

Electrochemical biosensors for continuous infection monitoring and early detection of sepsis

Saylee Jangam

Department of Bioengineering
Imperial College London

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**Imperial College
London**

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Abstract

The aim of this thesis was to develop electrochemical biosensors for the purpose of continuous infection monitoring. Lactate and C-reactive protein (CRP) were chosen as the biomarkers of interest to (i) detect early-warning signs of sepsis and (ii) monitor infection levels to support dose optimisation of antimicrobials, respectively.

A first-generation microneedle-based lactate oxidase biosensor was developed, and its dynamic range was extended using polyelectrolyte membranes. Koutecký–Levich analysis of the mass transport of lactate in the membranes revealed that polyurethane, cellulose acetate and Nafion performed best in extending the dynamic range. Nafion was chosen as the membrane for *in vivo* studies conducted with healthy volunteers producing lactate during exercise due to ease of fabrication methods and relatively high dynamic range. The *in vivo* studies showed good agreement between ISF (biosensor) and blood lactate, as well as between ISF and dialysate lactate (via microdialysis).

An electrochemical aptamer biosensor (E-AB) was developed for CRP using an aptamer reported by Huang *et al.* The aptamer was modified with a methylene blue redox reporter and optimised with respect to signal response. The aptamer was successfully deposited on the electrode, however, an electrochemical signal difference was not seen between bound and unbound states using multiple modalities. The same aptamer was modified with an osmium(II/III) redox label, developed by Ben Aissa *et al.*, and E-ABs fabricated. The aptamer was successfully deposited on the electrodes and able to produce a signal response at a potential of +0.2 V, however, there was some overlap with background signals.

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List of abbreviations

6-MCH – 6-mercaptohexanol
AA – Ascorbic acid
ABG – Arterial blood gas
AFP - Alpha fetoprotein
AIDS - Acquired immunodeficiency syndrome
ALISA – Aptamer Linked Immobilized Sorbent Assay
ALP – Alkaline phosphatase
AM – Acetaminophen
AMD – Age-Related Macular Degeneration
AMP – Adenosine monophosphate
AOx – Alcohol oxidase
ApDC – Aptamer-drug conjugates
AQ – Anthraquinone-2-carboxylic acid
AS1411 – An aptamer drug for leukemia
ASA – Acetylsalicylic acid
ATP – Adenine triphosphate
AUC – Area under curve
AuNPs – Gold nanoparticles
BCOP – Blood colloidal osmotic pressure
BSA – Bovine serum albumin
Bpm – beats per minute
CA – Cellulose acetate
CA-125 - Carbohydrate antigen 125
CDM – Continuous drug monitoring
CE – Capillary electrophoresis
CE-SELEX – Capillary electrophoresis Systematic Evolution of Ligands by Exponential Enrichment
CGM – Continuous glucose monitor
CHI – CH Instruments
CIM – Continuous infection monitoring
COVID – Coronavirus disease
CRP – C-reactive protein
CV – Cyclic voltammetry
DADMAC – Dimethyldiallylammoniumchloride
DET – Direct electron transfer
DI – Deionised
DK – Product of diffusion coefficient (D) and partition coefficient (K)
DLS – Dynamic light scattering
DNA – Deoxyribonucleic acid
DOX – Doxorubicin
DPV – Differential pulse voltammetry
E-AB – Electrochemical aptamer biosensor
ECM – Extracellular matrix
EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDC/NHS – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-2-(N-morpholino)ethanesulfonic acid (NHS)
 ELONA – Enzyme-linked oligonucleotide assay
 ES – Enzyme-substrate complex
 FACS – Fluorescence-activated cell sorting
 FAD - Flavin adenine dinucleotide
 FADH - Flavin adenine dinucleotide
 Fc – Ferrocenecarboxylic acid
 FDA – Food and Drug Administration
 FMN – Flavin mononucleotide
 GCE – Glassy carbon electrode
 GNP – Gold nanoparticle
 GO – Graphite oxide
 GO-DETA – Diethylenetriamine modified graphite oxide
 GOD – Glucose oxidase
 HDV – Hydrodynamic voltammetry
 HFIP – Hexafluoro-2-propanol
 HIV – Human immunodeficiency virus
 HIVRT – HIV Reverse Transcriptase
 HNL – Human neutrophil
 HPLC – High performance liquid chromatography
 HRP – Horseradish peroxidase
 hVEGF – Human vascular endothelial growth factor
 IDT – Integrated DNA Technologies
 IFN- γ - interferon-gamma
 IL-6 – Interleukin-6
 IL-8 – Interleukin-8
 IgG – Immunoglobulin G
 IrOx – Iridium oxide
 ISF – Interstitial fluid
 KDM – Kinetic differential measurements
 LCMS – Liquid Chromatography Mass Spectrometry
 LDH – Lactate dehydrogenase
 LMB – leuco methylene blue
 LMIC – Low- and middle-income countries
 LOD – Lactate oxidase
 LOESS - Locally estimated scatterplot smoothing
 MB – Methylene blue
 MBH – reduced form of methylene blue
 MES – 2-(N-morpholino)ethanesulfonic acid
 MEDIC – Microfluidic Electrochemical Detector for In vivo Continuous monitoring
 MIC – Minimum inhibitory concentration
 MIRACLE – Minimally Invasive Realtime Assessment of Continuous Lactate in Exercise
 mPD – m-phenylenediamine
 MWCNTs – Multi-walled carbon nanotubes
 NAD – Nicotinamide adenine dinucleotide
 NADH – Reduced form of nicotinamide adenine dinucleotide

NAMD - Nanoscale Molecular Dynamics
 NB – Nile blue
 NEWS – National Early Warning Score
 NHS – 2-(N-morpholino)ethanesulfonic acid (NHS)
 NICE – National Institute for Health and Care Excellence
 NR – Not reported
 OCT – Optical coherence tomography
 oPD – o-phenylenediamine
 Os – Osmium
 PBS – Phosphate buffer saline
 PC – Polycarbonate
 PCR – Polymerase chain reaction
 PCT - Procalcitonin
 PD – Pharmacodynamics
 PDGF – Platelet derived growth factor
 PDMS – Polydimethylsiloxan
 PEDOT – poly(3,4-ethylenedioxythiophene)
 PEDOT:PSS-PB – poly(3,4-ethylenedioxythiophene) polystyrene sulfonate prussian blue
 PEDOT:PSS-PB-EG-DVS – poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)
 potassium ferric ferrocyanide composite cross linked with ethylene glycol and divinyl sulfone
 PEG – Polyethylene glycol
 PEG-DE – Poly(ethylene glycol) diglycidyl ether
 PEI – Polyethylenimine
 PfLDH - Plasmodium falciparum lactate dehydrogenase
 pHEMA - Polyhydroxyethylmethacrylate
 PID - Proportional integral derivative
 PK - Pharmacokinetics
 PK-PD – Pharmacokinetics-pharmacodynamics
 polyA – poly adenine
 PPD - Polyphenylenediamine
 PPy – Polypyrrole
 PSA – Prostate specific antigen
 PSSA - Polystyrene sulfonic acid
 PTFE - Polytetrafluoroethylene
 PU - Polyurethane
 PVC – Polyvinyl chloride
 PVC-DOS – Polyvinyl chloride-dioctyl sebacate
 PVI – poly(1-vinylimidazole)
 PVP – poly(4-vinylpyridine) (PVP)
 RDE – Rotating disk electrode
 RE – Reference electrode
 RNA – Ribonucleic acid
 RT – Room temperature
 SAM – Self-assembled monolayer
 SARS – Severe acute respiratory syndrome
 SCE – Saturated calomel electrode

SD – Standard deviation
SELEX – Systematic Evolution of Ligands by Exponential Enrichment
SIRS – Systemic inflammatory response syndrome
SPR – Surface plasmon resonance
SPRI - Surface plasmon resonance imaging
ssDNA – Single stranded deoxyribonucleic acid
SWV – Square wave voltammetry
TCEP – Tris-(2-carboxyethyl) phosphine hydrochloride
TDM – Therapeutic drug monitoring
TEA – Triethanolamine
tHb – Total hemoglobin
THF – Tetrahydrofuran
TI – Therapeutic index
TMB – Tetramethylbenzidine
TNF- α - Tumour necrosis factor alpha
TTF – Tetrathiafulvalene
TTF-TCNQ – Tetrathiafulvalene-tetracyanoquinodimethane
UA – Uric acid
UHPLC – Ultra-high-performance liquid chromatography
UKAS – United Kingdom Accreditation Service
UV – Ultraviolet
VEGF – Vascular endothelial growth factor
VMD – Visual molecular dynamics
WE – Working electrode

Chapter 1 Introduction

1.1 A brief history of antimicrobials and the development of antimicrobial resistance

Antimicrobials are used to treat bacterial infections such as urinary tract infections, bacterial meningitis, and pneumonia. Antimicrobials are also used to prevent infections in surgeries and are prescribed to immunosuppressed patients, such as those undergoing chemotherapy or under treatment for HIV/AIDS. Some antimicrobials, such as doxorubicin and mitomycin, are also used as anticancer drugs. Antimicrobials also treat life-threatening bacterial infections such as sepsis, a bloodstream infection that can cause septic shock and organ failure if inadequately treated. In addition to their applications in healthcare, antibiotics are widely used in agriculture to treat and prevent infections in animals, maintain their health, and support their growth for meat production. In low- and middle-income countries, antibiotics are used to treat food- and water-borne infections such as typhoid fever caused by *Salmonella typhi*, as well as infectious diseases such as tuberculosis and malaria.^{1,2}

Antimicrobials were first discovered in 1928 and have since become the cornerstone of modern medicine. Since their discovery, they have greatly extended human life expectancy and reduced childhood mortality.^{3,4} Before antibiotics, 43% of people died from common infections and life expectancy was 20 years lower than it is today. The first antibiotic to be used clinically was salvarsan, salvation arsenic, for the treatment of syphilis. Following this came the discovery of the first naturally produced antibiotic, penicillin, in 1928. The antibiotic golden age, when many new antibiotic classes were discovered, was between the 1950s and 1960s, after which bacterial resistance started emerging (Figure 1.1). The first case of methicillin resistance *Staphylococcus aureus* was found in the 1960s, followed by

vancomycin-resistant *enterococci* in the 1980s, and vancomycin-resistant *Staphylococcus aureus* in the 2000s. The last new class of naturally produced antibiotic was discovered in the 1980s. The lack of sufficient new antibiotic development, combined with the inappropriate use of antibiotics, has led to a continued rise in antimicrobial resistance (AMR).

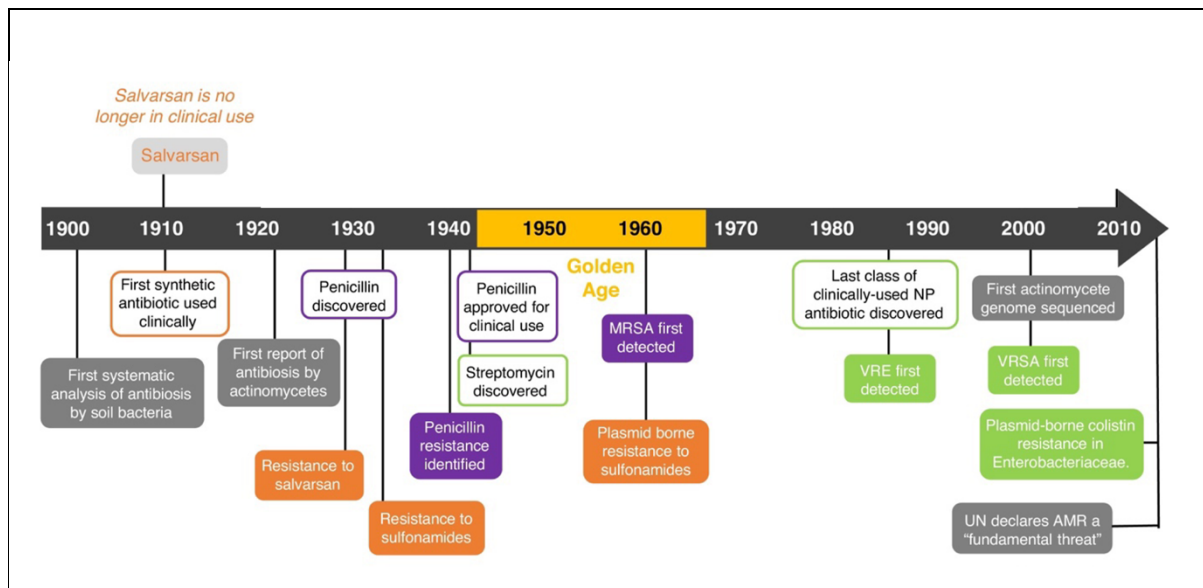


Figure 1.1 A brief history of antibiotic discovery and development of resistance from the 1900s to the 2000s (adapted from Hutchings et al.).³

AMR is the inevitable evolutionary process by which bacteria develop resistance towards an antibiotic, making treatment with that antibiotic ineffective. AMR poses a significant threat to global health because once our antimicrobial resources are no longer effective against infections, we will not be able to use them for infection prevention in surgeries or to treat life-threatening conditions like sepsis.

It is estimated that by 2050, 10 million people will die from antibiotic-resistant bacterial infections each year. In England, the number of antibiotic-resistant bloodstream infections have increased over the years, seeing a small decrease between 2019 and 2020, but have

been on the rise since then, as reported by the UK Health Security Agency (Figure 1.2).⁵ A large percentage of drug-resistant bloodstream infections come from *Escherichia coli* (*E. coli*), followed by *Klebsiella pneumoniae* (*K. pneumoniae*), and *Enterococcus faecalis* and *Enterococcus faecium* (*Enterococcus spp.*). This follows the general trend of rising antibiotic-resistant infections across the world; the post-antibiotic era is no longer just a threat, but an eventuality.⁵

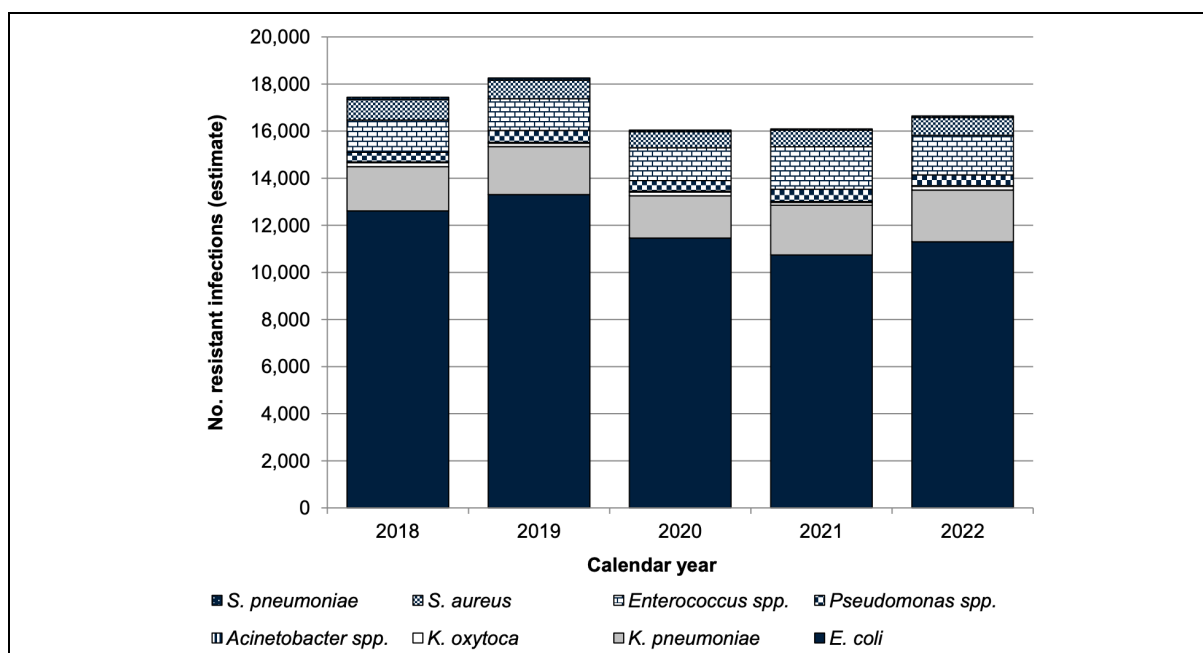


Figure 1.2 Number of antibiotic-resistant bloodstream infections in England from 2018 to 2022, as reported by the UK Health Security Agency.^{5,6}

1.2 Factors contributing to the rise of AMR

There are several factors contributing to the rise of AMR, including the overuse and misuse of antimicrobials, as summarised in Figure 1.3. Between 2000 and 2010, the global consumption of antibiotics increased by 40%,³ meaning that antibiotics were being prescribed in situations where they weren't necessarily needed, such as for viral infections. Another

factor contributing to the rapid depletion of working antibiotics is the fact that there are few new antibiotics in the pipeline. Since the end of the golden age of antibiotic discovery in the 1960s, no new classes of antibiotics have been discovered.⁷ This is because pharmaceutical companies have not prioritised the development of novel antimicrobials due to low profitability margins.

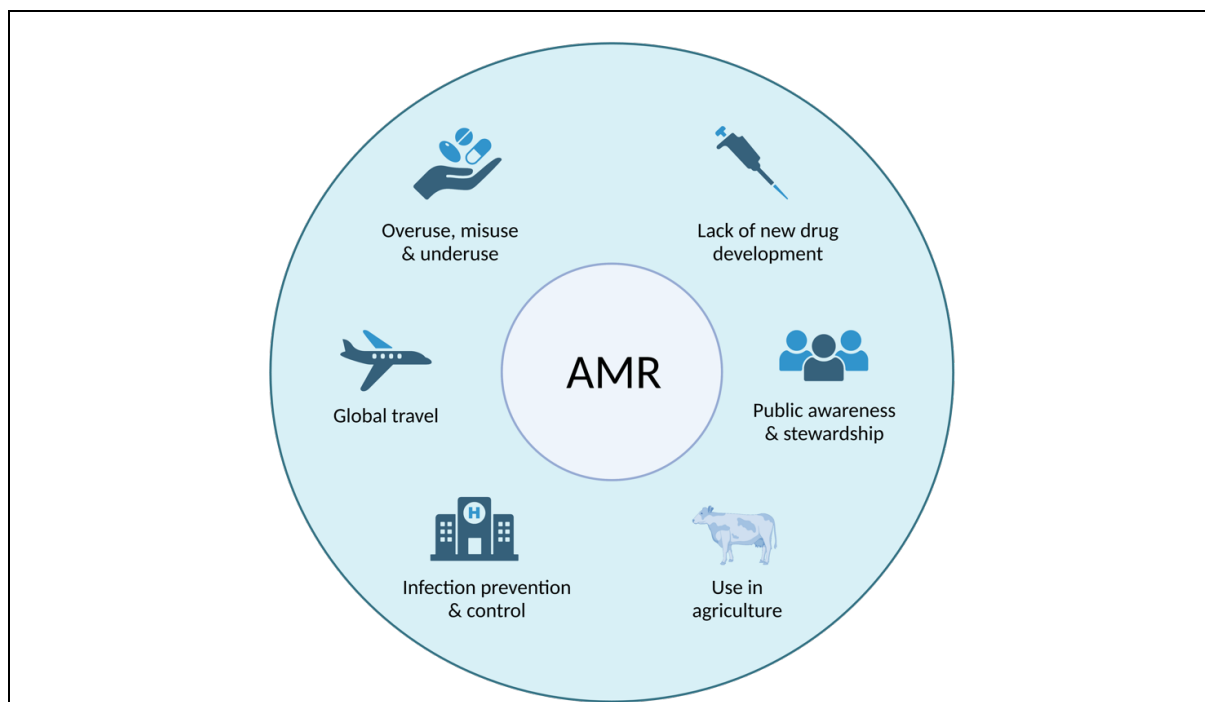


Figure 1.3 Factors contributing to the rise of antimicrobial resistance (AMR).

Other factors contributing to the rise in AMR include global travel leading to wider spread infections as witnessed in the COVID-19 pandemic, poor infection prevention and control in healthcare settings, and overuse in agriculture for farming animals and meat production. In addition, the unregulated use of antibiotics and availability of antimicrobials over the counter in some low- and middle-income countries has allowed further misuse of antimicrobials, allowing bacteria to develop resistance due to a lack of medical adherence. There is also a lack of public awareness and stewardship about the importance of safeguarding

antimicrobials and the onset of the post-antibiotic era. We need more education and outreach around this issue, as well as training around stewardship for healthcare practitioners.

1.3 Pharmacokinetics and pharmacodynamics

In addition to overuse and misuse, the underuse of antibiotics has also been reported in intensive care where up to 75% of patients are under prescribed antibiotics.⁸ This is due to the high inter- and intra-individual variability in the pharmacokinetic profiles of critically ill patients who are dosed using recommendations developed for non-critically ill patients with more predictable pharmacokinetic-pharmacodynamics (PK-PD).⁹ Administering too low a dose reduces the likelihood of clearing infection, which can cause clinical complications and provide selection pressure that increases the risk of the development of drug-resistant bacteria.

Pharmacokinetics (PK) is how the plasma drug concentration changes over time for a known dose of the drug.¹⁰ PK Encompasses the kinetics of absorption, distribution, metabolism, and excretion. Pharmacodynamics (PD) is how the drug affects the body, such as its effectiveness and the reduction in infection level it causes. The PK-PD ratio is the ratio of the free antibiotic concentration in plasma to the minimum inhibitory concentration (MIC) of the antibiotic¹¹ and is a target parameter in PK-PD studies. Table 1.1 summarises the different parameters used in the study of PK-PD for therapeutic drug dosing.¹¹

PK-PD parameter	Description
PK-PD ratio	The ratio of the free antibiotic concentration in plasma to the minimum inhibitory concentration (MIC) of the antibiotic.
Free antibiotic concentration	The free antibiotic concentration in blood plasma measured at 50% or 100% of the dosing interval.
Minimum inhibitory concentration (MIC)	The minimum plasma concentration of antibiotic that is required to prevent the growth of bacteria in culture.
Area under curve (AUC)	The “exposure” to a drug (i.e. total drug concentration), calculated from the area under the concentration-time curve.
C _{max}	The maximum concentration of antibiotic in blood plasma.
T _{max}	The time taken to reach maximum concentration, C _{max} .
Volume of Distribution (V _d)	The ratio of the amount of drug in the body (mg) to the plasma concentration of the drug (mg/L). A high V _d indicates the drug’s propensity to leave plasma and enter extravascular compartments.
Rate of clearance (C _L)	The rate at which the drug is excreted from the body, for example, through renal excretion.
AUC/MIC	The ratio of the total exposure of an antibiotic to the minimum concentration required to complete bacterial killing.
C _{max} /MIC	The ratio of the maximum concentration of antibiotic in blood plasma to the minimum concentration required to complete bacterial killing.
T>MIC	The amount of time where the free antibiotic concentration in plasma is above the MIC.

Table 1.1 An overview of PK-PD parameters used in dosing decisions.

Each PK profile typically shows an increase in drug concentration after dose administration, where the rate of absorption is higher than the rate of excretion, followed by a decrease in drug concentration where the excretion rate is greater than the absorption rate. A typical PK profile is shown in Figure 1.4. The MIC is the minimum plasma concentration of an antibiotic that is required to prevent the growth of bacteria. The free antibiotic concentration in plasma is measured at 50% or 100% of the dosing interval, which is defined as the interval between two subsequent doses. The amount of time where the free antibiotic concentration in plasma is above the MIC ($T > MIC$) is widely used as a PK-PD index that informs dosing recommendations. Antibiotic plasma levels below the MIC increase the risk of developing antimicrobial resistance and puts the patient at risk of developing sepsis; this would result in the patient requiring a higher dose of antibiotic or a combination of multiple antibiotics to overcome serious infection later on.

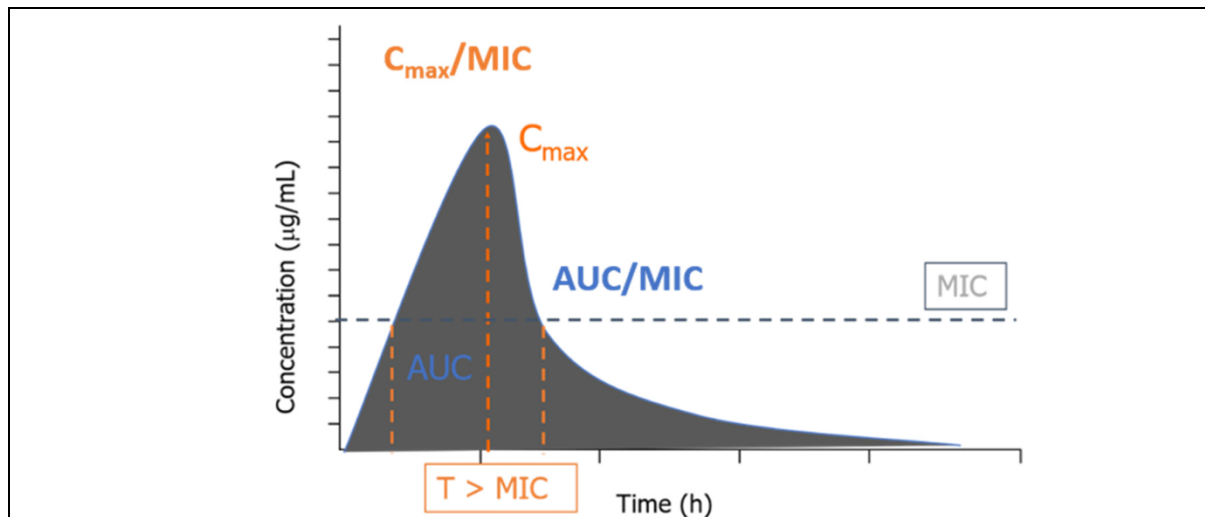


Figure 1.4 A typical PK profile of a drug, as reported by Pereira *et al.*¹²

There is inter-individual variability in PK-PD that is dependent on factors such as age, gender, race, weight, and any existing comorbidities. The PK-PD will change during the course of infections due to the pathophysiological changes that occur in critically ill patients, which

increases the volume of distribution (V_d) and the rate of drug clearance.¹³ Therefore, in addition to inter-individual variability in PK-PD, there is also considerable intra-individual variability in the PK profiles of critically ill patients.^{11,14} These differences point to the need for individualised drug dosing regimens that are currently only available for a small number of drugs through therapeutic drug dosing (TDM).

1.4 Sepsis

Sepsis is a life-threatening condition which requires broad spectrum antimicrobial treatment. Annually, there are more than 48 million cases of sepsis and 11 million cases of sepsis-related deaths globally.¹⁵ In the UK, there are 245,000 cases of sepsis annually, resulting in 48,000 deaths,¹⁶ and accounting for a third of all hospital admissions to intensive care. Sepsis disproportionately impacts lower- and middle-income countries, with a large proportion of sepsis cases occurring in neonates.^{17,18}

AMR is a key driver in the global burden of sepsis due to community- and hospital-acquired sepsis cases resulting from drug-resistant bacterial infections.¹⁵ AMR can negatively impact the treatment of sepsis in several ways, including the reduced number of effective antibiotics available to choose from when treating sepsis patients due to drug-resistant bacteria. In addition, sepsis can lead to a weakened immune system, increasing the risk of developing secondary bacterial infections, which can be caused by resistant bacteria, known as “super infections”. This can lead to complications in clearing the infection, requiring a stronger dose of antibiotic.

1.4.1 The difficulty in early diagnosis of sepsis

Due to the non-specific symptoms of sepsis and the overlap of symptoms with systemic inflammatory response syndrome (SIRS), diagnosis can be delayed. This results in delayed treatment, allowing bacterial infections to grow, increasing the risk of drug resistance and mortality. The symptoms of sepsis range from a high or low body temperature, a pulse rate of over 90 beats/min, reduced urine output, display of new confusion or drowsiness, and serum lactate of over 2.0 and 4.0 mmol/L for sepsis and septic shock, respectively. There are three broad stages of sepsis: early-stage sepsis, severe sepsis, and septic shock. Early-stage sepsis consists of a bacterial infection with symptoms of SIRS, severe sepsis is a progression of early-stage sepsis, whereby signs of organ dysfunction may be observed, and septic shock is where hypotension is presented, despite administration of fluids for recovery. The differences in symptoms between SIRS, early-stage sepsis, severe sepsis, and septic shock are outlined in Table 1.2, and these are used as diagnostic criteria for assessing the severity of sepsis.

In addition to the overlap of symptoms with SIRS, there is a large variety of symptoms, which can lead to delayed diagnosis and treatment. In a study of 904 patients with confirmed severe sepsis or early septic shock, 23% received inadequate antimicrobial therapy.¹⁹ This was defined either if the patient received an antimicrobial that was ineffective against at least one of the pathogens causing the infection within 24 hours of a confirmed diagnosis, or if the therapy was not administered early on after the diagnosis. In the group that was adequately treated with antimicrobials, the 28-day mortality was found to be 24%, much lower than the 39% mortality of the group of inadequately treated patients ($p < 0.001$).¹⁹

SIRS	Early-stage sepsis	Severe sepsis	Septic shock
- Temp. >38.3°C or <36.0°C - Pulse >90 beats/min - Respiratory rate >20 breaths/min - New confusion or drowsiness - White blood cell count >12 or <4x10⁹ cells/L - Blood glucose >7.7 mmol/L	- SIRS symptoms - Presumed or confirmed bacterial infection	- Early-stage sepsis symptoms - Serum lactate >2 mmol/L - Serum creatinine >176.8 µmol/L or urine output <0.5 mL/kg/h - Platelet count <100x10⁹ platelets/L - Bilirubin >34.2 µmol/L - Bilateral lung infiltrates - New need for oxygen to maintain saturation >90%	- Severe sepsis symptoms - Serum lactate >4 mmol/L - Hypotension: systolic blood pressure (BP) <90 mmHg, mean arterial BP <65 mmHg

Table 1.2 Symptoms of SIRS, early-stage sepsis, severe sepsis, and septic shock.

Early intervention is key to the optimal treatment of sepsis, as well as to improving patient outcomes and reducing the further development of AMR. In treating sepsis, broad-spectrum antibiotics are often prescribed to clear a wide range of possible infections. Early warning

signs of sepsis need to be recognised in order to start treatment and prevent further deterioration.

1.4.2 Diagnosis of sepsis

In the UK, sepsis is diagnosed as per the guidelines provided by the National Institute for Health and Care Excellence (NICE). A standardised framework, the National Early Warning Score (NEWS), has been developed to assess patients with suspected sepsis. This framework outlines the assessment of six physiological parameters in patients including pulse rate, blood pressure, respiratory rate, oxygen levels, temperature, and level of consciousness or confusion. Each of these parameters receive a score during assessment (on a scale of 1 to 10, with 1 being the least severe and 10 being the most severe) and an aggregate score is calculated to stratify the risk of sepsis and bacterial infection. The NEWS scoring system has been assessed for the early triage of patients with sepsis and septic shock in a study of 500 patients in the UK.²⁰ The study concluded that patients with an aggregated NEWS score of 3 or greater were at a high risk of septic shock, with the scoring system presenting a sensitivity of 92% and a specificity of 77%. In addition to using risk stratification scores, sepsis is diagnosed using laboratory methods such as blood tests for C-reactive protein and lactate, polymerase chain reaction (PCR) based molecular diagnostic tests for bacterial and fungal DNA in whole blood, and serum culture to identify bacterial infections.

In practice, if sepsis is suspected, doctors will prescribe broad spectrum antibiotics or antifungals or both to prevent deterioration. This happens without a confirmed diagnosis of infection and without knowledge of the type of pathogen causing the infection. The use of broad-spectrum antimicrobials can lead to the growth of antimicrobial-resistant bacteria.

To provide a faster diagnosis, molecular diagnostic tests are being investigated for use in the NHS to detect sepsis early and to identify the pathogen causing the infection for targeted treatment. The tests being investigated are the LightCycler SeptiFast Test MGRADE (Roche Diagnostics), SepsiTtest (Molzym Molecular Diagnostics), and the IRIDICA BAC BSI assay (Abbott Laboratories). While it was found that these tests had good accuracy and specificity, the implementation would require changes to current clinical workflows to achieve a rapid turn-around of results.²¹ It was found that it may also be difficult to obtain the required volume of blood from critically ill paediatric patients and neonates to carry out the tests. In addition, lactate measurements are periodically taken from acutely ill patients to monitor tissue perfusion; these measurements are made from whole blood drawn from arteries and assessed on the arterial blood gas (ABG) machines or in some cases, on handheld lactate analysers present in intensive care units. In a study comparing lactate measurements made using a handheld analyser, an ABG machine, and via central laboratory analysis methods, it was found that the median time from blood draw to lactate results was 5, 10 and 133 minutes for the handheld device, ABG machine, and via laboratory methods, respectively.²²

Point-of-care handheld analysers and ABG analysers offer quicker results and require a smaller amount of blood to be drawn for measurements. This can accelerate decision-making around treatment, particularly in patient populations where large blood draws are not possible. However, despite its convenience, the ABG procedure is painful as it requires arterial puncture and needle insertion or cannulation to draw blood, and is often difficult to perform, particularly in children and neonates, due to its invasiveness.²³ Arterial blood collection also presents an increased risk of clots, deep vein thrombosis (DVT), pulmonary embolism and stroke, as well as handling risks to those conducting the sampling process.

Another method of diagnosing sepsis is through bacterial culture, where blood drawn from the patient is sent to a central laboratory and is then cultured to identify the type of pathogen causing infection. There is invariably a time delay between testing and confirming sepsis due to the time taken to send samples to the laboratory, allow bacterial growth and analysis by trained personnel, which is often between 2 and 5 days.²⁴

1.5 Therapeutic drug monitoring of antimicrobials

Precision dosing, also known as Therapeutic Drug Monitoring (TDM), has been explored for some antimicrobials, such as penicillin, vancomycin and voriconazole.^{25–31} TDM involves assessing blood plasma concentrations of a given drug at multiple timepoints after administration. This is done by drawing venous blood and transporting this to a laboratory for sample preparation and stabilisation; it requires a validated assay, such as a chromatographic assay or immunoassay to measure drug concentration, as outlined in Figure 1.5. The drug concentration is then compared to the target therapeutic level – i.e., the concentration of the drug required to achieve the desired therapeutic effect – and a change is made in the next dose based on clinical judgement and dosing software recommendations.³²

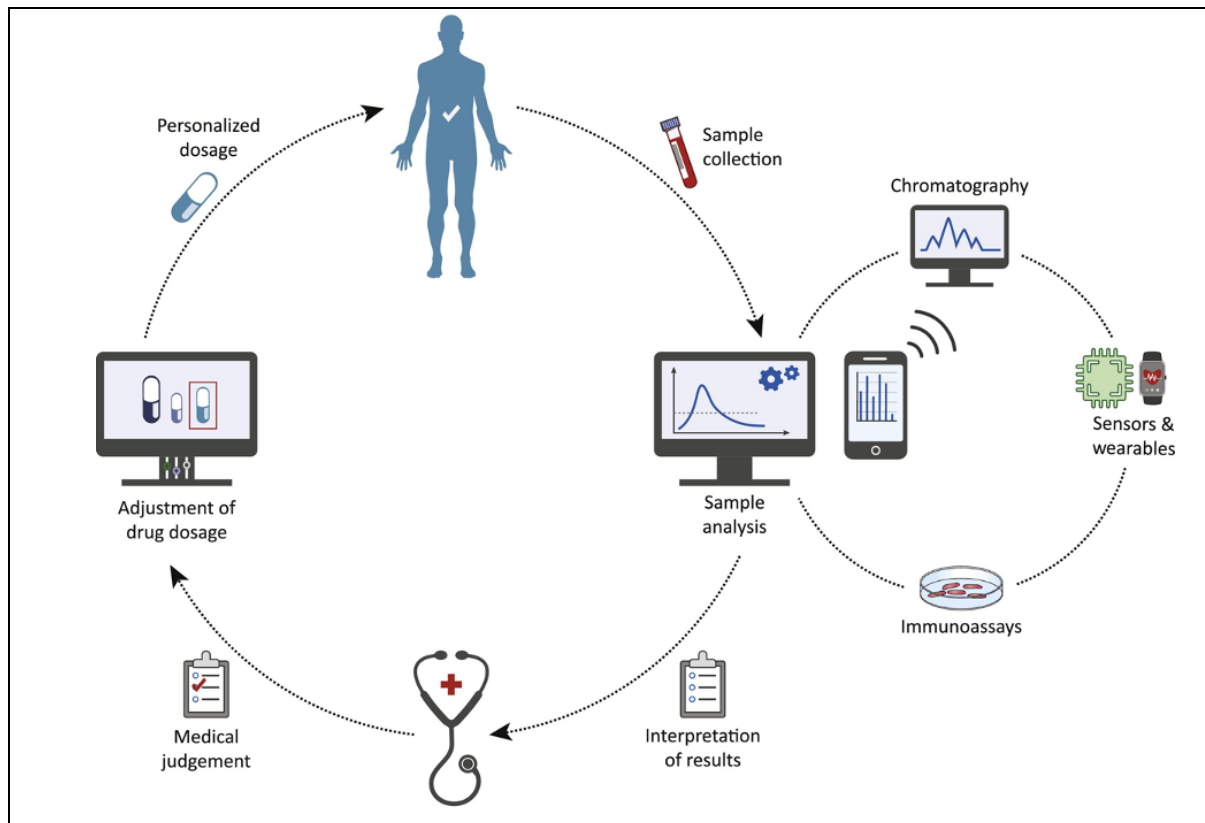


Figure 1.5 The process of therapeutic drug monitoring (TDM) involves sample collection, laboratory analysis, interpretation of results, and adjustments to drug dose at several timepoints after initial drug administration (as reported by Ates *et al.*).³³

Since TDM of antibiotics is carried out at selected timepoints beyond initial drug administration, the dosing strategy is based on patients' peak and trough drug concentrations, whereby the next dose is administered only after a trough in concentration has been reached, as shown in Figure 1.6. This approach often fails to achieve the targeted drug concentrations required to maximise antibacterial effect.³⁴ In addition, TDM carried out in the clinic is limited to drugs with a low therapeutic index (TI) or for drugs that are highly toxic.³⁴ TI is the ratio of drug concentration at which it is toxic to the concentration at which it is effective. For example, it is known that warfarin – an anticoagulant – has a low TI and therefore is a good candidate for TDM.³⁵ For a drug to be a candidate for TDM, there must be

no other measurable output of how effectively a drug is achieving its desired therapeutic effect and there must also be a reliable correlation between the drug concentration and clinical effect.³³

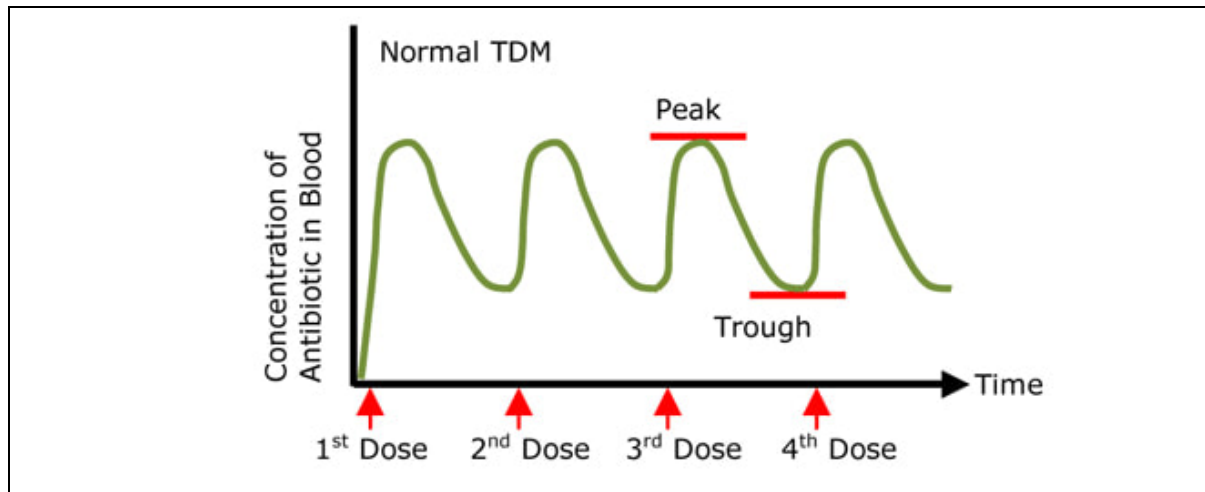


Figure 1.6 TDM of antibiotics involves dosing based on individual peak and trough drug concentrations, adapted from Watkins *et al.*³⁶

1.5.1 Challenges with TDM of antimicrobials

Despite the many benefits of TDM, it is not routinely used in practice for all drugs due to its high cost and labour intensiveness. It requires material, labour, and time; trained pharmacists and clinicians are needed, and blood samples must be collected, preserved, and processed several times a day for each patient to ultimately provide a dosing recommendation, as outlined in Figure 1.7. However, some studies argue that TDM can save huge costs for the NHS by avoiding inaccurate dosing.³⁷ Therefore, the potential for precision dosing of antimicrobials for curbing drug resistant infections, optimising the use of effective antimicrobials, minimising risks to patients, and saving costs for the NHS are huge.

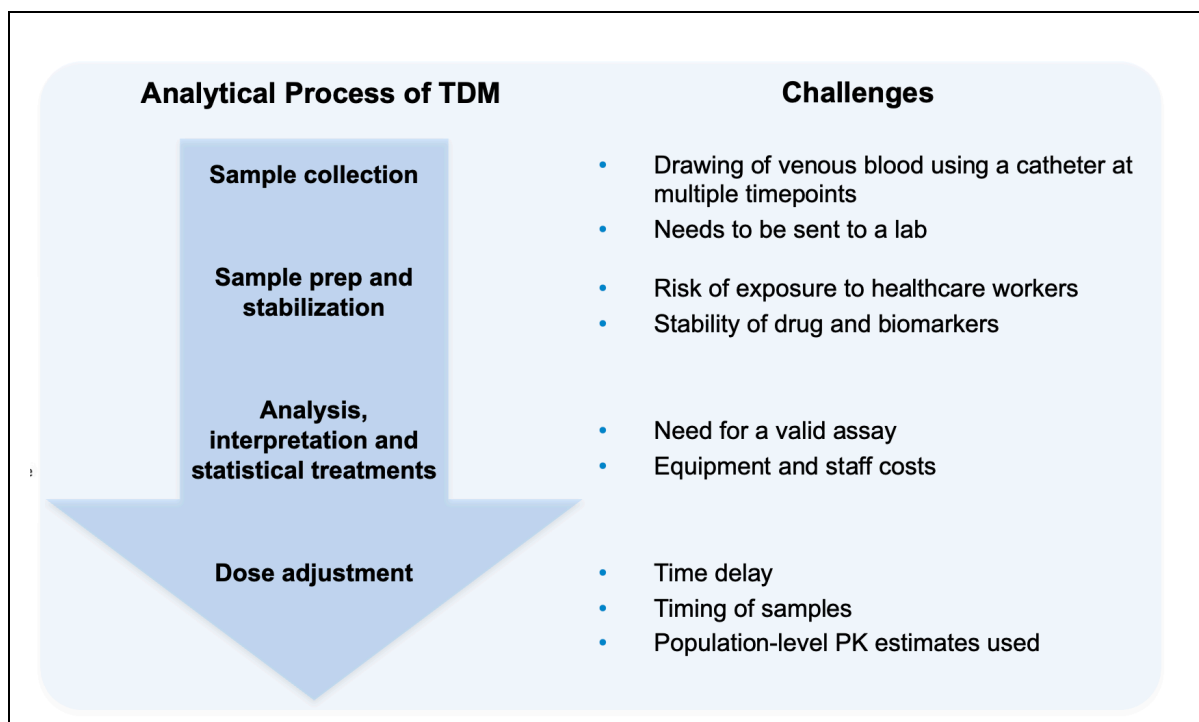


Figure 1.7 The challenges associated with TDM at each step in its process.

To overcome the challenges associated with TDM, a non-invasive or a minimally invasive approach needs to be developed wherein sampling and sample transport to a laboratory can be overcome by on-site measurements *in vivo*. This would provide timely results to support clinical decision making around dose adjustments. This is where electrochemical biosensors, such as those developed by Gowers *et al.*,³⁸ for the continuous monitoring of beta-lactam antibiotics in interstitial fluid (ISF), can be helpful to provide real-time antibiotic concentrations without the need for blood draws or laboratory analysis.

For antibiotics, precision dosing can be informed through two sensing measurements: (i) continuous real-time sensing of the antibiotic in blood plasma or interstitial fluid (ISF) and (ii) continuous real-time sensing of biomarkers of infection to infer the positive effects of an antibiotic dose i.e., the extent of bacterial killing achieved. These two sets of data can then be used to inform, using Bayesian modelling techniques, a closed-loop dosing software such

as that developed by Herrero *et al.* in their *in silico* study for the delivery of vancomycin in non-critically ill patients.¹⁰ The software predicts pharmacokinetics, such as a PK profile of plasma drug concentration, based on inputs from sensor measurements of antibiotic concentrations in ISF. The model uses a proportional integral derivative (PID) controller and sets PK-PD targets, such as maintaining an MIC of 1 mg/L in plasma, and adjusts the recommended dose based on sensor measurements of antibiotic concentration in ISF or plasma. A second parameter that could inform this model is measurement of biomarkers of infection such as CRP or lactate to infer infection levels in plasma and targets may need to be set which control the dose calculation. For the continuous measuring of systemic biomarkers and TDM, a temporal resolution of 1 min is sufficient since the total volume of blood is 5 L for the average adult, with a heart rate of 60 beats per minute (bpm), and a stroke volume (i.e., volume of blood ejected with each heartbeat) of approximately 100 mL.^{39,40} Therefore, in one minute, the total volume of blood ejected from the heart through ventricular contraction is approximately 6000 mL or 6 L, meaning that the blood has travelled one complete vascular circuit, allowing perfusion of biomarkers and drugs. A rate of measurement any faster than this will therefore not yield any more valuable information.

The process of closed-loop drug delivery is outlined in Figure 1.8, adapted from Herero *et al.*,¹⁰ wherein microneedle biosensors are used to measure concentrations in ISF.

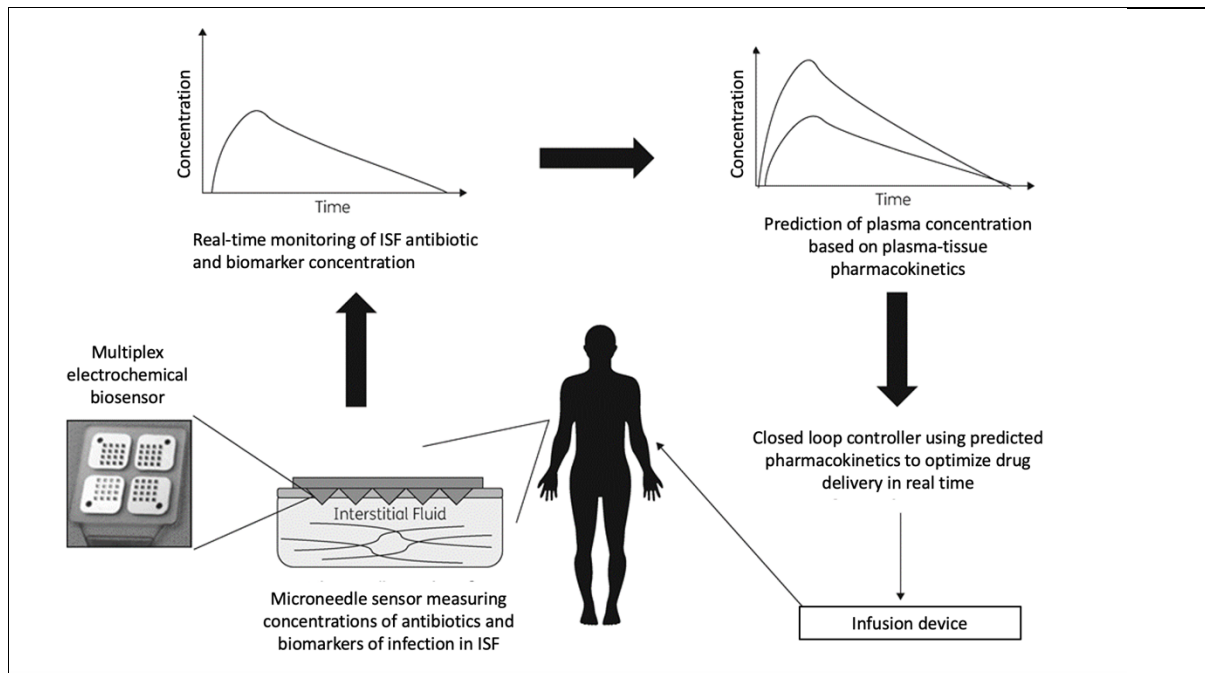


Figure 1.8 Real-time sensing of antimicrobials in interstitial fluid via microneedle biosensors to inform closed-loop drug delivery through an infusion device, adapted from Herrero *et al.*¹⁰

1.6 Biomarkers of infection

C-reactive protein (CRP) is a commonly used marker of infection and are used alongside leucocyte count and erythrocyte sedimentation rate to diagnose infections in the clinic. CRP production begins in the acute inflammatory stage of infection or injury and plasma concentrations rise above 200 $\mu\text{g/mL}$ within 24 hours, whereas concentrations in healthy individuals are generally just above 3 $\mu\text{g/mL}$.⁴¹ After the infection has been cleared, CRP levels decrease to normal levels. The vast difference in concentration between healthy and infected individuals makes CRP an attractive biomarker in monitoring infection, as demonstrated in Figure 1.9.^{42,43}

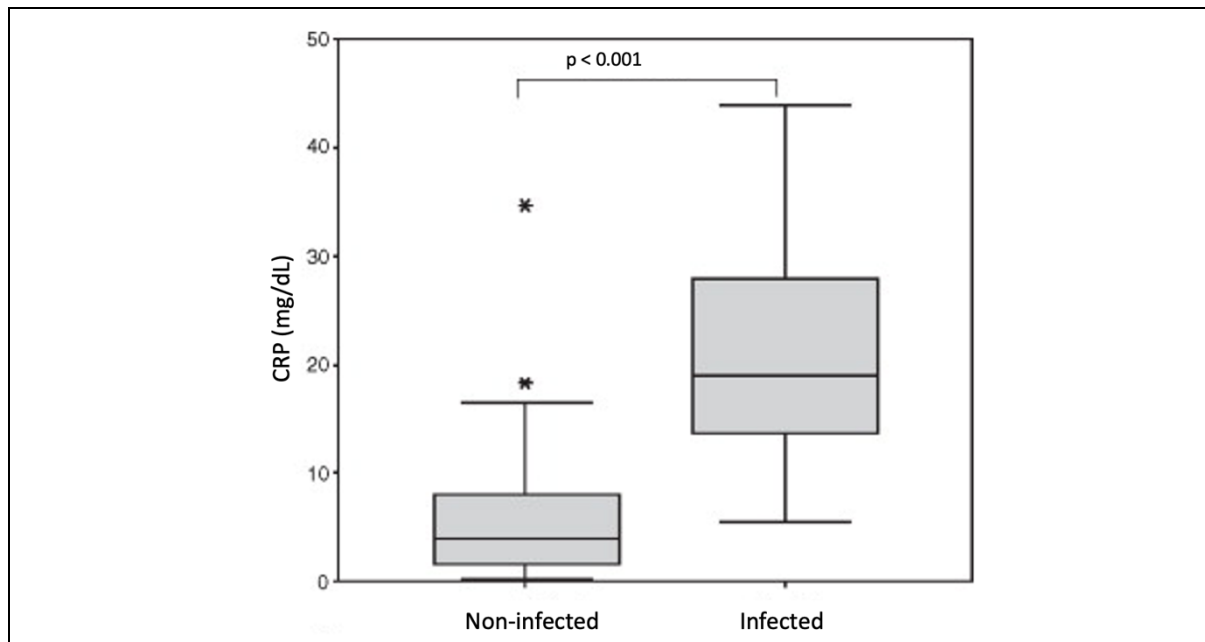


Figure 1.9 Box and whisker plot of CRP concentrations in healthy non-infected individuals vs infected individuals, adapted from Pova *et al.*⁴¹

Procalcitonin (PCT) is a promising biomarker of infection that has gained recent attention due to its investigated use in guiding antimicrobial therapy for COVID-19 and diagnosing other respiratory infections.^{44,45} Nobre *et al.* conducted a study wherein PCT was used to guide antibiotic therapy for sepsis patients. Serial measurements of PCT were taken for a group of sepsis patients and showed that the required duration of antibiotic therapy had reduced for the sepsis group vs the control group.⁴⁶ However, more clinical data is required to confirm the specificity of PCT for sepsis and infection before it can be relied on as a diagnostic marker.⁴⁷

Human neutrophil lipocalin (HNL) is another useful marker as it secreted by neutrophils in response to bacterial infection to prevent the uptake of iron(III) by bacterial siderophores. Bacteria depend on siderophores as this is their only source of iron, which is needed for

survival.^{48,49} The concentration of serum HNL in acute bacterial infections is significantly higher than in viral infections, thus making it a suitable marker for differentiating between acute bacterial and viral infections.^{50,51} In addition, lactate can be monitored as a marker of sepsis for early detection and can be used to monitor sepsis patients to prevent septic shock.^{51,52} It is produced through anaerobic respiration as a result of tissue hypoxia and inefficient lactate clearance, which indicates the presence of infection. Lactate is also used to monitor performance during exercise.⁵³

The biomarkers chosen for biosensor development in this thesis are lactate and CRP. Lactate was chosen as it a validated biomarker of sepsis and its change from baseline concentrations in sepsis patients is large and has previously been detected by sensors. CRP was chosen to support the optimised dosing of antimicrobials due its widespread use in infection monitoring and well-known concentration differences between healthy and infected individuals.

1.7 Measuring biomarkers in interstitial fluid

Interstitial fluid (ISF) is the extracellular matrix surrounding tissues in the body. It consists of water, sugars, fatty acids, coenzymes, hormones, neurotransmitters, salts, and cellular products.⁵⁴ It originates from blood plasma that has leaked out of capillaries to bring nutrients to the tissues and provide gaseous exchange between the blood and tissues. Therefore, there is a dynamic equilibrium between blood plasma and ISF that is impacted by cellular metabolism, as well as hydrostatic and osmotic pressures.⁵⁵

Hydrostatic pressure is the primary force that drives transport between the capillaries and the tissues. Hydrostatic pressure exerted by blood on the capillary walls is termed capillary

hydrostatic pressure (CHP), which drives fluid out of the capillaries. Hydrostatic pressure exerted by the interstitial fluid is lower than the CHP, causing fluid to move from the capillaries and into the ISF.

Plasma proteins form colloids in blood plasma, increasing the blood colloidal osmotic pressure (BCOP). Due to their large size, plasma proteins do not move out of capillary walls, causing a build-up of BCOP. While solutes do move across the capillaries into the ISF, the osmotic pressure of the ISF is lower than the BCOP. This means that fluid is reabsorbed into the lymphatic capillaries due to the osmotic pressure gradient created between the ISF and the capillaries. The volume of fluid that leaves the capillaries to form the ISF is greater than the volume of fluid that is reabsorbed into the lymphatic capillaries. For a 70 kg male who is not obese, the volume of ISF is about 11 L, accounting for 16% of total the body weight.⁵⁶ The interplay between the osmotic pressure gradient and the hydrostatic pressure gradient creates the dynamic equilibrium that exists between ISF and blood plasma.

1.7.1 Partitioning of analytes between blood and ISF

The plasma compartment makes up a quarter of the extracellular matrix (ECM) and the ISF makes up the remaining 75%.⁵⁷ The total volume of ISF in the body is somewhere between 9 to 13.5 L, of the 14 to 17 L that make up the ECM. The distribution of ions across the capillary walls into the ISF is controlled by the Gibbs-Donnan effect, wherein a difference in osmotic pressure and electrical potential is created between the two solutions (ISF and plasma) across a semi-permeable membrane (capillary wall), resulting in the uptake of water from the ISF, which maintains the arterial blood volume for tissue perfusion. The difference in osmotic pressure and electrical potential is created due to the presence of charged plasma proteins,

which cannot pass through the capillary walls, impacting the distribution of smaller ions across the membrane. The Donnan equilibrium maintains electroneutrality in both compartments.

As reported by Wolf *et al.* (shown in Table 1.3), the concentrations of smaller ions, such as Na^+ , K^+ and Cl^- , remain close to equal across both compartments, whereas albumin, a protein that can pass through the capillary walls, was at a much lower concentration in the ISF compared with the plasma.⁵⁸ For glucose in healthy individuals, the concentration range was similar across ISF and plasma, whereas for diabetic individuals, the concentration range of plasma glucose was almost double that of ISF glucose.

As reported by Friedel *et al.*,⁵⁴ bigger molecules and proteins (>70 kDa) in blood partition into the ISF via transcellular diffusion through the capillary endothelium, whereas ions and smaller molecules (<3 kDa) diffuse paracellularly, i.e., via junctions between the cells. Figure 1.10 demonstrates the size-based partitioning of analytes between blood and ISF, including the diffusion pathways used.

In commercially available continuous glucose monitors (CGM), developed for glucose measurements in the ISF of diabetic patients, a mathematical model is applied to overcome this difference between ISF and plasma glucose concentration as well as the time lag between the two. The application of linear regression, filters, and perturbation signals e.g., a change in voltage to understand how this affects sensor response and sensitivity, is used to compensate for the time lag and concentration differences in CGM devices.⁵⁹ The devices are also

calibrated against plasma concentration to understand correlation and error margins for ISF-plasma concentration differences.

Component	Plasma	ISF
Glucose (healthy)/ mM	4- 8	3.6- 6.5
Glucose (diabetic)/ mM	2- 40	2- 22
pH	7.4	7.39
Na ⁺ / mM	140	142
K ⁺ / mM	4.1	4.14
Cl ⁻ / mM	105	118
P _i / mM	1.16	1.12
pCO ₂ / Torr	40	46
HCO ₃ ⁻ / mM	24.2	29.4
Albumin/ mM	0.65	0.19

Table 1.3 The composition of various biomarkers across arterial plasma and ISF, as reported by Wolf *et al.*⁵⁸

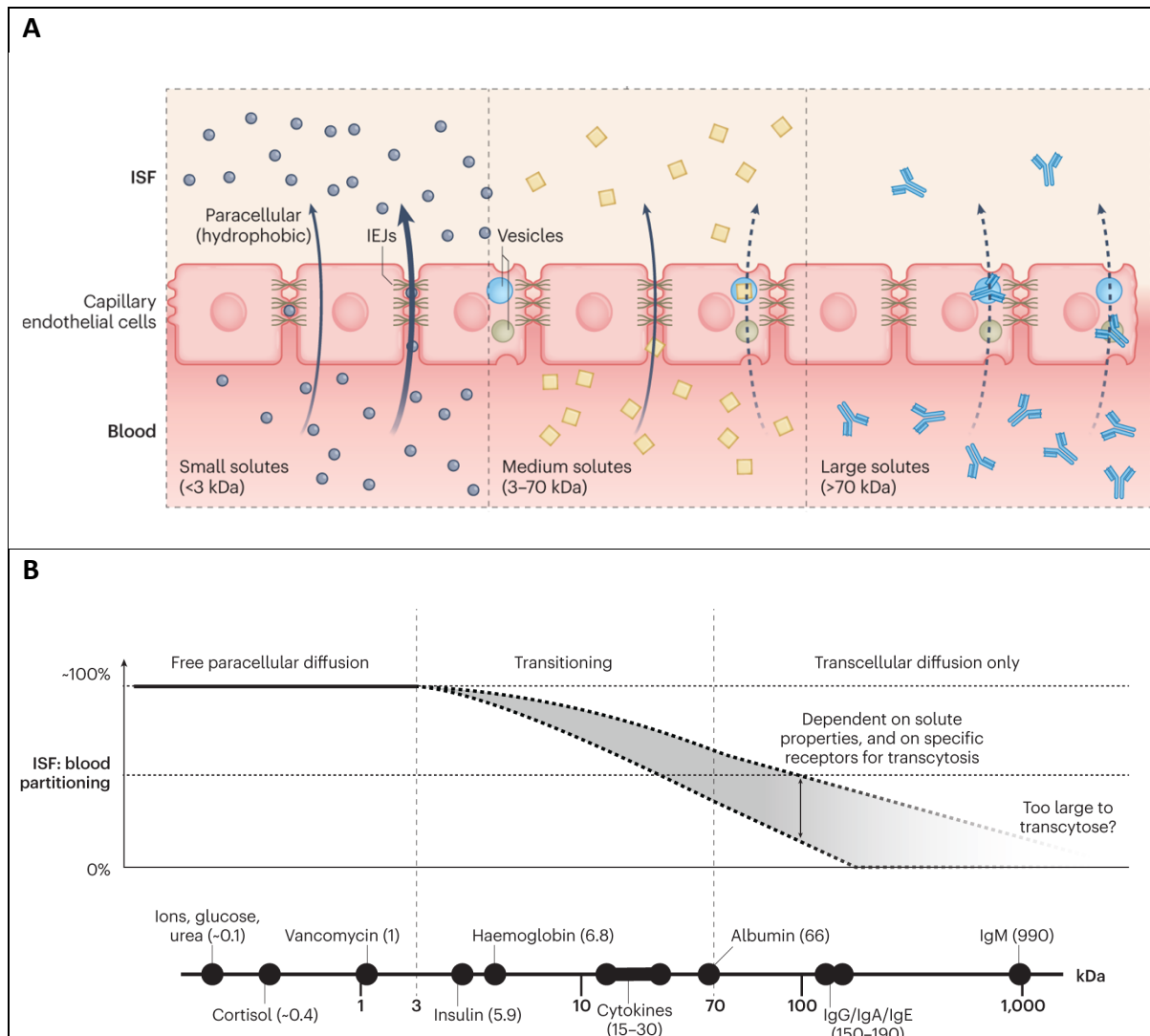


Figure 1.10 (a) Size-based paracellular and transcellular diffusion between the blood and ISF, (b) partitioning of different biomarkers based on molecular size in kDa, as reported by Friedel *et al.*⁵⁴

1.7.2 Accessing skin ISF

Since we know that ISF is a useful matrix to make measurements from, we are now going to explore methods of accessing ISF in the least invasive manner. In the skin, the ISF surrounds the cells of the epidermis and dermis, and contains useful biomarkers, such as glucose, lactate, and cortisol.^{60–65} ISF in the skin can be accessed by creating pores in the skin using mechanical methods such as an electrical vacuum pump or near infrared lasers that create

vacuum pressure in the skin. Such methods have been demonstrated to harvest ISF for electrochemical sensing of cortisol by Venugopal *et al.*⁶⁶ Suction blisters, wherein a negative pressure is applied to a small, selected area of the skin, have also been used to extract ISF for the detection of amino acids, nucleotides, and lipids.^{67,68}

These methods are invasive or require implantation, whereas less invasive solutions such as microneedles are available for accessing ISF. Microneedles penetrate <1 mm deep into the epidermis, without reaching any blood vessels or nerve endings, allowing them to be classified as minimally invasive, as shown in Figure 1.11. Microneedles for sensing applications can be hollow or solid; hollow microneedles are used to extract ISF for *in vitro* measurements, whereas solid microneedles are functionalised with sensing chemistry to enable on-site *in vivo* measurements. Prausnitz *et al.* initially developed hollow microneedles for drug and vaccine delivery into the skin, and since then, several studies have demonstrated the use of hollow microneedles for the extraction of biomarkers in ISF.^{61,69}

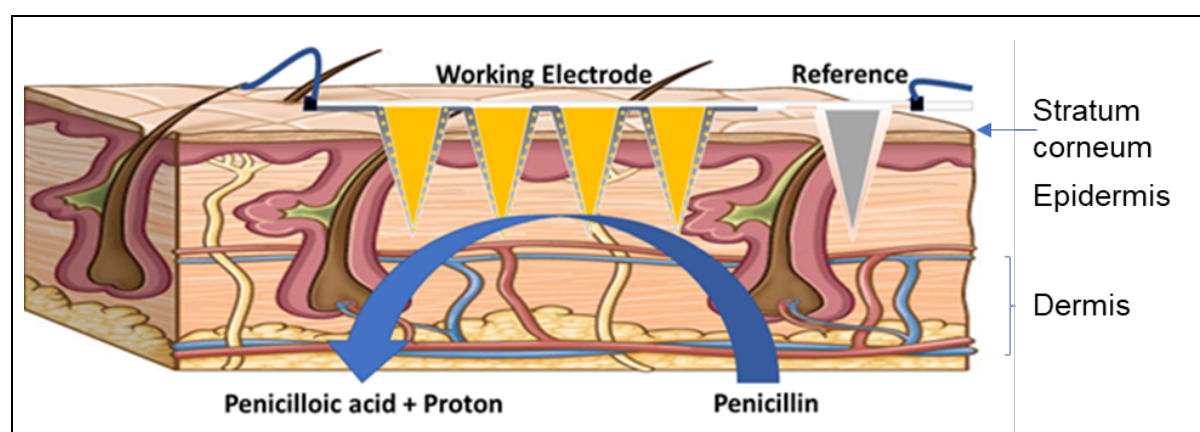
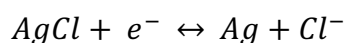


Figure 1.11 A microneedle biosensor measuring penicillin in ISF using the beta-lactamase enzyme, as reported by Gowers *et al.*⁷⁰

1.8 Electrochemical biosensors

Electrochemical sensors are a class of sensors that detect and measure the concentration of an analyte using an electrode as the transducer. An electrochemical cell, as pictured in Figure 1.12, consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE), in an electrolyte solution. The electrodes are connected to an external potentiostat, which can apply a potential to the WE with reference to the RE. The potentiostat can also measure the current at the WE, which is the current passing between the WE and CE. There are several types of WEs, including gold, glassy carbon, and platinum electrodes.⁷¹

The potential of the RE is stable and is used as a reference point against which the potential of the WE is measured. The stability of the RE comes from its composition; standard REs include the saturated calomel electrode (SCE), standard hydrogen electrode (SHE), and silver-silver chloride (Ag|AgCl) electrode. The Ag|AgCl RE consists of a silver wire coated with a thin layer of insoluble AgCl salt, placed in a chloride solution, such as potassium chloride. The equilibrium reaction at the Ag|AgCl RE is outlined in Equation 1.1.



Equation 1.1 Equilibrium reaction at the Ag|AgCl RE.

The CE completes the electrical circuit, meaning that the current flows between the WE and CE.⁷² If an oxidation reaction occurs at the WE, then a reduction reaction occurs at the CE. Typically, CEs consist of a platinum wire or mesh to allow for a greater surface area than that of the WE to ensure that the measured current at the WE is not controlled by the rate of electron transfer at the CE.

The Cottrell equation describes the relationship between the time dependent current, $I(t)$, the area of the electrode, A , the diffusion coefficient, D , the time for which the potential is applied, t , the concentration of the bulk solution, c , and the number of electrons transferred, n , as shown in Equation 1.2, where F is Faraday's constant for the case where the electrode reaction is limited by mass transport, i.e. the electron transfer is rapid. The initial condition is that there is no net electrode reaction (for thermodynamic or kinetic reasons). A potential step (Heaviside function)⁷³ is applied to take the potential to where the electrode reaction is limited by mass transport and the reactant surface concentration is zero.

$$I = nFAc \sqrt{\frac{D}{\pi t}}$$

Equation 1.2 The Cottrell equation.

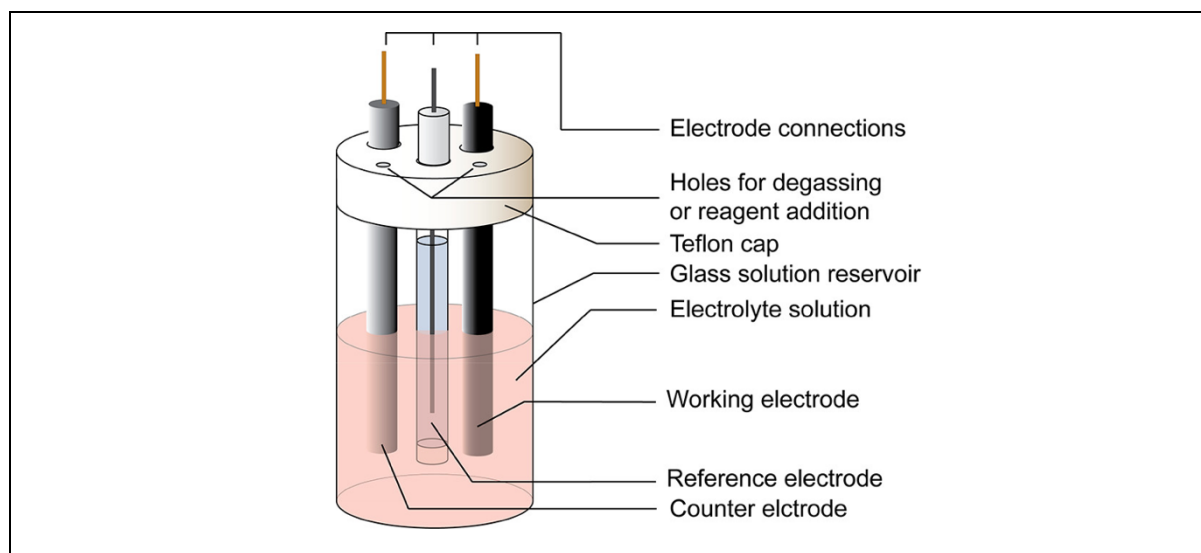


Figure 1.12 A three-electrode electrochemical cell comprising of the WE, RE and CE, adapted from Elgrishi *et al.*⁷²

1.8.1 Amperometric sensors

Amperometric sensors are sensors, in which the working electrode is held at a constant potential and the resulting current response is measured using amperometry. A well-known example of an amperometric sensor is a glucose biosensor in which glucose oxidase catalyses the oxidation of glucose to gluconic acid, with O_2 being reduced to H_2O_2 as a by-product. The H_2O_2 can be quantified by oxidation at a platinum electrode at an applied potential of +0.7 V vs Ag|AgCl. The current measured at the WE is proportional to the concentration of H_2O_2 which in turn relates to the concentration of glucose.⁷⁴ Enzyme based amperometric biosensors offer specificity for the target analyte and a direct transduction of the enzymatic reaction in current response. This allows a concentration-dependent relationship to be drawn with current. Amperometric sensors have been extensively reviewed for different applications.^{75,76}

1.8.2 Voltammetric sensors

Voltammetric sensing techniques include the measurement of current at the WE as a function of the applied potential over a period of time. Voltammetric methods include cyclic voltammetry, square wave voltammetry, differential pulse voltammetry, stripping voltammetry, and chronoamperometry. In the presence of the target being measured, a change in the current response is observed at the applied potential. The current response can be in the form of a peak observed at a specific potential, which is why applying a wide range of potential is useful in the first instance. The current response is directly proportional to the concentration of the analyte being measured. An example of a voltammetric sensor is the sensor developed by Karimi-Maleh *et al.* for nicotinamide adenine dinucleotide (NADH), wherein a carbon paste electrode was modified with nickel oxide nanoparticles.⁷⁷ The sensor

was tested using cyclic voltammetry, square wave voltammetry, and chronoamperometry in water, urine, and serum samples, demonstrating that measurements in samples were close to expected concentrations. Several other voltammetric sensors have been reviewed in literature, including sensors detecting paracetamol, biphenyl and cefixime.^{78–80}

In this thesis, two types of electrochemical sensors will be used. These are (i) enzyme based amperometric biosensors for lactate (discussed in Chapter 2) and (ii) aptamer based voltammetric biosensors for CRP (discussed in Chapters 3 and 4).

1.8.3 Enzyme-based microneedles biosensors

Enzyme-based microneedle biosensors, first reported by Sharma *et al.* for their GOD biosensor,⁸¹ are microneedles that are functionalised with an enzyme layer that catalyses a reaction with the target biomarker, such that its presence can be detected through the products of the reaction. For example, in the case of microneedles measuring β -lactam antibiotic concentrations, iridium oxide (IrOx)-coated gold microneedles were functionalised with β -lactamase enzyme which catalyses the hydrolysis of β -lactam antibiotics, causing a change in pH detected by the IrOx, as reported by Gowers *et al.* (see Figure 1.11).⁷⁰ Before the application of the enzyme layer, iridium oxide was deposited as the pH sensing layer, and the open circuit potential was obtained at increasing concentrations of penicillin, a β -lactam antibiotic, demonstrating a linear relationship.

An example of an amperometric enzyme-based microneedle biosensor is that developed by Mohan *et al.* for the subcutaneous measurement of alcohol.⁸² In this study, hollow microneedles were used through which wires of platinum and silver were inserted. One of

the platinum wires was functionalised with an interference layer – poly(o-phenylenediamine) (PPD) – followed by immobilisation of alcohol oxidase (AOx) enzyme. The AOx oxidises the alcohol, producing H_2O_2 as a by-product, which is oxidised at +0.6 V (vs. Ag|AgCl reference electrode).

1.9 Aims and objectives

To summarise, the aims of this thesis are to develop electrochemical biosensors for real-time infection monitoring to (a) detect the early warning signs of sepsis (i.e. measure lactate levels > 4 mM) and (b) support the personalised dosing of antimicrobials in critical care through continuous measurement of biomarkers of infection. To do this, this thesis will focus on the following objectives.

