



Dobrea, Alexandra (2026) *Development of a novel plug-and-play biomarker interrogation platform based on electrochemical biosensing for endometriosis research*. PhD thesis.

<https://theses.gla.ac.uk/86172/>

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk>

research-enlighten@glasgow.ac.uk

Development of a novel plug-and-play biomarker interrogation platform based on electrochemical biosensing for endometriosis research

Alexandra Dobrea, MEng

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF
DOCTOR OF PHILOSOPHY

JAMES WATT SCHOOL OF ENGINEERING
COLLEGE OF SCIENCE AND ENGINEERING



University
of Glasgow

Supervision: Dr Melanie Jimenez, Prof. Damion K. Corrigan and Prof. Rami Ghannam

DECEMBER 2025

*To my parents,
Luminita and Adrian Dobrea*

Abstract

Endometriosis is a debilitating disease affecting 1 in 10 of those assigned female at birth worldwide. Yet despite its prevalence, diagnosis currently takes ~ 9 years from symptom onset and necessitates laparoscopic surgery – an invasive procedure with many limitations. Producing a non-invasive diagnostic modality *e.g.*, based on molecular biomarkers is a priority, but no biomarker has been identified yet as a suitable replacement. Given its systemic nature, a multiplexed panel could provide a better insight into this disease, but that is currently hindered by a lack of tools that can concurrently detect the breadth of biomarkers of relevance to endometriosis in a cost and time effective manner in the community. Electrochemical biosensors provide an attractive avenue over conventional bioanalytical systems, as they are low-cost, mass manufacturable and, importantly, allow detection multiplexing *e.g.*, through fluidic integration.

This thesis aimed to produce a novel biomarker interrogation platform based on low-cost electrochemical biosensors for 2 key targets in endometriosis research: IL-6, an inflammatory cytokine, and cortisol, a small steroidal hormone. An investigation into the integration of a recently discovered cortisol aptamer into an impedimetric sensor highlighted different aspects of the fabrication process that are critical for optimal performance. A set of engineering tools were then produced to enhance the performance of the developed sensors, namely 1) a low-cost, easy-to-implement flow cell to improve accessibility to fluidic solutions within the electrochemistry community, 2) a fully automated platform for standardizing the fabrication of electrochemical biosensors and 3) a new methodology for impedance data fitting based on the Markov chain Monte Carlo method for improving the sensor readout reliability. As such, this work delivers a series of engineering solutions to the field of electrochemical sensing using biomarkers of interest for endometriosis research as the key application, in the hope that it will guide, inspire and facilitate further research into this disease.

Contents

Abstract	iv
Acknowledgements	xi
Declaration	xii
Abbreviations	xiii
1 Introduction	1
1.1 Motivation	1
1.2 Current diagnostic roadblocks	2
1.3 Electrochemical biosensors: opportunities and limitations	5
1.4 Target analytes chosen for this work	7
1.5 Electrochemical detection strategies	9
1.5.1 Electrochemical techniques	12
1.5.2 Electrochemical immunoassays	20
1.5.3 Electrochemical aptamer-based (E-AB) sensing	24
1.6 A crucial missing tool for researchers	26
1.7 Aims and research questions	29
2 Development of a low-cost electrochemical sensing strategy for cortisol	31
2.1 Introduction	33
2.2 Materials and methods	37
2.3 Results	41
2.4 Discussion and conclusion	52

3	A plug-and-play, easy-to-manufacture fluidic accessory to significantly enhance the sensitivity of electrochemical immunoassays	55
3.1	Prologue	57
3.2	Introduction	61
3.3	Materials and Methods	64
3.4	Results and Discussion	69
3.5	Conclusion	81
4	WAFFLE - An automated platform for enhancing the performance of electrochemical biosensors	83
4.1	Prologue	85
4.2	Introduction	89
4.3	Materials and Methods	92
4.4	Results and discussion	99
4.5	Conclusion	109
5	Conclusions and future outlook	110
	Appendices	120
A	The CSS.1 aptamer secondary structure	120
B	Validation of cortisol aptamer binding	121
C	Further information on cortisol aptasensor	122
D	Commentary of dose response shape and mechanism of enhancement seen in Chapter 3	124
E	Supplementary information - Flow cell publication	126
F	Commentary on the mechanism of enhancement seen in Chapter 4	132
G	Supplementary information - Waffle publication	134

List of Tables

2.1	Aptamer sequences used in this work	38
3.1	Limits of detection (defined as the first data point falling outside of the 99% confidence interval around the blank reading) before and after flow cell addition - a comparison between different sample types.	80

List of Figures

1.1	Laparoscopy - the current state of the art diagnostic procedure for endometriosis.	3
1.2	Biomarker tree for endometriosis	5
1.3	Typical outline of an electrochemical biosensing system	7
1.4	Cyclic voltammetry technique	13
1.5	Differential pulse voltammetry technique	14
1.6	Electrochemical impedance spectroscopy technique	17
1.7	Equivalent circuit components and their meaning	18
1.8	Chronoamperometry technique	19
1.9	Optical and electrochemical enzyme linked immunosorbent assays	23
1.10	The strand displacement mechanism for cortisol detection used in this work .	25
1.11	Biosensing assay automation tools reported in literature	28
2.1	Aptamer immobilization strategies explored	36
2.2	Electrode platform used in this work[183]	40
2.3	Translating the lateral flow assay demonstrated by Dalirirad <i>et al.</i> (2020) into an impedance-based detection format for rapid signal read-out	43
2.4	Aptamer buffer optimisation, stepwise tracking of functionalisation steps and calibration curve in spiked buffer	46
2.5	Repeat of previous dataset (Figure 2.4 c and d) with a different batch of electrodes from the same manufacturer	47
2.6	Optimisation work on the newer batch of electrodes	49
3.1	Comparison of the efficacy of oxygen plasma and sulfuric acid voltage cycling in removing contaminants from the gold electrode surface	70
3.2	Low-cost, easy-to-assemble flow cell developed in this work	73

3.3	Spontaneous reaction between the detection substrate TMB and the reference electrode and protocol developed to minimise it	76
3.4	Dose response of electrochemical IL-6 immunosensor showcasing the effect of anodisation of the Ag/AgCl reference electrode and adding the flow cell at the final step of the sensor preparation procedure	79
3.5	The effect of flow cell addition on the electrochemical IL-6 immunosensor challenged in 10% filtered and unfiltered serum and neat, unprocessed human serum	80
4.1	Typical steps involved in preparing a sandwich-type electrochemical immunoassay using the standard N-(3-Dimethylaminopropyl)-N -ethylcarbodiimide/N-Hydroxysuccinimide chemisorption mechanism	91
4.2	The Wee Ally for Flow Functionalisation of Low-Cost Electrodes (WAFFLE) Platform	98
4.3	Waffle Fluidic Characterisation	102
4.4	Comparison between manual and WAFFLE-made electrochemical sensors with antibodies attached via EDC/NHS coupling	103
4.5	Schematic representation of the steps involved in preparing the cortisol aptasensor and the effect of WAFFLE preparation	105
4.6	Comparison between different impedance responses fitted using a standard ‘black box’ proprietary software (PSTrace 5.10; middle column) and a modified version of Zfit using the Markov chain Monte Carlo (MCMC) algorithm (right column)	108
1	Secondary aptamer structure as predicted by mfold [182]	120
2	Calibration curve generated using an optical competitive cortisol ELISA assay, and equivalent calibrant wells with 200 nM cortisol aptamers added (gold stars). Cortisol addition resulted in an average 20% decrease in the optical signal output out of those wells demonstrating competitive binding against the cortisol antibodies present in the ELISA kit.	121

3	The effect of cortisol addition on the Nyquist plot of individual working electrodes for different experimental conditions discussed in Section 2.3. Data represents 3 working electrodes before (at the MCH backfilling step) and after cortisol addition (1 pg/ml) and demonstrated a decrease in charge transfer resistance upon target binding suggesting removal of receptor off the electrode surface.	122
4	Signal to noise ratios of cortisol aptasensors backfilled after the 3rd or 2nd step in the functionalisation procedure respectively i.e. after the anchor deposition or after the duplex assembly	122
5	Effect of 1 h incubation vs o'night for the SH configuration. Results in lower coverage and sensitivity not significantly improved	123
6	Fluorescence equivalent of data shown in Fiure 2.7	123

Acknowledgements

This work was conducted through the Biomedical Engineering departments of Glasgow University and the University of Strathclyde and could not have been possible without support from the EPSRC and Tenovus Scotland.

Firstly, I would like to express my deepest gratitude to the fantastic supervisory team which comprised of Dr Melanie Jimenez, Prof. Damion Corrigan and Prof. Rami Ghanam. Thank you for always being in my corner, supporting and encouraging me through this journey and allowing me the space to grow, be curious, explore and discover. You have all shown me what true leadership looks like and I will be forever thankful for everything that you have taught me, which shaped who I am today as a researcher.

I would also like to thank my friends and colleagues Jessie Howell, Alisdair Gordon, Nicole Hall, Perrine Lasserre, Alexander MacDonald, Adrian Butterworth, Daniel Macdonald, Niamh Docherty, Stuart Milne, Rowan Blake, Greta Mitchell, Nadia Avanessova for building me up and being a constant fountain of inspiration and insightful discussion.

Special thanks to Prof. Yoann Altmann for his help with the Markov chain Monte Carlo algorithm and Dr Veerappan Mani for his help proofreading and sound checking my first results chapter.

I would also like to thank Lindsey Whiteford and William Noon from FabLab@Strathclyde for their help with manufacturing the flow cells, inspiring me with their creativity and building me up.

I am also grateful to my parents, Luminita and Adrian Dobrea, who have always supported and loved me unconditionally, encouraged me to follow my dreams and instilled in me a deep sense of self-belief and the idea that through hard work anything is possible.

And last but certainly not least, to my amazing partner Fergus Hall. Thank you for always believing in me, looking out for and encouraging me and continually inspiring me with your beautifully inquisitive mind.

Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution

Alexandra Dobrea

Abbreviations

- ELISA - Enzyme-linked immunosorbent assay
- CV - Cyclic voltammetry
- DPV - Differential pulse voltammetry
- EIS - Electrochemical impedance spectroscopy
- SAM - Surface assembled monolayer
- TMB - 3,3',5,5'-Tetramethylbenzidine
- FF-Cy - Ferri-Ferrocyanide
- HRP - Horseradish peroxidase
- PoC - Point-of-care
- MCMC - Markov chain Monte Carlo

Chapter 1

Introduction

1.1 Motivation

Endometriosis is a chronic, menstrual-cycle dependent disease commonly presenting through episodes of debilitating pelvic pain, dysmenorrhoea and recurrent genitourinary and gastrointestinal distress, and is thought to affect approximately 10% of reproductive-age women and those assigned female at birth¹ worldwide - or 190 million people [2]. However, the true prevalence may be much higher with another estimate suggesting that about 6 in 10 cases of endometriosis remain undiagnosed [3]. While the focus on endometriosis typically centres around pain and its effects on fertility (30 – 50% of patients suffer from infertility [4]), this disease can permeate all aspects of a person's life from lost work productivity, higher rates of depression, missed opportunities, difficulty partaking in social situations. [5]. In economic terms, the financial burden of endometriosis is comparable to that of conditions such as type 2 diabetes and rheumatoid arthritis [6]. Despite its prevalence and high individual and economic burden, the average time in the UK to diagnosis is ~ 9 years from the first GP visit [7]. The reasons for this delay are multifaceted and include the non-specific presentation of this disease, societal normaliza-

¹While the majority of the endometriosis patient population are women, this disease can also in some cases affect people assigned male at birth [1].

tion of menstrual pain, lack of disease awareness [7], and invasive nature of the diagnostic procedure [8].

The current state-of-the-art for diagnosing endometriosis is symptom-prompted laparoscopic surgery (Figure 1.1), which combines diagnosis with therapeutic benefits, as endometrial implants can be visually identified and excised during the same session [9]. But, like any other surgical intervention, it features risks and success is highly dependent on the experience of the clinician performing the operation as well as the localisation and appearance of the endometrial lesions, as deep set or heterogeneous ones may be more challenging to identify [10, 11]. Furthermore, laparoscopy does not eliminate the risk of endometriosis recurrence, meaning that patients often have to undergo multiple invasive procedures throughout their lifetime [12, 13]. Other investigations such as ultrasound and magnetic resonance imaging are also recommended as first line diagnostic tools but cannot on their own definitively rule out endometriosis [9].

Therefore, producing an accurate, non-invasive diagnostic tool to replace surgical diagnosis and identify endometriosis earlier is one of the research priorities in the field at the moment [2, 14–16]. By accelerating the diagnosis timeline, such a test could improve treatment outcomes by limiting disease progression and tissue damage prior to surgical intervention, whilst simultaneously reducing the financial burden of endometriosis on both patients and healthcare systems [17].

1.2 Current diagnostic roadblocks

An alternative diagnostic route for endometriosis is based on the measurement of biomarkers present in bodily fluids accessible non-invasively (*e.g.*, blood or urine). The literature suggests that only through a combinatorial approach (*i.e.* multiple biomarkers, likely accompanied by imaging and clinical evaluation) may we get closer to meeting the criteria required for a replacement test [18] to surgical diagnosis [19–21]. However, at present, no biomolecular signature has been identified for endometriosis diagnosis and

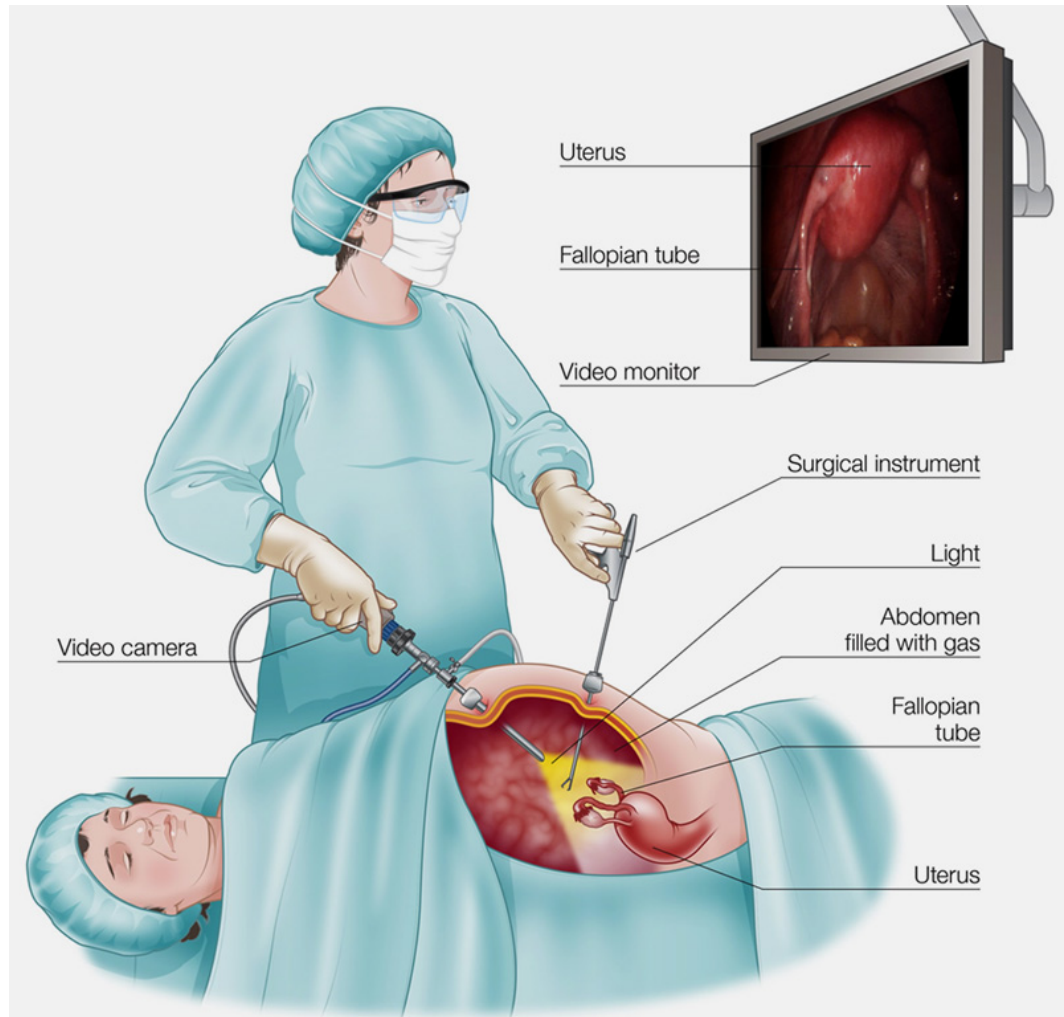


Figure 1.1: Laparoscopy or keyhole surgery - the current state of the art diagnostic procedure for endometriosis.

The general procedure is conducted under general anaesthetic and involves the use of a laparoscopic camera to visualise and confirm the presence of endometrial lesions and excision tool to destroy or remove endometrial implants. The abdomen is filled with carbon dioxide gas to facilitate instrument manipulation. Image source: <https://www.firmhospitals.com/laparoscopic-endometriosis>.

disease stratification [22]. Instead, multi-biomarker studies have hinted at a complex interplay between the systemic pathophysiology of endometriosis (which spans the immune, neurological and endocrine, gastrointestinal reproductive systems) and a person's genes and environment [23]. These competing influences have been mirrored in the large number of diverse biomarkers that have been linked to this disease (see biomarker tree in Figure 1.2).

This complex, systemic presentation of endometriosis together with the deeply personal experience of pain [24] and challenges in conveying that pain into clinical language [25], has encouraged the development of a range of home-based, self-tracking apps [26,

27](*e.g.*, Phendo [28], Endocare [29], Endi [30], Frendo [31]) that are recommended for women to monitor their symptoms and make their pain more recognisable in the clinical setting [32]. This is important as one of the leading causes of diagnostic delays in endometriosis is the normalisation and dismissal experiences around menstrual pain [33]. Additionally, these apps are featured in many endometriosis research studies that aim to for instance expand current modalities of disease stratification [34], understand the prevalence of certain symptoms across different phenotypes [35], or explore the relationship between factors such as exercise and pain symptoms [36].

Although the advantages and need for self-tracking systems for women’s health research is recognised [37] (*e.g.*, by patient advocacy groups like Citizen Endo [28] and the Endometriosis Society [38]), currently available systems are often limited to user-logged symptom tracking (mood, discharge, pain levels) [26, 27, 39] and wearable fitness trackers [40] that measure parameters such as heart rate variability, body temperature, sleep, physical activity. Expanding these at-home systems to include molecular biomarker tracking represents a promising opportunity to 1) empower women to identify the early signs of endometriosis and seek a diagnosis earlier and 2) provide researchers studying endometriosis with an additional dimension of data to move closer to a deeper understanding of this disease and eventually lead to a replacement or triage test to inform clinical decisions.

However, current state-of-the art techniques for biomarker quantification such as enzyme linked immunosorbent assays (ELISAs) and mass spectrometry are impractical for at-home, longitudinal (over a minimum of 2 menstrual cycles is recommended, but as many as possible is ideal [41]) screening across the multiple biomolecular classes that are believed to play a role in endometriosis (*e.g.*, hormones, proteins, nucleic acids) as they incur a high overall cost, limiting community-based studies.



Figure 1.2: Biomarker tree for endometriosis.

Showcasing the wide array of physiological functions and systems affected by endometriosis mirrored in the large number of potential biomarkers that have been identified for this disease. Image source: May et al. [42]

1.3 Electrochemical biosensors: opportunities and limitations

Electrochemical biosensors provide an opportunity for addressing the limitations of current bioanalytical tools owing to their low-cost, mass-manufacturability and ease of miniaturisation and multiplexing. These sensors have already had an impact on health-care and disease management (*e.g.*, home glucose monitoring) and have consistently shown a strong potential for tackling a wide array of medical [43–47] and environmental [48–51] challenges, reducing the time and costs associated with multi-analyte investigations across a spectrum of biomolecular classes [52, 53].

