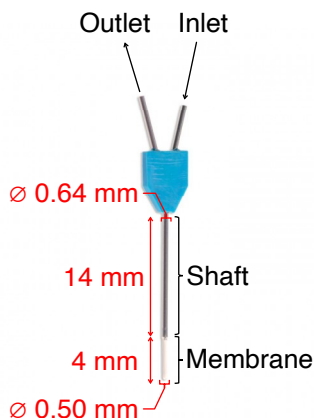


Figure 6.1: CMA 12 MD animal probe with important dimensions highlighted. The shaft and membrane diameter are 0.64 and 0.50 mm, respectively and the total length of the shaft + membrane is 18 mm. These dimensions were considered when designing the flow cell.

6.2.1 Microdialysis probe holder

The MD probe holder was designed with the aim to hold the CMA 12 animal probe securely in place and minimise movement of the MD probe in any direction. The MD probe holder consisted of a top and a bottom that slotted into each other around the probe. Figure 6.2 shows drawings of these two parts. There is a cut out in the two parts that is the same shape as the MD probe, to restrict sideways movement. To prevent the MD probe from falling out of the back of the MD probe holder, a triangular extruded wall was inserted. These design features were to limit movement of the MD probe inside the holder. To prevent the probe membrane from becoming damaged on insertion into the flow cell, the probe holder was designed so that it forms a tight fit with the flow cell body so that the holder itself cannot move sideways. The shelving on the side of the MD probe holder aligns with slots in the flow cell body to ensure that the MD probe will slot into the channel without touching any surface and possibly being damaged.

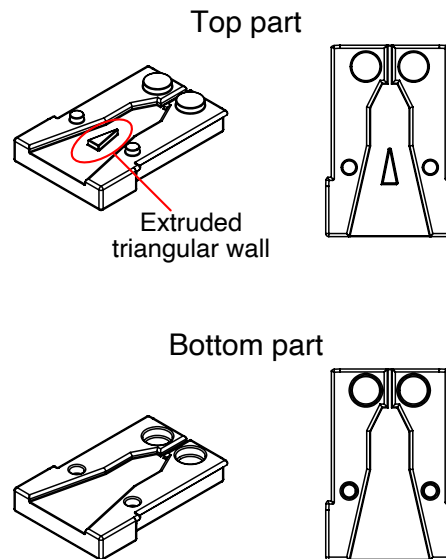


Figure 6.2: Drawings of the two parts of the MD probe holder. The top part of the MD probe holder, which consists of an extruded triangular wall to prevent the MD probe from moving out of the back of the holder. It also consists of extruded circular parts to fit into corresponding indentations in the bottom part of the MD probe holder. Once slotted together, the MD probe holder has a thickness of 7.80 mm, a width of 16.80 mm at the widest part and a length of 25.10 mm.

The MD probe holder was 3D printed using the ProJet MJP 2500 printer with Visijet M2S-HT90 material (3D systems, US). Figure 6.3 displays photos of the finished printed MD probe holder. The photos shown with the CMA 12 probe inserted demonstrate that the probe fits well in the holder.

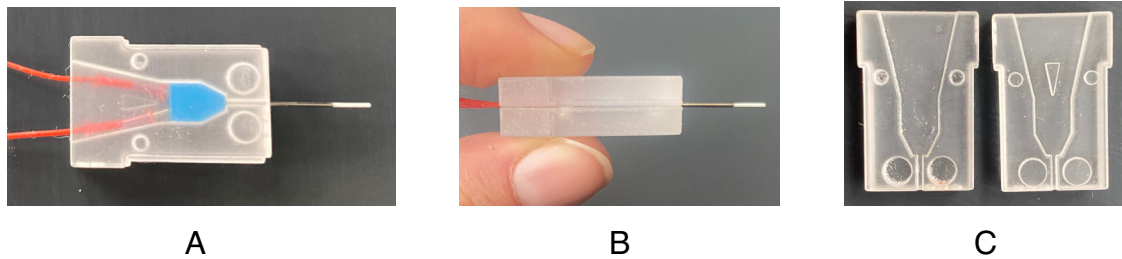


Figure 6.3: Photos of the MD probe holder. **A.** Top view of the two parts of the MD probe holder slotted together with the MD probe inserted. **B.** Side view of the two parts of the MD probe holder slotted together with the MD probe inserted. **C.** The top and bottom parts are shown.

6.3 Main body

This section describes the overall design considerations for the main body of the flow cell.

The main body of the flow cell was also designed in SolidWorks. It was designed to allow connection to the microfluidic platform described previously to enable easy manipulation of the solution flowing into the flow cell. To facilitate this connection, a CapTite C360-400 bonded-port connector (LabSmith, US) was used as the inlet, which pairs with 360 μm tubing. The outlet of the CapTite bonded-port connector is 250 μm diameter, therefore the flow cell channel size was chosen to be 300 μm diameter. It was slightly larger to allow for any alignment errors when inserting the bonded-port connector into the flow cell.

The channel size of the flow cell was then slowly increased from 300 μm to create a larger channel using a fillet. The slow increase was to ensure there were no unswept parts of the channel volume. The larger channel size was necessary to allow the MD probe (0.64 mm shaft diameter, 0.50 mm membrane diameter) to be inserted into the flow cell without risk of damage. It was also important to minimise the channel as much as possible to improve mixing of the solution and time response, while still maintaining a channel large enough to avoid damaging the MD probe.

A waste outlet channel was added so that the solution would have an easy path to exit the flow cell. This meant the solution would drip down from the main body into a container which could then be disposed of appropriately, rather than the solution leaking from the end of the main body. This would be an important consideration when handling human samples as well as samples containing organophosphorus compounds. As the main body and MD probe holder were designed to be a tight fit, another benefit to having a waste outlet was to prevent pressure build-up where the main body and the MD probe holder meet as otherwise this may influence the flow rate of the solution.

An example of the printed flow cell is shown in figure 6.4. This is design 1 of the flow cell, printed using ProJet MJP 2500 printer with Visijet M2S-HT90 material (3D systems, US). The key components are highlighted.

Following the initial design of the flow cell, which was tested and characterised, the flow cell was optimised further. The two designs are summarised below with details of specific dimensions.

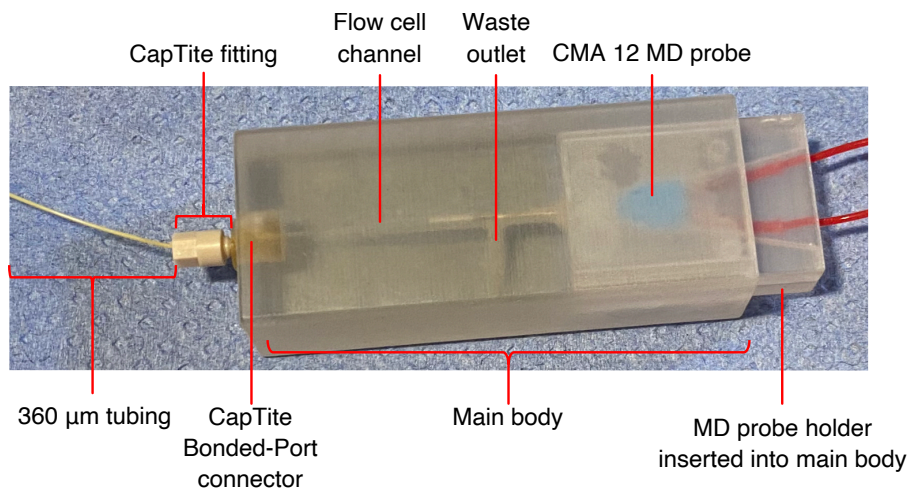


Figure 6.4: A labelled photo of design 1 of the flow cell. The flow channel and waste outlet can be seen through the transparent flow cell. The MD probe is placed in the holder and inserted into the flow cell. The CapTite bonded-port connector (LabSmith) has been inserted and glued to form the inlet of the flow cell. A CapTite fitting (LabSmith) and 360 μm outer diameter PEEK tubing is attached.

6.3.1 Design 1

The first design of the flow cell contained a 300 μm diameter channel increasing to a 1500 μm diameter channel over 10.71 mm. The length of the 1500 μm diameter channel was chosen as 16.99 mm. These parameters were initially chosen to allow the probe, which has a shaft diameter of 0.64 mm, to be safely inserted into the flow cell without risk of damaging the sides of the membrane or the tip of the membrane. The parameters were also chosen to ensure that the membrane was in a well mixed part of the solution. The volume of the flow cell channel was calculated approximately by considering the length of the channel up to the end of the membrane probe. For design 1, the channel volume was calculated to be approximately 21.2 μl .

The drawings of design 1 are shown in figure 6.5. A photo of the 3D printed flow cell was shown previously in figure 6.4.

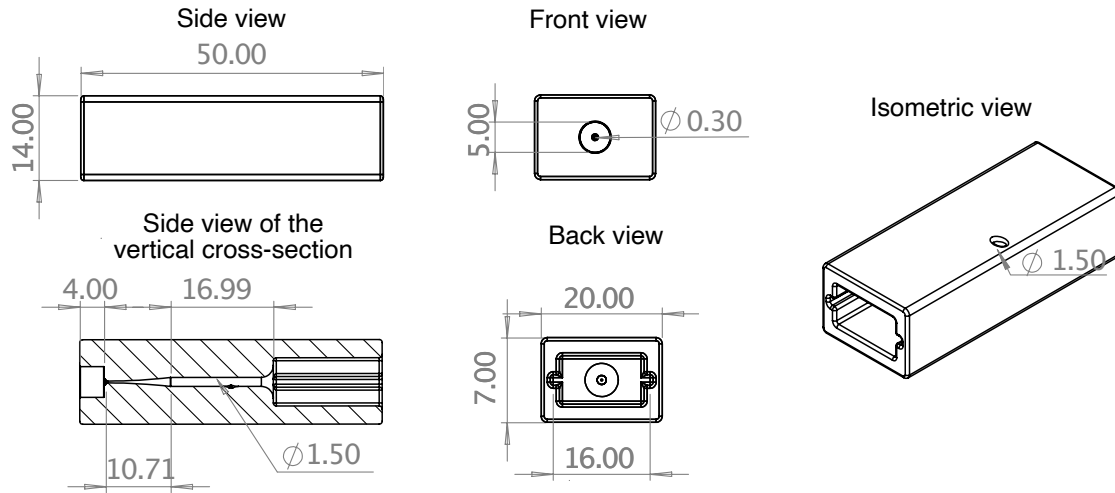


Figure 6.5: Drawings of the main body of flow cell design 1. Different views of the flow cell are shown. The dimensions given are in mm. The isometric view shows the waste outlet which has the same dimension as the main internal channel of the flow cell.

6.3.2 Design 2

In the second design, the internal volume of the flow cell was reduced further with the aim of improving the flow cell characteristics such as the response time. After design 1 was printed and tested, it became apparent that the channel diameter and length could be reduced further without risking damage to the probe as there was excess space around the MD probe and shaft. The diameter of the main channel was reduced to 1200 μm and the length of the main channel was reduced to 12 mm. The length over which the channel diameter was gradually increased from 300 μm to 1200 μm was kept the same (within 0.01 mm) at 10.70 mm. For design 2, the channel volume was calculated to be approximately 7.9 μl . The reduction in volume from design 1 was therefore 13.3 μl . This flow cell was printed with a ProJet MJP 2500 printer with Visijet M2S-HT250 material (3D systems, US). A different material was used here due to availability in the lab, however the properties were very similar.

Drawings of different orientations of design 2 are shown in figure 6.6, with dimensions given in mm.

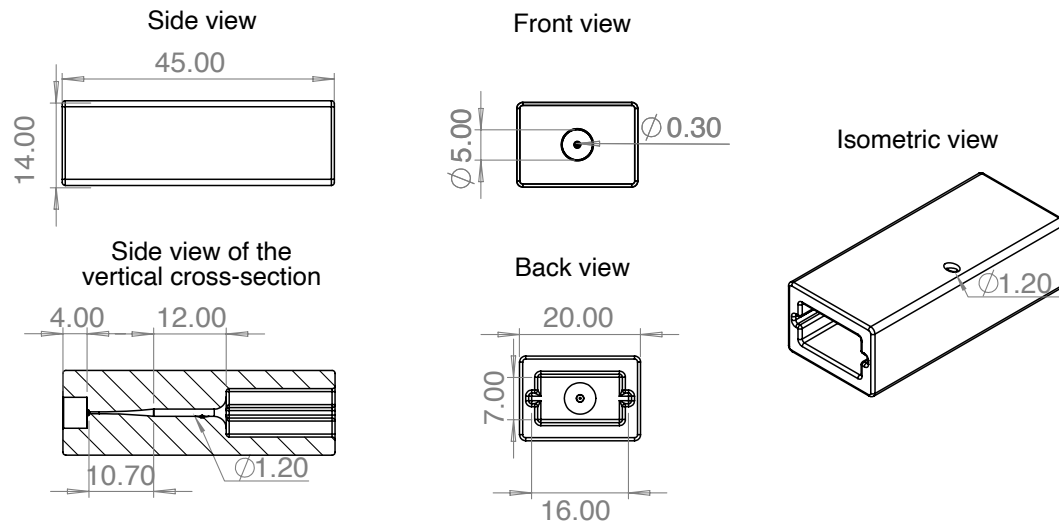


Figure 6.6: Drawings of design 2 of the flow cell main body. Different views of the flow cell are shown, with dimensions given in mm.

Photos of this design are shown in figure 6.7.

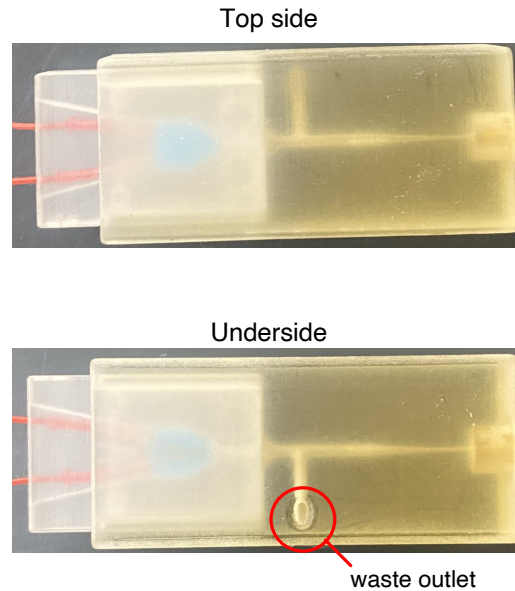


Figure 6.7: Photos of design 2 of the flow cell. The top side and underside of the flow cell are shown, printed with Visijet M2S-HT250 material (3D systems, US). The waste outlet can be seen from the underside. The MD probe holder, printed with Visijet M2S-HT90 material (3D systems, US), is placed in the holder and inserted in the flow cell main body.

6.4 Flow cell characterisation

In this section two flow cell designs are characterised in terms of the time response and the recovery of the analyte in solution. The time response was characterised using the time taken to reach 90% of a signal following a step concentration change, referred to as the T_{90} response time. The recovery of the analyte in solution was defined as the % of analyte in the dialysate output stream, compared to the external analyte concentration in the flow cell.

6.4.1 Experimental setup

As the flow cell has a relatively large volume, it was important that the flow rates used here were higher than used previously in this thesis otherwise there would be poor temporal resolution. In addition, as this device will ultimately be used for detection of AChE, it is beneficial to limit the reaction time between AChE and ACh, as it will ensure reactions are kept within an enzyme-limited region; this can be achieved by using higher flow rates. This increase in flow rate meant larger volume syringes were needed to allow the calibration steps to be run for a sufficient amount of time, and larger reservoirs were used to account for this change in volume of solution being used, otherwise the solution would need to be topped up frequently. Therefore, to perform these experiments, the microfluidic board was configured using 100 μ l syringes and 5 ml reservoirs instead of the 20 μ l syringes and 1 ml reservoirs used previously.

The outlet of the microfluidic platform was connected to the flow cell. A schematic showing the microfluidic platform setup for these characterisation experiments is shown in figure 6.8.

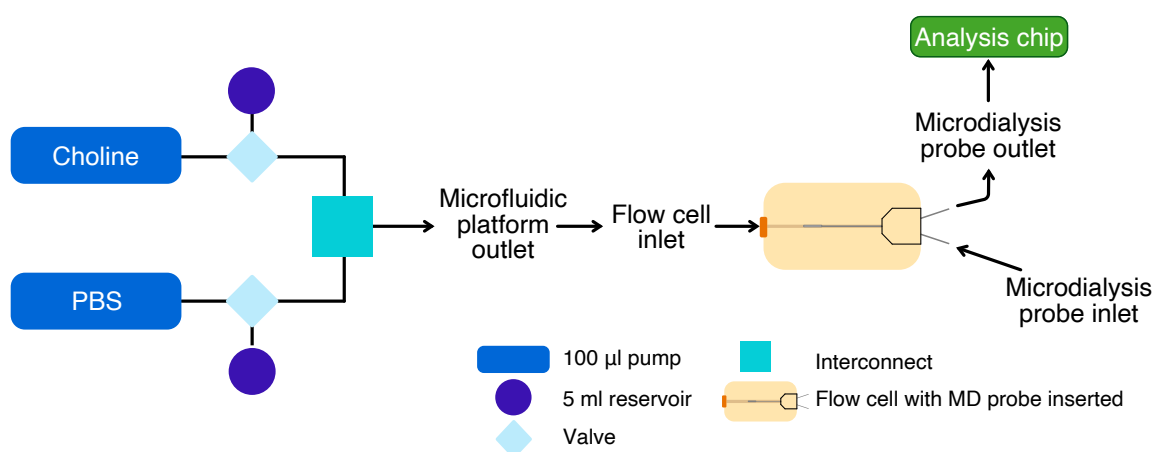


Figure 6.8: Schematic showing the microfluidic platform setup to characterise the flow cell. The microfluidic platform is configured using larger volume pumps, 100 μ l, and reservoirs, 5 ml, than previously used to enable a higher flow rate to be used for the flow cell, as it has a larger volume than the analysis chip.

To perform the characterisation experiments, choline was used as the analyte in the solution. While the aim is to use the flow cell to detect changes in AChE activity, the direct use of choline allowed simple interpretation of the flow cell characteristics. The choline biosensor was used as the detection mechanism, therefore, only two pumps were needed to vary the concentration of choline at the flow cell.

The flow rates of the pumps were varied to change the final concentration of choline at the outlet of the microfluidic platform and therefore at the inlet of the flow cell. There are two flow rates that were important in these experiments, which are highlighted in figure 6.9.

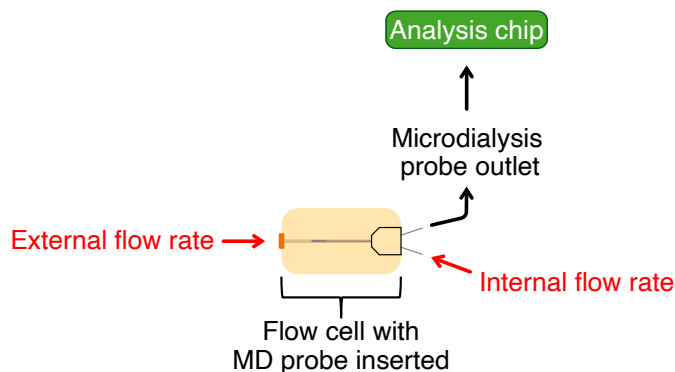


Figure 6.9: Schematic highlighting the two flow rates used to perform experiments with the flow cell coupled with MD. The external flow rate defines the flow rate of solution going into the flow cell from the microfluidic platform. The internal flow rate defines the flow rate of the perfusate in the microdialysis probe, which in turn, defines the flow rate going into the analysis system.