- (i) Develop a lactate oxidase (LOD) microneedle biosensor for the detection of early warning signs of sepsis wherein the sensor detects lactate levels at and above 4 mM (i.e. the threshold above which patients are diagnosed as septic), with a minimum dynamic range of 5 mM (Chapter 2).
- (ii) Compare the diffusion coefficients of lactate and H_2O_2 in different membrane materials to understand mass transport in the membrane and to choose a membrane to extend the dynamic range of the biosensor (Chapter 2).
- (iii) Measure lactate continuously *in vivo* using microneedle sensors with the ability to detect a rise and fall in lactate concentrations that is proportional to blood lactate measurements, and demonstrate good agreement between biosensor and blood lactate, and between biosensor and dialysate lactate (Chapter 2).
- (iv) Develop an electrochemical aptamer biosensor (E-AB) for the continuous measurement of CRP, wherein the sensor produces a concentration dependent

molecular recognition effect over the physiologically relevant CRP concentration range of 1 to 100 mg/L (Chapter 3).

- (v) Develop an osmium(II/III) based redox reporter labelled E-AB for CRP detection with a concentration dependent molecular recognition effect and demonstrate the ability of the aptamer to regenerate to at least 80% of its original signal response for continuous measurements (Chapter 4).

Chapter 2 Development and optimisation of lactate oxidase microneedle sensors for the early detection of sepsis and clinical validation in healthy volunteers

2.1 Introduction

2.1.1 An overview of lactate metabolism and clearance

Cells respire anaerobically in the absence or under low concentrations of oxygen, when energy is required rapidly e.g., during intense exercise or in response to rapid oxygen consumption in critical illness. Anaerobic glycolysis produces pyruvate from glucose, reducing two molecules of nicotinamide adenine dinucleotide (NAD^+) to NADH in the process, and producing two molecules of adenosine diphosphate (ATP) per glucose molecule. Lactate, or lactic acid, is produced from pyruvate, catalysed by lactate dehydrogenase (LDH) enzyme, causing the oxidation of NADH back to NAD^+ in the process.

Rapid oxygen consumption and poor circulation in tissues can lead to tissue hypoxia, wherein tissues experience a low level of oxygen. Tissues experiencing hypoxia resort to anaerobic glycolysis to meet the energy demands of cells, producing lactate.⁸³ This can cause lactate acidosis, in which lactate builds up in the bloodstream and causes the pH to become acidic.

The Cori cycle, as summarised in Figure 2.1, is a process by which lactate is converted back to glucose in the liver and kidneys, for use by the body's cells to release energy in aerobic or anaerobic respiration. Gluconeogenesis is the process by which lactate is oxidised to pyruvate, and this in turn is converted into glucose using energy from six ATP molecules.⁸⁴ This prevents the build-up of lactate in tissues which can lead to lactate acidosis. The process of anaerobic glycolysis and gluconeogenesis is summarised in Figure 2.1.

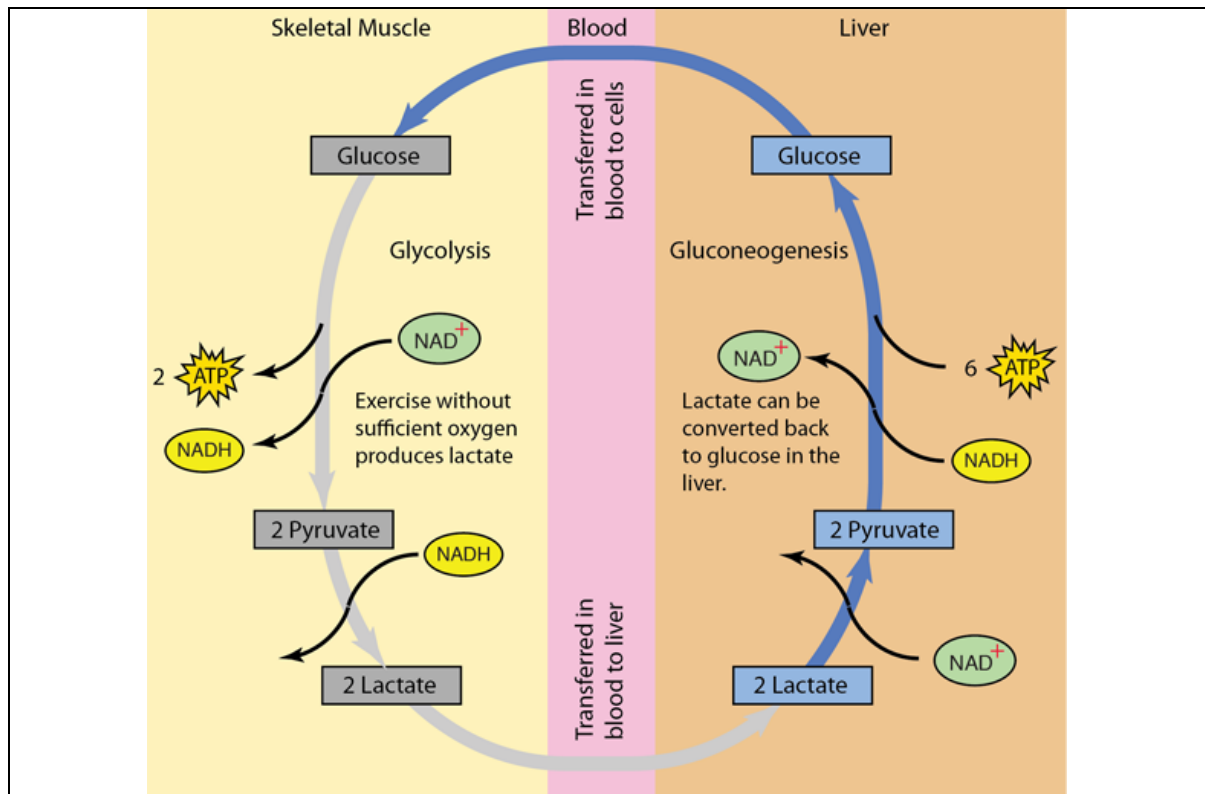


Figure 2.1 The Cori cycle is the process of anaerobic glycolysis and lactate clearance via gluconeogenesis.⁸⁵

In addition to tissue hypoxia, lactate build-up can be caused by decreased hepatic and renal lactate clearance, which may be caused by kidney failure in septic shock. Lactate acidosis can also be caused by certain medications, such as metformin – used to treat diabetes and insulin resistance – which can cause plasma lactate levels to rise.⁸⁶

Lactate production by anaerobic glycolysis and fermentation is balanced by lactate uptake and oxidation in the Cori cycle.^{84,87} This homeostasis, however, can be disrupted by critical illness. There is an interplay between tissue perfusion, hepatic and renal clearance, tissue hypoxia and the rate of glycolysis, which affects the concentration of lactate in blood, in turn affecting lactate concentration in interstitial fluid (ISF). A disruption in this balance indicates

altered cell function and in combination with symptoms of disease, can be used to assess a patient's disease state.

2.1.2 An overview of sepsis

There are several ways in which lactate production increases in sepsis. Glucose metabolism increases to meet the high energy demands of cells fighting infection.⁸⁸ There is a subsequent increase in lactate production due to rapid oxygen consumption and requirement for energy via anaerobic pathways. Sepsis also causes microcirculatory impairment, limiting oxygen supply and clearance, leading to tissue hypoxia, anaerobic glycolysis, and lactate production.^{89,90}

Jansen *et al.* report a study in which lactate has been used as a marker to guide therapy in intensive care units for a group of 171 patients.⁹¹ This study found that lactate-guided therapy, which included monitoring blood lactate concentrations periodically over eight hours after hospital admission, reduced mortality rates in sepsis patients from 43.5% to 33.9%. Lactate monitoring combined with guided therapeutic intervention, such as increased rates of fluid resuscitation and use of vasodilators, allowed significant reduction of mortality in the group where lactate was monitored versus the control group.

In sepsis, tissue hypoxia is related to organ failure and mortality. At rest, the concentration of lactate in arterial blood is between 0.5 and 2 mM⁹² and this can increase up to 25 mM during periods of intense exercise.⁹³ In critical illness, lactate levels above 4 mM are indicative of sepsis⁹⁴ and levels can rise to as high as 12 mM in some patients with septic shock.⁹⁵ As lactate is a commonly used biomarker for sepsis, point-of-care blood lactate measurement devices

have been developed, such as the ABL90 Flex Plus Analyser arterial blood gas (ABG) analyser (Radiometer Medical, UK), i-STAT clinical portable ABG analyser (Abbott Laboratories, USA), Lactate Pro analyser (EKF Diagnostics) and AccuTrend lactate analyser (Roche Diagnostics, USA). These devices allow discrete measurements to be made at a single timepoint and their use cases are outlined in Table 2.1. The i-STAT, AccuTrend and Lactate Pro analysers are handheld devices, shown in Figure 2.2, whereas the ABL90 Flex Plus, commonly used in clinical practice, is bulky and requires cannulation to draw blood.

Device	Analytes measured	Use case	Reference
i-STAT clinical portable analyser (Abbott Laboratories, USA)	Blood lactate and pH, bicarbonate ($[\text{HCO}_3^-]$), total carbon dioxide (TCO_2), blood oxygen saturation (sO_2), and partial pressures of O_2 (pO_2) and CO_2 (pCO_2).	100 μL finger-prick capillary blood taken and inserted into disposable i-STAT cartridge. - Used in hospital settings by trained personnel.	96,97
AccuSport (now AccuTrend) lactate analyser (Roche Diagnostics, USA)	Blood lactate.	- Simultaneous measurements of finger-prick capillary blood. - Used for performance monitoring in athletes and in laboratory settings.	98
Lactate Pro (EKF Diagnostics, USA)	Blood lactate.	- As low as 5 μL of finger-prick blood required for test strip. - Lactate monitoring in endurance training for athletes.	99
ABL90 Flex Plus Analyser (Radiometer, UK)	Blood lactate, glucose, and pH, PO_2 , PCO_2 , Na^+ , K^+ , Ca^{2+} , Cl^- , total haemoglobin (tHb), sO_2 , oxygenated haemoglobin (O_2Hb), deoxygenated haemoglobin (HHb), carboxyhaemoglobin (COHb), and methemoglobin (MetHb).	- Patient is cannulated and arterial blood drawn at various timepoints. - Requires 65 μL for a single measurement. - Requires trained personnel for cannulation and blood draw. - Used in critical care in hospital.	100

Table 2.1 A summary of the gold standard devices used in the measurement of lactate.

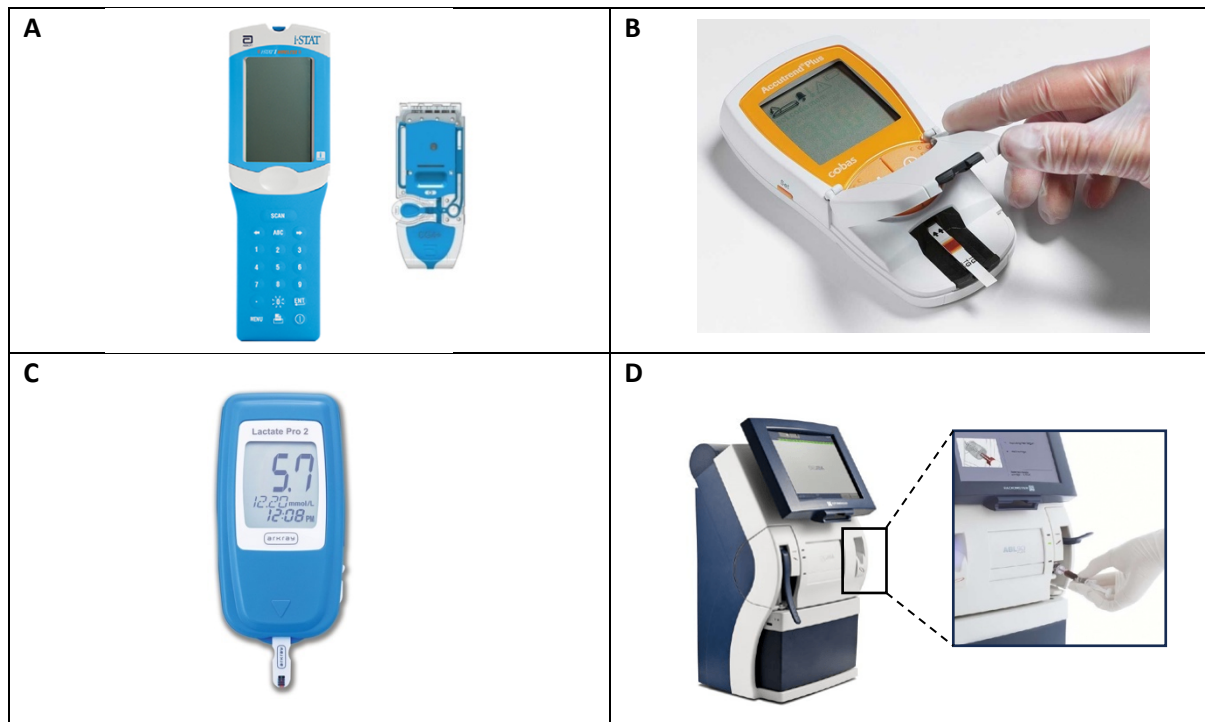


Figure 2.2 Images of handheld and laboratory-based lactate analysers; (a) i-STAT clinical portable analyser, (b) AccuTrend lactate analyser, (c) Lactate Pro analyser, and (d) ABL90 Flex Plus Analyser.

Measurements need to be taken several times over the period of treatment for critically ill patients and cannot provide information quickly enough to inform clinical decisions such as drug dosing or infection monitoring. In addition, each of the point-of-care devices require repetitive blood draws or finger-pricks to be made, which is not possible in some patient groups, such as children and neonates. A minimally invasive approach is therefore required to overcome the need for repetitive blood draws and trained clinical personnel.

Continuous measurement of lactate, afforded by minimally invasive electrochemical sensing, would allow (i) the early detection of the onset of sepsis, both in hospital and in community settings without requiring trained personnel, and (ii) the continuous monitoring of infection

to inform clinical decisions and drug dose optimisation, building a profile of lactate levels over time.

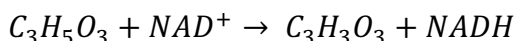
2.1.3 Lactate biosensors

Lactate biosensors are amperometric, meaning that a current response is obtained on application of a constant potential. Several different types of electrode materials have been used to fabricate lactate biosensors, such as gold, platinum, and carbon. Enzymes are used as the biorecognition element and are immobilised onto the surface of electrodes using several different methods, including crosslinking, physical adsorption, confinement by membranes, and electrical polymerisation. There are two types of enzymes used in enzyme coupled lactate biosensors: lactate oxidase (LOD) and lactate dehydrogenase (LDH).

2.1.4 Lactate dehydrogenase

LDH is a quaternary protein found in tissues and has a high catalytic activity.¹⁰¹ LDH catalyses the oxidation of L-lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD^+) which is in turn reduced to NADH, as shown in Equation 2.1. The NADH is then oxidised at the electrode surface at the applied oxidation potential in amperometry, and the current response is directly proportional to the lactate concentration. In this reaction, NADH acts as the electron mediator between the enzyme's active site and the electrode surface, as shown in Equation 2.2. The oxidation of NADH to NAD^+ is widely known to cause electrode fouling in biosensors, wherein radical intermediates and their reaction products adsorb onto the surface of electrodes, limiting the rate of electron transfer and causing a loss of electrochemical response.^{102,103} Therefore, the use of NADH detection is not preferable in lactate sensors.¹⁰⁴

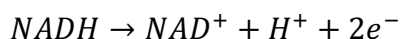
Lactate dehydrogenase



L-lactate

Pyruvate

Equation 2.1 Reaction at the active site of the lactate dehydrogenase enzyme.

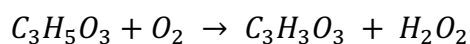


Equation 2.2 Oxidation of NADH at the electrode surface.

2.1.5 Lactate oxidase

LOD is a flavoprotein and is sourced from bacteria, such as *Aerococcus viridans* and *Mycrobacterium smegmatis*.¹⁰⁵ L-lactate is oxidised by LOD enzyme to pyruvate and H₂O₂ is produced as a by-product of oxygen reduction,¹⁰¹ as shown in Equation 2.3. H₂O₂ is an electroactive molecule and is oxidised at the electrode surface when a constant oxidation potential is applied in amperometry. H₂O₂ is oxidised to oxygen, releasing two H⁺ protons and two electrons, as shown in Equation 2.4. The released electrons generate a current that is proportional to the concentration of H₂O₂ that is oxidised, which in turn is proportional to the concentration of lactate that is oxidised by LOD. The H₂O₂ / O₂ redox pair acts as the electron mediator between the enzyme's active site and the electrode surface. LOD enzyme requires sufficient oxygen supply to complete the reaction in Equation 2.3, due to its co-factor, flavin mononucleotide (FMN), which can limit the rate of reaction if insufficient oxygen is present.

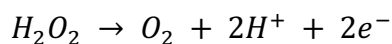
Lactate oxidase



L-lactate

Pyruvate

Equation 2.3 Reaction at the active site of the lactate oxidase enzyme.



Equation 2.4 Reaction at surface of the electrode.

2.1.6 Direct electron transfer enzymes

Direct electron transfer (DET) LOD enzymes have the ability to directly transfer electrons from the active site of the enzyme to the electrode surface, without the need for an additional electron mediator. This is useful because electron transfer is confined to distances of around a bond length or less (i.e., a distance of approximately 10^{-10} m). Freeman *et al.* have recently demonstrated that such DET LOD enzymes are able to detect lactate at low oxidation potentials of +0.2 V, preventing interference from other electroactive molecules at higher potentials.¹⁰⁶ However, DET enzymes are not currently commercially available.

2.1.7 A comparison of first, second and third generation lactate biosensors

LOD sensors are first generation biosensors as they are based on the detection of H_2O_2 . However, modifications can be made to how electrons are mediated at the electrode to optimise sensor performance, which may classify the sensor as a second or third generation biosensor.¹⁰⁷

First-generation lactate biosensors

First-generation biosensors directly detect the product of an enzymatic reaction, typically hydrogen peroxide for an oxidase enzyme and protons for a dehydrogenase. The enzyme catalyses a chemical reaction, producing an electroactive species, creating a change in signal which is then transduced by the electrode into current. A first-generation LOD biosensor is one in which H_2O_2 – the by-product of the oxidation of lactate – is oxidised at the electrode on application of an oxidising potential.

For this reaction to occur, there needs to be a constant supply of oxygen in the solution as oxygen acts as the electron acceptor in this reaction, as shown in Figure 2.3, where the primary electron mediator of LOD is the enzyme's prosthetic group, flavin mononucleotide (FMN). The co-factor then passes on the electrons to the secondary mediator, oxygen, in this case. In addition, other electroactive species can also be oxidised at this potential, causing potential interference in the signal response. First generation LOD biosensors include those reported by Rogers *et al.*¹⁰⁸ and Samper *et al.*¹⁰⁹ for on-line continuous monitoring of lactate using microdialysis.

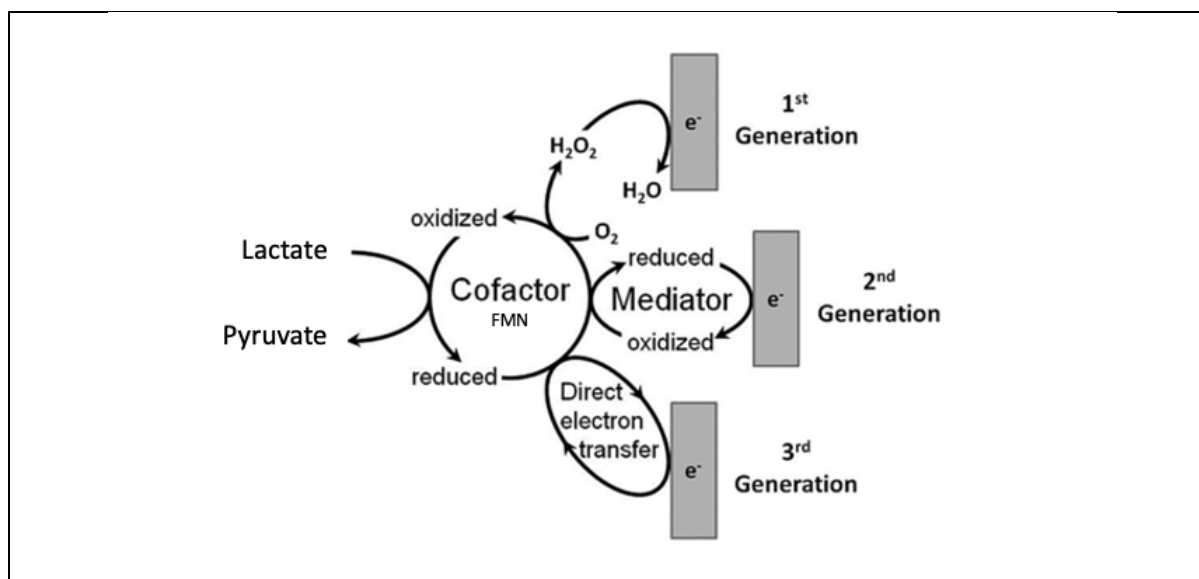


Figure 2.3 First, second and third generation LOD biosensors and their modes of electron transfer, adapted from Ferri *et al.*¹¹⁰

Second-generation lactate biosensors

To remove the need for oxygen as an electron acceptor, second generation biosensors use electron mediators to accept electrons, such as ferrocene/ferricenium, ferri/ferrocyanide and methylene blue. As shown in Figure 2.3, the mediator overcomes the need for oxygen by accepting electrons from the co-factor of the LOD enzyme, in turning, reducing itself before it is re-oxidised at the electrode. Mediators typically operate at a lower oxidising potential, meaning that the risk of interference from other molecules is lowered. However, these mediators can be toxic, and in any case need to have their toxicity evaluated, which limits the applications of second-generation biosensors.¹¹¹ Bollella *et al.* report a second-generation microneedle-based LOD sensor for lactate detection in ISF, wherein gold microneedles were functionalised with gold-multiwalled carbon nanotubes, followed by electropolymerisation of methylene blue (MB) as the redox reporter, and the drop-casting of LOD onto the sensor.⁶³ This technique allowed a low oxidation potential of +0.15 V to be applied in amperometry, with the ratio of interferent to lactate signal response measured to be low. The sensor was

tested in artificial ISF and human serum, producing a linear range of up to 100 μM lactate in both matrices.¹¹² However, it is worth noting that MB is highly toxic, particularly in combination with medications that contain serotonin or increase its production in the body, therefore, its use *in vivo* would need to be carefully regulated. In addition, organic dyes such as methylene blue can cause leaching out of catalytic material.¹¹¹ Other reported mediators used in biosensors include dimethylferrocene-modified linear poly(ethylenimine),¹¹³ and carbon nanotubes with tetrathiafulvalene (TTF).¹¹⁴

Third-generation lactate biosensors

Third-generation biosensors employ direct electron transfer (DET) wherein the electrons are directly transferred between the enzyme and the electrode, without the need for a mediator, as shown in Figure 2.3.¹¹⁰ To achieve DET, the enzyme's redox centre needs to be connected to the electrode surface and various molecular wires are being used to achieve this, such as osmium complex-linked polymers¹¹⁵ and multiwalled carbon nanotubes.¹¹⁶

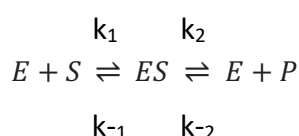
While third generation biosensors using DET enzymes are promising in decreasing interference from other compounds, there is literature that claims that the response from these sensors is a result of the adsorption of flavin and catalase on electrodes in glucose oxidase sensors.¹¹⁷ The source of these impurities is suspected to come from commercial enzymes, as reported by Bartlett *et al.*¹¹⁷

In this thesis, a first-generation biosensor was chosen due to (i) ease of transfer of the biosensor to the *in vivo* testing stage made possible by the lack of a need to check mediator toxicology, (ii) the lack of complicated transduction events, and (iii) the readily available

recombinant LOD enzyme (Sekisui Diagnostics, UK) with DET LOD enzymes not routinely or commercially available for research purposes. Despite first-generation biosensors needing the application of a relatively high oxidation potential for H₂O₂ oxidation, and this in turn causing interference from other electroactive molecules, we know this interference is likely to come from paracetamol for someone being treated in hospital for suspected sepsis and we can account for its contribution to signal response if the dose is known.

2.1.8 Enzyme kinetics

Enzymes are protein catalysts that increase the rate of reaction by decreasing the energy required to form the transition state of the reaction. Equation 2.5 provides an overview of a steady-state enzyme reaction, where *E* is the enzyme, *ES* is the enzyme-substrate complex (transition state), and *P* is the product. Steady-state enzyme kinetics can be modelled by the Michaelis-Menten equation if the following assumptions are met: (i) no product is present at the start of the reaction ($[P]_{\text{initial}} = 0$) and no back reaction can occur from *E*+*P* to *ES* (i.e., k_{-2} is vanishingly small), (ii) the binding and dissociation of *ES* is at equilibrium, (iii) $[E] < [S]$ and $[ES] < [S]$, meaning that *S* is in abundance in the reaction mixture and $[S]$ does not effectively change, and (iv) the enzyme is present as free enzyme or *ES* complex i.e., $[E]_{\text{total}} = [E] + [ES]$.



Equation 2.5 Steady-state enzyme reaction.

The Michaelis-Menten equation (Equation 2.6) demonstrates the rate of reaction per unit time, is limited by the concentration of the substrate, *S*. V_{max} is the maximum rate of an

enzyme-catalysed reaction, and K_m is the half-maximal concentration constant i.e., the concentration of substrate at half of V_{max} . The K_m of enzymes, known as the Michaelis constant, is determined experimentally and is reported by enzyme manufacturers.

The Michaelis-Menten equation is used interchangeably with the Hill equation which is also used to describe the kinetics of enzyme-controlled reactions, where n is the Hill coefficient. If n is equal to 1 in Equation 2.7, the Hill equation is equivalent to the Michaelis-Menten equation.²⁸ The parameter, n , represents cooperativity in the enzyme catalysed reaction, i.e. if $n > 1$, the binding of one substrate to the enzyme to form ES positively affects the binding of another substrate. If $n < 1$, negative cooperativity is indicated. For experiments in this thesis, n was equal to 1. Figure 2.4 shows an enzyme catalysed reaction with the rate of reaction V plotted against substrate concentration $[S]$, with K_m and V_{max} parameters deduced from the plot.

$$V = \frac{V_{max} [S]}{K_m + [S]}$$

Equation 2.6 The Michaelis-Menten equation.

$$V = \frac{V_{max} [S]^n}{(K_m)^n + [S]^n}$$

Equation 2.7 The Hill equation.

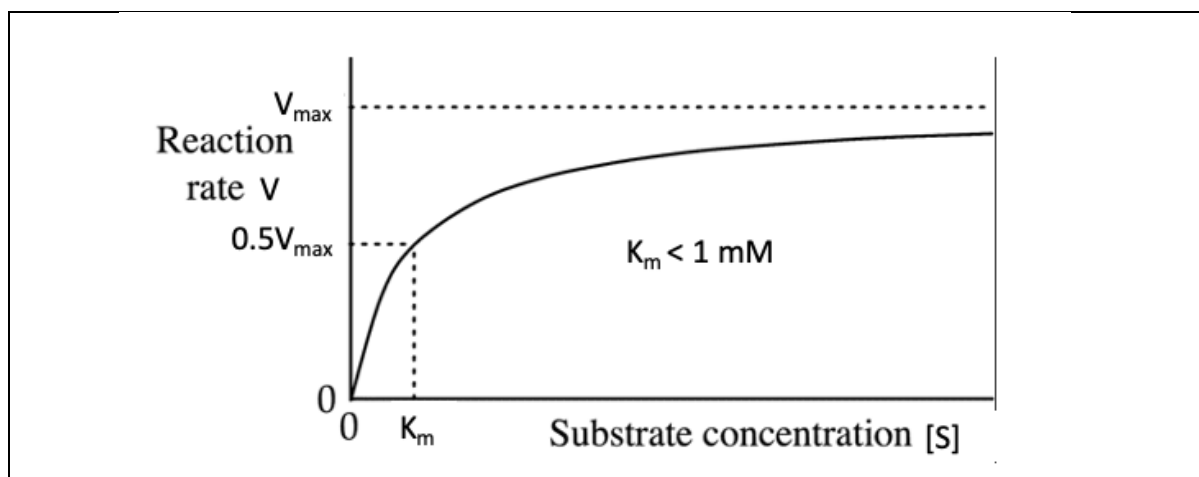


Figure 2.4 An enzyme's rate of reaction (V) against substrate concentration $[S]$, fitted with the Hill equation with enzyme K_m and V_{max} parameters.

For an enzyme based amperometric sensor, the current response can be plotted against the analyte concentration and fit with the Hill equation to determine the “effective” K_m of the sensor, as well as the “effective” V_{max} , which is the maximum current achieved when the sensor is saturated, i.e. the rate of reaction is limited by diffusion to the electrode.

2.1.9 Challenges with lactate oxidase enzyme

Lactate oxidase has a low K_m , which differs depending on the manufacturer, but lies within the range of 0.1 and 0.9 mM.^{118,119} LOD obtained from Sekisui Diagnostics (UK), produced by *Aerococcus viridans* and used here, has a K_m of 0.7 mM.¹²⁰ Lactate levels in blood serum can rise up to 15 mM in sepsis,⁸⁴ with the threshold for sepsis diagnosis being 4 mM, which means that an LOD biosensor would not be able to measure lactate in this concentration range for the early detection of sepsis *in vivo*.

For early sepsis detection, lactate sensors need to be able to measure concentrations up to 10 mM lactate. With the current sensors from literature, the higher end of this concentration range will not be reached due to the low K_m of the LOD enzyme. Therefore, there is a need to

extend the concentration range in which LOD biosensors detect lactate for the application of detecting sepsis early.

Through modification of the sensor surface, such as the application of films to optimise the mass transport of analytes, the dynamic range can be changed. The impact of the membrane is proposed to decrease the flux of lactate to the enzyme hydrogel layer, thereby allowing the active sites of the enzyme to not become saturated.

It is worth noting that for the early detection of sepsis, the sensor's lower limit of detection is not relevant as the aim is to detect high concentrations of lactate caused by hyperlactatemia in sepsis.

2.2 Membranes used to extend dynamic range of lactate biosensors

Several membranes have been used to coat immobilised LOD or LDH enzymes to improve the dynamic or linear range of lactate sensors for *in vivo* applications. Some of these include Nafion, polystyrene sulfonic acid (PSS), cellulose acetate (CA), polyurethane (PU), polypyrrole, poly(vinyl chloride) (PVC), sol-gels, and poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS). The use of membranes is effective in improving the "effective" K_m of sensors due to the diffusion coefficient of lactate being lower in the polymer matrix compared to in the solution, as a result of the decrease in flux.^{121,122} Since the lactate ion is an anion, using negatively charged conductive polymer membranes will slow down the diffusion of lactate through the membrane, through excluded volume effects, meaning that the enzyme is not saturated, and the dynamic range is extended. For example, Nafion is a negatively charged conductive polymer and since lactate is an anion, this reduces the partition

coefficient, K , which is the ratio of the concentration of lactate in the membrane to the concentration in solution i.e., $\frac{C_m}{C_s}$, as described by Donnan equilibrium.

2.2.1 Mass transport through a membrane

Consider a diffusion-limited sensor membrane at steady-state in one dimension. From Fick's second law of diffusion, we know that $\frac{dc}{dt} = D \frac{d^2c}{dx^2}$ where c is concentration (mol m^{-3}), t is time (s), x is position (m), and D is diffusion coefficient.¹²³ Equation 2.8 is Fick's second law of diffusion, which at steady-state, is equal to zero (Equation 2.9). When we integrate both sides of the equation, we get $\frac{dc}{dx} = c_1$ (Equation 2.10). We can solve for c (Equation 2.11) where, by using boundary conditions, When the electrode reaction is mass transport limited, the boundary conditions at the electrode surface are when $x = 0$, $c = 0$. Solving for c , we get $c_2 = c(0)$, and $c_1 = \frac{c\phi - c(0)}{\phi}$ (Equation 2.12). At $x = \phi$, at the boundary of the membrane – where ϕ is membrane thickness – $c(\phi)$ is $c_s K$ where c_s is the concentration in solution and K is the partition coefficient ($K = \frac{C_m}{C_s}$). Therefore, $c_1 = \frac{c_s K - c(0)}{\phi}$.

$$\text{At steady state: } \frac{dc}{dt} = D \frac{d^2c}{dx^2} = 0$$

Equation 2.8 Fick's second law of diffusion.

$$\text{Therefore: } \frac{d^2c}{dx^2} = 0$$

Equation 2.9 Fick's second law of diffusion at steady state.

Integrating both sides, we get $\frac{dc}{dx} = c_1$

Equation 2.10 Integration of Fick's second law of diffusion at steady state.

$$c(x) = c_1x + c_2$$

Equation 2.11 Concentration at a position 'x'.

Solving for 'c' at boundary conditions: $x = 0$ and $x = \phi$

At $x = 0$, $c_1 = 0$, therefore, $c_2 = c(0)$.

$$\text{At } x = \phi, c(\phi) = c_1\phi + c(0)$$

$$\text{Therefore, } c_1 = \frac{c\phi - c(o)}{\phi}$$

Equation 2.12 Solving for c_1 .

2.2.2 Charge effects on mass transport in the membrane

There are charge effects to consider when predicting mass transport through a membrane. As described by the Gibbs-Donnan effect, there is an unequal distribution of charged ions on either side of a semi-permeable membrane, due to the size of some ions making them impermeable. At equilibrium, the solution on either side of the membrane – despite there being an unequal distribution of ions – will be electrically neutral. The unequal distribution creates an electrochemical gradient, producing a transmembrane potential difference, as given by a Nernst equation like expression (Equation 2.13), where E is the reduction potential, E^0 is the standard potential, R is the gas constant, T is temperature, z is the charge on the ion, F is Faraday's constant and Q is the reaction quotient. In addition, the unequal

distribution creates an osmotic diffusion gradient, causing the movement of water into the compartment.

$$E = E^0 - \frac{RT}{zF} \ln Q$$

Equation 2.13 The Nernst Equation.

For a membrane coated sensor, ions will be distributed unequally across the membrane with ISF on one side and the electrode or hydrogel containing enzyme, on the other. Depending on the ions present in ISF, some may not permeate the membrane, causing the unequal distribution. This would affect the transport of other ions which can pass through the membrane, such as lactate and pyruvate.

Figure 2.5 demonstrates the concentration of H_2O_2 across the length of the diffusion-limited membrane across, denoted by ϕ , membrane thickness. The concentration increases linearly across the length of the membrane from the electrode surface (as demonstrated by Equation 2.11).

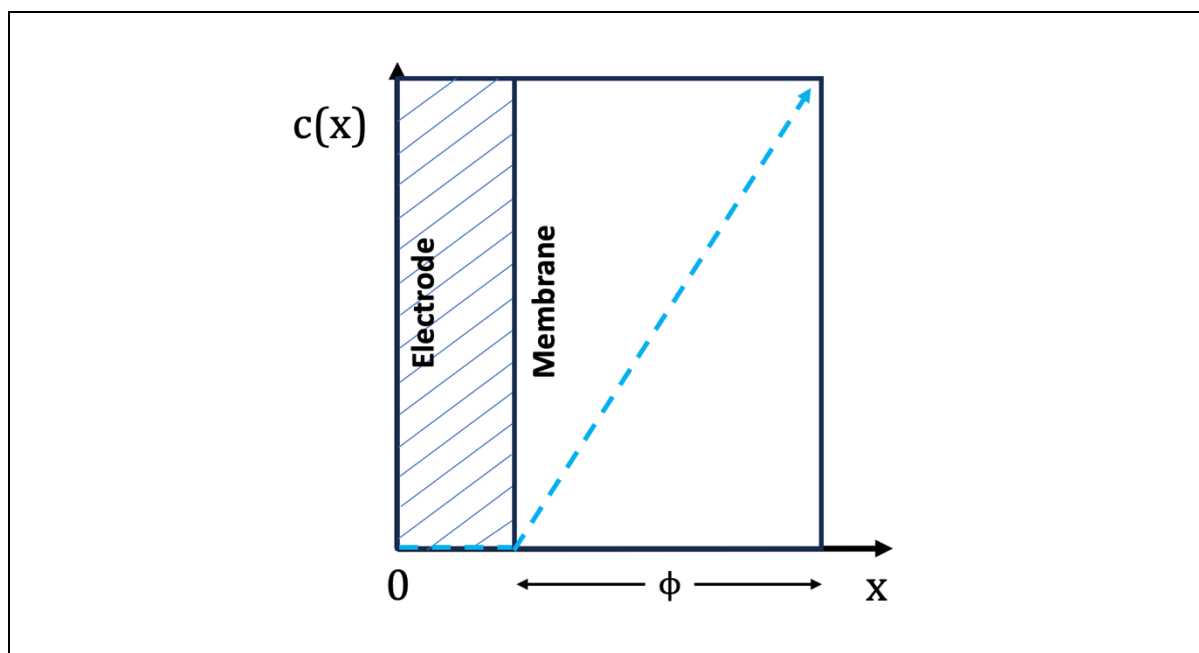


Figure 2.5 A simplified model of the mass transport of H_2O_2 through a membrane applied onto an electrode, where $c(x)$ is the concentration of H_2O_2 .

2.2.3 An overview of polymer membranes

Other polymer membranes, such as polyaniline, poly(vinyl) chloride (PVC) and polypyrrole, have also been used as films on lactate sensors. A summary of lactate sensors, membrane modifications, dynamic range, response time, sensitivity and stability is provided in Table 2.2.

Enzyme	Electrode	Membrane modification	Dynamic range/ mM	K_m / mM	T_{90} / s	Sensitivity	Stability	Ref
DET LOD	Gold micro-needles with carbon ink	3% w/v cellulose acetate	30 (lower limit NR)	19.7 ± 0.70	NR	NR	NR	¹⁰⁶
DET LOD	Planar gold	PEDOT:PSS-PB	NR	NR	NR	1.36 mA M^{-1} ₁	6 months at -20°C	¹²⁴
DET LDH	Gold	3% w/v cellulose acetate	0.1-40	NR	NR	$4.1 \text{ nA mM}^{-1} \text{ mm}^{-2}$	NR	¹²⁵

LOD	Platinum/ iridium wire	25 mg/mL polyurethane	1-10	NR	194 ± 15	NR	NR	¹²⁶
LOD	Carbon	Polyvinyl chloride, bis(2- ethylhexyl) sebacate (PVC-DOS)	1-10	NR	<86	-26.4 ± 0.7 nA mM ⁻¹		¹²⁷
LOD	Platinum	1% polyurethane, 1% w/v Nafion	0.5-4.0	1.75	35	53 µA mM ⁻¹ cm ⁻²	24 hours	¹²⁸
DET LOD	Au nanoclus- ters	Dialysis membrane	0.5-4.0	0.79	NR	106 A M ⁻¹ m ⁻²	3 months at 4 °C in dark	¹²⁹
LOD	Glassy carbon modified with N ₂ -doped carbon nano tubes	5 wt % Nafion modified with tetrabutyl- ammonium bromide	0.05-2	NR	≤2	(0.040 ± 0.002) A M ⁻¹ cm ⁻²	3 months at 4 °C	¹³⁰
LOD	Platinum	Nafion and poly -carbonate membranes	0.002-1	NR	NR	(0.537 ± 0.007) mA M ⁻¹	5 months at 4 °C	¹³¹
LOD, LDH	Indium- tin-oxide	Polyaliline	1-4	1.9	NR	38.5 µA mM ⁻¹	3 weeks at 4- 10°C	¹³²
LDH	Indium- tin-oxide	Polypyrrole- polyvinylsulph onate	0.5-7	NR	40	NR	2 weeks at 4- 10 °C	¹³³

Table 2.2 A summary of lactate biosensors (NR = not reported).

Of the reviewed membranes, we concluded that Nafion, PSS, PU and CA were shortlisted for dynamic range extension in this thesis. These membrane materials were further analysed using the Koutecký–Levich analysis, which is explored further in section 2.2.8. Koutecký–Levich analysis exploits the ability of hydrodynamic control to vary mass transport independently from potential and thus extract rate constants for electrode reactions.

2.2.4 Nafion

Nafion is a sulfonated fluoropolymer, consisting of a hydrophobic backbone of polytetrafluoroethylene (PTFE) and hydrophilic side chains with sulfonic groups at the end, as shown in Figure 2.6. The hybrid hydrophobic and hydrophilic properties of Nafion membranes means that in the presence of water, the hydrophilic side chains swell, while the hydrophobic membrane backbone does not, allowing for an equilibrium to be reached. Nafion is a commercially available coating with wide ranging applications, from fuel cells to biosensors.

The use of Nafion as a membrane on lactate sensors has been shown to improve operational stability,¹³¹ increase the dynamic range, decrease interference from other electroactive molecules, and protect the enzyme immobilisation layer. However, Nafion decreases sensitivity and current response.

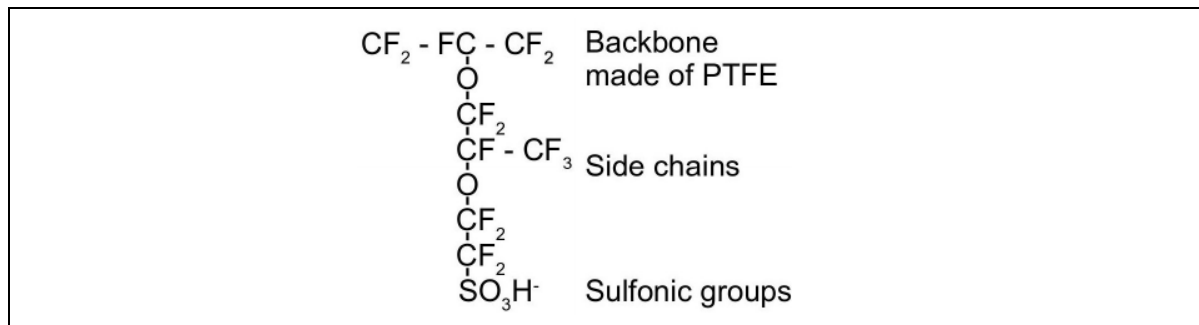


Figure 2.6 The chemical structure of Nafion with a SO_3H^- group at the end of the side chain.

There is also a need to deposit membrane materials conformally on electrodes to produce a reproducible response and reduce sensor-to-sensor variability.

2.2.5 Cellulose acetate

Cellulose acetate (CA) is a widely used coating across all types of sensors,¹³⁴ particularly electrochemical sensors to extend dynamic range. CA is a semi-synthetic derivative of

cellulose, a naturally occurring product, and is biocompatible. For this reason, several studies report the use of CA to extend the dynamic range of lactate sensors due to the ease of translation into *in vivo* settings. In addition, some studies have shown that CA can act as an interference rejection membrane, such as that reported by Arakawa *et al.* for their glucose sensors.¹³⁵ In this study, the response to interferents – ascorbic acid and uric acid – was shown to be 97.1% less compared to the glucose response, upon the addition of a 5% w/w CA membrane.

Freeman *et al.* have recently developed lactate sensors using gold microneedles functionalised with a mixed solution of carbon ink and DET LOD enzyme (DirectSens GmbH), followed by a membrane coating of 3% w/v CA solution in acetone.¹⁰⁶ The effective K_m is increased by coating the microneedles with 0%, 0.3% and 3% w/v CA from 0.56 ± 0.08 to 0.35 ± 0.10 , and 19.75 ± 0.70 mM, respectively, demonstrating that the increase in CA concentration increased the effective K_m and thus the dynamic range.

2.2.6 Polyurethane

Polyurethane (PU) is versatile a polymer synthesized from addition reactions of polyols and isocyanates, forming carbamate links. PU is widely used in biomedical applications due to its biocompatibility, mechanical properties, and low cost.^{136,137}

Gowers *et al.* have developed LOD sensors using 50 μm diameter platinum/iridium (90%:10%) wires. The tips of the wires were abraded to expose disc electrodes and the LOD enzyme was immobilised onto the m-phenylenediamine (m-PD) treated electrode surface.¹²⁶ The sensor was then coated with a membrane of 25 mg/mL PU, dissolved in THF, to extend the dynamic

range to 10 mM lactate. Thomas et al. have developed sensors embedded into contact lenses for monitoring lactate in tear fluid, using platinum working electrodes modified with LOD, followed by dip-coating in 1% w/v PU for dynamic range extension, and 1% w/v Nafion for interference rejection.¹²⁸ The addition of PU extended the dynamic range to 4 mM with a K_m of 1.75 mM.

2.2.7 Polystyrene sulfonic acid

Polystyrene sulfonic acid (PSSA) is a negatively charged polymer derived from polystyrene with sulfonate (SO_3^-) groups. It has been used in combination with poly(3,4-ethylenedioxythiophene) (PEDOT) to coat lactate sensors to extend dynamic range. Zhang *et al.* have reported lactate sensors made using the DET LOD enzyme (DirectSens GmbH) for monitoring lactate in exhaled breath condensate.¹²⁴ The sensors achieved a linear range between 0 and 100 μM lactate using a PEDOT:PSS-prussian blue (PB) membrane where the PB mediated H_2O_2 reduction. Perez *et al.* have developed lactate sensors based on multi-walled carbon nanotubes (MWCNTs) functionalised onto glassy carbon electrodes, with a polysulfonate membrane and ferrocene mixed layer, followed by immobilisation of LOD with horse radish peroxidase (HRP) enzyme. This achieved a linear range of 31.2 μM lactate.¹³⁸

2.2.8 Koutecký-Levich analysis

Koutecký-Levich analysis is a method by which the total current response of an amperometric biosensor can be investigated by its individual components, such as the current derived from the different processes along a reaction pathway. The measured current is directly proportional to the rate of reaction and is defined as the rate of change of charge. Flux, J , is the rate of flow of reacting electroactive species per unit area, A , and is defined by Equation

2.14, where i is current, n is the number of electrons, F is Faraday's constant and A is the surface area of the electrode. Since current is directly proportional to flux, the inverse of current, $\frac{1}{i_l}$, is a measure of the resistance to flux.

$$J = \frac{i}{nFA}$$

Equation 2.14 Flux.

For amperometric lactate oxidase sensors, the resistances that limit the rate of reaction are (i) the transport of lactate in the solution through the boundary layer above the immobilised electrode ($\frac{1}{i_l}$), (ii) the transport of lactate through the film membrane ($\frac{1}{i_{m,lactate}}$), (iii) the transport of H_2O_2 through the hydrogel ($\frac{1}{i_{m,H2O2}}$), (iv) the kinetics of the enzyme catalysed reaction between lactate and lactate oxidase ($\frac{1}{i_K}$), and (v) the kinetics of the electrode reaction of the oxidation of H_2O_2 ($\frac{1}{i_R}$). The sum of these de facto resistances add up in series to be the total resistance, i.e., $\frac{1}{i_{total}}$, which can be described by the Koutecký-Levich equation (Equation 2.15).

$$\frac{1}{i_{total}} = \frac{1}{i_l} + \frac{1}{i_{m,lactate}} + \frac{1}{i_{m,H2O2}} + \frac{1}{i_K} + \frac{1}{i_R}$$

Equation 2.15 The Koutecký-Levich equation at diffusion limited potential.

Since i_l is the current when mass transport is limited in the boundary layer, $\frac{1}{i_l}$ can be described by the Levich equation (Equation 2.16), which is used to predict the current produced by an RDE, where n is the number of moles of electrons transferred in the half

reaction, F is Faraday's constant (C mol^{-1}), A is the surface area of the electrode (m^2), D is the diffusion coefficient of the lactate in the boundary layer (m^2s^{-1}), ω is the rotational speed of the RDE (rad s^{-1}), ν is the kinematic viscosity of the solution (m^2s^{-1}), and C is the concentration of lactate (mol m^{-3}).

$$\frac{1}{i_l} = (0.620)nFAD^{\frac{2}{3}}\omega^{\frac{1}{2}}\nu^{\frac{-1}{6}}C$$

Equation 2.16 The Levich equation.

Koutecký-Levich analysis is conducted by plotting the reciprocal current (A^{-1}) against the reciprocal square root of the rotational rate of the RDE (rad s^{-1})^{-1/2}. The slope of the Koutecký-Levich plot is the mass transport in the solution (i_l) and the intercept represents a combination of the remaining current components summing up to i_{total} , as shown in Equation 2.17.

At the diffusion limited potential – the potential at which the current response is limited by mass transport to the electrode surface – $\frac{1}{i_l}$ becomes zero. The diffusion limited potential can be identified for an RDE sensor by conducting hydrodynamic voltammetry over the potential range in which the electrode reaction occurs. The potential at which the current becomes limited by mass transport i.e., where the current plateaus, is the start of the diffusion limited potential range, also known as the mass transport limited region, as shown in Figure 2.7(a). In this figure, the mass transport limited region can be seen to start at approximately +0.6 V. In this study, Huang *et al.* report the sensor's Koutecký-Levich analysis (Figure 2.7(b)), wherein amperometry was conducted at a potential of +0.7 V to find diffusion coefficients in the Nafion membrane of the glutamate oxidase sensors.¹³⁹

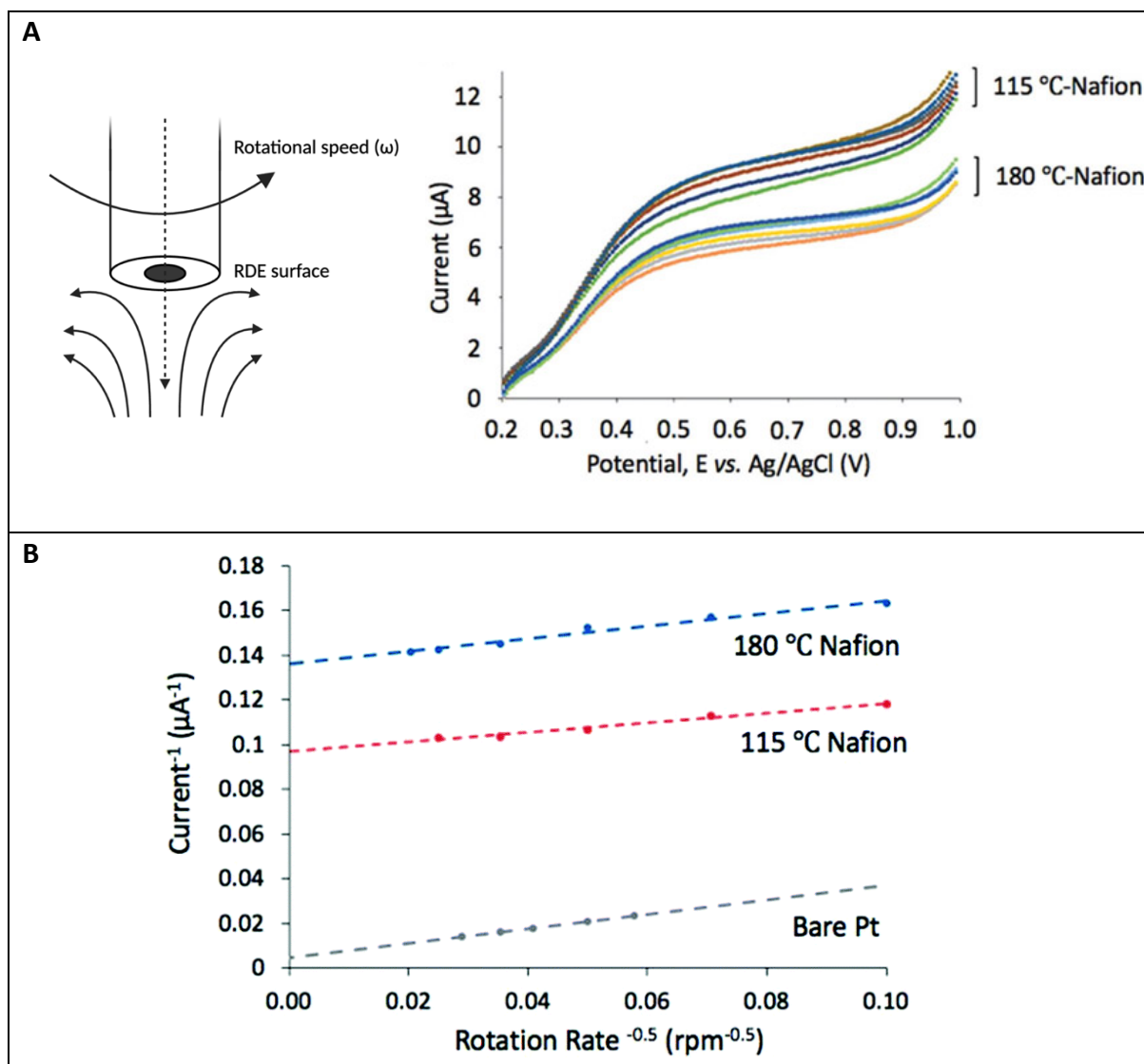


Figure 2.7 (a) Hydrodynamic voltammetry of Nafion-coated glutamate oxidase sensors in 0.2 mM H₂O₂ at increasing rotational speeds from 100 to 2400 rpm and (b) Koutecký-Levich plot of the sensors with Nafion membranes annealed at different temperatures (adapted from Huang *et al.*).¹³⁹

The rate of the electrode reaction of the oxidation of H₂O₂, $\frac{1}{i_R}$, defined by the Butler-Volmer equation, is negligible due to i_R being large at the diffusion limited potential, therefore, $\frac{1}{i_{total}}$, can be reduced to the sum of $\frac{1}{i_K}$, $\frac{1}{i_{m,lactate}}$ and $\frac{1}{i_{m,H_2O_2}}$, as shown in Equation 2.17.

$$\frac{1}{i_{total}} = \frac{1}{i_{m,lactate}} + \frac{1}{i_{m,H2O2}} + \frac{1}{i_K}$$

Equation 2.17 The Koutecký-Levich equation at diffusion limited potential.

The intercept of the Koutecký-Levich plot is therefore representative of the sum of the mass transport of lactate through the film membrane ($\frac{1}{i_{m,lactate}}$), the mass transport of H₂O₂ through the film membrane ($\frac{1}{i_{m,H2O2}}$), and the kinetics of the enzyme catalysed reaction between lactate and oxidase ($\frac{1}{i_K}$). Assuming the intercept ($\frac{1}{i_m}$) represents the current coming from the mass transport of lactate and H₂O₂ in the film membrane, represented in Equations 2.18 and 2.19, the inverse of the intercept (i_m) can be used (Equation 2.20) to estimate the diffusion coefficient (D) of lactate and H₂O₂ as a product of D and partition coefficient, K . Since K is unknown, we calculate the product DK , also known as permeability, and which will scale with the diffusion coefficient for any given membrane, as a function of its thickness. K is expected to be different for lactate and H₂O₂ and will vary with the membrane material. Table 2.3 summarises the limiting factors in the total current obtained from an RDE sensor.

$$\frac{1}{i_{m,lactate}} = \frac{\phi}{nFADKc}$$

Equation 2.18 The rate of transport of lactate through the film membrane.

$$\frac{1}{i_{m,H2O2}} = \frac{\phi}{nFADKc}$$

Equation 2.19 The rate of transport of H₂O₂ through the film membrane.

$$DK = \frac{i_m \phi}{nFAc}$$

Equation 2.20 The product of the diffusion coefficient and partition coefficient.

Limiting factor	Description	Equation
$\frac{1}{I_l}$	The transport of lactate in the boundary layer above the coated electrode surface.	$\frac{1}{i_l} = (0.620)nFAD^{\frac{2}{3}}\omega^{\frac{1}{2}}\nu^{\frac{-1}{6}}C$
$\frac{1}{I_{m,lactate}}$	The transport of lactate in the film membrane coated onto the electrode surface.	$\frac{1}{i_{m,lactate}} = \frac{nFADK}{\phi}c$
$\frac{1}{I_{m,H2O2}}$	The transport of H ₂ O ₂ in the film membrane coated onto the electrode surface.	$\frac{1}{i_{m,H2O2}} = \frac{nFADK}{\phi}c$
$\frac{1}{I_k}$	The kinetics of the lactate oxidase enzyme reaction with lactate.	$\frac{1}{i_k} = \frac{nFAk_2[E]C_L}{K_M}$ for $C_L < K_M$
$\frac{1}{I_R}$	The kinetics of the electrode reaction of the oxidation of H ₂ O ₂ .	$i = i_0 \left\{ e^{\left[\frac{\alpha_f n F (E - E_0)}{RT} \right]} - e^{\left[\frac{-\alpha_r n F (E - E_0)}{RT} \right]} \right\}$ where: α_f = forward transfer coefficient, α_r is reverse transfer coefficient, E = potential difference across electrode, E₀ = standard potential.

Table 2.3 A summary of limiting factors in total current from an RDE sensor.

In this thesis, the diffusivities of lactate and H₂O₂ in the shortlisted film membranes, estimated by their diffusion coefficients, were used to guide sensor optimisation and the extension of the sensor's dynamic range.

As shown previously, Huang *et al.* conducted a Koutecký-Levich analysis to determine the diffusion coefficient of H₂O₂ in permselective films coated onto their glutamate oxidase (GlutOx) sensors.¹³⁹ The permselective film, deposited as a combination of polypyrrole (PPy) with Nafion at annealing temperatures of 115 °C and 180 °C, were used to decrease interference from other electroactive molecules that may be present in brain extracellular fluid and that can be oxidised at a potential of +0.7 V vs. Ag|AgCl reference electrode. The permselective films were applied to the sensor surface before the crosslinked GlutOx enzyme layer and the analysis revealed that the diffusion of H₂O₂ was slower in the PPy-Nafion films annealed at 115°C than those annealed at 180°C. Huang *et al.* reported the diffusion coefficient of H₂O₂ as $(1.7 \pm 0.2) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for PPy-Nafion annealed at 180°C, and $(1.3 \pm 0.2) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for PPy-Nafion annealed at 115 °C. This effect can be explained by the Mackie-Mears obstruction model which describes the diffusion of a molecule in a polymer solution.¹⁴⁰ The model assumes that polymer chains are motionless relative to the diffusing molecule, which decreases the mean free path length of the molecule, and states that the diffusivity of the molecule is dependent on the concentration of the polymer solution, quantified as its volume fraction. Equation 2.21 depicts this model, where D is the diffusion coefficient in pure solvent, D_0 is the diffusion coefficient in polymer, and φ is the volume fraction of the polymer.

$$\frac{D}{D_0} = \left[\frac{1 - \phi}{1 + \phi} \right]^2$$

Equation 2.21 The Mackie-Mears model for diffusion of a molecule through polymer.

Zhang *et al.* reported mass transport rate constants, as estimated diffusion coefficients due to the unknown partition coefficient parameter, of lactate and H₂O₂ through the mixed gel membrane on their lactate oxidase sensors.¹²⁴ The mixed gel consisted of DET LOD enzyme in a PEG-DE hydrogel, mixed with poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) and Prussian blue (PB). The reported mass transport rate constant for lactate transport through the gel membrane was $1.29 \times 10^{-6} \text{ m s}^{-1}$. Chen *et al.* have also reported the diffusion coefficient of H₂O₂ in the PEDOT:PSS-PB film of their H₂O₂ sensors⁵² to be $9.2 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. The diffusion coefficients of H₂O₂ from these studies have been summarised in Table 2.4.

Enzyme	Membrane/matrix	Substrate	$DK/ \text{m}^2 \text{s}^{-1}$	Ref
LOD	PEDOT:PSS-PB membrane	H_2O_2	9.2×10^{-11}	¹⁴¹
LOD	PEDOT:PSS-PB-EG-DVS membrane (potassium ferric ferrocyanide composite cross linked with ethylene glycol and divinyl sulfone)	H_2O_2	1.56×10^{-10}	¹⁴¹
GlutOx	Polypyrrole-Nafion membrane annealed at 180 C	H_2O_2	$(1.7 \pm 0.2) \times 10^{-11}$	¹²¹
GlutOx	Polypyrrole-Nafion membrane annealed at 115 C	H_2O_2	$(1.3 \pm 0.2) \times 10^{-11}$	¹²¹
DET LOD	Bulk solution	Lactate	$(7.10 \pm 0.12) \times 10^{-10}$	¹²⁴
LOD	Bulk solution	Lactate	$(7.31 \pm 0.69) \times 10^{-10}$	¹²⁴
LOD	Collagen matrix	Lactate	3.54×10^{-10}	¹⁴²

Table 2.4 A summary of diffusion coefficients (DK) for lactate and H_2O_2 reported in literature.

2.2.9 Review of lactate sensors used for *in vivo* monitoring in ISF

Several studies have reported the use of lactate sensors for continuous monitoring producing lactate during intense exercise, however, very few studies report *in vivo* use of lactate sensors that involves on-site – i.e. implantable or wearable testing – in ISF in humans.

Recently (2023), Freeman *et al.* reported a third generation DET LOD microneedle sensor that was tested in humans producing lactate during intense exercise.¹⁰⁶ The sensor was coated with diffusion limiting membrane of 3% w/v cellulose acetate and achieved a high effective K_m value of 19.75 ± 0.70 mM during *in vitro* calibration. The sensor performed well *in vivo*, with good agreement observed between microneedle current and venous blood lactate, as shown in Figure 2.8(a), wherein the rise and fall in current followed the rise and fall in lactate in blood lactate with an approximate five-minute lag in ISF, as is reported by other *in vivo* studies measuring lactate in blood vs ISF. The sensor needed a low oxidation potential of +0.2 V due to the use of a DET enzyme, meaning that interference from molecules oxidising above this potential was avoided. However, interference was seen from ascorbic acid experimentally, as shown in Figure 2.8(b), which is present in serum at concentrations of <0.1 mM. The study does not report lactate concentrations derived from microneedle current. In blood, concentrations as high as 20 mM were seen during exercise and as low as 1 mM at rest across the five participants. The sensor was reported to have a dynamic range of > 10 mM lactate.

Other methods of continuous monitoring include flowing blood or dialysate in a continuous manner through a bedside LOD sensing system for *in vitro* lactate measurements. Rogers *et al.* report continuous on-line microdialysis, a method developed to detect lactate in dialysate recovered from injured human brain in real-time using a first generation LOD biosensor at the bed side.¹⁰⁸ The first generation LOD biosensor was fabricated on needle microelectrodes and recovered dialysate was passed through a microfluidic chip containing the sensors at a flow rate of $2 \mu\text{L min}^{-1}$. Simultaneous measurements of lactate and glucose, as shown in Figure 8(c), allowed correlations to be drawn; an initial fall in glucose concentration is seen, creating

oxidative stress in the surrounding tissue, and causing cells to undergo anaerobic respiration, producing more lactate, as is seen in the simultaneous rising concentration of lactate. The response time of the sensor was reported to be 68 s. No diffusion limiting membranes were used in the fabrication of sensors and the dynamic or linear range was not reported. Concentrations of lactate in brain dialysate ranged from 0.50 to 0.80 mM from sensor measurements.

Meyerhoff *et al.* report a first generation LOD biosensor for the continuous on-line monitoring of lactate in blood using an *in vitro* lactate sensing system (LSS).¹⁴³ Blood was collected using a double lumen catheter which connected to a flow chamber in the LSS. The blood was diluted 1:3 in saline and pumped through the flow chamber at flow rates of 43 to 92 $\mu\text{L min}^{-1}$. At the end of the flow chamber, an LOD membrane sensor was present, coated with a cellulose acetate and polycarbonate, which detected lactate continuously in the diluted blood. For comparison to gold standard methods, blood was collected at 30 mins before exercise, immediately before exercise, and at 6, 12, 18, 24, 36, 48, 60, 90 and 120 min after exercise, and analysed using an ESTAT lactate analyser (Eppendorf, USA). Figure 8(d) shows the sensor and gold standard measurements of lactate in blood wherein exercise was started at $t = 0$ s and stopped $t = 18$ min. Five minutes after stopping exercise, a fall in lactate concentrations can be seen in sensor measurements. Sensor measurements reached above 5 mM lactate after the peak of exercise and decreased to pre-exercise levels approximately 40 min after stopping exercise. The sensor was reported to have a linear range up to 15 mM *in vitro*, a response time of 100 s and a sensitivity of $(3.33 \pm 1.31) \text{ nA mM}^{-1}$. Good agreement can be seen between blood measurements made by the two methods with a time lag in sensor measurements of approximately 2 min.

While there was a limited number of studies measuring lactate *in vivo* in humans, several studies report the *in vivo* measurement in rats^{142,144} and artificial ISF.¹¹² Bollella *et al.* report a second-generation microneedle-based LOD biosensor consisting of gold-multiwalled carbon nanotubes (MWCNTs) and electrodeposition of methylene blue as an electron mediator.¹¹² The biosensor was tested in artificial ISF mimicking concentrations after a meal (10 mM) and after exercise (12 mM) *in vitro*. The 1 mm microneedles used in this study were the same needles used in this thesis (Torr Scientific, UK), i.e. polycarbonate bases metallised with gold and arranged as 4 x 4 arrays, before any functionalisation. The LOD biosensor demonstrated a high sensitivity of $(797.4 \pm 38.1) \text{ mA cm}^{-2} \text{ mM}^{-1}$ and a low linear range (10 to 100 μM) compared to physiological concentrations. However, the sensors responded to ascorbic acid and uric acid *in vitro* and were not tested *in vivo* or in clinically sourced human blood or ISF *in vitro*.

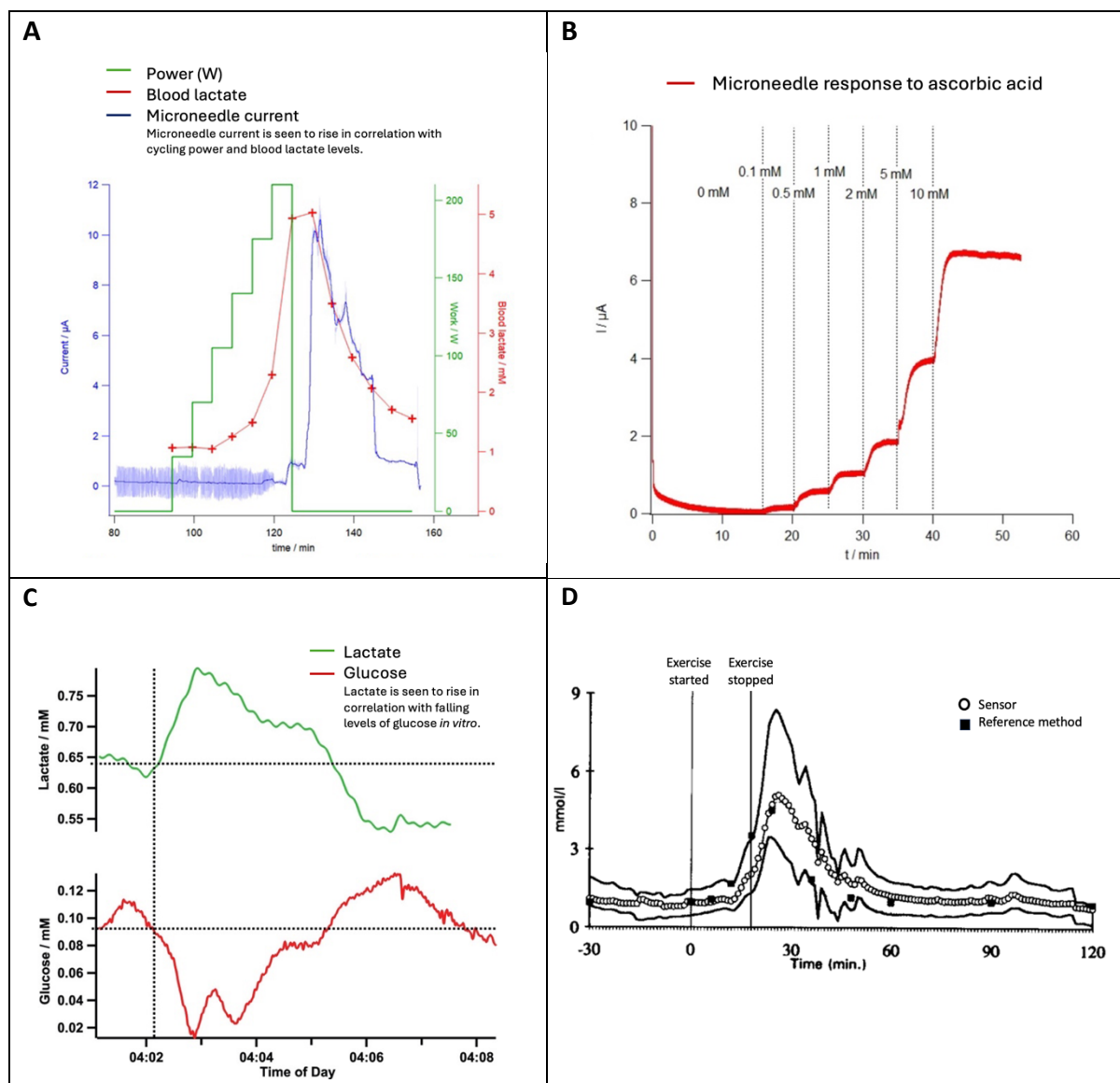


Figure 2.8 Examples of *in vivo* and *in vitro* lactate measurement in published work. (a) *In vivo* response of DET LOD microneedle sensors coated with CA and (b) the sensor's response to ascorbic acid, as reported by Freeman *et al.*,¹⁰⁶ (c) *in vitro* response of LOD and GOD biosensors to lactate and glucose, respectively, in dialysate recovered from injured human brain using on-line microdialysis, as reported by Rogers *et al.*,¹⁰⁸ (d) Blood lactate concentrations from an on-line lactate sensing system *in vitro* using sensor and reference methods over a period of exercise, adapted from Meyerhoff *et al.*, with the shaded area representing $\pm$ one standard deviation.¹⁴³

2.2.10 Summary

In this thesis, we report a minimally invasive microneedle-based lactate biosensor to continuously monitor lactate concentrations in interstitial fluid (ISF). The microneedle biosensor has a hydrogel layer of lactate oxidase enzyme which oxidises lactate to pyruvate, producing hydrogen peroxide, which is further oxidised at the electrode, using lactate sensor recipes adapted from Viggiano *et al.* and Vasylieva *et al.*^{145,146} To meet the physiologically relevant concentration window, the extension of the sensor's dynamic range will be demonstrated through the modification of the barrier membrane between the enzyme hydrogel layer and interstitial fluid. Figure 2.9 shows the proposed method of extending the dynamic range of sensors by using a diffusion limiting membrane.

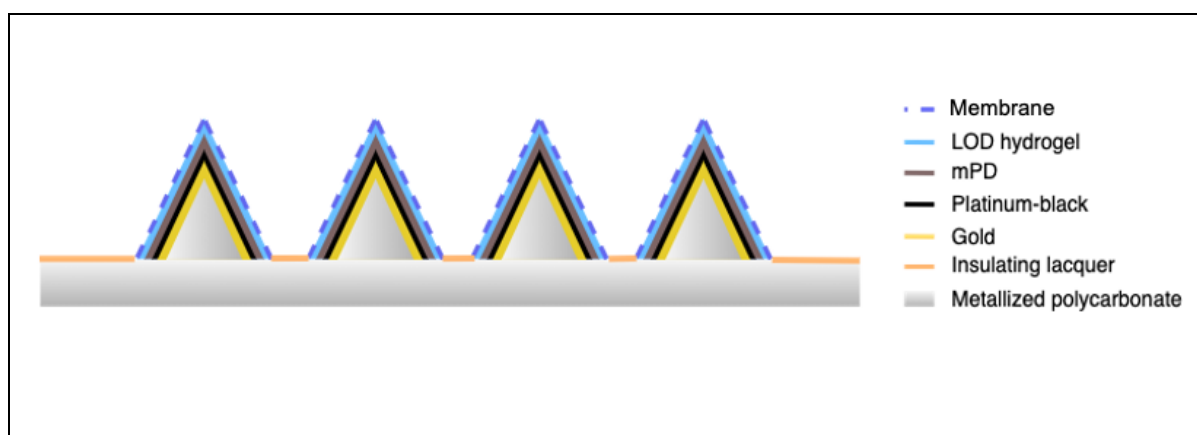


Figure 2.9 Proposed method of extending dynamic range.

Koutecký–Levich analyses featuring a range of membrane materials will be reported and the sensor's performance analysed over time. Finally, the sensor will be validated with *in vivo* results from a Phase I Clinical Study where exercise is used to generate lactate by healthy volunteers as a proof-of-concept before moving into monitoring of patients. Lactate generation in ISF will be compared to venous blood lactate and dialysate lactate.¹²⁶

In choosing a material to slow down the delivery of lactate of the enzyme hydrogel layer, we must consider that the top coating of the microneedle sensor will be in contact with skin and interstitial fluid. Therefore, it needs to be biocompatible, have high mechanical stability and permeability for the diffusion of lactate. Choosing a membrane that is already widely used such as Nafion can also save time and costs for approval and overall time to market. Products using Nafion as a key component, such as the PermaPure Nafion™ tubing and the LungFit® PH nitric oxide generator and delivery system are FDA approved for medical use.¹⁴⁷ There is a need to choose a fabrication method that deposit the chosen polymer evenly over the surface of the microneedles – such as through spray coating using a nebuliser – which is both scalable and reproducible.

2.3 Methods

2.3.1 Enzyme and reagents

Lactate oxidase (recombinant) enzyme was obtained from Sekisui Diagnostics (UK). The activity and K_m of the enzyme were reported as 20 - 60 U/mg at 37°C and 7.0×10^{-4} M (0.7 mM), respectively. All other reagents were obtained from Sigma Aldrich (UK). Electrical wires, insulating varnish, araldite glue and silver epoxy were obtained from RS Components (UK), Circuitworks and Chemtronics (USA). Where stated, a direct electron transfer lactate oxidase enzyme was used for comparison, and this was sourced via a collaboration with DirectSens GmbH (Austria).