Typically, electrochemical biosensors feature biological recognition molecules (*e.g.*, antibodies, DNA, polymers, enzymes), which may be attached to the surface of an electrical conductor (electrode - *e.g.*, gold, carbon, platinum) [54, 55], plastic wells [56], functional particles (*e.g.*, magnetic beads) [57, 58] and more [59]. The interaction between the target analyte and the biorecognition layer gives rise to a measurable change in the electrical properties of the system which can be probed using a potentiostat instrument and related back to the concentration of the target present in a sample (Figure 1.3).

Despite their inherent advantages, the translation of these sensors from the laboratory bench into widespread commercial use remains challenging [60], as discussed in greater detail by myself and colleagues in our recent publication [61]. Many improvements are still needed to ensure their deployability at the point-of-need such as increasing their detection sensitivity - particularly in complex bodily media [55, 62], storage stability, reproducibility and multiplexing capabilities [60]. Consequently, a large body of research has been dedicated to improving some of these aspects for instance using surface modifications [62], tuning the bioreceptor orientation [54] or decreasing the electrode size [63]. However, many of these approaches exponentially increase the sensor production cost and manufacturing complexity.

One solution to this challenge that has been explored has been embedding these sensors within low-cost microfluidic devices [51, 64–66]. The inherent synergy between electrochemical biosensors and microfluidics has allowed unprecedented levels of detection parallelisation, signal enhancement, automation and lowering of the quantity of expensive reagents used [65, 66]. Yet despite this, microfluidic use remains scarce in the electrochemical sensing community, with only around 6% of research outputs featuring fluidic integration (Web of Science, 2020–2025)². Some of the likely drivers for that are the high entry costs of microfluidic device fabrication and actuation (*e.g.*, syringe pumps, pressure controllers, photolithography equipment) and additional processing time these techniques

²Search terms: topic = (electrochem* OR impedimetric OR amperometric OR voltammetric OR potentiometric) AND biosens*) [AND (*fluidic* OR droplet OR lab-on-a-chip OR total analysis system)]. The search was constrained to research articles (excl. reviews) published in the English language between 2020-2025). [...] was used to determine the % of articles featuring fluidic integration

incur (*e.g.*, post-processing of devices) which can make it impractical or unfeasible for electrochemistry labs to adopt this on top of their sensor developmental workflows. Therefore, one of the goals of this thesis has been to address these limitations by producing a set of accessible tools that may promote microfluidic use in the community.

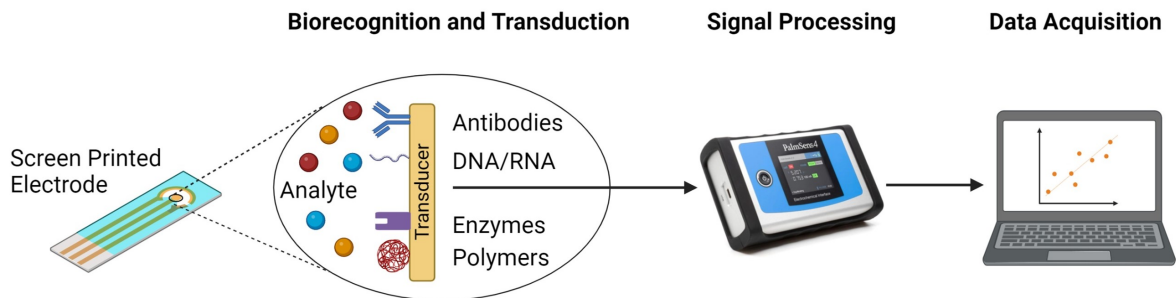


Figure 1.3: Typical outline of an electrochemical biosensor

The transducer with surface immobilized recognition molecules (antibodies, single stranded DNA or RNA, enzymes, and polymers – list not exhaustive). The transducer is exposed to the analyte solution which contains a patient’s sample with the target molecule. The interaction between the target and its receptor alters the electrical properties of the transducer and the output current from the transducer is amplified and processed using a microcontroller aboard the main analytical instrument (tabletop potentiostat) before being displayed on a laptop or tablet for analysis.

1.4 Target analytes chosen for this work

Given the vast array of potential biomarkers of endometriosis and the time required to develop sensors for these, the selection process was narrowed down using the following factors:

1. Sensitivity - the ability of a biomarker to identify positive cases
2. Specificity - the ability of a biomarker to identify negative cases
3. Whether they meet or approach the criteria for a replacement or triage³ test [21]:

³These thresholds were described by Vicky Nisemlat *et. al*[21] and represent clinically informed benchmarks for comparing the performance of different biomarkers. The exact purpose of triage tests can vary according to the clinical context and the patient’s priorities, but overall, the goal with them is to avoid unnecessary surgery and prioritise surgical treatment for those that show a high likelihood of having endometriosis. For biomarkers intended to rule in disease (SpIN), a specificity exceeding 95% is prioritised by the authors to minimise false-positive results, thereby ensuring that a positive test result provides strong evidence for the presence of disease. In this case, sensitivity is of lesser importance, as

- Replacement test = sensitivity ≥ 94 and specificity ≥ 79 (based on the accuracy of laparoscopy in successfully diagnosing endometriosis [67])
 - SnOUT triage test (rules out diagnosis if result is negative) = sensitivity ≥ 95 and specificity ≥ 50
 - SpIN triage test (rules in diagnosis if result is positive) = sensitivity ≥ 50 and specificity ≥ 95
4. Panel heterogeneity *i.e.*, pertaining to multiple biomolecular classes and performing a diverse array of functions congruent with the systemic pathophysiology of endometriosis.
 5. Non-invasive sampling method *i.e.*, urine, blood or saliva.
 6. Detectable by electrochemical methods⁴.

Based on these criteria, two highly promising markers from literature [18, 21, 68] were chosen for the initial developmental stages: IL-6 (an inflammatory protein) and cortisol (hormone). IL-6 has been extensively used in biomarker studies for endometriosis [18] and shown to meet the criteria for a SpIN test when used in combination with other biomarkers such as TNF- α , a pro-angiogenic inflammatory cytokine (Sensitivity: 70%; Specificity: 100%) or CRP (Sensitivity: 75%; Specificity: 100%) [18]. Additionally, IL-6 has been extensively targeted in electrochemical biosensing studies [69] and thus there is a wealth of literature available on its molecular binding mechanisms and how they may be exploited for electrochemical biosensing. Although another biomarker (urocortin) was closer to meeting the criteria for a replacement test on its own [18], it has never been

the principal objective is to maximise confidence in positive diagnoses (rather than detect every affected individual). Consequently, a minimum sensitivity value of 50% was considered sufficient by the authors. Conversely, biomarkers intended to rule out disease (SnOUT) require a sensitivity greater than 95% to minimise false-negative results, ensuring that a negative result reliably excludes disease. Although specificity remains relevant, it is of secondary importance because false-positive results can typically be resolved through subsequent confirmatory testing; therefore, a minimum specificity of 50% was deemed acceptable.

⁴The molecular sensitivity/thresholds depend on the specific biomarker and sample type. As currently no single biomarker or panel has been identified as a suitable replacement or triage test for endometriosis, there are no clinical concentration thresholds defined, thus these ranges were approximated based on literature reports of individual biomarkers identified for endometriosis that satisfy criteria 3-5 above.

targeted by electrochemical biosensing studies. Therefore, the choice to target IL-6 was motivated by an intention to de-risk the project and simultaneously ensure that the final product of this work is applicable not only to endometriosis but to other inflammatory diseases in which IL-6 plays a central role (*e.g.*, rheumatoid arthritis, sepsis, depression, cardiovascular disease and COVID-19) [69].

Cortisol is an emerging biomarker in endometriosis research [70–72]. Its role spans multiple areas of the body including the immune, reproductive, cardiovascular and digestive systems as it helps orchestrate the body’s ‘fight or flight’ response to stressors. Its diverse roles within the human body match the multifactorial nature of endometriosis and could provide interesting new insights into the pathogenesis and progression of this disease [72]. Animal studies to date have also indicated a promising link with endometriosis [70] and cortisol has very frequently been targeted by electrochemical biosensing studies [73].

Literature reports have indicated that clinically relevant ranges for serum IL-6 and salivary cortisol for endometriosis are around 0.5 - 50 pg/ml (IL-6) [74, 75] and 0.1 - 20 ng/ml (cortisol) [76, 77], which are suitable ranges for quantification by electrochemical measurements.

1.5 Electrochemical detection strategies

Although some of the advantages of electrochemical sensors have been listed in Section 1.3, the question remains of why this work aims to use these sensors specifically (rather than another platform of equal merit in terms of mass manufacturability, low cost and multiplexing potential for this specific application - such as a bio field-effect transducer or BioFET [78, 79]). While developing a BioFET implementation for the biomarkers chosen in this thesis would have been an equally worthwhile endeavour, the focus on electrochemical sensors arose for a few key reasons as follows.

Firstly, electrochemical sensors are, by comparison, less sensitive to sample ionic strength and Debye screening effects [79, 80] as they rely on different biorecognition transduction

mechanisms (*e.g.*, Faradaic reactions), meaning that the size of the bioreceptor used is less constrained [79] (Debye length in physiological sample is around 0.7 – 2.2 nm [81], smaller than that of an antibody which is typically around 12 nm [82]). This allowed more flexibility in choosing the bioreceptors and combining multiple different receptors into the same platform. Secondly, the manufacturing infrastructure is more mature for electrochemical sensors given the widespread use of glucose meters and electrochemical analysers such as the Abbott I-stat; whereas the synthesis process of the materials required for functionalising and operating BioFETs is currently still immature and prone to variability [78]. This was important to maximise the scalability of the solutions developed in this thesis on a faster time scale.

The standard detection layout for an electrochemical biosensor is the 3 electrode cell (although 2 or more than 3 electrodes may also be used). This setup consists of a working electrode, which is where the electrochemical reaction of interest is taking place, a counter electrode which effectively closes the electrical circuit and a reference electrode which maintains a constant voltage acting as an internal measurement reference point.

Silver/silver chloride is typically used as a reference electrode in electrochemical measurements because the reversible equilibrium between Ag, AgCl and Cl⁻ establishes a stable and predictable electrode potential [83]. Since the activities of the solid Ag and AgCl phases are effectively constant, the potential depends primarily on chloride activity. By maintaining a constant chloride concentration and drawing negligible current from the electrode, Ag/AgCl provides a reproducible electrochemical reference against which the potential of the working electrode can be accurately measured.

However, integrating a standard Ag/AgCl macroelectrode into a low-cost, portable system is not practical due to its size and geometry, meaning that it cannot be integrated within mass manufacturing systems such as roll-to-roll processing (which typically feature a planar 2D format). It is common therefore to use a quasi-reference electrode made of Ag/AgCl ink to replicate the behaviour of a true Ag/AgCl reference electrode [84–86], but this represents a compromise as the reference electrode is directly exposed to a sample which may have different chloride concentrations. However, that is often not significantly influencing the outcomes of a measurement in many situations as 1) the magnitude of signal drift is often sufficiently low (1 mV/h) [87] over typical experimental timescales (a

CV scan from -0.5 to +0.5 V for ferri-ferrocyanide for instance only takes a few seconds) so as not to substantially impact the measurement; 2) calibration and measurement can take place under the same conditions; and 3) the composition of the testing solution can be controlled for instance using consistent measurement buffers with the same chloride concentrations (which is what was done for example in Chapter 2 of this thesis). All experiments in this thesis used electrode arrays containing a Ag/AgCl ink quasi-reference electrode.

The 3 electrodes (working, reference and counter) are submerged in a conductive medium containing a redox active species with a supporting electrolyte (*e.g.*, NaCl, KCl) which electrically connects the 3 electrodes. The electrochemical properties of the medium may be probed using a suite of techniques such as cyclic voltammetry (CV), differential pulse or square wave voltammetry (DPV and SWV), chronoamperometry (CA) and electrochemical impedance spectroscopy (EIS) which provide different insights into the system properties such as redox reaction reversibility, capacitive behaviour, charge transfer across the electrode-liquid interface, the electrical double layer, reaction kinetics and more [83]. All of these techniques can be used for target species quantification with EIS, DPV and SWV featuring higher sensitivity compared to CA and CV but also higher noise interference. CV and CA may also be used to prepare the electrodes for affinity molecule attachment and subsequently enhance detection sensitivity. EIS particularly is very commonly used in the literature, often in tandem with techniques such as Raman spectroscopy [83] to study the effect of surface modifications (*e.g.*, alkane thiol, antibody or aptamer attachment) on discrete properties of the electrochemical system such as charge transfer resistance or double layer capacitance [88]. Since these techniques are employed extensively throughout this PhD, their fundamental principles are explained briefly in the following section.

1.5.1 Electrochemical techniques

Cyclic voltammetry

Cyclic voltammetry is a very powerful electrochemical technique used to study reduction and oxidation (redox) processes and has found extensive use in biosensing applications [89–93]. Figure 1.4 displays a typical cyclic voltammogram for a single electron transfer redox reaction - commonly referred to as the 'duck' plot. In a cyclic voltammetry measurement, the potential is scanned over a voltage window in which the redox reaction is known to take place (for instance for the redox couple ferri-ferrocyanide that is typically between -0.4 to 0.6 V vs Ag/AgCl). Following IUPAC convention, as the potential is scanned anodically from negative to positive potentials (from point A to point B), the concentration of reduced species (in this example ferrocene - Fe) is steadily oxidised to ferrocenium (Fe+). Once the voltage threshold for the oxidation reaction to occur is exceeded, there is an exponential increase in the current flow within the system as all the charges in the proximity of the electrode are depleted. Once the peak current is reached (point C), the rate of the reaction is limited by the arrival of additional Fc from the bulk solution, a slower process mirrored in the sharp decrease of the current down to point D where the scan direction is reversed. This results in the appearance of a matching reduction peak (point F), similarly followed by a decrease in the current until the end of the voltage window (point G). The theoretical separation between the oxidation and reduction peak in an electrochemically reversible system at 25°C is 57 mV [94]. This behaviour and the relationship between the electrochemical potential of the cell (E), the relative activities of the redox components (*e.g.*, concentrations) and the standard potential of the species (E^0) is captured by the Nernst equation:

$$E = E^0 + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \quad (1.1)$$

where R is the universal constant of gas (8314.3 J/mol K), T is the temperature (K), F is Faraday's constant (96485 C/mol) and n is the number of electrons exchanged in the redox reaction, which in the case of ferrocene and ferrocenium and ferri-ferrocyanide is 1. Where there are multiple sequential electron transfer reactions within the same voltage window (for instance in the case of the chromogenic ELISA substrate TMB interacting with the enzyme HRP in the presence of hydrogen peroxide, a 2-electron process [95]), 2 distinct peaks may emerge on the same forward and reverse scans. Throughout this work, cyclic voltammetry was used in conjunction with sulfuric acid (H_2SO_4) as a means of cleaning the electrode surface by lifting absorbed impurities and dissolving any organic contaminants. Typically the voltage is cycled until the peaks begin overlapping, which suggests that the electrode surface has been adequately cleaned [94]. Cyclic voltammetry has also been used within this work with ferri-ferrocyanide ($[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$, denoted in this text as FF-Cy), a standard and well characterised redox couple [96], to evaluate the electrode surface cleanliness and electron transfer characteristics.

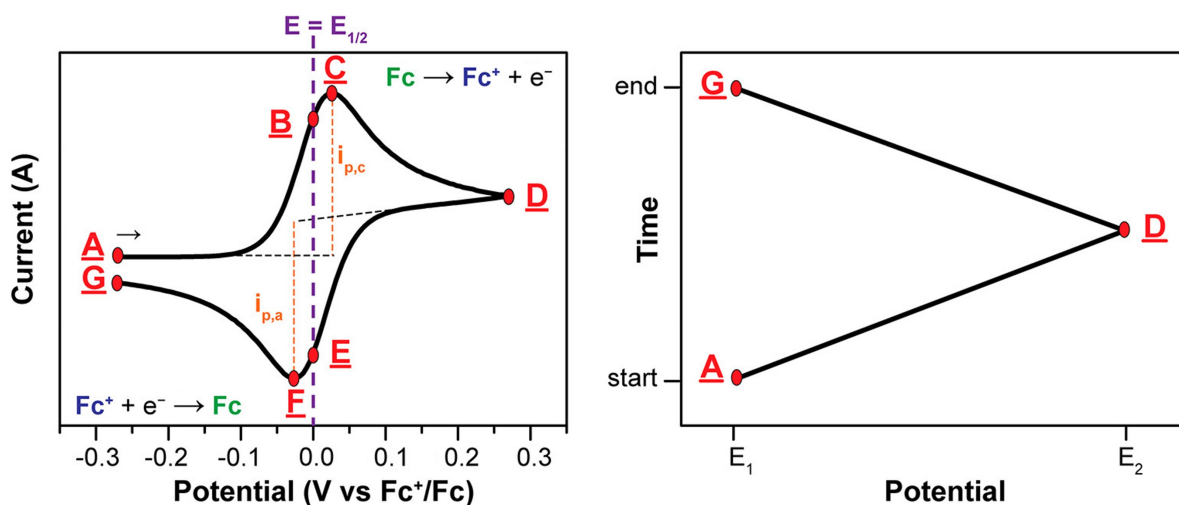


Figure 1.4: Cyclic voltammetry technique

Cyclic voltammogram (left) and the potential scanning regime used to generate it (right) [94]. The potential is scanned within a voltage window where the reaction of interest is known to take place which results in the appearance of the characteristic 'duck' shape in the case of a single electron transfer reaction such as the redox reaction of ferrocene (Fe) and its oxidised form, ferrocenium (Fe+) (displayed here). The graph highlights different points of interest detailed more in the text.

Differential pulse voltammetry

Differential pulse voltammetry is another popular electrochemical technique which involves the application of short (5-100 ms), low-amplitude (10-100 mV) pulses superimposed on a linear DC voltage ramp (Figure 1.5). A current measurement is taken before the pulse application and late in the pulse before the drop, and the difference between those values is plotted - hence the 'differential' name. While it can provide some similar insights to CV, DPV has a suppressive effect on the capacitive background current due to its differential nature yielding higher sensitivity and narrower voltammetric peaks which makes it easier to discern species with similar oxidation potentials [83]. Throughout this work, DPV has been used to study the cleanliness and electrochemical properties of electrodes as well as in the fluidics characterisation work to determine the minimum time required for different flow rates to completely remove redox active species for the electrochemical cell.