The flow rate of the perfusate in the MD probe, referred to as the internal flow rate, was held constant at 2 $\mu\text{L}/\text{min}$. The internal flow rate also defines the flow rate to the analysis chip as the chip is connected to the outlet of the MD probe. The flow rate of the solution being pushed through the flow cell and around the MD probe is referred to as the external flow rate. The external flow rate was varied to determine the best flow rate for probe recovery and response time, T_{90} .

To determine the time response of the flow cell and the probe recovery, a 3-point choline calibration up to 100 μM was performed. The different external flow rates trialled were 5, 10, 15 and 20 $\mu\text{L}/\text{min}$. To determine the probe recovery, a standard choline calibration up to 100 μM was also performed with the choline biosensor analysis system directly connected to the microfluidic platform, as described by figure 2.13, with a flow rate of 2 $\mu\text{L}/\text{min}$. This calibration was used to convert the flow cell current data into actual choline values by multiplying the current by the choline biosensor sensitivity. This gave the probe recovery.

6.4.2 Time response of the flow cell

To determine the time response of the flow cell, the T_{90} response times for both flow cell designs at different external flow rates were determined using the a step change in concentration from 0 to 50 μM choline. The internal flow rate was held constant at 2 $\mu\text{l}/\text{min}$.

Design 1

The normalised current responses recorded using design 1 of the flow cell are shown in figure 6.10.

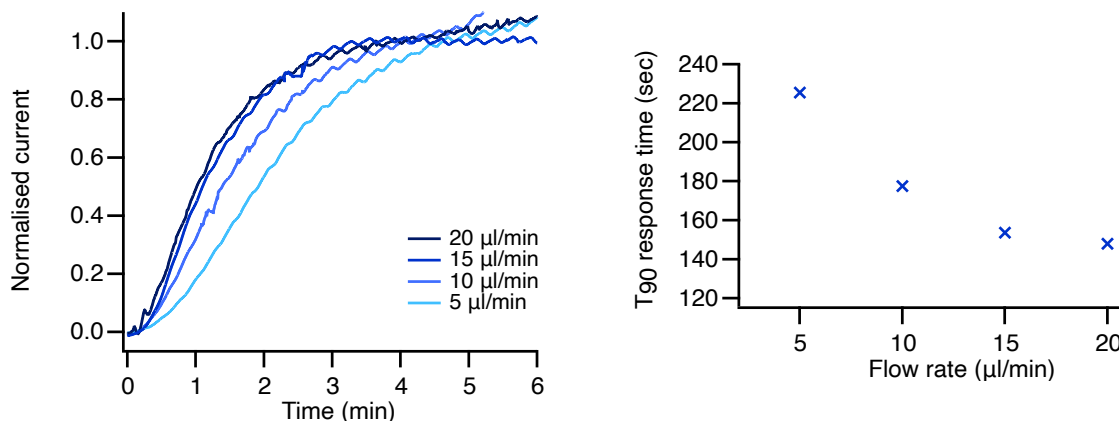


Figure 6.10: Comparison of T_{90} response times for different experimental conditions using the flow cell, design 1. Left: Normalised current response for a 50 μM choline step change for different flow rates. Right: The T_{90} response times shown against the corresponding flow rate.

The normalised current responses show that increasing the external flow rate improves the T_{90} value. By flowing faster down the flow cell, we reach a steady state across the probe sooner. The T_{90} shows a large improvement from an external flow rate of 5 $\mu\text{l}/\text{min}$ to 10 $\mu\text{l}/\text{min}$. At higher flow rates, the T_{90} appears to be reaching a plateau, suggesting that the T_{90} has reached a point where it is not limited by the external flow rate anymore. Rather, it seems that the T_{90} is now limited by the flow rate of the MD probe and the length of tubing between the MD probe outlet to the analysis chip. Flexible connection “trumpets” were used to connect the MD probe to 1/32” tubing which also creates some extra volume as it is not possible to have perfect alignment between the tubing and the MD probe outlet. It is possible that they connections are also limiting the T_{90} values.

Design 2

The normalised current responses recorded and resulting T_{90} response times using design 2 of the flow cell are shown in figure 6.11.

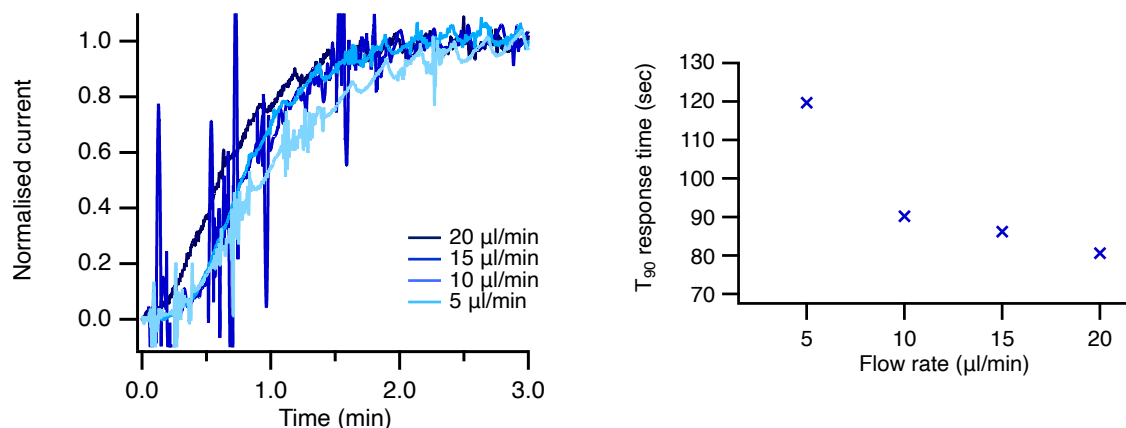


Figure 6.11: Comparison of T_{90} response times for different experimental conditions using the flow cell, design 2. Left: Normalised current response for a 50 µM choline step change for different flow rates. Right: The T_{90} response times shown against the corresponding flow rate.

The normalised current responses show that increasing the external flow rate improves the T_{90} value in agreement with the results obtained using design 1 of the flow cell. The response times follow a trend similar to that observed with design 1; changing the flow rate from 5 µl/min to 10 µl/min results in a large drop and therefore improvement in response time. Following this large drop, the response time then does not have a large dependence on the external flow rate. The trace in 6.11 is noisy compared to the trace in 6.10, this may be due to differences in noise between the sensors used, likely caused by electrical connections, and may also be due to the external lab environment as these experiments took place on different days.

The T_{90} response time for a 25 µM concentration step change of the choline biosensor when the analysis system was directly connected to the board (not through the flow cell) was calculated to be 58.8 s for a flow rate of 2 µl/min, to match the internal flow rate through the probe. This is only 21.8 s less than the T_{90} response time obtained for design 2 of the flow cell with 20 µl/min flow rate, which is very promising as it suggests that design 2 of the flow cell is well optimised.

6.4.3 Probe recovery

To determine the probe recovery for the different experimental conditions, the choline calibrations obtained using the flow cell were compared to the standard choline calibration obtained when the microfluidic platform was connected directly to the analysis chip. As the characterisation of the two flow cell designs were performed at different times, a different choline biosensor was used to perform the two characterisation experiments. The experimental setup and resulting choline calibrations obtained from the two different biosensors are shown in figure 6.12.

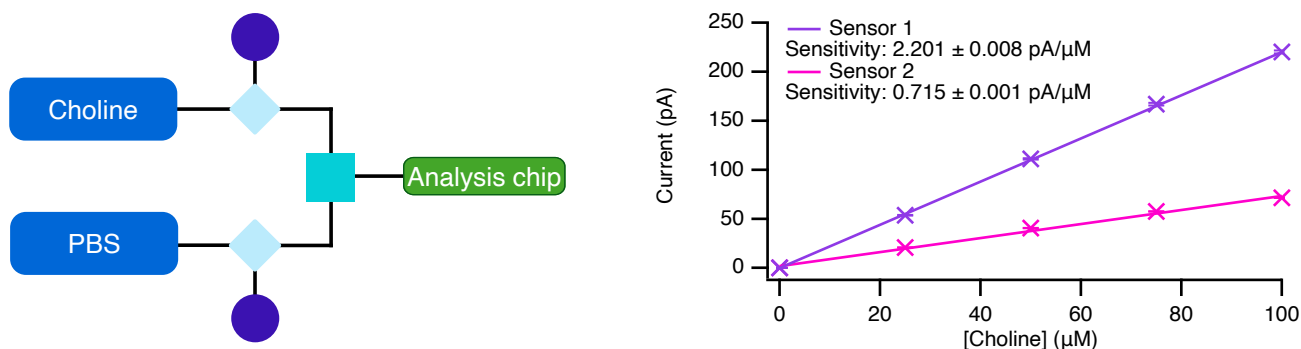


Figure 6.12: Experimental setup and resulting curve for a standard choline calibration. Left: Microfluidic platform setup to perform a standard choline calibration. Right: The resulting calibration curves and resulting sensitivity obtained for a choline calibration up to 100 μM for the biosensors used for the two characterisation experiments. Sensor 1 was used for the characterisation experiments with design 1. Sensor 2 was used for characterisation experiments with design 2. The markers and error bars represent the mean $\pm$ standard deviation for each concentration step. Linear regression has been used to fit the curves, the error on the sensitivities is given by the 95 % confidence interval of the regression.

To process the data obtained using the two flow cell designs the calibration curves were plotted in terms of current by calculating the average at each concentration step. The current was converted to the equivalent choline concentration by dividing the currents by the sensitivity of the relevant choline biosensor as shown in figure 6.12. The error on the calculated choline concentration was computed using error propagation, using the standard deviation of the average current of each concentration step and the error on the sensitivity of the choline biosensor found from the 95% confidence interval of the regression.

By calculating the measured choline in the dialysate, the resulting recovery of the probe can be calculated for the given experimental conditions.

Design 1

The measured choline concentrations in the dialysate for the different external flow rates of the flow cell printed using design 1 are shown in figure 6.13.

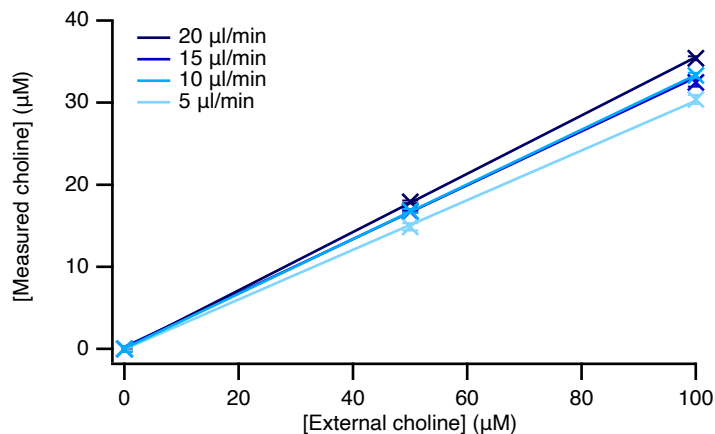


Figure 6.13: The measured choline concentration in the dialysate stream for different external flow rates for the flow cell printed using design 1. This data was used to determine the % probe recovery. The measured choline was calculated using the calibration shown in figure 6.12 for sensor 1. The markers and error bars show the average of each concentration step and the propagated error. Linear regression was used to fit the data.

Linear regression was used to fit the data, and the gradient was multiplied by 100 to determine the percentage recovery of the MD probe. A summary of the probe recovery % is given in table 6.1 for each flow rate.

External flow rate (μl/min)	Probe recovery (%)
5	30.2 ± 0.5
10	33.3 ± 0.5
15	32.8 ± 0.6
20	35.5 ± 0.3

Table 6.1: The probe recovery % for different external flow rates determined when using design 1 of the flow cell. The internal flow rate was held constant at 2 μl/min. The values were calculated using linear regression to fit the data shown in figure 6.13, the error is given by the 95 % confidence interval.

Results showed that, whilst 100% recovery was never reached, increasing the external flow rate and thus the ratio of external to internal flow rate, increased the probe recovery. This will likely be due to increased delivery of analyte past the probe. The reason for not reaching 100% recovery may be due to the short MD membrane length of 4 mm. While good probe recovery is desirable, 100% probe recovery may not be beneficial when sampling large concentrations of choline as there is risk that the choline biosensor could become saturated. This makes the probe recovery a potentially useful tuning factor for the system design.

Design 2

The measured choline concentrations for the different external flow rates of the flow cell 3D printed using design 2 are shown in figure 6.14.

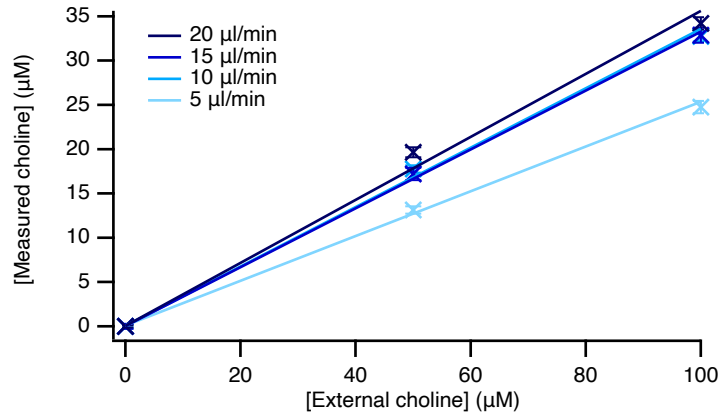


Figure 6.14: The measured choline concentration in the dialysate stream for different external flow rates for the design 2 of the flow cell. This data was used to determine the % probe recovery. The measured choline was calculated using the calibration shown in figure 6.12 for sensor 2. The markers and error bars show the average of each concentration step and the propagated error. Linear regression was used to fit the data.

The resultant % probe recovery was calculated by fitting the data using linear regression and multiplying the gradient by 100. The probe recoveries for the different flow rates when using design 2 of the flow cell are shown in table 6.2.

External flow rate (μl/min)	Probe recovery (%)
5	25.3 ± 0.7
10	33.5 ± 0.5
15	33.2 ± 0.8
20	35.6 ± 0.6

Table 6.2: The probe recovery % for design 2 of the flow cell for different external flow rates. The internal flow rate was held constant at 2 μl/min. The values were calculated using linear regression to fit the data shown in figure 6.14, the error is given by the 95% confidence interval.

The probe recovery was found to improve slightly when the external flow rate was increased, in agreement with the results from design 1. The results obtained for design 2 are very similar to those obtained for design 1, with a slightly more noticeable change from an external flow rate of 5 μl/min to higher flow rates. Once the linear flow rate across the membrane is sufficient to ensure the bulk concentration is available in the external environment to the membrane, then the device becomes limited by the flux through the membrane. That in turn, becomes limited by the length of membrane and internal flow rate, for a given type of membrane. Hence why the two designs show the same recovery. For these experiments, it seems that at higher flow rates that the system has become limited by the flux through the membrane.

6.5 Conclusion

This chapter presented the successful development of a microfluidic flow cell that can connect to the microfluidic platform and allows the incorporation of MD as the sampling technique. The microfluidic platform was adapted to have larger syringes (100 μl) and reservoirs (5 ml) and was successfully used to perform choline calibrations up to 100 μM . These calibrations demonstrated that the microfluidic flow cell coupled with MD and the adapted microfluidic platform can be used as a flowing system to allow the sampling of small analytes below the MWCO of the MD probe. Two versions of the flow cell were designed and characterised in terms of the probe recovery and response time, T_{90} .

These flow cell characterisation experiments show that decreasing the internal volume of the flow cell channel helped to improve the T_{90} response time of the system. The probe recovery benefited slightly from increasing the external flow rate in the flow cell, although recovery was ultimately limited by the membrane length and perfusion rate of the probe.

Results from both designs demonstrated that a low flow rate of 5 $\mu\text{l}/\text{min}$ led to a relatively large T_{90} response time and a slightly lower probe recovery. Noticeable changes were seen when increasing the external flow rate from 5 to 10 $\mu\text{l}/\text{min}$, with more limited improvement observed in the T_{90} and probe recovery when increasing the flow rate from 10 to 20 $\mu\text{l}/\text{min}$.

A limiting factor in the flow cell experiments going forward is that the syringes have a restricted volume. The syringes used for the flow cell experiments have a maximum volume of 100 μl . As previously shown in chapter 5, figure 5.7, refilling the syringes causes flow artefacts which is seen in the signal and means that part of the signal cannot be used in the analysis, so it is better to avoid these refill artefacts where possible. Given the need for long steps due to the large T_{90} values obtained when using design 1, it is important to use a flow rate that allows for a long continuous step where the pump volume cannot run out. Preliminary experiments using this flow cell to detect changes in AChE activities in chapter 7 used a total external flow rate of 10 $\mu\text{l}/\text{min}$ and design 1 of the flow cell. For design 1, the lower flow rate of 10 $\mu\text{l}/\text{min}$, which still has a relatively low T_{90} value and good probe recovery, is optimal to allow for long enough calibration steps without the need to refill syringes mid-step.

Final experiments to perform AChE calibrations, discussed further in chapter 7, used the flow cell printed using design 2 and a total external flow rate of 20 $\mu\text{l}/\text{min}$. Design 2 of the flow cell meant the steps did not have to be as long, as demonstrated by the lower T_{90} response time of approximately 80 s at 20 $\mu\text{l}/\text{min}$, therefore a faster flow rate could be used without the need for refilling. An added benefit to a faster flow rate is that the enzyme less time to react with the substrate. This was optimal as it is necessary to be in a region where not all of the substrate is being converted by the enzyme to see changes, particularly small changes, in enzyme activity. The benefit of this is discussed further in chapter 7.

Chapter 7

Detecting changes in acetylcholinesterase activity using the flow cell

7.1 Introduction

The development of the flow cell introduced in chapter 6 has created a flowing system in which reactions can be controlled by adjusting the various flow parameters. Furthermore, it allows sampling using microdialysis (MD), which can be separately controlled and will in turn allow sampling of complex medias. Following the experimental attempts described in chapter 5 to monitor changes in acetylcholinesterase (AChE) activity while sampling using microdialysis, the aim of this chapter is to sensitively detect changes in AChE activity in a range similar to that observed in plasma and blood.

Figure 7.1 shows a schematic demonstrating how the flow cell and MD will be used to determine AChE activity in a sample.