2.3.2 Electrodes and instrumentation

The electrodes used for the Koutecký–Levich characterisation of the sensor were platinum (Pt) rotating disk electrodes (RDEs) from Pine Research (UK). The diameter of the disk

electrodes was 5 mm with a 15 mm PTFE shroud. The reference electrode was Ag|AgCl, and the counter electrode was a platinum wire (IJ Cambria Scientific Ltd, UK). The RDE set-up consisted of the modulated speed rotator (Pine Research, UK), as shown in Figure 2.10, with an adjustable range of 50 to 10,000 rpm. The electrodes used for *in vivo* testing were gold microneedles (Torr Scientific, UK). The reference electrode was Ag|AgCl, and the counter electrode was a pad of gold microneedles on the same array. For all experiments in this chapter, unless otherwise stated, the CHI 1030 potentiostat (CH Instruments, USA) was used to run electrochemical readings. The software used to record measurements on the CHI potentiostat was the CHI1030 software.

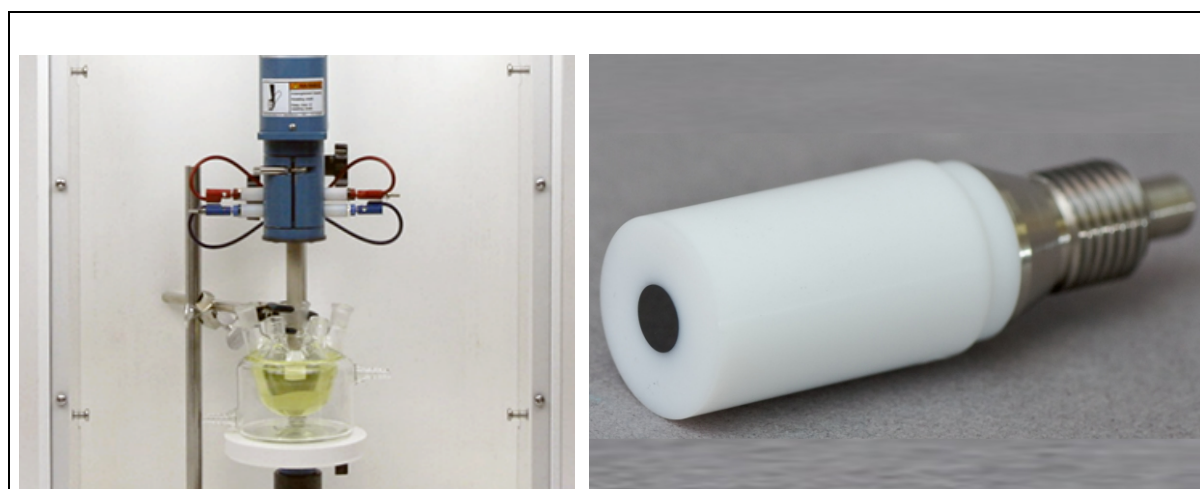


Figure 2.10 Modulated speed rotator set-up (left) and rotating disk electrode (right), adapted from Pine Instruments (UK).

2.3.3 Filtering and smoothing

A robust locally weighted scatterplot smoothing (LOESS) filter was applied to smooth signals with a high noise:signal ratio in Igor Pro software. LOESS filtering is a non-parametric local regression method which involves sampling neighbouring datapoints using a sliding window of points, k , and applying a weighted linear regression to this window. k can be specified

when applying the filter and for continuous data. In this thesis, k was chosen between 100 and 500 points, depending on the scale of the noise, and chosen such that the filter did not distort the underlying sensor data. The characteristic time is the reciprocal of the product of the mass transport boundary layer (Nernst layer) and the mass transport rate constant, so we can rationally select how to filter sensor data without distortion. For *in vivo* studies with higher noise:signal ratio, a higher k was used to smooth data to a higher degree.

The weighted average function used in LOESS gives the most weight to points closer to the point being filtered, and least weight to points further away. The weighted average function, also known as the tri-cube weight function, is shown in Equation 2.22, where $w(d)$ is weighted function at a distance d from the point being smoothed based on a scale from 0 to 1. The robust function of the LOESS filter on Igor Pro ignores outliers in the data when calculating the weighted average for each point, such as those arising from interruptions in sensor signal or applied potential. For a zero-degree polynomial, the LOESS filter acts as a moving average filter. The advantage of LOESS filtering is the ability to specify (i) the order of the polynomial used in the regression (zero, first or second), and (ii) the window size used in the weighting, allowing you to apply the filter to a range of noise levels observed in continuous data.

$$w(d) = (1 - |d|^3)$$

Equation 2.22 The weighted average function used in the robust LOESS filter.

2.3.4 Microneedle construction

Poly(carbonate) microneedle arrays were fabricated using injection moulding and metallized at Torr Scientific (UK). Each array – comprising of four pads – was sputter-coated with an initial layer of chrome (110 nm). Following this, three pads (forming the working electrodes) were coated with gold (150 nm) by e-beam evaporation. The fourth pad (forming the reference electrode) was coated with silver (150 nm) by e-beam evaporation (Figure 2.11). The metallized arrays were wired to gold contacts using silver epoxy (Circuitworks, Chemtronics) which was allowed to cure overnight, as shown in Figure 2.10. Insulating varnish (RS Components) was applied on top of the cured silver epoxy and to the base of the microneedles, leaving the tips exposed. Approximately 10 μL of insulating varnish was added to each pad on a microneedle array via syringe with a wide bore needle and allowed to dry at 30 °C for 2 hours.

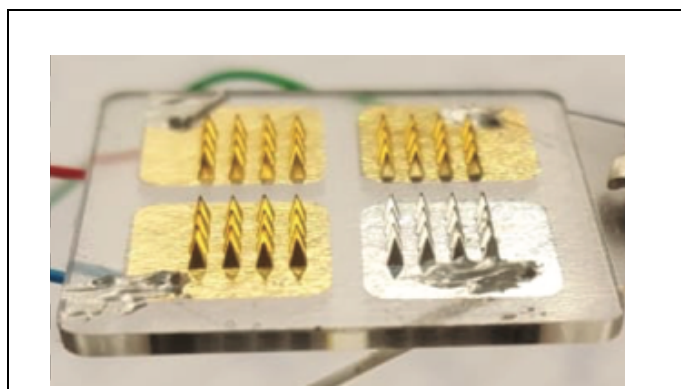


Figure 2.11 Silver epoxy used to connect gold contacts via wires to the microneedle pads.

2.3.5 Microneedle sensor fabrication

The biosensors are fabricated in several layers, as shown in Figure 2.9 (section 2.2.9). In addition to the microneedle, lactate sensors were fabricated onto rotating disk electrodes (RDEs) using the same methods of deposition described below. The following protocol has

been adapted from lactate sensor fabrication methods reported by Vasylieva *et al.*,¹⁴⁵ Viggiano *et al.*,¹⁴⁶ and Rogers *et al.*⁷⁴

The insulated microneedle arrays were cleaned with 70% ethanol in deionized water and dried with a stream of compressed air. Platinum-black (Pt-b) nanoparticles were then electrodeposited onto the gold working electrode pads to create a higher electrode surface area with greater catalytic activity (see Figure 2.12). To do this, a solution of 3% w/v chloroplatinic acid and 0.005% w/v lead acetate in deionised water was prepared and a constant current of -1.95 mA was applied per microneedle pad vs Ag|AgCl for 60 s. A platinum gauze electrode was used as the counter electrode (IJ Cambria, UK).

The microneedle array was then washed with deionized water and dried with a stream of compressed air. To stabilise the Pt-b nanoparticles on gold, the microneedle pads were cycled in 0.5 M sulfuric acid between -0.2 V and +1.3 V for 10 cycles at 0.1 V/s. The microneedles were then stored in deionised water until further functionalization.

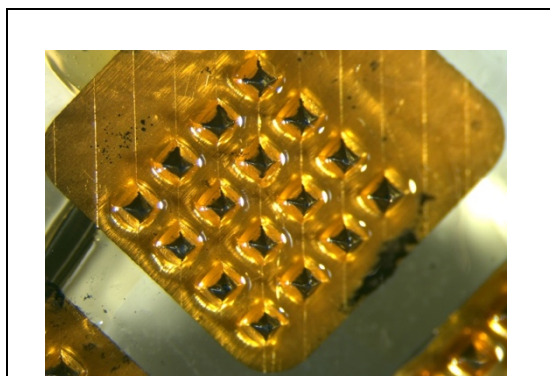


Figure 2.12 A gold microneedle pad functionalised with platinum-black nanoparticles.

To exclude interferents from reaching the electrode, a layer of m-phenylenediamine (mPD) was applied on the Pt-b coating. To do this, a solution of 100 mM mPD was prepared in 10 mM phosphate buffered saline (PBS) and 0 V applied for 20 s, followed by 0.7 V for 20 min and 0 V for 5 min (vs Ag|AgCl).

To create the Ag|AgCl reference electrode on the microneedle array, the silver pad on the microneedle was painted with Ag|AgCl ink using a 30G needle. The microneedles were dried facing down at 50°C for 1 hour. Following this, the pad was coated with approximately 20 µL of polydiallyldimethylammonium chloride (polyDADMAC) and the microneedles were dried face-down at 65 °C for 1 hour. Once dried, the pad was coated with 10 µL of poly(2-hydroxyethyl methacrylate) (pHEMA) and the microneedles dried face-down at 65C for 1 hour. The recipe for the reference electrode was followed as reported by Chen *et al.*¹⁴¹

To create the counter electrodes, the gold pads were electrodeposited with Pt-b and stabilised in sulfuric acid, as above.

A control hydrogel mixture was made containing 90 mg/mL bovine serum albumin (BSA), 60 mg/mL lactate oxidase, 2% (v/v) glycerol and 45.6 mg/mL poly(ethylene glycol) diglycidyl ether (PEG-DE, average Mn 500 Da) in 10 mM PBS. The control hydrogel mixture (10 µL) was applied to gold electrodes to be used as a control for in vivo studies. The array was clamped with the needles facing down to encourage the hydrogel to cover the tips and was cured at 55 °C for 2 h.

For the working electrodes, an enzyme hydrogel was made containing 30 mg/mL BSA, 60 mg/mL lactate oxidase, 2% (v/v) glycerol and 45.6 mg/mL PEG-DE in 10 mM PBS. This hydrogel mixture (10 μ L) was carefully applied to each working electrode and the hydrogel cured at 55 °C for 2 h with the microneedle facing down.

2.3.6 Applying a diffusion-limiting membrane

To extend the dynamic range of the sensors, a coat of 1% (w/v) Nafion was spray-coated onto the microneedles, and these were dried face-down at room temperature for 30 min. To spray-coat with Nafion, a nebulizer was used at a constant pressure of 1.5 atm. The thickness of the Nafion layer was controlled by the number of coats deposited by the nebulizer which stationed at the fixed distance and perpendicular to the electrode surface.

Other polymer membranes used to extend dynamic range, deposited via pipetting, included 25 mg/mL polyurethane (PU), 1% w/v cellulose acetate (CA) and 1% w/v poly(4-styrenesulfonic acid) (PSSA). 25 mg/mL PU was dissolved in tetrahydrofuran (THF) and 0.25 μ l/ml Brij 30 surfactant. The solution was covered to prevent evaporation and to left to stir for 2 hours before use. 1% w/v CA was dissolved in 100% acetone and 1% w/v PSSA was dissolved in DI water.

2.3.7 RDE sensor fabrication

To carry out Koutecký–Levich analysis and measure DK, the product of diffusion coefficient and partition coefficient, platinum RDEs (Pine Instruments, USA) were functionalised using the fabrication recipe described for the microneedles. Prior to functionalisation, RDEs were polished using hydrated aluminium oxide polishing pads and sonicated in deionised water for

10 min. Following this, the RDEs were electrochemically cleaned via cycling in 0.5 M sulfuric acid at -0.2 V to +1.5 V for 100 cycles at 0.5 V/s. RDEs were stored in deionised water until further functionalisation.

2.3.8 Sensor calibration *in vitro*

To calibrate the sensor, the microneedle array was immersed in a stirred solution of 10 mM PBS and standard additions of sodium L-lactate solution were made to vary concentration in the range of 0.2–10.0 mM. The microneedle array was left in 10 mM PBS for 1 hour before any additions were made to allow the current to stabilise. Chronoamperometry was carried out at +0.7 V.

2.3.9 Stability studies of the microneedle sensors

The long-term stability of the sensors was investigated over a period of three weeks. The sensors were calibrated with sodium L-lactate on day 1 and 21. The sensors were stored in the freezer at -20 °C in between calibrations.

2.3.10 Interference layer testing and optimisation

Interference testing was carried out on both working electrodes comprising the enzyme hydrogel and the control electrode comprising of hydrogel without enzyme. Microneedle arrays were immersed in a stirred solution of 10 mM PBS and standard additions of interferents such as acetaminophen (AM), uric acid (UA), and ascorbic acid (AA) were made to vary concentrations in the range of 0.1–0.4 mM. The microneedle array was left in 10 mM PBS for 1 hour before any additions to allow the current to stabilise. Chronoamperometry was carried out at +0.7 V.

Different interference layer materials – o-phenylenediamine (oPD), pyrrole and phenol – were electropolymerised on platinum-black coated microneedles under constant potential and their performance compared to that of mPD. 100 mM solutions of oPD, pyrrole and phenol were made in 10 mM PBS. For o-PD application, 0 V was applied for 20 s, followed by 0.75 V for 15 min, and then 0 V for 20 s (vs Ag|AgCl). For pyrrole application, 0 V was applied for 20 s, followed by 0.7 V for 15 min, and then 0 V for 20 s (vs Ag|AgCl). For phenol application, 0 V was applied for 20 s, followed by 0.9 V for 15 min, and then 0 V for 20 s (vs Ag|AgCl). The microneedles were stored in 10 mM PBS until further use.

The performance of the interference layer was assessed based on exclusion of peaks in cyclic voltammograms in 5 mM ferri/ferrocyanide after deposition of the exclusion layer, in addition to calibrations using lactate and common interferents.

2.3.11 Koutecký–Levich analysis

To extend the dynamic range of the sensors, a diffusion-limiting membrane, such as 1% w/v Nafion, was applied. For measurement of mass transport properties across this membrane, hydrodynamic voltammetry (HDV) was conducted from 0.0 V to +0.8 V (vs Ag|AgCl) at 1 mV/s. CVs were carried out in 10 mM PBS and in varying concentrations of lactate in the range of 0.1 – 0.5 mM. Amperometry was carried out at a constant potential of +0.7 V and the rotational speed was varied in the range of 49 – 625 rpm.

RDEs were prepared using the fabrication method described. Diffusion coefficients of lactate in various diffusion-limiting membrane materials were determined using the intercept from the Koutecký–Levich plot (Equation 2.20).

2.3.12 Error propagation in the calculation of mass transport rate constants

The absolute error associated with the calculation of the mass transport rate constants of lactate and H₂O₂ in the different film membranes was calculated using the intercept from the Koutecký–Levich analysis and the measured thickness of the membranes. Equation 2.23 shows how this error was calculated where I is 1/intercept (from the Koutecký–Levich plot), δ is thickness, dI is the standard deviation of I , $d\delta$ is the standard deviation of δ , c is the concentration in solution, n is the number of electrons being transferred in the reaction, F is Faraday's constant and A is the surface area of the electrode. dI was calculated, as shown in Equation 2.24, where x is the y-intercept, Δx is the error of the y-intercept (reported in the linear fit of the Koutecký–Levich plot), and n is -1 since the y-intercept is the inverse of current, I .

$$Uncertainty = \sqrt{\left(\frac{\delta}{cnFA}\right)^2 (dI)^2 + \left(\frac{I}{cnFA}\right)^2 (d\delta)^2}$$

Equation 2.23 Error propagation equation for uncertainty in the calculation of DK constants.

$$dI = |n|x^{n-1}\Delta x$$

Equation 2.24 Calculation of dI , the standard deviation of the inverse of the intercept (i.e. current).

2.3.13 Membrane thickness measurements

The thickness of the various diffusion-limited membranes was measured using a KLA Tencor AlphaStep D-600 stylus profilometer. Measurements were made along the longitudinal and

lateral axes, as shown in Figure 2.13. The average thickness values were then used to calculate diffusion coefficient from the Koutecký–Levich equation.

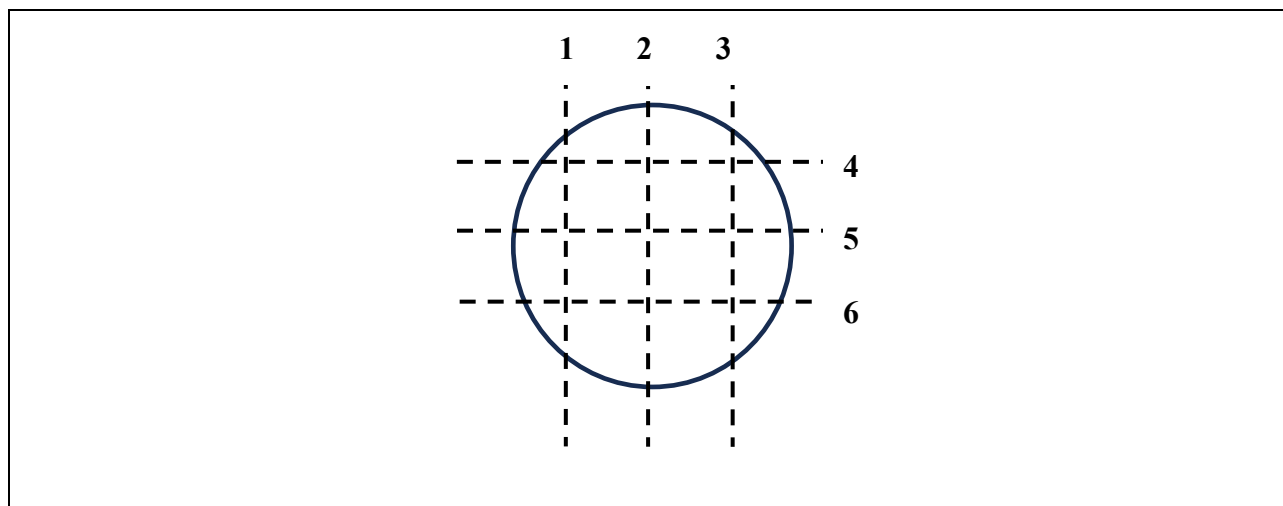


Figure 2.13 Method of profiling used in the calculation of film thickness of polyelectrolyte membranes.

2.4.14 Lactate microneedle measurements *in vivo*: a phase I clinical validation

In vivo studies were carried out with healthy volunteers performing moderate aerobic exercise on an exercise bicycle (Ergoselect 200, Ergoline Germany). The study was approved by London-Bloomsbury Research Ethics Committee (reference 20/LO/0364) and registered on Clinicaltrials.gov on 23rd January 2020 (NCT04238611). For each participant, two microneedle arrays were applied to the inner forearm, followed by the application of thumb pressure for 60 s, and constant pressure maintained by light tourniquet fastening over the back of the arrays.

The first array comprised of three working electrodes functionalised with enzyme hydrogel and 1% w/v Nafion. The second array comprised of one working electrode functionalised with

the control hydrogel (without enzyme) and 1% w/v Nafion, a Ag|AgCl reference electrode, and two counter electrodes. After application, the sensors were left to stabilise *in vivo* for 60 min. Following this, there was a period of rest for 15 min where baseline lactate was measured. The participant was then asked to cycle at moderate speed for 30 min with gradual increment in power of every 10 min. This was then followed by a rest period of 30 min.

Participants were disconnected from the arrays and a photograph taken of the site of application. Both sensing and control arrays were calibrated *in vitro* following the completion of the exercise regimen.

2.3.15 Venous blood measurements

Blood samples measuring venous lactate were taken at regular 5-minute intervals via a cannula and processed within 12 hours at a UKAS-accredited laboratory using an Architect Ci8200 serum analyser platform (Abbott, USA). When samples were taken, they were allowed to clot for 15 min and kept on ice until they were spun down and frozen at a -80°C . All samples were frozen within 45 min of being collected.

2.3.16 Microdialysis measurements

Microdialysis was carried out for one participant to measure dialysate lactate levels. A sterile CMA63 microdialysis probe (MDialysis, 10 mm membrane length, 20 kDa molecular-weight cut-off) probe was inserted subcutaneously into the forearm of a healthy volunteer by a qualified clinician after the skin was cleaned and an anaesthetic cream (EMLA cream 5%) applied 45 min prior to insertion. The probe was perfused with sterile T1 perfusion solution

(MDialysis) at 2 μ L/min using a CMA107 microdialysis pump (MDialysis). Dialysate samples were collected into microvials every 15 min. At the end of the study, the collected dialysate was amperometrically analysed for lactate concentration using an LOD biosensor (completed by S Gowers, Department of Bioengineering, Imperial College London), following a published protocol for microdialysis.^{108,126} The biosensor was calibrated in the perfusion solution (MDialysis) using standard additions of lactate.¹⁰⁹

2.3.17 Participant recruitment for clinical testing

Between 16 February and 10 July 2021, participants were identified through recruitment posters placed around Imperial College London advertising the study. Healthy adult (>18 years) volunteers with no significant medical history, who exercised regularly at moderate intensity for at least 30 min two times a week were eligible.

2.3.18 Impedance measurements and optical coherence tomography *in vivo*

To assess the penetration of the microneedles in the stratum corneum and epidermis, impedance vs time measurements were taken in skin at a frequency of 962 Hz (default frequency setting on CHI software). Different amounts of pressure were applied (no pressure, thumb pressure and tourniquet following thumb pressure) to see whether impedance decreased as it entered the skin and came into contact with ISF. Optical coherence tomography (OCT) scans were taken of the microneedle arrays applied to the inner forearm using thumb pressure for 60 s. All OCT images were taken in real-time while the microneedles were in the skin of healthy volunteers.

2.4 Results

2.4.1 Lactate sensor characterisation without membrane modification

Lactate sensors made with commercially available lactate oxidase enzymes have a low K_m and saturate very quickly at low concentrations of lactate, at the lower end of the physiologically relevant window of lactate concentrations in human serum and ISF. As shown in Figure 2.14, the K_m of an RDE lactate sensor made with lactate oxidase (Sekisui Diagnostics) is 0.47 mM, which is much lower than the 10 mM range seen in sepsis. There is therefore a need to extend the dynamic range of lactate sensors for the early detection of sepsis via optimisation of the membrane coating applied to the sensor. The dynamic range being targeted here is 5 mM; this is to cover the 4 mM lactate threshold, beyond which a patient is diagnosed as having sepsis. Table 2.5 outlines the V_{max} and K_m parameters of the Hill equation fit.

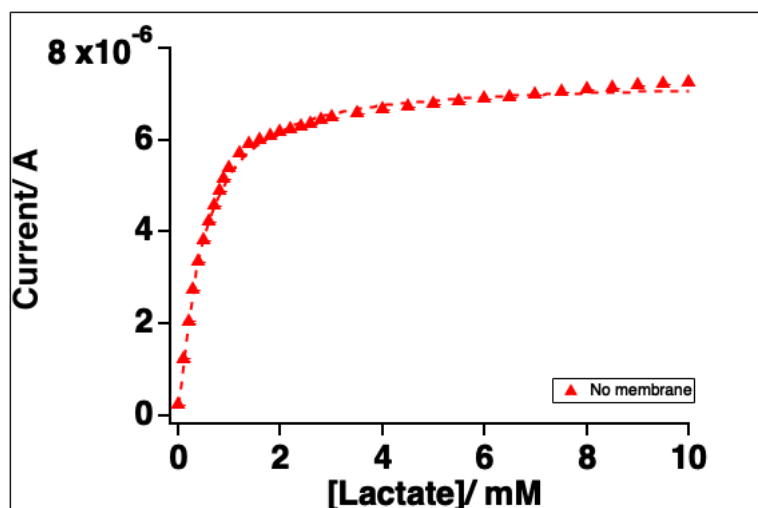


Figure 2.14 Calibration curve of an RDE lactate sensor without a membrane coating, produced via amperometry at a constant applied potential of +0.7 V vs Ag|AgCl (fitted with the Hill equation).

V_{max}/A	K_m/mM
$(7.25 \pm 0.04) \times 10^{-6}$	0.47 ± 0.01

Table 2.5 V_{max} and K_m values of an RDE lactate sensor without a membrane coating.

2.4.2 Membrane optimisation

Koutecký–Levich analysis was conducted for Nafion, CA, PU and PSSA coated lactate sensors. In this analysis, the concentration of the enzyme was assumed to be high enough, due to previous optimisation work,^{108,145,146} such that the enzyme reaction is instantaneous and not limiting. We would expect that at low concentrations of lactate (i.e. $c \ll K_m$), the Koutecký–Levich results more accurately reflect this constraint. In addition, the enzyme concentration needs to exceed the minimum concentration required to ensure that the lactate-enzyme reaction is not limiting, which would otherwise cause a concentration dependence in the Koutecký–Levich intercept. Factors that could limit the reaction are k_2 , the rate of the formation of product from the ES complex, and the number of active sites per enzyme molecule.

From the analysis, the product of the diffusion coefficient and partition coefficient, DK , of lactate and H_2O_2 in the membrane was estimated using Equation 2.20 (section 2.2.8 Koutecký–Levich analysis). A diffusion-limiting potential of +0.7 V vs Ag|AgCl was determined using hydrodynamic voltammetry conducted for all membrane-coated sensors (as shown in Figure 2.15 for an RDE sensor). This potential applied to all RDE sensors when conducting amperometry.

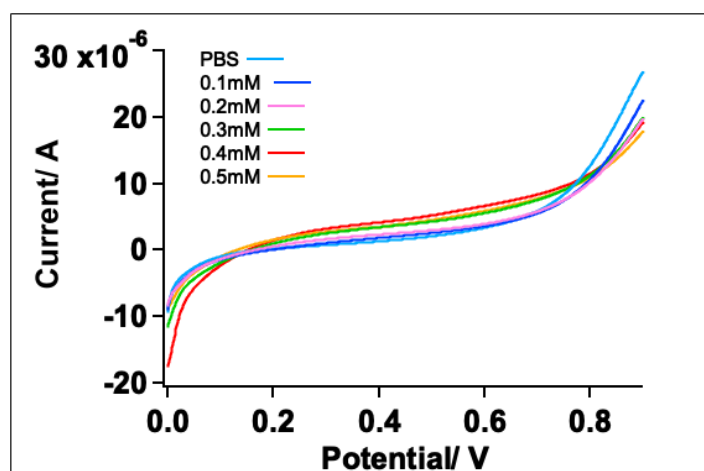


Figure 2.15 Hydrodynamic voltammetry of Nafion membrane-coated lactate RDEs in 0.1 to 0.5 mM sodium L-lactate solution with diffusion-limited current observed beyond +0.5 V. The scan rate used was 100 mV/s.

2.4.3 Nafion

The thickness of one coat of 1% w/v Nafion was calculated from measuring the thickness of six coats of Nafion. This is because of the measurement resolution of the profiler; measuring one coat of Nafion did not provide good results. An average of two profiles, as shown in Figure 2.16, was measured and the peaks at the edges were not included in the determining the average thickness of 757.30 ± 198 nm ($n=2$) for six coats. This was divided by six to determine the thickness of one coat (126.22 nm). Despite spray-coating the electrode with Nafion, a coffee-ring effect was seen in the thickness measurements.

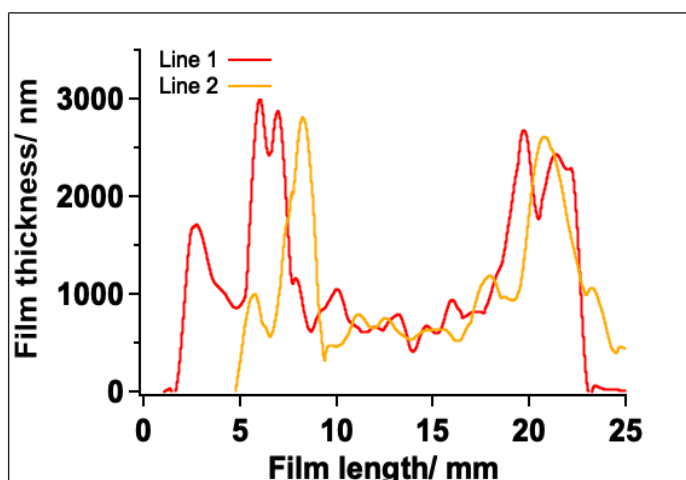


Figure 2.16 Membrane thickness profile of 6 coats of 1% w/v Nafion spray-coated onto a glass slide along the longitudinal axis.

Firstly, Koutecký–Levich analysis was conducted with Nafion-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM H_2O_2 to determine the mass transport rate constants of H_2O_2 through the film membrane. Potential was applied in the diffusion limited range to ensure that the electron transfer was not rate determining. Hydrodynamic voltammetry of the sensor in PBS, showing a mass transport limited region between +0.6 V and +0.8V. A Koutecký–Levich plot was obtained from amperometric measurements (Figure 2.17). Using intercepts from this plot, the diffusion coefficients of H_2O_2 in the Nafion membrane were calculated (Table 2.6). It was found that in 0.5 mM H_2O_2 , DK was $(1.06 \pm 0.278) \times 10^{-11} \text{ m}^2\text{s}^{-1}$.

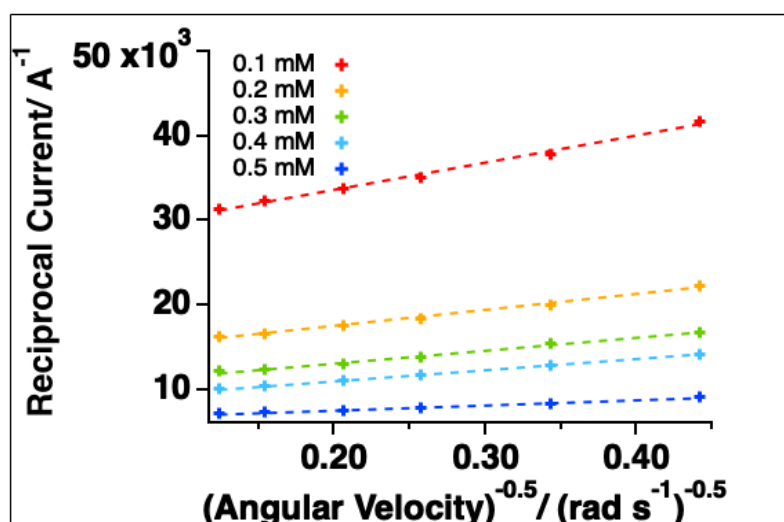


Figure 2.17 Koutecký–Levich plot in 0.1, 0.2, 0.3, 0.4 and 0.5 mM H₂O₂.

[H ₂ O ₂]/ mM	[H ₂ O ₂]/ mol m ⁻³	$DK/$ m ² s ⁻¹	Propagated error/ m ² s ⁻¹
0.1	0.1	1.23×10^{-11}	3.21×10^{-12}
0.2	0.2	1.22×10^{-11}	3.19×10^{-12}
0.3	0.3	1.11×10^{-11}	2.91×10^{-12}
0.4	0.4	9.94×10^{-12}	2.60×10^{-12}
0.5	0.5	1.06×10^{-11}	2.78×10^{-12}

Table 2.6 DK diffusion coefficient values for Nafion-coated RDE lactate sensor in H₂O₂.

Secondly, Koutecký–Levich analysis was conducted with Nafion-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate, which were below K_m of the sensor. A Koutecký–Levich plot was obtained (Figure 2.18) by plotting the reciprocal of the square root of angular velocity, to find the intercept. A linear relationship was observed between the reciprocal current and the reciprocal of the square root of angular velocity. The intercept of the plot was then used in the calculation of DK to provide an estimate of the diffusion coefficient of lactate through the Nafion membrane at each concentration (Table 2.7). It was found that in 0.5 mM lactate, DK was $(4.91 \pm 1.29) \times 10^{-13} \text{ m}^2\text{s}^{-1}$.

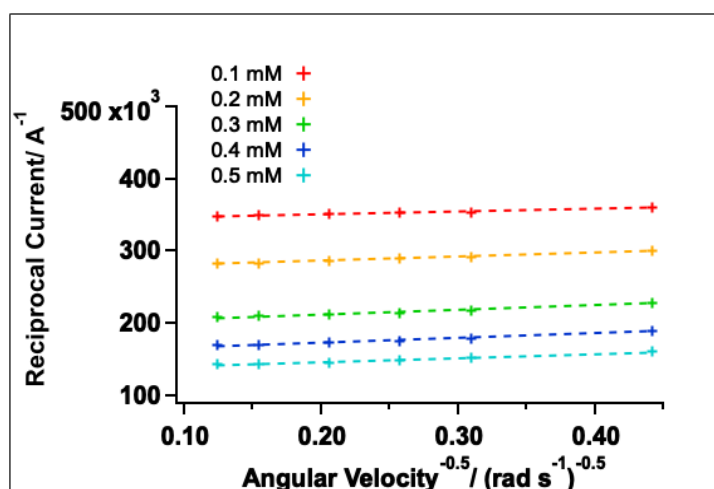


Figure 2.18 Nafion Koutecký–Levich plot in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate.

[lactate]/ mM	[lactate]/ mol m ⁻³	$DK/$ m ² s ⁻¹	Propagated error/ m ² s ⁻¹
0.1	0.1	9.67×10^{-13}	2.53×10^{-13}
0.2	0.2	6.05×10^{-13}	1.58×10^{-13}
0.3	0.3	5.54×10^{-13}	1.45×10^{-13}
0.4	0.4	5.20×10^{-13}	1.36×10^{-13}
0.5	0.5	4.91×10^{-13}	1.29×10^{-13}

Table 2.7 DK diffusion coefficient values of lactate in the membrane of Nafion-coated RDE lactate sensors.

It was found that the DK values obtained for H_2O_2 transport in the Nafion membrane were centred around $1.0 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ for all concentrations of H_2O_2 , whereas for lactate, DK values varied between 4.91 and $9.67 \times 10^{-13} \text{ m}^2\text{s}^{-1}$. Therefore, the assumption of rapid electrode reaction for the oxidation of H_2O_2 is reasonable. This suggests that the enzyme concentration could be higher to ensure we achieve a consistent DK , that is independent of concentration.

2.4.4 Cellulose acetate

The thickness of one coat of 1% w/v CA was measured to be $2772.91 \pm 1159 \text{ nm}$ ($n=6$). An average of six profiles, as shown in Figure 2.19, was calculated and the peaks at the edges were not included in the determining the average thickness.

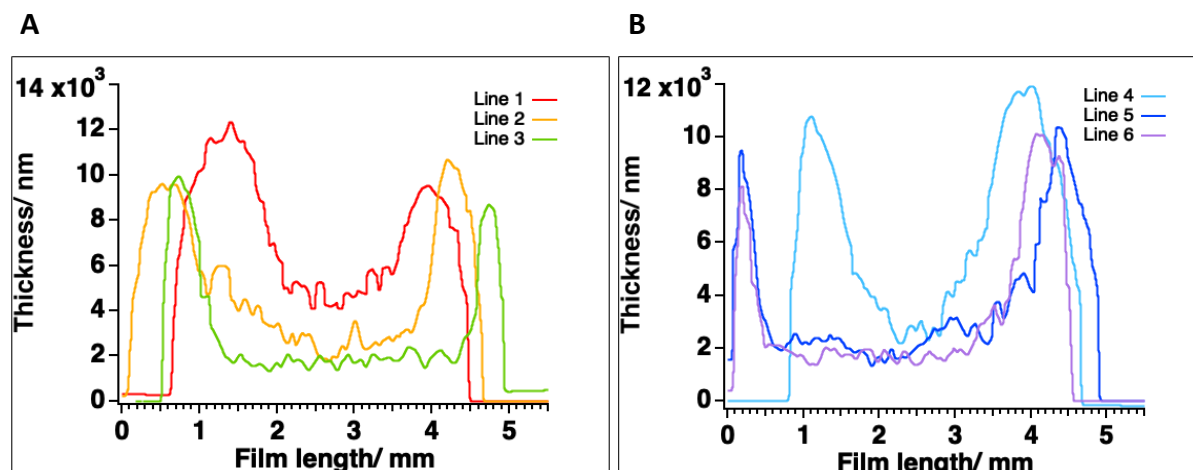


Figure 2.19 Membrane thickness profile of 1% w/v cellulose acetate pipetted onto a glass slide, along the (a) longitudinal and (b) lateral axis.

Amperometry was conducted in solutions of hydrogen peroxide to determine the mass transport rate constants. A Koutecký–Levich plot was obtained (Figure 2.20) by plotting the reciprocal current against reciprocal of the square root of angular velocity, to find the intercept. The intercept was then used in the calculation of DK to provide an estimate of the diffusion coefficient of H_2O_2 through the CA membrane at each concentration (Table 2.8). It was found that in 0.5 mM H_2O_2 , DK was $(3.36 \pm 1.41) \times 10^{-11} \text{ m}^2\text{s}^{-1}$, which was roughly three times that of H_2O_2 mass transport in the Nafion membrane.

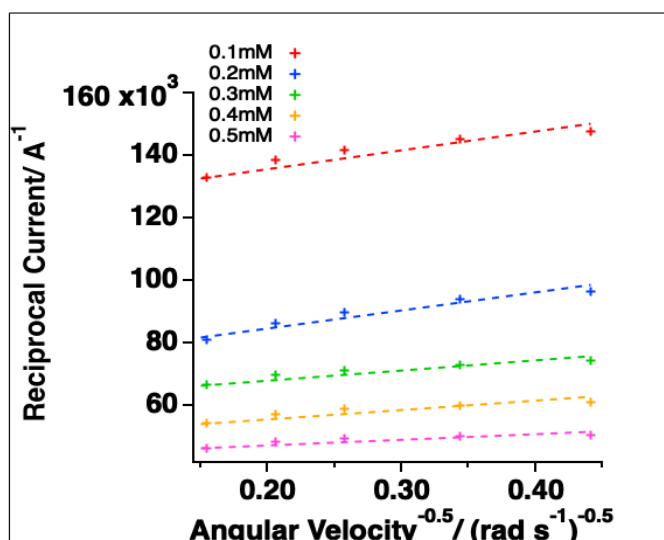


Figure 2.20 (a) Cellulose acetate Koutecký–Levich plot in 0.1, 0.2, 0.3, 0.4 and 0.5 mM H₂O₂.

[H ₂ O ₂]/ mM	[H ₂ O ₂]/ mol m ⁻³	$DK/ \text{m}^2\text{s}^{-1}$	Propagated error/ m^2s^{-1}
0.1	0.1	5.93×10^{-11}	2.48×10^{-11}
0.2	0.2	5.00×10^{-11}	2.10×10^{-11}
0.3	0.3	3.96×10^{-11}	1.66×10^{-11}
0.4	0.4	3.70×10^{-11}	1.55×10^{-11}
0.5	0.5	3.36×10^{-11}	1.41×10^{-11}

Table 2.8 DK diffusion coefficient of H₂O₂ values for cellulose acetate-coated RDE lactate sensor.

Koutecký–Levich analysis was conducted with CA-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate, which were below K_m of the sensor, however, no steps were seen in the amperometry. It was observed that the CA membrane had been peeling off the edges of the RDE surface, as shown in Figure 2.21, leading to the poor results observed in the amperometry in lactate solutions.

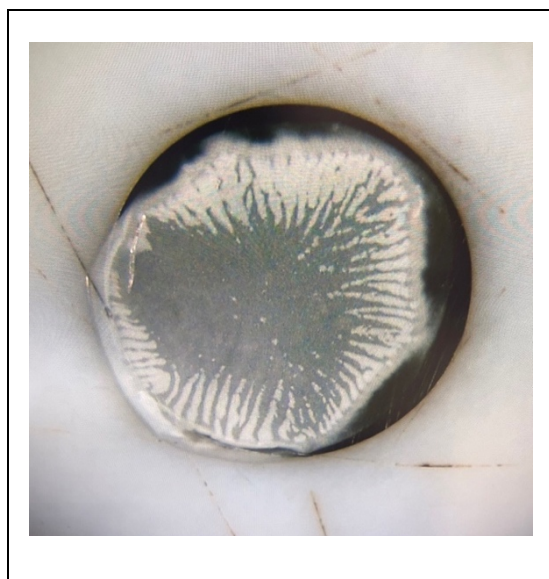


Figure 2.21 Cellulose acetate membrane peeling away from the edges of the electrode surface after conducting hydrodynamic voltammetry at high rpm.

2.4.5 Polyurethane

The thickness of one coat of 1% w/v PU was measured to be 3228.53 ± 1574 nm ($n=6$). An average of six profiles, as shown in Figure 2.22, was calculated and the peaks at the edges were not included in the determining the average thickness.

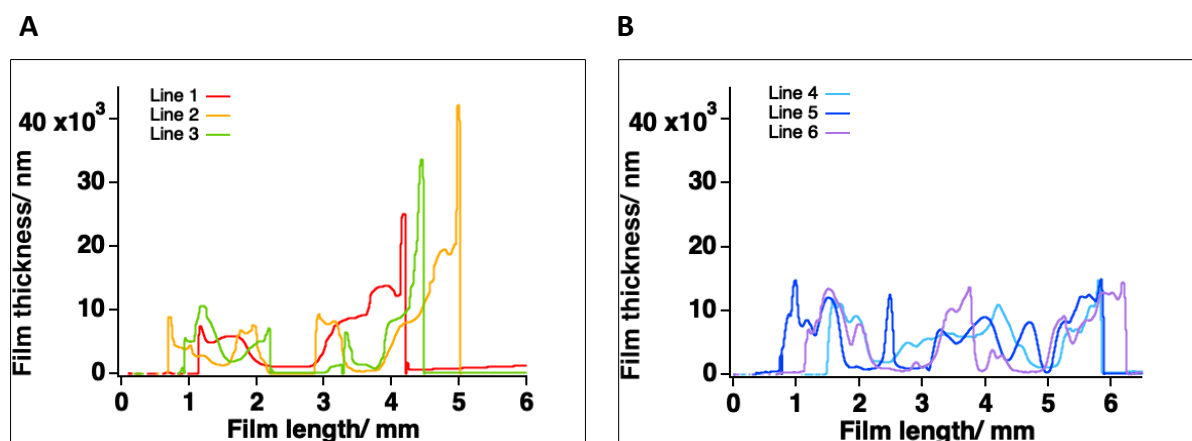


Figure 2.22 Membrane thickness profile of 25 mg/mL PU pipetted onto a glass slide, along the (a) longitudinal and (b) lateral axis.

Koutecký–Levich analysis was conducted with PU-coated RDEs in 0.5, 1, 1.5 and 2 mM H₂O₂ to determine the diffusion coefficients of H₂O₂ through the film membrane. A Koutecký–Levich plot was obtained amperometric measurements (Figure 2.23). Using intercepts from this plot, the diffusion coefficient of H₂O₂ in the Nafion membrane were calculated (Table 2.9). It was found that in 0.5 mM H₂O₂, DK was $(1.05 \pm 0.512) \times 10^{-11} \text{ m}^2\text{s}^{-1}$. This was comparable to the mass transport of H₂O₂ in the Nafion membrane and three times less than that in the CA membrane.

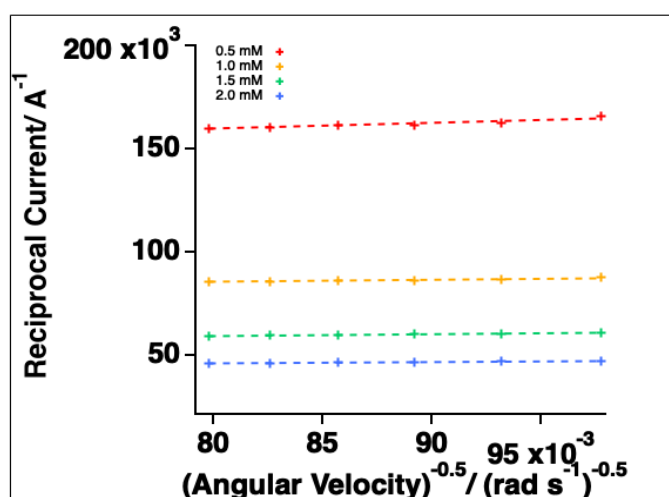


Figure 2.23 PU Koutecký–Levich plot in 0.5, 1.0, 1.5 and 2.0 mM H₂O₂.

[H ₂ O ₂]/ mM	[H ₂ O ₂]/ mol m ⁻³	$DK/$ m^2s^{-1}	Propagated error/ m^2s^{-1}
0.5	0.5	1.25×10^{-11}	6.11×10^{-12}
1.0	1.0	1.12×10^{-11}	5.45×10^{-12}
1.5	1.5	1.08×10^{-11}	5.26×10^{-12}
2.0	2.0	1.05×10^{-11}	5.12×10^{-12}

Table 2.9 DK diffusion coefficient of H₂O₂ values in PU-coated RDE lactate sensor membrane.

Koutecký–Levich analysis was conducted with PU-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate, which were below K_m of the sensor, however no steps were seen at concentrations

between 0.1 to 0.4 mM lactate. This is because, due to a higher K_m , the PU-coated lactate sensor only produced steps in amperometry at higher concentrations of lactate i.e. 0.5 and 1 mM lactate. A Koutecký–Levich plot was obtained (Figure 2.24) by plotting the reciprocal current against reciprocal of the square root angular velocity, to find the intercept. The intercept was then used in the calculation of DK to provide an estimate of the diffusion coefficient of lactate through the PU membrane at each concentration (Table 2.10). It was found that in 0.5 mM lactate, DK was $(9.20 \pm 4.49) \times 10^{-13} \text{ m}^2\text{s}^{-1}$.

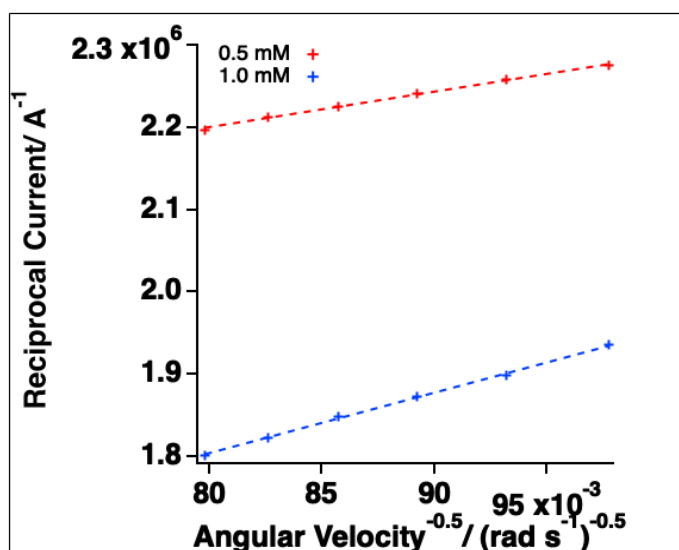


Figure 2.24 (a) PU HDVs in 0.1, 0.2, 0.3, 0.4, 0.5 and 1.5 mM lactate, and (b) Koutecký–Levich plot in 0.1 and 0.5 mM lactate.

[lactate]/ mM	[lactate]/ mol m ⁻³	DK/ m ² s ⁻¹	Propagated error/ m ² s ⁻¹
1.0	1.0	7.03×10^{-13}	3.43×10^{-13}
0.5	0.5	9.20×10^{-13}	4.49×10^{-13}

Table 2.10 DK diffusion coefficient values of lactate in PU-coated RDE lactate sensor.

2.4.6 Polystyrene sulfonic acid

The thickness of one coat of 1% w/v PSSA was measured to be 1314.40 ± 299 nm ($n=6$). An average of six profiles, as shown in Figure 2.25, was calculated and the peaks at the edges were not included in the determining the average thickness. Across the membranes, a coffee-ring effect was seen after deposition. Thickness measurements were made after depositing the membranes on glass slides, however, the effect is expected to be seen on electrode surfaces as well due to the evaporation occurring faster in the middle of a droplet as opposed to at the edge where curvature is seen.^{148,149}

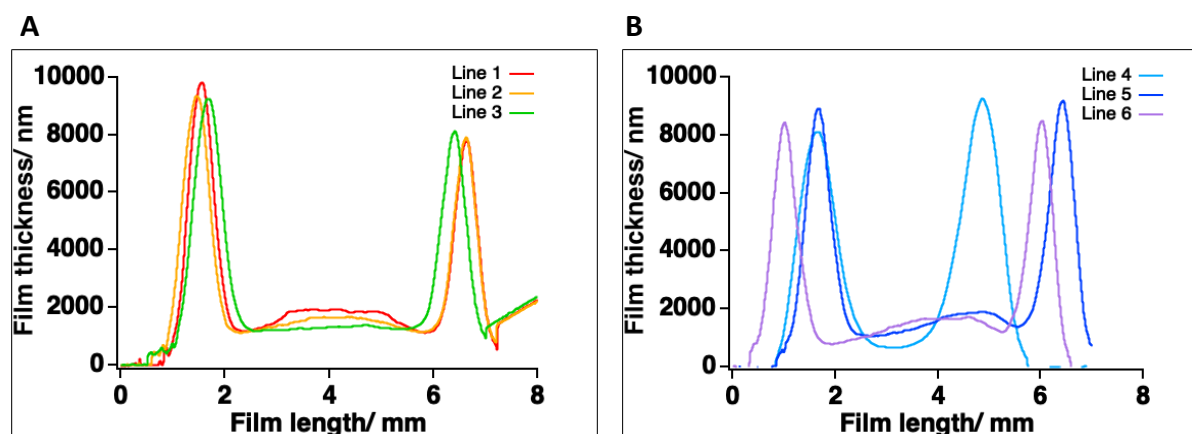


Figure 2.25 Membrane thickness profile of 1% w/v PSSA spray-coated onto a glass slide, along the (a) longitudinal and (b) lateral axis.

Koutecký–Levich analysis was also conducted with PSS-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM H_2O_2 to determine the diffusion coefficients of H_2O_2 through the film membrane. A Koutecký–Levich plot was obtained (Figure 2.26) by plotting the reciprocal current against reciprocal of the square root of angular velocity, to find the intercept. The intercept was then used in the calculation of DK to provide an estimate of the diffusion coefficient of H_2O_2 through the PSSA membrane at each concentration (Table 2.11). It was found that in 0.5 mM H_2O_2 , DK was $3.30 \times 10^{-10} \text{ m}^2\text{s}^{-1}$.

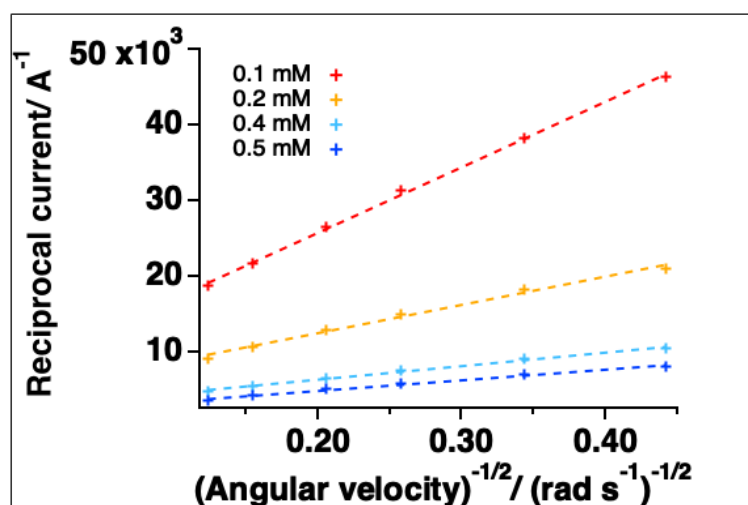


Figure 2.26 PSS HDV and Koutecký–Levich plot in 0.1, 0.2, 0.3, 0.4 and 0.5 mM H₂O₂.

[H ₂ O ₂]/ mM	[H ₂ O ₂]/ mol/m ³	DK/ m ² s ⁻¹	Propagated error/ m ² s ⁻¹
0.1	0.1	4.08 x 10 ⁻¹⁰	9.50 x 10 ⁻¹¹
0.2	0.2	3.54 x 10 ⁻¹⁰	8.67 x 10 ⁻¹¹
0.3	0.3	3.96 x 10 ⁻¹²	9.01 x 10 ⁻¹³
0.4	0.4	3.21 x 10 ⁻¹⁰	7.57 x 10 ⁻¹¹
0.5	0.5	3.30 x 10 ⁻¹⁰	8.04 x 10 ⁻¹¹

Table 2.11 DK diffusion coefficient of H₂O₂ values for PSSA-coated RDE lactate sensor.

Koutecký–Levich analysis was conducted with PSS-coated RDEs in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate, which were below K_m of the sensor. A Koutecký–Levich plot was obtained (Figure 2.27) by plotting the reciprocal current against reciprocal of the square root angular velocity, to find the intercept. The intercept was then used in the calculation of DK to provide an estimate of the diffusion coefficient of lactate through the PU membrane at each concentration (Table 2.12). It was found that in 0.5 mM lactate, DK was $3.54 \times 10^{-12} \text{ m}^2\text{s}^{-1}$.

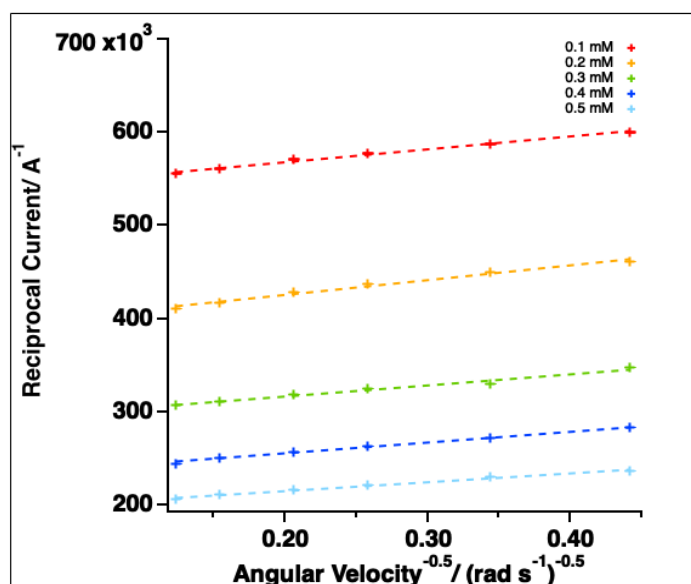


Figure 2.27 PSS HDV and Koutecký–Levich plot in 0.1, 0.2, 0.3, 0.4 and 0.5 mM lactate.

[lactate]/ mM	[lactate]/ mol m ⁻³	$DK/\text{m}^2\text{s}^{-1}$	Propagated error/ m^2s^{-1}
0.1	0.1	6.42×10^{-12}	2.03×10^{-9}
0.2	0.2	4.41×10^{-12}	7.00×10^{-10}
0.3	0.3	3.96×10^{-12}	5.19×10^{-10}
0.4	0.4	3.75×10^{-12}	6.94×10^{-10}
0.5	0.5	3.54×10^{-12}	5.14×10^{-10}

Table 2.12 DK diffusion coefficient values for lactate in the membrane of PSS-coated RDE lactate sensors.

Good linear fits were seen across the Koutecký–Levich plots for all membranes. The error associated with the DK value for mass transport of H_2O_2 is about 25% of DK in PSSA and Nafion, and about 50% of DK in PU and CA membranes, which is to be expected for handmade sensors. DK values for H_2O_2 are concentration independent. There is a concentration dependent effect on DK for the mass transport of lactate in the membranes, which was not expected as the enzyme concentration had been optimised in other studies.^{108,145,146}

2.4.7 Sensor characterisation

The RDE sensors were characterised further to develop an understanding of how DK had an impact on the effective K_m of the sensors, which would in turn impact the dynamic range (estimated to be approximately twice that of K_m). Sensors coated with 1% w/v PSS, 1% w/v Nafion, 3% w/v CA and 25 mg/mL PU were calibrated in PBS with standard additions of sodium L-lactate. Figure 2.28(a) shows the temporal response of the coated sensors and (b) shows the current response. Table 2.13 summaries K_m , V_{max} , T_{90} and DK for all sensors. V_{max} is consistent with DK values i.e. as DK increases, a rise is seen in V_{max} . As a membrane is coated onto the sensor, the sensitivity of the sensor drops as is evident by the decrease in V_{max} . Generally, as thickness of the membrane increases, K_m also increases, albeit with a loss in sensitivity (seen in V_{max}).

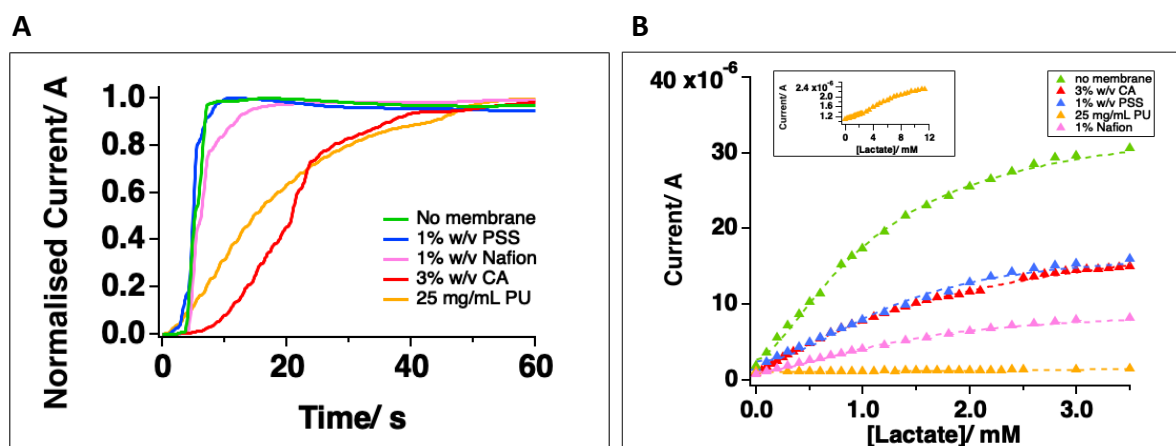


Figure 2.28 (a) Temporal response of lactate sensors without a membrane and coated with one layer of Nafion, polystyrene sulfonic acid (PSS), polyurethane (PU) and cellulose acetate (CA), (b) Current response of lactate sensors without a membrane and coated with Nafion, PSS, PU and CA.

Membrane	Charge	Thickness / nm	T_{90} / s	K_m / mM	V_{max} / μA	$DK_{lactate}$ in 0.5 mM lactate / $m^2 s^{-1}$	$DK_{H_2O_2}$ in 0.5 mM H_2O_2 / $m^2 s^{-1}$
No membrane (control)	N/A	N/A	2.7	1.09 ± 0.02	34.85 ± 0.20	N/A	N/A
1% w/v Nafion	Negative	757.30 ± 198	7.2	1.35 ± 0.03	9.31 ± 0.08	4.91×10^{-13}	1.06×10^{-11}
1% w/v PSSA	Negative	1314.40 ± 299	3.6	1.28 ± 0.04	17.21 ± 0.17	3.54×10^{-12}	3.30×10^{-10}
25 mg/mL PU	Neutral	3228.53 ± 1574	38.7	6.68 ± 0.18	2.76 ± 0.04	9.20×10^{-13}	1.25×10^{-11}
1% w/v CA	Neutral	2772.91 ± 1159	25.8	2.16 ± 0.17	23.72 ± 0.98	No steps seen in amperometry	3.36×10^{-11}

Table 2.13 Summary of sensor characteristics across different membranes.

It was decided that the sensors would be coated with 1% w/v Nafion due to the sensor's fast time response, in addition to relatively high K_m . Although PU and CA offered a higher K_m , the solvents used to dissolve these polymers were not compatible with the polycarbonate base of microneedles, therefore it was decided that Nafion was best placed for the final application of microneedle sensing *in vivo*.

2.4.8 Interference layer optimisation

To avoid interference from other electroactive molecules that would oxidise at +0.7 V applied during amperometry, an interference layer was applied before application of the enzyme hydrogel layer on microneedle sensors. M-phenyldiamine (mPD), phenol, pyrrole and o-phenyldiamine (oPD) were electropolymerized onto the platinum-black coated microneedles. The microneedles were analysed using cyclic voltammetry in 5mM ferri/ferrocyanide before and after the application of the interference exclusion layer to understand whether the layer was successful in blocking the response from the redox activity of the ferri/ferrocyanide redox couple.

mPD

Figure 2.29(a) shows CV scans of the microneedle sensor before and after electropolymerisation of mPD on its surface, with the red curve showing peaks from the oxidation and reduction of ferri/ferrocyanide at +0.36 and +0.30 V, respectively. The spikes at +0.17 V and -0.01 V are from the silver epoxy applied to the microneedle when attaching wires to the electrode arrays. To avoid exposing the silver epoxy, at least three layers of insulating lacquer are applied to the connection area, however, sometimes this may not be enough to avoid conductivity. After applying mPD to the same microneedle, hysteresis decreases and the peaks from the redox activity of ferri/ferrocyanide are no longer visible. In addition, the microneedle sensor was then functionalised with the LOD hydrogel and the sensor calibrated with standard additions of common interferents, such as acetaminophen (AM), ascorbic acid (AA) and uric acid (UA), as shown in Figure 2.29(b). The sensor responds to lactate but does not respond to AM, AA or UA. The control electrode – functionalised with

platinum-black, mPD and hydrogel without LOD enzyme – responds to neither lactate nor the interferents.

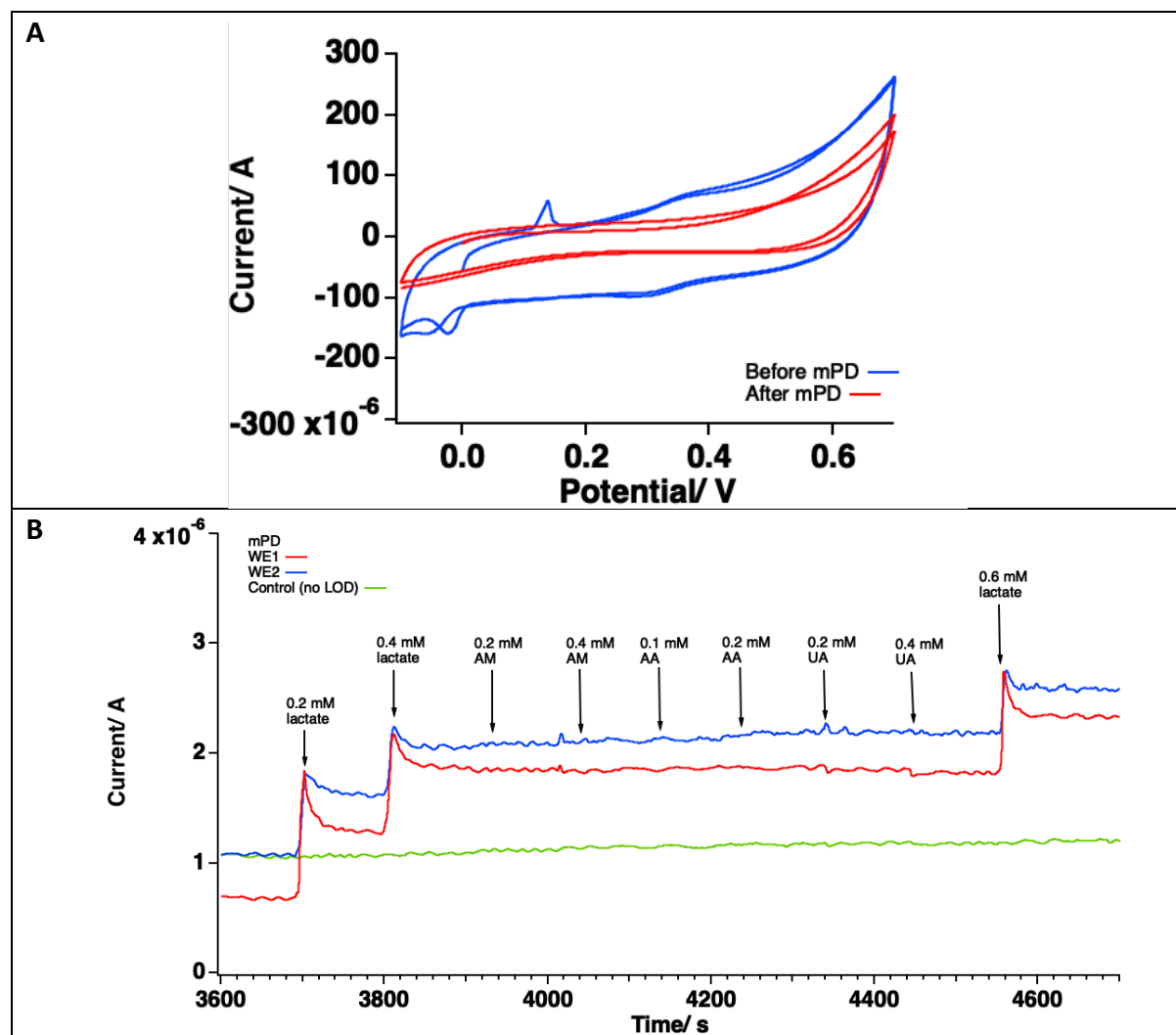


Figure 2.29 (a) Lactate sensor with an mPD interference layer tested for current response to interferents, acetaminophen, ascorbic acid, and uric acid, (b) CV in 5mM ferri/ferrocyanide solution before and after the application of mPD at a scan rate of 100 mV/s.

Phenol

Similarly, microneedles electrodeposited with phenol were cycled in 5 mM ferri/ferrocyanide solution and peaks were seen before the application of phenol at +0.36 and +0.30 V, as seen

in Figure 2.30(a). Peaks were no longer visible after the application of phenol and hysteresis had reduced.

The response to interferents was measured for microneedles (Figure 2.30(b)); it was found that the phenol-coated LOD sensors responded to both lactate and AM. The control electrode did not respond to lactate or ascorbic acid, but did respond to acetaminophen, as well as uric acid to a smaller degree.

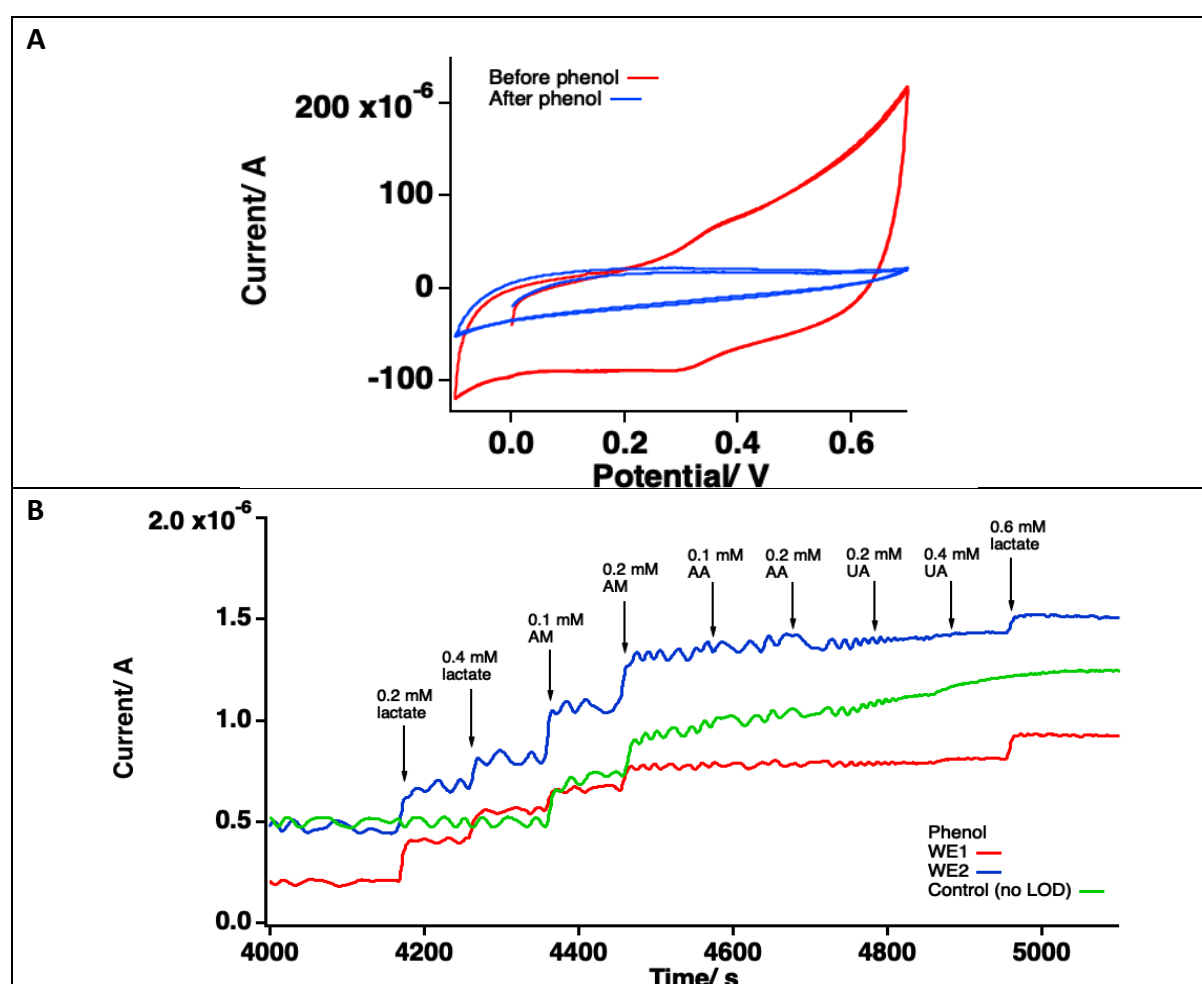


Figure 2.30 (a) Lactate sensor with a phenol interference layer tested for current response to interferents, acetaminophen, ascorbic acid, and uric acid, (b) CV in 5mM ferri/ferrocyanide solution before and after the application of phenol at a scan rate of 100 mV/s.

Pyrrole

The pyrrole-coated microneedle sensors blocked all activity at the sensor; no response was seen for additions of lactate or any of the interferents (Figure 2.31(a)). CV peaks were seen before and after electrodeposition of the pyrrole layer, meaning that the pyrrole layer was unable to block the redox activity of ferri/ferrocyanide at the electrode (Figure 2.31(b)). This was contradictory to the lack of sensor response seen towards lactate and interferents, suggesting that the layer is selectively permeable.

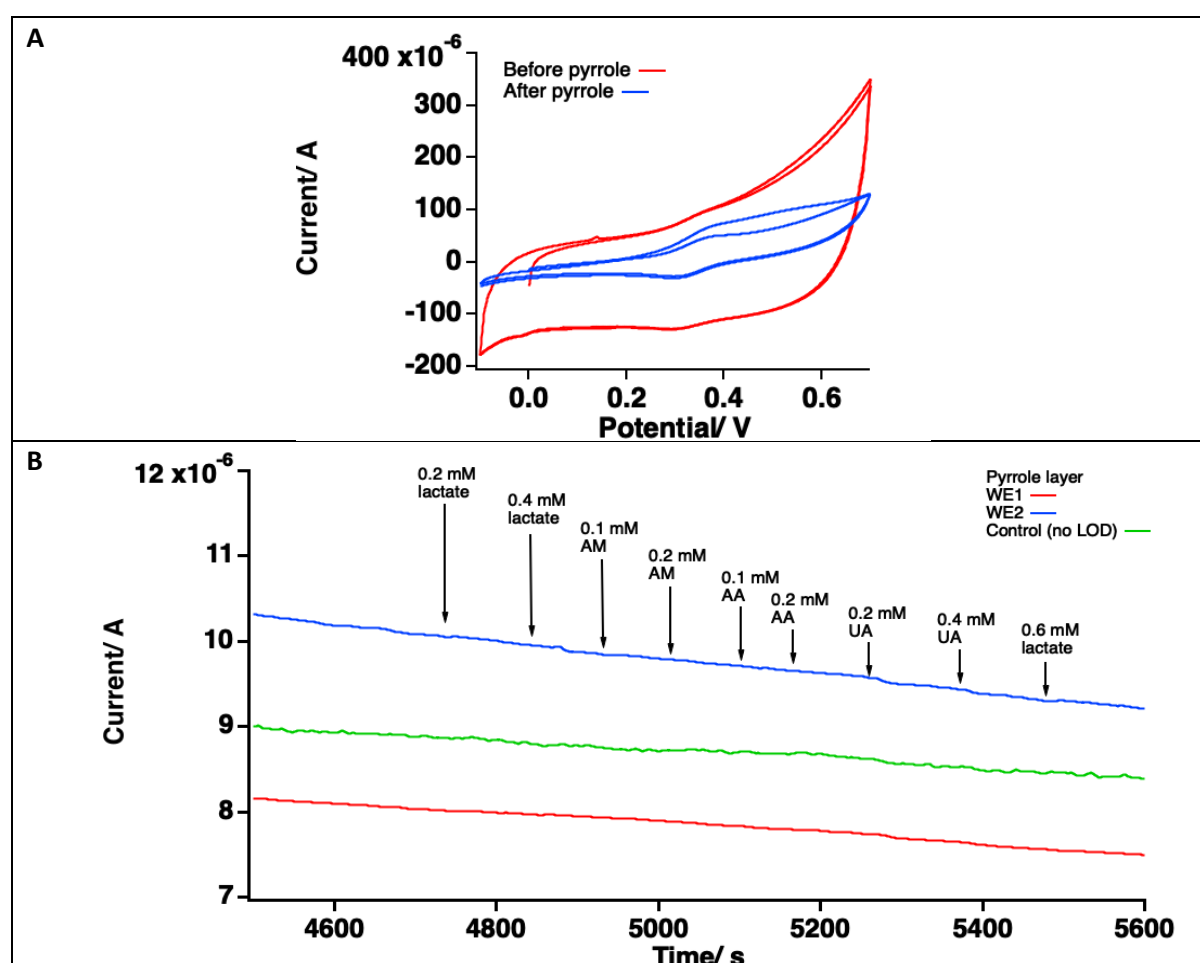


Figure 2.31 (a) Standard additions of acetaminophen (AM), ascorbic acid (AA) and uric acid (UA) to test interference with pyrrole-modified lactate oxidase microneedle sensor, (b) CV in 5mM ferri/ferrocyanide solution before and after the application of pyrrole at a scan rate of 100 mV/s.

oPD

Microneedles before functionalisation with oPD showed visible peaks at +0.36 and +0.31 V in ferri/ferrocyanide and after functionalisation, peaks were no longer visible (Figure 2.32(a)), meaning that oPD was successful at blocking the redox activity. However, the sensor still responded to acetaminophen, ascorbic acid and uric acid, as seen in Figure 2.32(b). The working electrodes responded to lactate, whereas the control electrodes did not.