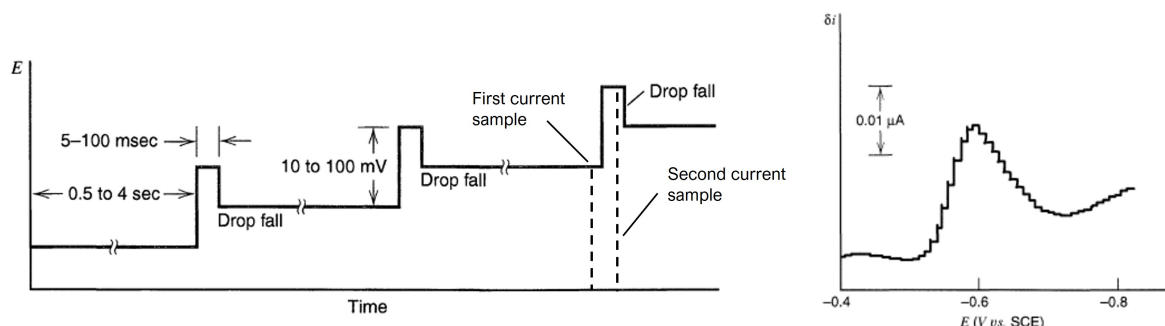


Figure 1.5: Differential pulse voltammetry technique

The voltage waveform applied to the electrochemical cell featuring short, small-amplitude step voltage pulses superimposed on a linear ramp (left) and the resulting current response in a differential pulse voltammetry measurement [83](right)

Electrochemical impedance spectroscopy

In electrochemical impedance spectroscopy (EIS), the electrochemical cell is exposed to a series of sinusoidal voltages (V) of varying frequencies (ω) and the resulting current (I) is measured. This allows the determination of impedance (Z), which is related to the voltage and current using a generalised form of Ohm's law:

$$V = I * Z \quad (1.2)$$

Typically, the two sinusoids (V and I) are out of phase from each other by a quantity known as the phase angle (ϕ). For a purely resistive circuit, the phase angle is 0 and for a purely capacitive one it is $\pi/2$; for a mix of the two it takes on a value in-between. For convenience, it is common to represent these quantities (V , I and Z) using complex notation. For instance the impedance is typically denoted as:

$$Z = Z' - j * Z'' \quad (1.3)$$

where Z' and Z'' are the real and imaginary components of impedance respectively. Purely resistive components are encompassed by the 'real' part of the impedance, while capacitive components contribute to the 'imaginary' part. Electrochemical impedance data is typically displayed in the form of a Nyquist plot where Z' and Z'' are plotted for varying frequencies (Figure 1.6). The multi-frequency aspect of this measurement allows probing of events taking place on multiple time scales. Namely, at high frequencies (left side of the Nyquist plot) one can visualise chemical events that occur quickly upon the application of voltage such as charge transfer across the electrode/liquid interface and double layer

formation. Conversely, at lower frequencies (right side of the Nyquist plot) the dominant mode of action is mass transfer which is a slower process.

As the electrochemical cell is essentially an impedance 'black box', it can be represented using equivalent electrical circuit models - series and parallel arrangements of resistors and capacitors. A very popular example is Randles equivalent circuit (Figure 1.6) - an electrical circuit widely used to model semi-infinite diffusion-limited Faradaic reactions on planar electrodes [97]. This model consists of a solution resistance (R_{sol}) in series with a parallel grouping of a charge transfer resistance (R_{ct}) and double layer capacitance (C_{dl}) with a Warburg impedance (W) - an element denoting the semi-infinite diffusion.

In EIS, electrical current is carried by different charge carriers in different regions of the electrochemical cell. Within the electrode, charge is transported by electrons, whereas within the electrolyte solution, it is carried by the migration of positively and negatively charged ions [83]. At the electrode-electrolyte interface, the applied alternating potential produces both capacitive (non-Faradaic) and Faradaic responses, if a redox substance is present (*e.g.*, ferri-ferrocyanide) [98].

The capacitive response arises from the periodic accumulation and depletion of electronic charge at the electrode surface, accompanied by the redistribution of ions within the adjacent electrical double layer. This behaviour is captured by the double-layer capacitor (C_{dl}) circuit equivalent (Figure 1.7). In parallel (and physically occurring in the same place *i.e.*, at the electrode interface), electroactive species undergo reversible electron-transfer reactions with the electrode, with the kinetic resistance to this process represented by the charge-transfer resistance (R_{ct}). The solution resistance (R_{sol}) represents resistance to ionic conduction through the bulk electrolyte, while the Warburg impedance (W) describes the contribution of mass transport arising from the diffusion of electroactive ions towards and away from the electrode surface.

For fitting circuit models on experimental impedance data, proprietary potentiostat data acquisition software such as PsTrace by PalmSens include equivalent circuit fitting functionalities. Similarly, open source versions based on the `fminsearch` MATLAB function such as Zfit and ZfitGUI by Jean-Luc Dellis are also available [99].

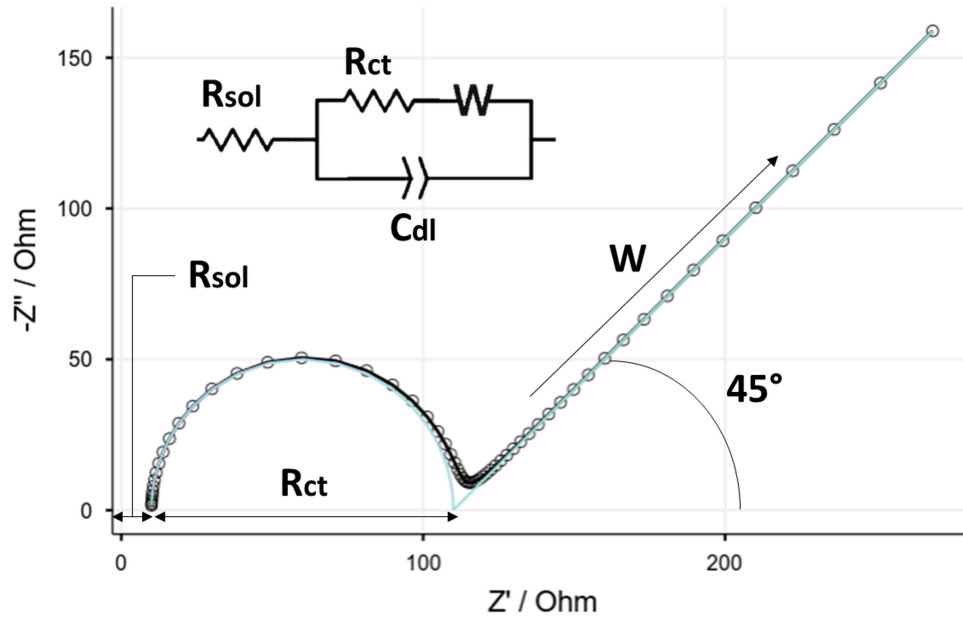
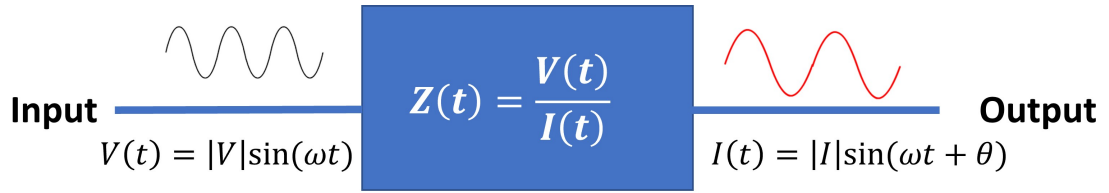


Figure 1.6: Electrochemical impedance spectroscopy technique

Example Nyquist plot and Randles equivalent circuit (simulated using an online tool available at: <http://lacey.se/science/eis/simulation-randles-circuit/>). An input sinusoidal voltage of varying frequencies (each point on the Nyquist plot represents a different frequency) is applied at the input and the output current is measured. The resulting real (Z') and imaginary (Z'') components of the impedance (calculated using Ohm's law) are then plotted and fitted using Randles equivalent circuit – an electrical model that captures charge transfer and diffusive phenomena taking place in the electrochemical cell by using electrical analogues such as charge transfer resistance (R_{ct}), double layer capacitance (C_{dl}), solution resistance (R_{sol}) and a Warburg element (W).

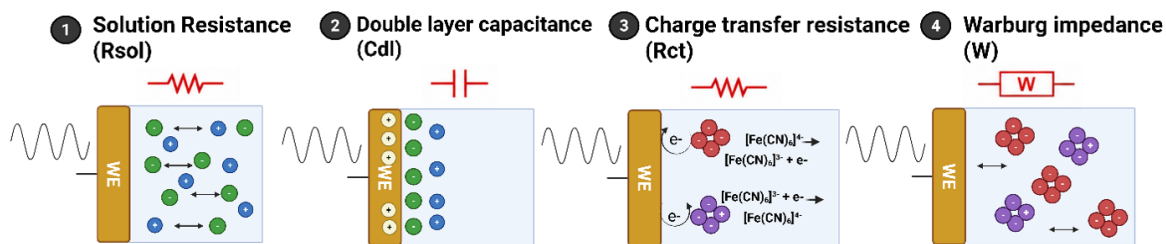


Figure 1.7: Equivalent circuit components and their meaning

Panel 1 - Ions (cations and anions) move through solution carrying an ionic current. R_{sol} represents the resistance to ionic conduction. Panel 2 - Electrons accumulating or depleting at the electrode surface attract counter ions in solution, leading to the creation of a double layer (Cdl). Panel 3 - Electrons are transferred between the electrode and the ferri-ferrocyanide redox species. The resistance to this process is captured by R_{ct} . Panel 4 - Redox species diffuse from the bulk solution to the electrode surface. Warburg element (W) models mass transfer limitation to that. Figure created using BioRender

Chronoamperometry

Another technique commonly used to measure the concentration of analytes of interest is chronoamperometry (Figure 1.8). In this technique, a step voltage is applied to the working electrode and the faradaic current that arises is measured as a function of time to gain information about chemical and diffusional processes taking place in the electrochemical cell. The behaviour of the Faradaic current response, i (A), is predicted by the Cottrell equation:

$$i = \frac{nFAC\sqrt{D}}{\sqrt{t\pi}} \quad (1.4)$$

where n is the number of electrons exchanged in the reduction/oxidation of one molecule of analyte, F is Faraday's constant (C/mol), C is the initial concentration of the redox active component (mol/cm³), A is the effective area of the electrode (cm²), D is the diffusion coefficient of the redox species (cm²/s), and t is time (s). Upon initial application of the

step voltage at $t = 0$ there is a sharp increase in the observed current as the charged particles in the proximity of the electrode are reduced or oxidised. After that the current gradually declines as the system enters the mass-transfer-limited phase, until it reaches a steady state value that depends on the ease of diffusion of the electroactive species from the bulk solution. Although not as sensitive as DPV and EIS, CA has found broad applicability in electrochemical biosensing studies [100], particularly with enzymatic-based detection systems [101–103], because of its simplicity and the fact that it can be integrated within point-of-care devices with relatively inexpensive and less complex electronic control systems [104].

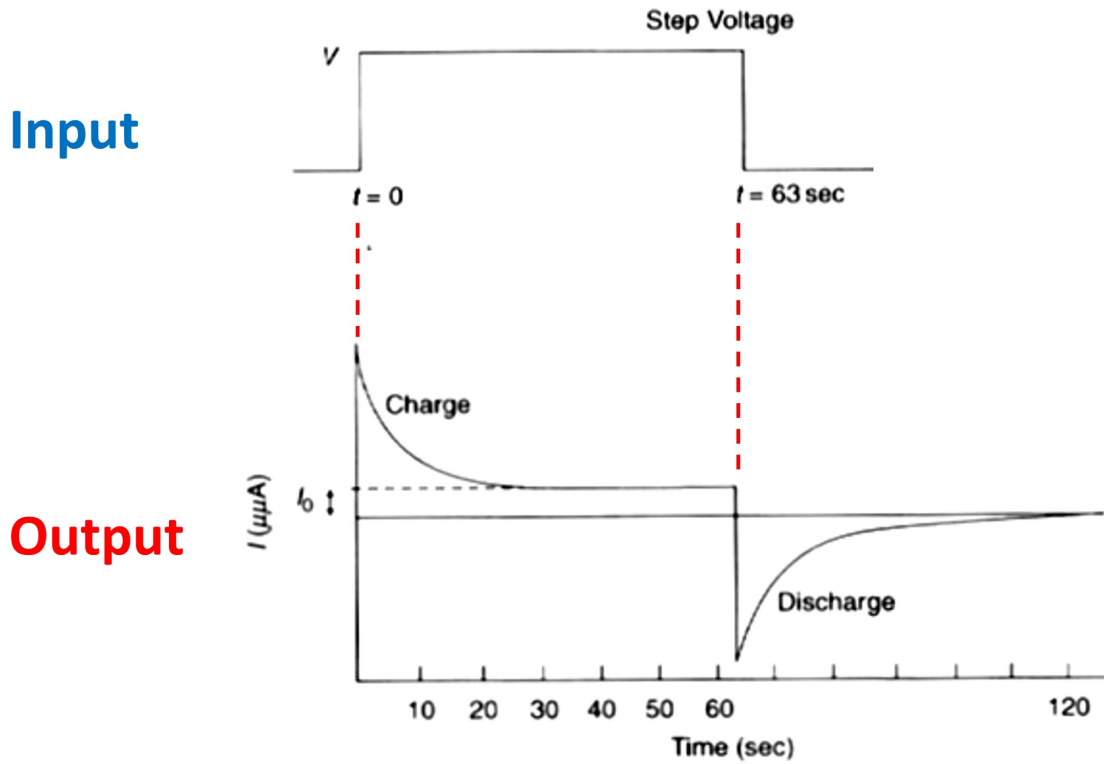


Figure 1.8: Chronoamperometry technique

Input and output in a chronoamperometry measurement. The input is a step voltage applied to the WE and the output is current as a function of time. Adapted from the book ‘Dielectric Phenomena in Solids’ by Kao [25]

1.5.2 Electrochemical immunoassays

The electrochemical biosensor literature is abundant in studies that have produced highly impressive sensors for IL-6 and cortisol using a variety of biorecognition (*e.g.*, nanobodies [105], aptamers [106, 107], molecularly imprinted polymers [108, 109], IL-6 receptors [110]) and signal transduction systems (*e.g.*, microelectrodes [111], nanomaterial decorated electrodes [112, 113]) [69]. Antibody-based detection systems have unsurprisingly been a popular choice among these for this application [114] because of their high selectivity and reliability, stemming from their extensive commercial use in bioanalytical products and wide breadth of documentation available for developing immunoassays [115–117]. Although antibodies may be used in conjunction with a whole suite of surface attachment chemistries and detection layouts, direct attachment to the sensing surface *via* spontaneous absorption (physisorption) presents the most cost effective option from a manufacturing standpoint as it does not rely on any further electrode modifications or hazardous chemicals [69]. However, the main disadvantage of this method is the chaotic orientation of the antibodies assembled on the detection surface which results in a lower detection sensitivity compared to oriented systems (*e.g.*, antibodies with thiol terminations, or immobilised *via* an intermediary with a cysteine terminal), which means that higher antibody concentrations are required per sensor to meet sensitivity requirements which drives up the cost of these assays [55].

Microfluidic strategies have been proposed as a low-cost avenue to increase the sensitivity of these sensors through minimization of environmental contamination, speeding up of reaction times and decreasing reagent volumes [65, 66, 118]. However, as mentioned in Section 1.3, presently there is still a high entry barrier for microfluidic manufacturing facilities into everyday laboratories – as not all may have access to clean rooms or specialist equipment [119]. Thus, one of the goals of this thesis was to produce a low-cost, accessible microfluidic strategy for increasing the sensitivity of electrochemical immunoassays based on physisorption (Chapter 3). This was demonstrated using a sandwich-based electrochemical immunoassay for IL-6, a common detection scheme employed in literature for its high detection specificity. Figure 1.9 highlights the difference between an optical and

electrochemical immunoassay and the typical steps involved in preparing these. Typically, in the optical format a primary antibody for the molecular target of interest is attached to the bottom of a 96 well plate which is subsequently backfilled using for example bovine serum albumin, SuperBlock or similar to prevent non-specific absorption to the walls of the plate. Following this, sample is added to the wells and incubated for a certain amount of time to allow the target to bind the primary antibodies. A secondary antibody is then added which forms an immunocomplex with the target and surface immobilised primary antibody. This secondary antibody may be pre-conjugated or become conjugated during the assay with an enzyme (*e.g.*, horseradish peroxidase or alkaline phosphatase) capable of converting a chromogenic substrate into a secondary form which is typically accompanied by a colour change in the detection medium. The amount of coloured substrate is measured using a standard absorbance reader and related back to the concentration of target present in a sample typically through the generation of a calibration curve. The electrochemical format follows a similar preparation sequence but the immunocomplex may be assembled on the surface of the working electrode and at the end, the chromogenic substrate (*e.g.*, 3,3',5,5'-Tetramethylbenzidine or p-Nitrophenyl Phosphate) may be measured electrochemically using techniques such as chronoamperometry by poisoning the potential step to a value within the mass-transport governed window of the redox reaction. This effectively removes the need for a highly expensive, lab-confined reader and reduces the amount of reagents used.

In between all of the immobilisation steps for both the optical and electrochemical version of this technique, the detection surface where the immunocomplex is being assembled is washed after each step using a buffer such as phosphate buffer saline (PBS) containing surfactants to remove any excess unreacted components before the next step is carried out. The necessity for wash steps and the involved, multi-step nature of this assay type are some of the main challenges that have so far limited its deployment (both in optical and electrochemical format) at the point-of-care (PoC). The answer to this challenge lies in a concerted approach combining biochemical advancements toward a one-step-only assay and engineering innovation on the automated, fluidic handling of reagents and sample processing to remove the need for trained operators and a laboratory environment.

Why an enzyme linked assay?

Immunoassays come in different formats that can be broadly classified into labelled and label-free [114, 120]. Labelled sensors typically involve the attachment of an electroactive reporter to the biorecognition element (non-competitive) or directly to the target analyte (competitive format). Upon target binding and immunocomplex formation, the reporter gives rise to a measurable signal which can be related back to the concentration of target present in a sample. Different molecular components can be used as a reporter, such as redox probes (*e.g.*, methylene blue [121], ferrocene (Fc) [122], thionine [123], Toluidine blue [124]), enzymes (*e.g.*, HRP [125], alkaline phosphatase [126]), nanoparticles (*e.g.*, quantum dots [127]) and nanozymes [128]. A core advantage of many labelled sensors is their ability to generate a one-to-many relationship between target recognition and signal generation *i.e.* a single binding event gives rise to multiple detectable species or events, which is particularly useful for low abundance (< 5 pg/ml) targets.

In contrast, label-free immunoassays rely on the immunocomplexes' ability to influence the properties of the detection interface for instance by blocking or opening charge access to the electrode surface or modulating the intercalation or electrostatic affinity of an external redox species [120]. This approach is very attractive from a final cost of assay perspective as it requires fewer processing steps and less reagent, and thus potentially a less complex manufacturing process. However, signals generated using this approach are typically of lower magnitude than labelled systems resulting in less sensitive assays. Additionally, label-free approaches tend to be more prone to non-specific binding events [120].

Although there are significant merits and drawbacks for each of these methods which have been summarised and discussed in detail in several review articles [114, 120, 129], it was decided in this thesis to employ an enzyme-linked immunoassay system for the detection of IL-6. This decision was made because IL-6 is a low abundance marker (< 5 pg/ml in serum in healthy individuals) and because enzyme-linked immunoassays are the

most common format available for this target [120] – meaning there is extensive literature, documentation and many validated off-the-shelf antibody kits available. The focus of this thesis was not to develop another biosensor for IL-6, but to create a reliable test bed assay for the fluidic automation tools developed in Chapter 4.