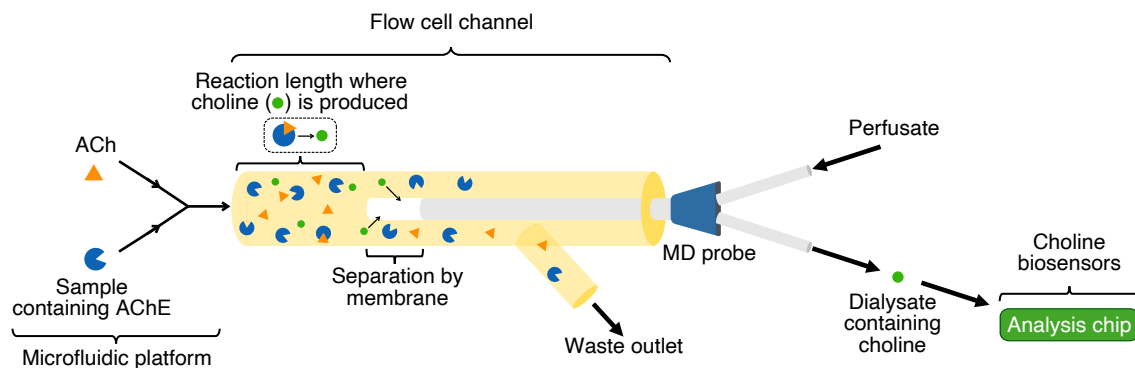


Figure 7.1: An overview of the detection mechanism to determine AChE activity using the flow cell. The ACh and an AChE-containing sample are combined using the microfluidic platform and mixed together in the reaction length of the AChE activity flow cell channel, where there is a limited time for the ACh to be hydrolysed by AChE into choline. The choline produced will be sampled by the MD probe, where it flows to the analysis chip housing the choline biosensors downstream. The amount of ACh hydrolysed and therefore the amount of choline produced is dependent on the AChE activity.

The schematic shows that the AChE-containing sample and acetylcholine (ACh) are mixed together using the microfluidic platform. In the flow cell channel, the length of which is controlled, the ACh and AChE react to produce choline, which diffuses into the membrane probe. In practice, not all of the choline will be recovered by the MD probe and some will flow out of the waste outlet. Some of the ACh will also be recovered by the MD probe and flow to the analysis system.

This chapter will begin by using the flow cell to detect changes in low level AChE activity before moving towards detecting higher AChE activity, closer to what is expected in the blood.

7.2 First approach to monitoring changes in AChE activity

My initial experiments used the flow cell 3D printed using design 1 from chapter 6.

7.2.1 Experimental setup

As stated in chapter 6, the flow cell was designed to connect to the microfluidic platform to allow easy manipulation of the solution at the inlet of the flow cell. To probe changes in AChE activity, three pumps were necessary: a PBS pump, an ACh pump and an AChE pump. The microfluidic platform configured for this purpose is shown in figure 7.2.

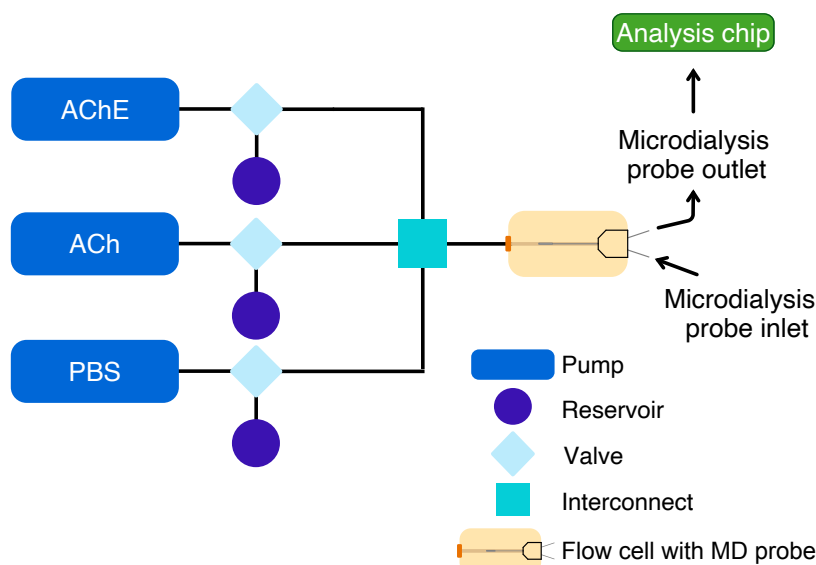


Figure 7.2: Microfluidic platform configured to detect different AChE activities by utilising the flow cell and sampling with microdialysis. The schematic shows the three different pumps required for this purpose - an AChE pump, an ACh pump and a PBS pump. The ACh pump flow rate is held constant at 50% of the total flow rate. The combined flow rate of the PBS and AChE pumps is always 50% of the total flow rate. The AChE and PBS pump flow rates are varied to change the activity of AChE at the flow cell. A different amount of ACh will be hydrolysed for a given AChE activity.

The ACh pump flow rate is held constant, to deliver a constant concentration of ACh into the flow cell. The AChE pump and PBS pump flow rates are varied such that the AChE activity in the flow cell is varied. The combined flow rate of the two pumps remains constant. A different AChE activity will result in a different amount of ACh being hydrolysed, given that the experimental parameters are in an enzyme-limited region. The ACh flow rate is always 50% of the total flow rate, and the PBS and AChE pumps combined flow rate is 50% of the total flow rate.

As the ACh stream is always needed to detect AChE activity in a sample, any sample will be diluted by the ACh flow stream. Therefore, the quoted AChE activities for the calibrations in this chapter are the resulting activities at the outlet of the AChE and PBS pumps, before the dilution by ACh, rather than the AChE activities in the flow cell.

7.2.2 Detecting low AChE activities

To initially detect changes in AChE activity, low AChE activities were investigated. A 3-step calibration was performed. The AChE values for calibration purposes are 0, 0.5 and 1.0 U/ml. The final AChE activities at the flow cell being 0, 0.25 and 0.5 U/ml (factoring in the dilution). The flow rate of the ACh was held constant at 5 $\mu\text{l}/\text{min}$, and the ACh concentration at the flow cell (factoring in the dilution) was 1 mM. The flow cell used for these experiments was design 1, which has a volume of approximately 21.2 μl . The total flow rate was held constant at 10 $\mu\text{l}/\text{min}$. Given the flow cell volume, this results in a reaction time of approximately 2.12 minutes. Therefore for 0.25 U/ml which

corresponds to $0.25 \mu\text{mol}/\text{min}/\text{ml} = 0.25 \text{ mM}$ of ACh being converted per minute, approximately 0.53 mM of ACh should have been converted to choline by the time the flow reaches the end of the membrane. For $0.5 \text{ U}/\text{ml}$ AChE (equivalent to 0.5 mM of ACh being hydrolysed per minute), there should be 1.06 mM of ACh converted, so it would be expected that approximately all of the ACh in the flow cell (1 mM) will be converted.

Calibrations were initially performed with an ACh ‘baseline’, that is, the PBS and ACh pumps are mixed together in a 1:1 ratio and no AChE is added. The baseline was then changed to just PBS, to determine if and how big the background signal from ACh is. The resulting current trace is shown in figure 7.3.

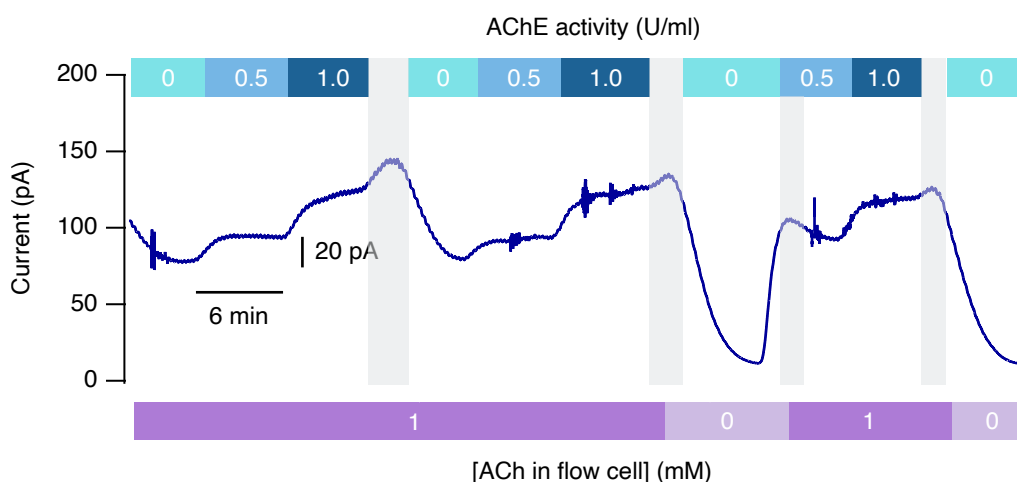


Figure 7.3: Current trace recorded for different AChE activities. Three cycles of the calibration are shown. For the first two calibrations, ACh was run through the board continuously and the background signal from ACh with $0 \text{ U}/\text{ml}$ AChE activity was recorded. For the final calibration shown, the background signal with no ACh added was recorded. This demonstrates that a large current is recorded from ACh itself. The 3 calibrations show the same activity steps are reproducible between repeats. They greyed out areas represent flow artefacts in the signal, where the calibration cycle begins again and pumps refill. The current was recorded at 200 Hz . The trace has been smoothed using a 201-point Savitzky-Golay filter.

As shown by the current trace, there are clear differences in the signal obtained for different AChE activities, a very promising result. Also shown are clear, stable steps for the different activities, which is something that was not observed in the microdialysis beaker experiments presented in chapter 5. This demonstrates that the flow cell is fit for purpose - the reaction is nicely controlled and as it is a flowing system, the probe is sampling a constant concentration of choline.

The change in baseline observed when switching from the 1 mM ACh baseline to just PBS shows there are still large signals observed from background levels of ACh, this must presumably be from enzyme sticking to the flow cell or MD probe membrane, or a combination of both.

The average of each current step was taken, shown in figure 7.4, with the baseline subtracted off each signal.

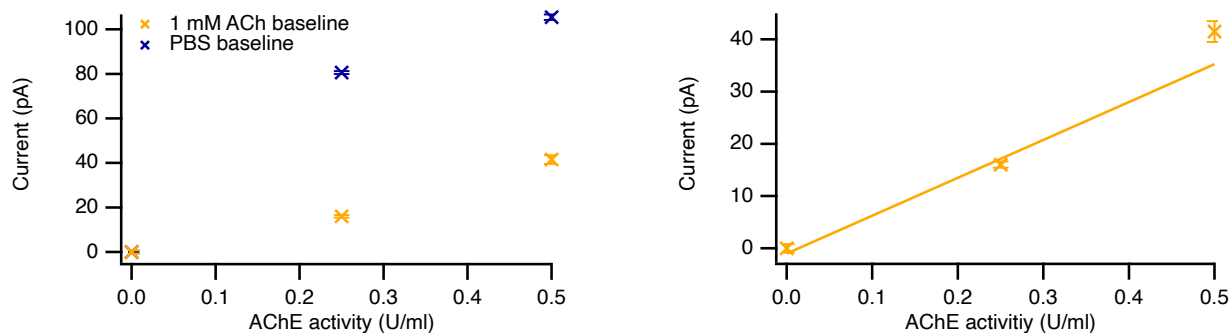


Figure 7.4: The recorded currents for different AChE activities obtained using the flow cell. Left: The average signal for each resulting current step was taken, two different baselines were considered as ‘zero’ and subtracted off the signal: the PBS baseline and the ACh baseline. Note, there are is a 0,0 point for both sets of data, the points overlap each other. Right: The calibration obtained by subtracting off the ACh baseline. Linear regression was used to fit this data. The markers and errors bars of both graphs show the mean $\pm$ standard deviation for each current step shown in the trace in figure 7.3

The left graph shows the calibration curves considering the two possible options for baseline, either using the ACh baseline as as the ‘zero’ signal (yellow) or using the PBS baseline as the ‘zero’ signal (blue). When the PBS baseline is considered as ‘zero’, the calibration is non-linear and when the ACh baseline is considered as ‘zero’, the calibration is linear, as shown by the right graph of figure 7.4, fitted using linear regression.

It was important to consider which baseline was truly representative of the system calibration. As ACh is being flowed through the system at all times for the AChE measurements, it made sense to consider the ‘zero’ signal as the one for ACh with 0 U/ml AChE activity. It is, however, important to consider if a linear or non-linear relationship is expected for this experimental setup. Given that the apparent K_m of the ChOx biosensor is typically 400 μ M, it is possible that the calibration would not be linear for the 1 U/ml AChE activity, corresponding to 0.5 U/ml at the flow cell, as it was expected that the choline levels could be 1 mM. However, based on the probe recovery experiments described in section 6.4.3, probe recovery will be approximately 30% for this flow cell design and flow rate, corresponding to 300 μ M choline recovered. Based on the calibrations performed in section 3.3.3, the dynamic range is, on average, 400 μ M, suggesting this experiment could be in the linear range. To test this experimentally, a 3-step choline calibration up to 1 mM was performed through the flow cell, in place of the AChE and ACh, to determine if this calibration is linear. To perform this calibration, the experimental setup was as shown in figure 6.8. The resulting choline calibration is shown in figure 7.5, compared to the AChE calibrations obtained after subtracting off the PBS baseline and the ACh baseline.

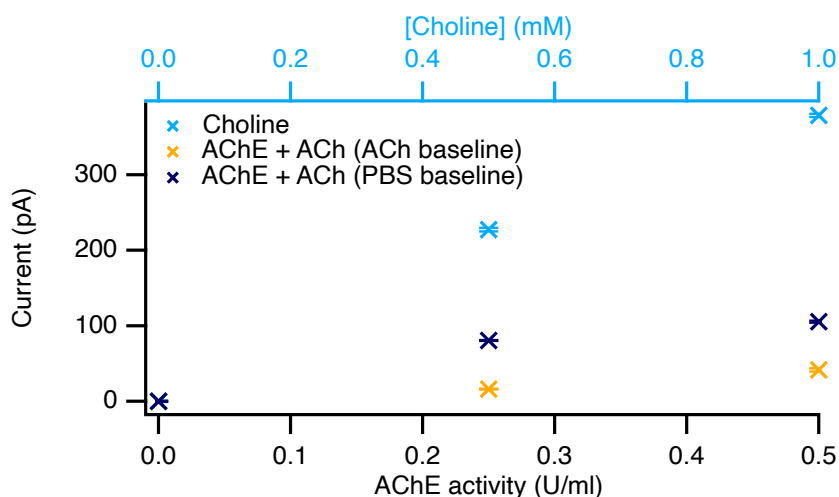


Figure 7.5: Calibration curves obtained following a choline calibration and AChE activity calibration using the flow cell. A 3-point 1 choline calibration up to 1 mM was performed using the flow cell coupled with microdialysis. A 3-point AChE activity calibration up to 0.5 U/ml was performed. Two calibration curves are shown for the AChE activity calibration; one with the ACh signal considered as the baseline and the other with the PBS signal considered as the baseline. All three series have a point at 0,0 which overlap each other on the graph. The markers and error bars represent the mean $\pm$ standard deviation of each current step.

The first noticeable result is that the signal obtained from the reaction with the 1 U/ml AChE activity (0.5 U/ml in flow cell) is considerably less than the signal obtained from the 1 mM choline concentration regardless of which baseline is considered. The large difference in the signal observed was initially surprising given that it was expected 0.5 U/ml AChE would fully convert the ACh in solution in the flow cell. The results suggests that not as much ACh is being hydrolysed. This could be due to poor mixing or a lower effective enzyme activity than quoted by the supplier (Sigma Aldrich). This could be possible as stated activity of the enzyme is determined under highly controlled conditions including pH, temperature and substrate concentration. Nevertheless, this was promising as it means the experimental setup has the capability to measure higher enzyme activities than initially expected. The results for the choline calibration show that at 1 mM choline, the relationship is starting to be non-linear. However, the lower concentrations of choline observed for the AChE activity calibration are still expected to be in the linear region. Given this analysis, the ACh baseline is considered the true ‘zero’ of the system for this experiment and going forward.

The results suggest that there is excess ACh using this experimental setup. Therefore, the next stage of the experiments was to try and detect higher levels of AChE activity, closer to the activities observed in the blood.

7.2.3 Detecting blood-related AChE activities

To detect higher AChE activities, the experiment was set up as presented in figure 7.2. The activity of AChE in whole blood in humans has been found to be 4.16 ± 0.14 U/ml ($n=5$) in a study conducted by Rudakova *et al* [126]. Therefore, in this experiment higher activities of AChE of 3.0 U/ml and 6.0 U/ml were used. The corresponding activities in the flow cell were 1.5 U/ml and 3.0 U/ml, after taking into account the dilution factor from ACh. To check the system was working as expected, the experiment started with the calibration for low activities (0.5 and 1.0 U/ml), followed by the higher enzyme activities. A possible concern with the previous experiment was that the increase in current observed between two enzyme activities may have partly been due to increasing enzyme sticking to the flow cell, causing more ACh to be turned over. To address this concern, in this experiment, the highest enzyme activity was sampled first, followed by the ACh baseline (no AChE activity), followed by the lowest enzyme activity. The resulting trace of this experiment is shown in figure 7.6.

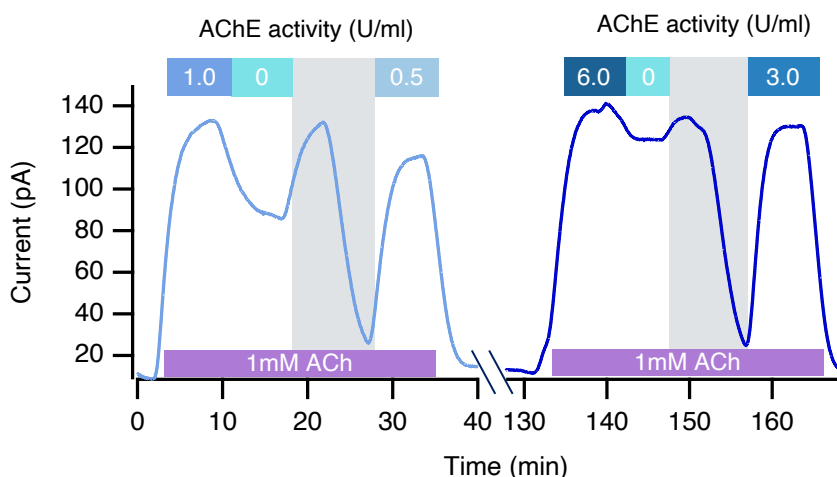


Figure 7.6: Current trace recorded during two different AChE activity calibration cycles. The first calibration cycle shows the results for 0.5 and 1.0 U/ml AChE activity, and the second one shows the results for 3.0 and 6.0 U/ml AChE activity. The greyed out area symbolises a refill step and a short PBS flush that is too short to resolve and should be ignored. The data was sampled at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

The trace shows a clear difference in the signal resulting from the enzyme activities 1.0 U/ml and 0.5 U/ml. There is also a small difference observed in the signal from 6.0 U/ml and 3.0 U/ml. These are promising results as it demonstrates that higher enzyme activities can be detected. However, there is a large increase in the ACh baseline between the two cycles, shown by the 0 U/ml AChE signal. The average current of each step has been plotted in figure 7.7.