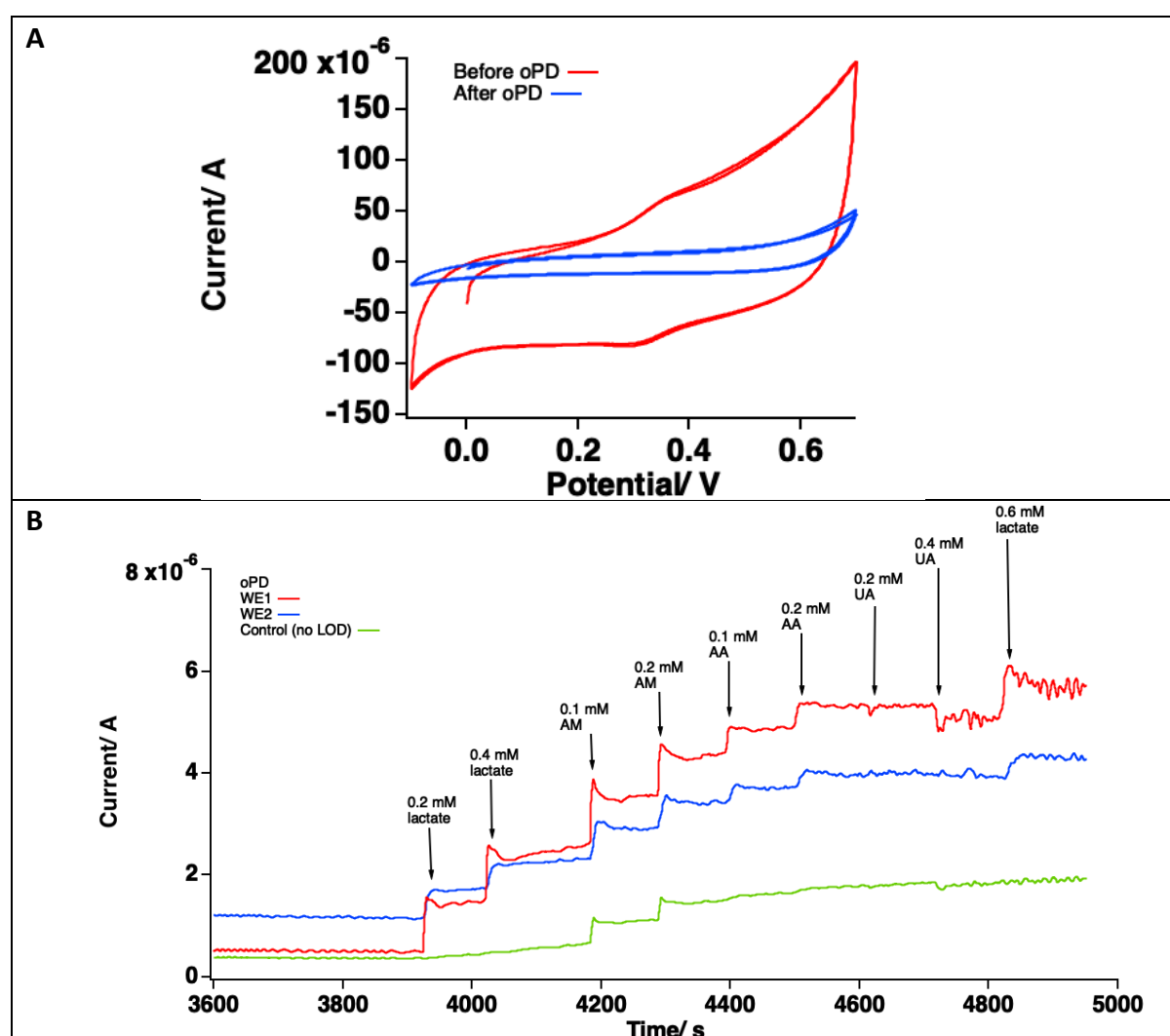


Figure 2.32 (a) Lactate sensor with an oPD interference layer tested for current response to interferents, acetaminophen, ascorbic acid, and uric acid, (b) CV in 5mM ferri-ferrocyanide solution before and after the application of oPD.

Due to the ability of mPD to block response to all the interferents tested during the optimisation process, and the loss of CV peaks in ferri/ferrocyanide after functionalisation with mPD, it was decided that microneedle sensors would be fabricated with mPD for the *in vivo* study.

2.4.9 Stability

Microneedle sensors fabricated with a Nafion diffusion-limiting membrane were calibrated on day 1 after manufacture and then stored in the freezer at $-20\text{ }^{\circ}\text{C}$ for 3 weeks. The sensor was re-calibrated on day 21 and it was seen that although there was a loss in sensitivity, the sensor was still responding to lactate over a similar dynamic range, as shown in Figure 2.33. The sensitivity of the sensor in its linear range was 34.54 nA mM^{-1} on day 1 and this decreased by 80.46% to 6.75 nA mM^{-1} on day 21.

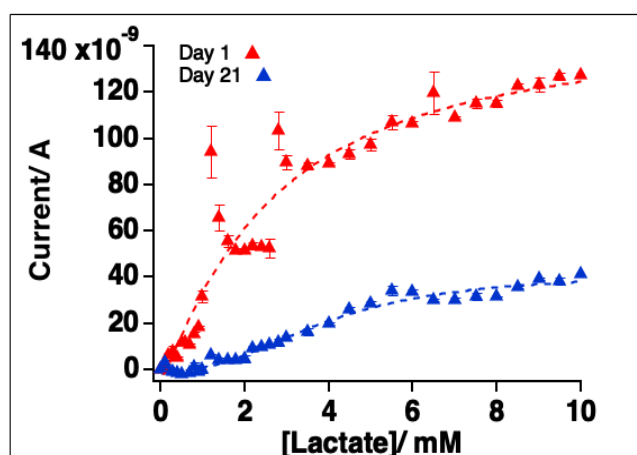


Figure 2.33 Stability of lactate microneedle sensor calibration for varying concentrations of lactate after 3 weeks. Current measured vs Ag|AgCl. RE is painted onto a silver microneedle pad and is plotted as change from baseline level. Marker Markers represent mean $\pm$ standard deviation of measurement. The calibration curve is fit with Hill equation.

2.4.10 Comparison of Sekisui LOD and DirectSens direct electron transfer (DET) enzyme

Response between sensors made with two different LOD enzymes – a recombinant enzyme from Sekisui Diagnostics (UK) and a DET enzyme from DirectSens GmbH (Austria) – was compared by conducting a Koutecký–Levich analysis of mass transport of H_2O_2 through the Nafion membrane covering the DET sensor. A potential of +0.5 V was applied at the electrode during amperometry at varying rotational speeds and a Koutecký–Levich plot was made, as shown in Figure 2.34. Using the intercept from this plot and the known thickness of 1% w/v Nafion, an estimate of the diffusion coefficient – DK – was calculated (see Table 2.14).

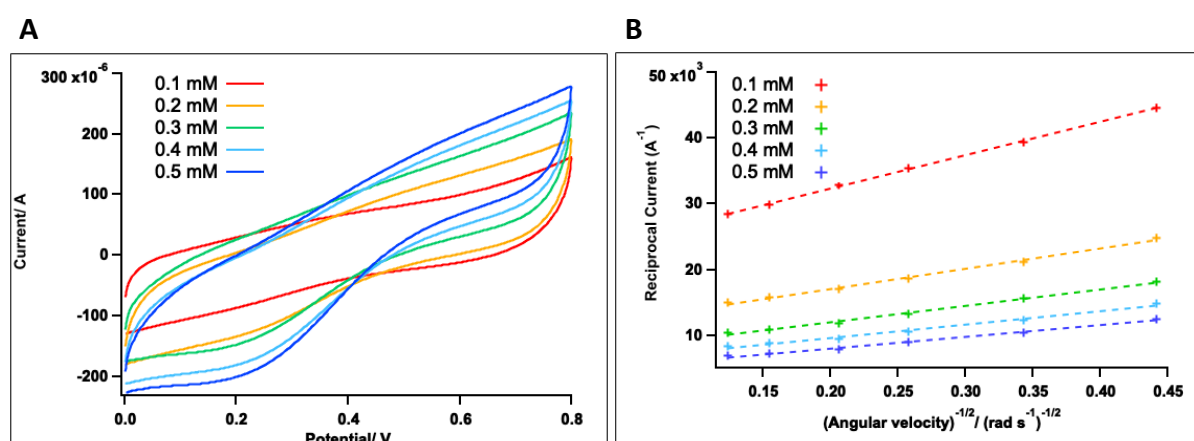


Figure 2.34 (a) HDVs and (b) Koutecký–Levich plot of DET LOD sensors coated with 1% Nafion in H_2O_2 .

$[\text{H}_2\text{O}_2]/\text{mM}$	$[\text{H}_2\text{O}_2]/\text{mol m}^{-3}$	$DK/\text{m}^2\text{s}^{-1}$	Propagated error/ m^2s^{-1}
0.1	0.1	1.49×10^{-11}	3.91×10^{-12}
0.2	0.2	1.52×10^{-11}	4.00×10^{-12}
0.3	0.3	1.58×10^{-11}	4.19×10^{-12}
0.4	0.4	1.48×10^{-11}	3.95×10^{-12}
0.5	0.5	1.48×10^{-11}	3.94×10^{-12}

Table 2.14 Diffusion coefficient (DK) of H_2O_2 values for Nafion-coated RDE DirectSens N137 lactate sensor.

The DK values of H_2O_2 through the diffusion-limiting Nafion membrane were compared with DK values of the same through the Nafion membrane on recombinant LOD sensors (Figure

2.35). It was found that diffusion of H_2O_2 is slightly faster in the Nafion membrane covering the DET sensor compared to the Sekisui LOD sensor. The diffusion coefficient stays relatively the same across lactate concentrations in the Nafion membrane covering the DET LOD sensor, whereas a decrease is seen in DK as the lactate concentration increases for the Sekisui LOD sensors. Based on the lower membrane DK covering the Sekisui LOD sensors, it was decided that the Sekisui LOD enzyme would be used for future studies.

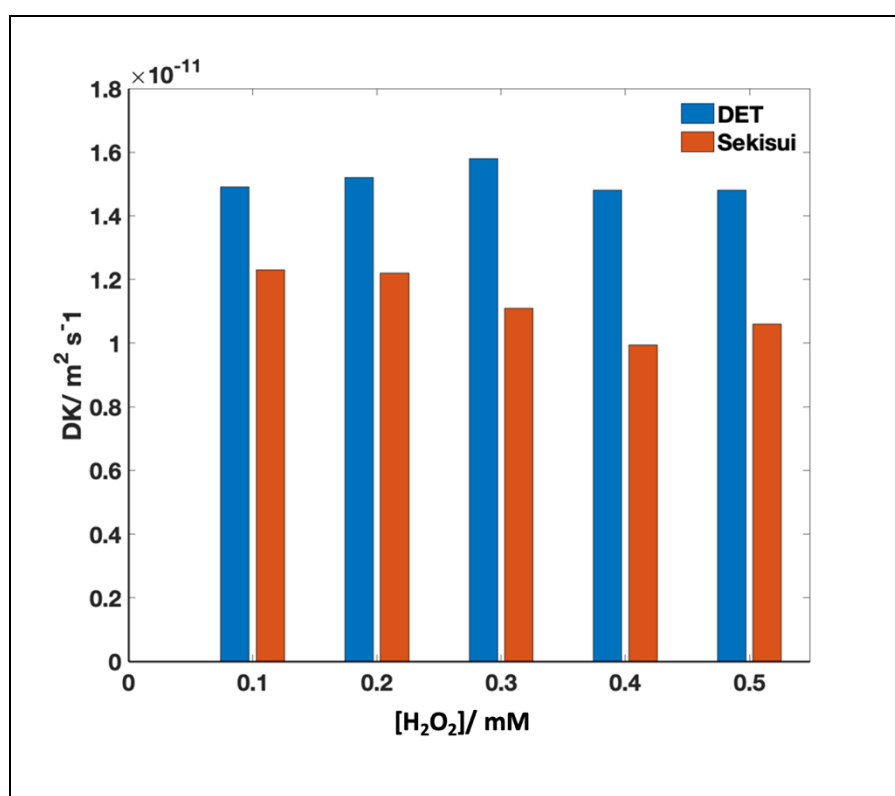


Figure 2.35 A comparison of H_2O_2 diffusivity in the Nafion membrane on DET and Sekisui LOD sensors.

2.4.11 Optimising K_m with the number of coats of Nafion applied to the microneedle sensor

To optimise K_m in order to meet the dynamic range requirement of measuring lactate in physiological concentrations, the number of coats of 1% w/v Nafion, the chosen diffusion limiting membrane, was increased from 0 (no membrane) to 3 coats. Calibration curves of

each sensor were plotted and fit with the Hill equation (Figure 2.36) to deduce V_{max} , K_m , and sensitivity (Table 2.16).

It was found that as the number of coats of Nafion were increased, the effective K_m of the sensors increased, however, the sensitivity decreased. This is expected as increasing the number of coats increases the thickness of the diffusion-limiting membrane and this limits the mass transport of lactate to the electrode, further limiting the signal response. A balance between the obtaining the required dynamic range for *in vivo* testing, and an optimal sensitivity needs to be reached. To maximise the dynamic range, the strategy of spray-coating three times with 1% w/v Nafion was chosen.

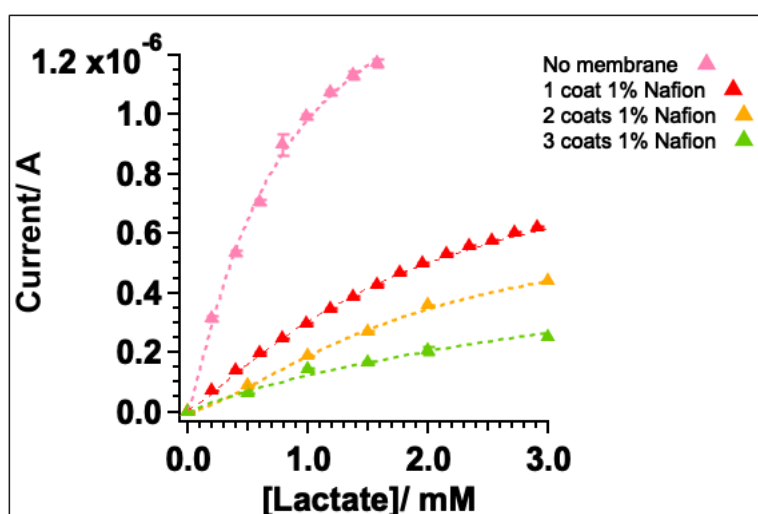


Figure 2.36 Calibration curves of microneedle LOD biosensors coated with 0, 1, 2 and 3 coats of 1% w/v Nafion. Curves are fitted with the Hill equation. Error bars represent $\pm$ standard deviation.

Number of coats	V_{max}/ A	K_m/ mM	Sensitivity/ $\mu A\ mM^{-1}$
0	$(1.67 \pm 0.15) \times 10^{-6}$	0.73 ± 0.13	1.43
1	$(9.77 \pm 0.49) \times 10^{-7}$	1.94 ± 0.16	0.25
2	$(6.60 \pm 1.04) \times 10^{-7}$	1.87 ± 0.46	0.19
3	$(8.49 \pm 2.26) \times 10^{-7}$	7.10 ± 4.18	0.12

Table 2.15 Hill equation fit parameters of microneedle biosensors coated with 0, 1, 2 and 3 coats of 1% w/v Nafion. Sensitivity is reported for the linear range of the sensors.

2.4.12 Continuous *in vivo* measurements of lactate in healthy volunteers

Lactate measurements were taken from five healthy volunteers at the Imperial College Clinical Research Facility at Hammersmith hospital under the ‘Minimally Invasive Realtime Assessment of Continuous Lactate in Exercise (MIRACLE)’ study, REC reference: 20/LO/0364 IRAS project ID: 274161. For all participants, venous blood lactate measurements were taken as gold standard measurements and analysed by photometric methods using the Architect Ci8200 serum analyser platform (Abbott, USA). For the fifth participant, microdialysis was conducted and lactate was measured in the dialysate, in addition to venous blood lactate, for comparison with lactate concentrations in ISF. Figure 2.37 demonstrates the set-up used for the *in vivo* measurements, wherein microneedle sensors were applied to the forearm of healthy volunteers on an exercise bicycle using medical-grade tape and a tourniquet to equalise pressure. A constant potential of +0.7 V was applied for amperometric measurements via the connected CHI potentiostat. The sensors were allowed to stabilise *in vivo* for an hour before starting a 15 min rest period, following which a period of cycling was started for the duration of 15 min. After cycling was stopped, the participant was asked to

remain on the bicycle for 30 min as a final rest period. Table 2.16 summarises the participants involved in the MIRACLE study.

Participant	1	2	3	4	5
Age group	30-34	20-24	25-29	30-34	30-34
Gender	Male	Male	Female	Male	Male
Baseline venous blood lactate (mmol/L)	1.52	1.96	1.23	1.40	1.07
Maximum venous blood lactate (mmol/L)	10.71	13.03	6.73	9.25	5.03
Venous blood lactate at end of rest (mmol/L)	2.90	4.71	2.06	2.41	1.56

Table 2.16 Participants recruited in the MIRACLE study.

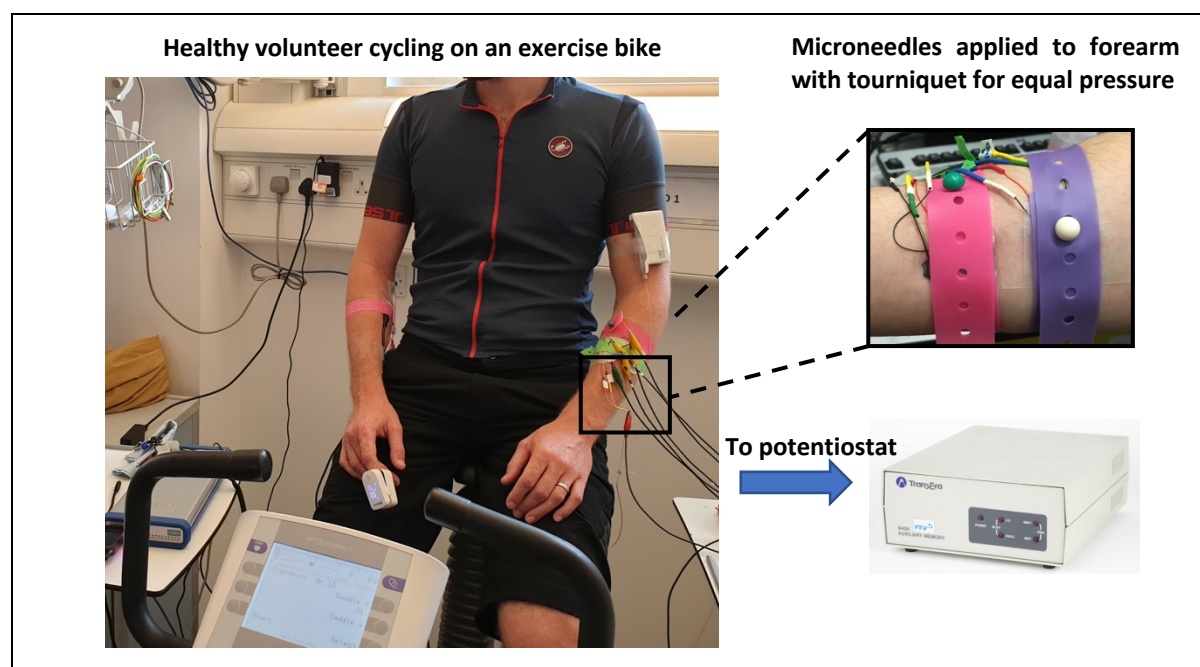


Figure 2.37 The set-up of the in vivo studies with microneedle sensors attached to volunteer's forearm and connected to an external potentiostat.

2.4.13 Biosensor response to lactate during exercise and rest

Biosensor response was measured amperometrically at +0.7 V vs Ag|AgCl for all five participants (Figure 2.38). Venous blood measurements were taken approximately every 10 mins and microdialysis was conducted on participant 5 to collect dialysate for local lactate measurement. Biosensors were calibrated *in vitro* after use and ISF lactate concentrations were derived from microneedle current using Hill equation fit parameters.

After starting exercise, lactate concentrations increased in ISF and blood across all participants, as well in dialysate for participant 5. Lactate concentration was seen to rise earlier in blood compared to ISF, with a time lag of between 8 to 11 min between ISF and blood. The exception to this was seen for participant 5, where a sharp rise in microneedle current is observed before lactate concentrations in blood begin to rise, which may be explained by localised generation of lactate in skin, causing a rise in ISF lactate before blood, as reported by Ming *et al.* in their *in vivo* studies.¹⁵⁰

Lactate concentrations as high as 13.03 mM were seen in blood during strenuous cycling (participant 2) and the lowest concentration of lactate during exercise was 5.03 mM (participant 5). In ISF, lactate concentrations ranged from 0.01 mM (participant 4) to 3 mM (participant 3). Almost all participants' lactate levels returned to the concentration measured at rest before exercise, if not close to baseline, nearing the end of the study time. At rest, lactate concentrations in blood ranged from 1.07 (participant 5) to 1.96 mM (participant 2).

When exercise was stopped, lactate concentrations in both blood and ISF were seen to be decreasing. In participants 1, 2 and 5, lactate clearance in ISF starts before it does in blood, as seen by the start of when the concentration starts falling, indicating that lactate clearance in ISF is happening at a faster rate than clearance in blood. This can be explained by clearance of lactate in local tissues surrounding the ISF, which means that the lactate could be reabsorbed by cells in the epidermis for clearance via the Cori cycle.

Dialysate lactate measured from participant 5 was also seen to rise and fall in line with blood and ISF lactate levels, however the change in lactate concentrations across the study was less than that seen in ISF and blood. Lactate concentrations in the dialysate ranges from 0.1 to 0.25 mM (excluding artefact seen at approximate 8600 s).

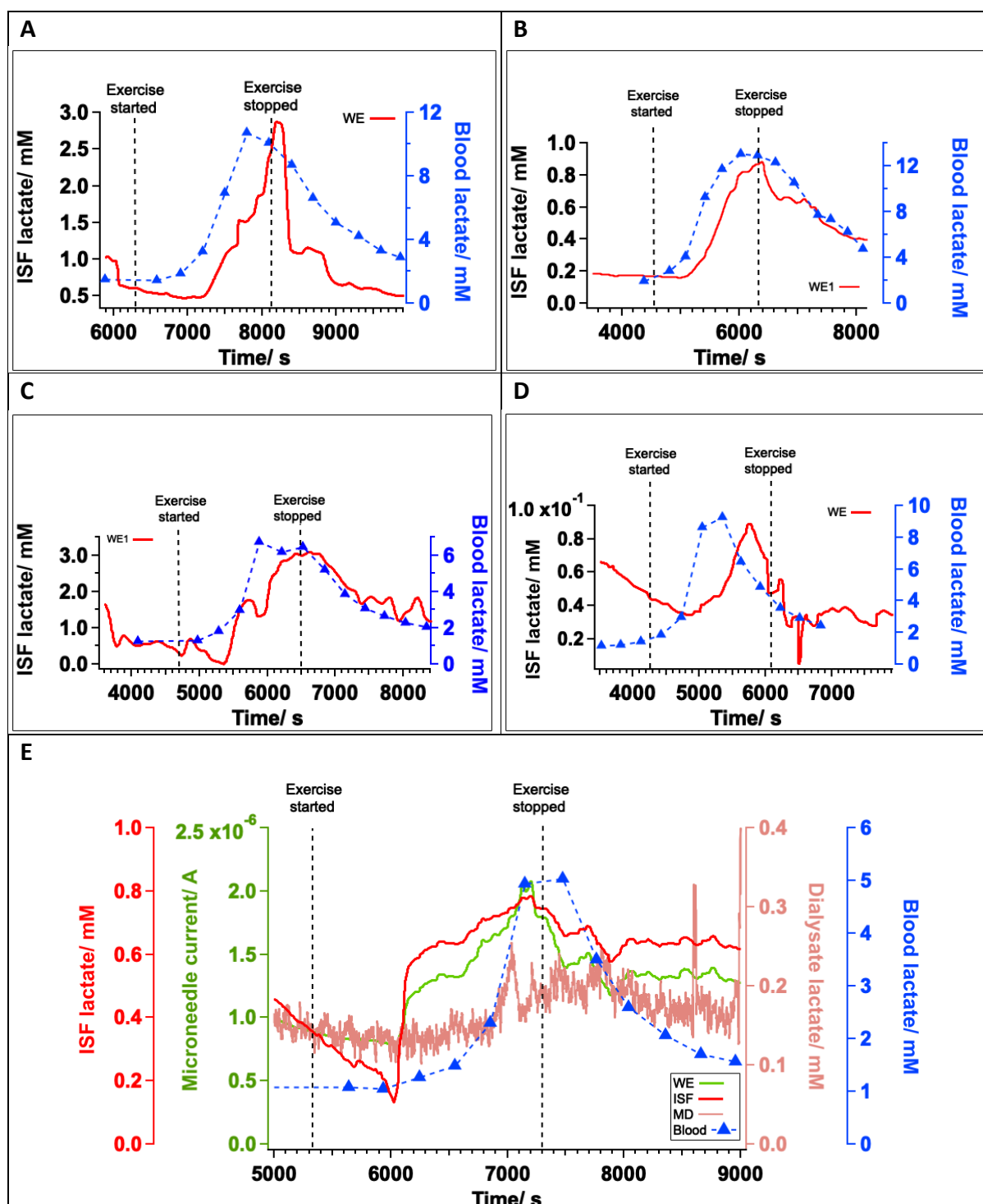


Figure 2.38 ISF (biosensor) and venous lactate vs time for (a) participant 1, (b) participant 2, (c) participant 3, (d) participant 4 and ISF, venous and dialysate lactate vs time for (e) participant 5. Microneedle current was measured at +0.7 V vs Ag|AgCl.

2.4.14 Calibration of microneedle biosensors *in vitro*

The LOD microneedle sensors used on the participants during the *in vivo* study were calibrated post-use with standard additions of sodium L-lactate. Post-use calibration data was collected as these parameters most accurately represent the *in vivo* quantitative performance. Figure 2.39 shows the dose-response curves of the five sensors (participant 1 to 5) fitted with the Hill equation. Table 2.17 summarises the V_{max} and effective K_m values derived from the fit.

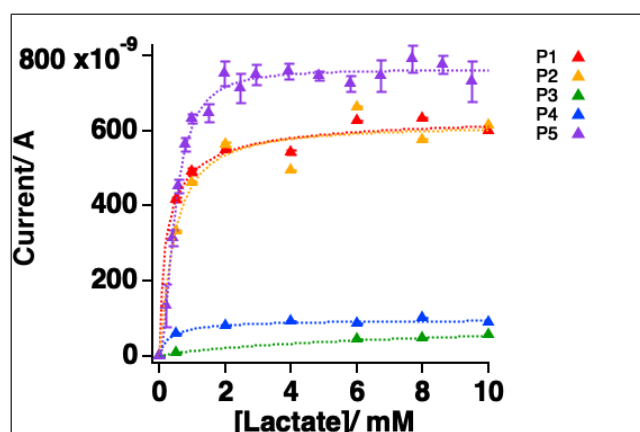


Figure 2.39 Post-use calibration curves of microneedle sensors used on participants (P1 to P5) during the *in vivo* studies. Curves are fitted with the Hill equation and error bars represent $\pm$ standard deviation.

Participant	V_{max}/A	K_m/mM
1	$(6.45 \pm 0.52) \times 10^{-7}$	0.23 ± 0.08
2	$(6.15 \pm 0.59) \times 10^{-7}$	0.43 ± 0.15
3	$(1.33 \pm 1.59) \times 10^{-7}$	16.24 ± 43.50
4	$(1.00 \pm 6.19) \times 10^{-7}$	7.15 ± 508
5	$(7.64 \pm 0.09) \times 10^{-7}$	0.48 ± 0.03

Table 2.17 V_{max} and K_m parameters of LOD microneedle sensors used in the *in vivo* studies.

The K_m of the microneedle sensors were lower than expected; higher K_m values were observed for RDE sensors used during the optimisation process. The K_m values for participants 4 and 5 were the highest out of the five sensors, however they had large very standard deviations. The V_{max} values were in the same order of magnitude across sensors, however, participants 3 and 4 had lower V_{max} values in comparison, indicating the loss of sensitivity which may have been caused by the Nafion membrane restricting mass transport to the electrode.

2.4.15 Comparing lactate concentrations between ISF, blood and dialysate

To understand the degree of agreement between lactate concentrations derived from the three methods used in the *in vivo* study i.e., biosensor (ISF), microdialysis (dialysate) and venous blood, Bland-Altman plots of agreement were plotted for lactate concentrations at rest and during exercise (Figure 2.40).

It was found that there was a systematic bias between lactate concentrations in blood vs ISF; as mean [lactate] increased, the difference between the two methods was also seen to increase across both the rest and exercise periods (as shown in Figure 2.40 (a) and (b)). The effect of increasing difference between measured concentrations was greater during the rest period. The mean difference between the two methods is not close to zero, meaning that the two methods are producing different measurements, however, there is some agreement as all points on the Bland-Altman plot lie within the 95% confidence intervals from the mean difference. The trend is that as [lactate] rises, the difference between sensor response (ISF) and venous blood becomes greater, meaning that either (i) the sensor is unable to detect

higher lactate concentrations or (ii) the concentration of lactate in the ISF compartment is proportionally lower than in blood.

A similar effect was seen in the Bland-Altman plot of blood vs dialysate at rest and during exercise (Figure 2.40(b) and (c)), however there are fewer data points to draw conclusions from. Compared to the mean difference between blood and ISF, the mean difference between blood and dialysate is closer to zero, particularly during the exercise period. All data points lie within the 95% confidence limits, indicating agreement between the two methods.

During exercise, the mean difference between ISF and dialysate lactate concentrations is almost zero, indicating that the two methods are producing similar results (Figure 2.40 (d) and (e)). At rest, the mean difference is similar to that observed between blood and dialysate at rest. Some random scatter of data points is observed at lower mean lactate concentrations between ISF and dialysate at rest, whereas during exercise, the same trend of proportionality between mean lactate concentrations and difference can be observed, albeit with fewer data points. This indicates that there is some systematic bias between the methods during exercise as higher lactate concentrations are measured. However, it is worth noting that during exercise, the mean difference is closest to zero and the confidence limits are the narrowest out of all comparisons, indicating that there ISF and dialysate concentrations are similar.

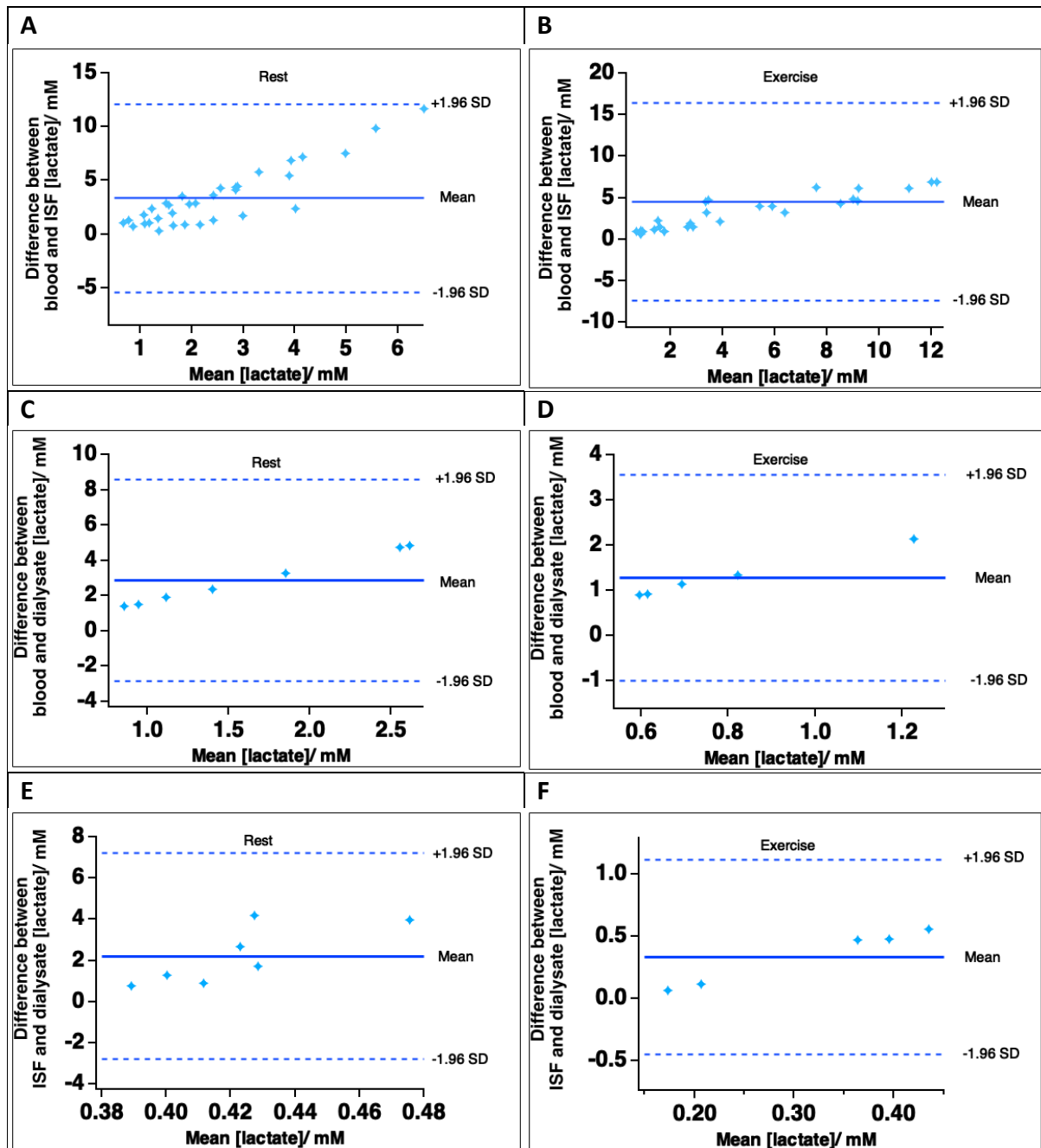


Figure 2.40 Bland-Altman plots of agreement between lactate concentrations in (a) blood and ISF at rest, (b) blood and ISF during exercise, (c) blood and dialysate at rest, (d) blood and dialysate during exercise, (e) ISF and dialysate at rest, and (f) ISF and dialysate during exercise.

To compare the ratio of lactate concentrations between the three methods, plots of the ratio of concentrations in blood vs ISF, ISF vs dialysate, and blood vs dialysate over time were made for each participant (Figure 2.41). Ratios of blood:ISF lactate concentration varied across the

participants, with similar ratios seen only between participants 1, 3 and 5 (Figure 2.41(a) and (c)). For these participants, the average blood:ISF ratio of lactate concentrations was 3.9 at rest and 6.4 during exercise. For participant 3, an anomaly was observed at $t = 5400s$, where a blood:ISF ratio of 37.6 was observed, which can be attributed to the microneedle current dropping too low after baseline correction of the raw data.

For participants 2 and 4, blood:ISF ratios were much higher than for participants 1, 3 and 5. Ratios were in the range of 11.4 and 31.0 for participant 2, and 3.3 and 170.2 for participant 4. The large range of ratios for participant 4 can be attributed to the fact that a rise in blood lactate is seen 10 mins before the rise in ISF appears. A time lag of 10 mins is reported between blood and ISF so this is expected,^{106,151} however, a much a lower time lag is observed for other participants.

On average across the study time, the concentration of lactate in blood is 3.75-fold higher than that in ISF (biosensor) and 12.80-fold higher than that in dialysate for participant 5. The ratios tend to increase for both blood:ISF and blood:dialysate at around the same time at $\sim 6500s$ for participant 5 (exercise started at 5325 s), and tend to fall around $\sim 7500s$ (exercise stopped at 7300 s), as shown in Figure 2.41 (c), which shows that the microneedle current, and in turn, the biosensor response to lactate does not increase proportionally with lactate concentrations in blood. The same is seen for dialysate with higher ratios compared to blood:ISF. The ratio of biosensor response to microdialysis response (ISF:dialysate) shown in Figure 2.41 (d) for participant 5, stays roughly the same after $t = 6250s$, with ratios in the range of 3.0 to 4.7, which indicates that the clearance of lactate after stopping exercise is not as steep as that observed in blood. This is evidenced by the lack of steeper decrease in

microneedle current in Figure 2.42(e) for participant 5, where the ISF lactate concentration does not return to its original levels before exercise. The difference in ratios for ISF:dialysate concentrations between rest (1.60) and exercise (3.86) for participant 5 indicate that lactate concentrations are in better agreement at lower concentrations at rest.

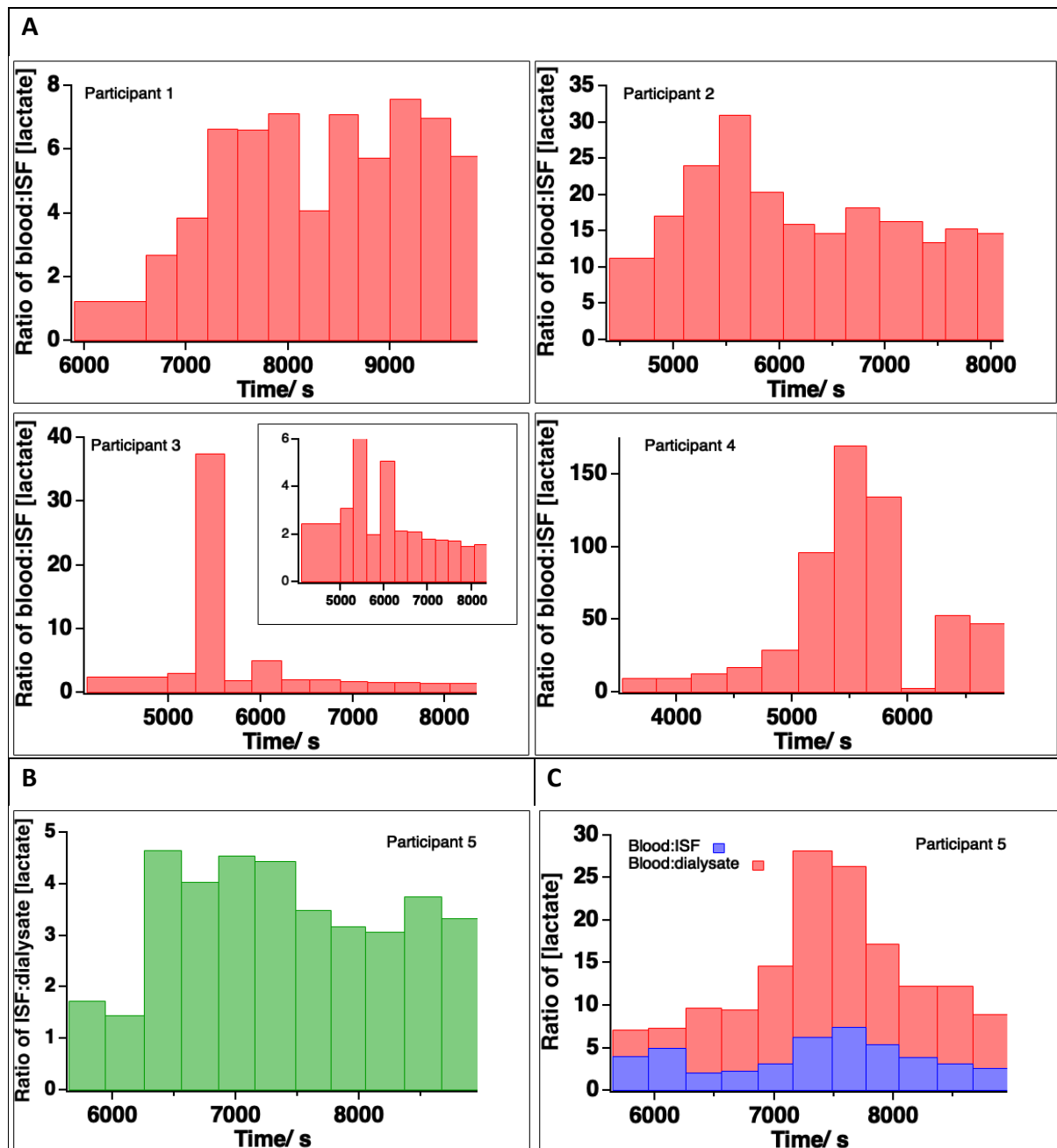


Figure 2.41 Ratio of lactate concentrations in (a) venous blood vs ISF across the first four participants, (b) ISF vs dialysate (participant 5), and (c) venous blood vs ISF and dialysate (participant 5).

2.4.16 Interference during *in vivo* studies

Working electrodes respond to interferents – acetaminophen (AM), uric acid (UA) and ascorbic acid (AA) – during the *in vitro* the calibration of microneedles. However, the step change to an interferent is smaller than the step change to lactate (as shown in Figure 2.42).

In the calibration, it can be seen that the control electrode responded to AM, UA, and AA, however, it did not respond to lactate. The working electrodes responded to both lactate and the interferents (AA, AM, and UA). The ratios of the current responses of AM:lactate, AA:lactate, and UA:lactate were 0.49 ± 0.10 , 0.54 ± 0.09 and 0.71 ± 0.12 from an average of responses of three microneedle sensors (± 1 SD). On average, the response to interferents made up 60% of the sensor's response to lactate for each 0.2 mM step change.

To overcome the overestimation of lactate concentrations as a result of measuring response to interferents, the control electrode could be used as the sentinel electrode in future *in vivo* studies. In the *in vivo* studies conducted herein, the control electrode consisted of gold microneedles, functionalised with the same recipe as the working electrodes, without the LOD enzyme and Nafion membrane.

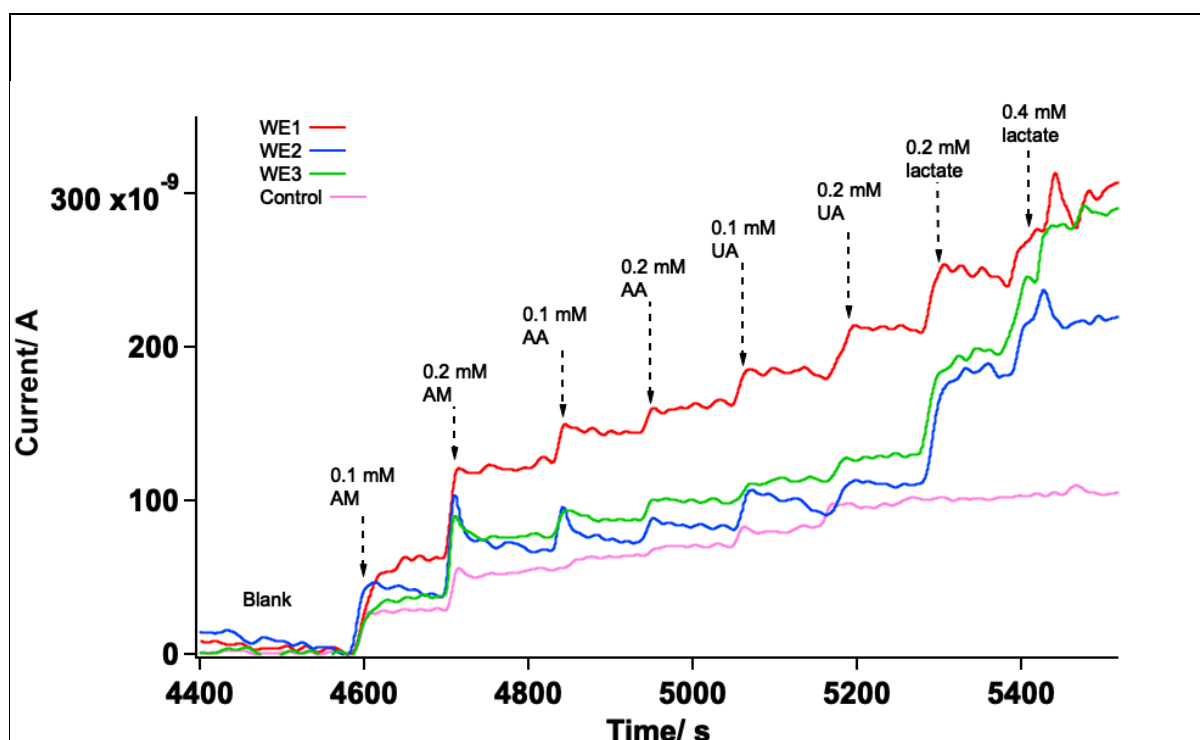


Figure 2.42 Interference calibration of microneedle sensors used in the *in vivo* studies with standard additions of acetaminophen (AM), ascorbic acid (AA), uric acid (UA) and lactate.

2.4.17 Implantation of microneedles into the epidermis

In the *in vivo* studies, functionalised microneedles were pushed into the skin of the forearm using thumb pressure for 60s, following which a tourniquet was applied over the back of the microneedle. Figure 2.43(a) shows an image of a healthy volunteer's forearm 1 minute after the microneedle array has been removed, with indentations in the skin capturing where the microneedles were during the study. Figure 2.43(b) shows the surface topography of the skin after removal of the microneedles, showing indentations from an individual pad of 16 microneedles. To understand how deeply the microneedles penetrate the skin, optical coherence tomography (OCT) images were captured of non-metallized polycarbonate microneedles applied to the skin. The microneedle arrays were non-metallized to allow light to enter from the back of the array. Microneedles were pushed into the skin on application of thumb pressure and images captured before and after the application, as shown in Figure

2.43(c). The length of each microneedle is 1 mm and the base of the microneedle is approximately 0.3 mm. Figure 2.43(b) shows the full length of the microneedle (1.28 mm) penetrating the skin.

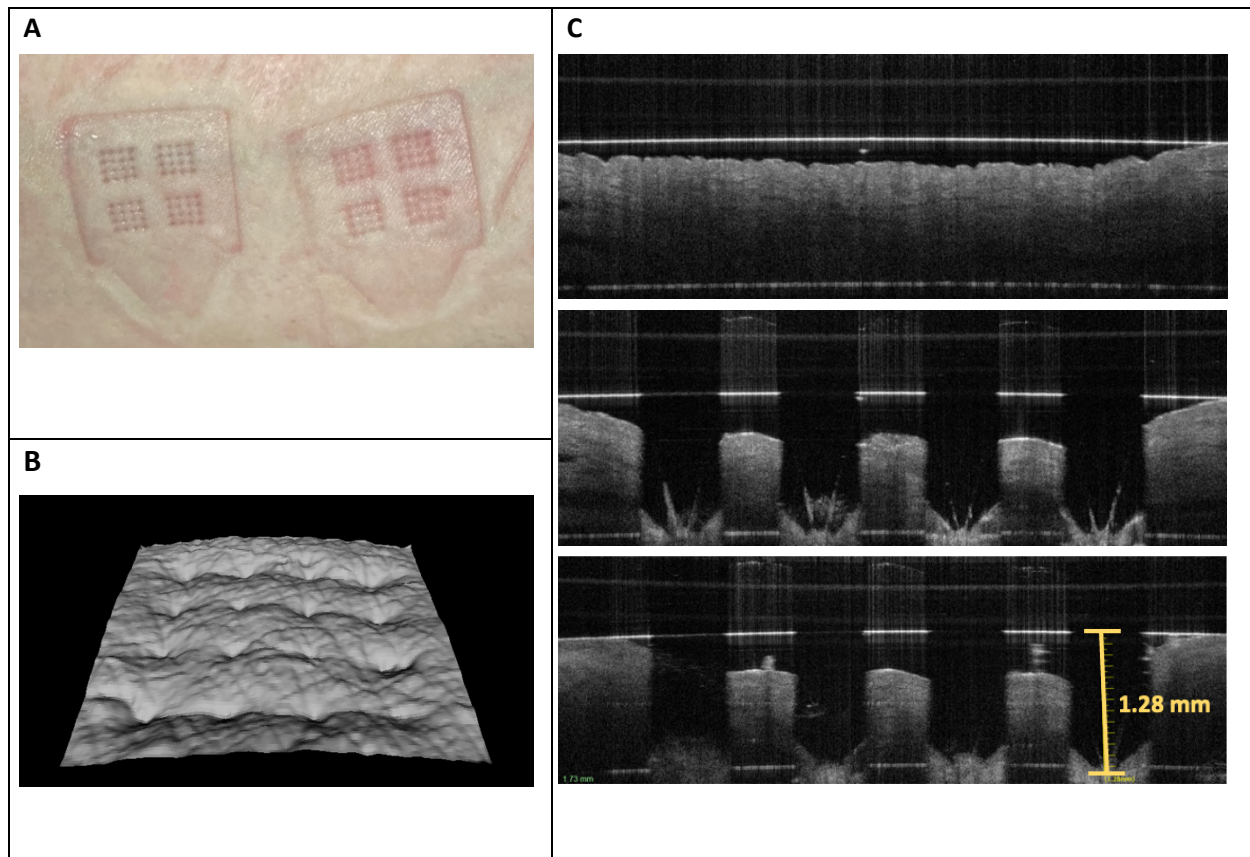


Figure 2.43 (a) Photo and (b) surface topography image of a participant's forearm taken 1 minute after removal of implanted microneedles in the skin, and (c) OCT images of a participant's epidermis before and after implantation of microneedles.

In addition, to compare the penetration of microneedles in the skin between thumb pressure application and using a mechanical applicator, as is available for commercialised sensors, the microneedles were applied under both conditions with trypan blue dye applied to the ends

of the needles. Figure 2.44 shows that applying thumb pressure for 10 seconds results in indentations in the skin, whereas using an applicator punctures the skin. The images taken after holding down the microneedle for 10 s and 10 min after application suggest that there might not be a need to hold down the microneedle if a mechanical applicator is used.

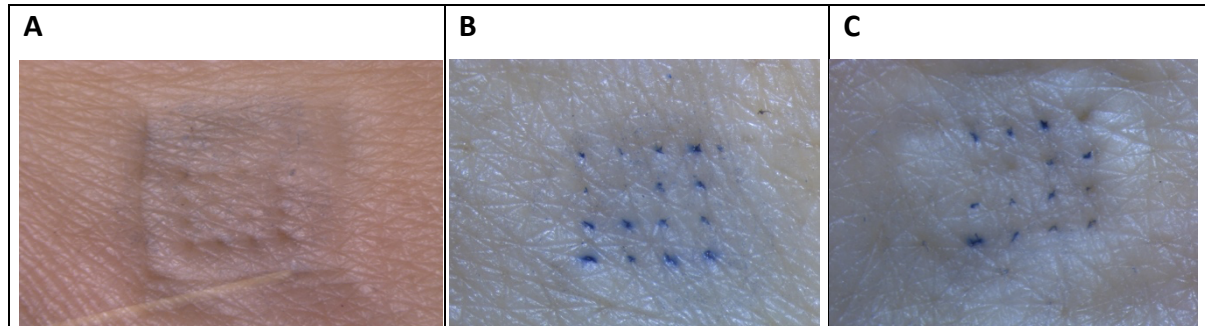


Figure 2.44 Microneedle applied using (a) thumb pressure and using an applicator, with images taken after (b) 10s and (c) 10 min of being held onto the skin after application (unpublished work used with permission, by I. Steer, Department of Bioengineering, Imperial College London, personal communication).

2.4.18 Impedance on application of microneedles *in vivo*

To understand whether the microneedles were accessing ISF, impedance was measured with respect to time, at a frequency of 962 Hz, as functionalised microneedles were placed on skin without any application of pressure, after which thumb pressure was applied, followed by the application of a tourniquet on the back of the microneedles, as conducted for the *in vivo* studies. Figure 2.45(a) shows that the impedance decreases after applying thumb pressure, and further decreases and stabilises on application of the tourniquet. There is a rapid drop in impedance going from “on skin” to “thumb pressure” which can be explained by the microneedles going from air into skin, thereby increasing the conductivity of the microneedle

electrodes in a fluid medium, such as ISF. There is a 1.5-fold decrease in impedance when the tourniquet is applied to the back of the microneedles, meaning that the microneedles have been pushed into the skin further, and have access to more ISF. Impedance was also measured for microneedles in PBS, where the impedance was much lower, as is expected in a liquid medium; the impedance was almost 500-fold lower in PBS compared to tourniquet-applied microneedle in skin (ratio of impedance in skin:PBS was 470:1). Figure 2.45 (b) shows impedance of the microneedle stabilising in PBS.

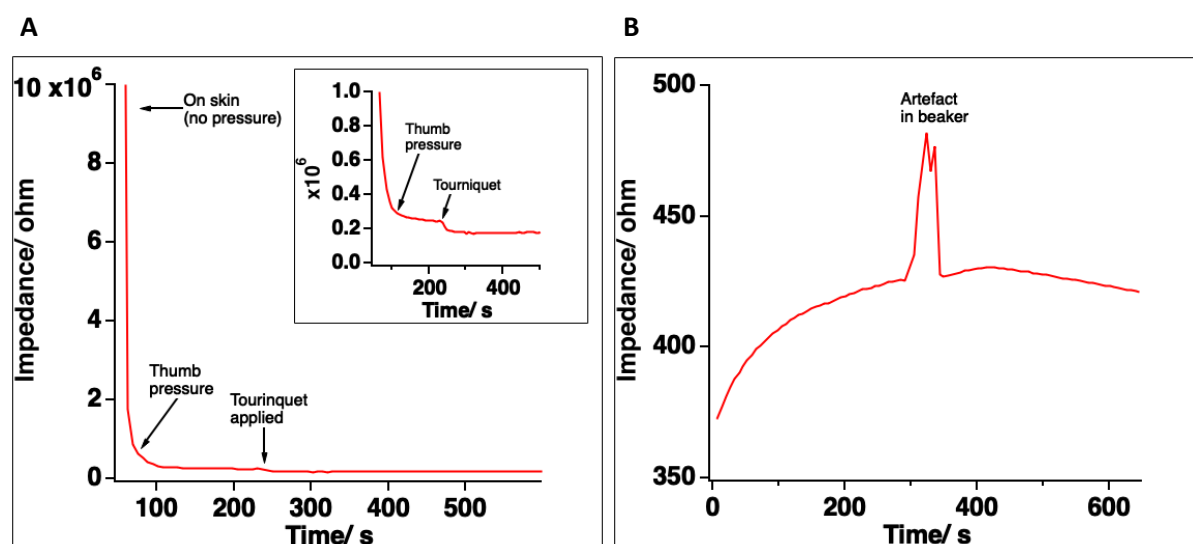


Figure 2.45 (a) Impedance of microneedle sensors when being pushed into skin and (b) impedance of microneedle sensors in PBS by comparison.

2.5 Discussion

2.5.1 Choice of Nafion as a diffusion-limiting membrane

The highest K_m achieved for the lactate sensors was 6.68 ± 0.175 mM, achieved by coating the sensors with 25 mg/mL PU. However, for the *in vivo* study, we decided not to use a PU coating due to the need for it to be dissolved in tetrahydrofuran (THF), which would dissolve

the polycarbonate base of the microneedles, causing the plastic to leach onto the sensor's surface which may cause fouling. In addition, THF has shown to cause skin irritation due to its moderate toxicity.¹⁵² The second highest K_m achieved amongst the membranes – 2.15 ± 0.17 mM – was achieved by coating the sensors with 2.5% w/v cellulose acetate. Despite CA being a good candidate as a sensor coating due to its biocompatibility, the film was mechanically unstable on the sensors and was seen to be peeling off after drying and during RDE experiments. In addition, acetone was used to dissolve the CA which would dissolve the outer layers of the polycarbonate base of the microneedles and cause the leaching of plastic.

Therefore, it was decided that the sensors would be coated with 1% w/v Nafion as this achieved the third highest K_m out of the studied membranes – 1.35 ± 0.03 mM – with a dynamic range of ~ 4 mM that would be suitable to cover the physiological threshold above which sepsis is detected. Nafion was diluted to 1% w/v in 100% ethanol which was compatible with the polycarbonate base of the microneedles. To improve the K_m of the sensor further, the number of coats of 1% w/v Nafion applied to the sensor were optimised. It was found that 3 coats of Nafion achieved the highest K_m of 7.10 ± 4.18 mM. To further expand this, it was decided that 5 coats of 1% w/v Nafion would be applied to the microneedle sensors for *in vivo* testing due to the proportional relationship seen between the number of coats and K_m . The effective K_m values achieved for the LOD sensors used *in vivo* were comparable to those reported in literature for first generation biosensors (0.79 to 1.75 mM in literature),^{128,129,131,132} but were lower than the effective K_m (19.75 ± 0.70 mM) reported by Freeman *et al.* for their third generation DET LOD biosensors.¹⁰⁶

The reported sensitivity of the Nafion sensor coated with 3 coats of 1% w/v Nafion ($0.12 \mu\text{A mM}^{-1}$) was lower than the sensitivity of LOD biosensors reported in literature, highlighting the need to explore ways to maintain sensitivity while the dynamic range is increased. The reported sensor had a faster T_{90} response time (7.2 s) compared to similar sensors reported in literature (35 to 194 s in literature),^{126–129,133} which is advantageous due to the need for continuous measurement of the changes in lactate concentrations *in vivo*.

2.5.2 Diffusion of lactate and H_2O_2 through the diffusion-limiting membrane

The purpose of using an estimated diffusion coefficient in this thesis, reported as DK , was to understand which membranes were providing the most resistance to the mass transport of lactate through the membrane, to aid in the decision of choosing a membrane for *in vivo* testing.

The diffusion coefficient estimate of H_2O_2 in the Nafion membrane reported herein – $1.2 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ – is in the same order of magnitude as that reported by Chen *et al.* for lactate in their PEDOT:PSS-PB film covering their LOD sensor ($9.2 \times 10^{-11} \text{ m}^2\text{s}^{-1}$).¹⁴¹ The diffusion coefficient of lactate in the Nafion membrane – $9.67 \times 10^{-13} \text{ m}^2\text{s}^{-1}$ – is three orders of magnitude lower than the diffusion coefficient of lactate in bulk solution outside LOD sensors reported by Zhang *et al.* ($(7.31 \pm 0.69) \times 10^{-10} \text{ m}^2\text{s}^{-1}$).¹²⁴ This is expected since the mass transport of lactate is limited by the membrane, therefore, diffusion is slower in the membrane than in the bulk solution. The same phenomenon is observed for the diffusion coefficient of H_2O_2 in bulk solution, DK is $(1.16 \pm 0.05) \times 10^{-9} \text{ m}^2\text{s}^{-1}$ (reported by Zhang *et al.*) in bulk solution vs $1.2 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ for H_2O_2 in the Nafion membrane. Rong *et al.* report a

diffusion coefficient of $3.54 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ for lactate in a collagen matrix¹⁴² and this is three orders of magnitude higher than that reported herein for lactate in the Nafion membrane ($9.67 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$).

Amongst the studied membranes, it was found that PU offered the most resistance to the mass transport of lactate in the membrane, with a DK value of $9.20 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ and a film thickness of $2772.91 \pm 1159 \text{ nm}$. The DK of lactate in the Nafion membrane – $4.91 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ – was in the same order of magnitude but almost half that of the DK of lactate in the PU membrane. This is reflected in the effective K_m of the sensors, with K_m being considerably higher in the PU membrane ($6.68 \pm 0.18 \text{ mM}$) than in the Nafion membrane ($1.35 \pm 0.03 \text{ mM}$). The diffusion coefficient estimate, DK , of H_2O_2 in the membrane stayed relatively the same across PU, Nafion and cellulose acetate membranes; observed to be between $(1.06\text{--}3.36) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. The DK observed in the PSSA membrane, however, was an order of magnitude higher than in the other membranes ($3.30 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$), meaning that diffusion of H_2O_2 was faster in PSSA.

The estimate of the diffusion coefficient, DK , derived from the Koutecký–Levich analysis of the membrane-coated sensors offers some limitations. Firstly, we estimate the diffusion coefficient as the product of the diffusion coefficient (D) and the partition coefficient (K). Since K is unknown, the estimate of the diffusion coefficient is always going to be influenced by a factor of K . The calculation of DK is dependent on the intercept of the Koutecký–Levich plot (I^{-1} vs $\omega^{-1/2}$) and the current is extrapolated to an infinite rotational speed to achieve this, which isn't possible in reality, and therefore, the intercept is based on this assumption. In

addition, we assume that the rate of reaction is not limited by enzyme concentration, which was seen to not be the case for *DK* of lactate.

2.5.3 Loss of hydrogen peroxide at high rotational speeds

Monbouquette *et al.* report the loss of H_2O_2 from the enzyme hydrogel layer of their amperometric glutamate sensors, reporting that the loss of generated H_2O_2 to the bulk solution can occur when the thickness of this layer is high, such as above $1\text{ }\mu\text{m}$.¹²¹ The study also reported greater loss of sensitivity as the thickness of the hydrogel layer was increased due to greater mass transport limitation, which is in alignment with our results reporting loss in sensitivity upon increasing thickness. In addition, although the thickness of the enzyme hydrogel was not measured in this thesis, the film thickness of the diffusion limiting Nafion membrane was measured to be 757.30 nm per coat. Despite the Nafion membrane covering the hydrogel layer and providing a protective barrier between the hydrogel and bulk solution, there may still be some loss of H_2O_2 at high rotational speeds. This is evidenced by the loss in current observed for some sensors during amperometry when the rotational speed was increased beyond 81 rpm, as shown in Figure 2.46. The opposite effect is seen in the current response as to what is expected, i.e. the current decreases as the rpm is increased. This effect was not seen across all RDE sensors coated with Nafion and this points to the variability in the thickness of the films across sensors.

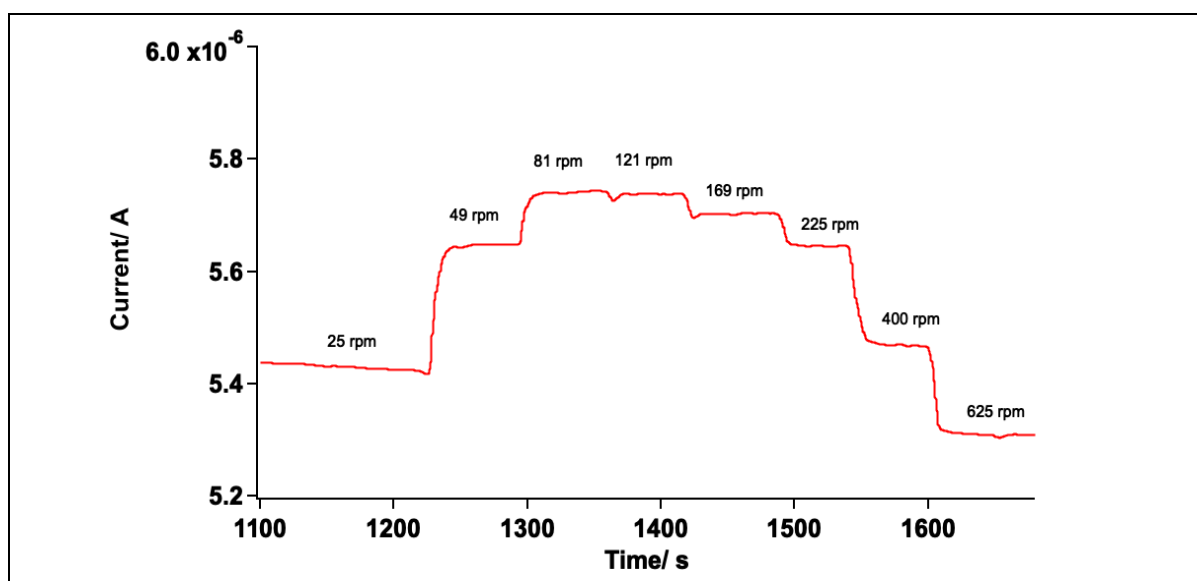


Figure 2.46 Amperometry of a lactate sensor coated with 1% w/v Nafion in 0.5 mM lactate, at varying rotational speeds showing a reduction in current beyond 81 rpm.

In this study, a nebuliser was used to evenly coat the sensors with the Nafion membrane, however a coffee-ring effect was still seen. Other studies have used drop casting^{124,142} and dip-coating¹⁴² methods to achieve an even thickness of the membrane across the sensor and reduce variability in thickness across sensors.

2.5.4 Lactate sensor response *in vivo*

A time lag of 8 to 10 min was observed between blood and ISF lactate concentrations for participants 1 to 4, which is in alignment with that reported in literature.^{106,151} The lag seen in ISF is due to time taken for the diffusion of lactate from the capillaries into ISF and has been widely reported for the transport of glucose between the two compartments. In the case of participants 1 and 2, the fall in lactate after stopping exercise was seen in ISF before it was seen in blood, and this trend has been seen for both lactate and glucose concentrations in ISF

vs blood in literature, indicating slower clearance in blood compared to ISF.^{106,153} This may be explained by the reabsorption of lactate from ISF and surrounding tissues back into the capillaries.

For participants 5, the local generation of lactate from tissues in the epidermis may account for the lack of a lag between ISF and blood lactate. Some *in vivo* studies have reported little lag for glucose measurements in ISF vs blood. There was no lag seen between dialysate measurements made using microdialysis, and this may be attributed to the fact that dialysate is sourced from deeper in the tissue than ISF, where the distance between the capillaries and tissues may be a lot less than that to the ISF compartment.

There was good agreement between ISF and blood lactate concentrations, as well as between ISF and dialysate, as evidenced by the Bland-Altman plots (Figure 39). Overall, a rise in lactate concentrations in ISF is seen after starting exercise and this is seen to decrease after stopping exercise. The sensor was able to measure the change in concentration of lactate over the course of the exercise regime and detected that lactate concentrations returned to near-baseline levels, i.e. those before exercise was started, which is an important characteristic if the sensor is to guide antimicrobial therapy for sepsis patients. The detection of this change in concentrations is important for the application of the early detection of sepsis, where lactate concentrations can range between 1 and 12 mM in ISF, and anywhere > 4 mM indicates sepsis.^{84,95} Lactate concentrations observed in blood (1.07 mM at rest to 13.03 mM after exercise) were similar to those seen in other *in vivo* studies measuring lactate during and after exercise.^{106,143} Meyerhoff *et al.* reported blood lactate measurements between 1 mM (at rest) and 5 mM (after exercise),¹⁴³ whereas Freeman *et al.* reported measurements

between 1 mM (at rest) and 14 mM (after exercise). Lactate concentrations observed in skin dialysate in this study (0.12 to 0.15 mM) were lower than those reported by Rogers *et al.* in dialysate from injured brain (0.55 to 0.80 mM).¹⁰⁸

The effective K_m of the sensors, and in turn, the resulting dynamic range provided by the sensors was low compared to that observed during the optimisation phase. This meant that the sensors were unable to detect high concentrations of lactate, and therefore, the ratio of blood:ISF was seen to increase along the course of each *in vivo* trial, wherein at higher concentrations of blood lactate after starting exercise, the ratio of blood:ISF lactate was seen to increase due to a lack of a large enough rise in concentrations of ISF lactate.

These concentration differences can be attributed to (i) the unequal partitioning of analytes across the semipermeable capillary walls, controlled by the Donnan equilibrium, (ii) local generation of lactate in the skin, (iii) the low K_m of sensors, meaning that large positive increases in lactate concentrations are not detected by the sensors even if they may be present in ISF, and (iv) calibrations in PBS not being representative of those *in vivo*. The latter is known to be a limitation in the development of biosensors i.e., conducting calibrations in PBS wherein this medium is not representative of ISF *in vivo* conditions, and therefore, the ISF lactate concentrations deduced from the Hill parameters of calibrations in PBS may not be fully representative.

Therefore, more optimisation to reduce the sensor-to-sensor variability in K_m needs to be carried out to ensure that the effective K_m of the sensors doesn't limit the measurement of higher lactate concentrations in the detection of sepsis.

2.5.5 Interference

mPD was chosen as an interference exclusion layer as it performed better than oPD, phenol and pyrrole at removing interference from ascorbic acid, uric acid, and acetaminophen. The peaks seen in ferri-ferrocyanide before the application of mPD were not visible after it had been electropolymerized on the gold electrode surface. However, despite the mPD layer working well during the optimisation phase on the microneedles, interference from AA, AM and UA was still seen at microneedles used in the *in vivo* study. Therefore, it is worth noting the calculation of ISF lactate from microneedle current is likely to be overestimated due to the working electrodes responding to interferents, and due to the lack of a sentinel electrode being used in the *in vivo* study herein.

Other *in vivo* studies, such as that reported by Freeman *et al.* applied a potential of +0.2 V vs Ag|AgCl at the working electrode, however, interference was still seen from AA. Thomas *et al.* reported the use of 1% w/v Nafion as a coating to remove interference from their LOD sensors, however, interference from AA was still present *in vitro*.¹²⁸ One way to overcome the effect of interference on working electrode current would be to use the control electrode (without enzyme) as the sentinel electrode in *in vivo* studies and subtract this from the working electrode current. Dias *et al.* report the use of a sentinel electrode in the measurement of lactate in brain slices using an LOD microelectrode sensor functionalised with mPD, allowing interference from ascorbate in the brain to be eliminated from working electrode current. In terms of sepsis detection, acetaminophen is likely going to administered to patients with suspected sepsis and therefore, this could pose a challenge, therefore using a sentinel electrode would be helpful. UA and AA are unlikely to change drastically during infection or sepsis.

2.5.6 Access to ISF using microneedles

Microneedle biosensors have shown to be effective for in vivo monitoring of analytes in ISF,^{38,106} however, there is some debate around whether microneedles are penetrating deep enough to access ISF in skin and whether they are displacing ISF during insertion of the needles. Cass *et al.* report microneedles of 1 mm in height and 500 μm in width, as is used in this thesis, and show that the microneedles are in the epidermis and enter some of the dermis through OCT imaging in the skin of a healthy volunteer (Figure 2.47), which is comparable to the imaging shown in this thesis.¹⁵⁴ The stratum corneum is known to be 20 to 30 μm thick and the epidermis is between 800 and 1500 μm thick,¹⁵⁴ so depending on the individual thickness of skin sections and variability of these patient-to-patient, the 1 mm microneedles may enter only the epidermis, or may pass through the epidermis and enter the top section of the dermis (as seen in Figure 2.48).

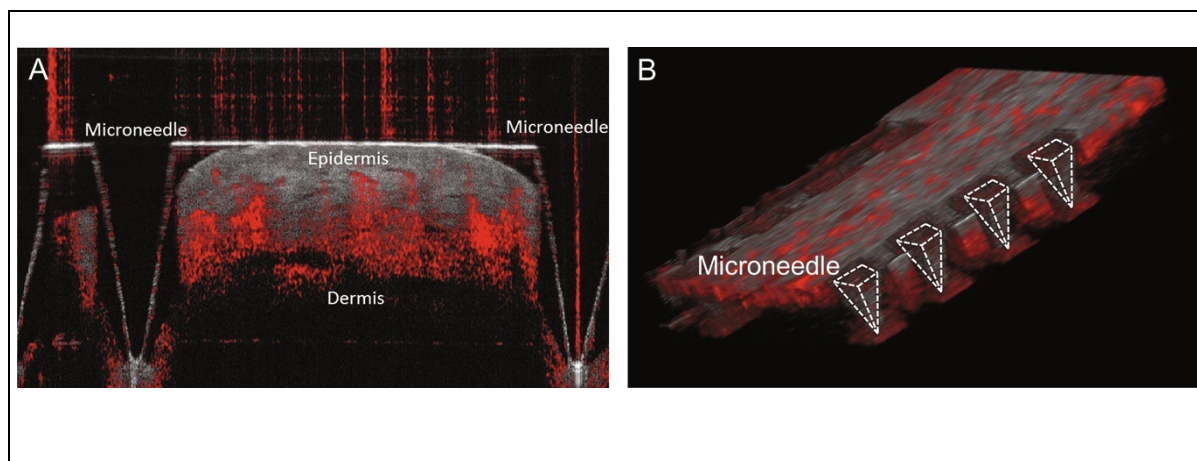


Figure 2.47 OCT image of 1 mm microneedles in a healthy volunteer, as reported by Cass *et al.*¹⁵⁴

Microneedle heights reported in literature range from 700 μm to 1.5 mm.^{38,76,106,155,156} The results presented in this study show an effect of decreasing impedance upon applying a constant tourniquet pressure to the back of the microneedle in skin, i.e. the impedance decreases in a liquid medium (ISF) vs air. This is in alignment with the OCT image data shown in this thesis and by Cass *et al.*¹⁵⁴

2.6 Conclusion and future work

The dynamic range of a first generation LOD sensor has been extended with a mass transport limiting membrane consisting of five coats of 1% w/v Nafion, achieving a K_m of 1.35 mM for RDE sensors and between 0.23 and 16.24 mM for the microneedle sensors. The diffusion coefficient of lactate and H_2O_2 through the mass transport limiting membrane was experimentally derived to be 4.91×10^{-13} and $1.06 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$, respectively, via Koutecký–Levich analysis.