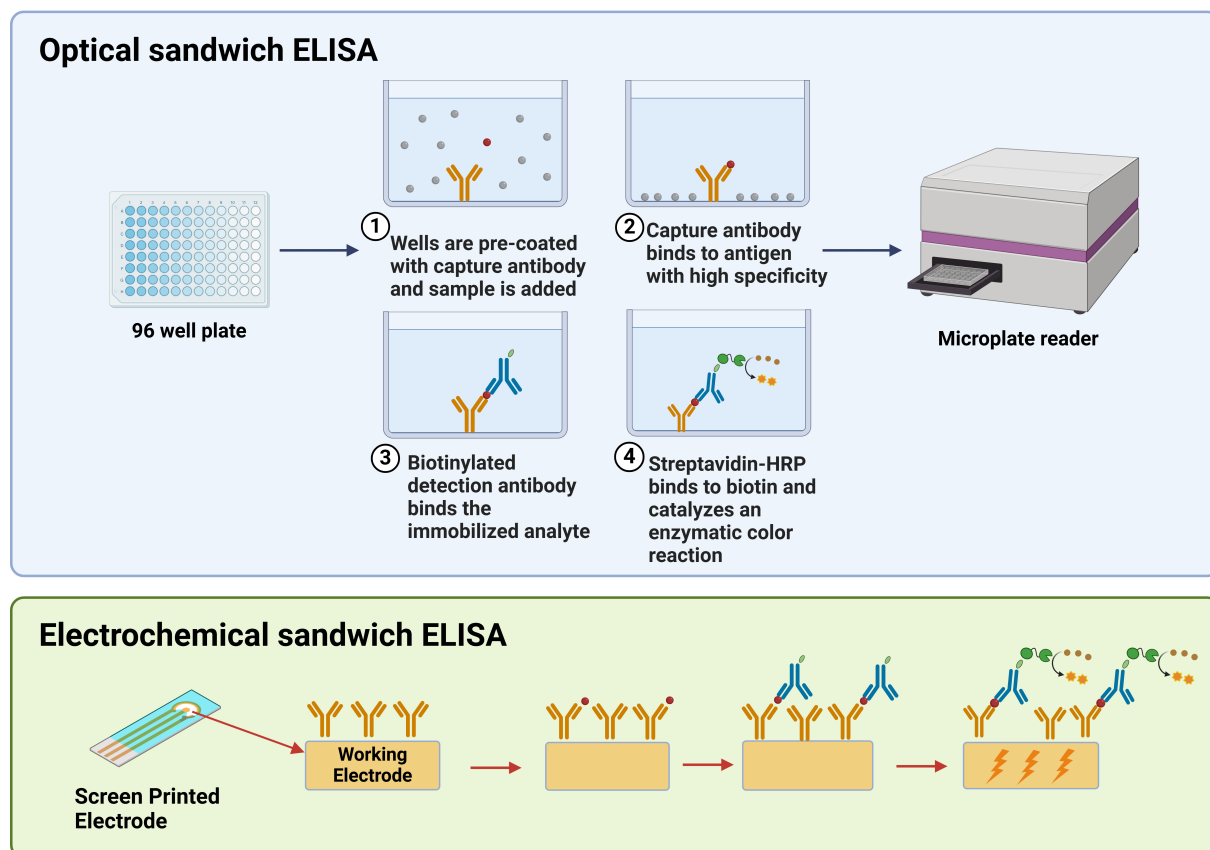


Figure 1.9: Optical and electrochemical enzyme linked immunosorbent assays
The typical layout of a sandwich based immunoassays with (top) optical and (bottom) electrochemical readout. These assays typically involve the immobilisation of a primary antibody specific to the target analyte to either a 96 well plate or the surface of the working electrode, backfilling to minimise non specific binding, sample addition, secondary antibody addition and enzyme attachment. Once the sandwich immunocomplex is assembled, a colorimetric substrate is added (*e.g.* 3,3',5,5'-tetramethylbenzidine or p-Nitrophenyl phosphate) which reacts with the enzyme label giving rise to a colour change in the detection medium which can be measured optically *e.g.*, with an absorbance reader or electrochemically using a potentiostat.

1.5.3 Electrochemical aptamer-based (E-AB) sensing

Another approach that has been particularly popular because of its high commercialisation potential is the use of aptamers as the biorecognition element. Aptamers are small oligonucleotide sequences which in the presence of their specific targets undergo 3D morphological changes and have numerous advantages over traditional antibody-based bio-recognition systems, namely they can be synthesized on a large scale at a significantly lower price point, and they have superior storage stability - only requiring a brief heating cycle to return to their primary active shape. However, compared to antibodies which have been predominantly used in commercial analytical assays for decades, aptamers are a newer development (the first papers were published in the 90's [130, 131]), with many unknowns and challenges remaining - particularly pertaining to their integration as affinity reagents within electrochemical detection systems [132, 133].

CSS.1 is an aptamer discovered by the Stojanovic group in 2017 and optimized for detachment from its complementary 'capture' aptamer in the presence of cortisol, a mechanism termed duplex dissociation or strand displacement [134]. This mechanism (Figure 1.10) has been used for cortisol sensing in a lateral flow format [135] and recently in an electrochemical format employing indium-tin oxide electrodes modified with mesoporous silica nanochannels for signal amplification [136]. CSS.1 was one of the first aptamers discovered to have strong affinity for cortisol ($k_d = 30\text{-}140\text{ nM}$ whereas previous reports could only reach $6\text{-}10\text{ }\mu\text{M}$ [137, 138]) and since its discovery it has been proven to bind cortisol using various methods including fluorescence tagging [134], label-free fluorescence, isothermal titration calorimetry and AuNP-based colorimetric assays [139], and thus was deemed a strong affinity molecule for building a label-free electrochemical sensor. Such a sensor has not been developed previously based on this aptamer sequence and its label-free format (based entirely on the conformational change in response to cortisol as opposed to using a redox-tag) would result in an ultralow cost sensing system compared to any other cortisol measurement system currently available [140]. The strand displacement mechanism is also attractive for potentially creating a continuous cortisol monitoring system (as in theory the cortisol aptamers may be replenished after each use). Such a system

could be invaluable for tracking a rapidly changing molecule such as cortisol which fluctuates in accordance with the circadian rhythm and is sensitive to acute stressors (*e.g.*, blood draw). However, many unknowns remain about the behaviour of CSS.1 within electrochemical sensors and the optimal conditions for its function within this detection layout. Chapter 2 explores some of the various experimental parameters of importance for optimal biosensor performance including buffer composition, aptamer concentration and direct *versus* indirect gold surface attachment which will hopefully inform and guide further research into this aptamer.

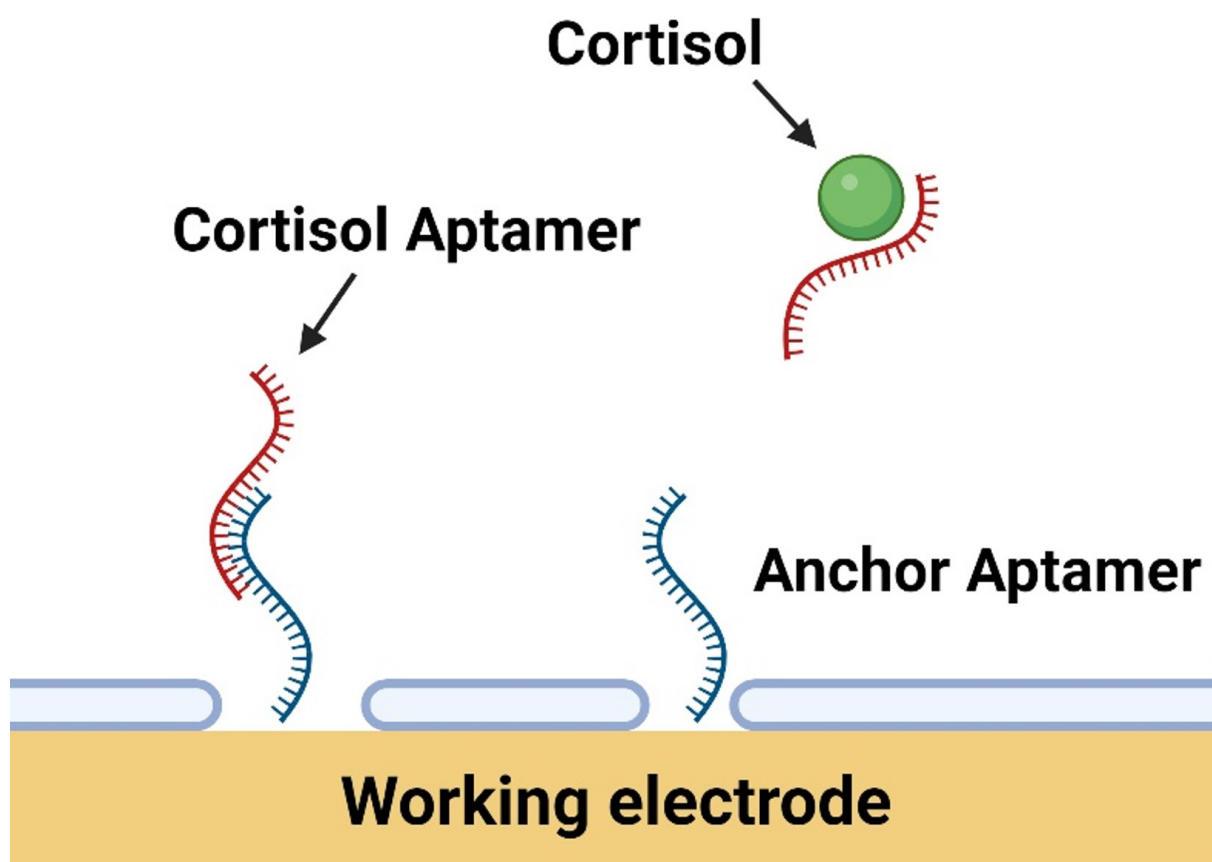


Figure 1.10: The strand displacement mechanism for cortisol detection used in this work

The strand displacement detection mechanism employed for cortisol detection in this thesis. An anchor aptamer is attached to the surface of a gold electrode via a thiol termination at the 5' end and connected to a cortisol specific aptamer (CSS.1) via a 13-mer complementarity zone towards the 3' end. The surface of the electrode is backfilled to prevent non-specific interactions. Upon sample addition, cortisol interacts with the aptamer duplex, releasing the cortisol-specific aptamer from the anchor which creates space for electric charges to more easily penetrate to the electrode surface which should result in a decrease of the charge transfer resistance of the surface as shown by Luan et al. with their electrode modified with mesoporous silica nanochannels [136].

1.6 A crucial missing tool for researchers

Both aptamer and antibody-based electrochemical sensors follow a similar sequence of manual preparation steps involving pipetting small reagent deposits on detection surfaces, followed by incubation and pipette washing. Unavoidably, this exposes the sensor to contaminants and evaporation, and each electrode may experience different shear stresses during washing causing variability. One potential solution to this is enclosing the reagents within a fluidic system and functionalizing the biosensors using consistent flow regimens [66]. This approach has gathered much interest in the sensing community [141–143] and different solutions have been proposed based for instance on the electrowetting-on-dielectric (EWOD) effect [144], flow-injection [145, 146] or centrifugal systems [147], droplet [148, 149] and paper-based [150] microfluidics or capillary-based devices [151] in an effort to transition away from manual processes prone to error and reliant on trained operators and expensive lab facilities. So far, EWOD has seen some commercial application in the area of electrochemical sensing but the devices are typically prohibitively expensive and too complex to be practical in a lab or clinic environment [60]. At the opposite end of the cost spectrum, paper and capillary-based fluidics feature very low cost but accurately tuning and controlling their flow profiles is more difficult compared to using a flow-injection system where the flow rate is determined by an external controller.

Some authors have developed platforms that can not only manage the multi-reagent process of functionalising electrochemical biosensors but also retain accessibility and a small form factor, ensuring their deployability at the point-of-need. For example, the eSiren system developed by Zhao and colleagues (shown in Figure 1.11 a) can be interfaced directly with off-the-shelf electrodes and employs a small pressure pump and 5-way solenoid valve to direct the sample and reagents from the on-chip storage reservoirs to the electrode surface to achieve the DNA-based detection of SARS-CoV-2 RNA [152]. The MiSens device (Figure 1.11 b) is another example of such a platform developed by a different group designed to be interfaced with a bespoke electrode cartridge and featuring a reagent carousel, pump, potentiostat and LCD display all of which can be controlled

wirelessly from a user-friendly interface. The authors have validated this platform with electrochemical detection schemes for prostate-specific antigen (PSA) [153], *E. coli* [154], and aflatoxin [155] - a toxigenic and carcinogenic fungal metabolite. Dulay and colleagues also proposed an impressive fluidic platform based on gold electrodes produced by lithography and interfaced with a polymeric microfluidic chip from the microfluidic ChipShop [156]. In their platform, displayed in Figure 1.11 c, each reagent reservoir is equipped with an in-line peristaltic pump which can be individually controlled from a user friendly interface. However, the nature and placement of the valves upstream of the detection chip inadvertently adds a large amount of dead volume into the system. This issue is mitigated in the design presented by Kadimisetty *et al.* (Figure 1.11 d) where the reagents are pre-loaded inside on-chip chambers and sequentially delivered to the sensing surface through a serpentine channel [157].

While the design of those platforms is well suited for their eventual deployment in the field, currently, to the best of our knowledge, there is no fully integrated, automated platform commercially available or reported in literature ⁵ that can allow flexibility to the electrode geometries and detection layouts used. Additionally, some concerns still remain regarding the stability and sensitivity of biofunctionalised sensors to hydrodynamic conditions which warrants further study [145].

In Chapter 4, a fully automated platform for electrochemical sensor preparation is described, that can be adapted to fit different electrode formats and explore the effect of fluidic control on sensor performance. This platform was specifically designed using a modular approach meaning that its components can be carried through into a point-of-care configuration once the focus shifts from sensor development to commercialisation. This platform may find use as part of the electrochemical biosensor developer's toolkit, and bridge the gap that currently exists between electrochemical biosensor research and their commercial use.

⁵While other similar platforms of equal merit to those discussed above have been produced for other measurement methods such as those with an optical readout [158, 159], the focus of this work was specifically on those used in conjunction with electrochemical biosensors.

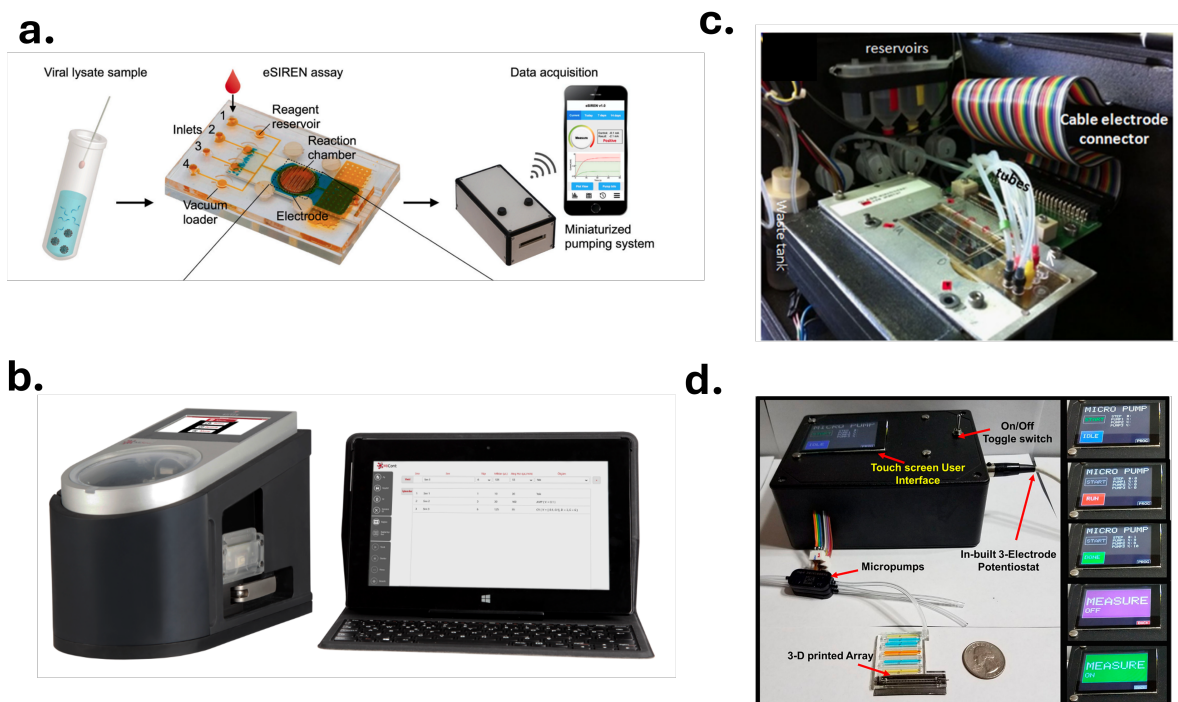


Figure 1.11: Biosensing assay automation tools reported in literature

(a) The eSIREN tool proposed by Zhao and colleagues [152] (b) The MiSens device with integrated reagent carousel and chip docking station on the side [153] (c) Automated microfluidically controlled platform for electrochemical biosensor preparation developed by Dulay *et al.* [156] (d) The electrochemiluminescence assay proposed by Kadimisetty *et al.* [157]

1.7 Aims and research questions

The overarching aim of this PhD is to develop an automated and reliable IL-6 and cortisol detection system that can be easily deployed to further endometriosis research. To achieve that, the following research questions were investigated:

1. Can reliable and low-cost electrochemical sensing of IL-6 and cortisol be achieved using commercial electrodes? (Chapters 2 and 3)

Technical objective(s): Development and exploration of low-cost electrochemical sensing strategies for cortisol and IL-6 using antibodies and aptamers.

Success criteria:

- Reproducible ($\text{CoV} < 15\%$ [160, 161]) attachment of biorecognition elements (aptamers and antibodies) to low-cost electrodes.
- Demonstration of electrochemical response to the target analyte across the relevant concentration range (0.5 - 50 pg/ml IL-6 [74, 75] and 0.1 – 20 pg/ml cortisol [76, 77, 162]) and discrimination from appropriate controls (blanks).

2. Can the sensitivity of electrochemical sensors be improved using easy-to-implement microfluidic solutions? (Chapter 3)

Technical objective(s): Development of a low-cost and accessible fluidic strategy for enhancing the performance of electrochemical assays.

Success criteria:

- Development of a microfluidic approach that can be quickly (< 5 min) manufactured using limited expertise and readily integrated with commercial electrodes.
- Demonstration of improved sensor sensitivity compared with an equivalent conventional electrochemical assay.

3. **Can the performance of these sensors be further improved with fluidic automation? (Chapter 4)**

Technical objective(s): Development of a fully automated platform for biosensor preparation to standardize fabrication and enhance the performance of electrochemical sensors during the research phase.

Success criteria:

- Development of a platform capable of automatically executing the required multistep sensor functionalisation workflow (minimum 8 steps for a sandwich assay).
- Demonstration of improved sensor-to-sensor variability ($\text{CoV} < 10\%$ [160, 161]) compared with manual preparation.
- Demonstration that automation maintains or improves sensor analytical performance.
- Successful application of the platform to up to 3 different electrochemical assays to demonstrate flexibility.

Chapter 2

Development of a low-cost electrochemical sensing strategy for cortisol

Highlights

- The CSS.1 cortisol aptamer sequence and its complementary strand were integrated for the first time in an impedimetric biosensor based on planar, unmodified gold electrodes.
- Different factors affecting sensor performance (*e.g.*, dilution buffer, reducing agent, sensor preparation procedure) were explored to maximise detection sensitivity.

Data partially presented in

Dobrea, A., Blake, R., Macdonald, D., McKenzie, C., Corrigan, D. K. & Jimenez, M. WAFFLE – an automated platform for enhancing the performance of electrochemical biosensors. *Lab Chip* 2026; 26 (6): 2035–2046.

Conference presentations

A. Dobrea, D. K. Corrigan, R. Ghannam, M. Jimenez (2023) Development of a label-free electrochemical cortisol aptasensor and automated plug-and-play sensor preparation strategy based on consumer-grade materials and processes., Aptamers 2023 Conference, Oxford, UK.

2.1 Introduction

Electrochemical biosensors are a very useful technique for decreasing the costs and time associated with biomarker investigations across a wide spectrum of biomolecular classes, and thus could accelerate research into systemic diseases such as endometriosis. This chapter describes the developmental work towards realizing such a sensor for cortisol - an emerging biomarker of endometriosis, using an aptamer-based affinity system.