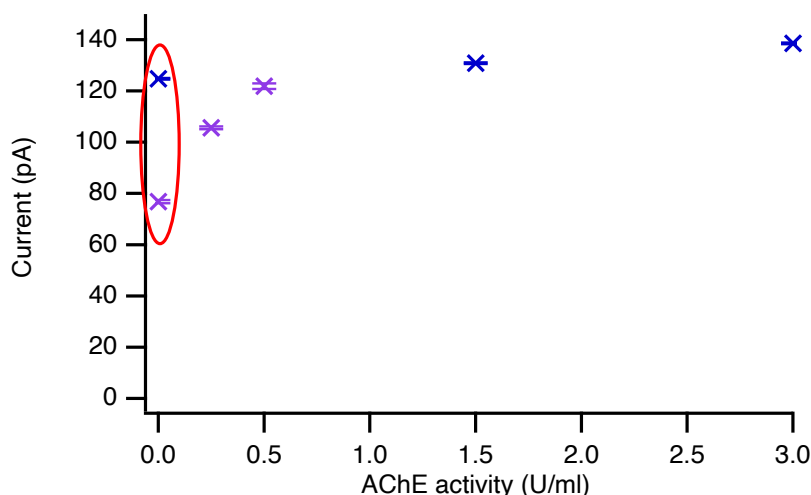


Figure 7.7: Calibration curves following a ‘low’ and ‘high’ AChE activity calibration. The low AChE calibration, shown by the light blue, shows a promising increasing trend. The high AChE calibration, shown by the dark blue, also shows an increasing trend, but it is a small change in signal. The red circle highlights the background ACh signal (no ACh). Between the two calibrations, there is a large increase in the background ACh signal, suggesting more enzyme is sticking to the flow cell. The markers and error bars represent the mean $\pm$ standard deviation of each current step.

These results are highly encouraging as they demonstrate that high enzyme activities can be detected. However, it is clear that there is an issue with enzyme sticking to the inside of the flow cell. This has been quantified by taking an average of the current step of the ACh baseline over time for multiple calibration cycles that are not shown in figure 7.6. The results of this are shown in figure 7.8.

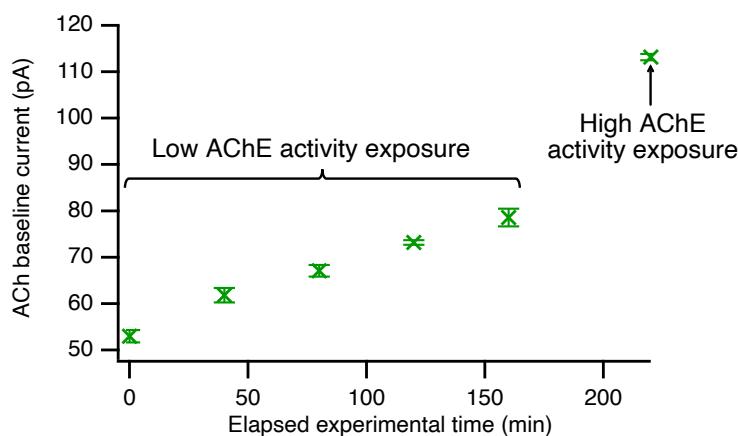


Figure 7.8: The recorded baseline ACh signal for repeated AChE calibrations. The markers and error bars represent the mean $\pm$ standard deviation of the ACh baseline signal, when no AChE is present in the flow. The first 5 markers show the baseline signal between low AChE activity calibrations. The last marker shows the baseline signal following exposure to a high AChE activity.

The results show that there is a steady increase in the ACh baseline signal during low AChE activity cycles. When the calibration cycle is changed to high AChE activities, there is a large increase in ACh signal observed, suggesting high enzyme activities cause further issues.

AChE sticking to the flow cell is problematic as it means the system can become substrate-limited due to more enzyme in the system. It also means the AChE calibrations will be unstable, and it will be hard to get an accurate measurement of the AChE activity in an unknown sample. If the amount of sticking can be limited, then the use of the flow cell has potential to sensitively detect changes in AChE activity. A large benefit to this method is that a stable signal is recorded for each change in activity, making it possible to visually see clear changes in AChE signal, without any further analysis. Methods to overcome the issues with the enzyme sticking are discussed in the next section.

7.3 Improved system for AChE detection

Improvements were made to sensitively detect higher levels of enzyme activity. These include:

- Using design 2 of the flow cell with a total external flow rate of 20 $\mu\text{l}/\text{min}$ resulting in less reaction time between AChE and ACh.
- Adaptation of the microfluidic platform to allow more calibration steps
- Addition of pluronic surfactant to prevent enzyme from sticking to the flow cell and/or microdialysis probe.

7.3.1 Experimental setup

For these experiments, the microdialysis pump was changed from the CMA 107 pump to a continuous pump assembled using LabSmith products, and the microfluidic platform was further adapted to allow continuous steps to be obtained at higher flow rates without having to refill pumps, further details are given in the following sections.

Microdialysis equipment

Previous microdialysis experiments, such as those discussed in chapters 4, 5 and 6, used an MDialysis CMA 107 pump. However, the ones used by the Boutelle group have been extensively utilised and needed servicing as they were not always reliable. Therefore, for the following experiments, the microfluidic platform was configured and tested within the Boutelle group to create a continuous pump using LabSmith equipment as shown in figure 7.9 to run as a microdialysis pump. Each time the valve switches, the flow is briefly affected. Therefore, for this application, I chose to use 40 μl syringes as this meant the valve needed to be switched less frequently. Smaller syringes are more accurate than larger syringes at lower flow rates, hence why 100 μl syringes were not used for the perfusion of the MD probe.

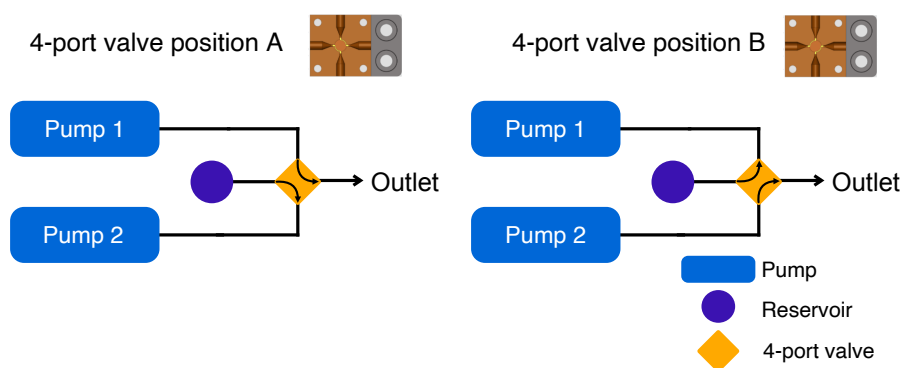


Figure 7.9: Schematic of the microfluidic platform configured to operate as a continuous pump. A 4-port valve is used to allow continuous flow to the outlet of the platform. The 4-port valve has two flow streams as shown. In position A, one flow path runs between pump 1 and the outlet, while the other flow path runs between pump 2 and the reservoir. In position A, pump 1 will push out to the outlet and pump 2 will pull back to refill with the solution from the reservoir. In position B, one flow path runs between pump 1 and the reservoir, while the other flow path runs between pump 2 and the outlet. When the valve is in position B, pump 1 will pull back to refill with the solution from the reservoir, and simultaneously pump 2 will push out to the outlet. By configuring the board in this way, and writing an automated uProcess script to control the pumps as described, continuous flow to the outlet is achieved, allowing the microfluidic platform to perform as a continuous pump.

The continuous pump required a 4-port valve, which could be set to position A and B as referenced in figure 7.9. In either position there are two flow paths that connect two ports. For example, in position A, one flow path is between pump 1 and the outlet and the other flow path is between pump 2 and the reservoir. In position B, one flow path is between pump 1 and the reservoir, while the other flow path is between pump 2 and the outlet. In position A, pump 1 pushes towards the outlet at a set flow rate, whilst pump 2 refills. In position B, pump 2 pushes towards the outlet at a set flow rate, whilst pump 1 refills. By having the microfluidic platform configured in this way, there is continuous flow to the outlet. An automated uProcess script was written to create this continuous flow.

Another benefit of using the microfluidic platform configured as a continuous pump is that it allows an easier connection to the CMA 12 MD probe used in these experiments compared with the CMA 107 pump. For the CMA 107 pump, a luerlock syringe is used. To connect to the syringe, either a needle or an adapter was required, both of which had a large volume. This was then connected to the 1/32" tubing using a flexible connector "trumpet" or an interconnect. The change from the large volume of the connector used (needle or adapter), to the 1/32" tubing meant there was a mismatch in pressure which resulted in leaks and, occasionally, unstable flow. By using the LabSmith board, the 1/32" tubing that was connected to the microdialysis probe could be directly connected to the LabSmith board overcoming this issue.

Microfluidic platform

A previous issue with the experimental setup was due to the volume of the pumps. The pumps have a maximum volume of 100 μl . Of this volume, only 90 μl is typically used due to variability in the pump calibration volume. For the previous experiments, the ACh pump runs at a constant flow rate of 5 $\mu\text{l}/\text{min}$, meaning it can only continuously flow for approximately 18 minutes. The T_{90} for design 1 of the flow cell at 10 $\mu\text{l}/\text{min}$ was approximately 180 s, so the step sizes chosen needed to be longer than this to resolve a stable signal. Given this, a step size of 6 minutes was used to ensure that the signal had plateaued and there was at least 1 minute of data to analyse to have a more accurate measurement. This meant that only three step changes in AChE activity could be made without refilling the ACh pump, including the ACh baseline measurement. More step changes were made possible by modifying the board. Three pumps were added to the adapted microfluidic platform in section 7.2.1, figure 7.2. This new, larger microfluidic board was configured as shown in figure 7.10. Two ACh pumps were used with with a 4-way valve; in the same way as the continuous pump described in section 7.3.1, one ACh pump pushes as the other one refills. There are two sets of PBS and AChE pumps, labelled set 1 and set 2 in the schematic. This is to allow continuous flow, so set 1 can push towards the flow cell while set 2 refills, and vice versa.

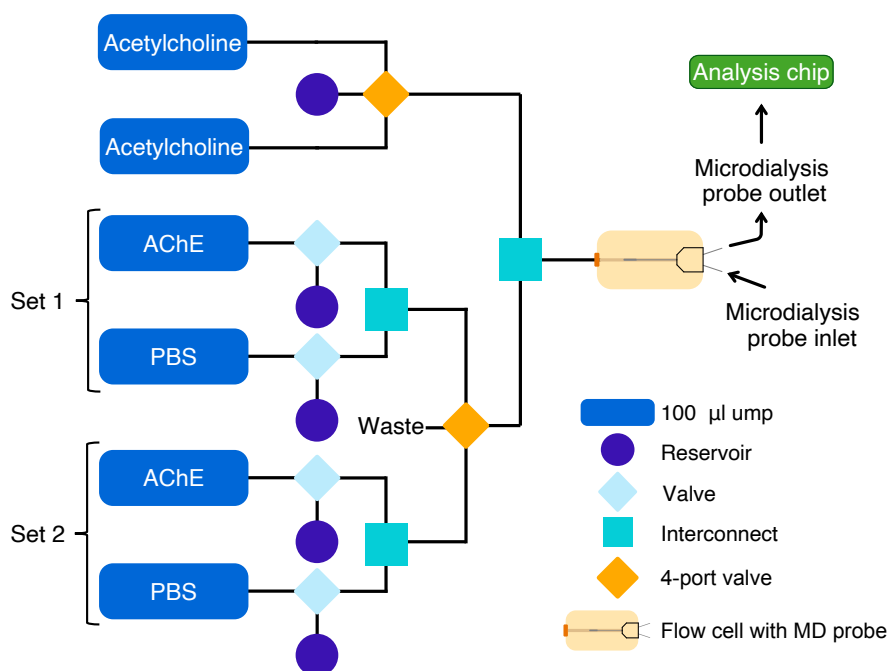


Figure 7.10: Schematic of the microfluidic platform configured to operate a continuous AChE activity calibration. The microfluidic platform has been further adapted and expanded from that described in figure 7.2. Additional ACh, PBS and AChE pumps were added, so that there are two sets of each pump. In this way, long calibration steps can be achieved with high flow rates and more calibration steps.

A flow chart describing the different calibration steps is shown in figure 7.11.

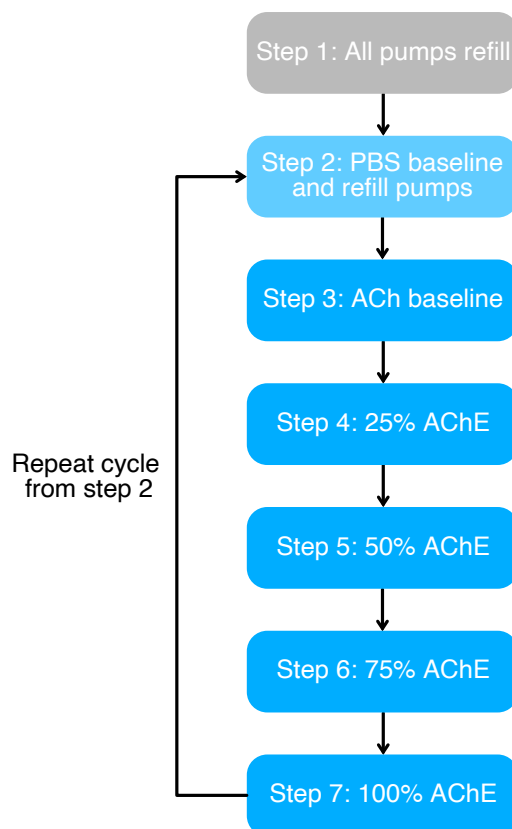


Figure 7.11: A flow chart showing the different steps of an AChE activity calibration. Using the microfluidic platform shown in figure 7.10, it is possible to perform multiple calibration steps without the need to refill the pumps between steps. This is achieved by having two sets of each pump. During step 2, a PBS baseline measurement is performed, which allows the ACh and AChE pumps to refill.

Step 1 is used the first time that the uProcess script is run to initially fill all of the pumps. The calibration then repeatedly loops steps 2 -7. The PBS baseline, step 2, has two parts, the PBS pump from set 1 pushes at 20 $\mu\text{l}/\text{min}$ for 3 minutes, then the PBS pump from set 2 pushes for 3 minutes. The calibration steps 3-7 alternate between using the set 1 and the set 2 pumps. This allows a continuous measurement to be made because the set 1 pumps can be used as the set 2 pumps refill, and vice versa, so the flow does not need to stop due to pumps refilling. A 4-way valve is used to switch between the two sets of pumps so that the set that is not being used can be flushed towards the waste outlet to ensure the respective tubing is filled with the correct solution prior to the next calibration step.

Again, in the following sections, a CMA 12 MD animal probe was used to sample choline resulting from ACh hydrolysis. The internal flow rate was held constant at 2 $\mu\text{l}/\text{min}$.

7.3.2 Preventing enzyme sticking and achieving high AChE activity calibrations

Initial experiments to detect changes in AChE activity showed promising results, however, the increase in baseline ACh levels, when the AChE activity was 0 U/ml, suggested that the enzyme was sticking to the tubing, flow cell channel or MD probe. As more enzyme sticks, it becomes harder to make a measurement, as more ACh gets converted resulting in smaller differences in the signal for different AChE activities.

Other research teams have reported success in preventing of proteins sticking to microfluidic devices by running surfactant simultaneously with the sample [127, 128]. To test this, 0.08 % w/v pluronic-F127 was added to the AChE reservoir and the AChE activity adjusted to account for this dilution. Repeated AChE calibrations were run over several hours to determine whether the enzyme was sticking. Whilst an initial increase in the ACh baseline current is observed following one AChE calibration (not shown), it then remains at a very stable level.

An example of the resulting calibration trace and curve for each sensor is shown in figure 7.12.

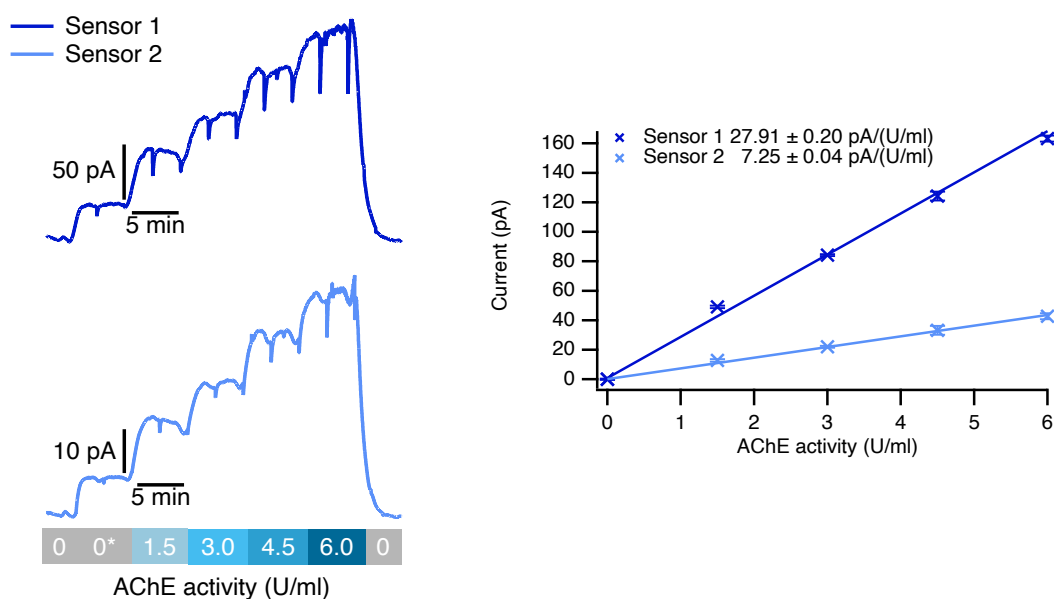


Figure 7.12: Calibration trace and curves obtained for an AChE activity calibration up to 6 U/ml with surfactant added. Left: Calibration trace for two biosensors during an AChE activity calibration. These were performed by sampling the hydrolysis product choline at the outlet of the MD probe inserted into the flow cell. 0* denotes the ACh baseline signal when there is no AChE activity. This baseline signal is subtracted off the other activity calibration steps in order to plot the corresponding calibration curve. The current was sampled at 200 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter. Right: The resultant calibration curves are shown. The markers and error bars represent the mean $\pm$ standard deviation of each activity step. The curves have been fitted using linear regression, from which the sensitivity is given. The error on the sensitivity is given by the 95% confidence interval of the regression. To perform these calibrations, 0.08 % w/v pluronic F-127 surfactant was added to the AChE reservoir.

The sensitivities were calculated by using linear regression to fit the calibration. The results over elapsed experimental time are shown in figure 7.13.

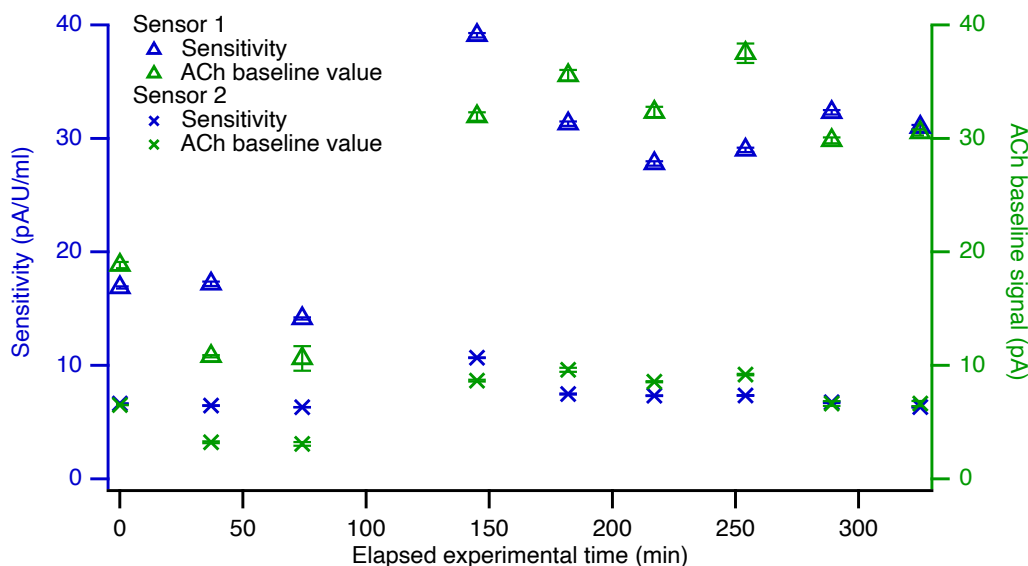


Figure 7.13: ACh baseline signal and system sensitivities during continuous AChE activity calibrations with surfactant added. 0.08% w/v was added to the AChE reservoir in an attempt to prevent the enzyme sticking to the flow cell. The results from two different biosensors are presented. The blue markers represent the sensitivities found by linear regression of the AChE calibration curves, the error bars represent the 95% confidence interval. The green markers and error bars represent the mean $\pm$ standard deviation of the ACh baseline calibration steps.