Following extension of the dynamic range, the sensor was clinically validated in healthy volunteers producing lactate during exercise.¹⁵⁰ The regime included a period of sensor stabilisation for 1 hour, followed by 15 min of rest to capture baseline lactate, 15 min of exercise, and another 30 minutes of rest to capture clearance. The results from the *in vivo* studies showed that the microneedle sensor can detect changes in ISF lactate concentrations with an increase in ISF lactate observed 6-15 mins after starting exercise, and a decrease observed 100-300 s after stopping exercise. The ISF lactate concentrations derived from microneedle current were compared with lactate concentrations from venous blood and dialysate. It was found that lactate concentrations were lower in ISF compared to blood, with

average ratios of blood:ISF lactate between 1.60 and 3.86. Concentrations of lactate were higher in ISF compared to dialysate, with average ratios of ISF:dialysate lactate between 1.60 and 3.86. Little to no lag was observed between ISF and dialysate, whereas a time lag of 8-10 min was observed between ISF and blood lactate.

In terms of future work, the DET LOD enzyme reported by Freeman *et al.* could be used to remove interference by application of a lower oxidation potential (+0.20 V).¹⁰⁶ In addition, more work needs to be carried out to reduce sensor-to-sensor variability in terms of the effective K_m achieved across batches. The low K_m values observed during the *in vivo* studies may be a result of insufficient Nafion being applied to the sensors, or a lack of an even coating across the sensor surface, which needs to be addressed before the next *in vivo* testing phase.

Another method of improving K_m may be to explore new solvents for dissolving PU and CA. Both of these membranes performed better than Nafion, however, they were not chosen for the *in vivo* studies due to incompatibility with the microneedle bases. PU, in particular, was seen to improve the dynamic range by a large amount. Another approach could be to cover the base of the microneedle such that no polycarbonate is exposed, allowing the solvent in which the membrane is dissolved to be separated from the base.

To ensure that the interference exclusion layer is deposited to a degree that prevents interference at the microneedle electrode surface, an additional step in the fabrication process could be added to check that interference has been removed by cycling in

ferri/ferrocyanide. If peaks are still seen, more mPD may need to be electropolymerised onto the electrode surface to ensure interference exclusion.

Drawing it back to the detecting of early warning signs of sepsis, the microneedle sensors (2 out of 5) detect in the range needed for sepsis detection (i.e., > 4 mM). More work is needed to reduce the sensor-to-sensor variability seen in the study, in order to ensure a high effective K_m across all sensors more reliably. In addition, more optimisation work of the control electrode needs to be conducted for use as a sentinel electrode to remove interference.

Chapter 3 Development of an electrochemical aptamer sensor for the continuous monitoring of C-reactive protein

3.1 Introduction

Aptamers are typically single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic Acid (RNA) molecules that have been selected to bind specifically to target molecules ranging from small molecules to proteins with high affinity. Prior to aptamer-target interaction, aptamers form complex secondary and tertiary structures through self-hybridisation, driven by non-covalent bonding, which create structures such as stem, loop, hairpin, and G-quadruplex structures, in addition to aptamers exhibiting a combination of these altogether.¹⁵⁷ This highly ordered structure allows aptamers to form hydrogen bonds and dipole-dipole interactions with their targets for which their shape and structure is complementary and specific (Figure 3.1).

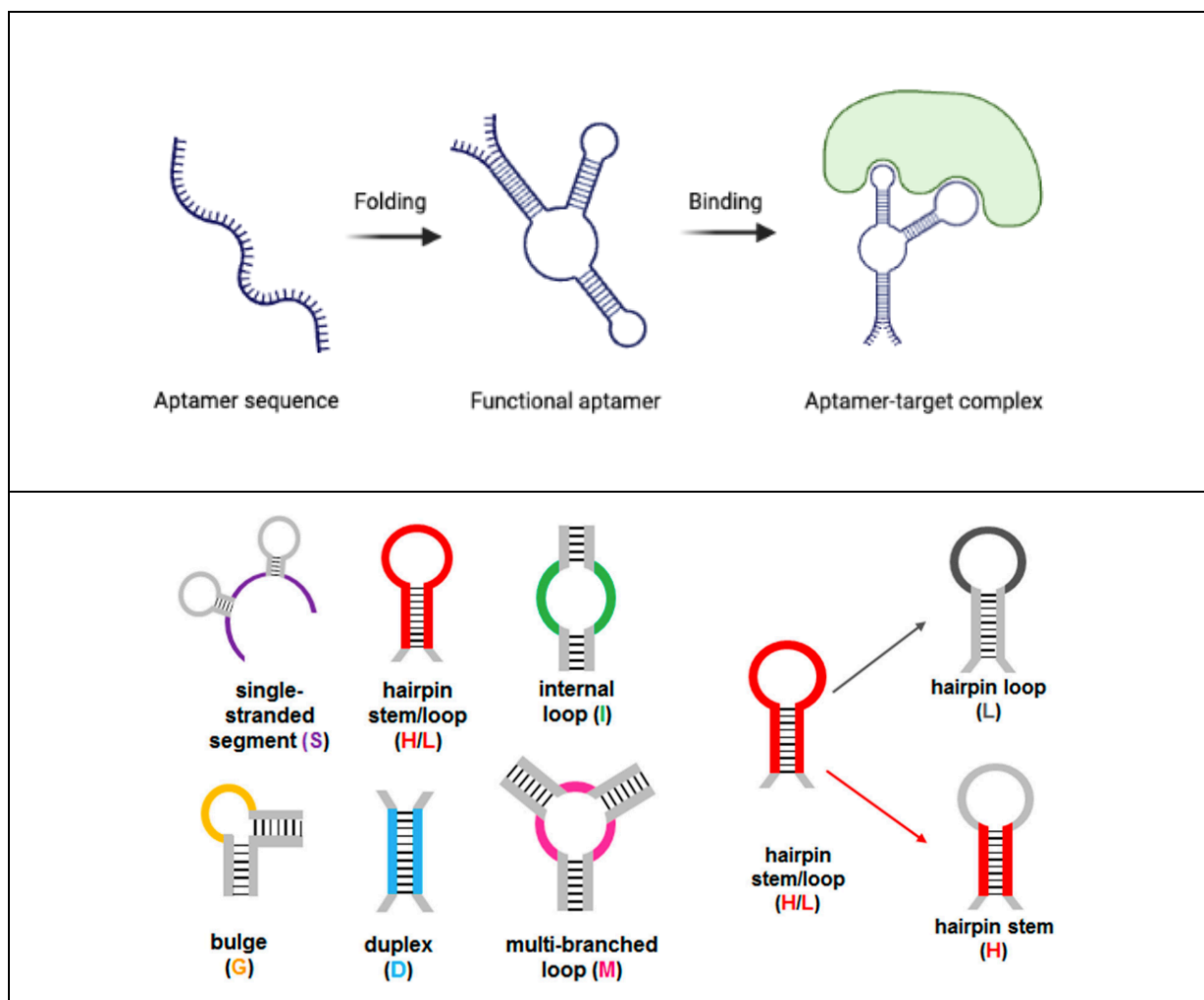


Figure 3.1 (a) An aptamer sequence folds to form a secondary and tertiary structure, forming the functional aptamer, which binds to its target, forming the aptamer-target complex, (b) Possible secondary structures of aptamers include single-stranded segments, hairpin loops and stems, internal loops, bulges, duplexes, and multi-branched loops.¹⁵⁸

3.1.1 SELEX

Aptamers were first discovered in the 1990s by Larry Gold (University of Colorado Boulder) and Andrew Ellington (University of Texas, Austin). Gold's group discovered SELEX for RNA aptamers, developing the first RNA aptamers for thrombin and T4 DNA polymerase¹⁵⁹. Ellington's group conducted SELEX for ssDNA aptamers.¹⁶⁰

Aptamers are selected from a randomised library of single stranded DNA or RNA molecules through a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). This library contains 10^{14} – 10^{15} variants of single stranded oligonucleotide sequences that are 30–100 nucleotides in length.¹⁶¹ SELEX is a process by which the target is exposed to the oligonucleotide library, allowing selective binding to occur, after which all unbound species are removed, resulting in a pool of target-specific aptamers which are then amplified using Polymerase Chain Reaction (PCR). This is iterated through several rounds of selection and amplification to concentrate the aptamer species with the highest specificity to the target. After several rounds of SELEX, the desired high-affinity aptamers are sequenced so that they can be cloned for future applications. The affinity of the aptamer to its target is determined using techniques such as Surface Plasmon Resonance (SPR) wherein the aptamer is exposed to the target and the ON and OFF rate of the aptamer is measured to determine a binding constant, K_D .

There are several ways of conducting SELEX, including capture-SELEX, nitrocellulose-based SELEX, Capillary Electrophoresis-SELEX (CE-SELEX), cell-SELEX, and Fluorescence-activated Cell Sorting (FACS) SELEX.

In the most common form of SELEX, capture-SELEX, the oligonucleotide library is exposed to complementary capture probes which have been modified with biotin, as shown in Figure 3.2. Once captured by the probes, the oligonucleotide library is combined with streptavidin coated beads, causing the streptavidin and biotin to bind, which immobilises the aptamer sequences – via the biotinylated capture probes – onto the surface of the beads. The library is then exposed to the target which causes the release of the bound aptamer-target

complexes from the beads. The beads are then removed from solution, either via centrifugation or by magnetic separation, and the supernatant is removed for elution of the target from its aptamer. Once eluted, the aptamers are amplified by PCR. The amplified library has a higher affinity to the target than the original randomised pool of sequences, and the double-stranded library is then digested to ssDNA or RNA sequences, which forms the initial library in the consecutive round of SELEX.¹⁶²

Another form of SELEX is the nitrocellulose membrane-based SELEX whereby the randomised oligonucleotide library is exposed to the target in vitro and the solution contains the bound aptamer-target complexes and unbound aptamer sequences. This mixture is passed through a nitrocellulose filter.¹⁶³ Due to the pore size of the nitrocellulose membrane, the bound aptamer-target complexes do not pass through the filter and the unbound sequences pass through. This separation method then allows the bound aptamer-target complexes to be washed off the membrane for elution and amplification. This process is then repeated until high affinity aptamer sequences are obtained and for nitrocellulose-based SELEX, this usually takes up to 12 cycles.¹⁶⁴

CE-SELEX is a form of SELEX that utilises capillary electrophoresis to separate the bound and unbound aptamers. The aptamer sequence library is combined with the target and the solution is injected into a CE capillary with the application of a potential difference between the terminals. The bound and unbound aptamers are separated due to the difference in the rate of movement of each species, which allows them to be collected in separate fractions. Once collected, the target is eluted from the bound species and PCR is used to amplify the aptamers. Mosing *et al.* used CE-SELEX to select an aptamer for HIV-1 Reverse Transcriptase

(HIVRT), a pharmacological target in the treated of HIV-AIDS.¹⁶⁵ Other methods of separation in SELEX utilise affinity columns, titre plates, and gel matrices.

Some SELEX procedures have a negative selection step, wherein the aptamer library is exposed to the non-target such proteins, ligands, or cells, allowing bound aptamers to be removed from the pool to reduce interference from these non-target molecules in future applications.

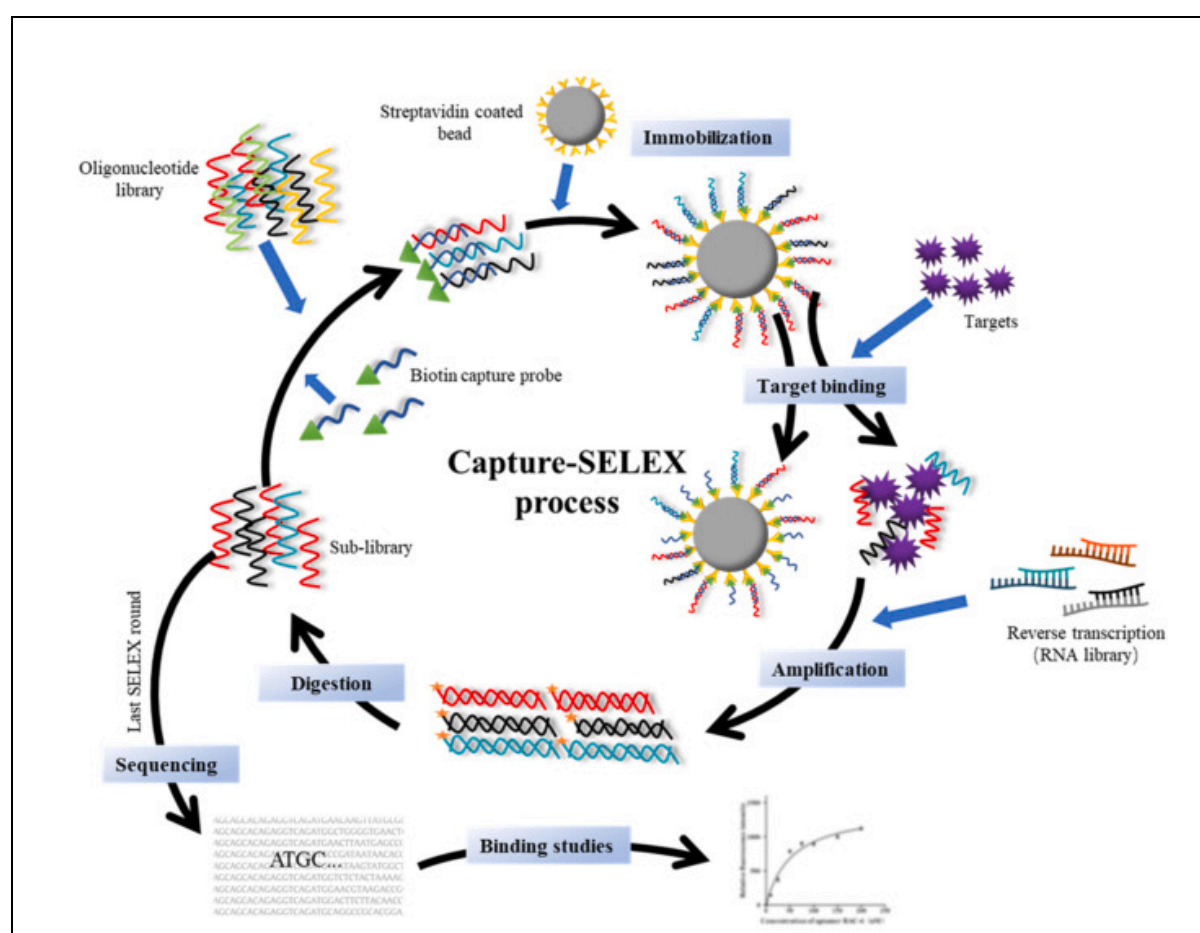


Figure 3.2 The process of capture SELEX, as demonstrated by Lyu et al.¹⁶²

Since the advent of SELEX, many aptamers have been developed for proteins, ligands, and cells of interest, including Adenosine Triphosphate (ATP), C-reactive protein (CRP) and tobramycin. Table 3.1 summarises published aptamers developed in recent years.

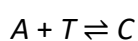
Target	K _D /binding affinity	Sequence length/ nt	Reference
Adenosine Triphosphate (ATP)	6 ± 3 µM	42	Szostak <i>et al.</i> ¹⁶⁶
Vascular Endothelial Growth Factor (VEGF)	0.2 µM	Between 29 and 36	Jellinek <i>et al.</i> ¹⁶⁷
Dopamine	2.8 µM	57	Mannironi <i>et al.</i> ¹⁶⁸
Thrombin	Not known	15	Macaya <i>et al.</i> ¹⁶⁹
Human Immunodeficiency Virus Type 1 (HIV-1) Rev protein	1-10 nM	36	Jensen <i>et al.</i> ¹⁷⁰
SARS-CoV-2	Not known	Between 57 and 88	Chen <i>et al.</i> ¹⁷¹
Hepatitis C non-structural protein	11.6 nM	72	Fukuda <i>et al.</i> ¹⁷²
CRP	3.51 nM	72	Huang <i>et al.</i> ¹⁷³
Tobramycin	12 ± 5 nM	26	Jiang <i>et al.</i> ¹⁷⁴
Penicillin	46.5% retention rate	98	Paniel <i>et al.</i> ¹⁷⁵
T4 DNA Polymerase	Not known	32	Tuerk and Gold <i>et al.</i> ¹⁷⁶

Table 3.1 Examples of aptamers developed for biosensing.

3.1.2 Binding affinity and regeneration

An aptamer (A) binds to its target (T) in a reversible process, forming an aptamer-target complex (C), as shown in Equation 3.1). The affinity an aptamer has for its target is measured through the aptamer association rate constant, k_{on} , and the dissociation rate constant, k_{off} . K_D , the equilibrium dissociation constant, also known as the binding affinity, is the ratio of k_{off} to k_{on} (as shown in Equation 3.2), where $[\gamma]$ is the surface concentration of the aptamer, $[T]$ is the concentration of the target, and $[C]$ is the concentration of the aptamer-target complex.

The rate constants for association and dissociation can be measured using several methods, including surface plasmon resonance (SPR), a tool used to track molecular interactions in real-time.¹⁷⁷ For the aptamer used in this thesis, Huang *et al.* carried out binding affinity measurements for their C-reactive protein (CRP) aptamer using SPR, where in the aptamer was immobilised onto an SPR sensor chip with blocker ssDNA molecules taking up free spaces on the chip.¹⁷³ A baselined measurement of the unbound aptamer was recorded on the SPR software, followed by measurement of the bound aptamer after a solution containing a known concentration of the target molecule was flowed over the chip at a constant flow rate of 20 $\mu\text{l}/\text{min}$ at 25 $^{\circ}\text{C}$. Next, the aptamer was regenerated using a wash buffer, and the response measured again. This was repeated for several concentrations of CRP (from 0.625 mg/L to 20 mg/L) and a Langmuir model was used to plot the responses and to determine the k_{on} and k_{off} rate constants, which were then used to determine K_D .



Equation 3.1 Aptamer (A) and Target (T) bind to form an aptamer-target complex (C). This is a reversible process.

$$K_D = \frac{[A][T]}{[C]} \quad (1) = \frac{k_{off}}{k_{on}} \quad (2)$$

Equation 3.2 The dissociation constant, K_D , is the product of the aptamer surface concentration and the target concentration, divided by the concentration of the aptamer-target complex (1). K_D is also the ratio of the dissociation rate constant to the association rate constant of the aptamer (2).

The wash buffer used to regenerate the aptamer was 10 mM tris buffer containing 50 mM NaCl and 2 mM CaCl_2 . Since the reported K_D of the aptamer is 3.51 nM, it can be assumed that the k_{off} rate constant is approximately nine orders of magnitude lower than the k_{on} rate constant, which means that the dissociation of target from the aptamer is not favoured and may require an active step such as heat treatment, pH shock or a change in the ionic strength of the solution.

3.1.3 Applications of aptamers

Aptamers have several applications, including therapeutics¹⁷⁸, targeted drug delivery, biomarker discovery, diagnostics and biosensing.

Aptamers as therapeutics and in targeted drug delivery

Aptamers are good therapeutic candidates due to being non-immunogenic and smaller in size, allowing them to enter biological compartments more efficiently. Aptamers can target both inter- and intracellular proteins, as well as cell surface receptors. As they are produced chemically, they can be easily scaled up in manufacture. Aptamer therapeutics are being widely researched, with several candidates currently in clinical trials. An example of this is the

drug AS1411 (Antisoma Research, Ltd.) which is a Guanine-rich DNA aptamer being tested for the treatment of acute myeloid leukaemia.^{178,179} Pegaptanib (also known as Macugen) was an aptamer drug approved by the FDA for the treatment of age-related macular degeneration (AMD).¹⁸⁰ The Pegaptanib aptamer targeted vascular endothelial growth factor (VEGF) which supports the regulation of pathological angiogenesis; the aptamer therefore blocks the activity of VEGF in AMD. However, Pegaptanib was later withdrawn due to high competition from more efficient drug candidates entering the market.¹⁸¹ Despite the potential of aptamer therapeutics, there are challenges that remain to be overcome, such as their small size – making them more susceptible to renal filtration – and their susceptibility for serum degradation. In addition, there are barriers such as the variability in pharmacokinetic properties which is difficult to predict. Aptamers have also been used in targeted drug delivery as aptamer-drug conjugates (ApDC) to target diseased cells with high selectivity and affinity.¹⁸² Aptamers are conjugated to drug molecules using covalent linkage or physical intercalation.

Aptamers in biomarker discovery

Aptamers have been used in biomarker discovery, particularly in probing molecules on the cell's surface. Cell-SELEX, a type of SELEX used in biomarker discovery, targets whole, live cells from cell culture. In cell-SELEX, a randomised library of ssDNA aptamer sequences is exposed to target cells and unbound aptamers removed. The bound aptamers are extracted and exposed to a negative cell line for counter selection to improve specificity, and the bound aptamers are removed. The unbound aptamers from this pool are then amplified and the process reiterated to produce a concentrated selection of aptamers specific to the cell surface proteins of the target cells. Cell-SELEX has produced aptamers for various cell lines, including

leukaemia, myeloid leukaemia, liver cancer, small cell lung cancer, and non-small cell lung cancer cells. Once these biomarkers have been identified, through isolation of bound aptamer species using an affinity matrix or photo crosslinking, they can be analysed via mass spectrometry.^{183,184,185}

Aptamers in point-of-care diagnostics

Aptamers have been used as the molecular recognition element in various diagnostic platforms, such as lateral flow assays, Aptamer-Linked Immunosorbent Assay (ALISA), Enzyme-Linked Oligonucleotide Assay (ELONA), free solution formulations and biosensors.¹⁸⁶

There are a range of colorimetric aptamer assays - including ALISA, Enzyme-Linked Oligonucleotide Assay (ELONA), also known as Enzyme-Linked Aptamer Assay (ALASA) - which use spectrophotometry to turn a qualitative optical readout into a quantitative one. ALISA assays are used in the high throughput screening of aptamers wherein the aptamer is biotinylated and incubated with target antigen molecules which are coated onto microtiter plates. After the removal of unbound aptamers, streptavidin-linked horseradish peroxidase (HRP) enzyme is added to the plate which binds to the biotinylated aptamer bound to the antigen, followed by an HRP substrate. The reaction between HRP and its substrate causes a change in colour.

The Enzyme-linked Oligonucleotide Assay (ELONA) is an enzyme-linked sandwich assay that uses aptamers as the recognition element in place of antibodies that are used in an ELISA.¹⁸⁷ Drolet *et al.* developed an ELONA assay to detect human VEGF where the capture reagent was a monoclonal antibody directed against hVEGF.¹⁸⁸ The aptamer was tagged with a

fluorescein molecule and used as the detect reagent, and a signal generated using chemiluminescent alkaline phosphatase.

In dot blot assays, the aptamer is biotinylated, and the antigen is immobilised onto a nitrocellulose or polyvinylidene fluoride membrane that is blocked by bovine serum albumin (BSA). Streptavidin linked HRP is added to the membrane, followed by an HRP substrate which creates a colour change, indicating the presence of the aptamer-binding event. Biotinylated aptamers can be conjugated to streptavidin-coated gold nanoparticles (Au-NPs) in dot plot assays, where these have been used to detect thrombin.

AuNPs-aptamer aggregates have also been used for diagnostic applications. Zhang *et al.* developed an aptamer-gold nanoparticle based colorimetric assay to detect cocaine. The cocaine aptamer is physically adsorbed onto the surface of GNPs and in the presence of cocaine, the aptamer leaves the surface of the GNP due to its high affinity to cocaine molecule, and this results in the salt-induced aggregation of GNPs which causes a colour change from red to blue.¹⁸⁹

In lateral flow assays, aptamers are conjugated to gold particles in a conjugate pad. Once the target antigen is in contact with the gold-aptamer complex, the aptamer-antigen complex flows through the conjugate pad and is captured by a capture aptamer in the test zone, causing a colour change and indicating the presence of the antigen. An example of this is the lateral flow assay developed by Xu *et al.* for thrombin. Two thrombin aptamers were used that bind to different sites on the protein molecule; the first aptamer was adsorbed onto GNPs on the conjugate pad, and the second aptamer was immobilised onto the test zone of

the lateral flow assay using biotin-streptavidin linker chemistry. Using the salt induced aggregation of GNPs due to the high affinity of the thrombin molecule to the second aptamer in the test zone, a colour change is seen and can be quantified using a test strip reader.¹⁹⁰

Aptamers in biosensing

While biosensing falls under the umbrella of diagnostics, herein we discuss electrochemical biosensors that use aptamers as the molecular recognition element. In electrochemical biosensing, aptamers are modified at the 5' or 3' terminal with a redox label such as methylene blue, ferrocene, or osmium (II/III) polymer, and a thiol group at the other terminal. The thiol is attached so that the aptamer can form a gold-sulphur (Au-S) bond with the gold surface of electrodes; the bond is commonly used in aptamer immobilisation on gold, particularly for electrochemical aptamer biosensors (E-ABs).¹⁹¹ The Au-S bond allows the formation of a self-assembled monolayer (SAM), which is complemented by the use of back-filler molecules, such as polyethylene(glycol) (PEG) and 6-mercaptohexanol (6-MCH), which fill in any empty spaces between the aptamers on the electrode surface.

The formation of SAMs of aptamers on gold surfaces depends on the strength of the Au-S and gold-gold (Au-Au) bonds¹⁹². The strength of the Au-S bond is dependent on factors such as pH, temperature, salt concentration and the oxidation state of the gold atoms¹⁹³. Inkpen *et al.* found that the Au-S bond is based on a physisorbed interaction – an interaction governed by van der Waals forces – on planar gold electrodes, and a chemisorbed interaction – an interaction governed by valence forces – when there is an excess of high energy gold atoms on bulk surfaces¹⁹⁴. As a result of these weaker physisorbed interactions, ligand exchange

from thiol-modified aptamers to other molecules containing sulphur in solution, such as glutathione and cysteine *in vivo*, is possible¹⁹⁵.

When the target molecule binds the aptamer, the resulting conformational shift causes the aptamer to either move closer or further away from the gold electrode surface, causing a shortening or lengthening of aptamer length. This causes the redox label to either spend more or less time in the vicinity of the electrode surface, causing a change in the rate of transfer of electrons between the label and the electrode. When the conformational change causes an aptamer to move closer to the electrode surface, an ON sensor is produced, wherein the signal increases upon binding to target, as shown in Figure 3.3. In the opposite scenario, an OFF sensor is produced, whereby the signal decreases upon binding to the target.

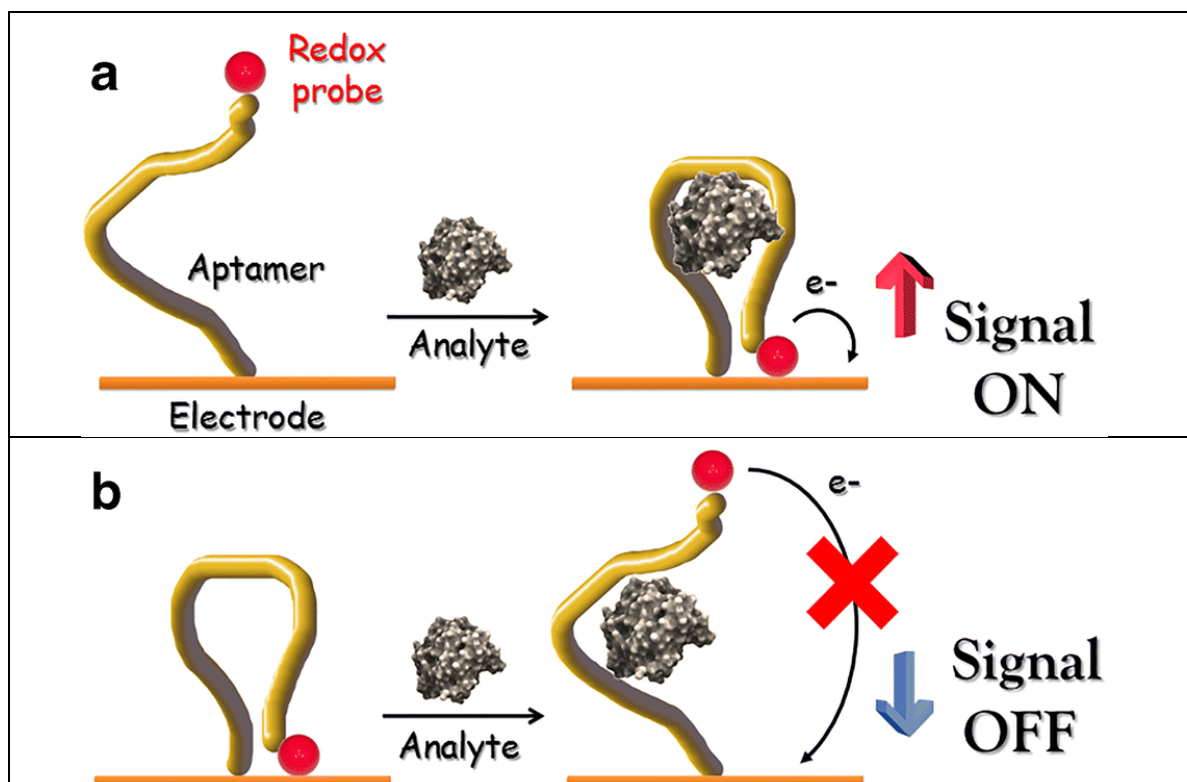


Figure 3.3 (a) An ON sensor wherein the redox label in the unbound state is further away from the electrode surface and once bound to target, the redox label is brought closer to the electrode, (b) An OFF sensor wherein the redox label is already close to the electrode surface and once bound to target, the redox label is brought further away from the electrode.¹⁹⁶

OFF sensors are associated with several challenges, including the formation of a negative signal which can be difficult to interpret, lower sensitivity due to a smaller change in signal and limited capability for multiplexing. On the other hand, OFF sensors can offer lower interference from the sample due to higher background signal, in addition to lower signal drift which can make the sensor more stable.

While ON sensors offer higher sensitivity due to a larger change in signal upon binding, making them easier to interpret, they often have higher signal drift. Due to a lower background signal in ON sensors, there is potential for interference from the sample, including non-specific

binding which can lead to false positives. ON sensors have greater capacity for multiplexing. ON sensors are generally easier to read and interpret due to there being an increase in the signal; in OFF sensors, the decrease in signal could be associated with other environmental conditions of the aptamer.

There are several ways to detect the conformational change caused by aptamer-target binding. Aptamers can be modified with different compounds for different diagnostic and sensing applications, including enzymes such as HRP and alkaline phosphatase (ALP) used to amplify response, gold or silver nanoparticles on which aptamers are immobilised, conductive polymers such as polypyrrole and polyaniline coated nanotubes or nanoparticles, and redox dyes such as methylene blue (MB) and ferrocene which cause a change in electron transfer rates.

Li *et al.* created a sandwich assay for the detection of thrombin and VEGF using aptamers that were immobilised onto a gold surface. Target proteins – thrombin and VEGF – were flowed over the sensor surface to form aptamer-target complexes. This was followed by the introduction of horseradish peroxidase-conjugated antibodies specific to the target proteins, followed by the addition of tetramethylbenzidine (TMB) which reacts with the HRP to form a dark blue precipitate. The enzymatic precipitation results in an amplified surface plasmon resonance imaging (SPRI) response, wherein a white light passed through a polariser generates p-polarised light, reflecting off the thin gold film assembly and being captured by a CCD camera as SPRI response.¹⁹⁷

Aptamers can also be conjugated with nanoparticles for detection of circulating cancer cells, as demonstrated by Liu *et al.* Ramos cells, cancerous B lymphocyte cell line, have a cell surface receptor TD05. Aptamers were selected for TD05 and immobilised onto gold nanoparticles. Aptamers for a second cell surface receptor, TE02, were selected and the aptamer was conjugated to biotin. The circulating Ramos cells were captured by the TD05-modified gold nanoparticles in the test zone of the immunoassay, and biotinylated TE02 aptamers were captured by the streptavidin coated conjugate pad in the test zone. The biotinylated aptamer-bound Ramos cells then bound to the streptavidin coated conjugate pad, immobilising the cell, and causing a positive line to appear in the test zone.¹⁹⁸

3.1.4 Redox labels in E-ABs

Redox labels are electroactive compounds that aptamers are modified with to measure electron transfer rates between the redox compound – such as methylene blue (MB) – and the electrode surface on which the aptamer is immobilised. Electron transfer occurs when the MB is reduced to MBH, or leuco-MB, when the applied potential is swept from 0V to -0.4 V vs. Ag|AgCl, for example. This change in potential causes the MB label to take up two electrons and a hydrogen ion from the electrode surface, causing it to be reduced and turn colourless, as shown in Figure 3.4. The rate of transfer of electrons from the electrode surface to the terminal of the aptamer where the MB label is, is affected by the distance between the MB label and the electrode surface, as well as the layer thickness of the SAM. The change in layer thickness is induced by the conformational change of the aptamer, as reported as Osypova *et al.*, where quartz crystal microscopy was used to confirm the loss of water molecules from the SAM, causing a decrease in layer thickness¹⁹⁹. In this study, it was shown that the greater the layer thickness, the lower the signal response.

When the aptamer folds upon binding to target, and this distance is reduced due to the conformational change of the aptamer, the MB label is brought closer to the electrode surface and the electron transfer rate increases, resulting in a greater current response. When this distance increases due to the conformational change, the MB is brought further away from the electrode surface, and the electron transfer rate is reduced, resulting in a smaller current response. This effect is demonstrated in Figure 3.5 wherein White *et al.* have shown the difference in signal response depending on whether the sensor is an ON or OFF sensor.²⁰⁰ The reduction of MB to leuco-MB is reversible; the reduced MB is able to lose the gained electrons and H⁺ ion once an oxidising potential sweep is applied, such as from 0.4 V to 0 V.

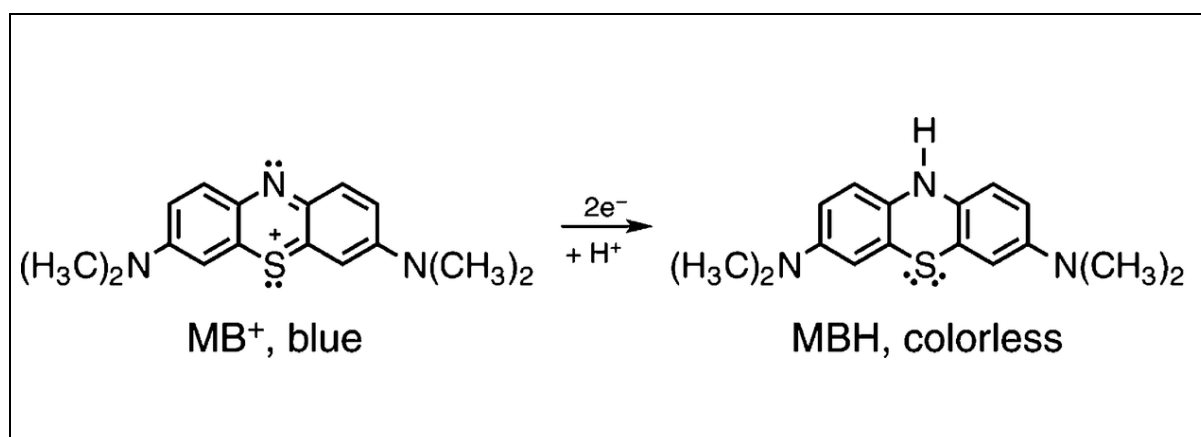


Figure 3.4 Methylene blue (MB⁺) is reduced to leuco-methylene blue (MBH) upon gaining two electrons and a H⁺ ion.²⁰¹

There are different types of redox labels that aptamers can be modified with to produce an oxidation or reduction current response. Aside from methylene blue, the most commonly used redox label in E-ABs, other labels such as ferrocene, Nile blue, anthraquinone, acetylsalicylic acid, and osmium redox complexes have been reported.²⁰²

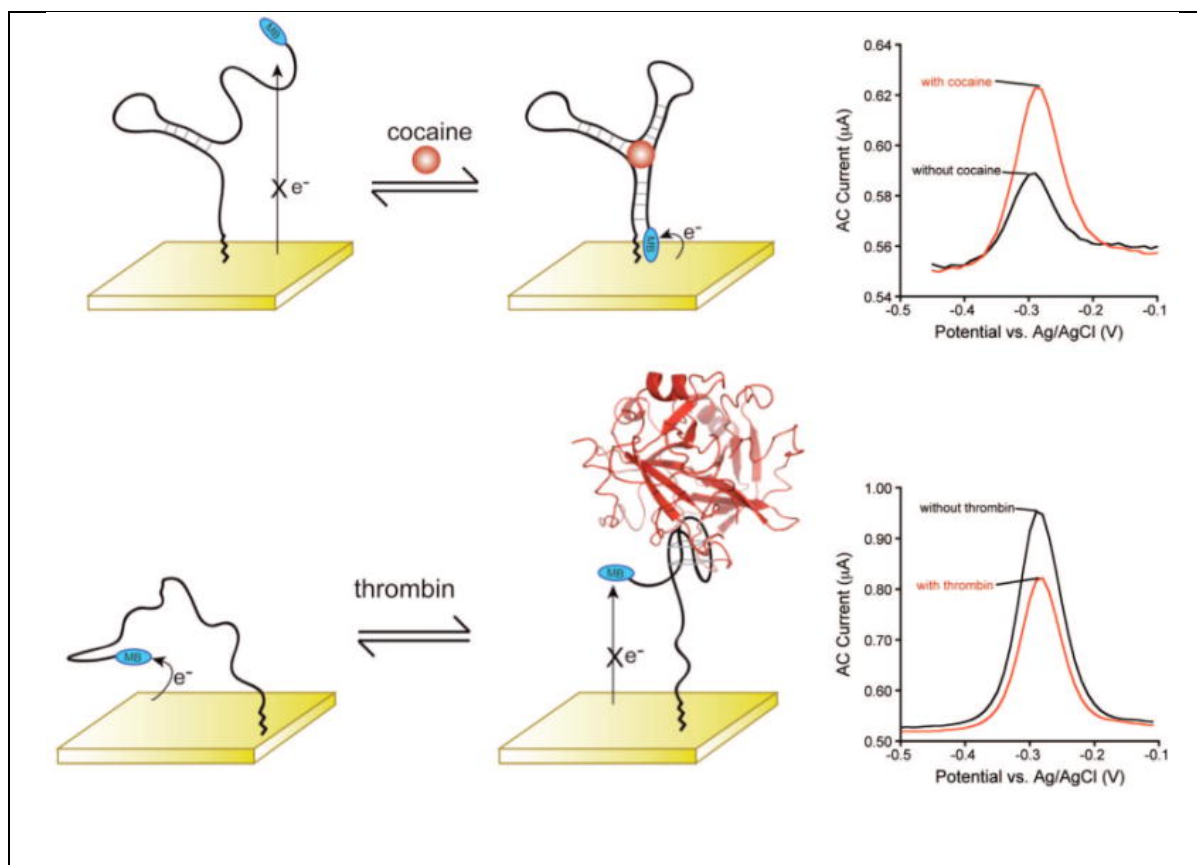


Figure 3.5 White *et al.* demonstrate how the conformational change of the cocaine and thrombin aptamers upon target binding result in different signal responses.²⁰⁰

An example of a MB-terminated aptamer used in the development of a biosensor is that for thrombin developed by Xiao *et al.* in Kevin Plaxco's group. The aptamer was immobilised onto polycrystalline gold disk electrodes with the MB group at the 3' end. The aptamer in its free state allows for high rate of electron transfer between the MB group and the gold electrode surface. Once bound to thrombin, the conformational change of the aptamer causes the MB group to move away from the electrode surface, inhibiting electron transfer and causing a reduction in measured current output. As the signal reduces upon aptamer-target binding, an OFF sensor is produced.²⁰³

Radi *et al.* reported a thrombin aptamer sensor with a ferrocene (Fc) label at the 5' end and a thiol modification at the 3' end.²⁰⁴ The Fc label was linked to the 5' end using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-2-(N-morpholino)ethanesulfonic acid (NHS) – EDC/NHS – chemistry wherein the original aptamer sequence had been modified with an amine (-NH₂) group. The aptamer was then immobilised onto polycrystalline gold electrodes and 2-mercaptohexanol (2-MCH) used as a back-filler molecule which improved signal intensity. Upon binding to thrombin, the measured current increases, indicating that the Fc-labelled aptamer is brought closer to the electrode surface, which increases the rate of electron transfer. This is an example of an aptasensor that would be classified as an ON sensor. Other studies have demonstrated achieving ON sensors using Fc-labelled aptamers, such as the aptasensor developed for potassium (K⁺) ions²⁰⁵ and adenosine triphosphate (ATP).²⁰⁶

Other redox labels include anthraquinone and acetylsalicylic acid; Wang *et al.* developed a multiplex immunosensor for the detection of multiple tumour markers using aptamers and antibodies. Aptamers for the tumour markers, prostate specific antigen (PSA), human α -fetoprotein (AFP), and carbohydrate antigen 125 (CA-125), were modified – through EDC/NHS chemistry – with carboxylated redox labels, ferrocenecarboxylic acid (Fc), anthraquinone-2-carboxylic acid (AQ), and acetylsalicylic acid (ASA). Antibodies for PSA, AFP and CA-125 were immobilised onto the diethylenetriamine modified graphite oxide (GO-DETA) electrodes and the active amide group on the Fc, AQ and ASA labels were used in an amide reaction to immobilise the aptamers onto the GO-DETA surface. Following this, the antibodies were immobilised on glassy carbon electrodes (GCE) and targets (PSA, AFP and CA-125) flowed over the surface for antibody-target binding. The GO-DETA electrodes with the antibodies were

then allowed to bind to the already-bound targets on the GCE, forming a sandwich immunosensor. As a result, the redox labels were immobilised, and a potential sweep was then able to electrochemically detect the presence of the target tumour markers.²⁰⁷

Similarly, Liu *et al.* immobilised MB- and AQ-labelled aptamers for interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α) on gold electrodes and stimulated cells to produce IFN- γ and TNF- α through a microfluidic device. The aptasensors observed a drop in peak current upon target binding during dynamic monitoring of the target molecules.²⁰⁸

Not as well explored but a promising target redox label nonetheless is a complex of osmium (II/III), a transition metal, has also been used to modify aptamers. A thrombin aptasensor developed by Galagadera *et al.* consisted of the aptamer being immobilised onto gold electrodes and the thrombin being labelled with an electroactive osmium complex, Osmium tetroxide bipyridine [OsO₄(bipy)].²⁰⁹

Furthermore, Nile blue (NB) has also been studied as a redox label for electrochemical sensors and compared to MB and AQ for its redox activity and signal production.²¹⁰ It was found that in addition to the type of redox label chosen, the performance of the aptasensor was dependent on the location at which the redox label was attached on the aptamer.²¹¹ The location of the redox label can affect the sensor's detection limit (by a factor of 10-fold, as reported by Mayer *et al.*²¹¹), ability to regenerate, stability, and reproducibility. The sensor response was also dependent on the thickness of the SAM layer, which is related to the coverage density of the aptamer on the electrode surface. The greater the aptamer density for an OFF sensor, the lower the observed difference in signal response before and after

target binding, resulting in lower % signal decrease due to presumed steric hindrance, as reported by Mayer et al. for their MB-terminated TNF- α aptasensor.²¹¹ The study also shows that the distally-attached MB label performed better than the proximally-attached and the internally-attached MB labels, which was thought to disrupt the formation of the SAM.

3.1.5 Aptasensors for continuous monitoring *in vivo*

Aptamers have been used in continuous monitoring of analytes due to their reversible conformational switching – in response to target binding – allowing for the real-time analysis of analytes *in vitro* and *in vivo*. The spectrum of molecular analytes that can be detected by aptasensors is much greater than enzymatic biosensors or biosensors based on the direct sensing of redox active analytes, as aptamers are able to bind to non-redox active molecules and produce a redox reaction due to being modified with a redox reporter.

To be able to measure analytes continuously and in real-time, the biosensor must be:

1. Selective towards its target analyte, without interference or inhibition from other molecules *in vivo*.
2. Sensitive towards small changes in concentration, depending on the application and detection window of interest, based on the Langmuir isotherm as a model for binding.²¹²
3. Regenerable, producing a reversible response to changing analyte concentrations, with a temporal resolution that matches physiological changes and an appropriate binding affinity for the target molecule.
 - a. The binding affinity needs to match the physiologically relevant detection window of the target analyte in humans.

4. Able to operate continuously *in vivo* without external support i.e., without washing or incubation steps.

Several aptasensors have been developed for the continuous real-time monitoring of markers of infection, such as the E-AB developed for interferon-gamma (IFN- γ) by Liu *et al.*²¹³ IFN- γ is a cytokine released by T cells in response to an infectious antigen and is a marker of inflammation. Continuous sensing of cytokines can also be applied in monitoring treatment for cancer patients, allowing side effects to be minimised. IFN- γ is also used in the detection of tuberculosis (TB). Albeit an indirect method of detecting TB, this is often less time consuming than direct methods such as sputum culture and skin tests. Liu *et al.* developed an aptasensor for IFN- γ using a Fc-terminated aptamer and immobilised this onto magnetic nanobeads through streptavidin-biotin chemistry. The beads were then immobilised inside a microfluidic chip under the application of a magnetic field, forming an OFF sensor i.e., the signal decreased upon target binding. The E-AB was able to detect IFN- γ in the range of 10-500 pg/mL *in vitro*. The binding affinity of the aptamer for IFN- γ was $2.1 \times 10^{10} \text{ M}^{-1}$. To probe whether the sensor was an ON or OFF sensor, Liu *et al.* carried out chronoamperometry scans at a constant potential of +0.2 V, close to the oxidation potential of the Fc-labelled IFN- γ aptamer, reporting that the current response was lower after target-binding, which confirmed that the E-AB was indeed an OFF sensor. In addition, the study also tested the E-AB with common interferents present in blood, such as bovine serum albumin (BSA), prostate specific antigen (PSA), carbohydrate antigen 125 (CA-125), immunoglobulin G (IgG), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF- α), and each produced less than 20% of the original signal produced during IFN- γ binding.

Other continuous monitoring aptasensors include those developed for therapeutic drug monitoring, such as the microneedle-based E-AB developed by Wu *et al.* for tobramycin, wherein the MB-terminated aptamer was immobilised onto the gold microneedles and the sensor tested *in vivo* in rat interstitial fluid (ISF).²¹⁴ Herein, it was shown that the microneedle-based tobramycin E-AB behaved as an OFF sensor at 150 Hz and as an ON sensor at 800 Hz. Wu *et al.* then used the same 3D-printed microneedle platform to develop a multiplex E-AB for doxorubicin and irinotecan – which are therapeutic drugs for leukaemia and Hodgkin's lymphoma – using aptamers for both targets. It was demonstrated that the microneedle E-AB platform could be used for *in vivo* continuous drug monitoring (CDM) of tobramycin in mice, finding a rise in tobramycin concentrations after drug administration and a fall that was similar to the expected half-life of the drug.²¹⁴

Another CDM application for an E-AB was developed by Ferguson *et al.* for doxorubicin (DOX) and kanamycin, wherein aptamers for these targets were immobilised inside a Microfluidic Electrochemical Detector for *In vivo* Continuous monitoring (MEDIC).²¹⁵ Inside the microfluidic chip, the MB-terminated aptamers are immobilised onto gold electrodes and there exists a continuous-flow diffusion filter which prevents large molecules and red blood cells from reaching the sensing site, preventing sensor occlusion. The E-AB was tested in buffer initially, producing a dynamic range of 0.1–10 μM DOX. To be able to measure at a temporal resolution that allows the detection of the changes in DOX concentrations *in vivo* in humans, the k_{on} and k_{off} rates of the DOX aptamer were studied, showing that the aptamer reached 90% saturation within 45s at a concentration of 600 nM DOX, and that it returned to 10% within 100s. The clearance rate of DOX in humans is between 6 and 26 mins, meaning that the temporal response of the sensor is compatible with the DOX clearance. The sensor was also tested with

live rats wherein the rats were injected with incremental doses of DOX and a placebo dose; rats given the real dose produced a response and the placebo rats didn't produce a response. However, it is worth noting that for E-ABs monitoring drugs, it is also important to measure drug metabolites or complexes of the drug with other proteins *in vivo*, which may be missed by the aptamer if only binding to the drug molecule.

Other aptamer sensors that monitor analytes continuously include the E-AB developed by Swensen *et al.* for the real-time continuous monitoring of cocaine.²¹⁶ Although not tested *in vivo*, the sensor demonstrated an increased signal response *in vitro* in continuously flowing undiluted bovine blood serum containing cocaine. The aptamer for cocaine was immobilised onto gold working electrodes in a microfluidic chip, and bovine blood serum flowed into the chamber containing the electrodes for voltametric detection. This produced a continuous concentration vs time plot, as shown in Figure 3.6. Long exposure time of the sensor to the serum caused a loss in signal after three hours, but for the purpose of this investigation, the half-life of cocaine in serum is 60-90 minutes, which is well under the 3-hour mark.²¹⁶

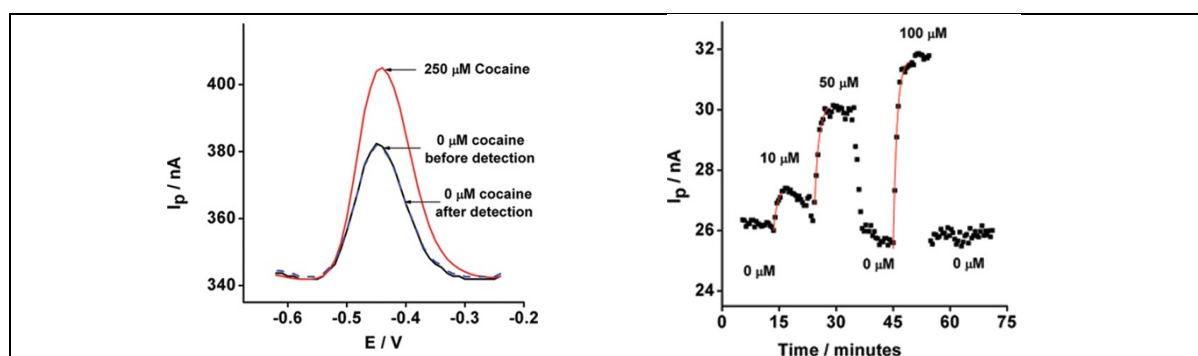


Figure 3.6 (a) The E-AB developed by Swensen *et al.* for cocaine detection is an ON sensor, (b)

Continuous monitoring of cocaine in bovine blood serum using an E-AB developed by Swensen *et al.*²¹⁶

Another continuous monitoring sensor was the wearable E-AB developed for cortisol monitoring by Singh *et al.* using a pseudoknot aptamer.²¹⁷ The wearable sensor was a flexible microneedle patch that monitors cortisol in sweat. The addition of the pseudoknot to the aptamer configuration was to restrict the aptamer to two states of configuration. Gold was sputtered onto polydimethylsiloxane (PDMS) to create the working electrodes and MB-terminated aptamer for cortisol immobilised onto the gold in the flexible microfluidic patch. The sensor was tested *in vivo*, and cortisol generated by participants doing exercise with the cold pressure test, demonstrating an induced stress response which was captured by the wearable sensors on the skin as a rapid increase in the current response.

3.1.6 Benefits of using aptamers in continuous monitoring

There are several benefits of using aptamers in continuous monitoring biosensors; one being their long half-life *in vivo*, meaning that they do not degrade quickly. They are also non-toxic and can be produced at scale due to being synthetically produced molecules. The affinity of the aptamer can be fine-tuned through SELEX and ELONA assays, and the region of the aptamer sequence where the target binds can be determined for further optimisation. Aptamers can be developed for a range of target molecules, including non-redox molecules, such as small molecules, peptides or proteins, and this versatility makes aptamers an attractive option for biosensor development.

3.1.7 The importance of binding affinity in continuous monitoring aptasensors

The binding affinity of an aptamer defines how strongly the aptamer binds to its target. The binding affinity constant, K_D , is a measure of the k_{on} and k_{off} rates, which indicate the rate at which an aptamer binds its target and the rate at which it unbinds the target on regeneration. Ideally, for continuous sensing, the binding affinity would be high enough that the aptamer binds its target selectively for the detection to occur, but not so strongly such that it is difficult to regenerate the aptamer quickly, for the next measurement. The regeneration and binding of the target needs to occur rapidly for the sensor to monitor changes in concentration. Without being able to regenerate quickly enough, important concentration events may be missed *in vivo*. Therefore, the greater the k_{off} rate, the greater the binding affinity of the aptamer. The K_D values of aptamers range from the pM to nM range and for continuous sensing, it is important that the aptamer is able to regenerate to capture the changing concentration of the target molecules *in vivo*. To measure concentrations of value, the K_D of the aptamer needs to be greater than the upper end of the dynamic range of the concentration range being measured in, as demonstrated in Figure 3.7.

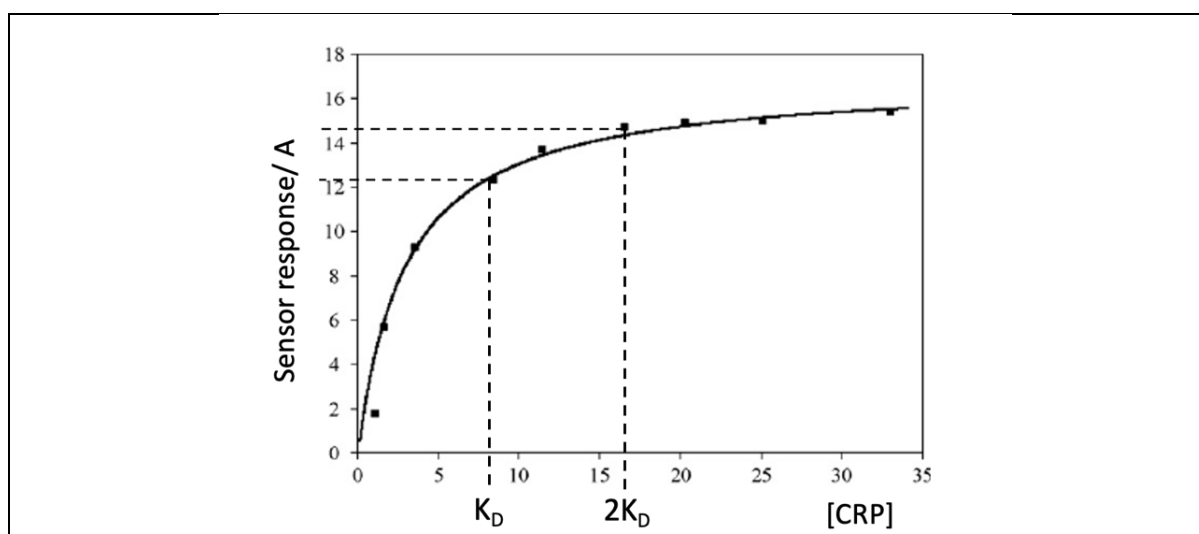


Figure 3.7 Ideal K_D of an aptamer, adapted from Dogan *et al.*²¹⁸

In terms of the temporal resolution requirements of the sensor, we recall that continuous measurement of systemic biomarkers does not need to exceed one measurement per minute (Chapter 1, section 1.5.1). CRP concentrations are seen to change over the course of days and reported studies have taken one measurement per day.^{219,220} However, to see small changes which may be used to optimise drug dose, a higher temporal resolution will be needed but this will not exceed one per minute.

3.1.8 Continuous Infection Monitoring (CIM) using E-ABs

Infection monitoring, like continuous drug monitoring, involves the real-time monitoring of biomarkers of infection to (i) understand infection levels in response to therapeutic intervention and optimise drug dosing based on the patient's individual response to a drug, (ii) combined with continuous drug monitoring sensors, to build a holistic picture of the patient's response to a drug and the concentration of the drug *in vivo*, and (iii) to detect early signs of infection, particularly in critical care, where sepsis can easily be missed.

There are E-ABs that detect a specific infectious disease, such as tuberculosis or malaria, and others that detect markers of infection, such as lactate, CRP, procalcitonin and cytokines.

CRP is an inflammatory biomarker produced by the liver and has been shown to be an important biomarker in detecting infections and sepsis. There are several biosensors that use antibodies to detect CRP.^{221–223} CRP sensing using aptamers include optical sensing and colourimetric assays. Ghosh et al. developed an optical aptamer sensor for CRP wherein a CRP aptamer was immobilised onto quantum dots using EDC/NHS chemistry. The other end of the aptamer was functionalised with thiol group and attached onto gold nanoparticles. Once

bound to CRP, the aptamer folding causes the quantum dot to be pulled nearer to the gold nanoparticle, which quenches fluorescence.²²⁴ Antonio *et al.* developed an aptamer based colourimetric assay for CRP, wherein the aptamer was adsorbed onto citrate-capped gold nanoparticles (AuNPs). On the addition of CRP saline buffer, the aptamer desorbs from the AuNPs and binds to the CRP, leaving the AuNPs bare in solution. In response, the Au-NPs form aggregates due to the presence of salts, and this causes a colour change from red to blue-purple, which is monitored using UV-Vis spectroscopy at different concentrations of CRP.²²⁵

Lactate is a validated biomarker of sepsis and several enzymatic electrochemical sensors have been developed for lactate.^{63,106,150} While there are no E-ABs developed for lactate – due to the popularity of lactate oxidase (LOD) and lactate dehydrogenase (LDH) based electrochemical sensors – there are E-ABs developed for LDH for malarial detection. Malaria is viral infection that kills over 600,000 people globally, disproportionately affecting low- and middle-income countries (LMIC). Lactate dehydrogenase (LDH) produced by *Plasmodium falciparum*, the mosquito vector, is a biomarker of malaria and is structurally distinct from human LDH. Several electrochemical sensors have been developed for *Plasmodium falciparum* LDH (PfLDH) to detect malaria, such as the E-AB developed by Jain et al. wherein a glassy carbon electrode was modified with graphene oxide to improve hydrophilicity, and an aptamer for PfLDH immobilised onto this.²²⁶ The PfLDH aptamer captures the PfLDH enzyme which oxidises lactate to pyruvate whilst reducing the co-factor Nicotinamide Adenine Dinucleotide (NAD⁺) to NADH. The generated NADH is then oxidised at +0.65 V and this signal response is measured against increasing lactate concentrations, to produce an ON sensor.

Galactomannan is a polysaccharide present in the cell walls of fungi and is therefore a biomarker of fungal infections such as invasive pulmonary aspergillosis (IPA), caused by *Aspergillus fungi*. IPA is of particular interest to detect early as it can be fatal for patients with primary infections such as tuberculosis and COVID-19; IPA had a notably higher incidence in patients with severe COVID-19 infection who were put on ventilation. Alenichev et al. developed a biosensor for galactomannan using dynamic light scattering (DLS).²²⁷

Procalcitonin (PCT) is a pre-cursor of calcitonin, a hormone involved in the regulation of calcium. PCT is produced in response to bacterial infections and has been used as a biomarker in the detection of sepsis and infectious diseases. PCT has also been studied as a marker in guiding antibiotic therapy in patients,²²⁸ and in monitoring COVID-19 patients, indicating that mortality was associated with high PCT levels.²²⁹ Pidal *et al.* developed an electrochemical immunosensor for the detection of PCT wherein the aptamer was modified with Alexa Fluor-405, a fluorescent label.²³⁰ The aptamer was immobilised onto graphene oxide (GO) and upon binding to PCT, the fluorescence was quenched, and the aptamer released from the GO surface. The quenching of the fluorescence was correlated with the concentration of the PCT, and good concordance was observed between concentrations obtained using the aptasensor and concentration of PCT deduced from a hospital-run test of patient samples.

3.1.9 Why CRP?

CRP was chosen for the development of an E-AB to continuously monitor infection to guide antibiotic therapy due to studies showing rapid rise in CRP levels in acute infection and up to 1000-fold increase of the baseline CRP concentration. As sepsis shares symptoms with systemic inflammatory response syndrome (SIRS), and CRP levels do not rise in SIRS,

measuring CRP can be a way of detecting the early signs of sepsis if other symptoms are missed. It is worth noting that baseline CRP levels vary between individuals based on age, gender, weight, lipid levels, blood pressure, genetics, and lifestyle factors such as smoking.²³¹ In Caucasian individuals, baseline CRP is around 0.8 mg/L, and concentrations above 10 mg/L indicate the presence of infection and inflammation.

In a cohort of children being treated for osteoarticular bacterial infections in critical care, the group was given oral and intravenous antibiotics. In this cohort of 37 patients, the average CRP peak concentration was seen at 156.91 mg/L and the average concentration at the time of discharge was 24.94 mg/L. In 92% of these patients, there was a 50% decline in CRP concentration over four days of treatment.²³² In group of 314 geriatric patients, it was observed that CRP concentrations greater than 100 mg/L at the point of hospital admission were associated with 2.5 times greater chance of mortality.²³³

CRP has been studied as a marker of infection to guide antimicrobial therapy. In a group of patients with community-acquired pneumonia, a decline in serum CRP concentration by 60% after 3 days and 90% after 7 days was indicative of improved patient outcomes and good response to treatment.²³⁴ Comparing to initial concentrations of CRP i.e. the concentration upon hospital admission, with serial measurements of CRP thereafter, would be beneficial in aiding treatment decisions around which antibiotics to use and how long to continue therapy for. In another study of 94 patients, where 49 were serially tested for PCT and 45 serially tested for CRP, it was shown that both markers were helpful in determining when to stop antibiotic use.²³⁵ In addition, the length of antibiotic use was shorter in the CRP-tested group

compared to the PCT-tested group, indicating that CRP may be a better marker to reduce antibiotic usage to curb the development of bacterial resistance.

The half-life of CRP in plasma is between 18 to 20 hours,²³⁶ meaning that dynamic changes in CRP levels are observed over a span of days, which if measured continuously, would bring valuable insights to the decision-making process around antibiotic treatment and dosing.

Herein, the development of an E-AB for CRP is described for the continuous monitoring of infection *in vivo*. The aptamer used in the development of the biosensor was reported by Huang *et al.* and its sequence is outlined in section 1 (under Sensor Fabrication). The aptamer has high binding affinity – 3.51 nM – for CRP and was chosen for the development of this biosensor due to ease of synthesis and sensor development.

The CRP aptamer was modified using two approaches; (i) with a methylene blue redox reporter, and (ii) with a novel osmium redox complex, to compare signal responses from the two redox labels. The osmium compound was developed by Professor Tony Cass's Group in the Department of Chemistry, Imperial College London, and attached to an amine-terminated CRP aptamer through amide coupling and is described in chapter 4.

3.2 Methods

3.2.1 Electrodes and instrumentation

The electrodes used were one-time use, disposable gold electrodes manufactured by Respire Diagnostics (UK). The working electrode was gold, the reference electrode was Ag|AgCl (30% AgCl, 70%Ag ink, Sun Chemical, USA), and the counter electrode was gold. The exact chloride

concentration at the RDE was not known. The electrode is pictured, and its dimensions are outlined in Figure 3.8.

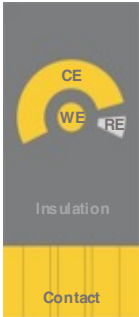
	Electrode	Surface area	Material
	WE	3.14 mm ²	Gold
	RE	2 mm ²	Ag AgCl
	CE	20 mm ²	Gold
	Contact	2 x 5 mm	Gold

Figure 3.8 Disposable sensors used in the fabrication of E-ABs (Respire Diagnostics, UK).

For all experiments in this chapter, unless otherwise stated, the PalmSens Emstat potentiostat by PalmSens BV GMBH (Germany) was used to run any electrochemical readings. Where stated, a CompactStat potentiostat by Ivium technologies (the Netherlands) was used. The software used to record measurements on the PalmSens Emstat was the PSTrace software and on the CompactStat, it was the IviumSoft software.

3.2.2 Filtering, smoothing and baseline correction

A Savitzky-Golay filter was applied to smooth the CV, DPV and SWV scan data in the PSTrace software (PalmSens, UK). The Savitzky-Golay smoothing method is a type of least squares fit wherein low order polynomials are used to smooth a set of data points. The level of smoothing is controlled by the order of the polynomial used and the number of points in the window. The window size was chosen based on the level of noise in the data, with PSTrace offering “low”, “medium”, “high” and “very high” filtering options, corresponding to window sizes of 5, 9, 15 and 25, respectively. For DPV and SWV data, a window size of 9 was chosen, whereas for CV data, higher noise-to-signal was observed, therefore, a window size of 15 was

chosen. After smoothing, the baseline was corrected on PSTrace software using the built-in linear baseline correction function. Two points were chosen on each scan to determine the baseline and a linear function was used for correction.

3.2.3 Synthesis of aptamers from Integrated DNA Technologies (IDT)

The aptamer developed against C-reactive protein (CRP) by Huang *et al.* was synthesized by (or purchased from IDT with modifications at the 5' and 3' ends to incorporate a thiol group for attachment to the gold electrodes and a redox reporter group (methylene blue or osmium) for electron transfer to the electrode. For the methylene blue-terminated aptamer, the methylene blue group was conjugated at the 3' end and the thiol at the 5' end.

Sequence published for CRP aptamer by Huang *et al.*¹⁷³:

5'-GG CAG GAA GAC AAA CAC GAT GGG GGG GTA TGA TTT GAT GTG GTT GTT GCA TGA TCG
TGG TCT GTG GTG CTG T-3'

Sequence of methylene blue-terminated CRP aptamer:

5'-/5ThioMC6-D/GG CAG GAA GAC AAA CAC GAT GGG GGG GTA TGA TTT GAT GTG GTT GTT
GCA TGA TCG TGG TCT GTG GTG CTG T/3MeBIN/-3'

3.2.4 3D modelling aptamer structure

The 2D secondary structure of the aptamer was derived using the aptamer sequence in the mfold software.^{237,238} The aptamer folding in mfold was generated under standard binding buffer conditions i.e. at 25° C, [Na⁺] = 137 mM, [Mg²⁺] = 2 mM. For each of the generated 2D structures, mfold reported an associated Gibbs free energy (ΔG) of self-hybridisation.²³⁹ The

three secondary structures with the lowest ΔG of self-hybridisation were selected by mfold due to being the most thermodynamically favourable.

Next, using the .ct files obtained from mfold, a 3D RNA structure was obtained using the RNAComposer server using a modelling technique developed by Ben Aissa *et al.*^{240,241} This is because there is no mean obtained from the 2D structure of the ssDNA from mfold, therefore the structure is treated as “mutated RNA” to obtain a 3D structure. The server assumed the primary structure of the DNA in its software to be that of RNA, such that every thymine base would now bind an uracil base, and the deoxyribose backbone was assumed to be made of ribose. Next, the 3D RNA structure was put into the Web 3DNA server, and a mutated 3D RNA structure was obtained.²⁴²

Using the interactive and graphical Visual Molecular Dynamics (VMD) software,²⁴³ the mutated 3D RNA structure was converted into a 3D DNA structure. Finally, the stability of the 3D DNA structure was improved by running it through the Nanoscale Molecular Dynamics (NAMD). The 3D structures of the ssDNA aptamers were then plotted for each of the three possible structures obtained from mfold, via the process outlined here (as shown in Figure 3.9), developed by Ben Aissa *et al.*²⁴⁴

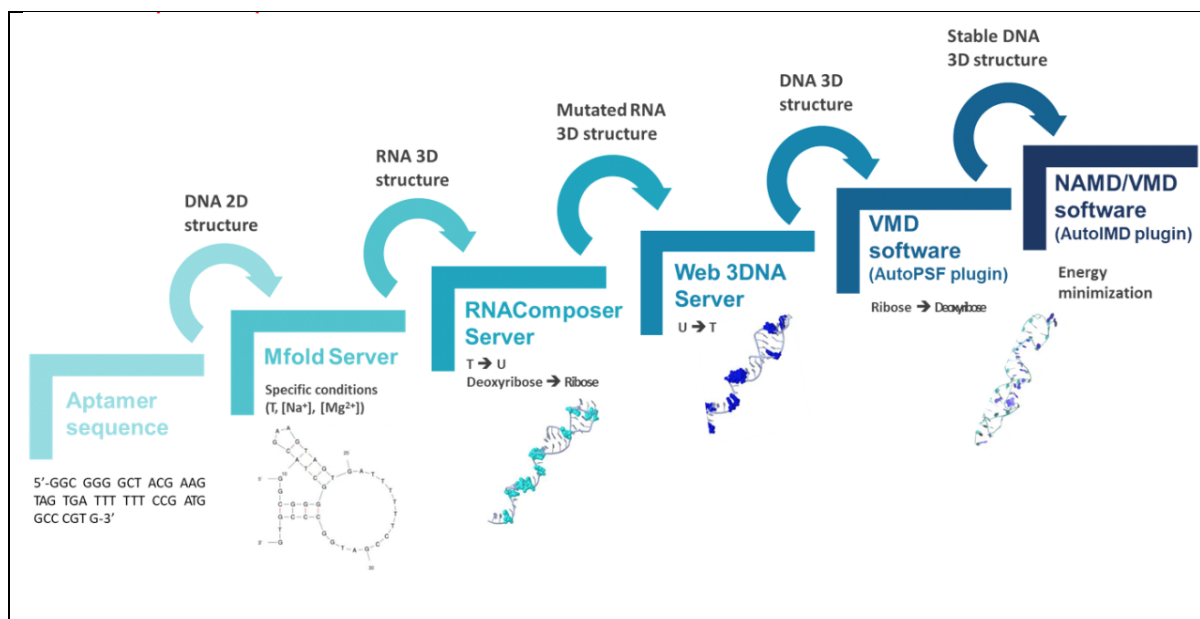


Figure 3.9 Aptamer 3D modelling technique developed by Ben Aissa *et al.*²⁴⁴

3.2.5 Electrode pre-treatment

Disposable gold electrodes (Respire Diagnostics Ltd) were rinsed with DI water and electrochemically cleaned by running cyclic voltammetry in 20 μL of 0.5 M H_2SO_4 for 10 cycles at a scan rate of 100 mV/s. Cyclic voltammetry was conducted between -0.2 V and +1.3 V vs Ag|AgCl, starting at 0 V. Following this pre-treatment, electrodes were stored in aptamer binding buffer (100 mM PBS with 2 mM MgCl_2). Figure 3.10 shows a typical voltammogram of the disposable gold electrode in 0.5 M H_2SO_4 , cycled 10 times. Peaks are visible at +0.64 V (reduction) and +0.94 (oxidation) vs Ag|AgCl. The area under the reduction peak, which corresponds to the reduction of gold oxide at the surface of the electrode, was calculated and divided by the scan rate (100 mV/s) to obtain the charge passed. The capacitance was then calculated using the charge passed, as demonstrated in Equation 3.3, where C is the measured capacitance at the electrode of interest (in μF), $\int I dt$ is the charge passed (in μC), and dE is the change in potential applied at the electrode (in V). As reported by Trassati *et al.*, Equation 3.4 was used to calculate the true surface area of the electrode by the ratio of

the capacitances,²⁴⁵ where C^* is the reference capacitance for the true unit surface area of an oxide,²⁴⁵ C is the measured capacitance at the electrode of interest, and A is the true surface area of the electrode. C^* was assumed to be $60 \mu\text{F cm}^{-2}$, as per Trassati *et al.*²⁴⁵ For the disposable gold electrodes studied herein, the true surface area was calculated to be 0.77 cm^2 or 77 mm^2 which is much higher than the surface area value reported by the electrode manufacturers of 3.14 mm^2 .

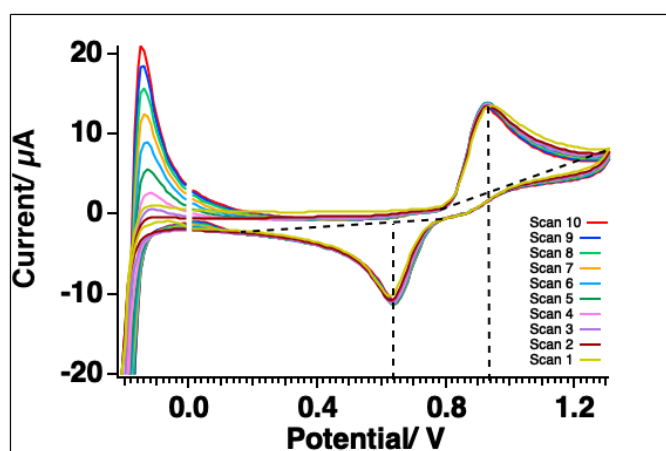


Figure 3.10 Cyclic voltammogram of a disposable gold electrode (Respire Diagnostics Ltd, UK) in $0.5 \text{ M H}_2\text{SO}_4$, being cycled at a scan rate of 100 mV/s for 10 cycles.

$$C = \frac{I dt}{dE}$$

Equation 3.3 Capacitance as a function of the charge passed and the change in potential.

$$A = \frac{C}{C^*}$$

Equation 3.4 The true surface area of an electrode.