Cortisol aptamer-based electrochemical sensor development

Cortisol is a small (~ 300 Da) steroid hormone secreted by the adrenal gland and regulated through the action of the hypothalamic-pituitary-adrenal (HPA) axis. Because of its small size and lipophilic nature, cortisol can migrate through the lipid bilayer of cells and thus can be found throughout the body and is measurable in blood (2 - 25 $\mu\text{g/ml}$), sweat (8 - 142 ng/ml), saliva (0.1 - 12 ng/ml), hair (18 - 153 pg/mg) [73] and even nails [163] and tears [164]. Although infamously known as the 'stress hormone', orchestrating the body's 'fight or flight' response to real or perceived stressors¹, cortisol performs a plethora of physiological functions of importance in health and disease, for instance in regulating inflammation and metabolism, controlling blood pressure and the sleep-waking cycle [165, 166]. As cortisol fluctuates in accordance with the circadian rhythm and is responsive to daily activities [167, 168] or standard sampling techniques (*e.g.*, blood draw) [169], gaining a true insight into its activity requires integration into wearable or minimally obtrusive portable devices, making it an ideal target candidate for electrochemical based sensing.

Given their low-cost and ease-of-scalability, aptamer-based electrochemical sensors specifically have been a prime target for development of cortisol monitoring systems [170–174].

¹A stressor is usually defined as a threat to homeostasis that exceeds the person's ability to resolve.

Mohammadi and colleagues [175] have provided an excellent overview of aptamer-based sensors for cortisol (including but not limited to electrochemical) from 2018-2023 which details some of the recent achievements on this topic. Of particular note is the rapid, label-free field-effect-transistor system developed by Wang *et al.* integrated into a wearable patch format complete with an LCD screen, pH and temperature sensors [176]. They demonstrate a limit of detection down to 0.36 pg/ml for cortisol using a newly discovered aptamer sequence, and used their system to test diurnal salivary and iontophoretically induced sweat cortisol levels in 88 participants. A similar wearable system design with an integrated mini potentiostat, named 'Captain Patch' was developed by another group recently, based on a redox-concatenated aptamer structure attached to gold electrodes produced in-house [177]. The surface of their electrodes was engineered to resemble the roughness of human skin featuring microscale ridges and grooves, a strategy they employ to boost the aptamer loading density and improve the signals generated by their sensor during voltammetric interrogation. Their system produced a limit of detection in spiked human sweat of around 181.3 ng/ml. Ganguly and colleagues also demonstrated an impressive autonomous cortisol monitoring platform in submicroliter volumes of sweat without any active sweat induction, capable of acquiring real-time cortisol level data for up to 8 hours [178]. Their transduction platform is a nanoporous ZnO-modified screen printed electrode, upon which a thiolated aptamer monolayer encapsulated in a hydrophilic polymer matrix was deposited. This system was shown to outperform in terms of saturation behaviour an equivalent sensing system based on cortisol antibodies. Singh *et al.* also demonstrated sensitive sensing of cortisol in undiluted serum samples by attaching their methylene blue-tagged aptamers to a gold electrode *via* a nanocomposite antifouling layer [179]. Although redox-tagged aptamers are advantageous over label-free variants in that they do not require any external redox probes to study the detection surface, consequently simplifying the overall device design, any modifications to the aptamer structure and underlying electrode platform (*e.g.*, nanostructures) incur additional production costs and manufacturing steps which can negate some of the cost advantages of aptamer-based systems [132].

The focus of this chapter was the development of a low-cost, label-free, impedimetric aptasensor for cortisol based on the CSS.1 cortisol aptamer discovered by the Stojanovic group in 2017 [134]. When this work started, the only use of the CSS.1 aptamer and its complementary strand within a biomarker quantification system was the lateral flow detection format published by Dalirirad and colleagues [135]. In their implementation, the cortisol aptamer duplexes were immobilised onto gold nanoparticles using thiol chemistry, and the spaces inbetween aptamers are backfilled using MCH. The functionalised nanoparticles are added to samples containing cortisol and then applied to a nitrocellulose pad with a test and control zones. When the aptamer duplex comes in contact with cortisol in a sample, the cortisol-specific aptamer is detached from its complementary strand. This allows the strand still immobilised to the AuNP to bind to a complementary sequence present on the test zone, leading to particle aggregation proportional to the amount of cortisol present in the sample. This aggregation is visually confirmed by a distinctive colour development in the test band which is quantified using a camera and subsequent image analysis by ImageJ. While lateral flow systems have the advantage of being low-cost, electrochemical biosensors allow for lower detection limits and, particularly important for hormones like cortisol which fluctuate in accordance to the circadian rhythm and daily activities, are amenable to being integrated within continuous monitoring systems. To the best of our knowledge, this aptamer has only once to date (in 2024) been integrated with an electrochemical transduction system [136] which featured a indium-tin oxide electrode decorated with mesoporous silica nanochannels, a sensitive but high cost system. The aim of this work was to decrease the cost of a detection system based on this aptamer and concurrently, shed light into its behaviour within electrochemical detection systems featuring unmodified planar electrodes. Two distinct detection methods were trialled: one based on the strand displacement mechanism of a surface immobilised duplex (the scheme which this aptamer and its complementary strand were originally developed for) and one based on the direct attachment of CSS.1 to the electrode surface. The difference between those two methods and the general fabrication process for both of these methods is summarised in Figure 2.1. While the strand displacement method has the advantage of potentially forming the basis of a wearable continuous cortisol monitoring system (as in theory the cortisol aptamers may be replenished after each use), the direct attach-

ment has the advantage of simplicity and low cost, and could be easily manufactured and deployed within a portable cortisol monitoring system. To that end, the cleaning method of electrodes, buffer composition, thiol reduction and duplex assembly method, and optimal aptamer concentration for detection were investigated. Aptamer sequences with a fluorescein (6-FAM) at the 3' end were also used to optically validate some of the electrochemical results². The assay was developed for quantification in impedance mode due to the high sensitivity and non-destructive nature of this technique [132].

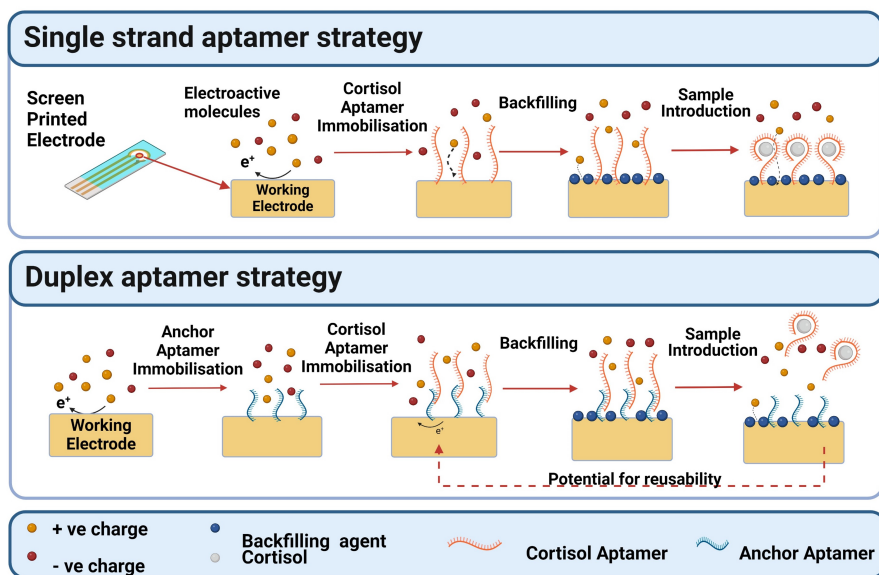


Figure 2.1: Aptamer immobilization strategies explored

Top panel shows protocol for electrode functionalization using the single strand aptamer layout where the target induces a morphological change in the aptamer which blocks the access of charges to the electrode surface consequently altering the current passing through the electrode. Bottom panel shows the duplex aptamer immobilization protocol and detection strategy. In this case, the presence of the target displaces the cortisol aptamer from its anchor, allowing more charges to reach the surface of the electrode.

²It was demonstrated in prior literature using the same aptamer structure as the one used in this thesis that attachment of a fluorescent probe at either end of the sequence does not interfere with its binding capability [134, 136]

2.2 Materials and methods

Chemicals

1,4-Dithiothreitol (DTT), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), sodium chloride, Trizma base (Tris), 6-Mercapto-1-hexanol (MCH), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (monobasic; m.w. = 138), Na_2HPO_4 (dibasic; m.w. = 142), hydrocortisone, potassium ferri-cyanide and ferrocyanide, sulfuric acid (98%), magnesium chloride (MgCl_2), phosphate buffer (PBS) tablets were purchased from Merck. Nuclease-free water was sourced from Fisher Bioreagents and ultrapure water (18.2 M Ω .cm) was obtained from an Elga Purelab Chorus water purification system.

Aptamers

HPLC-purified DNA aptamer sequences (Table 2.1) were sourced from Sigma-Aldrich. To attach the thiolated aptamers to the surface of the gold working electrodes, the disulfide bond of the thiol groups was reduced to its active sulfhydryl form by incubating the aptamers in 100 mM DTT solution in sodium phosphate buffer (pH 8.3 - 8.5) at room temperature for 1 hour. By-products of this reaction were then filtered using a NAP-5 column (Cytiva LifeSciences). The output of the filtration columns was fractionated into individual Eppendorf tubes (2 droplets per tube) and aptamer concentrations were quantified using a Qubit 4 fluorometer with an ssDNA Assay Kit from InvitrogenTM (Q10212). The final working concentration of aptamers (200 nM) was obtained by diluting the filtered aptamer solution in a buffer containing 0.15 mM NaCl, 0.05 M Tris, 0.25% Triton X-100 (pH = 8.0) in nuclease-free water as reported elsewhere in literature [135]. Aptamer working concentration was chosen based on literature accounts of sim-

ilar detection systems [180, 181]. Where a duplex aptamer strategy was chosen, a 1:2 ratio of capture to cortisol aptamer was used. To induce folding of the aptamer into its three-dimensional shape, the cortisol aptamer solutions were heated to 95°C for 5 minutes using a benchtop mini-PCR machine (miniPCR bio) and immediately cooled to 4°C right before use.

Table 2.1: Aptamer sequences used in this work

The folding structure of the cortisol aptamer predicted using mfold [182] can be found Appendix A A.

Component	5'-3' Sequence	No. bases
ssAptamer (SH)	(thiC6) - CTC TCG GGA CGA CGC CCG CAT GTT CCA TGG ATA GTC TTG ACT AGT CGT CCC	51
Capture Aptamer (C)	(thiC6) - TTT TTT TTT TTT TTT TTT TTG TCG TCC CGA GAG	33
Cortisol Aptamer (A)	CTC TCG GGA CGA CGC CCG CAT GTT CCA TGG ATA GTC TTG ACT AGT CGT CCC	51
Capture Aptamer (A) - FAM	(thiC6) - TTT TTT TTT TTT TTT TTT TTG TCG TCC CGA GAG - (6FAM)	33
Cortisol Aptamer (A) - FAM	CTC TCG GGA CGA CGC CCG CAT GTT CCA TGG ATA GTC TTG ACT AGT CGT CCC - (6FAM)	51

Aptamer biosensor functionalization

The electrode format used throughout this work is an 8 individually addressable³ gold (Au) working electrode (WE) platform with common Au counter and Ag/AgCl pseudoreference electrode (Figure 2.2a) which connects to a PalmSens 4 potentiostat *via* a 3D printed electrical connector made in house shown in Figure 2.2b. The functionalization strategy of these electrodes is depicted graphically in Figure 2.1. Before attachment of biorecognition molecules, the electrodes were cleaned by voltage cycling between 0 and

³The electrode platform was designed that way because of a trade-off between generating an adequate number of repeats per condition, time required to measure them all in one experimental session and to minimize the overall size of the platform.

1.6 V in 0.1 M sulfuric acid for 10 cycles. Following the DTT reduction of the sulfhydryl group, the thiolated aptamers were immobilized by placing 2 μ l of aptamer solution on each working electrode and incubating it overnight ($\sim$ 18 h) in a humidity enclosure at 4°C. The next day, sensors were washed using 3 consecutive rinses with 1 ml of aptamer buffer to remove any unbound compounds. This wash step was repeated after each immobilisation step of the fabrication procedure. Once the single strand aptamer was attached to the surface, the electrode surface was passivated by depositing 1 μ M MCH in molecular-grade water on the working electrodes. The electrodes were incubated at room temperature for approx. 2 hours before rinsing unbound MCH with aptamer buffer. If the duplex strategy was employed, the cortisol aptamer was immobilized to its complementary strand by placing 2 μ l of the aptamer solution on each working electrode and incubating at room temperature for a further 2 hours. This was followed by an MCH backfilling step similar to that performed on the single strand aptamer. To observe the dose dependent response of the functionalized biosensor, different hydrocortisone concentrations were prepared in aptamer buffer and 100 μ l of each concentration were placed on top of the working electrodes and incubated for 1 h at room temperature. The electrical properties of the electrode-electrolyte interface in response to cortisol addition were probed using 400 μ l of 2 mM $Fe[CN]_6^{3-} + Fe[CN]_6^{4-}$ (Ferri-Ferro Cyanide solution) in 1xPBS drop cast on electrode platforms and running an electrochemical impedance spectroscopy measurement before and after cortisol addition. During development, an EIS measurement was also taken after each step of the functionalisation procedure to track the binding events. The EIS measurement was taken over a frequency window from 0.1 Hz to 100 kHz (50 frequencies) against the open circuit potential of the cell with an AC potential of 10 mV.

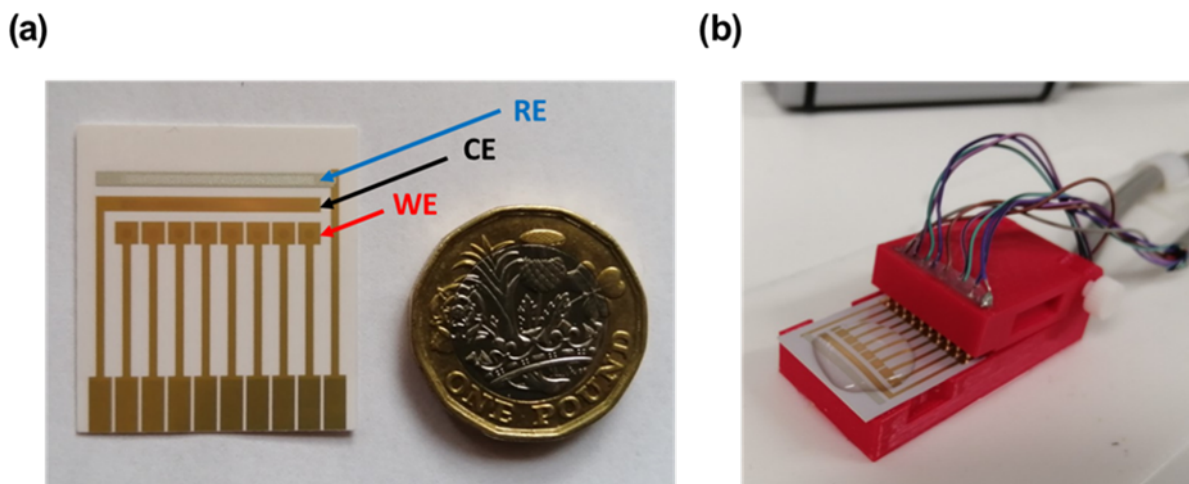


Figure 2.2: (a) Electrode platform used in this work. This was reproduced from [183] with permission from Springer Nature.

Image (a) shows the counter (CE) and reference (RE) electrodes and the 8 working electrodes (WE) and (b) the experimental set-up where the PCB is placed inside a custom 3D printed connector and the sample media is placed on top of all the electrodes for measurement.

Data fitting and statistical analysis

The Nyquist plots generated by the EIS experiments were fitted to Randles equivalent circuit using the Levenberg–Marquardt algorithm. High frequency noise from the instrument was filtered by setting a threshold on 7906 Hz which is where the noise appeared to stop (only frequencies below this were used for the analysis). The average chi squared value for the fits was $0.005 (\pm 0.001)$. All experiments were performed using $n = 8$ working electrodes unless otherwise noted in the figure caption. When multiple experimental conditions were compared, one-way analysis of variance (ANOVA) with Fisher post-hoc test was performed using Minitab 19 to detect any statistically significant differences between the different groups. Statistical significance was accepted for $p < 0.05$.

2.3 Results

Figure 2.3 presents the first reported attempt at translating the fundamental principles underlying the CSS.1 duplex dissociation system demonstrated by Dalirirad et al. [135] into an impedimetric format based on low-cost unmodified planar gold electrodes. As an additional test condition, a thiol modified cortisol aptamer was directly attached to the surface to investigate whether the conformational change of the aptamer alone in response to cortisol presence is enough to generate a detectable impedance change. Such a system would substantially simplify the sensor preparation process, reducing the fabrication cost and potential sources of variability.

In this first test, it was observed that after the first washing step following the immobilisation of thiolated aptamers, the impedance would substantially decrease likely due to a strong change in the hydrophilicity of the surface generated by the presence of the Triton-X surfactant in the aptamer buffer (Figures 2.3 a,b). A hydrophilic surface exhibits a hydrated interfacial layer that can improve electrolyte wetting and thus facilitate the transport of redox species to the electrode surface, thereby enhancing electron-transfer kinetics and reducing R_{ct} , an effect that has been observed in prior work [184–186]. Moreover, the surface of the electrode platform became so strongly hydrophilic that it was impossible to drop cast reagents (aptamers or redox media) onto it without them leaking over the side of the electrode platform. Therefore, the following experiment, depicted in Figures 2.3 c and d, was performed with a buffer devoid of this surfactant which allowed significantly easier handling of the electrode platform and a clear discrimination of the individual SAM assembly steps on the electrode surface. Each step of the functionalisation procedure resulted in an increase of the charge transfer resistance (R_{ct}) suggesting effective assembly of the detection layer on the electrode surface. It was also noted however that the baseline level of charge transfer resistance (R_{ct}) was higher which suggested that either the cleaning protocol chosen for the electrodes was not strong enough to effectively remove surface contaminants across this batch of electrodes and/or the absence of surfactant resulted in aptamer aggregation resulting in non-uniform surface

attachment across different electrodes. Consequently, a new electrode cleaning protocol based on oxygen plasma was trialled. Oxygen plasma has been previously demonstrated to be a more effective electrode cleaning method compared to electrochemical techniques [187, 188] as it does not rely on a liquid/electrode interface. However, that is not a blanket rule across all gold electrode substrates as demonstrated in depth in section 3.3 of this thesis. Figures 2.3 e and f demonstrate that in the case of this particular electrode batch, plasma cleaning produced substantially less variable electrode baseline resistances (CoV = 8.4%) compared to acid cleaning (CoV = 28.2%).

Although in some cases the R_{ct} decreased following the addition of cortisol as illustrated in the Nyquist plots shown in Appendix C – Figure 3, which indicates removal of aptamers off the electrode surface in both the duplex and single aptamer cases, it was overall difficult to discern any significant differences between the response of fully functionalised electrodes and those exposed to cortisol, both in the case of directly attached cortisol aptamers and those attached *via* a thiolated 'anchor' aptamer. In terms of variability, the duplex functionalisation method resulted in slightly lower CoVs (19.0%) compared to the single strategy (27.3%) and thus from this point onward it was decided to focus primarily on the duplex system as opposed to the target-induced conformational change system as the latter effect would likely be more challenging to accurately detect without further electrode modifications to boost sensitivity given the small lengthscale of this effect compared to the removal of an entire 15 kDa structure off the surface of the electrode (based on prior work, the R_{ct} value could be expected to drop by up to 42-63%, or in absolute values 2.5-15 k Ω , as a result of sample induced aptamer strand displacement [189, 190]). This allowed a more targeted approach going forward.