The continuous use of the system to perform AChE activity calibrations for a time period of approximately 5 hours is an encouraging result. A large jump in sensitivity is observed in both sensors at around 145 min, following this the system response to AChE activity is very stable. The baseline ACh level is also shown, found by taking an average of the current step with 0 U/ml AChE present. While a jump in the baseline ACh signal is also observed at the same time as the sensitivity jump, the ACh baseline level can be seen to be stable over several hours. A possible explanation for the shift in signal may be due to an air bubble in the flow cell, which was present at the beginning and then moved past the MD probe membrane. An air bubble in the flow cell would result in a lower MD probe recovery and thus a lower signal. Regardless, the results show a stable baseline and sensitivity values for both sensors either side of the shift in signal.

Importantly, figure 7.13 shows that these are repeatable calibrations and the baseline resulting from ACh does not show a continuous increase, a trend that was observed previously as demonstrated by results presented in figure 7.8. Furthermore, these calibrations were performed at a high enzyme activity, in a range expected for whole blood, and the stability of the ACh baseline demonstrates that the addition of pluronic surfactant is a very effective solution to prevent sticking of AChE, making the system suitable for whole blood monitoring.

Initially, the pluronic surfactant was only added to the AChE reservoir, however, during the calibration the AChE flow rate varies with the PBS pump flow rate to make different AChE activities in the flow cell. Therefore, the surfactant concentration going through the flow cell was varied. To keep the surfactant concentration constant, it was also added to the PBS pump. The PBS and AChE flow rates always total 10 $\mu\text{l}/\text{min}$, and the ACh pump flow rate is constant at 10 $\mu\text{l}/\text{min}$, therefore the final concentration of the surfactant in the flow cell is 0.04% w/v as it is diluted by the ACh pump.

To try and reduce any sticking further, the pluronic surfactant was also added to the ACh pump to make the final concentration of surfactant inside the flow cell 0.08 %. However, this blocked the flow cell inlet becoming blocked and it was no longer added to the ACh pump.

7.4 Conclusion

This chapter has described the use of the microfluidic flow cell, the development of which is described in chapter 6, to sensitively detect changes in AChE activities by MD sampling. Initial experiments showed that changes in both low and high AChE activities could be observed, achieved using design 1 of the flow cell. However, these experiments were not repeatable due to enzyme sticking to the inside of the flow cell and possibly the MD probe, creating an increasing ACh baseline signal despite there being no AChE present in the flow stream. This was made worse when using higher AChE activities, which is problematic given the aim of this monitoring device is to sample whole blood where high levels of AChE activity are present.

Issues with enzyme sticking were overcome by adding pluronic surfactant to the sample streams. While there was still a background signal observed for ACh, likely from some enzyme sticking, the surfactant was successful in preventing additional AChE from sticking to the flow cell channel or MD probe or a combination of both. This resulted in repeatable calibrations in high enzyme activity ranges, suitable for monitoring AChE activities found in whole blood.

The flow cell has allowed clear, stable current steps to be recorded for different AChE activities. This was not possible before the development of the flow cell. This is a huge improvement as now changes in AChE activity can visually be seen in the raw signal, without having to analyse specific time points or the rate of reaction. This shows potential for the flow cell to be utilised for continuous monitoring of AChE activity in samples exposed to OP compound, as any effect on the AChE activity should be seen in the raw trace. This will be further investigated in chapter 8.

Design 2 of the flow cell has meant that faster flow rates can be used, limiting the reaction time between the AChE and ACh, allowing higher activities to be observed. This was demonstrated by performing multiple 6 U/ml calibrations which were presented in this chapter, in figures 7.12 and 7.13. Design 2 of the flow cell has also led to faster response times, which allows calibration steps to be shorter, reducing the need to refill the pumps, therefore, reducing the number of refill artefacts.

Faster response times will also benefit real-time measurements, as changes in AChE activity will be made apparent sooner.

The system has been optimised to sensitively detect high levels of AChE activities. This is an excellent result given the challenges with current monitoring systems to sample high levels of AChE meaning large dilutions are required [37]. The next chapter investigates the use of this system for validation with OP-exposed samples and with blood samples.

Chapter 8

Monitoring inhibition of acetylcholinesterase

8.1 Introduction

In this chapter I examine the inhibition of acetylcholinesterase (AChE) using organophosphorus (OP) pesticides. The pesticide chosen was paraoxon due to its wide use and availability. This work was carried out at the Defence Science and Technology Laboratory (DSTL), Porton Down under control of substances hazardous to health (COSHH) forms and risk assessments developed by Dr. Nick Coe and Tom Mann. Stock pesticide solutions were prepared by Dr. Coe and Tom Mann and given to me in concentrations <1 mg/ml.

To perform the experiments detailed in this chapter, the wireless electronics described in section 2.5 were used. This was to avoid taking large, bulky equipment to another site. Additionally, it enabled more flexibility in the experimental setup as it is a portable device so the flow cell and electronics could be placed next to the microfluidic platform without need for long extension tubing.

8.1.1 Electronic housing

For these experiments the electronic housing was modified from the previously used electronic enclosure described in section 2.5 and in a similar way to that described in section 4.3. For this work, I wanted to use two choline biosensors at any given time to provide security in the measurements, for example to protect against failure of a sensing electrode or connection. However, having more than two biosensors was unnecessary, therefore only one potentiostat was needed, requiring a smaller enclosure to be made. The box was amended to hold a 3-electrode chip. This size of chip was chosen in case any solution needed to be collected and it was easier to contain and collect waste through the unused biosensor port. This electronic housing was modified from the original described in section 2.5, to allow connections through the lid of the box to protect the electrical wires from being held in awkward positions which

weakens the connection to the electrode. This was achieved by printing small holes in the lid of the box through which the electrical connections could be placed. Again, extruded plastic walls were added to hold the PCB, Bluetooth module and battery firmly in place. Figure 8.1 shows drawings of the box with the various dimensions. The box was designed using SolidWorks (Dassault Systèmes).

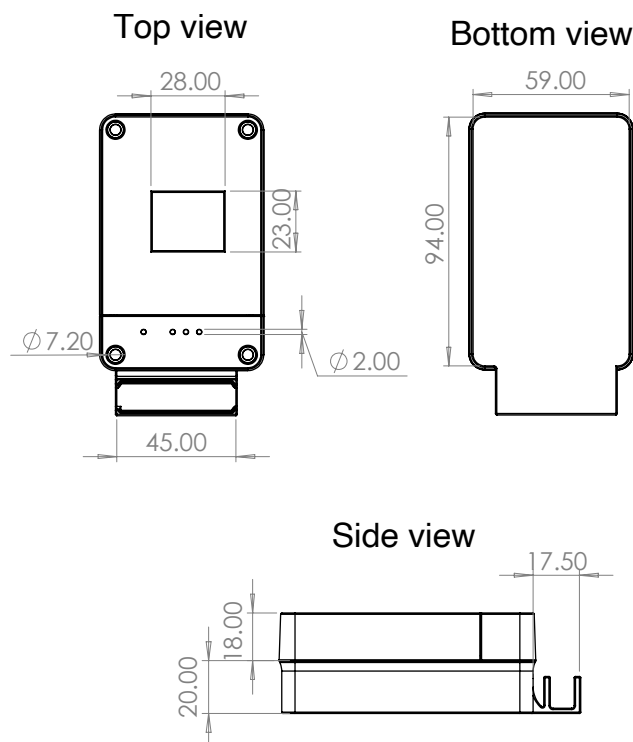


Figure 8.1: Drawings of the electronic enclosure adapted for experiments at DSTL. The top, bottom and side view of the box are shown. The dimensions given are in mm. The box is taller than the original electronic enclosure to facilitate easier connections from the electrodes to the potentiostats. 2 mm holes were printed into the lid of the box to insert electrical connectors through, which were later glued in place. The box was designed to hold a 3-electrode microfluidic chip.

As was the case for the electronic housing described in 4.3, this enclosure is taller to allow for the UFL leads to bend. The box was printed using an original Prusa i3 (Prusa Research, Czech Republic). Photos of the 3D printed enclosure are displayed in figure 8.2.

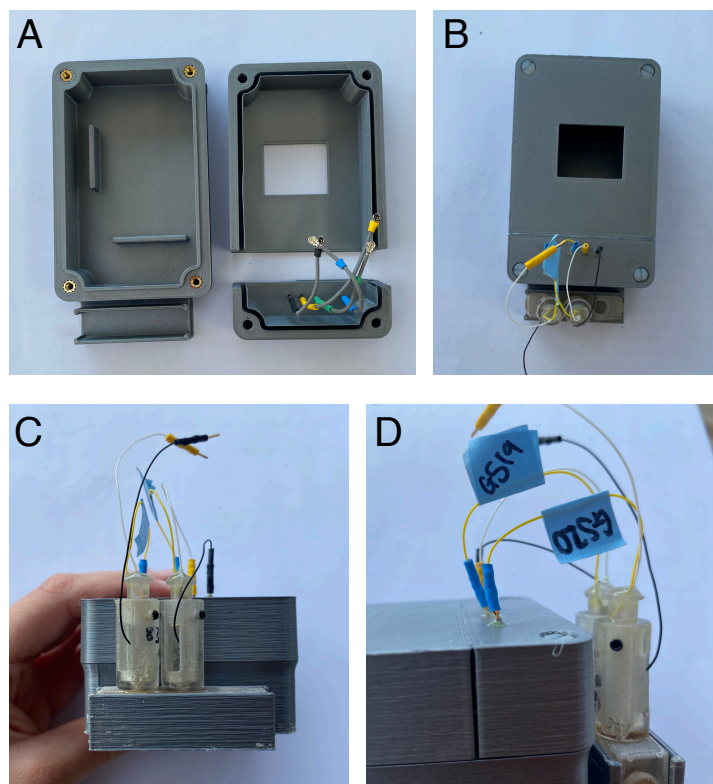


Figure 8.2: Photos of the 3D printed electronic enclosure adapted for use for experiments at DSTL. A. The inside of the box and lid. The UFL leads are shown attached to the small section of the lid. The extruded plastic walls can also be seen inside the box to secure the potentiostat in place. **B.** The exterior of the box, with the chip and biosensors inserted. The connection of the biosensors through the lid of the box is shown. **C.** Front view of the box, with the 3-electrode chip and two biosensors inserted. **D.** A close up of the biosensor connections through the lid of the box.

8.1.2 Experimental setup

This section provides a brief, general overview of the equipment used, with specifics of the experimental setup given in the relevant sections of this chapter.

For microdialysis (MD) experiments, a CMA 12 animal probe was used (MWCO 20 kDa, membrane length 4 mm). The MD probe was perfused with a 0.01 M PBS solution at a flow rate of 2 $\mu\text{l}/\text{min}$. To perfuse the probe, the microfluidic platform configured to run as a continuous pump as described in section 7.3.1 was used. For these experiments, the second iteration of the flow cell was used, details of which are described in chapter 6. AChE activity calibrations were performed using the microfluidic platform configured as shown in figure 7.10. The external flow rate inside the flow cell was always 20 $\mu\text{l}/\text{min}$ for these experiments, with the ACh pump flow rate held constant at 10 $\mu\text{l}/\text{min}$.

8.2 Monitoring inhibition of AChE

This section explores the inhibition of AChE following exposure to paraoxon.

8.2.1 Effect of introducing an OP compound into the analysis system

To inhibit AChE, an OP pesticide called paraoxon was used. However, previous studies have found that paraoxon inhibits choline oxidase (ChOx) and some research has utilised this to detect paraoxon in a sample [129] [130]. Paraoxon has a molecular weight of 275.20 g/mol and, given that the MD probe used has a MWCO of 20 kDa, the paraoxon should be able to diffuse into the probe towards the analysis system containing the biosensor. To investigate this, a test was performed to determine if the paraoxon was diffusing into the dialysate stream and affecting the performance of the biosensor. The microfluidic board was set up with 4 pumps, as shown in figure 8.3. Pumps A and B contained 0.01 M PBS and 100 μ M choline, respectively, with no added paraoxon. Pumps C and D contained 0.01 M PBS and 100 μ M choline, respectively, both with a certain concentration of paraoxon added. A uProcess script was written that alternated between a 3-step choline calibration with no added paraoxon, using pumps A and B, and a 3-step choline calibration with added paraoxon, using pumps C and D. This was repeated with three different concentrations of paraoxon, which were 100, 10 and 1 μ M.

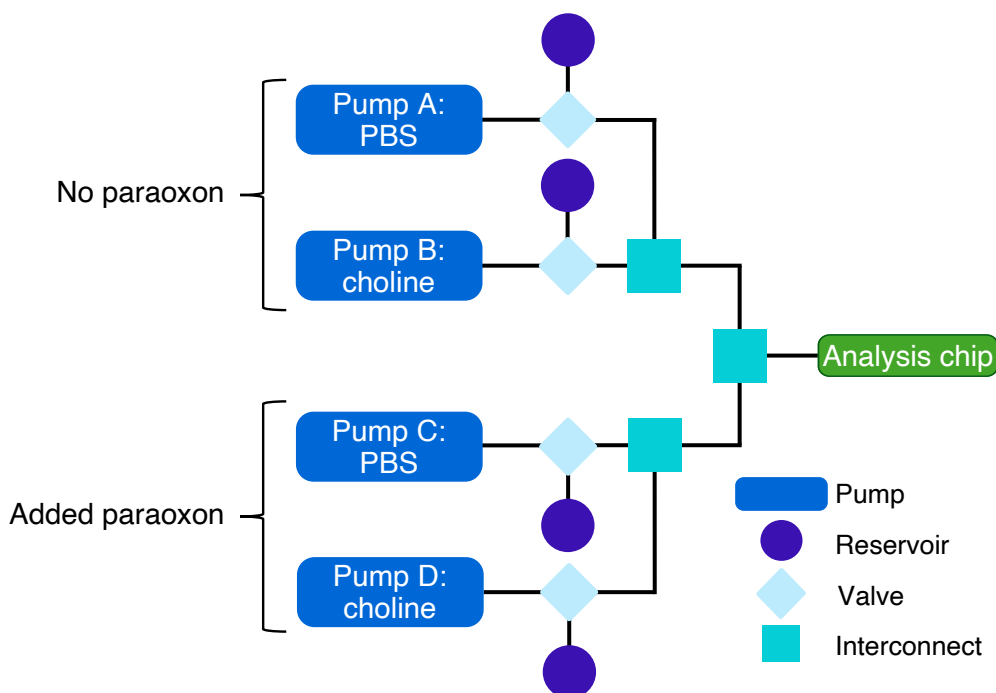


Figure 8.3: Microfluidic platform configured to alternate between a choline calibration with no paraoxon added and a choline calibration with paraoxon added. A uProcess script was written to perform an automated calibration that alternated between a 3-step paraoxon-containing choline calibration and a 3-step choline calibration with no added paraoxon, to determine if the paraoxon affected the sensitivity of the choline biosensor.

The calibration curves were plotted for the choline calibrations with no added paraoxon, C_0 , with 100 μM paraoxon, C_{100} , with 10 μM paraoxon, C_{10} , and with 1 μM paraoxon, C_1 , as shown in figure 8.4.

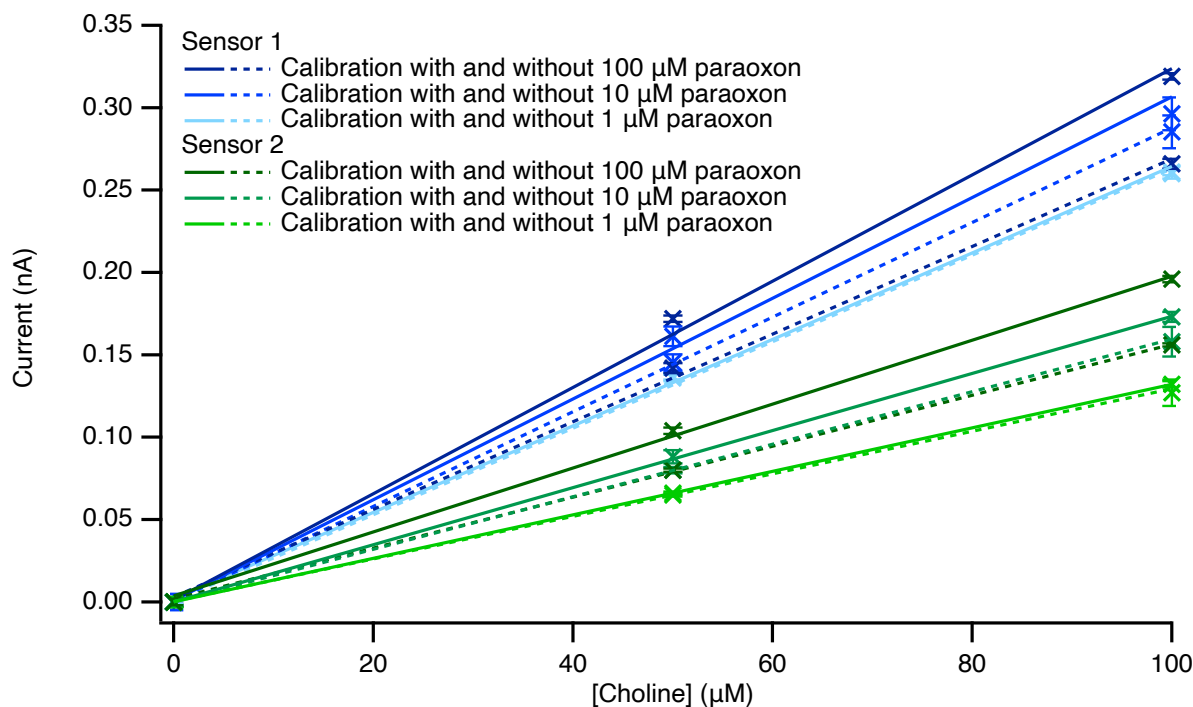


Figure 8.4: Choline calibration curves obtained when performing a calibration with and without paraoxon. The calibrations C_0 refer to the calibrations with no paraoxon added. The C_0 calibration immediately before the respective paraoxon-containing calibration has been plotted, as shown by the line colour. The curves C_{100} , C_{10} and C_1 refer to the choline calibration performed with 100 μM , 10 μM and 1 μM of paraoxon added, respectively. The lines were fitted using linear regression to give the sensitivities of each setup. The error on these sensitivities are given by the 95% confidence interval. The sensitivities are shown in table 8.1.