3.2.6 Deposition of aptamer onto electrodes

The lyophilized aptamer was reconstituted in sterile DI water volume to a concentration of $100 \mu\text{M}$. A 1:1 v/v dilution of aptamer solution ($100 \mu\text{M}$) with 20 mM tris(2-

carboxyethyl)phosphine (TCEP) solution was prepared for a final aptamer concentration of 50 μM and 10 mM TCEP (i.e. TCEP concentration at 200x excess). This mixture was incubated at room temperature for 1 hour to allow the thiol group on the aptamer to reduce for bonding to gold.

Following reduction, the aptamer was diluted to the desired concentration (between 1 and 10 μM) in binding buffer and a temperature pre-treatment was performed before immobilisation. The aptamer solution was heated at 95 $^{\circ}\text{C}$ for 8 min in a thermocycler and then removed to cool down at room temperature for 20 mins. The tubes containing the aptamer solution were covered with aluminium foil due to the redox labels being sensitive to light.

Once cooled, 10 μL of the aptamer solution was pipetted onto the working gold electrode and incubated in a humidity chamber in the fridge overnight at 4 $^{\circ}\text{C}$. Following overnight incubation of the aptamer, the aptamer solution was rinsed off using binding buffer and 10 μL of 2 mM 6-mercapto-hexanol (6-MCH) was pipetted onto the gold working electrode and incubated overnight in a humidity chamber in the fridge at 4 $^{\circ}\text{C}$. Following overnight incubation of the 6-MCH, the 6-MCH solution was rinsed off the electrode using binding buffer and the electrode stored in binding buffer until further use. The 6-MCH is used as back-filler or blocking reagent used to block the unoccupied binding sites of the gold surface forming a self-assembled monolayer (SAM).

3.2.7 Sensor characterisation

The objectives behind electrochemical characterisation of the sensors is to (i) find the potential at which a conformational change occurs in the aptamer, (ii) find whether the aptamer has successfully deposited onto the electrode, (iii) calculate the quantity of deposited aptamer, (iv) find if there is a concentration dependent relationship with signal response, and (v) optimise the fabrication process and conditions under which the sensor is characterised for performance testing.

3.2.8 Cyclic Voltammetry

Cyclic voltammetry is a commonly used electrochemical technique in which the potential is scanned linearly at a specific scan rate ' v ' (in mV/s) from E_{vertex1} to E_{vertex2} , and vice versa, as shown in Figure 3.11. The resulting current is measured as a function of potential. If $E_{\text{vertex1}} < E_{\text{vertex2}}$, the scan is an oxidation scan e.g., scanning from 0.1 to 0.4V. If $E_{\text{vertex1}} > E_{\text{vertex2}}$, the scan is a reduction scan (e.g., from 0.4 V to 0.1 V). The forward and reverse scan (oxidation and reduction, or vice versa) is one cycle of a CV scan. Methylene blue is known to have a redox potential of -0.25 V, therefore, the potential range used during CV was between 0 V and -0.4 V vs Ag|AgCl. This was to ensure that there was no electrochemical reaction before the starting potential.

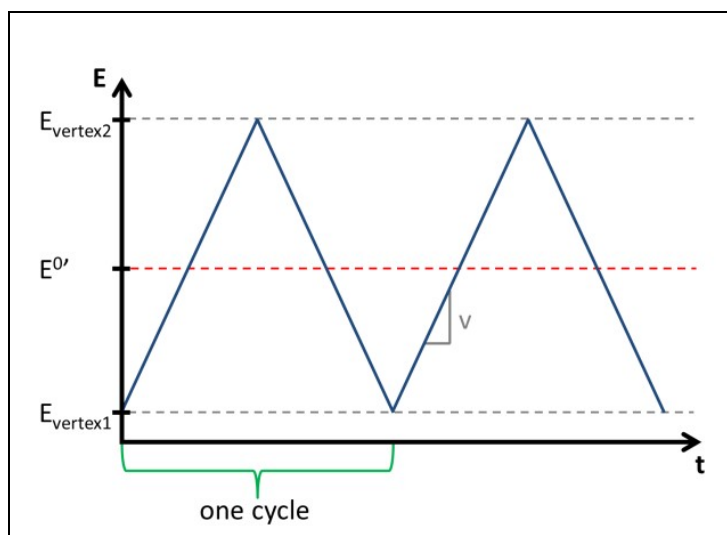


Figure 3.11 A CV scan consists of a forward and reverse scan, at a scan rate ' v ', from E_{vertex1} to E_{vertex2} .

In this thesis, CVs were conducted to calculate the surface coverage of aptamer on electrodes, to understand current dependence on scan rate (via the Randles-Sevcik plot), and to optimise scan rate with respect to signal response. To optimise response, CV scan rates were varied in the range of 25 mV/s to 400 mV/s and CV scans captured. The E_{step} used for each CV scan rate is outlined in Table 3.2.

Scan rate in CV (mV/s)	E_{step} (V)
10	0.0001
20	0.001
25	0.001
50	0.001
100	0.001
150	0.01
175	0.01
200	0.01
300	0.01
400	0.01
500	0.01

Table 3.2 Scan rate used during CV scans and the associated E_{step} for each scan rate.

3.2.9 Differential Pulse Voltammetry

In Differential Pulse Voltammetry (DPV), short pulses of 1-100 mV (E_{pulse}) are applied for 10-100ms (t_{pulse}). The pulse duration is short compared with the pulse interval, and the current is measured at the start (i_1) and end (i_2) of each pulse, and the difference is calculated ($i_2 - i_1$), as shown in Figure 3.12. The difference in current is plotted as a function of potential. The interval of a pulse, t_{int} , is calculated by dividing the E_{step} (mV) by the scan rate (mV s^{-1}). DPV is a more sensitive technique than linear sweep voltammetry due to the removal of capacitive current (i.e. charging current) by calculating the difference between the current obtained at the start and each of each potential pulse.

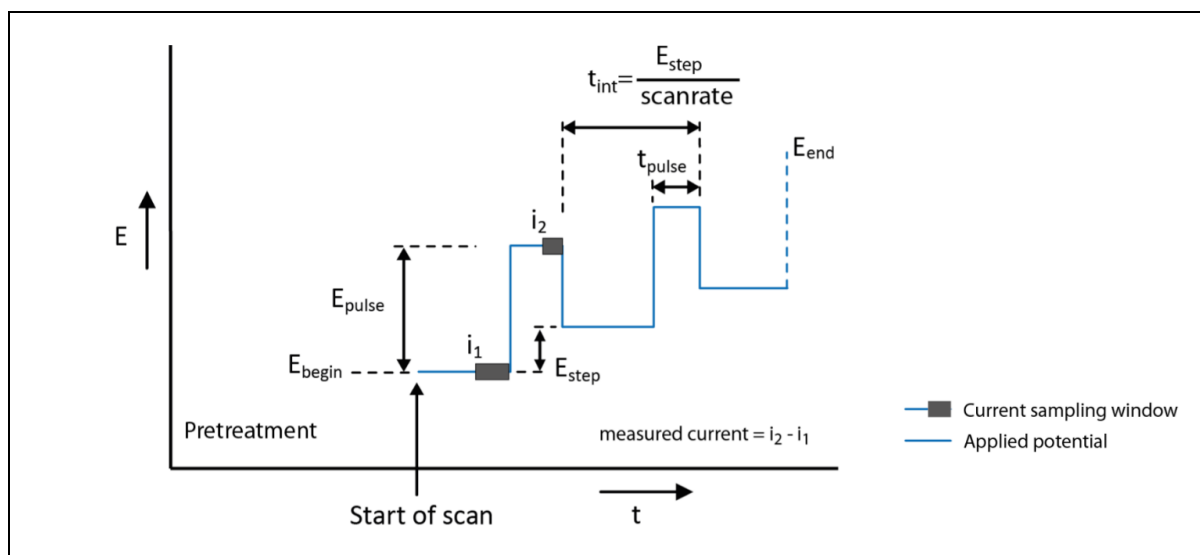


Figure 3.12 Current is measured at the start and end of each pulse in a different pulse voltammetry (DPV) scan; the size and rate of the pulses can be controlled.

To optimise signal response, DPV scan rates were varied in the range of 25 mV/s to 400 mV/s and DPV scans captured. The E_{pulse} used for each scan rate is outlined in Table 3.3.

Scan rate in DPV (mV/s)	E_{pulse} (V)
10	0.0001
20	0.001
25	0.001
50	0.001
100	0.001
150	0.01
175	0.01
200	0.01

Table 3.3 Scan rate used during DPV scans and the associated E_{pulse} for each scan rate.

3.2.10 Square Wave Voltammetry

Square Wave Voltammetry (SWV) is a form of DPV wherein t_{pulse} is equal to $t_{\text{int}}/2$. The t_{int} is the inverse of the frequency (Hz). SWV is a voltammetric technique in which a symmetrical square wave is superimposed on a base staircase potential, and the resulting square wave is applied to the working electrode, as shown in Figure 3.13. The resulting current is the difference between the forward current (i_{forward}), measured at the end of the forward-square wave pulse, and the reverse current (i_{reverse}), measured at the end of the return-square wave pulse.

To optimise signal response, the frequency used in SWV was varied in the range of 10 Hz to 100 Hz and SWV scans captured. For each frequency, an E_{step} of 0.001V (1 mV) was used.

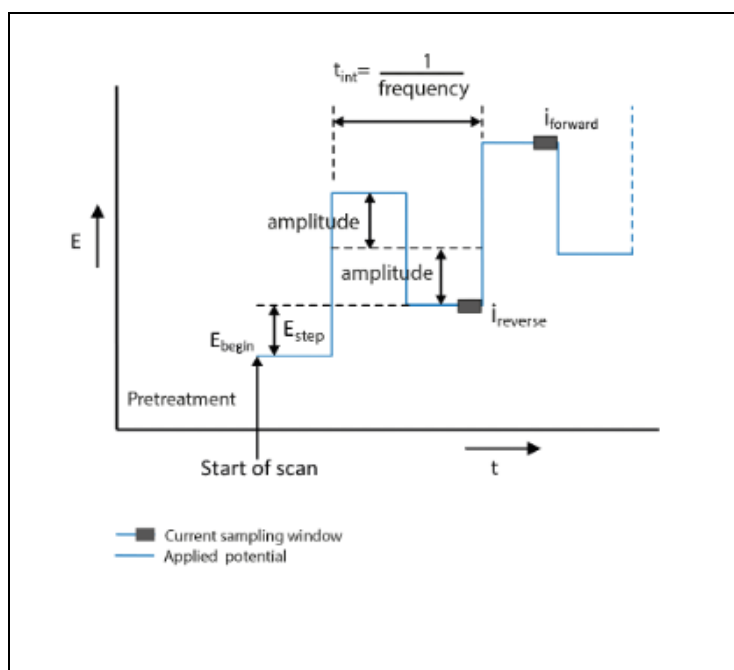


Figure 3.13 Superimposition of a symmetrical square wave onto a base staircase potential, resulting in a square wave that is applied to the working electrode, and the current response measured.

3.2.11 Chronoamperometry

Chronoamperometry is an electrochemical technique in which the potential is stepped at a constant rate (E_{dc}) between two potentials (E_1 to E_2), and the resulting current is measured at constant time intervals ($t_{interval}$), as demonstrated in Figure 3.14.

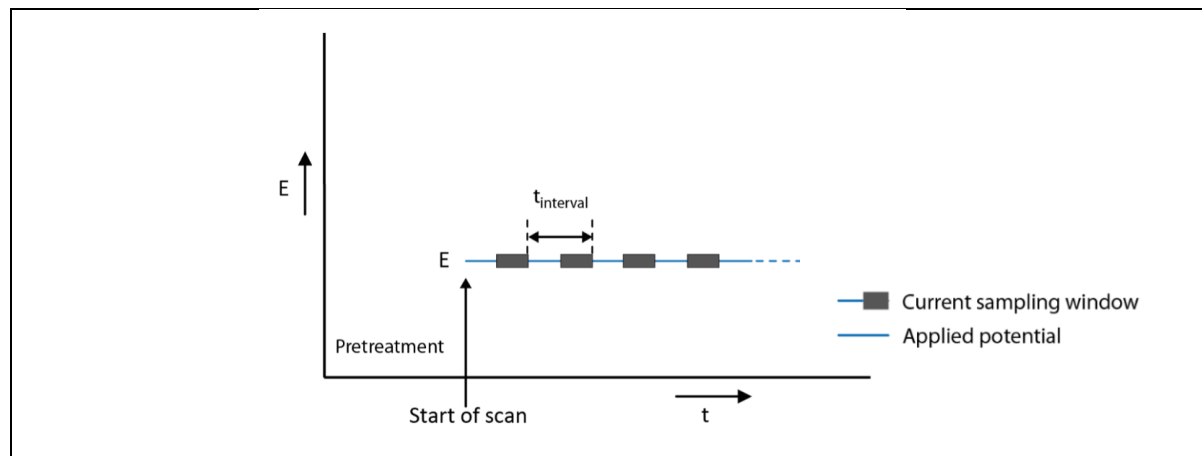


Figure 3.14 The application of a constant potential step, E_{dc} , at time interval t_{int} gives rise to a current response in chronoamperometry.

The potential range chosen for the chronoamperometry was based on the peaks obtained in the SWV scans and the sampling rate ($t_{interval}$) used was 0.001 s. The start (E_1) and end (E_2) potential of the entire oxidation or reduction peak was chosen as the potential range for the chronoamperometry scans. E_1 was chosen such that there is no electrode reaction at this potential and E_2 was chosen so that the desired reaction is so fast that the back reaction can be legitimately neglected. This is demonstrated in the Butler-Volmer equation, as shown in Equation 3.5, where i is current passing through the electrode (A), i_0 is the exchange current density (A), α_f and α_r are the forward (oxidation) and reverse (reduction) transfer coefficients, E is the potential difference across the electrode (V), E_0 is the equilibrium potential (V), n is the number of electrons, F is Faraday's constant ($C\ mol^{-1}$), R is the constant ($J\ K^{-1}\ mol^{-1}$) and T is the temperature (K).

$$i = i_0 \left\{ e^{\left[\frac{\alpha_f n F (E - E_0)}{RT} \right]} - e^{\left[\frac{-\alpha_r n F (E - E_0)}{RT} \right]} \right\}$$

Equation 3.5 Butler-Volmer equation.

Chronoamperometry measurements were recorded to compare the amount of charge passed through the electrode surface before (bare electrode) and after aptamer deposition, in addition to before (blank) and after CRP incubation. The charge was calculated by integrating the current with respect to time (t_1 to t_2), obtained from the oxidation and reduction chronoamperometry scans, as shown in Equation 3.6.

$$\text{Charge } (Q) = \int_{t_1}^{t_2} I \, dt$$

Equation 3.6 Calculation of charge from the integration of current with respect to time.

3.2.12 Aptamer concentration optimisation

Aptamer concentrations were made up to 1, 2.5, 5 and 10 μM by diluting with binding buffer and aptamer solutions were deposited onto electrodes using the deposition method above. Following sensor fabrication, sensors were interrogated using CV, DPV and SWV methods.

3.2.13 Sensor calibration

The aptasensor was tested electrochemically in binding buffer in the unbound state at a CRP concentration of 0 mg/L. The aptasensor was then incubated with 1, 10 and 100mg/L CRP in aptamer binding buffer (100 mM PBS with 2 mM MgCl_2) serially for an hour each time, before being rinsed off with aptamer binding buffer. After each incubation, the aptasensor was

tested electrochemically with CV, DPV and SWV techniques. Peak analysis was conducted for each scan wherein the baseline was corrected and if needed, a low robust LOESS filter applied, followed by a comparison of peak height, peak position, and peak-to-peak separation. A calibration curve was then plotted of peak current vs CRP concentration for each aptasensor.

3.3 Results

3.3.1 3D aptamer model

The mfold software was used to predict the 2D structure of the CRP aptamer using minimum energy requirement parameters, thereby plotting the most thermodynamically stable 2D structures of the ssDNA molecule, with respect to the random coil.^{237,238} Three thermodynamically stable 3D models of the aptamer sequence were developed using 2D structures from mfold and a 3D modelling technique developed by Ben Aissa *et al.*²⁴¹ The mfold software predicted three 2D configurations of the aptamer sequence, with an associated Gibbs free energy (ΔG) value.^{237,238} Configuration A (Figure 3.15(a)) was predicted to be the most thermodynamically stable at a ΔG of $-23.14 \text{ kJ mol}^{-1}$. Configuration B (Figure 3.15(b)) was the second-most thermodynamically stable ($\Delta G = -22.80 \text{ kJ mol}^{-1}$) and configuration C (Figure 3.15(c)) was the least thermodynamically stable of the three ($\Delta G = -19.83 \text{ kJ mol}^{-1}$). The differences between the three 3D structures were not significant due to the thermal energies being close to the -20 kJ mol^{-1} mark at room temperature. Therefore, we can expect a combination of these aptamer structures on the surface of electrodes after functionalisation.

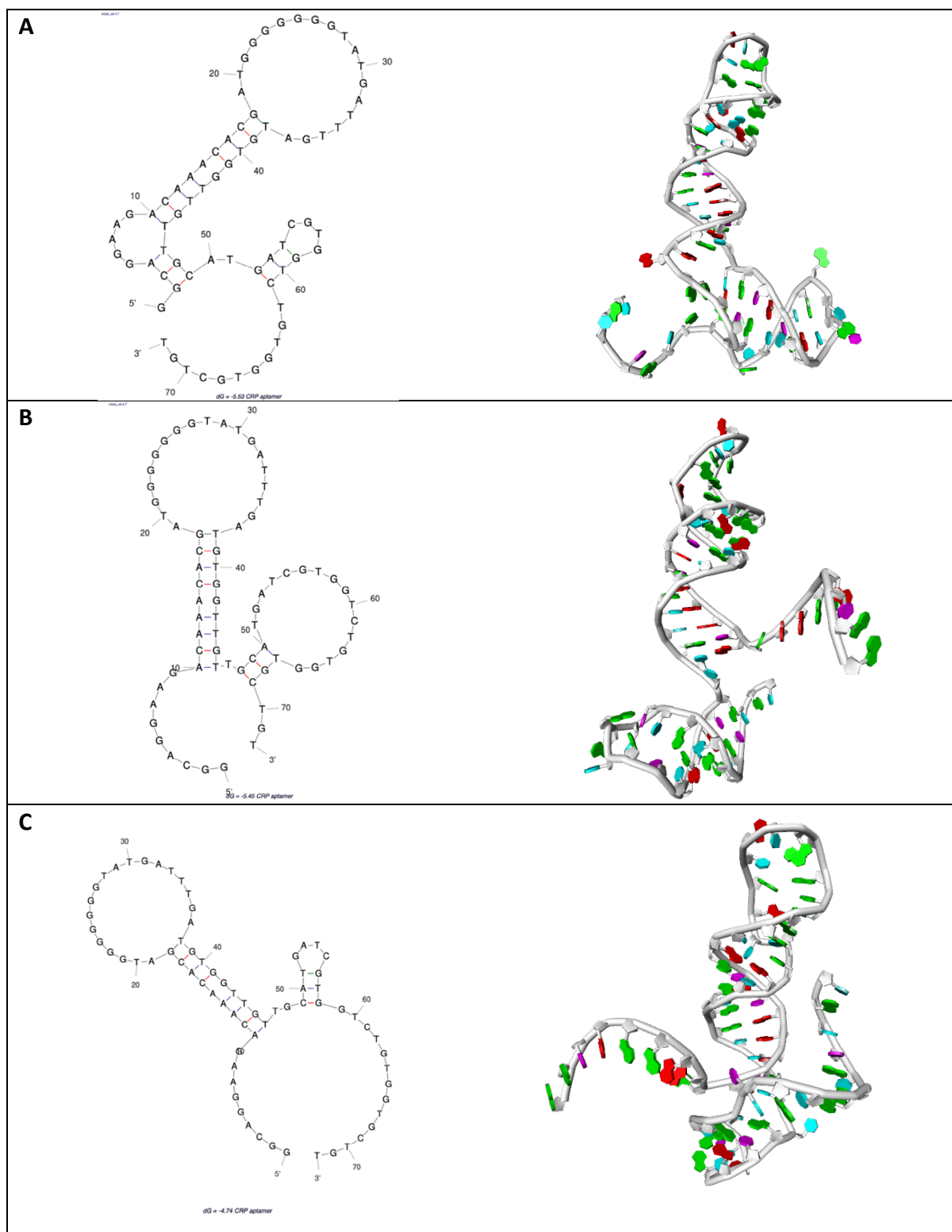


Figure 3.15 2D (left) and 3D structure (right) of the three most thermodynamically stable configurations of the CRP aptamer with thermal energies of (a) $\Delta G = -23.14$ kJ mol⁻¹, (b) $\Delta G =$

-22.80 kJ mol⁻¹ and (c) $\Delta G = -19.83$ kJ mol⁻¹. 3D modelling work completed by Ben Aissa *et al.*²⁴¹ (used with permission).

3.3.2 Sensor characterisation results

Once functionalised, the sensors were electrochemically interrogated at each step of the fabrication process to understand when the peaks arise. No DPV peaks were observed after immobilisation of the aptamer alone, the peak at -0.13 V was seen after the immobilisation of the 6-mercaptohexanol (6-MCH) back-filler on aptamer-functionalised electrodes (as shown in Figure 3.16(a)). The peak height of the DPV signal response in buffer (blank) after incubation with 1 mg/L CRP saw a decrease of 29% decrease. The peak position did not shift after incubation with CRP.

No SWV peaks were observed after immobilisation of the aptamer alone, the peak at -0.23 V occurred after the immobilisation of the 6-MCH back-filler on aptamer-functionalised electrodes (as shown in Figure 3.16(b)). The peak height of the SWV signal response after incubation with 1 mg/L CRP solution saw a decrease of 57%. The position of the peak shifted by +10 mV after incubation with CRP.²⁴⁶

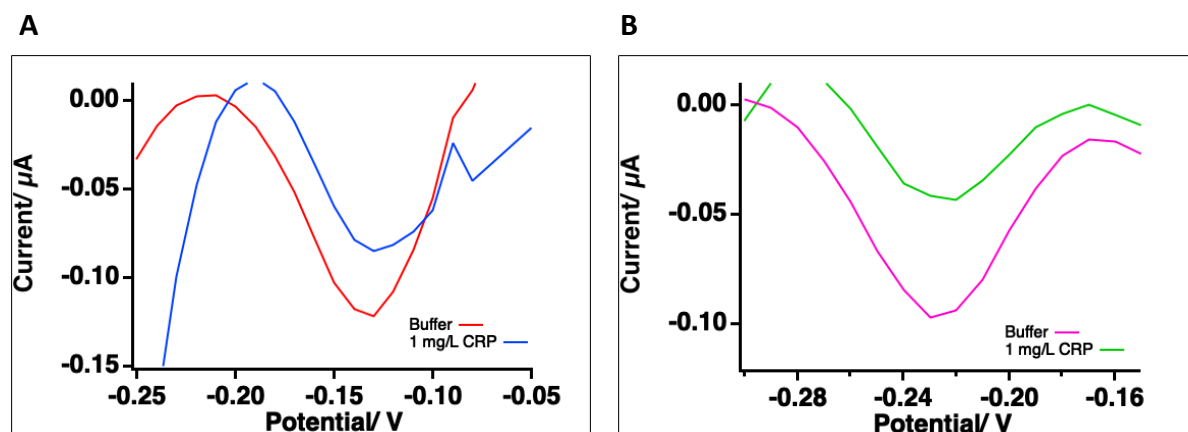


Figure 3.16 Sequential characterisation after aptamer and 6-MCH immobilisation in buffer, followed by incubation with 1 mg/L CRP solution by (a) DPV and (b) SWV (where buffer = blank reading) ($n=1$).

This response is in line with a signal OFF sensor and the lack of a response after functionalisation with aptamer only can be attributed to the aptamer folding over onto the surface of the electrode, and subsequently “standing up” once the back-filler has been immobilised to occupy free spaces, as is observed in other studies.²⁴⁶

3.3.3 Scan rate optimisation

The scan rate used in DPV scans was optimised with respect to the signal response from 10 to 25 mV/s. No anodic or cathodic peaks were visible when a scan rate of 10 mV/s ($E_{\text{pulse}} = 0.1$ mV) was used in DPV, as seen in Figure 3.17. There was an 87% increase in the anodic peak heights between the 20 and 25 mV/s and the peak positions were at -0.312 V and -0.318 V for signal responses at 20 and 25 mV/s, respectively. There was a peak shift of -6 mV upon increasing scan rate from 20 to 25 mV/s.

The cathodic signal response at the scan rate of 25 mV/s was 127% higher than that at 20 mV/s, and the peak positions were -0.281 and -0.278 V at 20 and 25 mV/s, respectively. There was a peak shift of +3 mV upon increasing scan rate from 20 to 25 mV/s. For scan rates greater than 100 mV/s, the E_{pulse} increased to 10 mV and beyond. Any pulse height greater than 1 mV produced poorer resolution of data. A scan rate of 25 mV/s was therefore ($E_{\text{pulse}} = 1 \text{ mV}$) was chosen for future CV interrogation of the E-ABs.

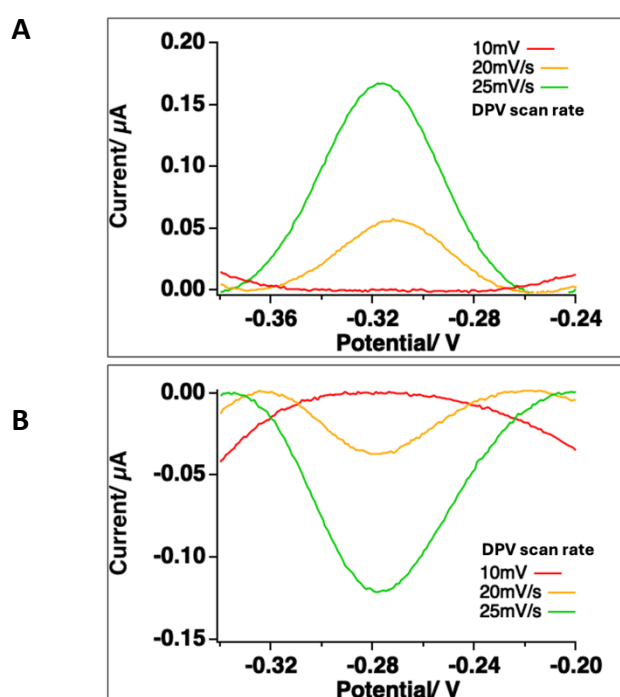


Figure 3.17 Optimisation of signal response by altering scan rates in DPV from 10 to 25 mV/s for (a) the anodic response and (b) cathodic response (n=1).

3.3.4 Current dependence on scan rate

To investigate the relationship between peak height and scan rate, the scan rate was increased from 20 to 200 mV/s (Figure 3.18). Both the anodic and cathodic peak heights of the CV increased as the scan rate was increased from 20 to 200 mV/s. Ohmic resistance was

present and can be seen via the linear slope of the CV scans in Figure 3.18(a). The ratio of peak heights at 25 mV/s:200 mV/s was 0.28 for the oxidation sweep and 0.02 for the reduction sweep. There was a peak-to-peak separation between the anodic and cathodic scans and these separations are listed in Table 3.4.

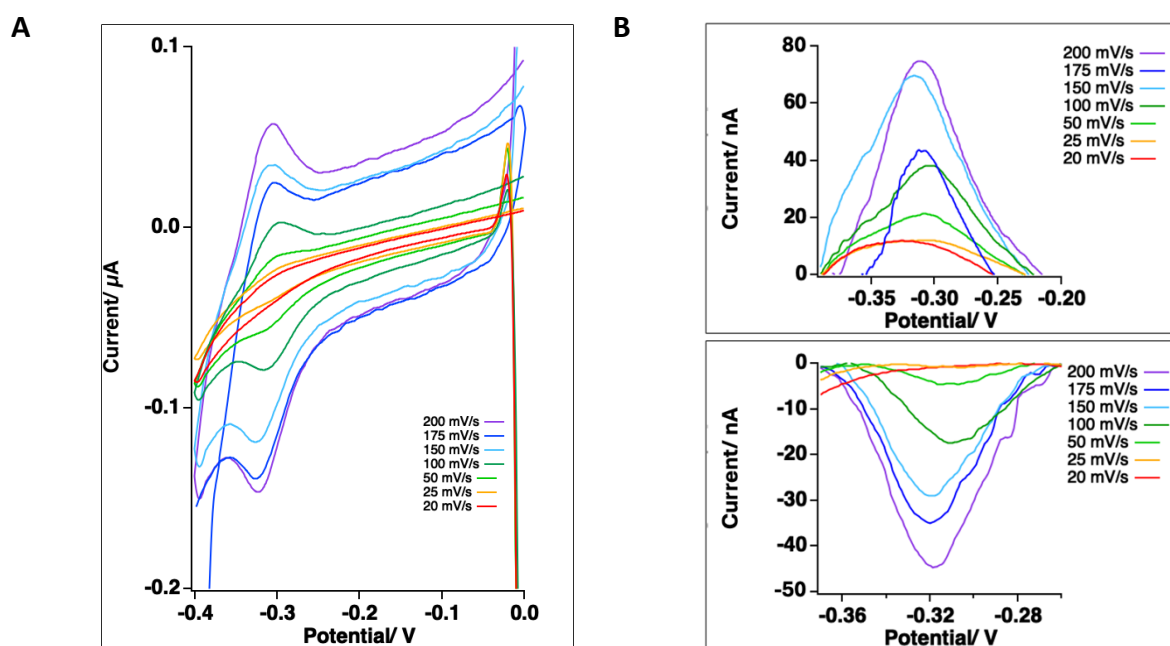


Figure 3.18 (a) Current dependence on scan rate in CV at scan rates of 20 to 200 mV/s ($n=1$), and (b) extracted CV peaks in the oxidation (top) and reduction (bottom) sweep ($n=1$).

Scan rate/ mV/s	Peak-to-peak separation/ mV
25	-3
50	-8
100	-9
150	-3
175	-12
200	-6

Table 3.4 Peak-to-peak separations in CV at each scan rate.

To explore the process occurring at the electrode, the data from the CV scans was fitted to the Randles-Sevcik equation (Equation 3.8), where I_p is peak height (A), n is the number of electrons in the reaction, F is Faraday's constant, A is surface area of the electrode (cm^2), C is the concentration of the redox species (mol cm^{-3}), v is the scan rate (V s^{-1}), D is the diffusion coefficient of the redox species ($\text{cm}^2 \text{s}^{-1}$), R is the gas constant and T is the temperature. The peak height was plotted against (i) the scan rate (Figure 3.19(a)) and (ii) the square root of scan rate (Figure 3.19(b)) and the log of peak height was plotted against the log of scan rate ($\log(I_p)$ vs $\log(v)$) (Figure 3.19(c)). All plots were fitted with a linear regression and slope, intercept and R^2 parameters are in Table 3.5.

$$I_p = 0.446nFAC\left(\frac{nFvD}{RT}\right)^{\frac{1}{2}}$$

Equation 3.7 The Randles-Sevcik equation.

In the plot of current against scan rate, a linear relationship is observed which confirms that the reaction is surface- or adsorption-controlled. A linear relationship between the peak height and the square root of the scan rate (i.e. the Randles-Sevcik plot) for an adsorbed redox species denotes that the reaction is quasi-reversible, meaning that the redox label is unable to oxidise and reduce to the same amount each time. This is, evidenced by the lack of symmetry in the Randles-Sevcik plot between the oxidation and reduction data. This means that the label is unable to fully oxidise or reduce to the same amount each time. To be fully reversible, the oxidation and reduction peaks need to be equal which isn't the case for MB-mediated electron transfer.

To further understand whether the reaction is quasi-reversible, we can examine the peak-to-peak separation of scans. For a quasi-reversible reaction, peak-to-peak separation changes with scan rate, whereas for a surface or adsorption-controlled reaction, no peak-to-peak separation exists.⁷² In the data seen here, there is a peak-to-peak separation, as seen in Figure 3.18(b) previously, which confirms quasi-reversibility.

The slope of the $\log(I_p)$ vs the $\log(v)$ determines whether the process is controlled by diffusion or adsorption. For an adsorption-controlled process, the slope is close to 1.0, whereas for a diffusion-controlled process, this is close to 0.5.²⁴⁷ As shown in Figure 20(c), the relationship between $\log(I_p)$ and $\log(v)$ is linear for both oxidation and reduction. The slope of the oxidation scan is 0.83 ± 0.12 , which is close to 1.0, meaning that the process is adsorption-controlled, as expected for E-ABs. The slope of the reduction scan is higher than 1.0 – i.e. 1.85 ± 0.11 – therefore it is difficult to interpret.

Table 3.5 outlines the parameters of the linear fits from the peak height against scan rate plot, the Randles-Sevcik plot, and the log of peak height log of scan rate.

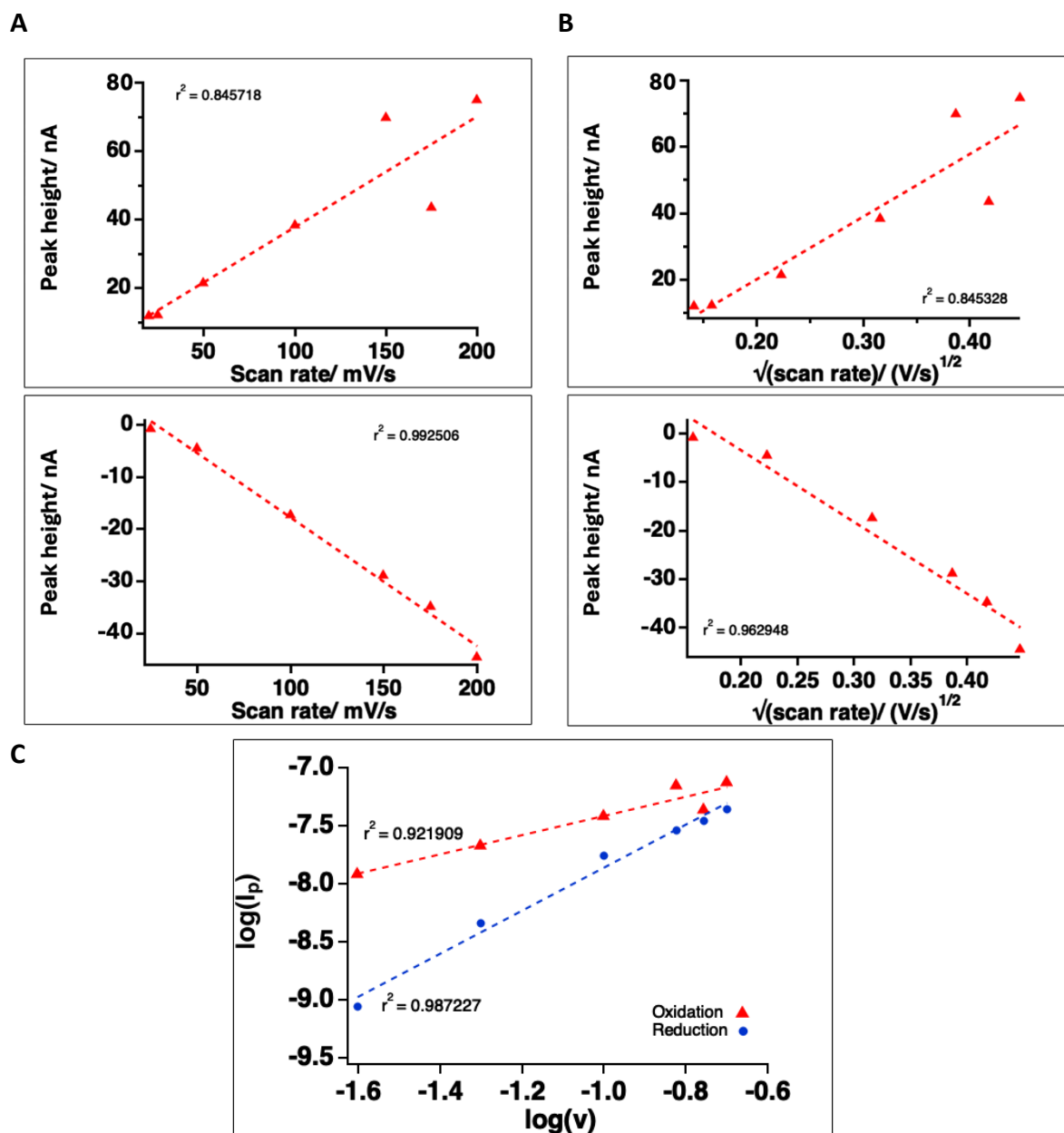


Figure 3.19 (a) Peak height against scan rate in anodic (top) and cathodic (bottom) scans, (b) Randles-Sevcik plot i.e. peak height against the square root of scan rate in anodic (top) and cathodic (bottom) scans, and (c) log of peak height log of scan rate.

Plot	Scan	Slope	Intercept	r ²
Peak height vs scan rate	Anodic	(32.24 ± 6.16) x 10 ⁻⁵	(56.48 ± 76) x 10 ⁻⁴	0.845718
	Cathodic	(-24.57 ± 1.07) x 10 ⁻⁵	(67.64 ± 14.2) x 10 ⁻⁴	0.992506
Peak height vs √(scan rate)	Anodic	0.19 ± 0.04	-0.018 ± 0.012	0.845328
	Cathodic	-0.15 ± 0.01	0.026 ± 0.005	0.962948
log(I _p) vs log(v)	Anodic	0.83 ± 0.12	-6.59 ± 0.13	0.921909
	Cathodic	1.8463 ± 0.105	-6.013 ± 0.113	0.987227

Table 3.5 Parameters of linear relationship between peak height and scan rate, peak height and square root of scan rate, and log of peak height vs log of scan rate.

3.3.5 Frequency optimisation

Frequency was increased from 1 to 150 Hz and the peak currents recorded (Figure 3.20). No peaks were visible at a frequency of 1 Hz and a secondary peak was visible at a potential of -0.35 V, in addition to the primary MB peak, at frequencies higher than 30 Hz. This is expected to come from dissolved oxygen species in the solution as this was not de-gassed during the experiment. Therefore, only the peak coming from the MB-terminated aptamer at -0.20 V was extracted and analysed.

The peak heights of the SWV scans scaled with frequency. A 15-fold increase in peak currents was seen from 10 Hz to 30 Hz, and the anodic peak position at 10, 20 and 30 Hz were -0.201, -0.203 and -0.208 V, respectively. There was a peak shift of -7 mV as the scan rate was increased. The cathodic peak positions at frequencies of 10, 20 and 30 Hz were -0.203, -0.205

and $-0.209\ \mu\text{A}$, respectively. There was a peak shift of $-6\ \text{mV}$ as the scan rate was increased from 10 to 30 Hz. The frequency used in SWV scans for the CRP aptasensor characterisation was optimised at 30 Hz to prevent the second peak from contributing to the signal.

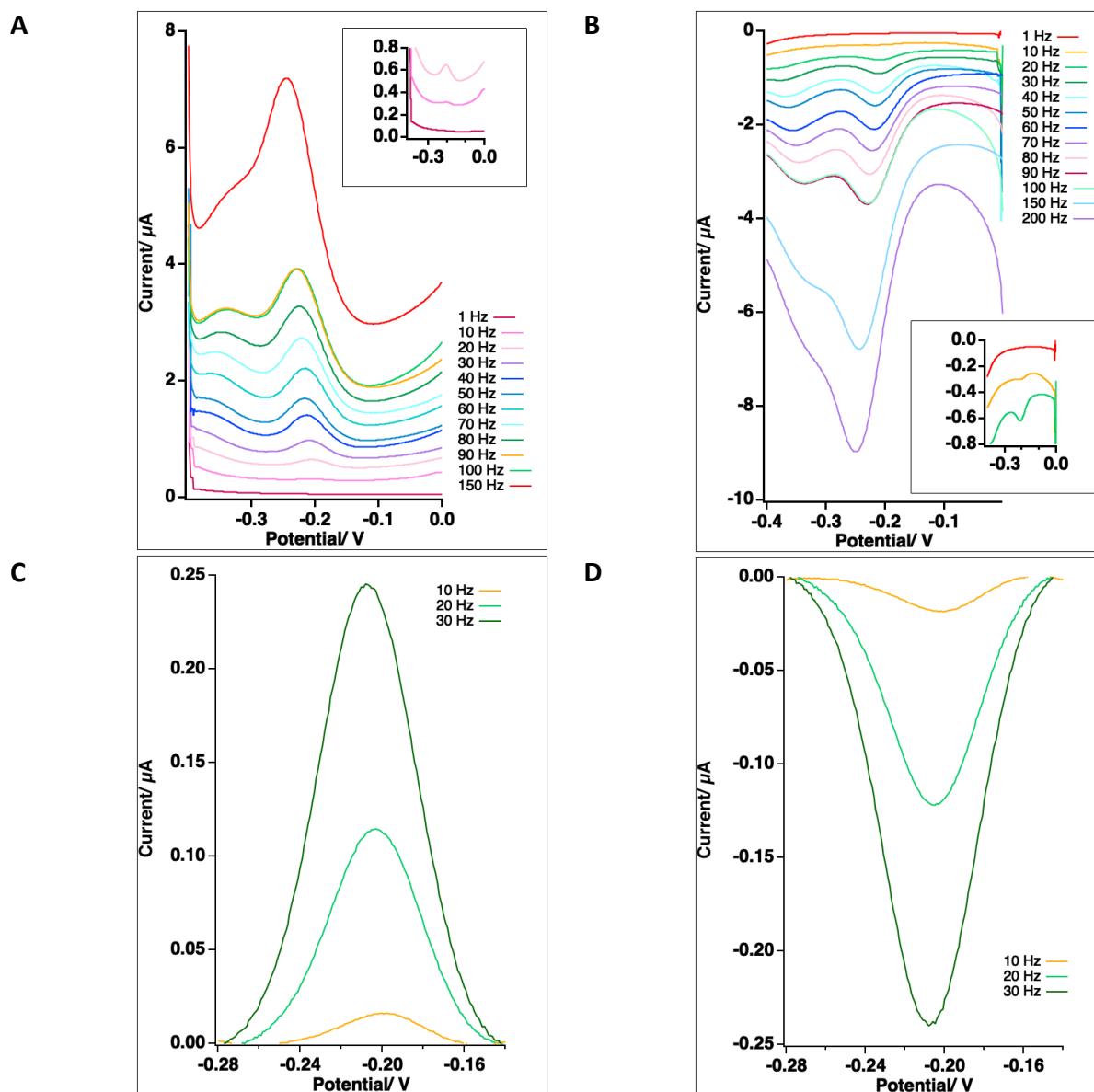


Figure 3.20 Optimisation of signal response by altering frequency in SWV scans (a) in anodic and (b) cathodic response from 1 to 150 Hz ($n=1$), (c) anodic and (d) cathodic peak heights at 10, 20 and 30 Hz ($n=1$).

3.3.6 Aptamer concentration optimisation

The concentration of the aptamer deposited during the fabrication process was increased from 1 to 10 μM to achieve the highest signal response (Figure 3.21). In the anodic and cathodic DPV scans shown in Figure 3.21(a), the electrode deposited with 10 μM aptamer demonstrated the highest current response overall. The DPV peaks from 10 μM aptamer-functionalised electrode were seen at -0.355 V (anodic) and -0.149 V (cathodic). In addition, the anodic response from 10 μM aptamer-functionalised electrode was 90.7%, 82.3% and 79.0% greater than the response from the electrodes treated with 1, 2.5 and 5 μM aptamer, respectively. There was a peak shift of -20 mV when the aptamer concentration was increased from 1 to 10 μM .

The cathodic DPV peaks from the 10 μM aptamer-functionalised electrode were 83.0%, 90.4% and 80.9% greater than the response from the electrodes treated with 1, 2.5 and 5 μM aptamer, respectively. There was a peak shift of -8 mV when the aptamer concentration was increased from 1 to 10 μM .

In the anodic and cathodic SWV scans shown in Figure 3.21(b), the electrode deposited with 10 μM aptamer demonstrated the highest current response overall. The SWV peaks from 10 μM aptamer-functionalised electrode were seen at -0.249 V (anodic) and -0.253 V (cathodic). In addition, the anodic response from 10 μM aptamer-functionalised electrode was 79.6%, 70.2% and 73.9% greater than the response from the electrodes treated with 1, 2.5 and 5 μM aptamer, respectively. There was a peak shift of +9 mV when the aptamer concentration was increased from 1 to 10 μM .

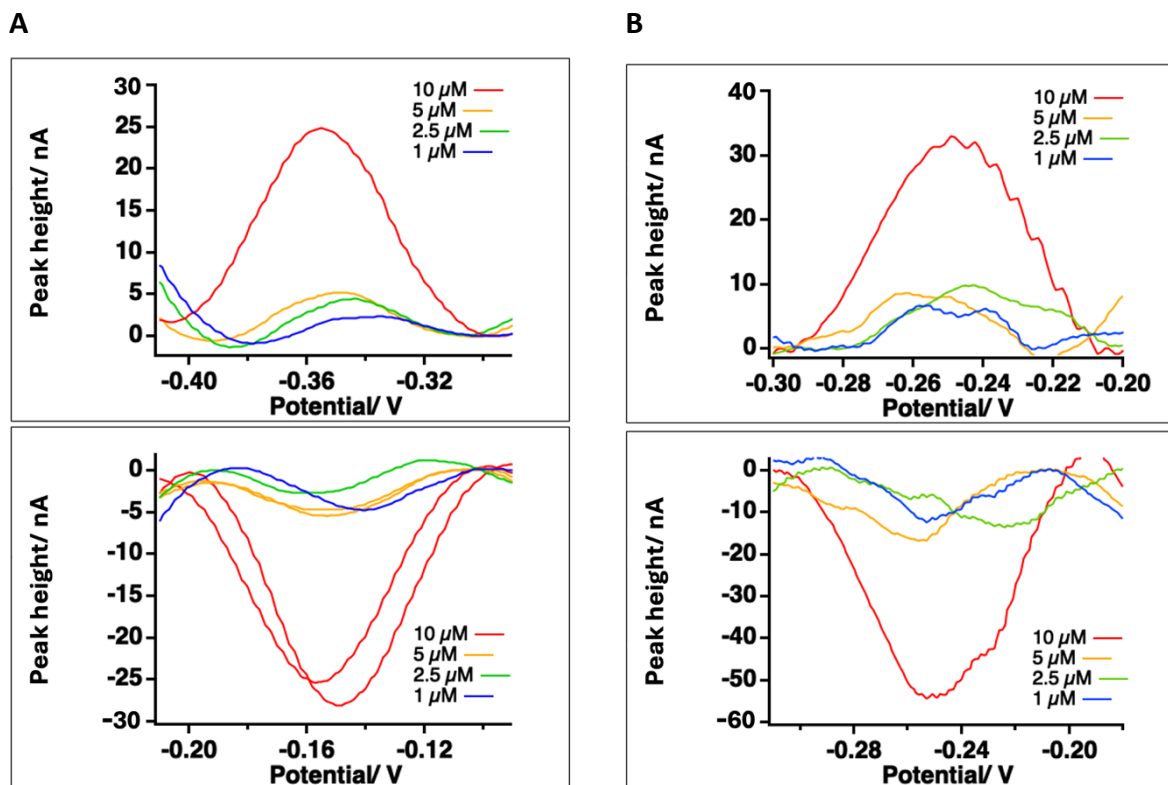


Figure 3.21 Optimisation of signal response by altering aptamer concentration from 1 to 10 μM in (a) DPV and (B) SWV ($n=1$).

No clear anodic and cathodic peaks were visible in the CV scans of aptasensors made with 1, 2.5 and 5 μM aptamer (as shown in Figure 3.22(a)). The anodic peak height and position of the aptasensor signal response made with 10 μM aptamer were 0.036 μA at -0.327 V. The area under the peak was calculated as 0.004 $\mu\text{A V}$ (Figure 3.22(b)).

To determine the quantity of aptamer deposited, the surface coverage (Γ_{ap}) of the aptamer (in mol cm^{-2}) was calculated by integrating the area under the CV peaks and dividing this by scan rate, which achieved a value for charge, Q (C), in equation 3.8, where n is the number of electrons, F is Faraday's constant (C mol^{-1}) and A is the surface area of the electrode (m^2).

$$\Gamma_{ap} = \frac{Q}{nFA}$$

Equation 3.8 Calculation of surface coverage of aptamer.

The surface coverage of the aptamer, Γ_{ap} , at the E-AB fabricated with 10 μM aptamer was calculated to be $4.44 \times 10^{-10} \text{ mol cm}^{-2}$ which is an order of magnitude higher than reported values of aptamer coverage ($3\text{--}5 \times 10^{-11} \text{ mol cm}^{-2}$).²⁴⁸

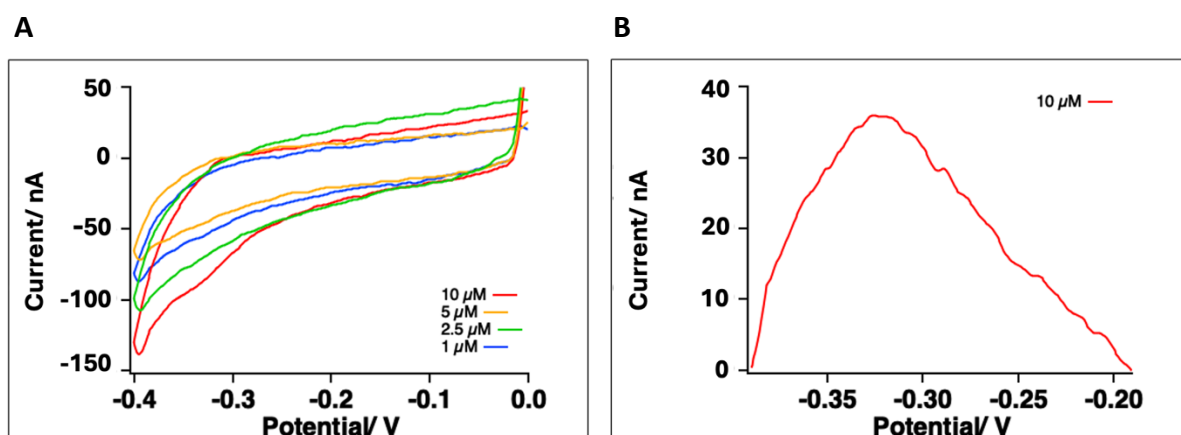


Figure 3.22 (a) Optimisation of CV signal response by altering aptamer concentration from 1 to 10 μM and (b) anodic peak extracted from CV scan ($n=1$).

3.3.7 Aptasensor calibration

The optimised aptasensor was calibrated at 0, 1 and 10 mg/L CRP concentrations using a scan rate of 25mV/s in DPV, a frequency of 30Hz in SWV, and a scan rate of 150V/s in CV.

The ratios of the peak heights of the anodic DPV responses were 0.96 (blank:1 mg/L CRP) and 0.89 (blank:10 mg/L CRP). There was a peak shift of +4 mV from 0 to 10 mg/L CRP. The sensor behaves as an ON sensor in the oxidation DPV scan, as seen in Figure 3.23(a). The ratios of the peak heights of the cathodic DPV responses were 2.71 (blank:1 mg/L CRP) and 4.0 (blank:10 mg/L CRP). There was a peak shift of +7 mV from 0 to 10 mg/L CRP. As seen in Figure

3.23(a), the sensor behaves as an ON sensor in the oxidation scan of DPV and as an OFF sensor in the reduction scan.

The ratios of the peak heights of the anodic SWV responses were 0.99 (blank:1 mg/L CRP) and 0.95 (blank:10 mg/L CRP). There was a peak shift of +3 mV from 0 to 10 mg/L CRP (Figure 3.23(b)). The ratios of the peak heights of the cathodic SWV responses were 1.0 (blank:1 mg/L CRP) and 0.95 (blank:10 mg/L CRP). There was a peak shift of +1 mV from 0 to 10 mg/L CRP. In both the oxidation and reduction scans of the SWV calibration, the differences in peak heights were not significant.

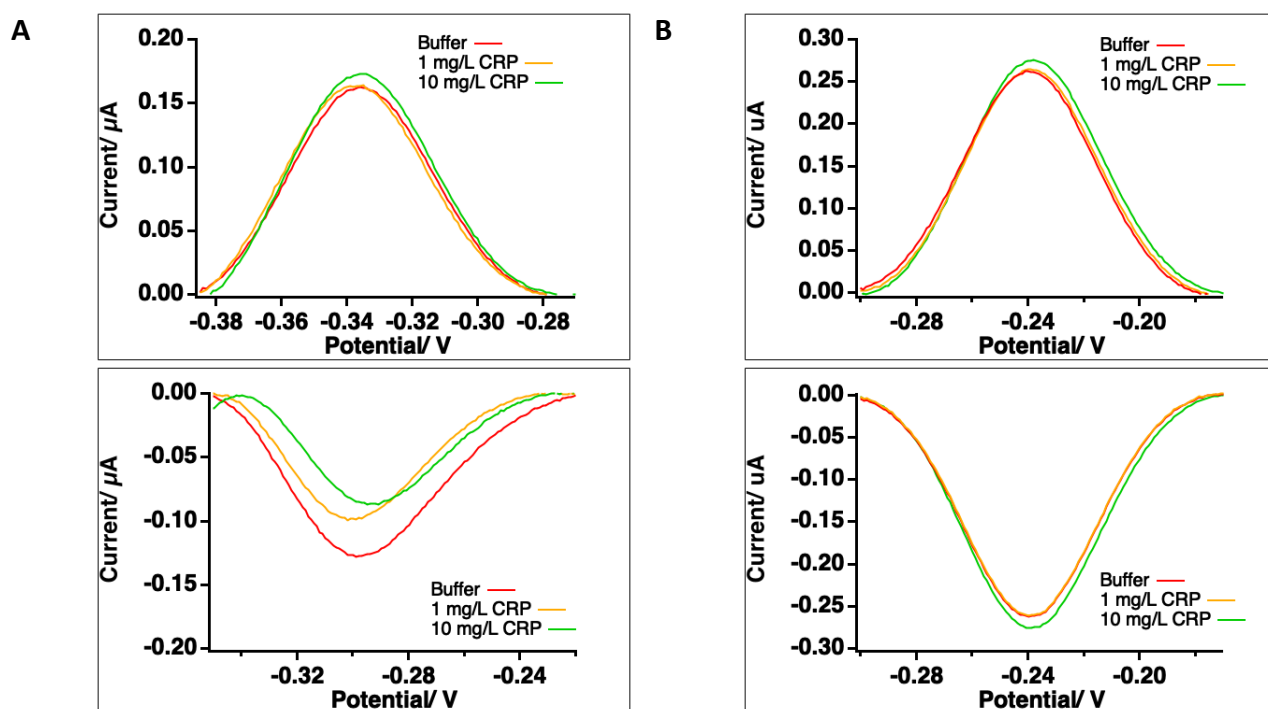


Figure 3.23 Aptasensor calibration at 0, 1 and 10 mg/L CRP concentrations using (a) DPV and (b) SWV (where buffer = blank reading) (n=1).

Apart from the cathodic DPV scans, a molecular recognition effect was not seen in the DPV or SWV scans. The ratios of the peak heights are close to 1.0 which indicates that not much of a difference was seen between the different concentrations of CRP. The concentrations of 1

mg/L and 10 mg/L CRP were chosen due to the physiological range of CRP present *in vivo* (<1 mg/L at baseline and >100 mg/L in acute infections). The K_D of the aptamer is 3.51 nM which means the aptamer can detect concentrations up to roughly twice that of K_D i.e. 7.02 nM. If we were to assume that the E-AB can detect up to 10 nM of CRP, this would mean it detects up to (3.5×10^{-25}) mg/L CRP ($M_w(\text{CRP}) = 3.5 \times 10^{-20}$ g/mol), which is 25 orders of magnitude lower than the concentrations being tested in the *in vitro* experiments in this thesis. The sensor would be highly saturated at 1 and 10 mg/L concentrations of CRP which can explain why there isn't a significant difference between peak heights observed during the calibrations.

In general, the lower the K_D of the aptamer, the greater the affinity of the aptamer for its target and the greater the sensitivity of the E-AB. However, a low K_D can limit the upper concentration limit (as seen in the work presented here) and limit the dynamic range in which the E-AB can measure its target.

While the concentration dependency on signal response was low, the calibration datapoints were plotted to understand the relationship, if any (Figure 3.24). Both the DPV and SWV calibration curves were fit with a linear regression and parameters are outlined in Table 3.6. A linear relationship was observed, albeit with a very low sensitivity, between CRP concentrations and current response. The linear regression and determination of any molecular recognition effect was limited by there being only three concentrations that the E-ABs were interrogated in.

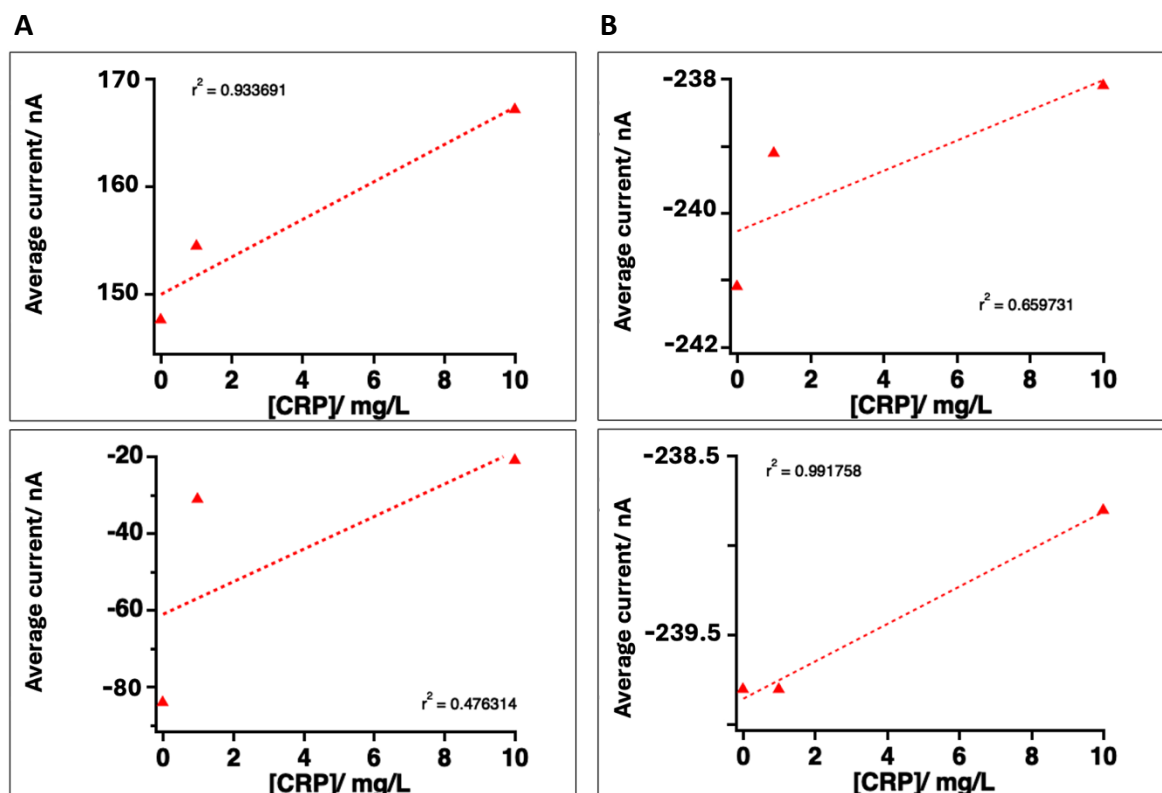


Figure 3.24 (a) Aptasensor calibration curve using DPV peak heights in oxidation and reduction sweeps, (b) aptasensor calibration curve using SWV peak heights in oxidation and reduction sweeps (n=1).

Technique	Scan	Slope	Intercept	r^2
SWV	Anodic	$(2.25 \pm 1.62) \times 10^{-4}$	-0.24 ± 0.00094	0.659731
	Cathodic	$(10.43 \pm 0.95) \times 10^{-6}$	-0.24 ± 0.000055	0.991758
DPV	Anodic	$(1.7 \pm 0.47) \times 10^{-3}$	0.15 ± 0.0027	0.933691
	Cathodic	$(4.3 \pm 4.5) \times 10^{-3}$	-0.061 ± 0.026	0.476314

Table 3.6 Linear regression fit parameters for calibration curves by SWV and DPV techniques.

CV was conducted with the optimised aptasensor made with 10 μM aptamer at CRP concentrations of 0, 1, and 10 mg/L (Figure 3.25). The ratios of the anodic peak heights were 1.43 (blank:1 mg/L CRP) and 0.81 (blank:10 mg/L CRP). There was a small peak shift of -1 mV between the blank and 10 mg/L CRP peaks. The ratios of the cathodic peak heights were 1.04 (blank:1 mg/L CRP) and 1.0 (blank:10 mg/L CRP). There was no significant difference between the peak heights observed for the cathodic scan and the peak shift was -5 mV as the CRP concentration increased from 0 to 10 mg/L CRP.

The surface coverage of aptamer, Γ^* , was calculated in mol cm^{-2} (as shown in Figure 3.25(c)). The average surface coverage was $(3.80 \pm 1.26) \times 10^{-10} \text{ mol cm}^{-2}$ in the oxidation sweep, and $(1.48 \pm 0.05) \times 10^{-9} \text{ mol cm}^{-2}$ in the reduction sweep.

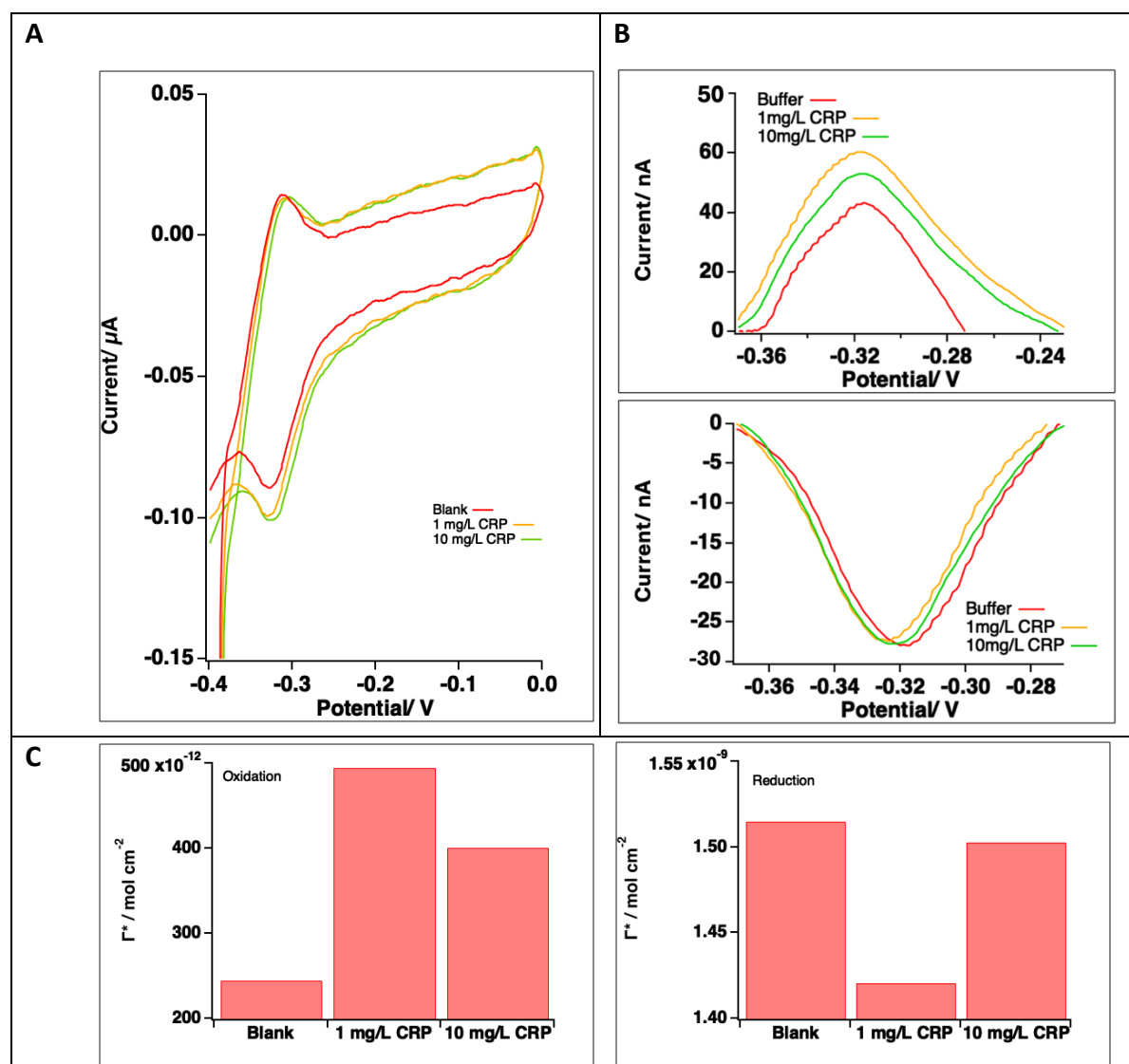


Figure 3.25 (a) Aptasensor calibration using CV at 0, 1 and 10 mg/L CRP concentrations ($n=1$), (b) baseline corrected signal responses and (c) surface coverage of aptamer (Γ^*).

Calibration curves using the CV peak heights were plotted. There was a concentration dependent molecular recognition effect in the oxidation sweep of the CV scan i.e. the peak height increased relative to concentration (Figure 3.26(a)), however, this relationship wasn't observed in the reduction sweep due to a lack of significant difference between peak heights at the different concentrations (Figure 3.26(b)). The calibration curves were fit with a linear regression and the parameters of the fit are outlined in Table 3.7.

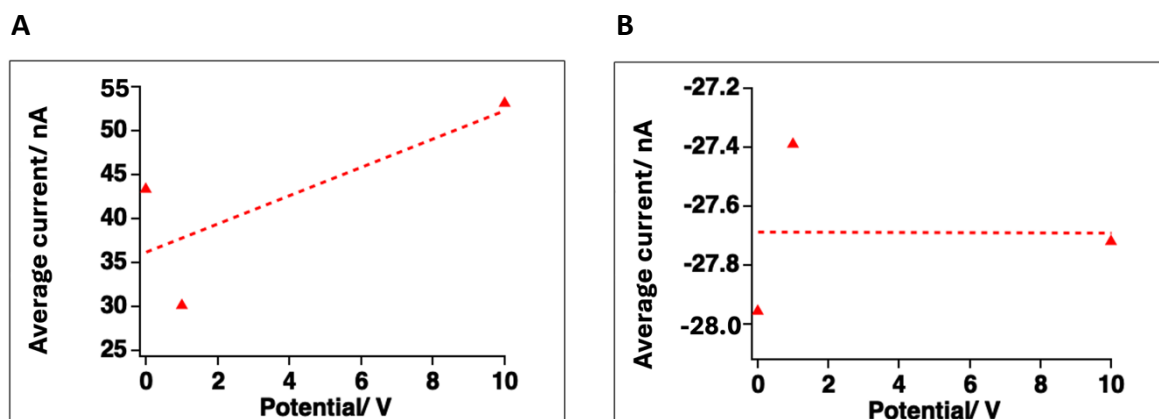


Figure 3.26 Aptasensor calibration curve using CV peak heights in (a) oxidation and (b) reduction sweeps (n=1).

Scan	Slope	Intercept	r ²
Anodic	0.0016 ± 0.0014	0.036 ± 0.0078	0.584849
Cathodic	(-0.031 ± 5.16) × 10 ⁻⁵	-0.028 ± 0.0003	3.62 × 10 ⁻⁵

Table 3.7 Linear regression fit parameters for calibration curves by CV.

3.3.8 Characterisation using chronoamperometry

Chronoamperometry was carried out at the functionalised electrodes in blank, 1 mg/L CRP and 10 mg/L CRP. The charge passed through the electrode was calculated by integrating the area under the curve (Figure 3.27(a)). In the oxidation sweep of the chronoamperogram, the charge passed at the electrode increased relative to the CRP concentration (Figure 3.27(b)). The sensor behaves as a signal ON sensor. In the reduction sweep, there isn't a clear relationship due to the charge passed being higher at the 1 mg/L CRP concentration than at 10 mg/L CRP, however the sensor is still seen to behave as a signal ON sensor.

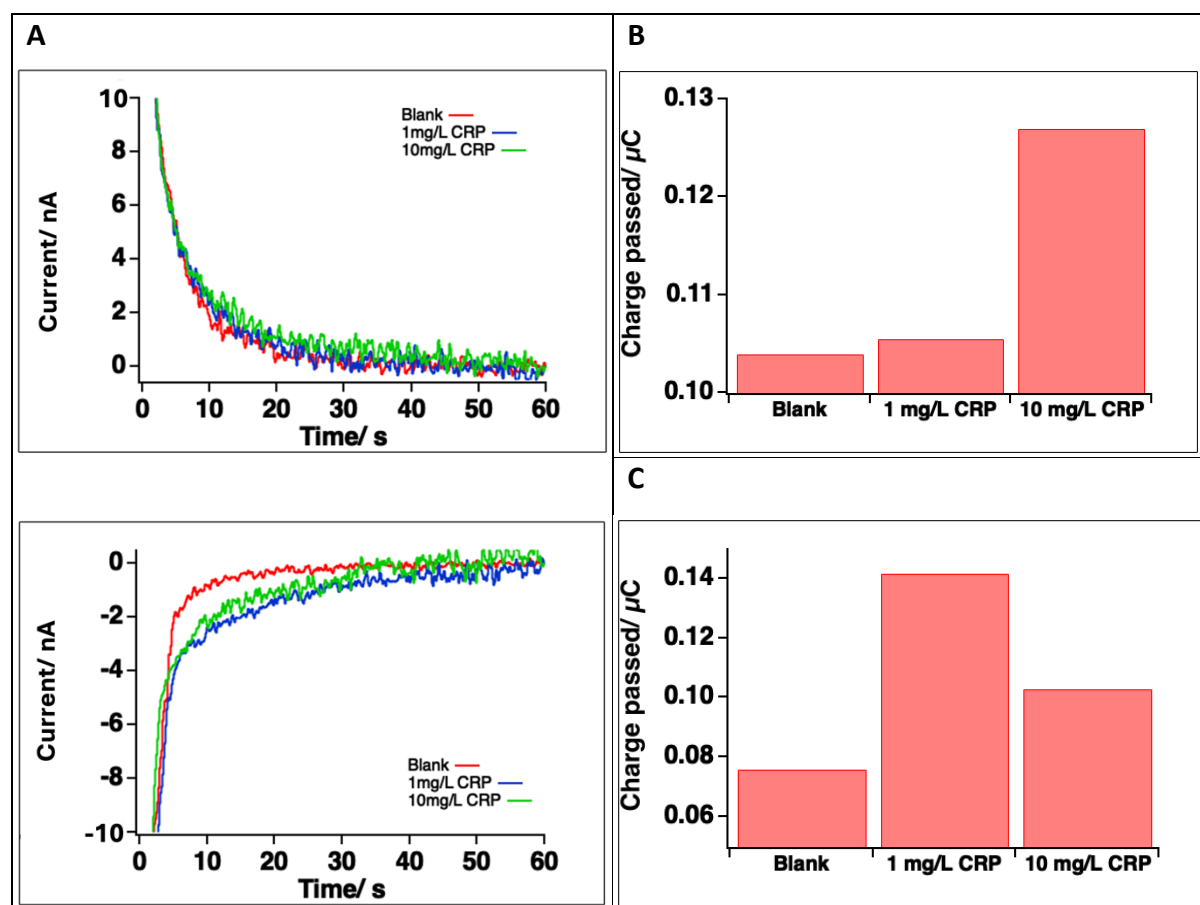


Figure 3.27 (a) Chronoamperometry of E-ABs in the unbound state (blank) and after binding to CRP at 1 and 10 mg/L concentrations ($n=1$), and charge passed through the electrode in the unbound state (blank) and after binding to 1 and 10 mg/L CRP concentrations in the (b) oxidation sweep and (c) reduction sweep.

3.3.9 Sensors made with and without TCEP reduction step

It was thought that TCEP may be reducing the gold surface of the electrodes or adsorbed species on the electrodes, which were then oxidised at the positive potentials being applied during the electrochemical interrogation of the sensors. To understand whether the reduction of the thiol groups on the aptamer makes an impact on the performance of the sensor, E-ABs were fabricated with and without the TCEP reduction step.

No anodic DPV peaks were visible for the CRP aptasensors made without the TCEP reduction step. Sensors that had not been reduced by TCEP produced a larger change in signal response upon binding to CRP, as shown in Figure 3.28. The reduction peak height ratios were 1.08 (blank:1 mg/L CRP) and 1.12 (blank:10 mg/L CRP) for the TCEP reduced E-AB, and 1.49 (blank:1 mg/L CRP) and 1.53 (blank:10 mg/L CRP) for the unreduced E-AB. There was a peak shift of +15 mV for the unreduced aptasensors and -5 mV for the reduced aptasensors.