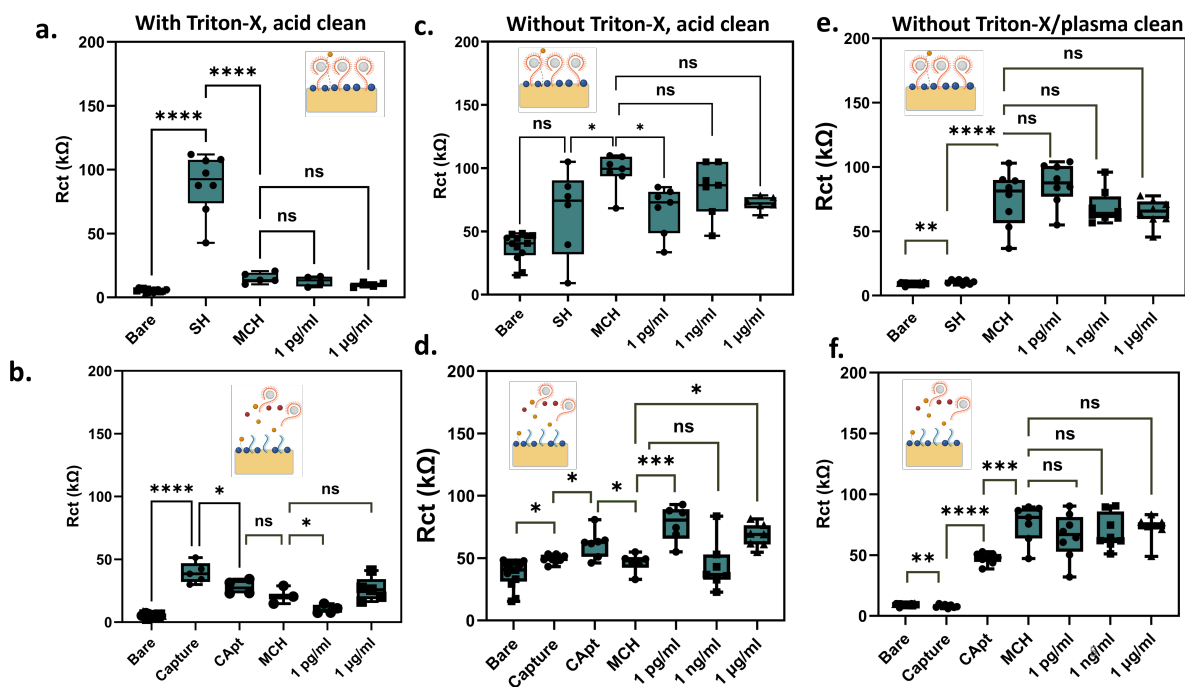


Figure 2.3: Translating the lateral flow assay demonstrated by Dalirirad et al. (2020) into an impedance-based detection format for rapid signal read-out

Two different strategies were investigated (a,c,e) Direct attachment of a thiolated cortisol aptamer (SH) to the electrode surface (b,d,e) Attachment of the cortisol aptamer (CApt) to the gold surface *via* an anchor aptamer (Capture). The surface was backfilled with 6-Mercapto-1-hexanol (MCH) to prevent non-specific binding and the assembly of the surface monolayer was tracked using Rct. (c,d) The effect of removing Triton-X from the buffer composition due to difficulty handling electrodes treated with this surfactant. (e,f) The effect of plasma compared to acid cleaning on the variability of electrodes. Individual sensors were identified using different data markers (circle, square and triangle) and each data panel a-f represents a different set of sensors. Experiments were conducted using $n = 8$ working electrodes per tested condition (if less than 8 markers appear it is because of electrode damage *i.e.*, gold stripping during the measurement rendering the WE unusable). Statistical differences between the different groups was assessed using t-tests where ns - non-significant, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ and **** - $p < 0.0001$. Box plots represent the upper and lower interquartile ranges, the horizontal line denotes the median and the whiskers represent the minimum and maximum values recorded.

Aptamer buffer optimisation

As the aptamer buffer composition is one of the determining factors of aptamer function, the next step was to optimise its composition, as the buffer reported by Dalirirad *et al.* might not be directly transferable to an electrochemical detection system. Typically, aptamers function optimally in the buffer that they have been selected in [191]. That is one of the core advantages of aptamers compared to other detection molecules as they can be selected specifically for detection in specific sample matrices such as blood, urine or saliva. Figure 2.4 a showcases the effect of different buffers on the SAM assembly on the electrode surface, comparing the buffer used by Dalirirad *et al.*, PBS, the SELEX buffer of the study that first reported the discovery of CSS.1 (20 mM HEPES, 1 M NaCl, 10 mM MgCl_2 , 5 mM KCl, pH 7.5) [134], with varying concentrations of different Tween-20 surfactant - a more gentle non-ionic surfactant compared to Triton-X. It was found that the SELEX buffer resulted in less variable assemblies of thiolated aptamers on the surface of electrodes (CoV = 15% compared to 27% for PBS and 21% for the Tris-based buffer [135]) and particularly, the SELEX buffer containing 0.1% v/v Tween-20 led to the clearest discrimination between blank cortisol samples and two different concentrations of cortisol (Figure 2.4 b). This condition was then repeated using a wider concentration range (0.1 - 10 ng/ml) and the individual functionalisation steps were tracked using EIS to confirm SAM assembly (Figure 2.4 c). The aptasensor behavior demonstrated a decrease in the charge transfer resistance with increasing cortisol concentration (Figure 2.4 d) which was congruent with the expected behaviour of an impedance detection scheme based on strand displacement. With increased detachment of cortisol aptamers from the detection surface, the physical barrier they impose to electrical charges reaching the electrode surface is removed, resulting in a decrease in the apparent charge transfer resistance of the electrode/liquid interface.

However, repeating this result with a different batch of electrodes from the same manufacturer resulted in a very different sensor response where the different concentrations of cortisol could not be clearly distinguished from the baseline. The issue of batch-to-batch variation with electrochemical sensors has been previously highlighted [192]. It is

likely that the gold material was changed by the manufacturer as the SAM assembly for the different functionalisation steps was substantially different compared to the previous electrode batch as shown in Figure 2.5, with wider Rct distributions and likely ineffective surface attachment as suggested by the decrease in impedance caused by the backfilling step - suggestive of aptamer removal by the MCH. Thus, a series of experiments were performed to unpick the different contributing factors and reoptimise the system to the new electrode platform.

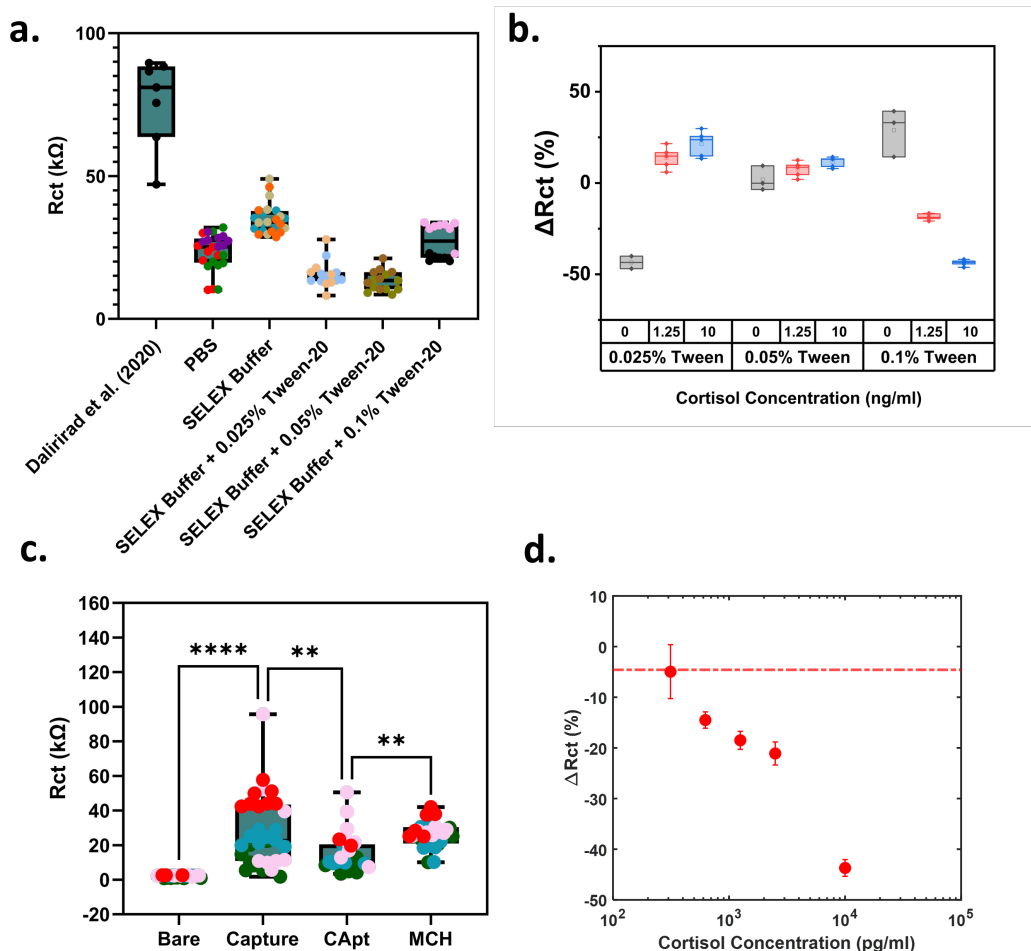


Figure 2.4: Aptamer buffer optimisation, stepwise tracking of functionalisation steps and calibration curve in spiked buffer

(a) Comparing the effect of different buffers on the attachment of aptamers to the electrode surface ($n = 24$ working electrodes except the first buffer and SELEX buffers with Tween which only had 7 and 16 respectively due to gold stripping). Electrodes belonging to the same array of 8 have been grouped using the same marker colours. The SELEX buffers (Yang et al., 2019) led to lower variability compared to PBS and the buffer reported by Dalirirad (2020). The SELEX buffer + 0.1 % Tween-20 yielded the lowest variability per electrode (indicating the variability came from differences between different electrode platforms rather than the SAM) and reasonably high aptamer coverage compared to the other concentrations of Tween-20 tested as well as the best separation between the different cortisol concentrations tested. All groups were statistically different from each other except 0.025 and 0.05% Tween buffers, and PBS and the 0.1 % Tween buffer (b). Only the 0.1% Tween cortisol response showed a duplex dissociation behavior expected for this detection layout ($n = 3$ working electrodes, each condition represents a different sensor array). (c) SELEX BUFFER WITH 0.1 % TWEEN-20, plasma cleaned electrodes, overnight incubation steps - tracking the individual SAM assembly steps using EIS ($n = 30$ working electrodes; Electrodes belonging to the same array of 8 have been grouped using the same marker colours.) and (d) dose-response of the biosensor to different concentrations of cortisol ($n = 3$ working electrodes per concentration, meaning 18 working electrodes total for the full calibration curve; each concentration dot is the average over the 3 measurements, whiskers represent 1 standard deviation and the horizontal dashed line is the average blank ± 1 standard deviation). Box plots represent the upper and lower interquartile ranges, horizontal line the median and the whiskers represent the minimum and maximum values recorded.

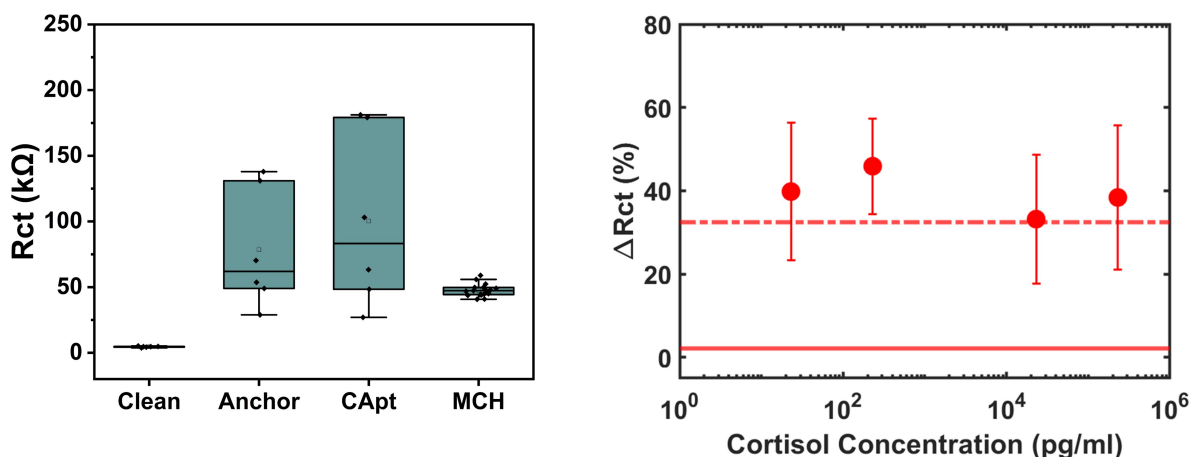


Figure 2.5: Repeat of previous dataset (Figure 2.4 c and d) with a different batch of electrodes from the same manufacturer

(left) $n = 6$ working electrodes for all except MCH step which had an $n = 16$ (2 different devices); Box plots represent the upper and lower interquartile ranges, horizontal line the median and the whiskers represent the minimum and maximum values recorded. (right) $n = 3$ working electrodes per concentration. Each concentration dot is the average over the 3 measurements, whiskers represent 1 standard deviation and the horizontal solid line is the average blank and the dashed line is the average blank ± 1 standard deviation).

DTT vs TCEP

Although recommended by the aptamer manufacturer for reducing the disulfide bond at the end of the aptamer sequence in preparation for gold immobilisation [193], DTT has been previously demonstrated to preferentially bind to gold surfaces, competing for the available binding sites with thiolated molecules [194]. TCEP is typically used as an alternative to DTT in biochemical applications as it does not exhibit gold affinity and is a more powerful and stable reducing agent [195]. Figure 2.6 a displays the effect of different concentrations of TCEP compared to DTT in the attachment of aptamers to the electrode surface showing a significant decrease in overall variability, suggesting a more effective and repeatable reduction of the disulfide bonds and aptamer attachment. An additional advantage of TCEP over DTT is that it does not require filtration, fractionation and quantification of aptamer solutions prior to gold attachment. This simplification of the preparation process reduces not only the aptamer loss inherent with filtration, but also removes an additional potential source of error and variability by removing some of

the handling steps. From the concentrations of TCEP tested, 10 mM was determined to be optimal as it resulted in higher aptamer loading density compared to 5 mM, and was non-significantly different from 20 mM. These results also showed good agreement with microscopy data taken of fluorescent-tagged aptamers attached to the electrode surface using the same protocol (Appendix C - Figure 6). Maximising aptamer loading was chosen as an optimisation criteria as it is known to be a key driver of sensor sensitivity [196–198] and stability [136, 199, 200] as denser SAMs are more resistant to the deleterious effects of DNAses, enzymes which are ubiquitous in living systems.

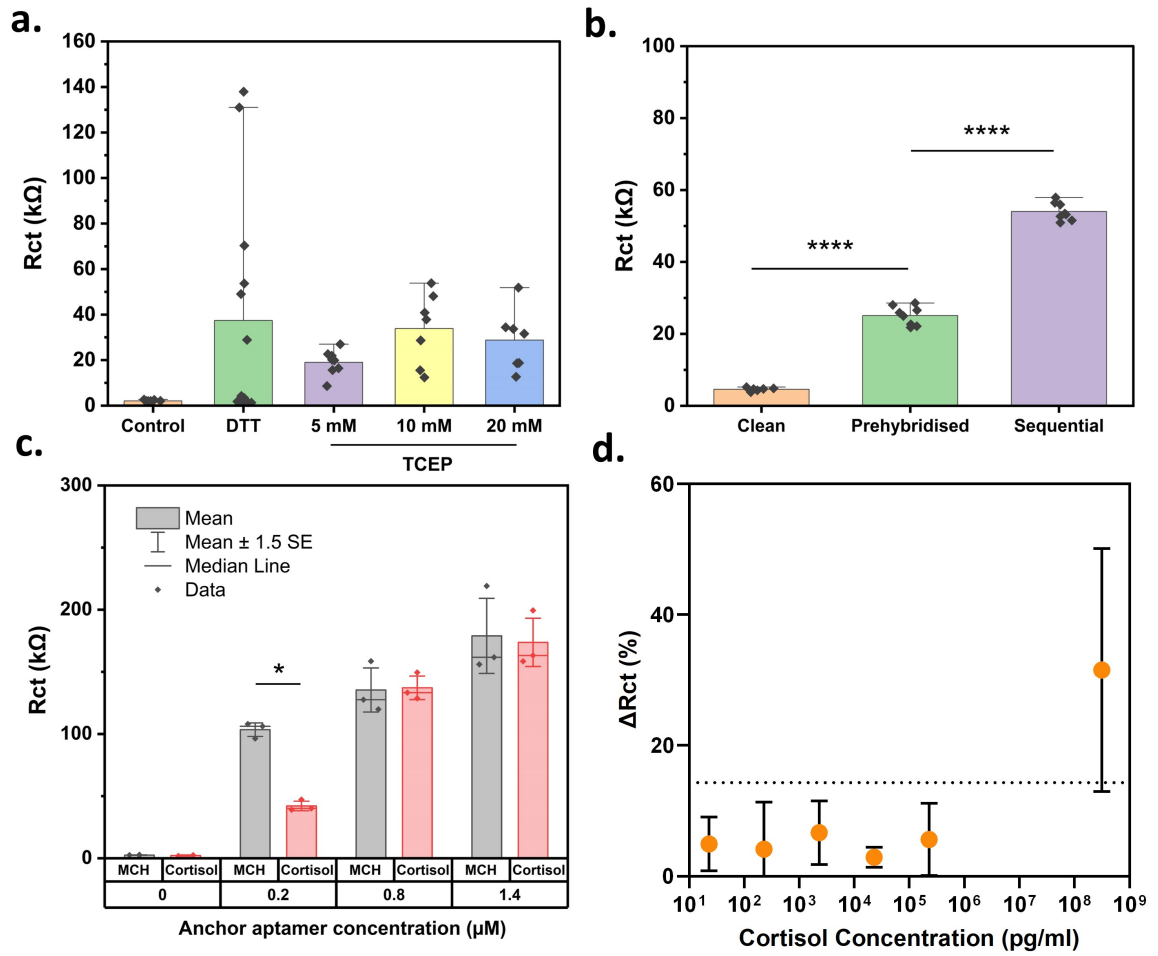


Figure 2.6: Optimisation work on the newer batch of electrodes

(a) Comparison between control protocol (DTT reduction of disulfide bonds as per manufacturer's instructions) and different concentration of TCEP ($n = 8$ working electrodes; each condition represents a different sensor array). (b) The difference between aptamer duplexes sequentially assembled on the detection surface and pre-hybridised in liquid form prior to surface attachment ($n = 8$ working electrodes; each condition represents a different sensor array). (c) The response of different aptamer layers deposited at different concentrations of anchor aptamer (ratio of capture to cortisol aptamer was kept constant at 1:5) to cortisol addition (23 ng/ml) ($n = 3$ working electrodes per aptamer concentration condition). (d) Final dose response of the optimised sensor ($n = 3$ working electrodes per concentration point). * - $p < 0.05$, **** - $p < 0.0001$. Each concentration dot is the average over the 3 measurements, whiskers represent 1 standard deviation and the horizontal dashed line is the average blank $\pm$ 1 standard deviation.