The repeated C_0 calibrations refer to each choline calibration with no added paraoxon preceding the respective calibration with paraoxon. The calibrations were alternated to track sensitivity over time or in case any inhibition was reversible. A line fit was used to fit the calibration curves, and the sensitivity of each setup was found, as shown in table 8.1.

[Paraoxon] (μM)	Sensor 1		Sensor 2	
	C_0 (nA/mM)	C_x (nA/mM)	C_0 (nA/mM)	C_x (nA/mM)
100	3.23 ± 0.01	2.66 ± 0.04	1.93 ± 0.03	1.55 ± 0.03
10	3.06 ± 0.09	2.9 ± 0.1	1.73 ± 0.04	1.60 ± 0.07
1	2.62 ± 0.03	2.63 ± 0.02	1.32 ± 0.03	1.29 ± 0.06

Table 8.1: The sensitivities of the choline calibrations with and without paraoxon. The sensitivities were found using linear regression, the error is given by the 95% confidence interval.

The ratio of the biosensor sensitivity when paraoxon was in solution to the biosensor sensitivity without paraoxon, C_x/C_0 was found. The ratios expressed as percentages are shown in table 8.2.

	Sensor 1	Sensor 2
[Paraoxon] (μM)	C_x/C_0 (%)	
100	82 ± 1	80 ± 2
10	95 ± 4	92 ± 5
1	100 ± 1	98 ± 5

Table 8.2: A summary of the effect of different concentrations of paraoxon on the choline biosensor.

The results show that, by taking the average of the two sensors, the choline calibration with 100 μM paraoxon added causes a $21 \pm 1\%$ reduction in the biosensor sensitivity, 10 μM paraoxon causes a $6.5 \pm 1.5\%$ reduction in sensitivity and the 1 μM paraoxon causes a $1 \pm 1\%$ reduction in sensitivity. This suggests that high concentrations of paraoxon do inhibit the ChOx immobilised on the biosensor, despite there being only a short interaction time. As these experiments were run over the duration of the day, I would expect some drop in the biosensor sensitivity throughout the day. This accounts for some of the drop in sensitivity of the C_0 calibrations displayed in table 8.1, which dropped by approximately 20% and 35% of sensor 1 and sensor 2, respectively. However, this drop in signal may also be due to the larger concentrations of paraoxon, which may have had a long-term effect on the sensitivity of the biosensors. This could be further investigated to determine whether the drop in signal is due to the biosensor stability or due to exposure to a high concentration (100 μM) of paraoxon. However, this is not a concern for this thesis as the final paraoxon concentrations to inhibit AChE used in section 8.2.2 were 750 nM and 1 μM and in section 8.2.3 were between 1 and 100 nM; based on the results for 1 μM paraoxon, the effect of this concentration and below on the choline biosensor should be negligible.

8.2.2 Continuous monitoring of inhibition of AChE

Continuous monitoring is of high interest to determine exposure to OP compounds, track an individual's health and to determine if treatment is working. The current monitoring standard, the Ellman's assay, only allows for point measurements. The assay presented in this thesis therefore has the advantage of monitoring changes in AChE activity continuously. This section describes how the microfluidic platform and flow cell, coupled with MD, were utilised to perform a continuous experiment to monitor real-time inhibition of AChE by paraoxon. 4 U/ml AChE samples were mixed with a certain concentration of paraoxon. The mixed solutions were immediately added to the microfluidic platform and analysed to continuously track the inhibition of AChE.

To perform these experiments, the board was configured as shown in figure 8.5. Two choline biosensors were placed in the analysis chip at the MD outlet to detect the choline resulting from ACh hydrolysis in the dialysate.

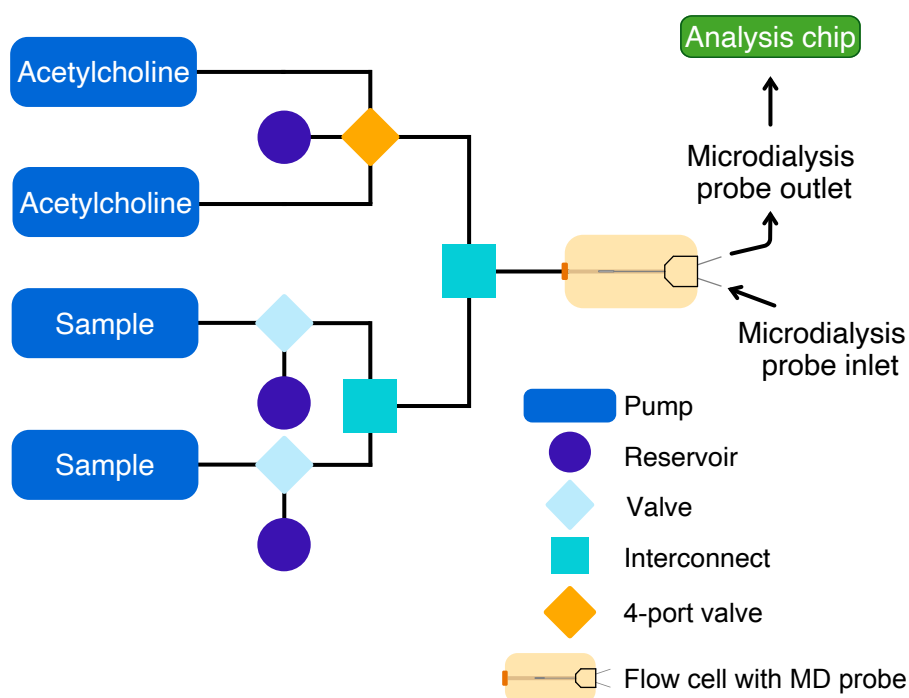


Figure 8.5: Microfluidic platform configured to continuously monitor AChE activity in a sample. Two pumps are used to run ACh through the board and two pumps are used to run the sample, which refers to the paraoxon and AChE mixture, through the board. The ACh and sample are run through the microfluidic platform in a 1:1 ratio, both at a flow rate of 10 $\mu\text{l}/\text{min}$. The 4-port valve is used as described in section 7.3.1, figure 7.9, to allow the refilling of one pump whilst the other pump pushes towards the flow cell. This is the most efficient way to allow continuous flow of one analyte as it requires just one valve and one reservoir. Similarly two pumps are used for the sample to allow one pump to refill whilst the other pushes to the flow cell. Here, two reservoirs and two 2-way valves are used as in other cases, for example the AChE activity calibration, the reservoirs are filled with different solutions. For either configuration, having two pumps with the same solution allows one pump to push while the other refills, to keep continuous flow and avoid refill artefacts.

The board is configured with two sets of identical pumps, to ensure there is a constant flow going into the flow cell. The pumps used for these experiments had a maximum volume of 100 μl . The ACh and sample are run through the board in a 1:1 ratio, both at a 10 $\mu\text{l}/\text{min}$ flow rate, meaning for each pump a step can last for a maximum of 10 minutes. The pump then needs to refill which is performed at a flow rate of 200 $\mu\text{l}/\text{min}$, therefore it takes 30 s for the pump to refill. However, a continuous measurement is desired so by having a second pump, this can push while the first pump refills, in the same way that the continuous pump works.

When placing a new paraoxon-AChE mixture into the reservoirs, the board was flushed once to ensure that only that solution was in the tubing and there was no mixing with the previous solution. The measurement was then started.

The different paraoxon concentrations used to inhibit AChE were 1 μM and 750 nM. The paraoxon-AChE solution was mixed and immediately placed in the two ‘sample’ pumps for analysis, where the pesticide is inhibiting AChE in real-time and the resulting inhibition is detected. Prior to these measurements, a calibration was performed, varying the AChE activity from 0-4 U/ml, to determine

the system sensitivity to AChE activity. The resulting current traces from the paraoxon-AChE samples were converted to AChE activity using this calibration. To see how the AChE activity was affected, I have expressed the AChE activity in terms of % of the original AChE activity (4 U/ml). The results for the two sensors used are presented in figure 8.6, showing the trace for each pesticide inhibition.

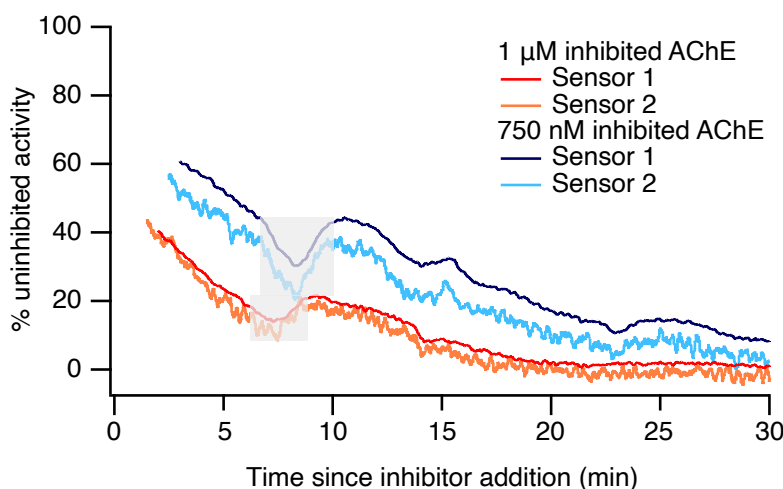


Figure 8.6: Traces recorded for two choline biosensors during dynamic inhibition experiments. The top graph shows the results for sensor 1, in blue, and the bottom graph shows the results for sensor 2, in green. The dark colours represent 4 U/ml AChE exposed to 1 μ M paraoxon and the light colours represent 4 U/ml AChE exposed to 750 nM paraoxon. The greyed out areas represent the time at which the pumps used to deliver the sample and ACh were switched to the second pump in each pair. The current was recorded at 10 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter.

These results are very encouraging as both sensors are in close agreement with one another. The trace shows the 1 μ M paraoxon-inhibited AChE has less initial activity compared to 750 nM paraoxon-inhibited AChE, this is a great result, showing potential for the device to distinguish between different concentrations of inhibitor exposure. Approximately 35 minutes after the AChE was exposed to the inhibitor, the 1 μ M inhibited AChE activity has been reduced to zero, and the 750 nM inhibited AChE activity has been reduced to near zero, although it still retains some activity. These results very nicely demonstrate that the device can be used to measure dynamic changes in AChE activity in real time, showing potential for the device to be used to investigate rates of inhibition for different concentrations of inhibitor.

8.2.3 Static inhibition of AChE

To validate my monitoring system, the results obtained using my assay were compared to the standard assay used currently, which is based on a modified Ellman's assay. To compare the two assays, rather than looking at the dynamic changes in AChE following exposure to the OP compound, the OP compound and AChE solution were mixed together and left so the AChE had reached maximum inhibition, dependent on the OP concentration. Different concentrations of paraoxon were used to inhibit 4 U/ml AChE. An overview of the experimental approach is shown in figure 8.7.

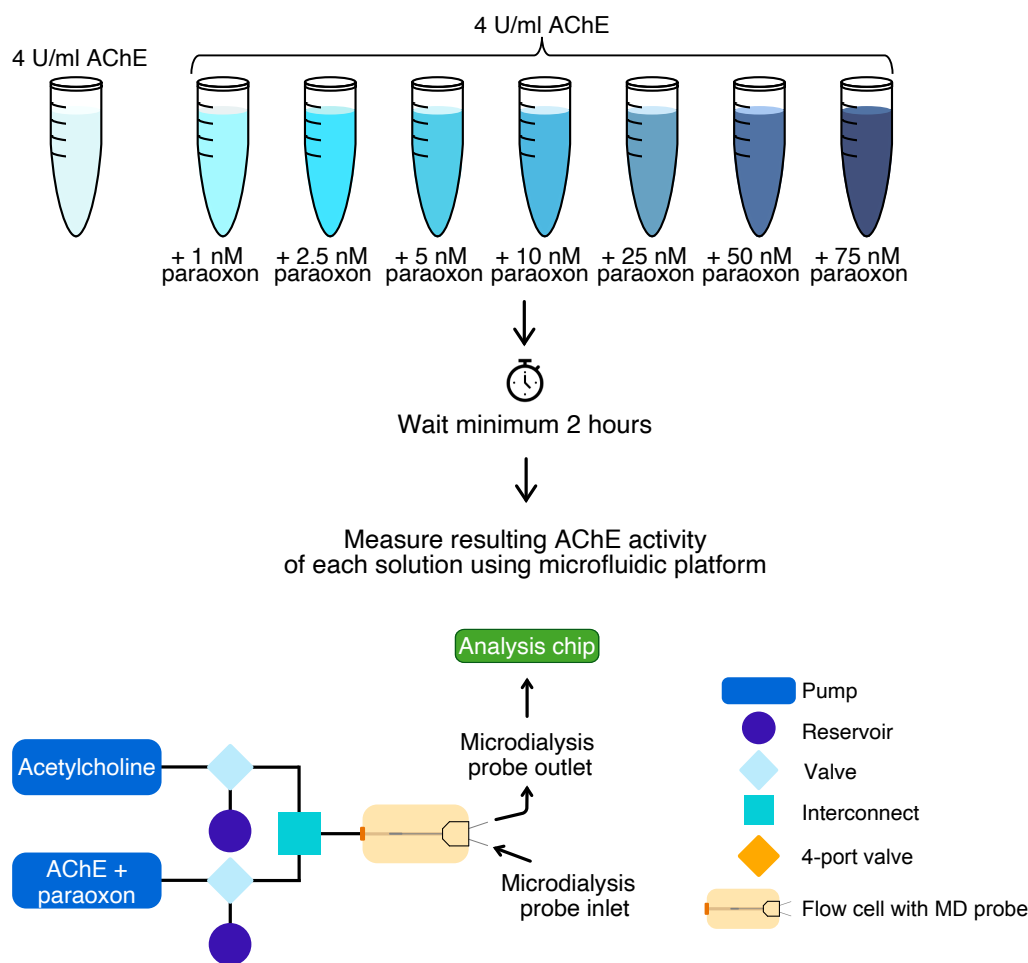


Figure 8.7: Experimental approach for monitoring inhibition of AChE. Different concentrations of paraoxon were added to a solution containing 4 U/ml AChE. The solution was left for a minimum of 2 hours, allowing the available paraoxon to inhibit the AChE. Following this, the different inhibited solutions were run through the microfluidic board, configured as shown, for a minimum of 6 minutes. As the measurements obtained were single measurements, and the signal should be stable, only one pump was needed for each the ACh and the inhibited AChE sample.

Each chosen concentration of pesticide was mixed with 4 U/ml AChE and left for at least 2 hours. Based on the dynamic experiments described in section 8.2.2, we can see that the activity of AChE began to plateau 30 minutes after inhibition. Therefore by leaving the enzyme-pesticide solution for 2 hours, the pesticide should have inhibited the AChE to its full capacity. The amount of AChE that is inhibited will depend on the paraoxon concentration. The activity of the AChE was then measured

to see if the enzyme had been inhibited and if so by how much. To do this, the microfluidic board was configured as shown in figure 8.7.

Here, as it was expected that the inhibition of AChE would have plateaued and therefore that the AChE should be constant, the measurement over a long time was not necessary as before, and a 6 minute measurement of the sample was expected to be plenty to measure the AChE activity. This meant only one sample pump and one ACh pump was needed. Using the microfluidic board, the pesticide-enzyme solution was mixed with the ACh solution in a 1:1 ratio. This meant the pesticide-enzyme solution was diluted 50%. The concentration of ACh mixed with it was 2 mM.

The measurements performed using a modified Ellman's assay were performed by Dr. Nick Coe who also provided the following details on the measurement. The equipment used was an Ultrospec 6300Pro (Amersham Biosciences), with a heated (37°C), multi-change carousel attached to a Stuart circulating water bath. In a 4 ml polystyrene cuvette (Sarstedt), 2.25 ml of a 0.33 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution (Sigma) in 0.2 M potassium phosphate buffer (pH 8.0, VWR), was left to warm to 37°C for 10 minutes. After this time, the sample was diluted 1:40 in 0.01 M PBS and 93 μ l was added to the DTNB in the cuvette, along with 300 μ l of a 1 mM acetylthiocholine iodide solution. The mixture was pipetted up and down twice to mix the solution. The change in absorbance was measured for 78 seconds at 412 nm after an initial equilibrium period of 20 s.

To look at the level of inhibition, the ratio of the signal for the inhibited enzyme to the signal for the uninhibited enzyme was taken. The ratio is plotted against the paraoxon concentration in figure 8.8.

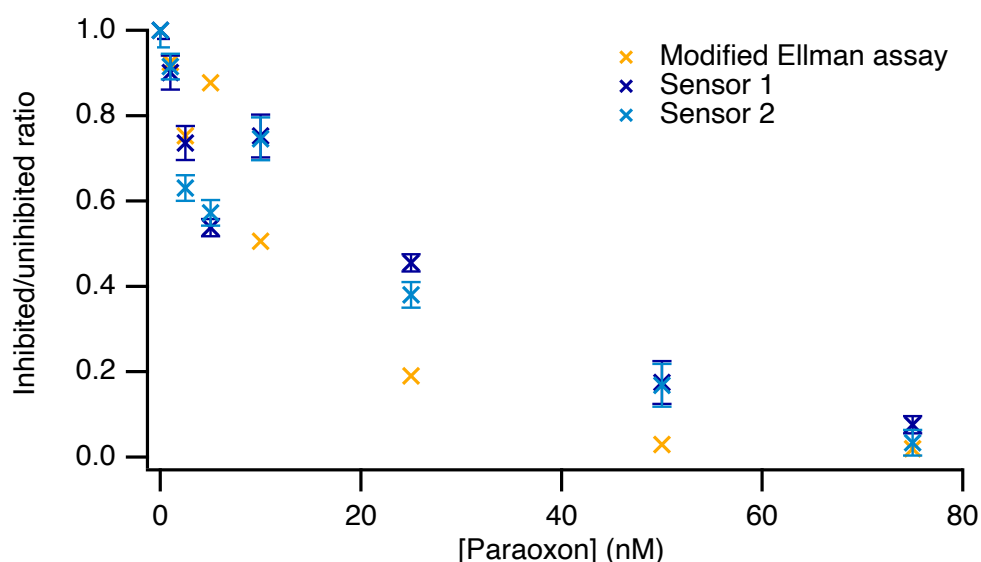


Figure 8.8: Results showing the ratio of inhibited:uninhibited AChE obtained using my assay and the standard assay for different paraoxon concentrations. My assay consisted of the microfluidic flow cell coupled with MD and the microfluidic platform. Two choline biosensors were used to sample the dialysate stream. The results obtained using my assay are shown in dark blue for sensor 1 and light blue for sensor 2. The markers represent a 3 minute sample of each current step. The error bars were found using error propagation, by taking into account the error on the uninhibited measurement and the standard deviation of the sample. The results from the two sensors used in my assay show similar results. The results obtained using the modified Ellman’s assay are shown in yellow. A clear and similar trend is shown in both assays.

With the exception of the data point at 5 nM paraoxon, the Ellman’s assay shows a distinct trend. Promisingly, the two sensors used in my assay also show a similar trend. Despite some differences between the two measurements, the biosensor measurements agree with the Ellman’s assay at the lowest and highest paraoxon concentrations. In addition, the two biosensors are mostly in agreement with each other, which is a very positive result. There does appear to be a shift in the data points at the 10 nM paraoxon value for the two sensors; it is possible that these data points just have an offset. A clear difference in the ratio is evident for the different paraoxon concentrations when using my assay, showing very nicely that my assay can be used to detect different levels of exposure to OP compounds.