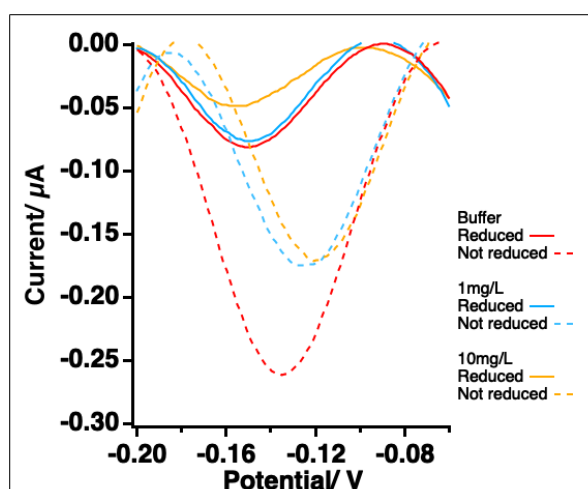


Figure 3.28 DPV reduction scans of aptasensors made with the TCEP reduction step (solid line) and without TCEP reduction (dashed line), in buffer and after incubation with 1 and 10 mg/L CRP (where buffer = blank reading) (n=1).

The SWV signal response of aptasensors made without the TCEP reduction step was much higher than aptasensors made with the TCEP reduction step, as seen in Figure 3.29. This is observed across the different states of the aptasensor i.e. in buffer and after incubation with CRP. The oxidation peak height ratios were 1.3 (blank:1 mg/L CRP) and 1.26 (blank:10 mg/L CRP) for the TCEP reduced E-AB, and 1.44 (blank:1 mg/L CRP) and 1.25 (blank:10 mg/L CRP)

for the unreduced E-AB. There was a peak shift of +15 mV for the unreduced aptasensors and -2 mV for the reduced aptasensors. The reduction peak height ratios were 1.14 (blank:1 mg/L CRP) and 1 (blank:10 mg/L CRP) for the TCEP reduced E-AB, and 1.27 (blank:1 mg/L CRP) and 1.37 (blank:10 mg/L CRP) for the unreduced E-AB. There was a peak shift of +15 mV for the unreduced aptasensors, and no peak shift was seen for the reduced aptasensors.

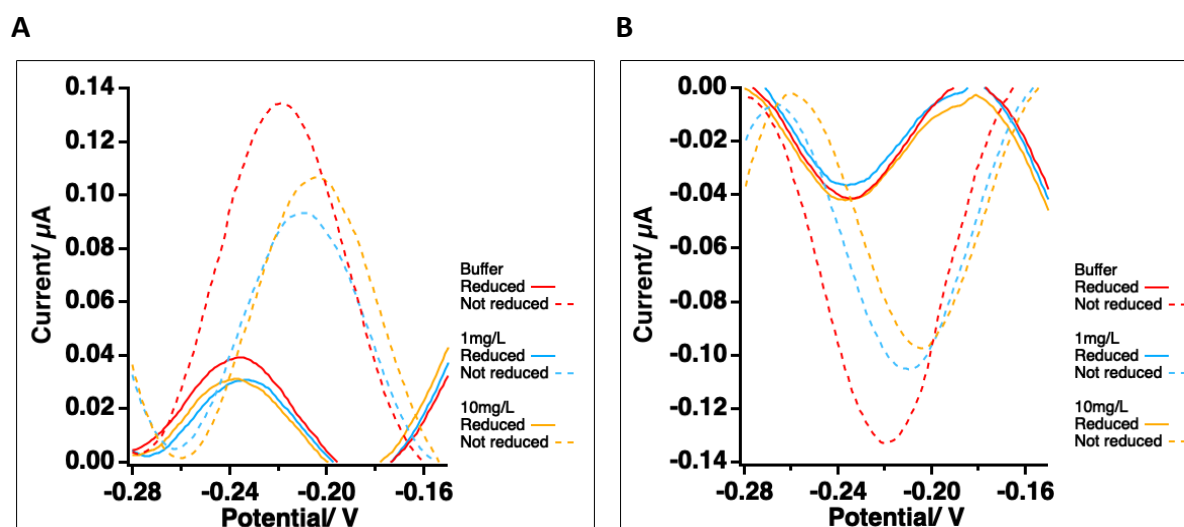


Figure 3.29 SWV (a) oxidation and (b) reduction scans of aptasensor reduced by TCEP (solid line) and not reduced by TCEP (dashed line), in buffer and after incubation with 1 and 10 mg/L CRP (where buffer = blank reading) (n=1).

3.3.10 Cycling pre-treated bare electrodes in ferri-ferrocyanide

The purpose of cycling pre-treated bare electrodes in 5 mM ferri/ferrocyanide was to understand whether the sensor produced any background noise or signal drift in a standardly-known CV of ferri/ferrocyanide. The electrodes were pre-treated via cycling in sulfuric acid as part of the electrochemical cleaning process and cycled at a scan rate of 25 mV/s and an E_{step} of 1 mV. Peaks were seen at +0.12 V and +0.08 V in the CV scan, as shown in Figure 3.30, with a peak separation of 0.04 V. Some ohmic resistance can be seen in the CV as per the observed

linear slope which has also been observed in the voltammograms of the E-AB response, indicating that this resistance may be coming from the electrode or from wiring or contact surfaces in the electrochemical set-up. For E-AB voltammograms, this drift has been corrected when extracting peaks.

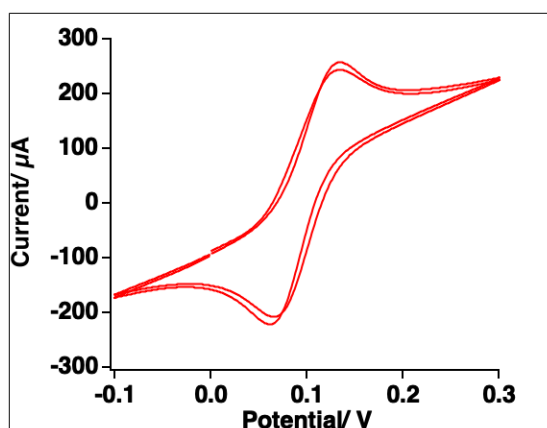


Figure 3.30 CV in 5 mM ferri-ferrocyanide solution under the same scanning conditions (at 25 mV/s scan rate and 1 mV E_{step}) using a disposable gold electrode (Respire Diagnostics).

3.3.11 Sensors fabricated with bulk gold rod electrodes and analysed in de-gassed solution

This purpose of fabricating E-ABs on rod electrodes was to validate whether the peaks seen in previous data – gathered in the presence of oxygen i.e. not under de-gassed conditions – were a result of the aptamer, rather than the reduction of oxygen species in the solution. As peaks are seen in the de-gassed solution (as seen in Figure 32 and 33), it can be confirmed that the previously seen peaks are not a result of oxygen reduction.

Figure 3.31(a) shows DPV scans of the sensors in buffer (blank) and after incubation with 1 and 10 mg/L CRP. The oxidation sweep of the DPV scan demonstrates signal OFF behaviour, whereas the reduction sweep demonstrates signal ON behaviour, which contrasts the

behaviour seen thus far. The ratio of the DPV peak heights in the oxidation sweep were 1.12 (blank:1 mg/L CRP) and 1.18 (blank:10 mg/L CRP), and in the reduction sweep, the ratios were 0.78 (blank:1 mg/L CRP) and 0.77 (blank:10 mg/L CRP). Figure 3.31(b) shows SWV scans of the sensors where the oxidation and reduction sweeps demonstrate signal OFF behaviour, which is in alignment with the behaviour seen thus far. The ratio of the SWV peak heights in the oxidation sweep were 1.10 (blank:1 mg/L CRP) and 1.15 (blank:10 mg/L CRP), and in the reduction sweep, the ratios were 1.15 (blank:1 mg/L CRP) and 1.18 (blank:10 mg/L CRP). It is worth noting, as evidenced by these ratios, that the difference seen between peak heights across both DPV and SWV scans were not significant after incubation with CRP, indicating a low sensitivity of the rod electrode-based sensors.

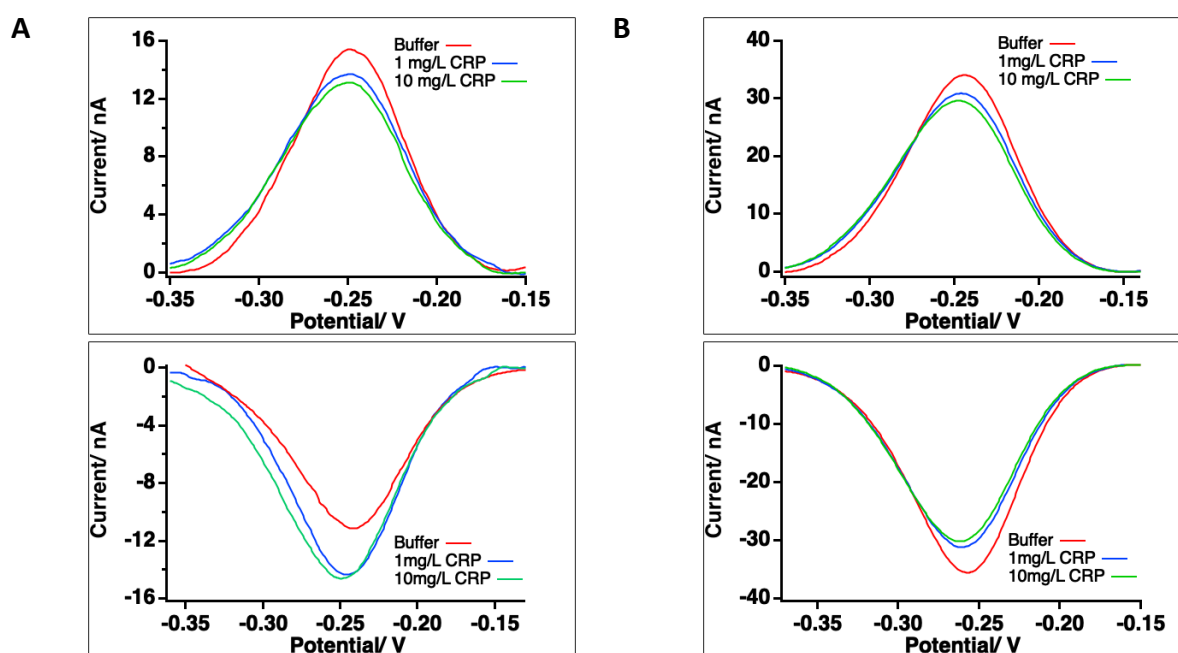


Figure 3.31 (a) DPV and (b) SWV scans of E-ABs made with gold rod electrodes in buffer (blank), 1 mg/L CRP, and 10 mg/L CRP (n=1).

The rod electrode-based sensors were also interrogated using CV, as shown in Figure 3.32. Unlike the behaviour seen so far, the sensor behaved as a signal ON sensor in the CV scans;

the oxidation peak height ratios were 0.68 (blank:1 mg/L CRP) and 0.81 (blank:10 mg/L CRP), and the reduction peak height ratios were 0.68 (blank:1 mg/L CRP) and 0.43 (blank:10 mg/L CRP). Peaks were seen at -0.22 V and -0.25 V in the oxidation and reduction sweep, respectively. There was some ohmic resistance observed in the CV scans with artefacts around the -0.07 V and -0.13 V marks. The artefacts were removed when extracting peaks from the scan (Figure 3.32(b)).

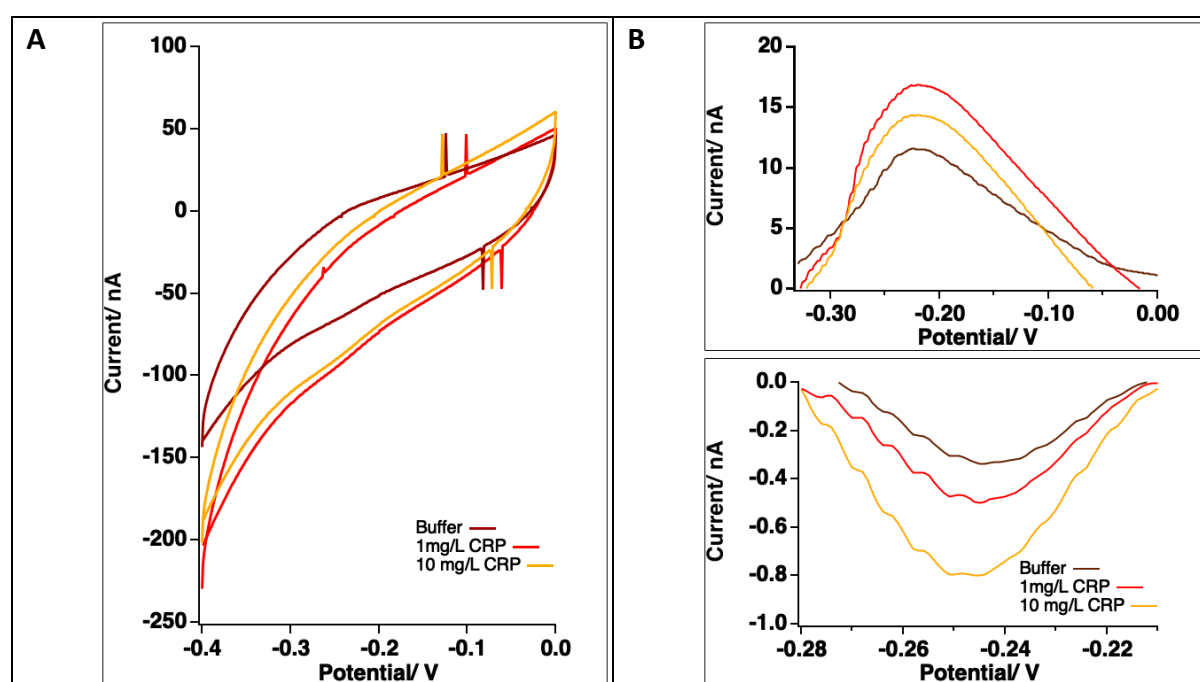


Figure 3.32 (a) CV scans of E-ABs made with bulk gold rod electrodes in buffer, 1 mg/L CRP, and 10 mg/L CRP, and (b) peaks extracted from CV scans in buffer, 1 mg/L CRP, and 10 mg/L CRP (buffer = blank reading) (n=1).

Analysis of the peak height and position shows that the sensor behaves as an ON sensor in CVs, however, the correlation isn't consistent in the oxidation scan as it is in the reduction scan. The sensor behaves as an OFF sensor in SWV scans, wherein the signal decreases upon increasing concentration of CRP. In DPV scans, the sensor behaves as an OFF sensor in the

oxidation scan and as an ON sensor in the reduction scan. This is inconsistent with previous data shown herein, wherein the sensors behaved as OFF sensors throughout all scans.

Calibration curves from DPV, SWV and CV peaks were plotted in Figure 3.33. Due to there being only three calibration points to fit to, a linear regression was chosen as the most suitable fit. Polynomial fits were attempted, however, the highest polynomial to which the data could be fitted to was a second order polynomial i.e., largest possible polynomial fit for a data set containing n calibration points is an $(n-1)$ order of polynomial, as reported by Miller and Miller *et al.*²⁴⁹ Table 3.8 summarises the slope, intercept and r^2 parameters of the linear regressions.

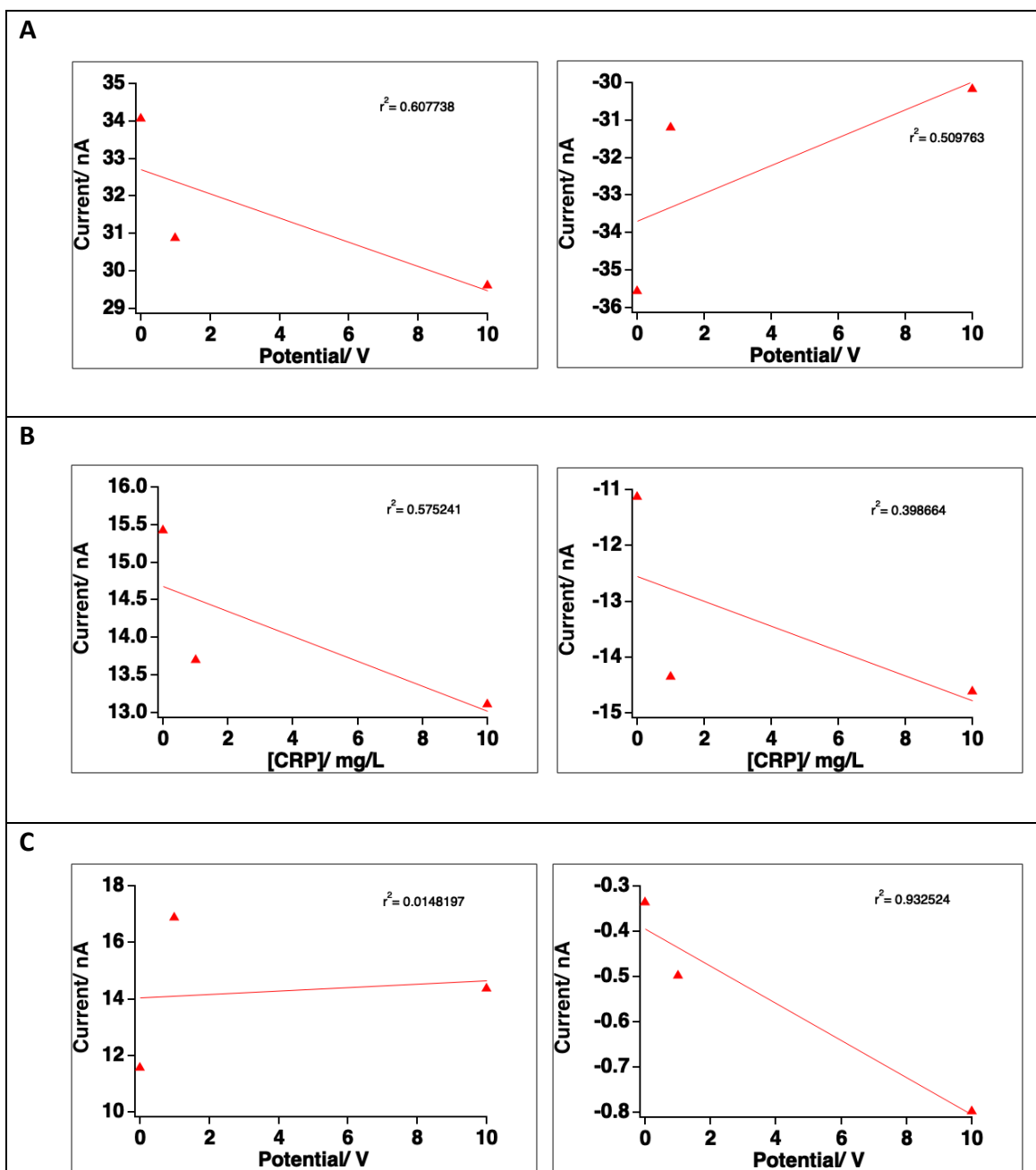


Figure 3.33 Calibration curves produced using (a) SWV, (b) DPV and (c) CV peaks obtained from scans of E-ABs made with bulk gold electrodes ($n=1$).

Technique	Scan	Slope	Intercept	r^2
SWV	Anodic	-0.32 ± 0.26	32.71 ± 1.51	0.607738
	Cathodic	0.37 ± 0.36	-33.68 ± 2.11	0.509763
DPV	Anodic	-0.17 ± 0.14	14.68 ± 0.83	0.575241
	Cathodic	-0.22 ± 0.27	12.56 ± 1.58	0.398664
CV	Anodic	0.06 ± 0.48	14.05 ± 2.78	0.0148197
	Cathodic	-0.04 ± 0.01	-0.39 ± 0.06	0.932524

Table 3.8 Linear regression fit parameters for calibration curves by SWV, DPV and CV techniques.

3.3.12 Stability of sensors made with gold rod electrodes

E-ABs fabricated with gold rod electrodes were tested in de-gassed solution after 15 days of storage at 4°C. The signal decreased by 11.3% and 26.1% for the anodic and cathodic sweep, respectively, in the DPV scans, as shown in Figure 3.34(a). The signal decreased by 15.1% and 39.2% for the anodic and cathodic sweep, respectively, in the SWV scans, as shown in Figure 3.34(b). A signal decrease is expected after storage due to repeated electrochemical interrogation having shown to disturb the SAM, as well as possible aptamer loss at the electrode surface over time.²⁵⁰

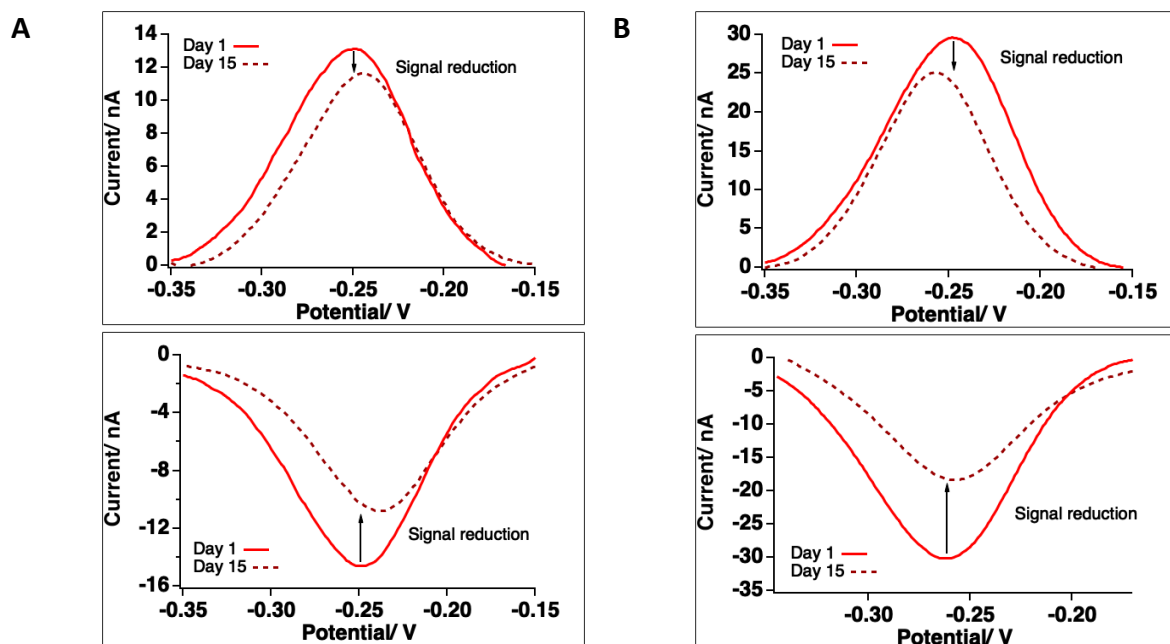


Figure 3.34 (a) DPV scans of E-ABs made with bulk gold rod electrodes after 15 days in storage and (b) SWV scans of E-ABs made with bulk gold rod electrodes after 15 days in storage (n=1).

3.3.13 Chronoamperometry of gold rod-based E-ABs

Chronoamperometry was conducted for the rod electrodes functionalised with CRP aptamer and 6-MCH back-filler. The charge passed through the electrode was higher when the electrode was bare compared to after functionalisation. Figure 3.35 (a) shows the chronoamperometry scans and (b) shows the charge passed at each electrode before (bare electrode) and after functionalisation (with aptamer and 6-MCH). There was an 85.5% decrease in the charge passed in the oxidation sweep and a 62.2% decrease in the reduction sweep after functionalisation with aptamer and 6-MCH back-filler. This result is unexpected as after functionalisation as the charge passed at the electrode is expected to increase.

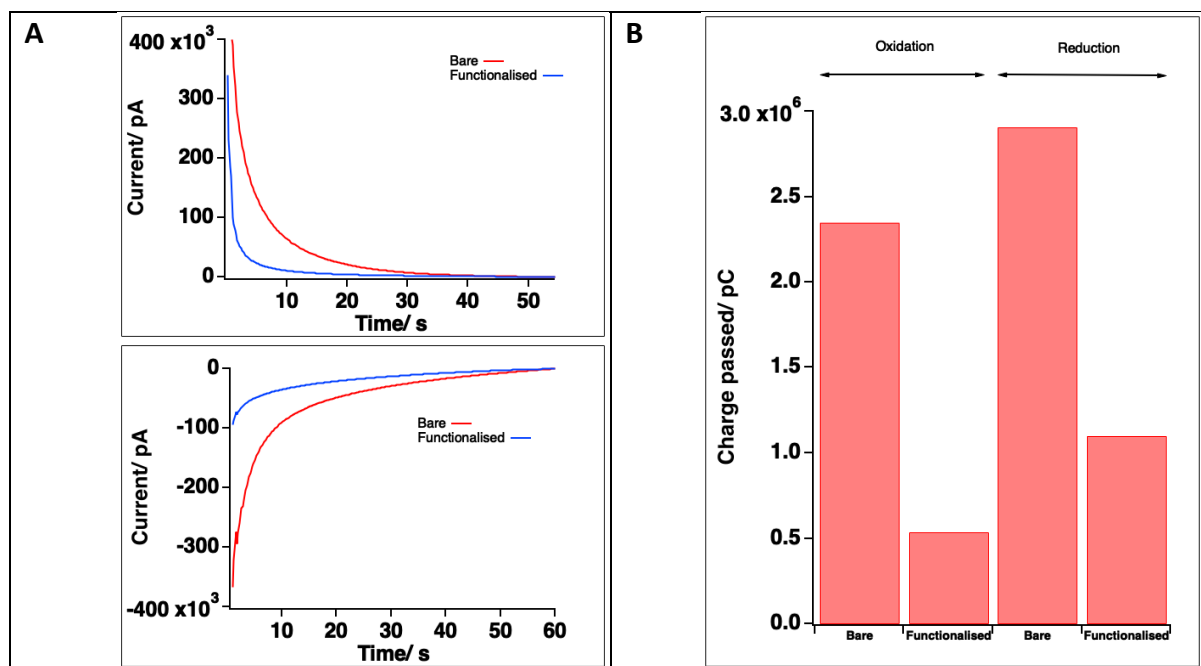


Figure 3.35 (a) Chronoamperometry of bulk gold electrode E-ABs and (b) charge passed before and after functionalisation (n=1).

3.4 Discussion

We report an E-AB sensor for detecting CRP using a methylene blue-terminated aptamer (sequence reported by Huang *et al.*).²⁵¹ It was confirmed that the aptamer had been successfully deposited onto the electrodes due to interrogation of the E-AB at each step of the fabrication process, wherein SWV and DPV peaks were only present after deposition of the 6-MCH back-filler onto the aptamer-functionalised gold electrodes. Peaks were observed between -0.16 V and -0.32 V vs Ag|AgCl across CV, SWV and DPV techniques, which is in alignment with the peak potentials reported in literature for methylene blue-terminated E-ABs.^{252–254} Peaks from methylene blue have been reported to be as low as -0.2 V which is 4 mV higher than the lowest reported peak in this thesis (-0.16 V vs Ag|AgCl in DPV).²⁵⁵

The deposited aptamer concentration was optimised with respect to signal response, with the highest signal being achieved when the deposited aptamer concentration was 10 μM . The surface coverage of the aptamer, calculated during the optimisation phase, was $4.44 \times 10^{-10} \text{ mol cm}^{-2}$, which is an order of magnitude higher than that reported by Kelley *et al.* for MB-terminated aptamers deposited onto gold electrodes ($4.1 \times 10^{-11} \text{ mol cm}^{-2}$) and that reported by Furst *et al.* ($3.0 - 5.0 \times 10^{-11} \text{ mol cm}^{-2}$).²⁴⁸

The scan rate was optimised with respect to signal response, with the greatest signal being achieved at 25mV/s for DPV scans, and 150 mV/s for CV scans. The frequency was optimised with respect to signal response, with the greatest signal being achieved at 30 Hz for SWV scans. These optimised parameters were used for all experiments in this thesis.

Concentration-dependent molecular recognition effect

The concentration-dependent molecular recognition effect was limited by the lack of large differences seen between signals at 0 and 1 mg/L CRP, and 0 and 10 mg/L CRP. The ratios (blank:CRP) of the anodic responses of the sensor were very close to 1.0 in the DPV and SWV scans, as well as in the cathodic SWV scan. For the DPV cathodic scan, the ratio of blank:CRP was greater than 1.0, meaning that the sensor exhibited signal OFF behaviour.

The lack of a significant difference in signal response between different concentrations of CRP can be attributed to the aptamer being saturated at concentrations over 7.01 nM CRP (i.e., twice that of K_D , which was reported to be 3.51nM).²⁵¹ For an E-AB to measure CRP concentrations up to 100 mg/L in infection,⁴¹ the K_D needs to be at the upper end of the

dynamic range. 100 mg/L CRP is equivalent to 5×10^8 nM CRP, which is the desirable K_D of the aptamer for the application of monitoring infection in humans.

The K_D of the aptamer used in this thesis is eight orders of magnitude lower than where it needs to be. Most K_D values for aptamers used in E-ABs lie between 11.6 nM and 800 μ M.^{172,256} At a higher K_D , we expect that the E-AB will not be able to measure CRP concentrations at the lower end of the concentration range which may pose challenges in detecting changes from baseline to infectious levels.

Another reason that a concentration-dependent effect is not seen could be that the redox reporter is far away from the CRP binding site. Since the aptamer consists of 72 bases and falls on the upper end of aptamer lengths reported in literature,^{169,170,174,176} the aptamer conformational change upon CRP binding may not have an impact on electron transfer rates if the distance of the redox reporter from the electrode surface is unchanged.

In addition, to observe a concentration-dependent molecular recognition effect, it would have been useful to optimise scan rate (and thereby E_{pulse}) for DPV, and frequency for SWV, with respect to signal gain, as opposed to the magnitude of the signal response. The conditions under which the largest difference between the bound and unbound state of the aptamer is seen would have been most useful to achieve, in order to produce a concentration dependent effect to build a calibration curve.

In addition, for SWV readings, it would be useful to conduct the kinetic differential measurements (KDM) technique, wherein the response is recorded at multiple different

square wave frequencies, and the difference in response from two frequencies is subtracted to overcome the drift seen and optimise signal gain in SWV measurements. This works in practice because the responses drift in concert.^{256–258} As reported by Ferguson *et al.*,²⁵⁸ the two frequencies are chosen based on the “cross-over frequency”, below which the sensor behaves as a signal OFF sensor, and above which it behaves as an ON sensor at higher frequencies. Choosing to sample at frequencies at either side of this cross-over frequency allows drift to be corrected via KDM, producing more reproducible responses. The cross-over frequency can be found by plotting the inverse of peak height vs the inverse of frequency for both the bound and unbound aptamer at multiple SWV frequencies.²⁵⁹

Charge passed at the electrode

Chronoamperometry was carried out at different concentrations of CRP, and it was found that charge passed at the electrodes in the anodic scan increases with the concentration of CRP. This is expected for a signal ON sensor as electron transfer rates are greater in the bound state of the aptamer. For the cathodic scan, charge passed was greater in the bound state than the unbound state, however, the charge decreased when the concentration of CRP was increased. This may be an error in the measurement, or it may be a result of decreased surface coverage of aptamer; repeated electrochemical interrogation has shown to disrupt the SAM of E-ABs.²⁶⁰ Similarly, the charge passed at the bulk gold rod-based E-ABs was seen to be higher at the bare electrode than the functionalised one, which could be explained by the back-filler occupying more sites on the electrode than the aptamer. This may be possible since the 6-MCH back-filler has a molecular weight (134.24 g mol⁻¹) that is much lower than the 72-base long CRP aptamer (24,436.6 g mol⁻¹, as reported by manufacturers), meaning that the diffusion of the back-filler may happen at a faster rate than the aptamer in solution. To reduce

the risk of back-filler over occupying the electrode, either (i) the aptamer needs to be truncated to a shorter length to decrease molecular weight, or (ii) a higher molecular weight back-filler needs to be chosen.

In addition, where chronoamperometry was used to assess whether functionalisation and binding had occurred in this thesis by determining the charge passed at the electrode, it could also be used as a method to conduct calibration-free measurements of E-ABs, as reported by Arroyo-Currás *et al.*²⁵⁷ Chronoamperometry measures the lifetime of a current transient generated by the application of a potential step, whereby the redox reporter is fully oxidised or reduced (depending on the direction of the potential step). The lifetime measurement is a direct result of electron transfer kinetics, unlike voltammetric methods. The lifetime can be determined by fitting the current transient to an exponential fit, and this can then be plotted against target concentration. Since the lifetime of the current transient is based on relative concentrations of bound and unbound aptamer, and not on the absolute concentration, it has been reported that the lifetime stays the same across sensors, negating the need for calibrations for each sensor.²⁵³

Stability and regeneration

It is known that the negative potential window needed to oxidise and reduce the MB label can cause the desorption of bound aptamer at gold surfaces.²⁶¹ In addition, repeated electrochemical interrogations can also contribute to this by inducing field-induced oscillations. For an E-AB to make continuous measurements over a period of time, it needs to be regenerable and stable without significant loss of the initial signal to provide correct concentration estimates.

The aptasensors developed in this thesis were stable after 15 days of storage in binding buffer at 4°C, with an average signal decrease of 18.7% in DPV and 27.2% in SWV. Karuppaiah *et al.* report that their MB-terminated cortisol E-AB remains stable for five regeneration cycles, with a signal decrease of only 3.5% between the 1st and 5th cycle.²⁶² Wang *et al.* report a 38% loss in signal of an MB-terminated E-AB for vascular endothelial growth factor (VEGF) after 6 days of storage,²⁶³ which is comparable to the loss seen for the MB-terminated CRP sensors in this thesis. Regeneration was not attempted for the MB-terminated E-ABs, however, this has been explored for the osmium(II/III) redox reporter-terminated E-ABs in Chapter 4.

In addition, a limitation exists in assuming that the K_D is the same as that reported by Huang *et al.* who determined the binding affinity based on aptamer-binding to a gold surface using SPR technique. K_D may be slightly different on a disposable gold electrode with the presence of a hydrophilic back-filler.

Current dependence on scan rate

In this thesis, the Randles-Sevcik plot confirmed that the oxidation and reduction of the MB label is quasi-reversible, which points to the challenge of stability over time. The plot of the log of peak current vs the log of scan rate revealed a slope of 1.0 (oxidative) and 1.83 (reductive), indicating that the process was adsorption-controlled, as is expected for surface-immobilised aptamers. Sidhu *et al.* identified the diffusion-limited transport of their target, *Listeria spp.* Bacteria, to their E-AB electrode surface, confirmed by the slow increase in peak current with respect to an increase in the scan rate,²⁶⁴ as well as by the slope of their $\log(I_p)$ vs $\log(v)$ plot being close to 0.5. Other E-AB studies have used the Randles-Sevcik equation to

calculate the surface roughness factor – i.e., area – of their electrodes, if the diffusion coefficient is known.

Bare electrode studies with TCEP

It was found that TCEP reduction of thiols was not needed to enable SAM formation on gold electrodes. Data reported in this thesis shows greater signal response from sensors fabricated with non-reduced thiols vs reduced thiols. This could be attributed to the reduction of MB by TCEP in the fabrication of E-ABs,²⁶⁵ meaning that the label needs to be re-oxidised to produce the same peak current subsequently. Further analysis of the stability of the gold-thiol bond, using non-reduced thiol groups, needs to be conducted over time and after repeated electrochemical interrogation cycles to assess impact on stability.

On disposable gold electrodes, it was observed that a positive signal response was being achieved after treatment of the electrode with TCEP overnight, with the possibility of false positive results or contribution from background signal. To remove this possibility, sensors were fabricated with and without the TCEP reduction step, and lower signal responses were seen from E-ABs wherein the reduction step was included, indicating that this may not be needed to deposit aptamers.

In addition, it was suspected that background signal may be coming from dissolved oxygen species. This hypothesis was tested by de-gassing the solution before testing the sensors, in which it was observed that the sensors treated with TCEP did not produce a signal response, meaning that the background signal from TCEP-treated disposable gold electrodes was coming from the redox reaction of the oxygen species in the solution.

3.5 Conclusion and future work

The main drawback of this study was that the K_D of the aptamer was too low for the application of monitoring infection levels in humans, and also too low for the concentrations chosen to incubate the sensor with to see any significant and reproducible difference in peak current. An important strategy to overcome the low K_D would be to increase the binding affinity of the aptamer using truncation methods combined with the enzyme-linked oligonucleotide assay (ELONA assay). Truncation would allow the identification of the binding region on the aptamer and using a shorter sequence would also allow the aptamer to have a lower molecular weight, in line with the molecular weight and size of the back-filler molecule used herein (6-MCH).

The ELONA assay measures the affinity of an aptamer for its target molecule using a sandwich assay wherein colourimetry is used to measure absorbance. Aptamer truncation is a trial-and-error process, whereby the binding region can be identified from a process of eliminating sequences that do not result in good absorbance and thereby, affinity. One strategy is to start truncating the aptamer from one end, such as the 3' end, as conducted by Stoltenburg *et al.* for their protein A (presented on *Staphylococcus aureus* bacterial walls) aptamer.²⁶⁶ This allowed the K_D of the protein A aptamer to be optimised from 101.4 ± 5.9 nM (full length) to 11.3 ± 1.4 nM (truncated). The reduction in K_D indicates an improvement in binding affinity. For our CRP aptamer, we want to achieve the opposite effect; we want to reduce binding affinity by increasing K_D to be able to detect CRP at higher concentrations.

Since only two different concentrations of CRP were calibrated with in this study, in addition to a blank reading, this posed a limitation on the relationships that could be drawn from the

calibration data. Future work should involve testing the E-AB with a larger set of concentrations, as well as testing over a larger range of CRP concentrations, particularly below K_D concentration in order to see significant difference in peak currents.

Since we know that MB offers quasi-reversible redox behaviour, another strategy could be to modify the aptamer with a different redox label for comparison, such as ferrocene or an osmium(II/III) polymer, so that the signal gain can be optimised. This strategy, combined with truncation to achieve a higher K_D , would allow the E-AB to measure CRP at the concentrations of interest. Once a reproducible and significant difference in peak current is observed, the sensor would need to be interrogated in artificial or clinically obtained ISF with standard additions of CRP to predict how sensors would respond *in vivo*.

Since this aptamer was obtained from one study,²⁵¹ there are other reported sequences of CRP aptamers that could be synthesized to compare sensor responses.^{267,268} However, only sequences with high binding affinities would be worth pursuing. And finally, to fully achieve infection monitoring in the long-term, it would be beneficial to monitor more than one biomarker of infection to produce reliable inputs for the closed loop drug delivery model.

Chapter 4 Development of an osmium-based redox reporter-labelled aptamer sensor for the continuous monitoring of CRP

4.1 Introduction

4.1.1 Redox labels used in biosensors

One way to optimise the sensitivity of an electrochemical aptamer biosensor (E-AB) is to optimise the redox label, particularly one which can (i) transduce the signal at a lower potential to avoid interference from other molecules and (ii) be chemically stable under a changing chemical environment, such as pH *in vivo*. Other considerations when optimising the redox label for an E-AB application is the size of the complex, which can create steric hindrance and affect aptamer secondary structure. The complex may also interact with the phosphate backbone of the single-stranded DNA (ssDNA) aptamer, with hydrophobic complexes inducing interactions with the hydrophobic alkyl thiols in the backbone.²⁶¹

Redox labels undergo reversible oxidation and reduction by losing or gaining electrons, respectively, in response to an applied potential. Redox labels used in electrochemical biosensors include methylene blue (MB),²⁶⁹ ferrocene (Fc),²⁷⁰ ferricyanide/ferrocyanide,²⁷¹ tris(bipyridine) ruthenium(II),²⁷² thionine,²⁷³ tris(bipyridine)-osmium(II),²⁶¹ ferrocene-modified polysiloxane,²⁷⁴ polyacrylamide,²⁷⁵ polypyrrole,²⁷⁶ polyquinone,²⁷⁷ polyvinylpyridine (PVP),²⁷⁸ and polymetallocyanines.²⁷⁹ The use cases of some of these redox labels are summarised in Table 4.1.

Redox label	Redox potential (E^0)	Analyte(s) measured	Sensor performance	Ref
Ferrocene (Fc)	+0.25 V vs Ag AgCl	Chloramphenicol	• Low detection limit of 0.22 pM. • Linear range up to 100 nM • pH dependency not reported.	²⁸⁰
Methylene Blue (MB)	-0.25 V vs Ag AgCl	Tobramycin, vancomycin	• Wide dynamic range (up to 100 μM) • Tested in rats with good acceptance between blood and ISF • Photo sensitive • pH dependent.	²⁶⁹
Anthraquinone (AQ)	-0.37 V vs Ag AgCl	Interferon gamma (IFN- γ)	• Sensors able to continuously monitor IFN-γ over 2 hours. • High redox potential • pH dependency not reported.	²⁸¹
Ferricyanide/ Ferrocyanide ([Fe(CN) ₆] ^{3-/4-})	+0.30 V vs Ag AgCl	Thrombin	• Impedimetric measurements • High sensitivity • Linear range from 5-35 nM • pH dependency not reported.	²⁷¹
Tetrathiafulvalene (TTF)	+0.05 V vs Ag AgCl	Cocaine, kanamycin	• Chemically stable • Redox potential close to 0 V • pH independent • Tested in pH-fluctuating living rat bladder.	²⁸²
Ruthenium(II) complex	N/A	Platelet-derived growth factor (PDGF)	• Luminescent probe • Non-covalent binding to DNA backbone	²⁷²

			• Limit of detection is 0.8 pM • Small effects of pH.	
Osmium(2,2'-bipyridyl)poly(4-vinylpyridine) ([Os(bpy) ₂ (PVP) ₁₀ Cl]Cl)	+0.40 V vs Ag AgCl	Glucose	• Used in commercially available CGMs • Low toxicity of Os(II/III) • Improved sensitivity, less interference.	283284
[Os(dmebpy) ₂ Cl(ampy)] ⁺ (Os(II/III))	+0.20 V vs Ag AgCl	Tobramycin, procaine	• pH independent • 35% less signal loss • 50-times higher electron transfer.	261
Thionine	-0.35 V vs Ag AgCl	Alpha fetoprotein	• Dynamic range up to 100 µg/mL • Limit of detection was 0.050 µg/mL • Label free method • pH dependency not reported.	273

Table 4.1 A summary of the different types of redox labels used in E-ABs.

The ideal potential for a redox label is one where the least interference is observed from other electroactive molecules, such as acetaminophen, ascorbic acid, and uric acid. For the purpose of infection monitoring of patients, it is likely that paracetamol may be prescribed in hospital and therefore, circulating acetaminophen may contribute to signal interference. The potential at which acetaminophen oxidises is dependent on pH; at pH 7, Nematollahi *et al.* report the potential to be +0.45 V vs saturated calomel reference electrode (SCE) at a glassy carbon working electrode.²⁸⁵ In addition, interference from dissolved oxygen is a possibility, however, this could be considered an offset to the biosensor response as large changes in oxygen concentration are not expected *in vivo*. The redox potentials of the O₂/H₂O₂ and the O₂/H₂O couples are +0.281 V and +0.815 V, respectively, vs the standard hydrogen electrode

(SHE) at pH 7.0, however significant overpotentials are observed experimentally.²⁸⁶

Therefore, an ideal potential for a redox label is somewhere between 0 V and +0.1 V.

While there are a vast number of redox labels reported in literature, as summarised in Table 1, the most commonly used labels in E-ABs are ferrocene, methylene blue and osmium complexes. These are explored in greater detail below.

4.1.2 Ferrocene label

Ferrocene behaves as a redox label, showing reversible redox behaviour in organic and aqueous solutions, as shown in Figure 4.1. It is commercially available with several derivatives,²⁸⁷ offering ease of synthesis and conjugation to aptamers in E-AB fabrication.²⁸⁰ The redox potential of the oxidation of Fe(II)/Fe(III) couple is +0.25V vs Ag|AgCl. Despite its wide use, ferrocene-labelled E-ABs can experience rapid signal loss due to the ferrocenium ion (Fc^+) undergoing ligand exchange reactions with chloride ions present *in vivo*.²⁶¹

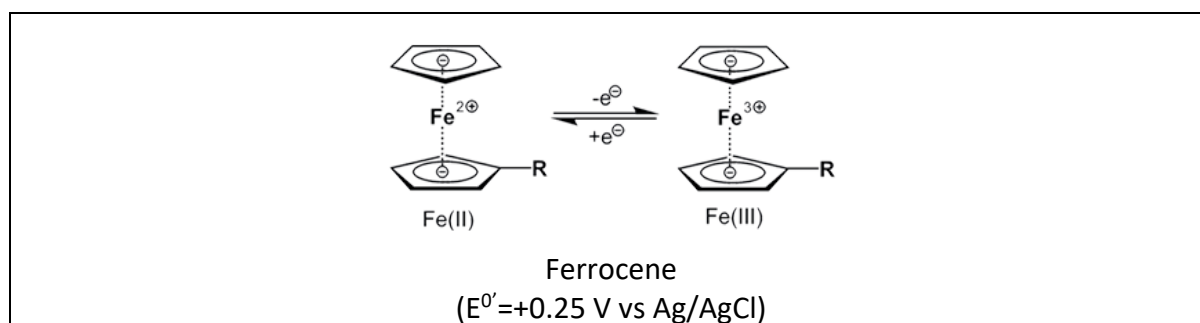


Figure 4.1 The reversible oxidation of the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}) in ferrocene redox labels.

4.1.3 Methylene blue label

Methylene blue (MB), the most commonly used redox label in aptamer sensors,^{269,288,289} has several drawbacks, including the need for pH regulated environments to enable electron transfer, the reported loss of signal over time after repeated electrochemical interrogation,²⁵⁰ and the photosensitive nature of the label. In addition, the negative potential range required to capture a signal response has shown to cause reductive desorption of the self-assembled monolayer of thiol-aptamers on gold electrodes over time.²⁹⁰ Therefore, there is a need to explore alternative redox labels for aptamer modification in the development of E-ABs. Figure 4.2 shows the reversible reduction of MB to leucomethylene blue (LMB) at a redox potential of -0.25 V vs Ag|AgCl.

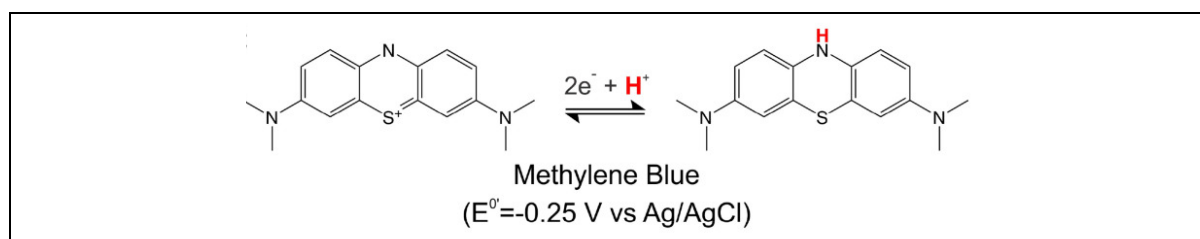


Figure 4.2 The reversible reduction of MB to LMB, adapted from Pellitero et al.²⁶¹

The effect of pH on MB sensor response has been reported by Li *et al.* wherein sensor responses from tetrathiafulvalene (TTF) and MB-labelled cocaine and kanamycin E-ABs were compared in different pH environments.²⁸² Figure 4.3 shows that the proton-dependent MB label had a large positive peak shift as the pH decreased, whereas the TTF-labelled sensor did not show peak shift based on pH.²⁸² However, there are other reports of pH affecting the TTF^+/TTF and TTF^{2+}/TTF^+ redox couples, due to the H^+ proton transfer that occurs in the redox reaction.²⁹¹

This demonstrates the importance of designing a redox label that is pH independent to ensure the sensor responds under pH fluctuations in unbuffered environments, such as sweat and saliva.

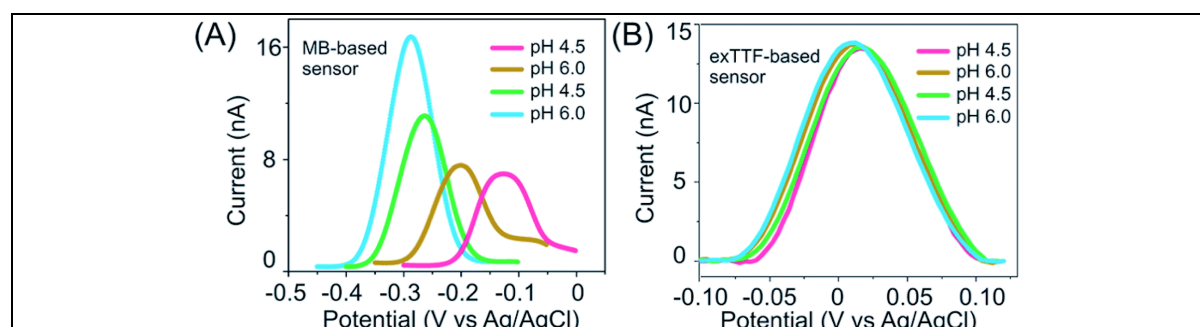


Figure 4.3 pH dependent sensor response in (a) MB-labelled and (b) TTF-labelled kanamycin E-ABs, as reported by Li *et al.*²⁸²

4.1.4 Osmium complex label

Most applications of osmium redox labels in literature involve cross-linking of the osmium complex to polymer matrices or hydrogels, as opposed to linking the complex to the aptamer.²⁹² Adam Heller's group (University of Texas at Austin, USA) first reported the use of osmium in redox hydrogels consisting of poly(1-vinylimidazole) (PVI) complexed with an osmium reporter - $[\text{Os}(\text{bpy})\text{zCl}]^+$ - and crosslinked this with poly(ethylene glycol) diglycidyl ether (PEG-DE).^{293,294} The resulting redox hydrogels were used to fabricate amperometric lactate oxidase (LOD) and glucose oxidase (GOD) sensors with low operating potentials of +0.2 V vs SCE, due to which interfering signals from acetaminophen, ascorbate and urate were negligible compared to sensor response. Forster *et al.* have reported combinations of metallopolymer, osmium bis(2,2'-bipyridyl) and ruthenium(II) with poly(4-vinylpyridine)

(PVP) and PVI, finding the osmium based metallopolymer to be more photostable on glassy carbon electrodes than ruthenium(II).²⁹⁵

Osmium labelled PVP polymers have since been used as redox labels in GOD sensors commercially.²⁹⁶ Herein, the $\text{Os}^{2+/3+}$ ion mediates the transfer of electrons between the enzyme and the electrode surface, as shown in Figure 4.4. The substrate, glucose, is oxidised to gluconolactone, in turn reducing the enzyme's co-factor, flavin adenine dinucleotide (FAD), to FADH_2 . In the presence of oxygen, FADH_2 can oxidise back to FAD. In the presence of an electron mediator like the $\text{Os}^{2+/3+}$ ion at the electrode surface, Os^{3+} is ready to receive electrons from FADH_2 , reducing itself to Os^{2+} , and then transfers these to the electrode's surface, overcoming the need for oxygen in electron transfer.

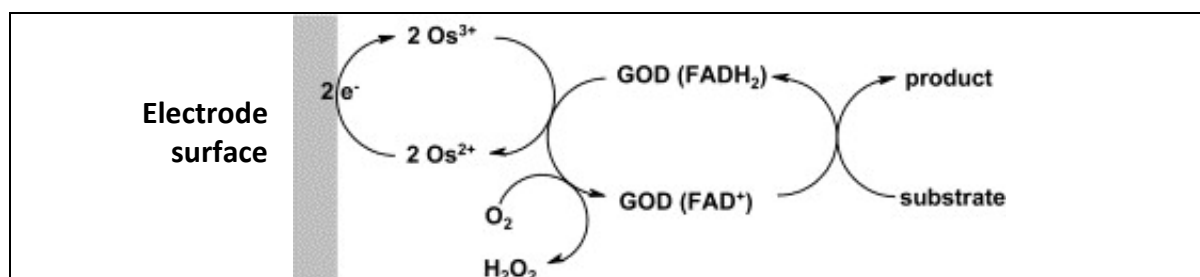


Figure 4.4 Reaction scheme of osmium mediated GOD reaction at the electrode surface, as reported by Reiter *et al.*²⁹⁷

Osmium redox labels offer the advantage of lower redox potentials, meaning less noise from interfering electroactive molecules, and robust labelling schemes, as reported in the fabrication of GOD sensors. Osmium redox labels are also not sensitive to pH, unlike MB redox labels, which makes them more chemically stable in different pH environments. In addition, the tuneable potential of Os(II)/Os(III) couple and its higher stability compared to other metal complexes makes osmium based redox reporters an attractive choice for E-ABs.²⁹⁸ However, osmium labels do come with their drawbacks; they can be more toxic than MB, depending on

their oxidation state i.e., the higher the oxidation state, the more toxic the label. At an oxidation state of Os(II) or Os(III), toxicity is reported to be low.^{299,300} Due to the large size of osmium labels, they can cause steric hindrance, however, this has proven to be beneficial in some circumstances, preventing the adsorption of the redox label on graphite electrode surfaces.²⁹²

Pellitero *et al.* report E-AB sensors for tobramycin and procaine, made using an Os(II/III) redox label, $[\text{Os}(\text{dmebpy})_2\text{Cl}(\text{ampy})]^+$, with a redox potential of +0.20 V vs Ag|AgCl (Figure 4.5). Herein, the reporter was immobilised directly onto the surface of gold electrodes, and the aptamer was coupled to the reporter using EDC/NHS chemistry. The sensor performance was independent of pH, unlike MB-labelled E-ABs, and electron transfer rates were reported to be 50-times faster than conventional MB sensors. The use of $[\text{Os}(\text{dmebpy})_2\text{Cl}(\text{ampy})]^+$ as a redox reporter has also shown to improve stability of the monolayers upon repeated electrochemical interrogation, with 35% less loss of signal compared to sensor responses from MB-labelled E-ABs.²⁶¹

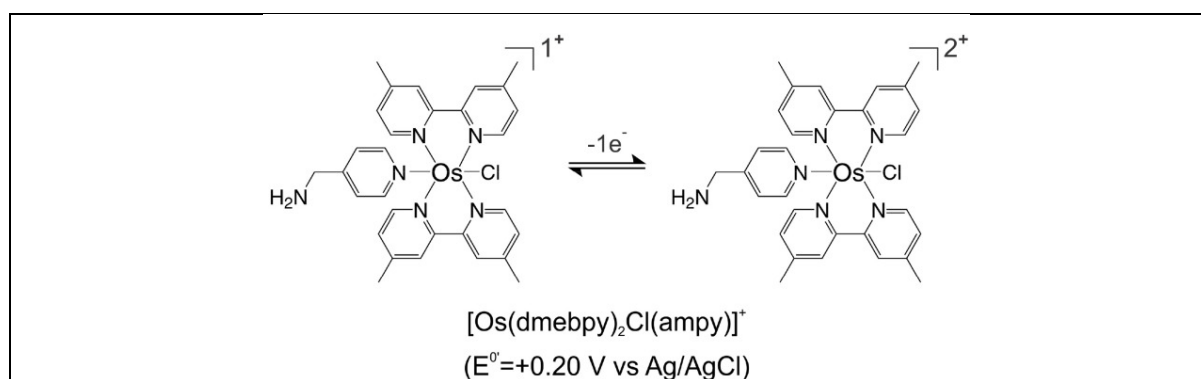


Figure 4.5 Reversible electrochemical redox reaction of Os(II/III) complex, $[\text{Os}(\text{dmebpy})_2\text{Cl}(\text{ampy})]^+$, adapted from Pellitero *et al.*²⁶¹

4.1.5 Synthesis of osmium(II/III) bipyridyl complex and modification of CRP aptamer

In this chapter, we discuss the synthesis of an osmium redox label $\text{Os(II)(bipy)}_2\text{ClNH}_2$ modified with an amine group, and the conjugation of this label to a carboxylic acid-terminated aptamer using EDC/NHS chemistry. The proposed synthesis of the redox label and the modification of the aptamer is shown in Figure 4.6. We optimise the sensor fabrication protocol and test the sensor's performance in aptamer binding buffer (blank) and after binding to CRP using CV, DPV and SWV methods.

Melow *et al.* report the synthesis of an osmium-based redox complex used for the electrochemical detection of glucose and lactate. The synthesis protocol, outlined in the Methods section and shown in Figure 4.6, was followed to produce an intermediate compound, $\text{Os(II)(bipy)}_2\text{Cl}_2$, wherein ammonium hexachloroosmate(IV) $((\text{NH}_4)_2\text{OsCl}_6)$ was reacted with 2,2'-bipyridine in ethylene glycol. Following this, as reported by Conghaile *et al.*, 4-aminomethyl pyridine (4-AMP) was used to substitute one of the Cl ligands with an amine group on the osmium complex, producing $\text{Os(II)(bipy)}_2\text{ClNH}_2$.³⁰¹ The carboxyl terminated aptamer was then coupled using EDC/NHS chemistry with the amine-terminated osmium bipyridyl complex.

Meng *et al.* report several different types of surface immobilisation strategies for nucleic acid sensors.³⁰² Of those based on gold, the gold-thiol self-assembled monolayer (SAM) is the most commonly used immobilisation method, with thiol-modified nucleic acids and thiol derivatives being used to create the Au-thiol bond. In addition, poly adenine (polyA) anchoring, electrostatic adsorption and hydrophobic interactions are other ways to immobilise nucleic acids on gold electrodes that have been explored. For the immobilisation

of the osmium-labelled aptamer in this chapter, the aptamer was modified with a thiol group at the 3' end and this was reduced by tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) before immobilisation and formation of the SAM on gold, as reported by Kevin Plaxco's group (University of Santa Barbara, USA).³⁰³

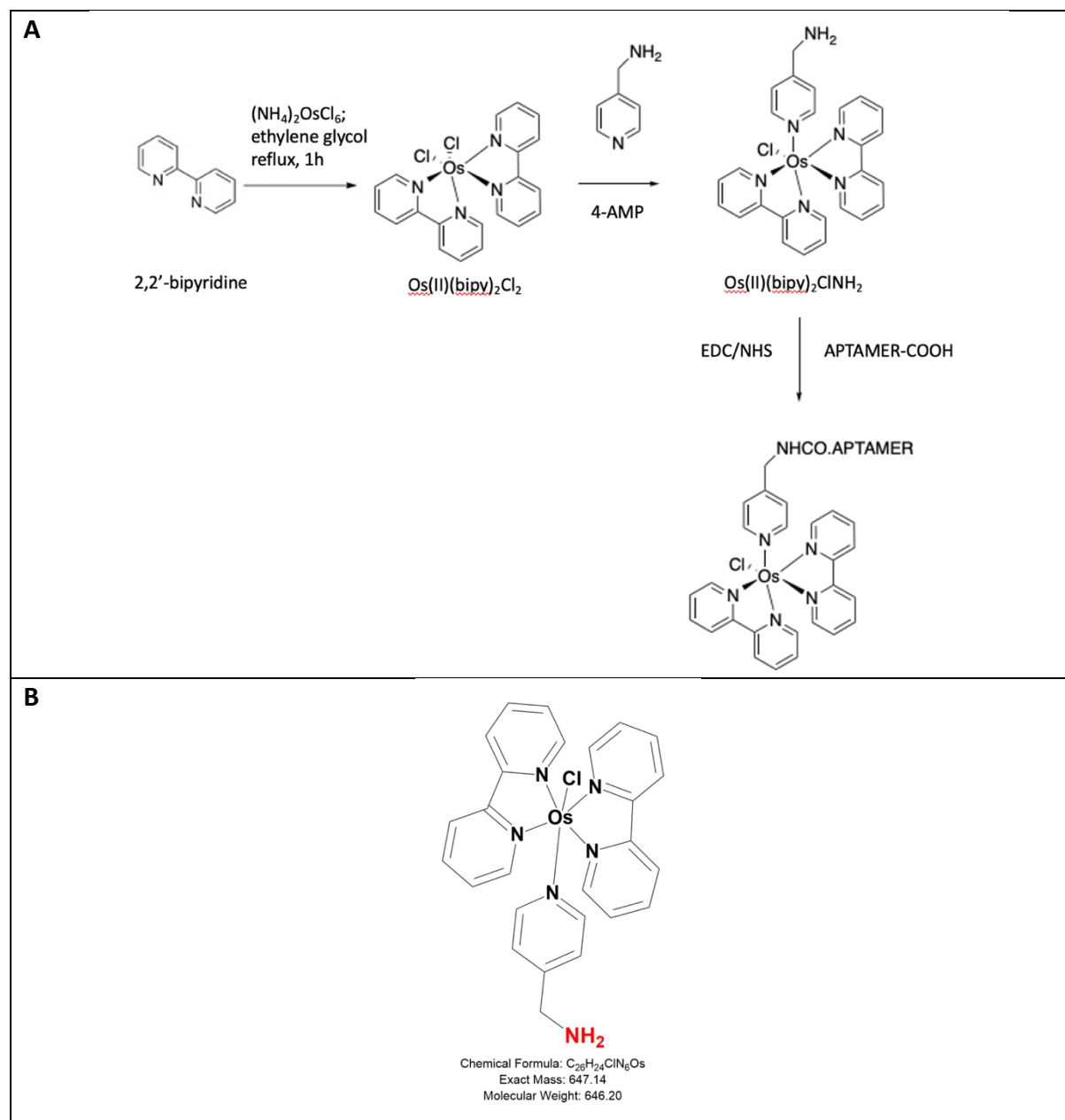


Figure 4.6 (a) Synthesis of an amine-terminated osmium-bipyridyl complex, $\text{Os(II)(bipy)}_2\text{CINH}_2$, and proposed chemistry for amide coupling with carboxyl terminated aptamer, and (b) molecular weight of the amine terminated osmium-bipyridyl complex.

4.2 Methods

4.2.1 Electrodes and instrumentation

The electrodes used were one-time use, disposable gold electrodes manufactured by Respire Diagnostics (UK), as described previously in Chapter 3 for the MB-terminated aptamers. For all experiments in this chapter, unless otherwise stated, the PalmSens Emstat potentiostat by PalmSens BV GMBH (Germany) was used to run any electrochemical readings. Where stated, a CompactStat potentiostat by Ivium technologies (the Netherlands) was used. The software used to record measurements on the PalmSens Emstat was the PStace software and on the ComptactStat, it was the IviumSoft software.

4.2.2 Smoothing and baseline correction

A Savitzky-Golay filter was applied to smooth the CV, DPV and SWV scan data in the PStace software (PalmSens, UK), as described previously in Chapter 3 for the MB-terminated aptamers. After smoothing, the baseline was corrected on PStace software using the built-in linear baseline correction function. Two points were chosen on each scan to determine the baseline and a linear function was used for correction.

4.2.3 Synthesis of osmium(II/III) redox reporter-conjugated aptamers

The CRP aptamer published by Huang *et al.*²⁵¹ was synthesized from IDT with a 3' thiol modification and a 5' carboxylic acid (-COOH) modification. The carboxylic acid group was chosen for subsequent coupling to an amine group on the redox reporter. The following aptamer sequence was sent to IDT, adapted from the sequence published by Huang *et al.*²⁵¹

Sequence of carboxylic acid-terminated CRP aptamer:

5'-/5Carboxy1/GG CAG GAA GAC AAA CAC GAT GGG GGG GTA TGA TTT GAT GTG GTT GTT
GCA TGA TCG TGG TCT GTG GTG CTG T/3ThioMC3-D/-3'

4.2.4 Synthesis of Os(II)(bipy)₂Cl₂ redox reporter

Os(II)(bipy)₂Cl₂ in the reaction scheme, outlined in Figure 4.6, was synthesized by Professor Tony Cass' group in the Department of Chemistry at Imperial College London. The synthesis protocol was adapted from Melow *et al.*³⁰⁴ and Groppi *et al.*,²⁹² wherein ammonium hexachloroosmate(IV) ((NH₄)₂OsCl₆) was refluxed with 2,2'-bipyridine (in the ratio 1:2) for 45 minutes in ethylene glycol in a round bottom flask under N₂ gas. The reaction mixture was then cooled for 2 hours and added drop-wise to an aqueous solution of 0.5 M sodium dithionite, under constant stirring, to reduce the osmium complex to the Os(II) state. The mixture was then cooled further on ice and filtered using a glass frit. The filtered crystals were then washed with DI water and dried under a vacuum for 1 hour, followed by overnight drying in an oven at 65 °C. Once dried, the compound was reacted with 4-aminomethyl pyridine (4-AMP) to produce an amine group on the compound for conjugation with the carboxyl-terminated aptamer via EDC/NHS coupling.

4.2.5 Amide coupling of Os(II)(bipy)₂ClNH₂ redox reporter to carboxyl-terminated aptamer

The COOH-terminated aptamer was reconstituted in 175 µL sterile DI water to make a concentration of 200 µM aptamer. A solution of 800 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 200 mM 2-(N-morpholino)ethanesulfonic acid (NHS) was prepared in 200 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer. To 87.5 µL of the 200 mM aptamer solution, 87.5 µL of the prepared EDC/NHS solution was added and

mixed well. The concentration of aptamer, EDC and NHS in this mixture then became 100 μ M, 400 μ M, and 100 μ M, respectively. The solution was incubated for an hour at room temperature to allow amide coupling.

0.12 mg of Os(II)(bipy)₂Cl₂ was weighed out and following incubation, the aptamer-EDC/NHS solution was added to this mixture with the Os(II)(bipy)₂Cl₂ redox reporter being at least 10x excess in concentration to the aptamer. The solution of aptamer and Os(II)(bipy)₂Cl₂ were mixed well overnight on a rotator at room temperature. This allowed the amine group on the Os(II)(bipy)₂Cl₂ complex to react with the carboxyl group of the aptamer.

4.2.6 Filtering Os(II)(bipy)₂ClNH₂ redox reporter solution

To filter the Os(II)(bipy)₂ClNH₂-bound aptamer from the unbound Os(II)(bipy)₂ClNH₂ in solution, a 0.5 mL Amicon Ultra centrifugal filter (Millipore) was used. 170 μ L of the Os(II)(bipy)₂Cl₂-coupled aptamer solution was aliquoted out of the stock solution and diluted by a factor of two in MES buffer. The diluted aptamer solution was added to the filter tube and the tube centrifuged at 13,000 rpm for 10 mins. The filter allows molecules of < 30 kDa to pass, therefore, any unbound Os(II)(bipy)₂Cl₂ would pass through as filtrate and any Os(II)(bipy)₂Cl₂-coupled aptamer would stay in the filter as residue solution. Subsequent filtrations were carried out to purify the Os(II)(bipy)₂Cl₂-coupled aptamer solution by diluting the aptamer residue with 170 μ L MES buffer and spinning the filter tube at 13,000 rpm in the centrifuge. After seven runs of filtration, the leftover aptamer solution was collected by spinning the inverted filter into a fresh tube at 13,000 rpm for 45s and further aliquoted for sensor fabrication.

4.2.7 Liquid Chromatography–Mass Spectrometry (LCMS) of Os(II)(bipy)₂Cl₂ before coupling

An LCMS was carried out to verify that the structure of the Os(II)(bipy)₂ClNH₂ label. A standard LCMS protocol was followed using the Agilent 1290 Infinity II (Agilent, USA) ultra-high performance liquid chromatography (UHPLC) instrument. A 100 µL solution of 2.5 µM Os(II)(bipy)₂Cl₂ complex was prepared in sterile DI water. The mobile phase was triethylamine (TEA) hexafluoroisopropanol (HFIP) buffer in the ratio of 15mM:400mM (TEA:HFIP) and methanol. The % of methanol followed a gradient regime and the columns used were the AdvanceBio Oligonucleotide UHPLC guard columns (Agilent, USA).

4.2.8 Sensor fabrication

Electrode pre-treatment

Disposable gold electrodes (Respire Diagnostics, UK) were electrochemically cleaned, as described in the methods section in Chapter 2 (MB).

Deposition of aptamer onto electrodes

Aptamers were deposited onto the pre-treated electrodes, followed by 6-MCH back-filler, as described in the methods section in Chapter 2 (MB).

Sensor characterisation

The objectives behind electrochemical characterisation of the sensors is to (i) find the potential at which the redox label undergoes oxidation and reduction, (ii) find whether the aptamer has successfully deposited onto the electrode, (iii) calculate the quantity of deposited aptamer, (iv) find if there is a concentration dependent relationship with signal

response, and (v) optimise the fabrication process and conditions under which the sensor is characterised for performance testing.

4.2.9 Electrochemical interrogation

Cyclic Voltammetry

CV was conducted to understand current dependence on scan rate and to calculate the area under peaks to determine the immobilised concentration of aptamer. CV was conducted as described in the methods section in Chapter 2 (MB).

Differential Pulse Voltammetry

DPV was conducted to understand the peak potential (E_p) and peak current (I_p) of the E-AB before and after binding to CRP. DPV was conducted as described in the methods section in Chapter 2 (MB).

Square Wave Voltammetry

SWV was conducted to understand the peak potential (E_p) and peak current (I_p) of the E-AB before and after binding to CRP. DPV was conducted as described in the methods section in Chapter 2 (MB).

Chronoamperometry

Chronoamperometry measurements were recorded to compare the amount of charge passed at the electrode surface before and after aptamer deposition, in addition to before and after CRP incubation. The charge was calculated by integrating the current with respect to time,

obtained from the oxidation and reduction scans of chronoamperometry, as described in the methods section in Chapter 2 (MB).

4.2.10 Optimisation of aptamer concentration

Aptamer concentrations were made up to 1, 2.5, 5 and 10 μM by diluting with aptamer binding buffer and aptamer solutions were deposited onto electrodes using the deposition method above. Following sensor fabrication, sensors were tested using CV, DPV and SWV methods, also outlined in the methods section in Chapter 2 (MB).

4.2.11 Sensor calibration

The aptasensor was tested electrochemically in blank, 1, 10 and 100mg/L CRP serially for an hour each time, before being rinsed off with binding buffer. After each incubation, the aptasensor was tested electrochemically with CV, DPV and SWV techniques.

4.2.12 Regeneration of aptamer after CRP binding

To regenerate the aptamer from the aptamer-CRP complex, a regeneration solution of 10 mM Tris buffer was made containing 50 mM NaCl and 2 mM CaCl_2 . 10 μL of this solution was placed onto the aptasensor working electrode and incubated for 1 hour. The solution was then rinsed off with aptamer binding buffer and the aptasensor interrogated electrochemically to compare the response of the regenerated aptamer with the unbound (blank) aptamer response.

4.3 Results

4.3.1 Liquid Chromatography–Mass Spectrometry

LCMS was used to confirm the presence of $\text{Os(II)(bipy)}_2\text{Cl}_2$ ($\text{C}_{26}\text{H}_{24}\text{ClN}_6\text{Os}$) in solution before amide coupling with the carboxyl terminated aptamer. Figure 4.7 shows the LCMS spectra at mass-to-charge (m/z) ratios between 615 and 660. Peaks are seen at m/z ratios of 644.1113, 645.1206, and 646.1041, which are comparable to the molecular weight of $\text{Os(II)(bipy)}_2\text{ClNH}_2$ i.e., 646.20 g/mol. This confirmed that $\text{Os(II)(bipy)}_2\text{Cl}_2$ was successfully synthesized with an amine group for coupling to the aptamer.

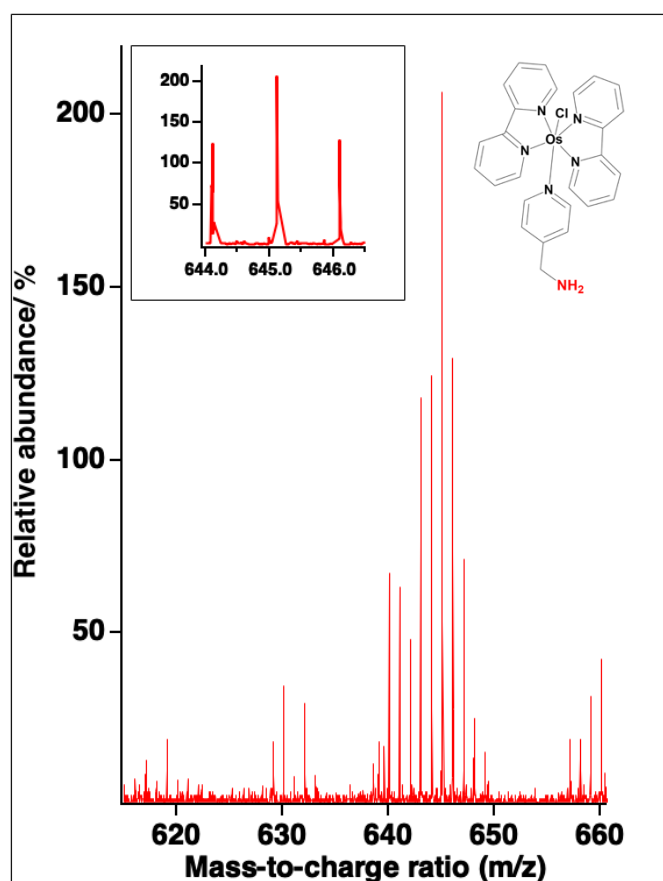


Figure 4.7 LCMS spectra of $\text{Os(II)(bipy)}_2\text{Cl}_2$.

4.3.2 Electrochemistry of $\text{Os(II)(bipy)}_2\text{ClNH}_2$ and $\text{Os(II)(bipy)}_2\text{ClNH}_2$ -coupled aptamer solutions

After the EDC/NHS coupling step, where $\text{Os(II)(bipy)}_2\text{ClNH}_2$ was coupled to the carboxyl-terminated aptamer, filtration of the solution to remove any unbound $\text{Os(II)(bipy)}_2\text{ClNH}_2$ was carried out. CV, DPV and SWV scans were taken of the $\text{Os(II)(bipy)}_2\text{ClNH}_2$ solution (osmium redox label) and the $\text{Os(II)(bipy)}_2\text{ClNH}_2$ -coupled aptamer (osmium-aptamer solution) on pre-treated disposable gold electrodes, as shown in Figure 4.8.