Order of immobilisation

Next, the order of the biosensor assembly steps was varied to study the effect on the SAM. Particularly it was of interest whether prehybridisation of the aptamer strands prior to being attached to the surface as opposed to sequentially assembling them on the surface would be beneficial. For clarity, in the sequential version, the anchor aptamer is incubated on the surface of the electrode overnight and the next day the surface is washed and the cortisol aptamer is attached before backfilling the surface with MCH. Conversely, in the prehybridised workflow, the anchor and cortisol aptamers are incubated together for one hour during the TCEP reduction step and drop cast on the working electrodes and incubated overnight before washing and backfilling the surface the next day. The advantage of a prehybridised system is the elimination of one of the preparation steps and thus one potential source of variability in the sensors. It was found, as displayed in Figure 2.6 b that prehybridisation resulted in a lower aptamer loading density compared to sequential hybridisation on the electrode surface. This effect was also observed with the fluorescein-tagged aptamers (Appendix C - Figure 6). It is likely that this is due to the fact that smaller molecules (the anchor is only 10 kDa, whereas the cortisol aptamer is an additional 15 kDa) pack more tightly on the surface of the electrode. Whereas larger molecules such as the aptamer duplexes may incompletely attach to the surface due to steric interference and be more readily displaced by washing and subsequent backfilling with MCH. Therefore, since the aim was to maximise the aptamer loading density onto the surface of the electrode, the sequential functionalisation method was taken forward. Although it is possible that a more tightly packed SAM in itself can lead to poorer detection by blocking cortisol access due to steric interference, this can be modulated experimentally by decreasing the concentration of aptamers deposited on the surface or by co-incubating the anchor aptamers with MCH to ensure they are more spaced out [197].

Another order that was optimised (Chapter 4) relevant to this discussion, is the backfilling step. Appendix C - Figure 4 shows the effect of backfilling the surface of the electrode as the second step of the functionalisation procedure (after anchor aptamer attachment) or as the third step (after cortisol aptamer attachment and duplex formation).

Interestingly, backfilling after anchor aptamer attachment (same way that it is typically done in immunoassays) leads to a more insulating layer but a poorer discrimination between blank and positive (23 ng/ml) cortisol samples. However, the overall trend follows the behaviour expected from a strand displacement system where the average ΔR_{ct} was lower compared to the blank. Conversely, backfilling after the duplexes have been formed on the detection surface leads to a significant 30% increase in ΔR_{ct} with cortisol addition compared to the blank which is suggestive of a strand retention mechanism, where the conformational change of the aptamer in response to cortisol creates a barrier to electron transfer across the interface. However, given the noise on the blank measurement, this change was not sufficient to breach the 3 standard deviation zone required to determine the limit of detection. One area of future work could be to attempt the co-immobilisation of aptamers with the MCH backfiller, which has previously been demonstrated to be a robust method of controlling the aptamer deposition density [197]. Additionally, while MCH is one of the most commonly used alkanethiols for this application [198], different lengths of alkanethiols are also available (shorter ones such as 3-mercapto-1-propanol or longer such as 11-mercapto-1-undecanol). Longer alkane thiols produce more ordered SAM structures with improved stability and antibiofouling characteristics [198], but they can also produce more insulating layers which hinder electron transfer [201].

Aptamer concentration optimisation

One possible way of modulating the aptamer deposition density on the electrode surface is through varying its deposition concentration. Whereas maximising the aptamer loading is critical to ensuring good detection sensitivity, there is an upper limit beyond which steric effects become dominant and impede target binding and/or aptamer conformational change. The effect of varying the concentration of the anchor aptamers and its effect on detection sensitivity is displayed in Figure 2.6 c. It was possible to clearly discriminate between the different levels of aptamer coverage, mirrored in the increasing charge transfer resistance with increasing aptamer concentration. It was noted that

200 nM concentration was the only condition which resulted in a significant decrease in ΔR_{ct} in response to cortisol addition (50 ng/ml). Thus, this concentration was taken forward. Unfortunately, repeating this condition over a wider concentration range did not produce a distinct dose response with none of the concentration points breaching the 99% confidence zone around the blank reading. It is likely that the poor sensor response was due to the insufficient sensitivity of the transduction platform, which featured an unmodified gold electrode. The effectiveness of the cortisol aptamer at capturing cortisol was validated using it in competition with cortisol antibodies from a standard optical cortisol ELISA kit where standard curves with and without the aptamer present at its working concentration (200 nM) were generated. This result is shown in Appendix B. Cortisol aptamer addition resulted in a net 30% decrease in the observed colorimetric signal produced in the wells which confirmed that the aptamer was binding as expected. In future work, the signal output from this sensor could be improved as discussed in the introduction, through attaching a redox active tag to one of the terminals of the cortisol aptamer and detecting its presence through a technique such as differential pulse voltammetry. Additionally, the active surface area of the electrode could be increased (and thus the current and available surface for aptamer binding increased) as described by Wan and colleagues [202] by electrodepositing gold nanostructures using chronoamperometry - a method successfully used by Raykova et al. for the aptamer-based electrochemical detection of tetracycline residues in cow's milk [203].

2.4 Discussion and conclusion

Cortisol is a key biomarker linked to endometriosis, and in combination with other biomarkers (*e.g.*, IL-6) has the potential to unlock new insights into the pathophysiology and development of this disease, yet no commercially available platform currently exists that can detect both in a cost-effective format. This chapter was focused on the develop-

ment of a low-cost electrochemical biosensor for cortisol based on aptamers.

The cortisol assay was based on an aptamer structure previously reported in literature and demonstrated to perform well in a lateral flow assay and more recently on an electrochemical sensing strategy employing mesoporous silica nanochannel modified electrode [136]. The goal of this work was to explore whether this detection principle could be replicated in a low-cost format using unmodified gold electrodes. While initial data following optimization of the dilution buffer showed promise with a detection limit down to ~ 200 pg/ml, switching to a different electrode batch from the same manufacturer (featuring a different gold material) produced sensors with a much more variable response and poor detection sensitivity. This was followed by a series of experiments aiming to unpick the different contributing factors that may play a role in the performance loss on the new gold substrate. The factors included the reducing agent used for attaching the aptamers to the electrode surface, the sequence of the monolayer assembly (whether the aptamer duplex was prehybridized prior to surface attachment or hybridized on the surface as well as the order of the steps) and the concentration of aptamers used. The results of these experiments in impedance mode were validated using fluorescently tagged aptamers.

It was found that dilution in the original selection buffer with a 0.1% v/v concentration of Tween-20 and using 10 mM TCEP compared to DTT while preparing the aptamer sequences for gold attachment produced the most consistent sensors. Sequential assembly of the duplexes on the electrode surface (as opposed to pre-hybridisation in liquid phase) resulted in the highest and thus most efficient aptamer loading density on the electrode surface. Titrating the anchor aptamer concentration down to 200 nM also produced the most sensitive sensor response to cortisol. Whereas these experiments did not produce a sensor with a well-defined cortisol response in the end, they uncovered some important aspects regarding the integration of this aptamer sequence into electrochemical biosensors based on unmodified gold electrodes. This line of work was then re-opened and tackled from an engineering angle in Chapter 4 using an automated biosensor fabrication platform which was shown to decrease the variability of the sensors and lead to a more pronounced discrimination between positive and negative cortisol spiked samples suggesting the need for automation strategies in these types of assays.

Potential avenues for improvement which could be explored in the future include the

co-incubation of the anchor aptamer or aptamer duplex with the backfilling agent to more precisely control the spacing of the aptamer units on the detection surface [197, 204]. Additionally, different sizes of backfilling agents [205] or intermediate linkers [206] may be trialled. In this case, MCH was used due to its popularity in aptamer-based electrochemical sensing literature and its size relative to the aptamer sequences used, but smaller variants such as mercaptopropionic acid (MCP) could be explored. Another avenue which could be explored is varying the length of the aptamer and thus adjusting the spacing between the aptamer binding site and the electrode surface [207] or potentially adding sequence intercalators [208]. It is likely that a substantial signal boost will be provided by using an internal redox active probe (*e.g.*, ferricyanide) rather than an external one as used in this work, however that was avoided here due to that technique being under patent protection [209].

Integration within fluidic devices has also been previously demonstrated to enhance assay sensitivity [210] and automate the manual steps associated with preparing these sensors in research settings [65] which invariably introduce variability that can negatively impact measurement reliability [211]. This approach was explored in Chapters 3 and 4 as the last tested strategy to improve the sensor response.

Chapter 3

**A plug-and-play,
easy-to-manufacture fluidic accessory
to significantly enhance the
sensitivity of electrochemical
immunoassays**

Highlights

- A flow cell design was created to enhance the sensitivity of electrochemical assays which can be fully assembled and integrated with an electrochemical biosensor in < 1 min.
- The proposed implementation of the flow cell increased the sensitivity of the electrochemical immunosensor for IL-6 by a factor of 7 in neat unprocessed human serum.
- The flow cell highlighted for the first time a spontaneous peroxidase-like reaction taking place at the Ag/AgCl pseudoreference electrode and a protocol was proposed and demonstrated to minimise it.

Published work

Dobrea, A., Hall, N., Milne, S., Corrigan, D. K. & Jimenez, M. A plug-and-play, easy-to-manufacture fluidic accessory to significantly enhance the sensitivity of electrochemical immunoassays. Sci. Rep. 14, 1–11 (2024).

3.1 Prologue

In Chapter 2, different sensor preparation factors were explored to improve cortisol detection using an impedimetric aptamer-based biosensor. Unfortunately, following this optimisation, a convincing and clinically useful dose response could not be produced. This workstream could have been continued down the biochemical and surface chemistry route (in turn, varying all the relevant parameters, adding and removing steps until a meaningful response could be seen), but this would have taken a significant amount of time and increasing the assay complexity would quickly begin negating the cost benefits of an aptamer-based platform. A more straightforward and low-cost approach that has been previously shown to improve the performance of electrochemical assays is their embedding within fluidic systems. This has been demonstrated to simultaneously boost assay sensitivity while remaining selective to the target of interest and improve fabrication variability through the elimination of manual handling steps. To assess if this approach could be beneficial for the cortisol assay, an easy-to-assemble, low-cost flow cell (an enclosed chamber consisting of laser cut PMMA and double sided tape, installed on top of the sensor) was developed and tested with a more well-established electrochemical immunoassay for IL-6 to demonstrate proof-of-concept. Through this work, it was realised that although (micro)fluidics present a suite of practical advantages such as increased sensitivity, low reagent volumes, minimizing contamination, multiplexing, *etc.*, it remains an underutilized resource with less than 6% of the research outputs on electrochemical biosensors featuring a fluidic component (Web of Science, 2020-2025).

Two factors which may contribute to that are 1) the high upfront technological entry barrier (setting up an in-house manufacturing facility, purchasing and commissioning equipment, training users) and 2) the recurring time cost. Because it is very common for electrochemical biosensing labs to manufacture their own biosensors or order custom ones in large quantities, there is a wide range of designs and geometries reported in literature. Additionally, during the initial development stages, these geometries may also undergo rapid prototyping cycles before landing on a suitable design. Given the custom

and rapidly evolving nature of these platforms, there are very few off-the-shelf fluidic options available to be directly interfaced with them, which often forces production to take place in house. Depending on manufacturing process, this can be quite costly and time-consuming (*e.g.*, photolithography) as specialized expertise and equipment is often required *e.g.*, ovens, degassing pumps, oxygen plasma machines. Rapid prototyping processes such as 3D printing offer more flexibility and have a lower entry cost but require post-processing (*e.g.*, ethanol bath, drying) and have low throughput incurring a high recurring time cost.

This recurring time cost consists not only of fluidic system manufacturing but also implementation within biosensing research workflows. Typical electrochemical biosensor preparation and testing from start to finish can range from 3 to 8 hours depending on the detection mechanism employed. To maximize adoption, fluidic integration should incur minimal disruption to the sensor fabrication workflow (*i.e.*, add very little time) and be readily manufactured in bulk quantities, even by non-fluidic experts. For instance, Asri *et al.* presented a manufacturing process for integrated microfluidic and gold leaf sensors using crafting-grade materials and processes such as transparency and heat and bond films, conductive silver pen, a laminator and an electronic craft cutter. Faster and easier manufacturing and prototyping may be realized by using high throughput processes such as laser cutting. While previously confined to specialized manufacturing labs, laser cutters have now become a standard consumer crafting equipment and are widely available through community-based ‘makerspaces’ such as the FabLab network (www.fabfoundation.org). This chapter presents a low-cost, easy-to-implement fluidic device based on consumer grade fabrication processes (laser and electronic craft cutting), shown to produce an up to x7 increase in the detection sensitivity of the IL-6 immunoassay in spiked undiluted human serum.

First, the cleaning protocol of the electrodes had to be re-optimised. Whereas previously in Chapter 2 oxygen plasma could produce highly consistent biosensors with low inter-electrode variability, the new gold substrate seemed to respond much better to voltage cycling in sulfuric acid, with a concentration of 0.1 M being optimal. Secondly, fluidic integration highlighted for the first time, a spontaneous peroxide-like reaction occurring at the Ag/AgCl pseudoelectrode on board the sensor platform affecting sensing

reliability. This was determined to be due to the presence of metallic silver in the Ag/AgCl paste and its effect was minimized through the anodization of the reference electrode in a 1 M NaCl solution for 1 minute at 1 V.

Finally, it was found that by adding the flow cell at the final step of the sensor preparation workflow (enzymatic amplification of the colorimetric substrate), a significant increase in the signal generated by the sensor could be observed due to the confinement of the detection media in the proximity of the electrode surface, where it is more readily available for detection (for further detail on dose response shapes and possible mechanism for this enhancement please see Appendix E). The manufacturing processes presented here is fast (fluidic device assembly and electrode attachment time < 1 min) and highly flexible and thus well suited to be easily adapted to any planar electrode design. Furthermore, the implementation suggestion (*i.e.*, only adding it at the final step of the sensor preparation process) adds minimal disruption to the biosensor fabrication workflow which encourages widespread adoption within the community. The fluidic system assembly principle described here can also represent a building block for more complex fluidic designs. This was demonstrated later in Chapter 4 which describes the development of a fully integrated platform for automating the entire sensor fabrication workflow, tested with both IL-6 and the cortisol assay described in Chapter 2. Additionally, to increase the relevance for clinical use, future work could focus on integrating plasma separation strategies directly on-chip [212] (for instance by embedding a filtration membrane within the flow cell inlet or inside a dedicated filtration chamber) for automated sample processing rather than relying on centrifugation.

This work was published in 2024 in Scientific Reports and has been included in its entirety following this prologue. The supplementary materials can be found in Appendix E of this thesis and at the following URL. The supplementary videos may be accessed at <https://www.nature.com/articles/s41598-024-64852-5Sec26>. My contribution included

designing and manufacturing the flow cells, conceiving and conducting the experiments and analysis, data interpretation and manuscript writing and editing under the guidance of Dr Melanie Jimenez and Prof. Damion Corrigan. Permission to reproduce this work in this thesis has been granted by Springer Nature.

3.2 Introduction

The early detection of biomarkers is instrumental in ensuring the best possible patient outcomes and minimizing the economic burden on healthcare systems through a personalized and preventative approach to disease. One of the gold standard techniques for quantifying biomarker levels in patient samples are enzyme linked immunosorbent assays (ELISAs); ELISAs exploit the high affinity between antibodies and their specific antigens to optically detect a biomolecular target, and are routinely used in care settings to detect a variety of conditions from bacterial [213, 214] or viral infections [215, 216], to autoimmune disorders [217], and cancer [218]. Despite their wide use, the incurred high running costs and instrumentation footprint make standard ELISA approaches poorly suited for deployment at the point-of-care [219].

Electrochemical immunosensors have been long hailed as the potential answer to this problem [220]. These sensors harness the reliability and selectivity of antibodies, small instrumentation size, low sample/reagent volumes, and the ability to be mass produced at low costs. Compared to their optical counterpart, electrochemical biosensors detect the antibody-antigen binding event through changes in the electrical profile of the medium *e.g.*, using amperometry, voltammetry, potentiometry or impedance spectroscopy. Another key strength of this sensing mechanism resides in its multiplexing capabilities [221], which is critical for diseases that have a diverse biomolecular signature, and its strong point-of-care potential has already been demonstrated for *e.g.*, cardiovascular diseases [222] and cancer [43].

Translating ELISAs into an electrochemical format has however not been devoid of challenges. Without proper signal amplification strategies the sensitivity of these sensors has generally been quite low, particularly in complex media such as blood [55]. Consequently, a large body of research has been dedicated to improving the signal output using for instance surface modification with polymeric or metallic nanomaterials to increase the conductive and catalytic activity [62], modulating the orientation of surface immobilized antibodies [54, 55] *e.g.*, through pH, click chemistry or covalent immobiliza-

tion via SAM assembly (EDC/NHS), using nanobodies (shortened version of an antibody only including the active site), nanoparticles (AuNPs, IrOx etc.) and magnetic beads for increased surface area and conductivity [223, 224], or decreasing the electrode size into the micrometer realm [63]. Importantly however, all these modifications exponentially increase the production cost and manufacturing complexity of these sensors. Microfluidic strategies have also been proposed as a low-cost avenue to automate and increase the sensitivity and throughput of these sensors through minimization of environmental contamination, speeding up of reaction times (*e.g.*, through temperature control), decreasing the volume of sample and reagents, and gaining the ability to measure multiple biomarkers simultaneously through spatial separation of the fluid path [65, 66, 118, 225, 226]. Myriad microfluidic devices have been designed and presented mainly using polydimethylsiloxane (PDMS), glass etching, 3D printing, paper-based flow, CNC machining, thin film deposition and digital microfluidics [65, 227–229]. While these manufacturing methods all have their merits and the choice of material and fabrication process is highly dependent on the nature of the final application, they still present some substantial limitations from a user adoption perspective in electrochemical biosensor development. PDMS devices for instance require an involved and somewhat lengthy fabrication process [119] (mold design and production, degassing, curing, mold detachment, etc.), tend to swell over time when exposed to certain reagents and are prone to protein absorption [230]. 3D printed devices produced using stereolithography or fused-deposition techniques require post-treatment and curation to ensure that the photocuring agents do not foul the electrochemical transducer, and the surface finish is sufficiently smooth to not interfere with the flow or create air pockets [231]. Thin film deposition of microfluidic channels, while ideal for a final product, typically rely on high volume production methods and thus have limited prototyping flexibility and high start-up costs – the same applying to CNC machining [232]. Digital microfluidics have high power requirements and need complex engineering to set up, and thus might not be suitable or accessible for everyday research [227].

While the clear benefits of microfluidic integration in electrochemical sensing have been previously demonstrated, microfluidic use in the community remains scarce, with only 4% of research outputs on electrochemical biosensors having a microfluidic element (Web of Science, 2020-2024). To improve user compliance, integration of a microfluidic element should have minimal disruption to the sensor testing workflow and should be easy to manufacture in bulk, even by non-microfluidic experts. Asri et al. [233] recently reported for instance a simple and accessible way to manufacture both microfluidic devices and electrodes for electrochemical biosensors using consumer grade materials (gold leaf, silver ink pen, double sided tape, photocopier transparency film and heat and bond film) and processes (lamination, electronic craft cutting) for glucose sensing. Easier fluidic manufacturing could be achieved by working with existing, commercial electrodes and using consumer grade processes such as laser cutting, which is widely available within community-based ‘makerspaces’ such as the FabLab network (www.fabfoundation.org) [119].

In this study, we explore this avenue and propose a new approach based on PMMA laser cutting for easy-to-manufacture and easy-to-assemble fluidic chambers that can improve the sensitivity of commercial electrodes. This approach is versatile, easily adaptable to different electrode designs, and can improve signals even in the absence of pumps to drive the flow – which can facilitate adoption by non-microfluidic experts. The principle is demonstrated here for an ELISA-based electrochemical detection of IL-6, an inflammatory cytokine produced by the immune system in response to an immunological challenge such as injury or infection. With relevance in a range of conditions such as sepsis, COVID-19, cancer or Alzheimer’s, IL-6 has garnered a high level of interest in the electrochemical biosensor community [69]. Using the proposed fluidic chamber, which is only added during the final step of electrochemical sensing to minimize interference, we demonstrate an increase in sensitivity (defined here as the change in current over the linear portion of the sensing range) of 5.5-fold in buffer, 5.8-fold in 10% v/v serum and 7-fold in neat, freshly extracted human plasma. This work consequently presents a rapid, low-cost and minimally invasive approach to enhance the signal of electrochemical sensors that can

further enable deployment for point-of-care testing. The approach presented is unique in that it was specifically developed to facilitate uptake by the electrochemical biosensor community and we believe it can break down the barriers associated with microfluidic integration in these systems.