8.3 Monitoring AChE activity in blood

In the previous section, I demonstrated that the microfluidic analytical device has been successfully used to monitor changes in AChE following exposure enzyme-containing sample to OP compounds. Since the goal for the device is to monitor AChE activity in whole blood, the next step was to demonstrate that AChE activity measurements in blood were possible. Up until now, blood has not been run through the microfluidic platform.

8.3.1 Experimental setup

For these experiments, the microfluidic platform was configured as shown in figure 8.9. Similarly to the static measurements, the measurement in blood for this purpose did not need to be a long measurement, as the AChE activity will be stable throughout the blood. There are two measurements that will take place. Measurement 1 is the signal obtained from background levels of choline already present in blood. To perform this measurement, the blood sample is run at a flow rate of 10 $\mu\text{l}/\text{min}$ and the PBS pump is run at a flow rate of 10 $\mu\text{l}/\text{min}$. The PBS pump is used to mimic the dilution effect of the ACh pump that will be used in measurement 2. Measurement 2 is the signal obtained due to the reaction of the added ACh with the AChE in the blood, which is dependent on the AChE activity. For this measurement, the blood sample and ACh pumps are both run at a flow rate of 10 $\mu\text{l}/\text{min}$.

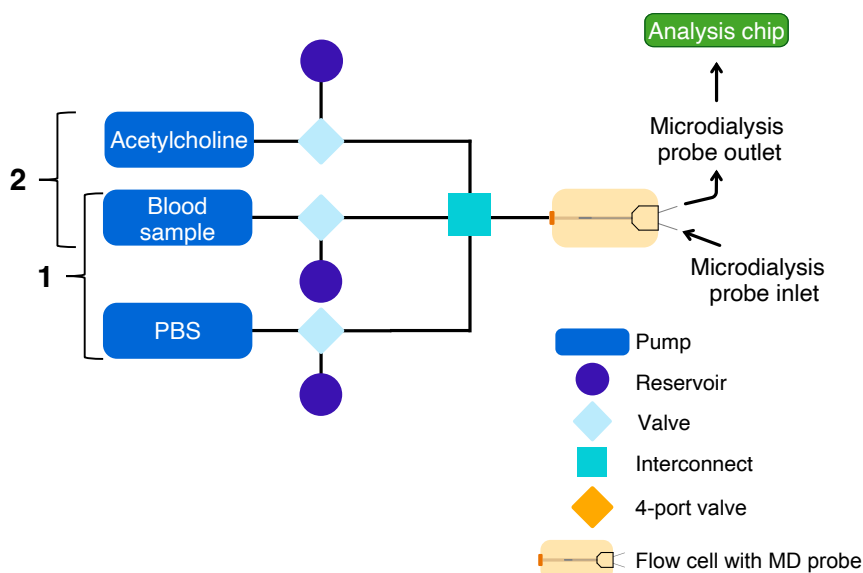


Figure 8.9: The microfluidic platform configured to sample AChE activity in a blood sample. To sample blood, two measurements will be taken. Measurement 1 uses the blood sample pump and PBS pump, to measure the background signal in blood from basal choline levels. Measurement 2 uses the blood sample pump and ACh pump to measure the signal from AChE activity in blood.

To perform these measurements, a CMA 12 MD animal probe was used (MWCO 20 kDa, membrane length 4 mm), perfused with 0.01 M PBS at a flow rate of 2 $\mu\text{l}/\text{min}$. The outlet of the probe was connected to the analysis chip housing two choline biosensors, for the detection of resulting choline in the dialysate stream from ACh hydrolysis.

As it was not clear initially if the blood would interfere with the microfluidic platform or the analysis system, the blood sample was initially diluted different amounts. In all cases, 0.08% pluronic surfactant was added to the final dilutions of the samples to ensure the experimental setup was the same as the pre-tests and the AChE activity calibration, and to help prevent sticking of large proteins to the flow cell and MD probe.

To convert the measured signal resulting from ACh hydrolysis from AChE activity, the microfluidic platform was configured as shown in figure 7.10 to perform AChE calibrations. By obtaining the system sensitivity to AChE activity, it was possible to convert from current to AChE activity in the blood samples.

8.3.2 First attempts to measure AChE activity in blood samples

Blood samples (Cambridge Biosciences) were diluted. Two different blood dilutions were tested, 1 in 40 and 1 in 20. The choice of the 1 in 40 dilution was to match what is used in the Ellman's assay. The 1 in 20 was then performed to move closer to whole blood sampling. To monitor the AChE activity in the diluted blood, the microfluidic platform was set up as shown in figure 8.9.

A 3-point AChE activity calibration was performed through flow cell before and after the measurements. The calibration was used to convert the measured current for the different blood dilutions to an AChE activity. The resulting activity is plotted against the dilution factor in figure 8.10.

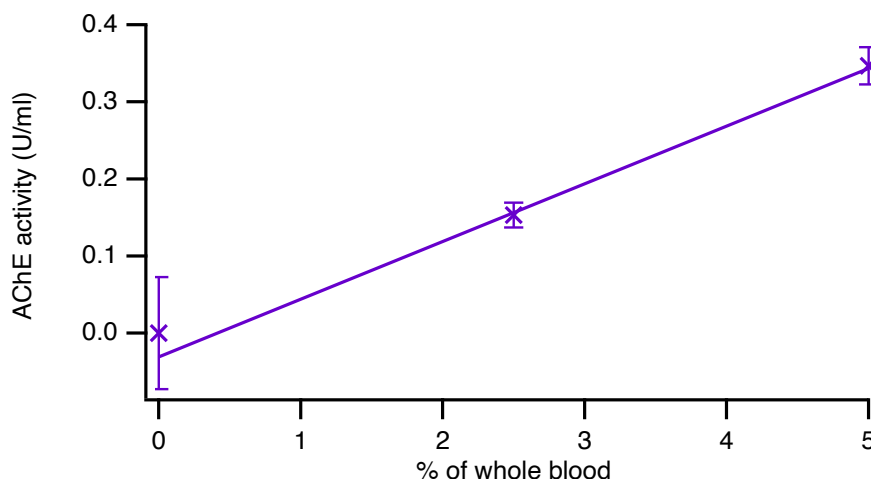


Figure 8.10: Resultant AChE activity measured from a blood sample that had been diluted using 2 different dilution factors. A blood sample had been diluted to a final concentration of 2.5% and 5% blood. The resultant AChE activity is plotted against % of whole blood. The markers and error bars represent the mean $\pm$ standard deviation of each current step. The data was fitted using linear regression.

The results show a linear fit. Although this was expected as the 1 in 20 dilution should give double the activity compared with the 1 in 40 dilution, it shows the sensing system is working very well even in blood samples. The gradient of the line gives the activity of the enzyme per % of whole blood, meaning the activity of the undiluted whole blood can be found. The data was fitted using linear regression, with a gradient of 0.0748 ± 0.0103 U/ml/% of whole blood, where the error is given by the 95% confidence interval. Based on these experimental results, the activity of the whole blood is 7.48 ± 1.03 U/ml. The activity of this whole blood sample was not measured with the standard assay routinely used. The results do potentially seem high for AChE activities in the blood [126], however considering there is also BChE in the blood which may also be hydrolysing the ACh, these results are

in the correct range. Furthermore, this value is within the range of other samples tested in this thesis with my assay and with the modified Ellman's assay, as discussed in the following chapters.

8.3.3 Attempts to monitor near undiluted blood samples

The experiment was repeated with two different samples of whole blood. These blood samples were collected as part of routine health screening at DSTL. The donors provided informed consent prior to donating the blood (0.5 ml). The aim was to try to dilute the blood less to get closer to using whole blood in the assay. The dilutions used gave a final blood concentration of 50% and 92% for each blood sample. The microfluidic platform was configured as shown in 8.9, and the same experimental protocol was followed.

The final dilution of 92% was the closest to whole blood that was possible while still allowing the addition of a 1% pluronic surfactant solution for a final concentration of 0.08% w/v surfactant.

The resulting current trace is shown for the 92% blood measurements in figure 8.11.

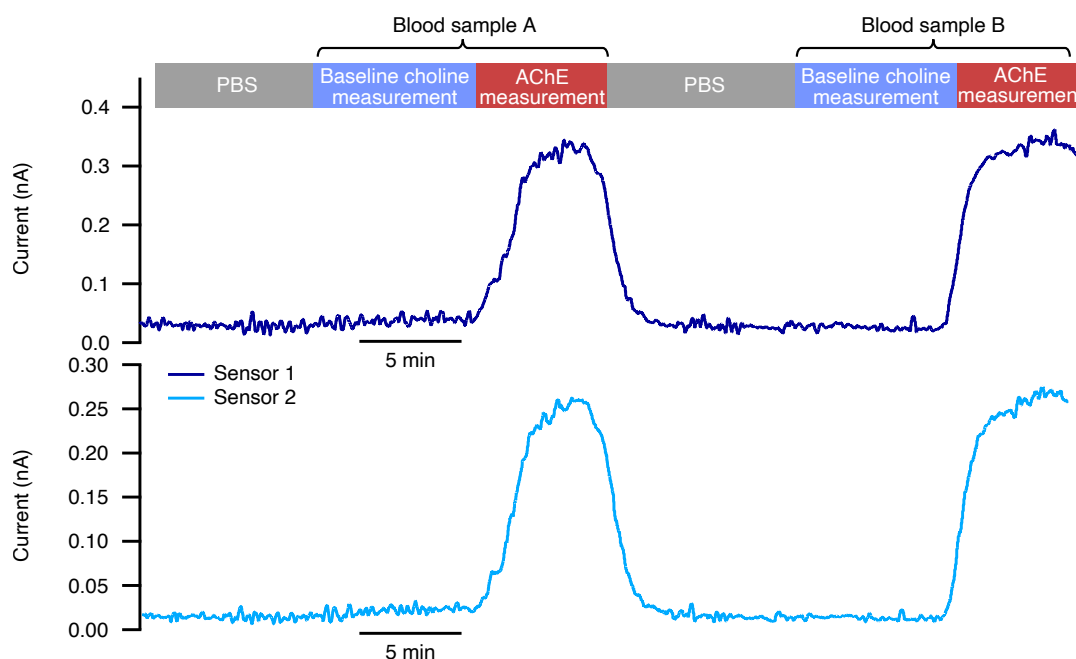


Figure 8.11: The current traces for two biosensors recorded during measurements of two blood samples. Two blood samples (92%) blood were analysed using the monitoring system comprising of the flow cell with integrated microdialysis. Initially, a baseline choline measurement was made. During this period, no ACh is added to the sample stream. Instead, PBS is added to the sample stream to create the same dilution effect as the ACh. The signal goes up when the ACh is added to the sample stream, as it is hydrolysed by the AChE in the blood, generating choline which diffuses into the dialysate stream and is detected by the choline biosensors at the outlet of the MD probe. The current trace was recorded at 10 Hz. The traces were smoothed by using a 201-point Savitzky-Golay filter.

The basal choline measurements allow us to measure the choline level but also the background signal from blood, as it is possible there would be some interference from blood. The low baseline signal is very promising. It suggests that there is little interference from other electroactive species in

the blood and also suggests that, in these blood samples, the basal choline levels are low.

After subtracting the basal choline signal and the ACh background signal, determined from the 0* step in the calibration shown 8.12, the resulting currents recorded from the blood measurements were in the range of 0.22 - 0.25 nA for sensor 1 and 0.19 - 0.21 nA for sensor 2. To convert this current to activity, the calibration curves had to be used. Initially, an AChE activity calibration up to 4 U/ml was performed, the trace of which is shown in figure 8.12.

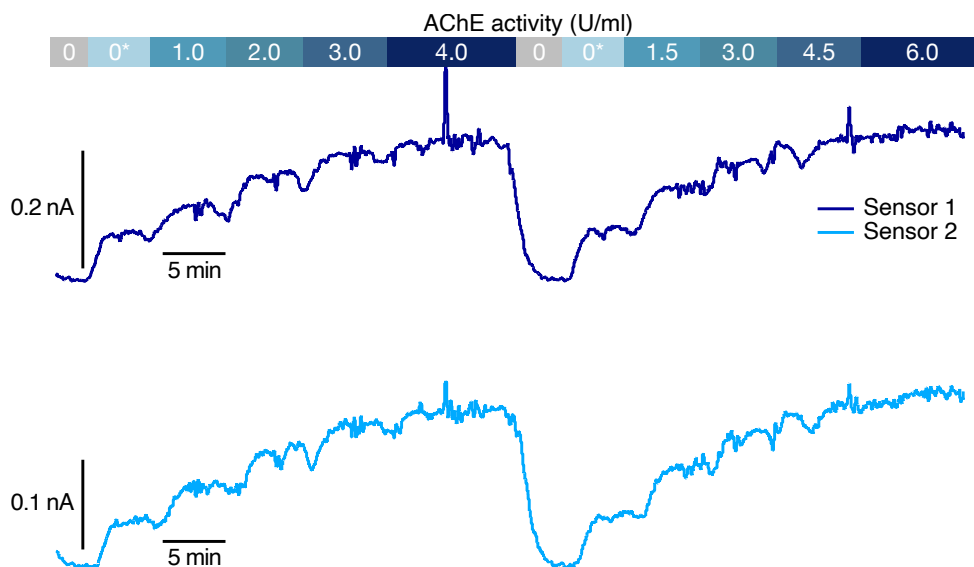


Figure 8.12: The current traces recorded during an AChE activity calibration up to 4 U/ml and an AChE activity calibration up to 6 U/ml. The traces recorded for two biosensors are shown. The calibration stream was flowed into the flow cell at a rate of 20 $\mu\text{l}/\text{min}$. The resultant choline from ACh hydrolysis diffused into the dialysate stream and was detected at the choline biosensors connected to the outlet of the MD probe. 0* denotes the ACh baseline, when ACh was being run through the flow cell with 0 U/ml AChE. The current trace was recorded at 10 Hz. The traces were smoothed by using a 201-point Savitzky-Golay filter.

From the calibration curves the 4 U/ml AChE measurement resulted in currents of approximately 0.16 and 0.12 nA for, sensor 1 and 2 respectively, after the background ACh signal had been subtracted off. Therefore, the signal from the 92% blood samples was beyond the range of the calibrations. To try and obtain a calibration in the correct range, another calibration was performed up to 6 U/ml, shown by the second calibration trace in figure 8.12. From the trace, it is clear that there is a plateau in current at higher activities. These results suggest that either the biosensor or the experimental parameters were not in the right range. To further investigate this, the calibration curves were plotted for the AChE calibration up to 6 U/ml, as it was clear that the blood measurements were in an enzyme activity range closer to the higher activity levels investigated. The resulting calibration curves are shown in figure 8.13 for the two biosensors. The curves have been fitted using both Michaelis-Menten (MM) kinetics and a linear regression.

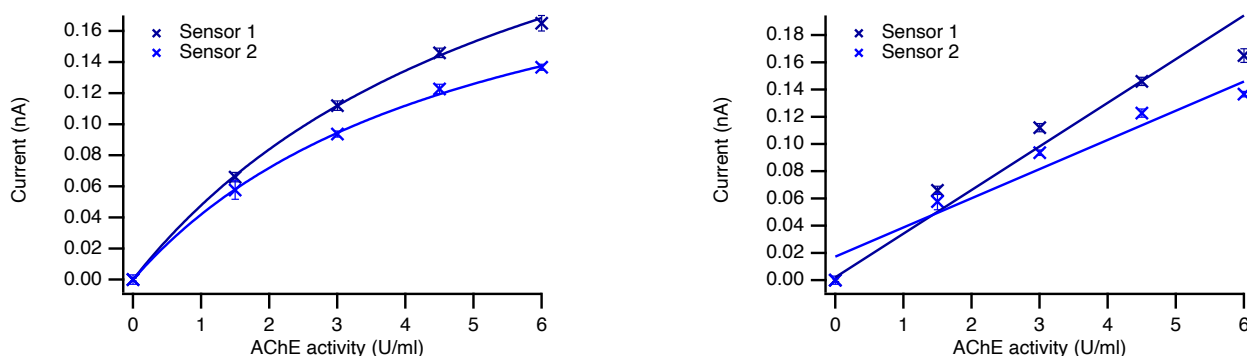


Figure 8.13: Calibration curves obtained for an AChE activity calibration up to 6 U/ml. Left: The calibration curves have been fitted using MM kinetics. Right: The calibration curves have been fitted using linear regression. The markers and error bars for both graphs show the mean $\pm$ standard deviation for each current step.

The graph on the left shows the curves fitted with MM kinetics. This has resulted in a good fit of the data, however as the curves are only just starting to leave the linear region, there are no points on the plateau and, therefore, it is an inaccurate way to obtain V_{max} and K_m . The graph on the right shows the curves fitted using linear regression. The data points do not appear to be linear, particularly for sensor 1. The resulting gradients are 0.034 ± 0.03 nA/U/ml and 0.0214 ± 0.0006 nA/U/ml for sensor 1 and sensor 2, respectively. The two fits suggest that the data follows MM kinetics, meaning it is likely that the choline biosensor is in the enzyme-limited range due to the higher activities generating more choline that is diffusing into the probe and starting to saturate the biosensors at the probe outlet. Regardless, an attempt was made to use the two fits to calculate the resulting activity in blood.

Using the MM fit, the calculated AChE activities ranged from 12 - 25 U/ml, with propagated errors as large as 50%. This suggests that the apparent K_m and V_{max} cannot be used to calculate the concentration for a given signal unless the full MM curve is obtained. The linear fit resulted in more reasonable activities for sample A of 7.0 ± 0.3 and 9.0 ± 0.3 U/ml for sensor 1 and 2, respectively

and for sample B of 7.6 ± 0.3 and 9.7 ± 0.4 U/ml for sensor 1 and 2, respectively. However, using a calibration curve that does not have an accurate fit is not ideal.

The results suggest that the AChE activity values of the blood samples were outside of the calibration range. Trying to extrapolate outside of the calibration range is inaccurate. To overcome this issue, measurements of the same blood were performed with 50% blood dilutions. The resulting current ranges obtained for these AChE measurements after the ACh background had been removed were much lower (in the region of 0.1 nA). These currents correspond to lower AChE activities, which appear to be within the linear range of the biosensors. To determine the activities in these blood dilutions, the calibration performed up to 4 U/ml AChE was used, but only 0-3 U/ml AChE activities were considered when performing the linear regression, as 4 U/ml was beyond the linear range. It should be noted that the currents obtained for this calibration were slightly higher compared with the currents obtained for same corresponding AChE activities in the 6 U/ml calibration. This could be due to the slight increase in ACh baseline, or due to the sensors degrading. It could also be due to errors associated with making two different solutions, for example pipetting errors. The resulting calibration curves are shown in figure 8.14.