In the CV of the $\text{Os(II)(bipy)}_2\text{Cl}_2$ solution (Figure 4.8(a)), peaks were seen at +0.320 V and +0.200 V. Negative peak shifts of -0.180 V and -0.140 V were seen in the anodic and cathodic scans, respectively, after coupling to the aptamer. The ratios (before:after coupling to aptamer) of the CV peak heights were 2.29 (anodic) and 3.33 (cathodic).

In the DPV scan of the $\text{Os(II)(bipy)}_2\text{Cl}_2$ solution (Figure 4.8(b)), peaks were seen at +0.230 V (both anodic and cathodic). Negative peak shifts of -0.165 V and -0.085 V were seen in the anodic and cathodic scans, respectively, after coupling to the aptamer. The ratios (before:after coupling to aptamer) of the DPV peak heights were 3.54 (anodic) and 1.59 (cathodic).

In the SWV scan of the solution (Figure 4.8(c)), peaks were seen at +2.20 V (both anodic and cathodic). Negative peak shifts of -0.095 V and -0.120 V were seen in the anodic and cathodic scans, respectively, after coupling to the aptamer. The ratios (before:after coupling to aptamer) of the SWV peak heights were 1.76 (anodic) and 2.22 (cathodic).

A mean negative peak shift of -0.147 V (anodic) and -0.115 V (cathodic) was seen after coupling to the aptamer across CV, DPV and SWV scans. Since the aptamer-coupled redox label was in solution, and not immobilised, we expect there to be a peak-to-peak separation, as is observed in the CV and DPV scans.

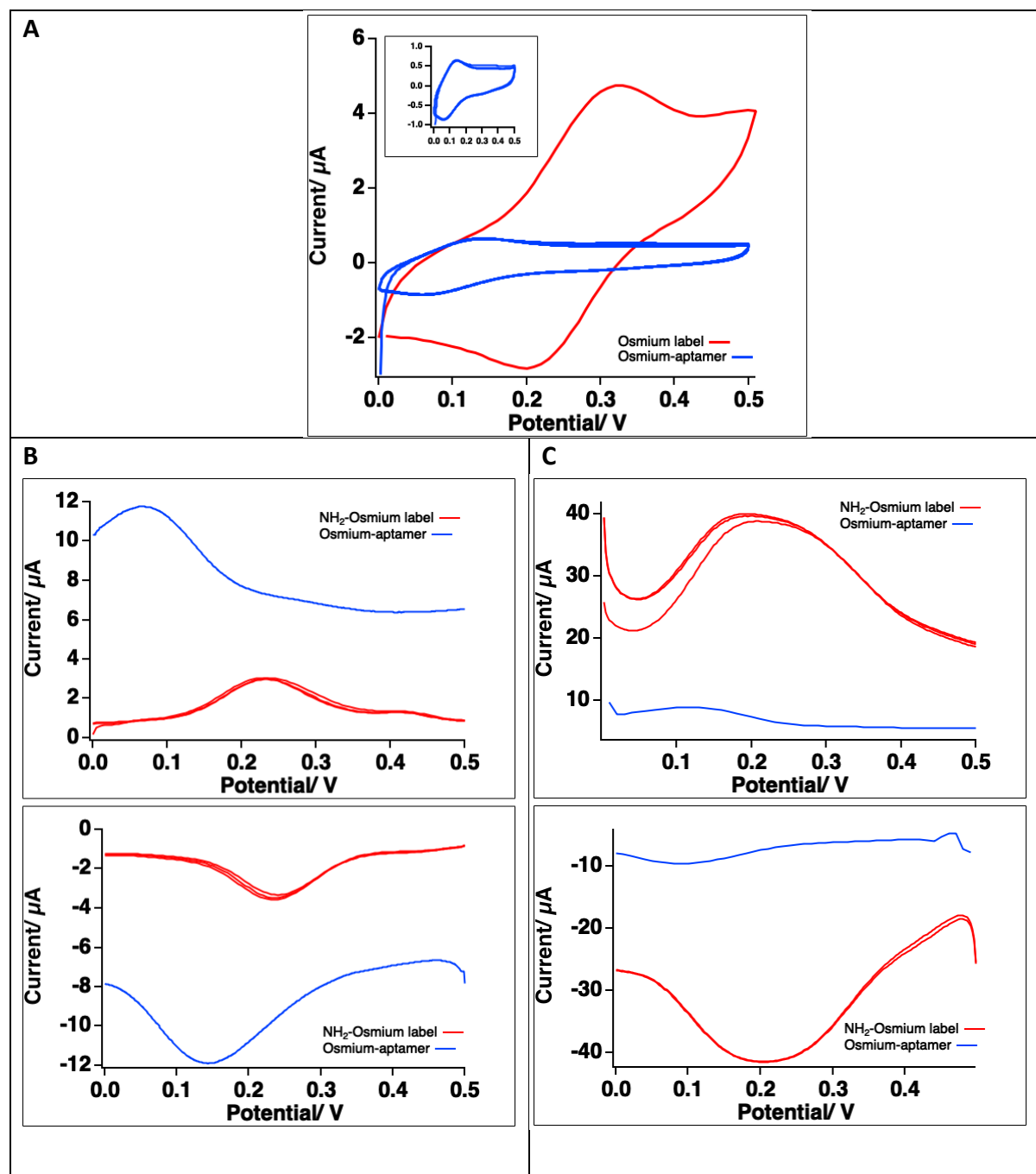


Figure 4.8 (a) CV, (b) DPV, and (c) SWV of $\text{Os(II)(bipy)}_2\text{ClNH}_2$ before (red) and after (blue) conjugating to aptamer sequence.

Table 4.2 outlines the peak potentials of the $\text{Os(II)(bipy)}_2\text{Cl}_2$ complex before and after coupling to the aptamer, and their relative peak shifts.

Method	Anodic peak potential before coupling/ V	Anodic peak potential after coupling/ V	Anodic peak shift/ V	Cathodic peak potential before coupling/ V	Cathodic peak potential after coupling/ V	Cathodic peak shift/ V
CV	+0.320	+0.140	-0.180	+0.200	+0.060	-0.140
DPV	+0.230	+0.065	-0.165	+0.230	+0.145	-0.085
SWV	+0.220	+0.125	-0.095	+0.220	+0.100	-0.120

Table 4.2 Peak potentials of the $\text{Os(II)(bipy)}_2\text{Cl}_2$ complex before and after coupling to the aptamer, and their relative peak shifts.

The large changes in peak potential observed after coupling to the aptamer can be explained by the amide bond created by the EDC/NHS coupling reaction between the redox label and the aptamer. The redox label, before coupling, is a free diffusing species, whereas after coupling to the aptamer, it is possible that the aptamer adsorbs onto the gold electrode or spontaneous Au-thiol bonds form, immobilising the aptamer onto the gold surface.

This demonstrates that the $\text{Os(II)(bipy)}_2\text{ClNH}_2$ -coupled aptamer's electrochemical behaviour was similar to the complex before coupling.

4.3.3 Scan rate optimisation in DPV

To maximise the absolute current response in the unbound state, the scan rate was varied from 10 to 100 mV/s in DPV. A scan rate of 25 mV/s was chosen for future DPV experiments due to the high signal response, as seen Figure 4.9. It was observed that peak current was

highest at 25 mV/s, and decreased at the higher scan rate of 100 mV/s. No anodic peaks were observed at scan rates between 25 and 100 mV/s, and no cathodic peaks were observed at any of the scan rates.

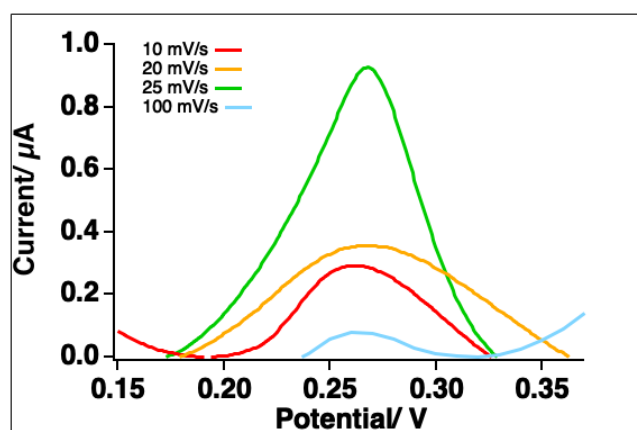


Figure 4.9 Optimisation of signal response by altering scan rates in DPV from 10 to 100 mV/s for (a) the anodic response and (b) cathodic response ($n=1$).

For CVs, a scan rate of 25 mV/s was chosen. This is because, although signal response was higher at higher scan rates, a low resolution of data points per unit volt was being obtained due to a lower E_{pulse} (Figure 4.10). The E_{pulse} was 1 mV at 25mV/s and 10 mV at scan rates higher than 150 mV/s.

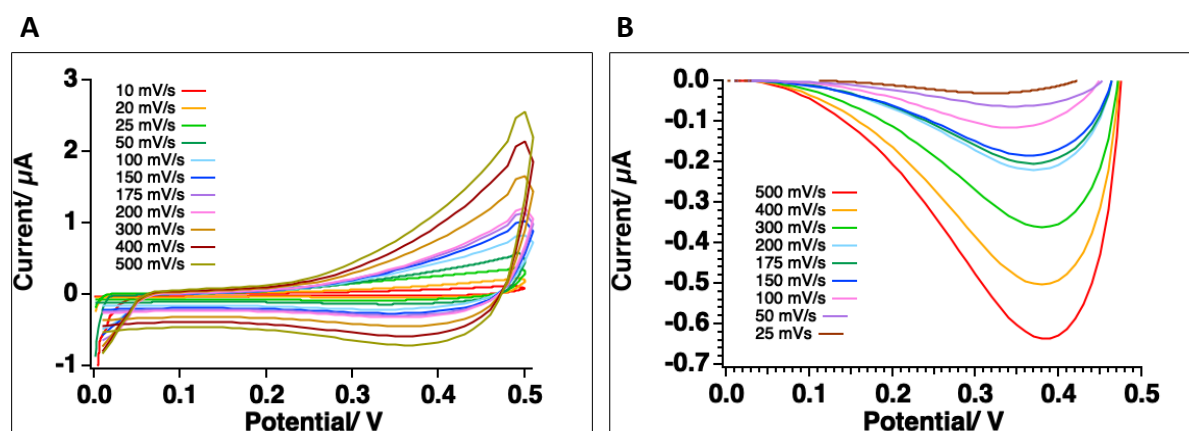


Figure 4.10 Optimisation of signal response by altering scan rates in (a) CV from 10 to 25 mV/s, and (b) extracted anodic CV peaks ($n=1$).

4.3.4 Current dependence on scan rate

To investigate the relationship between peak height and scan rate, data from the CV scans was fitted to the Randle-Sevcik equation (see equation 4.1), where I_p is peak height (A), n is the number of electrons in the reaction, F is Faraday's constant, A is surface area of the electrode (cm^2), C is the concentration of the redox species (mol cm^{-3}), v is the scan rate (V s^{-1}), D is the diffusion coefficient of the redox species ($\text{cm}^2 \text{s}^{-1}$), R is the gas constant and T is the temperature. The peak height was plotted against the square root of scan rate (Figure 4.11 (a)). Table 4.3 outlines parameters of the linear regression applied to the plot of the peak height vs square root of scan rate (i.e. the Randle-Sevcik plot).

$$I_p = 0.446nFAC\left(\frac{nFvD}{RT}\right)^{\frac{1}{2}}$$

Equation 4.1 The Randle-Sevcik equation.

A linear relationship in the Randles-Sevcik plot generally indicates that the species is freely diffusing. For a known adsorbed species, such as for an E-AB, the linear fit denotes that the reaction is quasi-reversible, meaning that the redox label is unable to oxidise and reduce to the same amount each time.

We also see a linear relationship between peak height and the scan rate. This confirms that the electrochemical process is quasi-reversible, and the difference seen between the anodic and cathodic peak heights at each scan rate show that the redox label is not oxidising and reducing the same amount each time. The cathodic peak heights are consistently lower in magnitude at each scan rate.

It is worth noting that the anodic intercept of the Randles-Sevcik plot is very close to zero (0.10) and the cathodic intercept is relatively close to zero (0.21). This indicates that the current response is coming from the redox reaction of the osmium(II/III) redox reporter, as opposed to from non-faradaic contributions.

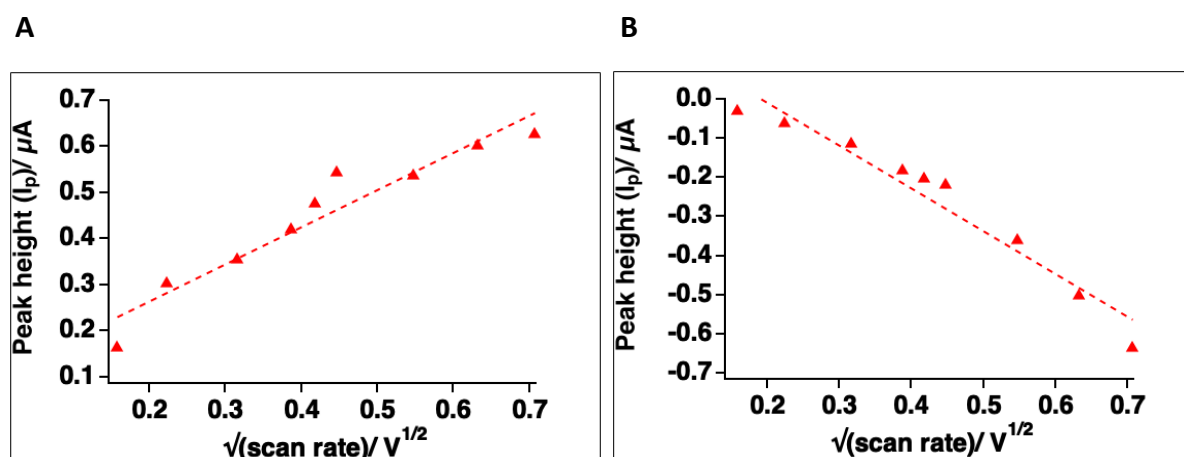


Figure 4.11 Randles-Sevcik (peak height against square root of scan rate) plots for (a) anodic and (b) cathodic scans ($n=1$).

Parameter	Anodic	Cathodic
Gradient	0.80 ± 0.090	-1.09 ± 0.100
y-intercept	0.10 ± 0.041	0.21 ± 0.046
r^2	0.920024	0.944283

Table 4.3 Parameters of linear relationship between peak height and square root of scan rate (V/s)^{1/2}.

For known adsorbed species, Equation 4.2 describes the expected linear relationship between current response (I_p) and scan rate (ν), where the Γ^* is the surface coverage of the adsorbed species (mol cm^{-2}).⁷² Therefore we expect to see a proportional relationship between peak current and scan rate, as well between peak current and surface coverage of the aptamer.

$$I_p = \frac{n^2 F^2}{4RT} v A \Gamma^*$$

Equation 4.2 The relationship between peak current, scan rate and surface coverage of aptamer.

Peak current was plotted against scan rate (Figure 4.12(a) and (b)) and a linear relationship was observed. This means that the reaction is surface- or adsorption-controlled. Fit parameters from the linear regression applied to the peak current vs scan rate plot are outlined in Table 4.4.

The slope of the $\log(I_p)$ vs the $\log(v)$ determines whether the process is controlled by diffusion or adsorption. For an adsorption-controlled process, the slope is close to 1.0, whereas for a diffusion-controlled process, this is close to 0.5.²⁴⁷ As shown in Figure 4.12(c), the relationship between $\log(I_p)$ and $\log(v)$ is linear for both oxidation and reduction. The slope of the oxidation scan is 0.43 ± 0.04 , which is close to 0.5, indicating that it could be a diffusion-controlled process which is an unexpected result for an E-AB.

The slope of the reduction scan is 1.00 ± 0.02 , meaning that the process is adsorption-controlled, as expected for E-ABs. Fit parameters from the linear regression applied to the $\log(I_p)$ vs the $\log(v)$ plot are outlined in Table 4.5.

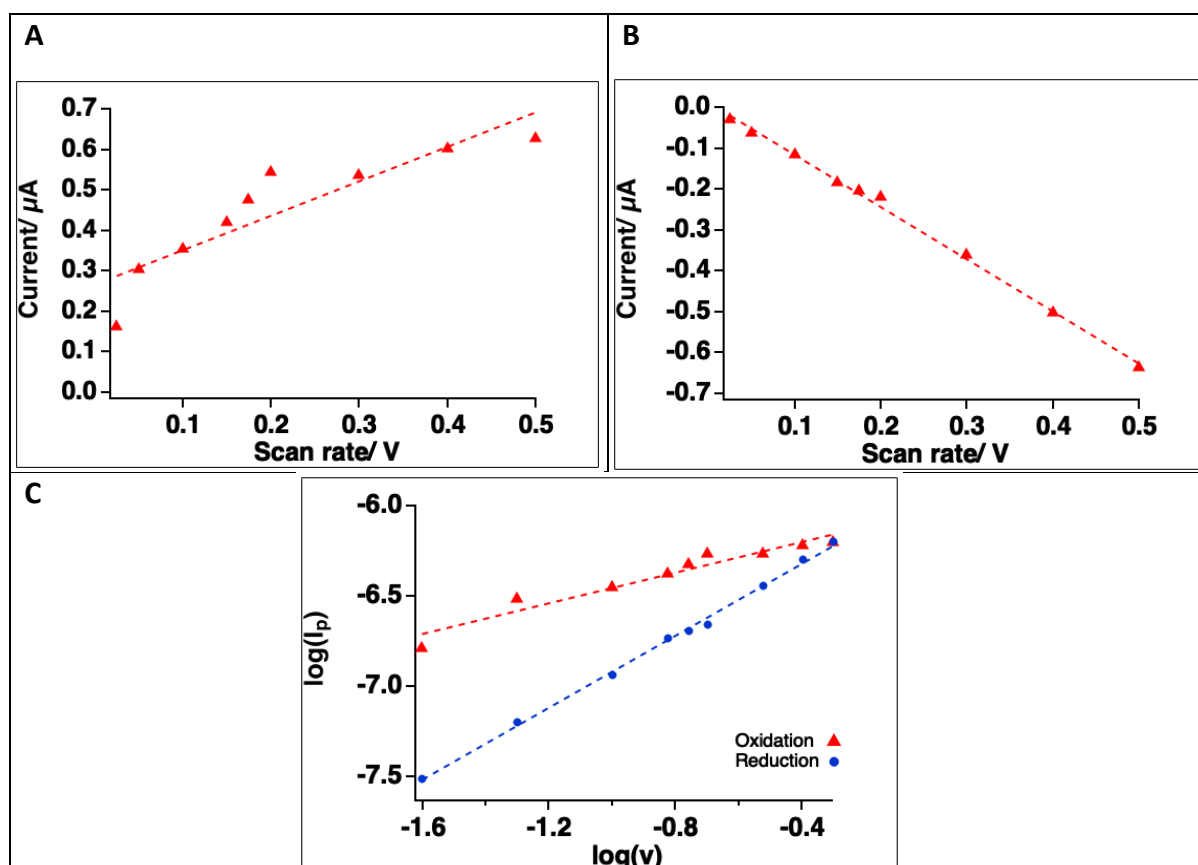


Figure 4.12 (a) Oxidation and (b) reduction peak height against scan rate, and (c) log of peak height against log of scan rate ($n=1$).

Parameter	Anodic	Cathodic
Gradient	0.85 ± 0.157	-1.27 ± 0.027
y-intercept	0.27 ± 0.041	0.01 ± 0.007
r^2	0.807649	0.996769

Table 4.4 Parameters of linear relationship between peak height and scan rate (V/s).

Parameter	Anodic	Cathodic
Gradient	0.43 ± 0.042	1.00 ± 0.019
y-intercept	-6.03 ± 0.039	-5.92 ± 0.017
r^2	0.935815	0.997483

Table 4.5 Parameters of linear relationship between $\log(I_p)$ vs $\log(v)$.

4.3.5 Frequency optimisation

The frequency used in square wave voltammograms to interrogate E-ABs has shown to impact the signal gain of the sensor.³⁰⁵ To maximise the absolute current response in the unbound state, the frequency used in SWVs was varied from 10 to 100 Hz (E_{step} of 1 mV).

A positive peak shift was observed as the frequency was increased from 20 Hz to 100 Hz in the anodic SWV scans in Figure 4.13(a). However, there was no difference seen between the peak height at 20 Hz and that at 100 Hz. For the cathodic scans in Figure 4.13(b), there was an increase in peak height from 10 Hz to 30 Hz, beyond which, an increase in frequency caused a decrease in the peak height. A frequency of 30 Hz was chosen for future SWV experiments.

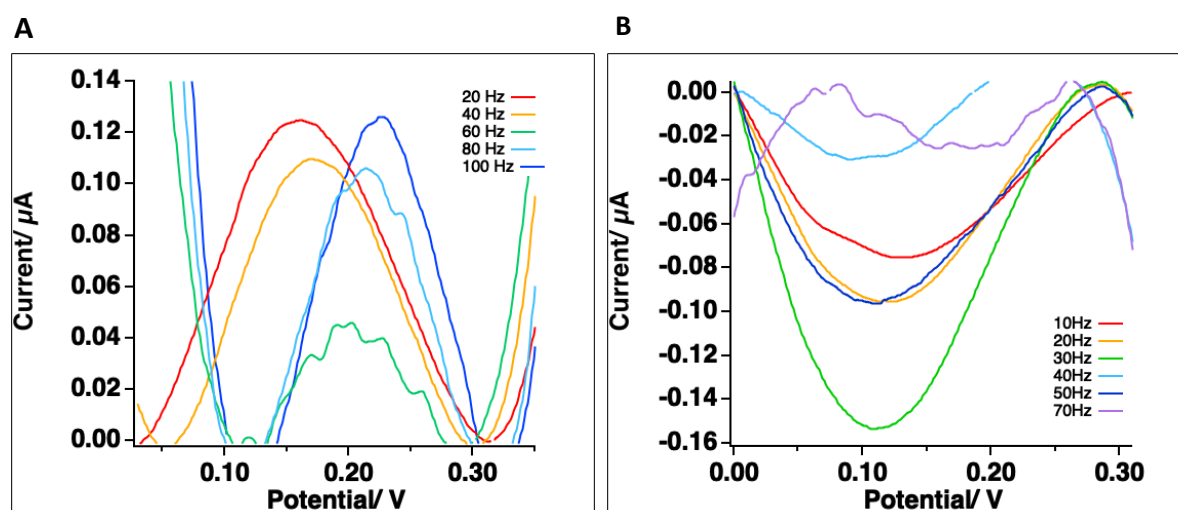


Figure 4.13 Optimisation of signal response by altering frequency in SWV (a) in anodic scans from 20 to 100 Hz and (b) in cathodic scans from 10 to 70 Hz ($n=1$).

4.3.6 Aptamer concentration optimisation

It has been shown that the aptamer concentration used in the fabrication of E-ABs has an impact on the surface coverage of the aptamer on the electrode.³⁰⁶ The surface coverage of the aptamer has a proportional relationship with the sensor response. To optimise the surface coverage, the concentration of the aptamer deposited during the fabrication process was varied from 1 to 10 μM and E-ABs were interrogated with DPV, SWV and CV methods. The surface coverage of aptamer on the electrode was calculated from the area under the CV peaks for each concentration of aptamer deposited.

In the DPV scans shown in Figure 4.14(a), the control electrode was a pre-treated bare gold electrode and peaks were observed at +0.29 V (anodic) and +0.11 V (cathodic) in aptamer binding buffer. The DPV peaks were substantially lower from the control electrode than the peaks from the aptamer-functionalised electrodes, with peaks from the electrode deposited with 5 μM aptamer showing the highest current response overall. The DPV peaks from aptamer-functionalised electrode deposited with 5 μM aptamer were seen at +0.05 V (anodic) and +0.25 V (cathodic). In addition, the response from 5 μM aptamer electrode was 87.7% and 95.9% greater than the response from the control electrode. A higher peak-to-peak separation was seen in the DPV scans, with an average peak separation of 0.17 V.

In the SWV scans in Figure 4.14(b), the electrode functionalised with 2.5 μM aptamer achieved the highest current response. There was an increase in peak heights from 1 μM to 2.5 μM , beyond which a decrease in peak height was seen as the aptamer concentration was increased further. This may have been due to the steric hindrance produced from the large size of the osmium bipyridyl complex, with overcrowding of the aptamer on the electrode

surface as the concentration increased. The aptamer may not have been able to reach its optimal conformation due a lack of free spaces on the electrode for the back-filler molecule, which is essential to the aptamer to “stand up”. Another explanation for a decrease in signal response could be the hybridisation between complimentary ssDNA strands, meaning that the redox reporter is taken further away from the surface of the electrode due to the conformational change, allowing it to be shielded away.

In the CV scans shown in Figure 4.14(c), the largest peaks are seen for the E-AB fabricated with 5 μM aptamer and no peaks are seen for the control electrode. Surface coverage of the aptamer, deduced from the area under the CV peaks, is plotted against aptamer concentration in Figure 4.14(d). The greatest surface coverage of aptamer was seen for the E-AB made with 5 μM aptamer during the fabrication process, therefore, this concentration was chosen for all future experiments, also due to the high current response observed across all voltametric interrogation techniques at this aptamer concentration.

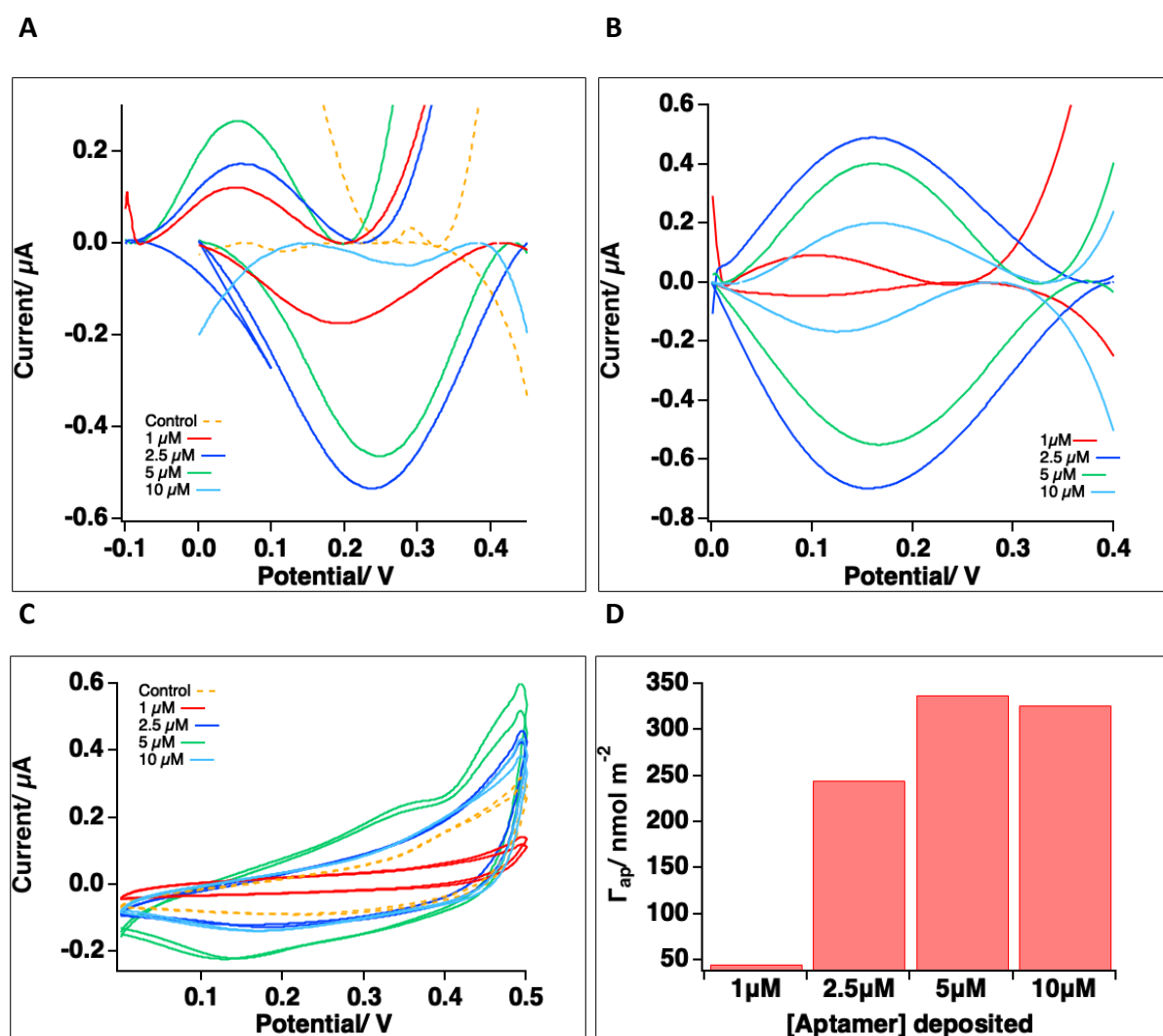


Figure 4.14 Optimisation of signal response by altering aptamer concentration ($n=1$) from 1 to 10 μM in (a) DPV (at 25 mV/s), (b) SWV (at 30 Hz), (c) CV (at 25 mV/s) scans, and (d) surface coverage of aptamer based on concentration deposited.

The surface coverage of aptamer achieved ($\sim 3.25 \times 10^{-11} \text{ mol cm}^{-2}$) at an aptamer deposition concentration of 5 μM is comparable to aptamer surface concentration values published in literature ($3\text{--}5 \times 10^{-11} \text{ mol cm}^{-2}$).²⁴⁸

4.3.7 Sensor response to CRP binding

Osmium(II/III) redox reporter-terminated aptamer was immobilised onto pre-treated disposable gold electrodes, followed by immobilisation of 6-mercapto-1-hexanol (6-MCH) back-filler. E-ABs were tested in aptamer binding buffer (blank) and then incubated with 1 and 10 mg/L CRP for 1 hour, and subsequently tested in buffer again. A decrease in peak height was observed across both the DPV and SWV scans, as shown in Figure 4.15, meaning that the E-AB was a signal OFF sensor.

A decrease of 96.2% in peak height was observed after incubation with 1 mg/L CRP in the oxidation scan of the DPV, and a decrease of 97.8% was seen after incubation with 10 mg/L CRP in the reduction scan of the DPV. No oxidation peaks were seen after incubation with 10 mg/L CRP and no reduction peaks were seen after incubation with 1 mg/L in the DPV scan.

There was a peak-to-peak separation of 120 mV in the DPV scan for the blank reading, and a positive peak shift of +30 mV in the oxidation scan, after binding to 1 mg/L CRP, and a negative peak shift of -110 mV in the reduction scan, after binding 10 mg/L CRP.

As for the SWV scans, a signal decrease of 99.0% was seen in the oxidation scan after incubation with 10 mg/L CRP. No oxidation peaks were seen after incubation with 1 mg/L CRP and no reduction peaks were seen after incubation with CRP. However, after incubation with 10 mg/L CRP, a negative peak shift of -10 mV was seen in the oxidation scan. A small peak-to-peak separation of 10.3 mV was seen between the oxidation and reduction SWV peaks for the blank reading.

Due to there not being sufficient data to draw from, a calibration curve for the E-AB could not be plotted using the DPV or SWV peaks.

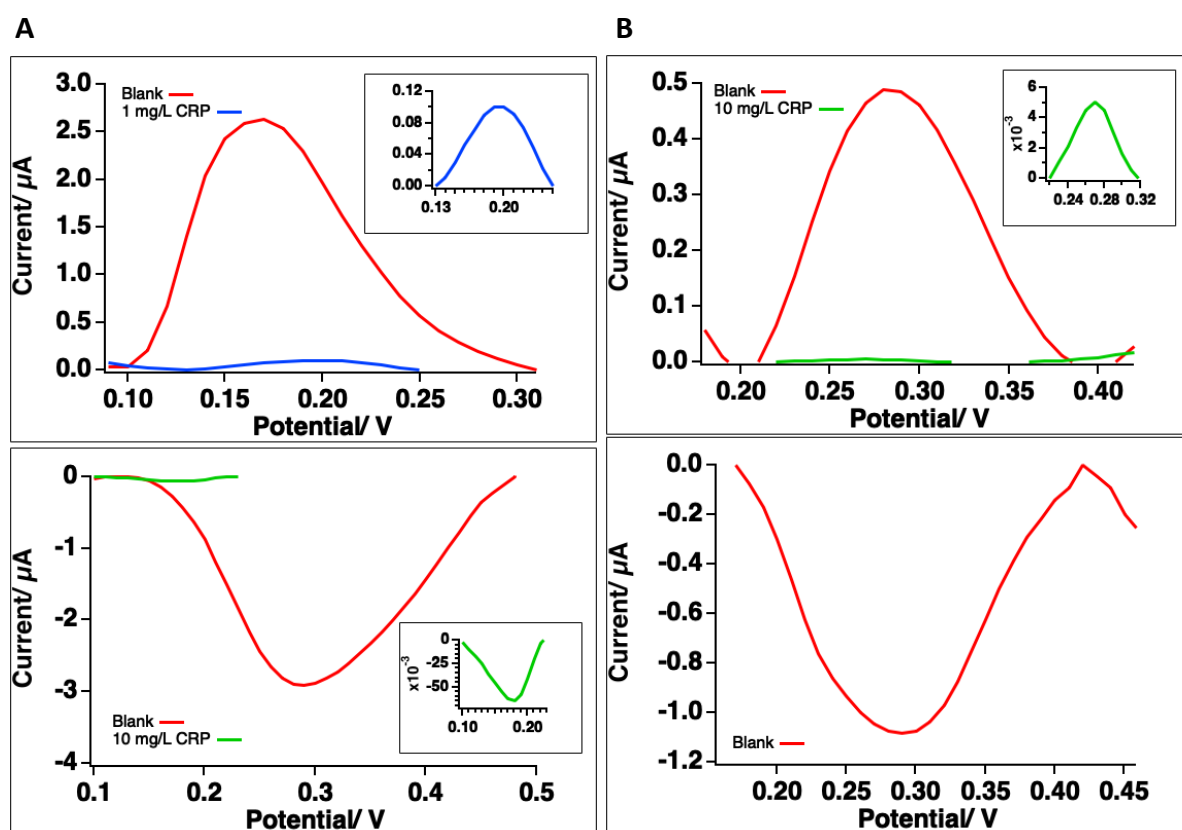


Figure 4.15 Aptasensor response to CRP binding ($n=1$) using (a) DPV at 0, 1 and 10 mg/L CRP concentrations and (b) SWV at 0 and 10 mg/L CRP concentrations.

4.3.8 Background signal from bare electrodes

Background signals from pre-treated bare gold electrodes (before any functionalisation) were seen in DPV and SWV scans, as shown in Figure 4.16. The bare gold electrodes were interrogated in aptamer binding buffer.

In the DPV scans, the oxidation and reduction peak heights were 15.65 and -3.93 μA , respectively, which were 6 and 1.4 times higher than the peak heights seen for the

functionalised sensor response (Figure 4.16(a)). The positions of the DPV background peaks are at +0.18 and +0.32 V for the oxidation and reduction scans, respectively, which are very similar to the positions of the sensor response peaks at +0.17 and +0.29 V for the oxidation and reduction scans, respectively. With the peaks being at similar positions and the background oxidation peak almost six times greater than the sensor oxidation peak, it is difficult to interpret whether the sensor response is a result of the redox label.

In addition, the DPV peak widths of the background signal are larger than the sensor response peak widths. For the background signal, the oxidation and reduction peak widths were 0.43 V and 0.34 respectively, compared to the sensor peak widths of 0.22 V (oxidation) and 0.33 V (reduction).

For SWV scans, the background oxidation and reduction peak heights were 19.07 and -16.61 μA , respectively, compared to previously seen sensor signal responses of 0.49 and -1.08 μA (Figure 4.16(b)). The background signals were almost 39 (oxidation) and 15 (reduction) times greater than the sensor signal responses.

The positions of the SWV background peaks are at +0.26 V and +0.25 V for the oxidation and reduction scans, respectively, which are very similar to the positions of the sensor signal peaks at +0.28 and +0.29 V for the oxidation and reduction scans, respectively. The SWV peak widths of the background signal are also much larger; 0.45 V and 0.47 V for oxidation and reduction, respectively, compared to sensor signal peak widths of 0.16 V and 0.34 V, with background signal peaks covering almost the full potential range applied to the electrode (0 to +0.5 V).

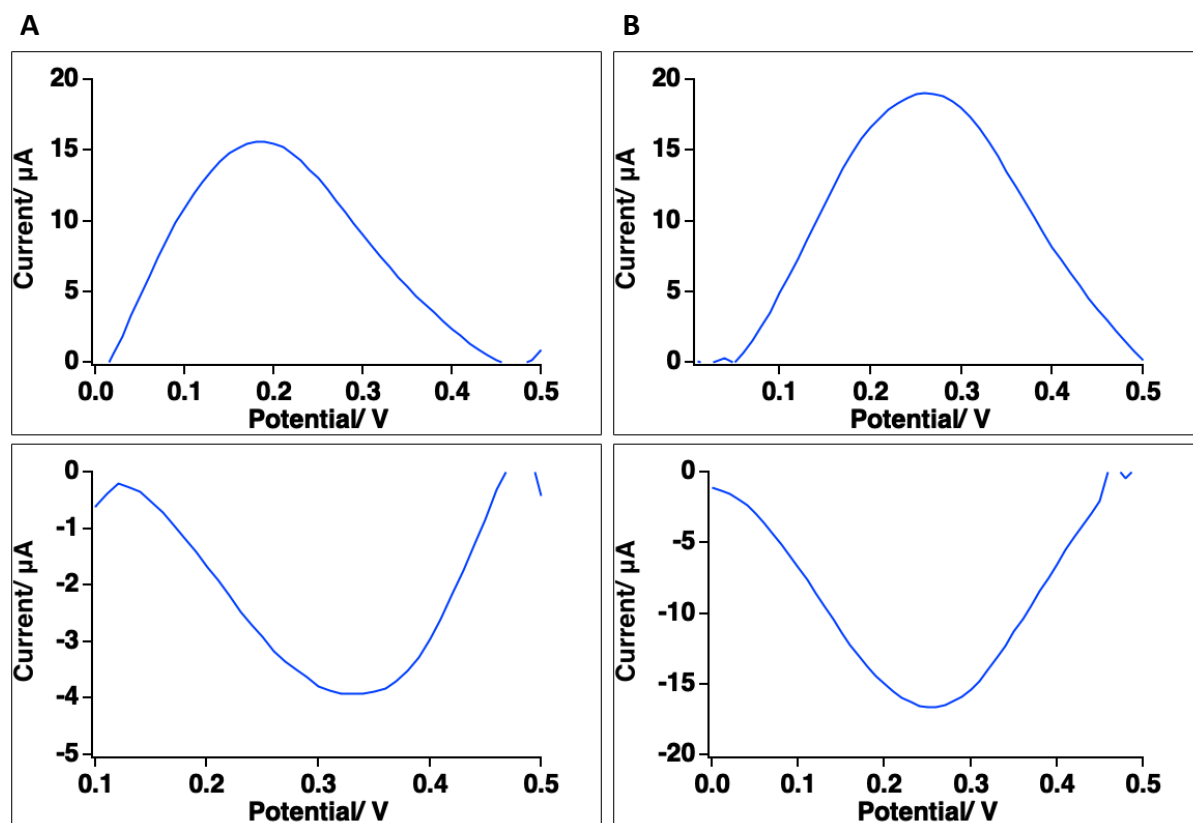


Figure 4.16 (a) DPV and (b) SWV scans of disposable bare gold electrodes in aptamer binding buffer after pre-treatment (n=1).

4.3.9 Osmium(II/III) redox reporter interaction with ssDNA backbone of aptamer

E-ABs were fabricated using unlabelled carboxyl-terminated aptamers on disposable gold electrodes. It was suspected that the positively charged osmium(II/III) redox reporter – $\text{Os(II)(bipy)}_2\text{ClNH}_2$ – may be electrostatically interacting with the negatively charged phosphate backbone of the ssDNA aptamer. If this interaction happened during the EDC/NHS coupling step of the aptamer with the redox reporter, then some non-specific oxidation and reduction of the redox reporter may be happening during the electrochemical interrogation of the sensors. Despite the filtering of the osmium(II/III) redox reporter-bound aptamer to remove any unbound osmium(II/II) reporter species from the solution before the EDC/NHS coupling step, it is possible that some may have electrostatically interacted with the ssDNA

backbone even after filtering and not have been removed. Figure 4.17(a) is the DPV scan of the unlabelled aptamer sensor before (red line) and after (blue line) incubation with the osmium(II/III) redox reporter. The oxidation and reduction peaks appear after incubation, suggesting that the osmium(II/III) redox reporter has bound to the aptamer in a non-specific manner, or potentially adsorbed onto the surface of the electrodes.

Figure 4.17(b) is the SWV scan of the unlabelled aptamer sensor before (red line) and after (blue line) incubation with the osmium(II/III) redox reporter. Peaks are visible in the oxidation and reduction scans of the unlabelled aptamer sensor, which is difficult to interpret given that there is no redox label attached to the aptamer. However, after incubation with the redox label, the peaks are much larger, owing to the electrostatic interaction effect. There is also a peak shift after incubation of -0.97 and -14.98 mV for the oxidation and reduction scans, respectively.

Figure 4.17(c) is the CV scan of the unlabelled aptamer sensor before (red line) and after (blue line) incubation with the osmium(II/III) redox reporter. There are no peaks in the oxidation and reduction scans of the unlabelled aptamer sensor, as expected, and after incubation with the redox label, oxidation and reduction peaks are seen at +0.24 and +0.15 V, respectively.

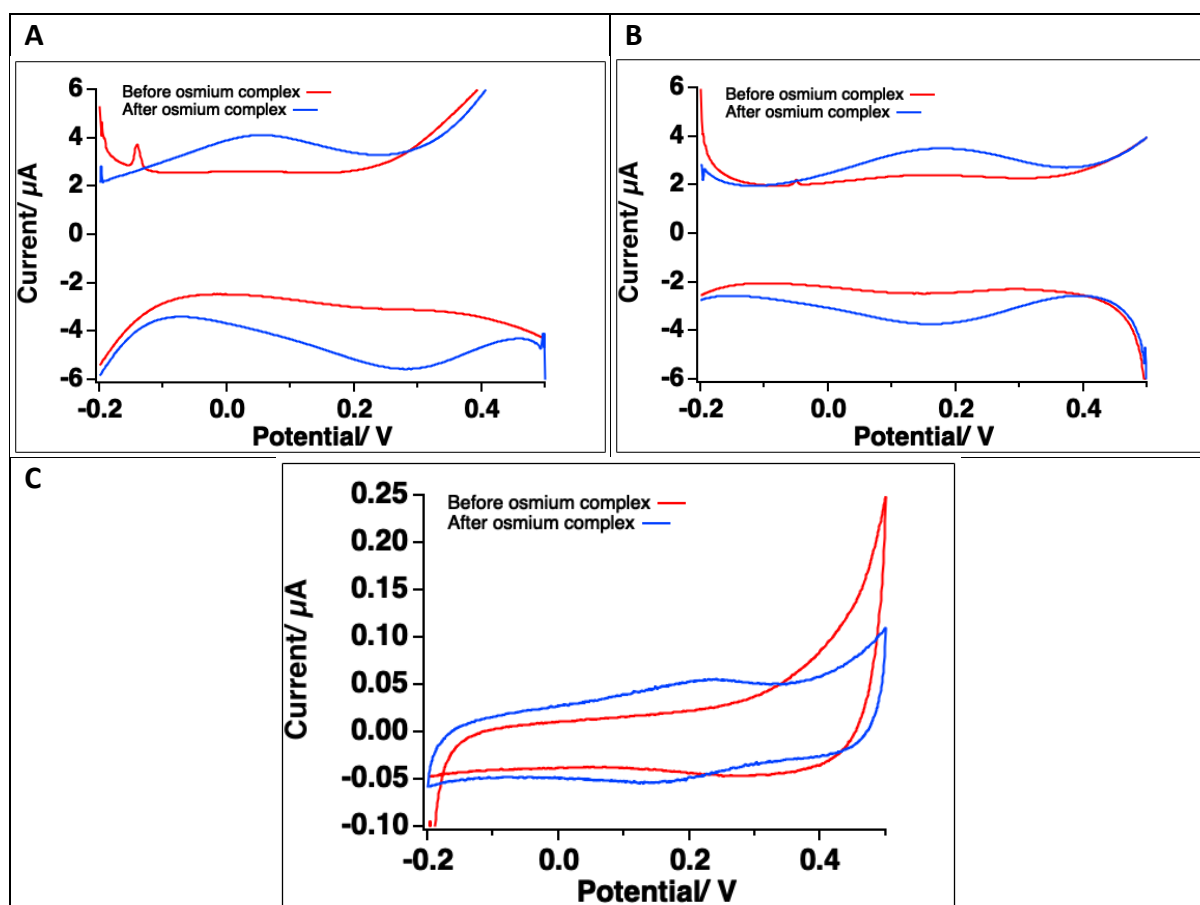


Figure 4.17 (a) DPV, (b) SWV and (c) CV scans of unlabelled carboxyl-terminated E-ABs before after electrostatic interaction with Os(II)(bipy)₂Cl₂ complex (n=1).

4.3.10 Regeneration of aptamer from bound state

For the continuous sensing of CRP, the aptamer needs to be regenerated from its bound state to accept new target molecules rapidly under changing concentrations. To verify that the aptamer-target (A-T) complex is able to return to its unbound aptamer state (A + T), a regeneration buffer, consisting of 10 mM tris buffer, 50 mM NaCl and 2 mM CaCl₂, as reported by Huang *et al.*²⁵¹ (who also reported the sequence of the CRP aptamer) was used to incubate sensors for 1 hour before conducting SWV. It was found that the sensor behaved as a signal OFF sensor after incubation with 10 mg/L CRP, as expected. After regeneration for 1 hour, the signal response decreased from the bound state response, indicating that the aptamer had

not returned to its unbound state i.e., regeneration had not been successful, as shown in Figure 4.18.

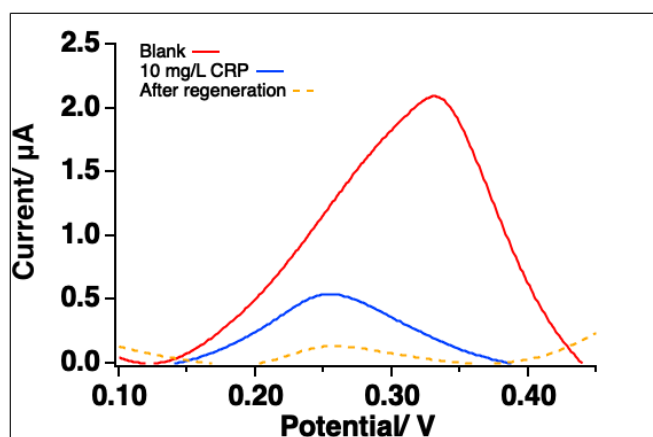


Figure 4.18 Anodic SWV scan of E-ABs in blank (unbound state), after incubation with 10 mg/L CRP, and after incubation with regeneration buffer for 1 hour (n=1).

4.3.11 Chronoamperometry and charge passed

To understand the amount of charge passed at each state of the sensor i.e., (i) bare electrode before any functionalisation, (ii) after functionalisation in aptamer binding buffer (blank), (iii) after incubation with 10 mg/L CRP, and (iv) after incubation with regeneration buffer, chronoamperometry was conducted at a sampling rate of 0.001 s until the current reached zero (at ~60s). Figure 4.19(a) and (b) show the oxidation and reduction chronoamperometry scans, respectively.

The area under the chronoamperometry scans was integrated to find charge passed ($Q = It$) and this was plotted against each sensor state in Figure 4.19(c).

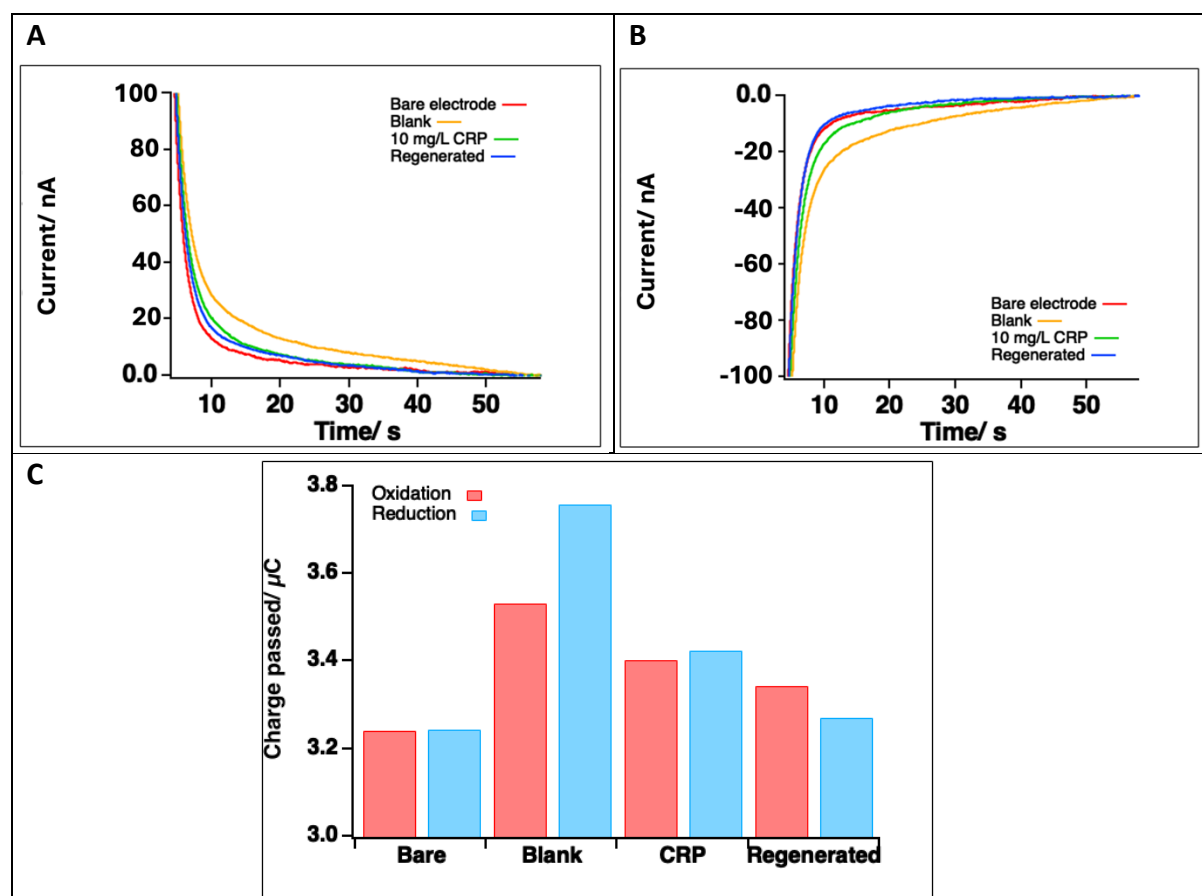


Figure 4.19 (a) Oxidation and (b) reduction chronoamperometry of E-ABs before functionalisation (bare electrode), after functionalisation (blank), after binding to CRP and after regeneration ($n=1$), and (c) charge passed through the electrode at each sensor state.

It can be seen that the charge passed increases after functionalisation of the electrode, and as expected for a signal OFF sensor, the charge passed decreases once the aptamer has bound to CRP. Once the aptamer has been incubated with regeneration buffer, we expect to see an increase in charge passed, due to the aptamer returning to its unbound state. However, we see a decrease in charge passed after regeneration, which aligns with the SWV of the sensor response after regeneration (as shown in Figure 4.18 previously). This may be due to inadequate regeneration, pointing to the need for the optimisation of the regeneration buffer and the conditions under which the aptamer is regenerated.

4.3.12 TCEP-reduced vs not reduced aptamer immobilised onto gold electrodes

It was suspected that the TCEP used to reduce the thiol groups at the 3' end of the aptamer was causing the reduction of the gold surface of the electrodes, in turn, producing peaks from the re-oxidation of the surface, as shown in Figure 4.20. This was suspected to contribute to the background signal in the E-ABs, since aptamer was deposited on the electrodes with TCEP in the solution.

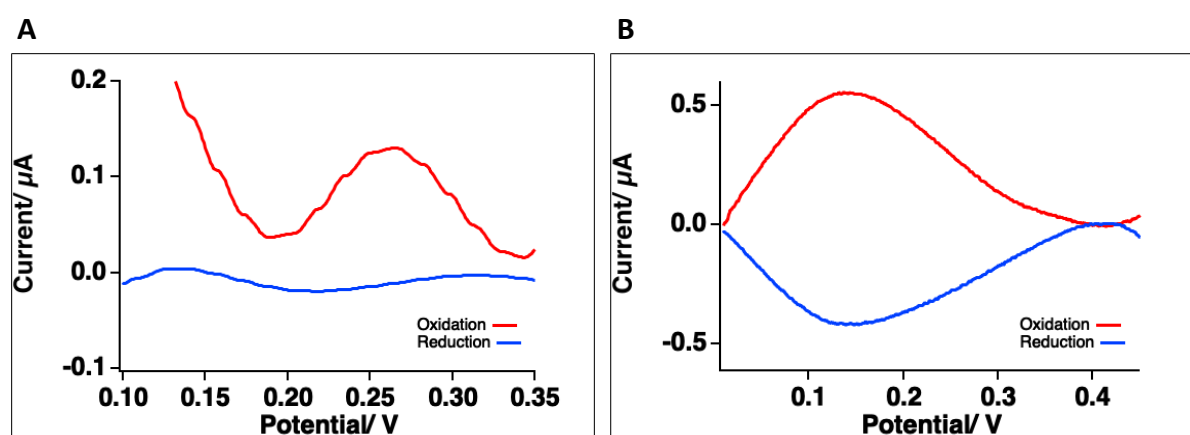


Figure 4.20 Bare electrodes treated with TCEP and interrogated ($n=1$) using (a) DPV and (b) SWV.

To understand whether TCEP was needed to ensure Au-thiol bonding and formation of the SAM, it was decided to fabricate sensors with and without the TCEP reduction step, to compare the performance of the sensor. Figure 4.21(a) shows the DPV of sensors fabricated with (solid line) and without (dashed line) TCEP reduction of thiol. The sensors fabricated without the reduction step had much a much lower signal response, with a decrease in signal response of 85.9% and 88.7% for electrodes 2 and 3, respectively.

Figure 4.21(b) shows the SWV of sensors fabricated with (solid line) and without (dashed line) TCEP reduction of thiol. Similar to the DPV response, the sensors fabricated without the reduction step had much a much lower signal response in SWV, with a decrease in signal response of 77.4%, 60.5% and 95.5% for electrodes 1, 2 and 3, respectively. Figure 4.21(c) shows the CV of sensors fabricated with (solid line) and without (dashed line) TCEP reduction of thiol. Similar to the DPV and SWV responses, the sensors fabricated without the reduction step had much a much lower signal gain in CV, with a decrease in signal response of 85.6% and 92.5% for electrodes 1 and 2, respectively. These trends indicate that reducing the thiol on the aptamer achieved higher signal gain.

To understand whether the TCEP reduction step had an impact on the formation of the SAM, the surface coverage (Γ_{ap}) of the aptamer (in nmol m⁻²) was calculated by integrating the area under the CV peaks and dividing this by scan rate, which achieved a value for charge, Q (C), in Equation 4.3, where n is the number of electrons, F is Faraday's constant (C mol⁻¹) and A is the surface area of the electrode (m²). When the thiol groups on the aptamer are reduced by TCEP, a greater surface coverage of aptamer was achieved, as shown in Figure 4.21(d). It was therefore concluded that the reduction of thiols by TCEP was a necessary step in achieving good surface coverage and better signal gain.

$$\Gamma_{ap} = \frac{Q}{nFA}$$

Equation 4.3 Calculation of surface coverage of aptamer.

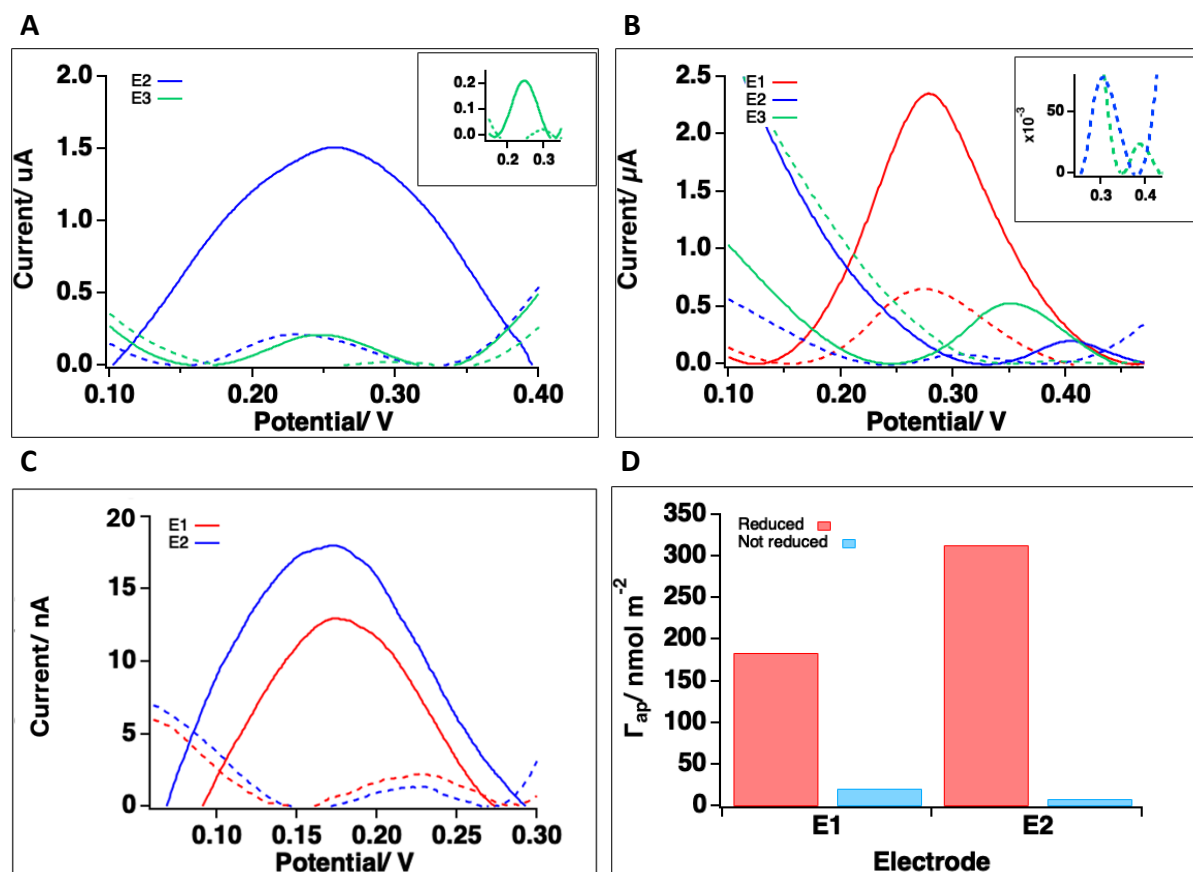


Figure 4.21 Sensor response from (a) DPV, (b) SWV, (c) CV scans of thiol-modified aptamer reduced and not reduced by TCEP (solid line = reduced, dashed line = not reduced) and (d) surface coverage (Γ_{ap}) of the osmium(II/III) redox reporter modified aptamer on the gold electrode (E1 = electrode 1, E2 = electrode 2, E3 = electrode 3) (n=1).

4.4 Discussion

The Os(II)(bipy)₂Cl₂ compound has been successfully synthesized and conjugated to the carboxylic acid terminated aptamer, evidenced by CV, DPV and SWV scans of the Os(II)(bipy)₂Cl₂-aptamer solution on gold electrodes. The solution has been filtered using spin columns and analysed before and after filtering using CV, DPV and SWV. The aptamer has successfully been deposited onto disposable gold electrodes by thiol-Au coupling, and a sensor response is obtained by DPV, CV and SWV. The peak potential for the osmium(II/III) redox label observed in this thesis – +0.17 to +0.30 V vs Ag|AgCl – is similar to that seen in

literature (oxidative peaks at +0.35 V and reductive peaks +0.25 V vs Ag|AgCl).³⁰⁷ The same study, by Boland *et al.*, reports the redox potential of [Os(2,2'-bipyridyl)₂(4-aminomethylpyridine)Cl]PF₆ to be +0.29 V vs Ag|AgCl).³⁰⁷

The application of a relatively low redox potential would mean that interference from other compounds at this oxidation potential would be limited. This has shown to be particularly useful in commercial GOD sensors utilising osmium(II/III) polymer based redox probes.³⁰¹

Concentration-dependent molecular recognition effect

A concentration-dependent molecular recognition effect could not be determined due to a lack of calibration data points, however, a much larger change in peak current was observed as a result of CRP binding, as compared to the MB-labelled E-AB reported in Chapter 3 which saw little to no change after CRP binding. The change in peak current after CRP binding was negative, meaning that the sensor was a signal OFF sensor. Due to the large change in current, the osmium(II/II) redox reporter labelled E-ABs developed in this thesis are therefore promising in achieving a high signal gain, however, to fully understand this relationship, a larger number of CRP concentrations would need to be calibrated with, starting with concentrations below K_D of the aptamer. Similar to the MB-labelled E-AB, the signal gain of the sensor needs to be optimised, as opposed to the magnitude of the signal response in blank.

Regeneration of aptamers

The charge passed at the electrode – calculated from the area under chronoamperometry scans – is greater after the immobilisation of aptamer than at the bare electrode. This is

expected due to the presence of the redox label at the electrode resulting in greater electron transfer. The charge passed decreases after CRP binding, as expected for signal OFF sensors, and decreases even further after the aptamer is regenerated using Tris buffer at high salt concentrations (50 mM NaCl and 2 mM CaCl_2) at room temperature. This is unexpected as the aptamer, if regenerated, would return to its original unbound state, producing a signal response larger than the bound state aptamer for a signal OFF sensor.

One of the reasons why regeneration may not have been effective in this study is because an active step, such as heat treatment, was not used. Several studies report the use of an active step, such as the use of hot water by Baker *et al.* to regenerate their cocaine E-AB, retaining 90% of the initial signal even after 40 regeneration cycles.³⁰⁸ Other active methods use surfactants such as Triton-X and Tween-20 to achieve regeneration, as well as pH disrupting solutions. In our study, an 93% decrease in peak current was observed after just one regeneration cycle. The loss in signal was much higher than that reported by other studies.

Since the reported K_D of the aptamer is 3.51 nM, it can be assumed that the k_{off} rate constant is approximately nine orders of magnitude lower than the k_{on} rate constant, which means that the dissociation of target from the aptamer is not favoured and would therefore, require an active step such as heat treatment, pH shock or a change in the ionic strength of the solution.

Osmium(II/III) redox reporter interaction with ssDNA backbone

The osmium(II/III) redox reporter was seen to have bound to the negatively charged ssDNA backbone, producing a peak current after having interacted with unlabelled CRP E-ABs. Despite the filtering step to remove any uncoupled intermediate osmium(II/III) compound

(Os(II)(bipy)₂Cl₂) from the Os(II)(bipy)₂ClNH₂ solution, there was still some leftover uncoupled intermediate compound in the solution. This meant that when mixed with the unlabelled aptamer for amide coupling to the redox reporter, some unbound intermediate osmium(II/III) compound may have electrostatically interacted with the ssDNA backbone of the aptamer non-specifically, contributing to the peak current during electrochemical interrogation of the E-ABs. This background signal needs to be removed in future studies to avoid over-estimating peak currents. In addition, a more robust filtering step needs to be incorporated in the fabrication process to remove unbound compound.

Current dependence on scan rate

The linearity in the Randles-Sevcik plot indicated that the redox reaction of the osmium(II/III) redox reporter was quasi-reversible, as was seen for the MB label in Chapter 3. The intercept of the plot being close to zero indicated the peak current was being derived from the redox reaction of the osmium(II/III) redox reporter, as opposed to from non-faradaic contributions. To build an aptamer that regenerates to 80% or higher of the initial peak current response, the redox reaction needs to be reversible, indicating that further study is required to understand why the label is quasi-reversible and how this may be overcome.

Bare electrode studies with TCEP

It was found that there was a background signal coming from the electrodes treated with TCEP overnight. The incubation period was chosen to simulate the aptamer solution, which contains TCEP, being left overnight on electrodes for optimal aptamer deposition. It was

suspected that TCEP was causing the reduction of gold or gold oxide at the electrode, and subsequent oxidation and reduction peaks were being seen in aptamer binding buffer at the electrode. To assess whether TCEP is needed for the reduction of thiol to form the gold-thiol bond, E-ABs were made with and without the TCEP reduction step and their signal responses compared.

Unlike the MB-labelled E-ABs, osmium(II/III) labelled E-ABs performed better when the thiol group on the aptamer was reduced with TCEP before immobilisation. This is evident in the large increase seen in surface coverage of aptamer after TCEP reduction. The aptamer surface coverage reported in this thesis for TCEP-reduced E-ABs – $1.84 \times 10^{-11} \text{ mol cm}^{-2}$ and $3.14 \times 10^{-11} \text{ mol cm}^{-2}$ – are in the same order of magnitude as those reported in literature ($10^{-11} \text{ mol cm}^{-2}$).²⁴⁸ In addition, osmium(II/III) redox reporter labelled E-ABs made with the TCEP reduction step were in fact producing signals twice as large as the E-ABs made without the TCEP reduction step. To understand signal gain however, the E-ABs would need to be calibrated with CRP to assess whether the reduction of the thiol groups have an impact on the difference in signal seen between target concentrations. Wu *et al.* report that TCEP interacts with the gold surface of AuNPs, demonstrating that heat is released during their interaction, meaning that TCEP has an affinity for gold on AuNPs.³⁰⁹ This indicates that TCEP may also be interacting with the planar disposable electrodes used in this thesis.

4.5 Conclusion and future work

We report an osmium(II/III) redox label terminated aptamer for CRP. The resulting E-AB can measure CRP at potentials between +0.17 V and +0.3 V vs Ag|AgCl, which is higher than the

negative potentials required for MB oxidation and reduction, allowing the SAM to remain intact over repeated interrogations. In addition, the low oxidation and reduction potential required for the osmium(II/III) redox label is expected to minimise interference from other electroactive species.

Unfortunately, due to time constraints, the LCMS of the aptamer after coupling to the redox label could not be carried out. This is an important step that would confirm the structure of the osmium-conjugated aptamer and that the amide coupling has been successful.

There is literature that suggests that the location of the redox label makes a difference to the response of the sensor and one forward direction for the project could be reversing the positions of the redox label and the thiol group to check whether the conformational change of the aptamer after target binding is affected by label position and aptamer orientation.

In addition, to understand the concentration dependent molecular recognition effect of CRP on the E-AB, a larger number of CRP concentrations need to be calibrated with over a larger concentration range for CRP, covering concentrations below K_D .

While the aptamer reported by Huang *et al.* has been selected specifically for CRP, it has not been applied to an E-AB setting previously, and it might be interesting to apply the osmium redox label across to other, more well-known sequences of aptamers that have shown to produce a significant signal change upon binding to target, such as the cocaine, kanamycin, thrombin, and tobramycin aptamers.^{209,214,308} Some groups have reported the immobilisation of the redox reporter on electrode surface, before the aptamer is immobilised on the surface.

This allows the redox reporter to transfer electrons closer to the electrode's surface, allowing greater signal gain to be achieved.

Chapter 5 Conclusion

We report an LOD microneedle biosensor modified with 5 coats of 1% w/v Nafion with a variable dynamic range (estimated to be twice that of K_m) of between 0.46 and 32.5 mM lactate and an effective K_m of between 0.23 and 16.25 mM. Nafion was chosen as a diffusion limiting membrane due to its stability on the sensor surface, solvent compatibility with the polycarbonate base of the microneedles, and its resistance to the mass transport of lactate due to its negative charge and buildable thickness via spray-coating. The diffusion coefficient of H_2O_2 and lactate in the Nafion membrane, estimated as DK , was $(1.06 \pm 0.278) \times 10^{-11}$ and $(4.91 \pm 1.29) \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ in 0.5 mM lactate. DK was relatively consistent across all concentrations of H_2O_2 , however, a concentration dependent effect on DK was observed for lactate, wherein as concentration was increased, DK decreased. This indicates that the enzyme concentration could be increased to ensure that the reaction is not limited by k_2 and the number of active sites available at any given time.

To demonstrate the sensor's response *in vivo*, lactate was measured using the LOD microneedle biosensor in five healthy volunteers performing exercise and at rest. The K_m achieved by the sensors used *in vivo*, as reported above, was lower than that in the optimisation phase ($7.10 \pm 4.18 \text{ mM}$). The dynamic range of the sensors used *in vivo* was lower than that required (5 mM) to measure lactate at and above the threshold for sepsis, for three out of five biosensors (dynamic range < 1 mM lactate). However, the biosensor (ISF) demonstrated a proportional rise and fall in lactate compared to blood lactate measurements; lactate concentrations were lower in ISF than blood during exercise and rest. There was little to no time lag observed between ISF and dialysate measurements, whereas

a time lag of 8-10 min was observed between ISF and blood lactate. In addition, good agreement was observed between biosensor and blood concentrations, and biosensor and dialysate concentrations, wherein all data were within the 95% confidence limits.

In terms of future work, sensor-to-sensor variability needs to be reduced so that a reproducible K_m can be achieved for the microneedle sensors. One way of doing this might be to develop a way to apply a consistent thickness of the membrane on the sensors, overcoming the coffee-ring effect seen in the membrane, so that DK across the membrane, and the resulting K_m , is reproducible and high enough across sensors. The LOD concentration could be further optimised to ensure that this is not the rate limiting step. Interference from AA, UA and AM was observed during *in vitro* calibration of the sensors, meaning that the control electrode employed in the *in vivo* studies should be used as a sentinel electrode to correct for this in future work. The stability of the exclusion layer, mPD in this case, needs to be investigated over time. Furthermore, it would be useful to assess the response of the microneedle biosensor using a DET enzyme *in vivo*, as this would allow interference to be removed at the lower applied potential, as well as to explore new solvents to dissolve PU and CA, due to their promising performance during the optimisation phase.

We report the development of E-AB for the continuous measurement of CRP using methylene blue as a redox reporter. The aptamer has successfully been deposited onto the electrode, and peaks are seen at -0.16 and -0.30 V vs Ag|AgCl across different modalities, however, the sensor does not produce a concentration dependent molecular recognition effect. This was evidenced by the lack of a difference in signal response across different CRP concentrations.

The main reason behind this was the low K_D of the aptamer (3.51 nM), meaning that the aptamer was not able to detect CRP concentrations above approximately 10 nM, which is equal to 35×10^{-25} mg/L CRP. In terms of future work, the sensor needs to be calibrated with much lower concentrations of CRP, in the range of 0.5 to 7 nM. To achieve measurements in the physiologically relevant concentration window, the K_D of the aptamer needs to be increased by truncation of the aptamer and assessing the change in K_D . Alternatively, another aptamer with a reported higher K_D (in the order of magnitude of 10^{18}) could be used to fabricate E-ABs or SELEX could be used to select an aptamer for in vivo applications.

Lastly, we report the modification of the same aptamer with an osmium(II/III) based redox reporter for CRP detection. Whether the aptamer produced a concentration dependent molecular recognition effect could not be determined due to a lack of reproducible data gathered across sensors. The aptamer has successfully been deposited onto the electrode, and peaks are seen at +0.17 V and +0.3 V vs Ag|AgCl across different modalities.

The aptamer was regenerated using Tris buffer with a high salt concentration and the loss in signal was greater than 90% from the unbound state. This was below the 80% signal retention threshold set for regeneration, meaning that this was not successful and was proven to be insufficient for continuous measurements. In terms of future work, an active step could be included in the regeneration process to achieve a smaller loss in signal. The possible background noise from electrostatic interaction of the unbound osmium(II/III) redox reporter with the ssDNA backbone needs to be eliminated to gather more reliable data and therefore, an optimised filtration step is necessary. In addition, LCMS of the aptamer coupled with the

osmium(II/III) redox reporter needs to be carried out in order to understand how successful the coupling using EDC/NHS was.

Overall, the work presented in this thesis progresses existing research towards achieving continuous infection monitoring in critical care, which could have a huge impact on how infection is detected, monitored, and treated in the future. By successfully demonstrating (i.e. a proof-of-concept) that lactate can be monitored *in vivo* using microneedle sensors in the ISF of healthy volunteers, we can be confident that the sensor, if optimised correctly, can be further tested in patients with infection as the next step in the clinical validation process. If successful in ill patients, this sensor could be adopted in the clinical pathway of identifying infections early and monitoring progression over time in hospital.

Furthermore, by exploring another biomarker of interest, CRP, we explore monitoring infection levels through multiplex sensing, which would be more robust and enable personalised dosing of antimicrobials based on (a) real-time drug concentrations (via another sensor measuring the drug) and (b) real-time infectious biomarker concentrations. Personalised dosing of antimicrobials, in the context of AMR, would be hugely beneficial in optimising the use of current antimicrobials, preventing further development of resistance.

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