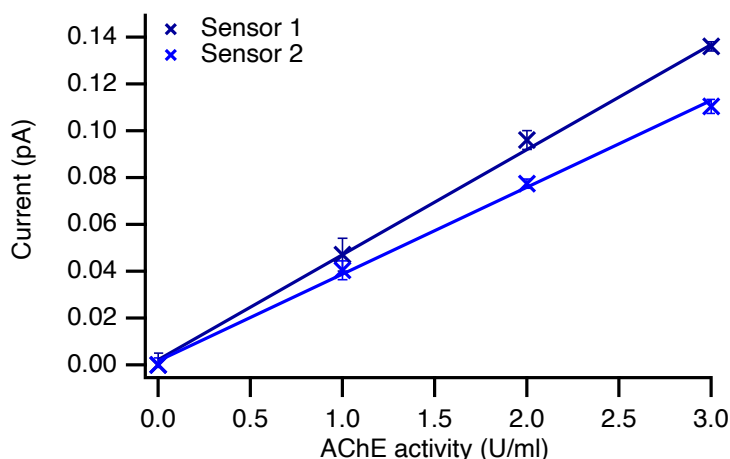


Figure 8.14: Calibration curves for a linear section of the system response to changes in AChE activity, up to 3 U/ml. The markers and error bars represent the mean $\pm$ standard deviation for each current step. The data has been fitted using linear regression.

These values were used to convert the recorded current for the 50% blood dilutions to AChE activity. These values were then multiplied for a factor of 2 to deduce what the AChE activity in undiluted (100%) blood would be. The resulting activities are shown in table 8.3.

Blood sample	AChE measurement (U/ml)		
	Sensor 1	Sensor 2	Ellman's assay
A	5.2 ± 0.2	5.4 ± 0.4	6.71
B	6.7 ± 0.4	6.7 ± 0.4	6.93

Table 8.3: AChE activity measurements in blood using my assay and the Ellman's assay.

The values obtained across both sensors are the same or otherwise very similar, demonstrating that the results were repeatable.

These blood samples were tested using a modified Ellman's assay. This one varied slightly from the one previously described. A Randox Daytona+ clinical analyser is used. The reagent concentrations used were as before, 2.25 ml of a 0.33 mM DTNB in 0.2 M potassium phosphate buffer, 300 μ l of 1 mM acetylthiocholine iodide solution and the sample was diluted 1 in 40. The reagents were dispensed automatically by the analyser and the mixture was incubated in the cuvette for 20 s before the change in absorbance was measured for 182 seconds at intervals of 13 seconds at a wavelength of 415 nm and a temperature of 37°C. The resulting activities obtained are also shown in table 8.3. The results obtained from my assay do differ from those obtained by the Ellman's assay, however, they are of the same order of magnitude which is highly promising. Furthermore, my assay is in agreement with the Ellman's assay that sample B has a higher AChE activity than sample A. This demonstrates that differences in AChE activity between samples can be detected with my assay.

To further test the capabilities of the monitoring system to perform sensitive measurements in whole blood, a polyurethane (PU) coating was added to the biosensor. The aim of this was to extend the dynamic range of the choline biosensor, in the hope of achieving sensitive detection for higher AChE activities without the need to dilute the sample. The AChE activity calibration was altered so that the activity varied from 0 to 8 U/ml in 2 U/ml steps, to be within the range of higher activities levels observed in the blood. The ACh concentration was increased to 6 mM in the reservoir corresponding to 3 mM at the flow cell to account for the increased activity as the reaction needs to be in the enzyme-limited region to detect changes in AChE activity. Two biosensors were used for this experiment, one with a PU coating and one without a PU coating, to determine if the calibration plateauing was due to the choline biosensor saturating or due to the experimental parameters. The calibration traces and corresponding curves are shown in figure 8.15.

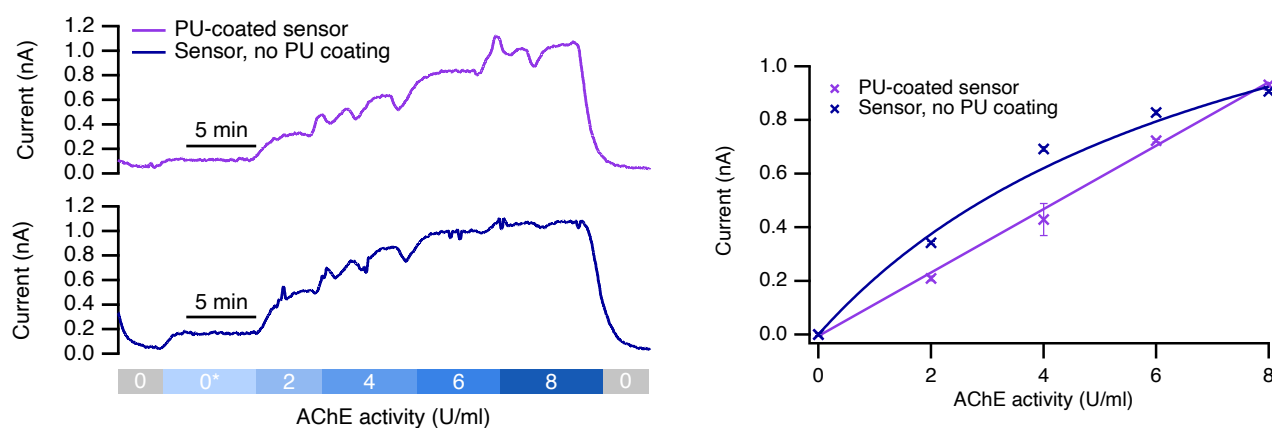


Figure 8.15: Current trace and corresponding calibration curves obtained during an AChE activity calibration up to 8 U/ml using a biosensor with a PU coating and a biosensor without one. Left: The current trace recorded for two biosensors; one with a PU coating and one without. The unstable signal on the 4 U/ml and 8 U/ml steps are due to the refilling of the syringe pumps. In this case, only one AChE pump shown in figure 7.10 was used to perform this calibration as the other pump was producing unstable flow, therefore the flow was briefly stopped while the pump refilled. The current was recorded at 10 Hz. The trace has been smoothed using a 201-point Savitzky-Golay filter. Right: The corresponding calibration curves. The biosensor with no PU coating exhibits non-linear behaviour, suggesting the biosensor is beginning to saturate. This data has been fitted using MM kinetics. The data obtained using the PU-coated biosensor is linear and has been fitted using linear regression. The markers and error bars represent the mean $\pm$ standard deviation of each current step.

The traces demonstrate that a linear calibration is achieved for the PU-coated sensor, and a non-linear calibration is achieved for the biosensor without a PU coating. This suggests that at these high levels of AChE, the choline produced as a result of ACh hydrolysis and the amount of choline diffusing into the probe is beyond the dynamic range of the biosensor (without PU). The linear calibration obtained with the PU-coated biosensor shows the capabilities of this monitoring system to detect higher activity levels.

Using this experimental setup with these sensors, a further 2 samples of blood were measured, referred to as samples C and D. These samples were also collected as part of routine health screening at DSTL from consenting individuals. As before, the final whole blood percentage was 92% to allow the addition of surfactant. Only the PU-coated biosensor signal was analysed. Described previously, if the full MM curve is not obtained to accurately calculate the apparent K_m and V_{max} of the biosensor, then it is not an accurate way to convert between current and concentration.

The activity of the two blood samples (92% whole blood) were found to be 2.9 ± 0.2 and 4.0 ± 0.2 U/ml for sample C and D, respectively. This corresponds to 3.2 ± 0.2 and 4.3 ± 0.2 U/ml for sample C and D, respectively, for 100% blood. Using the same modified Ellman's method described above, as part of the health screening, the activity of sample C was determined to be 6.93 U/ml. Sample D was not analysed in the health screening, however, the individual's baseline was found to be 6.39 U/ml based on previous routine measurements. The results suggest that my assay is measuring slightly

lower AChE activity than expected, however, further samples need to be analysed to determine if this is the case.

8.3.4 Monitoring blood inhibition following exposure to an OP compound

The next step was to investigate whether it was possible to measure the effect of OP compounds in blood samples.

The blood samples C and D were diluted to 50%. The total blood sample was approximately 500 μ l, from which a 92% blood measurement was already made as described in the previous section, with and without the addition of ACh. This used 200 μ l of the total blood sample. This meant approximately 300 μ l of sample was left. For this experiment, samples had to be diluted to allow addition of both surfactant and paraoxon, and it was also necessary to have a large enough sample, ideally 100 μ l, to be able to run a 6 minute measurement but also have some more sample available in case there were any issues when running the sample through the microfluidic platform, for example there could be equipment failure. For these reasons combined with the limited sample volume, the blood was diluted to 50% for these inhibition experiments.

The paraoxon was left to inhibit the blood for at least 2 hours. The ‘baseline’ measurement of the 50% diluted blood was not made due to a limited sample volume and based on the 92% sample, the baseline signal is very small ($< 1\%$) relative to the signal obtained due to the addition of ACh in the system and therefore is not necessary for this dilution.

The activity of the 50% blood dilution with no inhibitor was found to be 2.68 ± 0.07 and 2.63 ± 0.05 U/ml for sample C and D, respectively. This would equate to 5.36 ± 0.14 U/ml and 5.26 ± 0.10 U/ml in 100% blood for sample C and D, respectively. As these were the same samples used in the previous section the results can be compared to the results obtained when measuring the activity in the 92% whole blood sample. In order to compare the measured values for the different dilutions of blood samples C and D have been multiplied by 2 and 1.09 for the 50% and 92% dilution, respectively. These calculated values of AChE activity with propagated errors are presented in table 8.4.

% whole blood used for measurement	Calculated AChE activity (U/ml)	
	Sample C	Sample D
50	5.36 ± 0.10	5.26 ± 0.10
92	3.20 ± 0.20	4.30 ± 0.20

Table 8.4: The AChE activity measured in different dilutions of two blood samples.

Noticeably, for sample C, the result obtained from the 50% whole blood sample (5.36 ± 0.10 U/ml) is high relative to that obtained from the 92% whole blood sample (3.2 ± 0.2 U/ml). For sample D, the signal from the 50% whole blood sample (5.26 ± 0.10 U/ml) is above the measurement obtained from the 92% whole blood sample (4.3 ± 0.2 U/ml), but the difference seen here is less extreme compared

to sample C. These differences in measure AChE activity suggest the system potentially needs further refinement. The probe recovery may be different in the 92% blood compared to the 50% blood and compared in 0.01 M PBS, the solution in which the calibration is performed, due to the viscosity of different blood dilutions and liquids. To gain a better understanding of probe recovery in blood, it could be spiked with known concentrations of choline and the resulting recovery calculated.

The resultant, absolute AChE activities measured following inhibition with 50 nM and 100 nM paraoxon and without paraoxon are shown in table 8.5 for both samples, without the dilution factor considered.

[Paraoxon] (nM)	AChE activity measurement (U/ml)	
	Sample C	Sample D
0	2.68 ± 0.07	2.63 ± 0.05
50	0.89 ± 0.03	0.35 ± 0.07
100	0.60 ± 0.10	0.84 ± 0.04

Table 8.5: The AChE activity of blood samples diluted by 50% following exposure to OP compounds.

Compared to the AChE activity recorded for the 50% blood dilution with no inhibitor, the residual % activity following 50 nM paraoxon exposure was 33.2% and 13.3% for samples C and D, respectively. The residual % activity following 100 nM paraoxon exposure was 22.4% and 31.9% for samples C and D, respectively. For both samples, it is clear that inhibition has taken place and a resultant drop in activity in blood has been detected. These are very promising results that demonstrate very clearly that the device is usable for its intended purpose. The results for sample D are surprising as the residual activity increases with increasing paraoxon exposure. It is possible that the samples were mixed, which the results indicate was the case. The two blood measurements show similar uninhibited AChE activities, so it would be expected that they would be inhibited a similar amount.

8.4 Conclusion

In this chapter, experiments were carried out as part of a collaboration with DSTL, Porton Down. The monitoring system, including the microfluidic platform, flow cell and wireless electronics, was taken to DSTL for the purpose of testing my assay with OP compounds and real blood samples to compare my results with the current standard assay used.

The analysis system was successfully employed in an external environment with relative ease, demonstrating that this device is portable between different locations without the need for any additional equipment.

The developed assay was successfully used to detect changes in AChE activities following exposure to paraoxon. These changes were detected, both continuously and discretely. The continuous measurement shows great potential for this device to be employed to further study inhibition kinetics.

Furthermore, it could also be utilised to study the success of treatment. The setup for discrete monitoring also showed great potential and has a number of benefits in detecting AChE activity levels in samples that may have been exposed to OP compounds.

The monitoring system was utilised to monitor near-whole blood levels (92%). Baseline choline measurements allowed us to see any baseline level from possible interferents in the blood, however, the very small signal recorded during this measurement demonstrates that the analytical system is highly satisfactory in blocking out interferents. The capability to run blood through the microfluidic platform and analysis system for the first time demonstrates the promising capabilities of this analytical device. It was unknown if this would work and potentially other preparation steps might have been needed, but following careful design, the system works very well for its intended purpose.

There is more research needed to investigate the differences between my assay measurements and the standard assay measurements. It is likely that in blood the probe recovery is different, therefore more experiments are needed to determine the probe recovery when blood is being sampled. However, these are very exciting result, demonstrating the potential for the assay to be used in a wide range of applications.

Chapter 9

Conclusion

9.1 Overview

The aim of this thesis was the development and use of a system for the continuous monitoring of acetylcholinesterase (AChE) activity. This thesis has described the different stages in producing the final monitoring system, including the challenges that were overcome.

The developments of two different methods to detect choline, the product of the reaction between acetylcholine (ACh) and AChE, using choline oxidase (ChOx) has created flexibility in the choline monitoring system. The fabricated choline biosensor has excellent sensitivity with a mean of 4.5 ± 1.6 nA/mM. This was a great result given the typical sensitivities of that the glucose and lactate biosensors without a polyurethane (PU) layer are both 1 nA/mM. The in-flow ChOx system has been found to give a better sensitivity of 24.68 ± 0.09 nA/mM for the chosen experimental parameters of a 30 cm length of tubing, a 2.5 μ l/min flow rate and 0.2 mg/ml ChOx concentration. Although this could be useful in future applications, the biosensor sensitivity was already beyond what was needed and provides a more compact device for monitoring choline concentrations.

As part of clinical studies, the choline biosensor was successfully used *in vivo* during cardiac arrest and resuscitation attempts. It also required the modification and development of the electronic enclosure for wireless monitoring, further refining this system for use in crowded monitoring environments, such as operating theatres, and external settings.

Changes in AChE were monitored successfully using the microfluidic platform modified for the addition and manipulation of ACh and AChE streams. Following this, attempts were made to detect AChE activities using microdialysis (MD) in solution. Delivering ACh externally to a solution containing AChE and using one MD probe for the pick up of the product choline showed promising results. However, an unstable, increasing signal meant that it was not possible to see a clear difference in signal between AChE activities in the raw data. It became apparent that to get reliable and repeatable results, the system needed to be highly controlled.

Retrodialysis for delivery of ACh was also trialled with both a one probe and a two probe system as a potential means to monitor *in vivo*. Results from using one probe were promising for low level AChE activities, however an increasing background signal from ACh was observed, likely due to enzyme sticking to the MD probe. The two probe system showed that using one probe to deliver ACh and the other to pick up choline did produce a signal, however, repeatable and reliable results were hard to obtain. It was also challenging to deliver a high enough ACh concentration to sensitively detect changes in AChE activity.

To overcome these a microfluidic flowing system that incorporated an MD probe was designed. Unlike the microfluidic platform and analysis chip previously developed within the Boutelle group, the flow cell importantly provided means for an analysis system that allowed sampling with MD. Following the initial design, the flow cell was optimised. The two iterations of flow cells were characterised for different flow rates in terms of time response and probe recovery. The final design gave an excellent time response of approximately 80 s, and a probe recovery of $35.6 \pm 0.6\%$ at an external flow rate of 20 $\mu\text{l}/\text{min}$ in the flow cell, and a flow rate of 2 $\mu\text{l}/\text{min}$ of the dialysate in the MD probe. The probe recovery is a potentially useful tuning parameter as limiting probe recovery could prevent the choline biosensor from saturating if necessary.

The flow cell was used to detect changes in AChE activity. Preliminary results were promising as a stable signal was recorded for each different AChE activity, which had not been achieved before. This meant differences in AChE activity could be seen in the raw data.

Initially, there were still problems with enzyme sticking to the flow cell channel and the MD probe. However, this was overcome by adding a pluronic surfactant to the flow stream to limit the amount of enzyme sticking. This resulted in repeatable and reliable AChE calibrations at activities in the range observed in whole blood.

In experiments carried out at DSTL, Porton Down, the flow cell coupled with MD and the choline biosensor analysis system was used to probe changes in AChE activity following exposure of an AChE sample to organophosphorus (OP) compounds. Different concentrations of OPs were used to inhibit AChE samples. The system was compared to the standard method that is based on the Ellman's assay. Dynamic and static experiments were performed. The dynamic experiments demonstrated the potential for the device to be used as a continuous monitoring system.

Finally, the system was successfully used to sample whole blood AChE levels. Almost undiluted blood samples (92% blood) were successfully run through the microfluidic platform and the flow cell without interfering with the analysis system. It became apparent that the choline biosensor was saturating for higher AChE activities, therefore a polyurethane (PU) membrane was added to the choline biosensor surface to extend its linear range. The addition of the PU membrane meant the choline biosensor produced linear calibrations in the higher AChE ranges (up to 8 U/ml), an excellent result. This meant the AChE levels in near whole blood (92%) were successfully measured.

Blood samples were diluted to 50% and exposed to OP compounds. The resulting decrease in AChE activity was successfully measured, demonstrating that this system can be used to monitor changes in AChE activity in biological samples.

9.2 Future research

This research has demonstrated the potential for the analysis system to perform real-time and continuous measurements of AChE activity for *in vitro* experiments. There are a number of directions this research can now take.

- **Further validation of the AChE monitoring system in blood and comparison to Ellman's assay**

While these preliminary experiments show great potential, further validation is needed to understand how the assay compares to the standard currently used based on the modified Ellman's assay. This would likely involve repeats of the static inhibition experiments to compare to the Ellman's assay results. It would also be highly beneficial to continue to monitor in blood and refine the system for this purpose.

- **Dynamic experiments**

The system can be used further for the measurement of OP inhibition kinetics. It would be interesting to investigate the differences between different OP compounds. It is also of great interest to understand treatment dynamics, to investigate the use of different oximes to treat OP exposure.

The system is ready for use to detect the effect of OP compounds directly in unknown liquid samples.

- **Further optimisation of the flow cell**

While it has not been a problem for the experiments discussed in this thesis, further optimisation of the flow cell may be desirable. There is potential to make the flow cell channel smaller, reducing the volume further to improve temporal resolution and the time delay to see changes in signal. By reducing the flow cell volume, less blood will be needed.

It is also imaginable to have an entirely self-supporting field version. Many aspects of the analysis system would need to be refined. This would need to include autocalibration of the sensors, and the flow cell could be coupled with small pumps to allow the flow of samples into the flow cell and allow perfusion of the MD probe.

- **Retrodialysis of ACh and moving towards an *in vivo* assay**

It would be highly beneficial to further investigate the use of retrodialysis of ACh through a one probe or a two probe system. In particular, it would be useful to test the capabilities of the flow cell in performing retrodialysis using the one probe system, as the addition of surfactant has proven to reduce sticking of AChE, therefore, it may help to reduce the background signal seen from ACh. The flow cell could also be optimised to include two probes. This could be useful to investigate the potential for monitoring using MD in the blood vessel *in vivo*.

- **Monitoring different biological targets**

Monitoring AChE levels in other tissues is highly desirable to investigate the capabilities of monitoring in other locations. While monitoring the blood provides valuable and useful information, the ability to monitor in subcutaneous tissue could open up potentials for this to be a wearable device. AChE levels could also be investigated in muscle. MD would provide a means to perform these measurements.

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