3.3 Materials and Methods

Chemicals and electrodes

Sulfuric acid (98%), potassium ferricyanide (99+%, for analysis), potassium ferrocyanide trihydrate (99+%, for analysis), human serum (H4522) and PBS tablets were all purchased from Merck. Ultrapure water (18.2 M Ω .cm) was obtained from an Elga Purelab Chorus water purification system. Primary and secondary IL-6 antibodies, IL-6 standard and x40 horseradish peroxidase concentrate (HRP) were all purchased from R&D Systems Inc (Minneapolis) as part of a Human IL-6 DuoSet ELISA kit (DY206). Reagent diluent x10 (1% BSA in PBS, pH 7.2-7.4, 0.2 μ m filtered, DY008B), TMB substrate (DY999B) and PBS plate-coating buffer (DY006) were also purchased from the same manufacturer. The Au electrodes used in these experiments were manufactured by FlexMedical Solutions (Livingston, UK) and feature 8 working electrodes and a common reference and counter electrode on each chip. The Ag/AgCl (CHI111) and Au (CHI101) macroelectrodes used for the anodization experiments were purchased from IJ Cambria Scientific Ltd. The 0.2 μ m membrane syringe filters were from Whatman (cat. number 6780-2502).

Cleaning experiments

In these series of experiments, two common cleaning methods were compared to determine the optimal protocol for removing contaminants from the Au electrode surface and facilitate sensing: sulfuric acid voltage cycling and oxygen plasma etching. Three different acid strengths were investigated including 0.1, 0.2 and 0.5 M. Before use, the electrodes were rinsed with ultrapure water and dried under stream of argon. Following this, 600 μ l of acid was drop cast onto the electrode platform ensuring that all electrodes were adequately covered. The potential was then scanned on each working electrode between -0.5 and 1.6 V using a scan rate of 0.1 V/s for 10 cycles using a PalmSens4 potentiostat equipped with a MUX-R2 multiplexer unit from PalmSens. The software PStTrace 5.9 was used to control the potentiostat. After cleaning, the electrodes would again be rinsed with ultrapure water and dried with argon making them ready for use. For plasma cleaning, we used a Zepto plasma asher (200 W) connected to an oxygen gas cylinder set to different time and power settings. 3 electrodes were placed inside the asher chamber during the cleaning process. To characterize the electrodes, we used a 2 mM solution of $Fe[CN]_6^{3-} + Fe[CN]_6^{4-}$ (Ferri-Ferro Cyanide) redox couple in 1xPBS (made from tablets) and interrogated the system using cyclic voltammetry, differential pulse voltammetry and impedance spectroscopy. For the cyclic voltammetry measurement, we scanned the potential between -0.4 and 0.5 V in 0.01 V steps using a scan rate of 10 mV/s. The same voltage window, step size and scan rate were used for the DPV characterization. EIS measurements were performed against the open circuit potential of the cell, over the range 0.1 – 100,000 Hz (83 frequencies) with an AC potential of 10 mV. To extract the Rct values, data was fitted in PsTrace 5.9. using Randles equivalent circuit with a constant phase element (CPE) instead of an ideal capacitor (Figure 3.1 a inset) to account for non-ideal sensor behaviour. The Rct values for individual electrodes were averaged and the error was calculated as 1 standard deviation from the mean.

Fabrication and assembly of the flow cell

The flow cell was constructed using 2 distinct layers: a inlet/outlet layer made from 3 mm laser cut acrylic with two holes (1.3 mm diameter) and a channel layer made from double sided tape cut using a Cricut® Maker 3 to delineate a channel exposing all electrodes. The PMMA sheet for the inlet/outlet layer was purchased from Stockline Plastics Ltd (Glasgow, UK) and the 230- μ m thick double sided polyester tape was from RS Components (RS Stock No.: 468-380). The geometry for the inlet/outlet layer was created in Serif DrawPlus X8, which can upload the design directly to the laser cutter. The geometry for the channel layer was created in the Cricut Design Space App which is available with all Cricut cutters. To cut the inlets layer we used an Epilog Mini 50-Watt laser cutter set to 30% speed, 100% power, 5000 Hz frequency. For the channel layer, since the tape was on a roll format, it had to be unraveled and placed on a temporary release silicone film before being placed on a Strong Grip Cricut® cutting mat (12'' x 12''). Masking tape was also added at the edges to ensure that the silicone film remains in place during cutting. The cutting settings used were the standard Cricut® ones for double-sided adhesive which resulted in a clean cut of the double-sided tape. For introducing the reagent into the flow cell, we used mini female luer adaptors from the Microfluidic ChipShop (Fluidic 631) and a pipette to mini luer adaptor from the same supplier (Fluidic 391). The adaptors were attached to the flow cell via adhesive rings cut to size (Fluidic 699).

IL-6 sensor preparation procedure and interrogation

After cleaning with 0.1 M sulfuric acid, some of the silver pseudo reference electrodes were anodized by submerging in a 1 M NaCl solution and applying a 1 V potential for 60 s with a 0.1 s time interval which changed them to a dark brown color. This was to prevent the spontaneous reaction between the Ag/AgCl pseudo reference and the TMB substrate observed during preliminary experiments. To begin the sensor preparation, 2 μ l of 100 μ g/ml primary IL-6 antibody diluted in ELISA plate coating PBS buffer was pipetted on each working electrode. The electrodes were then incubated overnight at 4 °C inside a humidity chamber. The next day, the electrodes were gently washed 3 times with 1 ml of 1xPBS to remove any unbound antibodies. This wash step was repeated after each incubation step. Next, the electrodes were incubated with reagent diluent (1% BSA in PBS) for 1 hour at room temperature to minimize any non-specific interactions with the detection surface. Standard dilutions of the target were then prepared in the medium of interest (PBS, filtered or unfiltered dilute serum and plasma), placed on the individual electrodes and incubated for 1 h at room temperature. IL-6 secondary antibodies were then prepared in 1x reagent diluent at double the concentration stated in the certificate of analysis (in our case this was 100 nM, but this may change between different batches) and incubated for a further hour. HRP was then diluted to 1x in reagent diluent and placed on the electrodes for 20 minutes, washed off and then 400 μ l of TMB substrate was added covering all electrodes (including the counter and the reference) and incubated for 20 more minutes. If the flow cell was used, the release liner would be peeled off the bottom of the channel layer and the flow cell manually installed using the edge of the electrode platform and installation guide lines laser engraved onto the top side of the flow cell to ensure proper alignment (Video S2 - available at <https://www.nature.com/articles/s41598-024-64852-5Sec26>). The flow cell was then filled with 40 μ l of TMB using a pipette and a pipette to mini luer adaptor (Fluidic 391). The last 2 incubations steps were performed in the dark (electrode storage boxes were wrapped in tinfoil). After the final incubation

step, the presence of target was indicated by the production of a blue product (oxidised form of TMB) which was measured using chronoamperometry by applying -0.2 V for 60 seconds with a 0.1 s time interval. The stable value of the current after 60 s was then extracted from PSTrace and plotted in MATLAB R2022a.

Extraction of plasma from blood samples

The study was approved by the ethics committee of the University of Strathclyde (UEC23/05). Whole human blood was acquired from Research Donors (<https://researchdonors.co.uk>) in a 10 ml lavender topped K2EDTA blood collection tube. To extract the plasma, the blood was centrifuged for 5 minutes at 4100 x g and then the supernatant was extracted into a clean 15 ml Eppendorf tube and used the same day.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis

Electron microscope scans were acquired using a Hitachi TM-1000 tabletop microscope and elemental analysis was conducted using an Oxford EDS unit connected to the main SEM unit. The microscope and EDS unit were connected to a laptop with the TM-1000 and SwiftED EDS data acquisition software installed. The acquisition time for the EDS data was 8 seconds and all measurements were carried out with an n=10, taken at different locations along the sample.

Data fitting and statistical analysis

The peaks in the voltammetry data was extracted using the PStTrace 5.9 software and the impedance spectra was modeled using a standard Randles equivalent circuit with a constant phase element instead of an ideal capacitor and fitted in PStTrace using the Levenberg-Marquardt algorithm to extract the equivalent circuit parameters (*e.g.*, Rct). The chronoamperometry dose response curves were fitted using a 4-parameter logic regression model by least square fitting. The limit of detection was determined as the first experimental point outside of the ± 3 standard deviation zone around the blank value (0 pg/ml IL-6). Further details on the means and standard deviations of all blank readings presented in this work can be found in Table S3. Graphics were produced in Origin 2022 graphing software and MATLAB R2022a.

3.4 Results and Discussion

Reliable cleaning procedures of commercial electrodes are critical for reproducible electrochemical sensing

Gold (Au) is an excellent material for electrochemical sensing due to its high stability, chemical inertness and high electrical conductivity which yields highly sensitive sensors with low background noise. As presented in Figure 3.1, commercial electrodes out of the box (OOTB) can however have contaminants on their surface, introduced either during the manufacturing process or during storage and transport, which can lead to a large variability in the electrode's response. To prevent this, cleaning procedures must be deployed to remove these impurities; this is particularly important in sensors employing

non-covalently attached antibodies, where the sensitivity may already be lower due to the non-uniform orientation of the attached antibodies and consequent blocking of some of the active binding sites [54]. Sulfuric acid voltage cycling and plasma etching are two popular non-destructive techniques for electrode cleaning [234–237] that were tested in this work. Both techniques work by removing the top layer of gold and any attached contaminants without substantially altering the surface morphology [238].

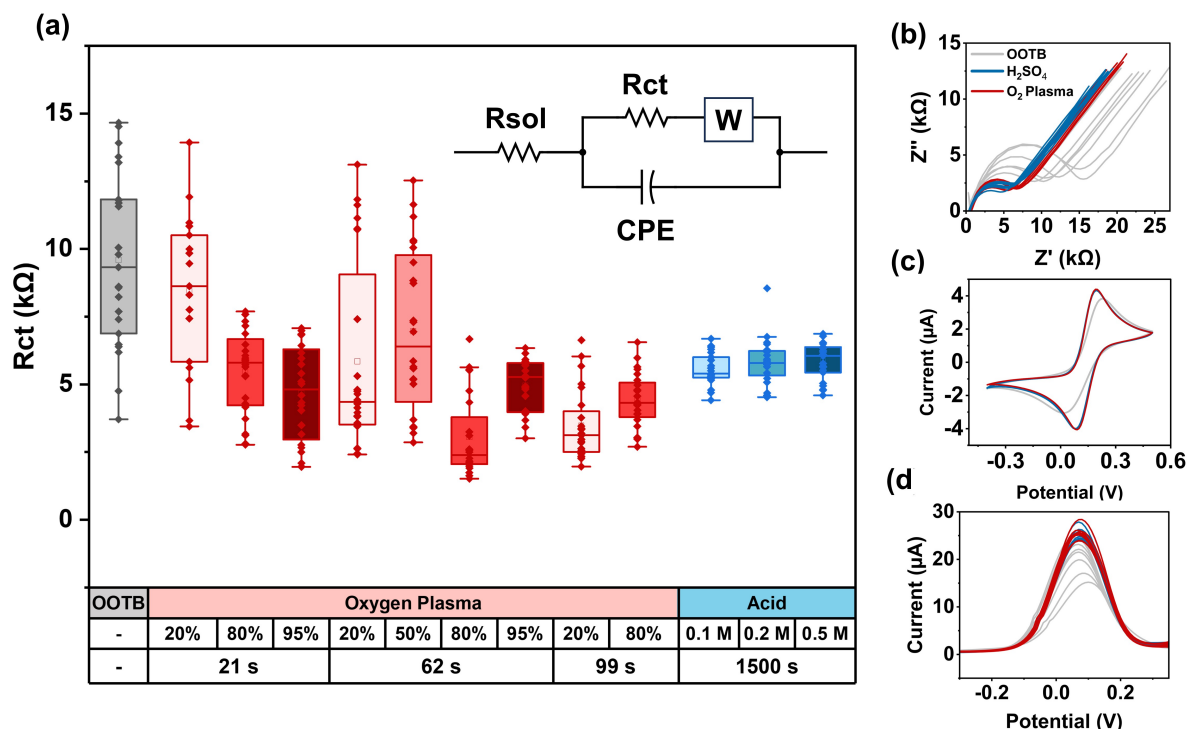


Figure 3.1: Comparison of the efficacy of oxygen plasma and sulfuric acid voltage cycling in removing contaminants from the gold electrode surface

(a) The charge transfer resistance (R_{ct}) of the surface of commercial electrodes is measured through impedance spectroscopy without treatment (out of the box electrodes, OOTB), with plasma treatment at different powers (% of 200 W) and exposure times, and different sulfuric acid molarities. Data was fitted using Randles equivalent circuit (inset) to obtain the R_{ct} values (R_{sol} =solution resistance, CPE=constant phase element, W=Warburg impedance). Datapoints represent different thin-film electrode platforms with 8 working electrodes ($n=24$ except 20% power 21s which had $n=16$). Each box delineates the interquartile range (IQR), the straight line denotes the median, the hollow square represents the mean and whiskers denote the range within $1.5 \times IQR$. (b) Examples of Nyquist plot, (c) Cyclic voltammogram and (d) Differential pulse voltammogram of an electrode without treatment (OOTB, grey) in comparison with one treated with the optimal plasma (95%, 62 s, red) and acid cycling cleaning (0.1 M, blue) protocols identified ($n=8$). The response was measured using 2 mM ferri-ferrocyanide in 1xPBS.

Evaluation of the electrode surface using cyclic voltammetry, differential pulse voltammetry and impedance spectroscopy in a standard redox media ($2 \text{ mM } Fe[CN]_6^{3-} + Fe[CN]_6^{4-}$ in 1xPBS) shows that treatment with both plasma and acid cleaning significantly ($p < 0.01$, Student's t-test) reduced the charge transfer resistance (R_{ct}) from $9593.8 \pm 3189.1 \, \Omega$ to $4961.3 \pm 1025.7 \, \Omega$ (62s 95%) and $5569.7 \pm 599.6 \, \Omega$ (0.1 M) respectively, suggesting improved accessibility of charges to the electrode surface (Figure 3.1 a and b). The peak separation voltage of the cyclic voltammogram (ΔE_p) was also reduced from 201.1 ± 35.0 to 107.9 ± 6.6 and 106.6 ± 6.6 mV respectively (Figure 3.1 c), indicating improved reaction reversibility and electrode cleanliness. In an ideal system, where the reaction is chemically and electrochemically reversible, ΔE_p should be ~ 57 mV (at 25°C) [239]; deviation from this value could be attributed to imperfections in the electrode surface or the presence of trace amounts of contaminants remaining on the surface after cleaning.

Although plasma cleaning is a faster process ($\sim$ minutes to clean multiple electrodes) and is thought to have a more uniform and reproducible effect on the electrode surface compared to liquid solvents [187, 240], acid cleaning yielded lower coefficients of variability (CoVs) compared to any of the plasma settings investigated (Table S1). This could be due to the formation of gold oxides on the topmost layer of gold following O_2 plasma exposure behaving in a contaminant-like fashion [237] or acting as a protective layer which consequently decreased the cleaning efficacy. The best settings identified for plasma in terms of CoV was a 95% power (or 190 W) for 62 s, but this already started damaging the electrode connector pads which began to shrink and fold in, likely because of overheating in the areas where the largest amount of gold was present. In terms of differential pulse voltammetry (DPV) peak currents (I_{peak}) – and as shown in Figure 3.1 d– both plasma and acid treatment substantially reduced the variability of the sensors. Although plasma etching typically produced higher currents compared to acid (Figure S2 - Appendix E), the variability between different sensors was much higher (12.76% compared to 4.96% for acid cleaning). With the aim of minimizing variability between electrodes, acid cleaning was deemed the best approach here. No statistically significant difference was observed in terms of the parameters of interest across the different acid molarities tested for sulfuric acid treatment, thus the lowest molarity (0.1 M) was selected for the rest of the work.

Fluidic chambers can be designed and manufactured for easy integration with commercial electrodes

The benefits of minimizing sample volumes during electrochemical measurements have already been demonstrated [118, 241]. While this is traditionally done via microfluidic integration, most fluidic manufacturing processes are challenging to couple with commercial (plastic) electrodes such as the ones used in this work (Figures 3.2 a and b). In Figure 3.2 c, we introduce the flow cell – a fluidic chamber that consists of only 2 layers: an inlet/outlet layer with 2 holes for the inlet and outlet adaptors made of laser cut 3 mm acrylic, and a channel layer made of 230 μm thick double-sided tape, cut using an electronic cutter (cf Materials and Methods). The two layers are designed so that the working, counter and reference electrodes are all covered, while the layers are easily bondable (via the double side tape) to plastic electrodes. The use of laser and electronic cutting enables rapid alteration of the fluidic design to fit different electrode formats available off-the-shelf. The top layer is designed to work with off the shelf connectors, here pipette to mini-luer adaptors for sample addition. This allows fluid manipulation in a pump-free manner, substantially cutting down the cost and complexity of the system while retaining the advantages of having a microfluidic device (lower reagent volumes, minimal contamination, and reduced evaporation of the liquid). The flow cell can be used in different ways by changing the connectors *e.g.*, it can be connected to a syringe pump for automated sample/reagents injection. The bonding strategy proposed can withstand flow rates of dozens ml/min without leaking, although when working with sensitive reagents such as antibodies, flow rates at the lower end of the working range may be preferable (< 1 ml/min). The total cost for one flow cell is only £2.7, cost that can be further lowered if other fluidic connectors are used (here off-the-shelf connectors are used as they are easily accessible and versatile for non-experts) or directly embedded in the platform design (see Table S2 for a breakdown of the materials cost).

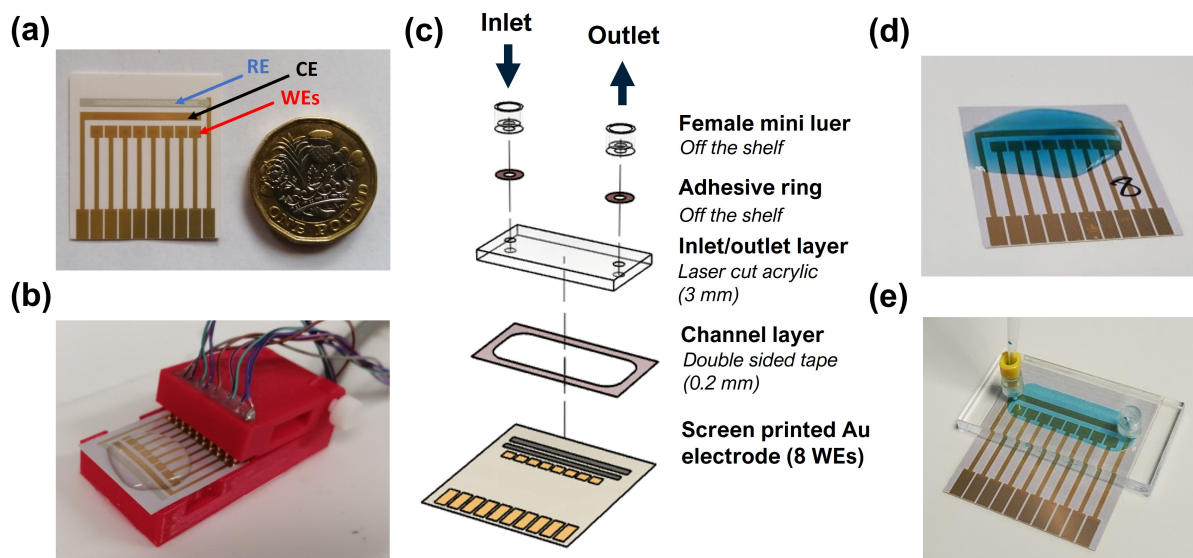


Figure 3.2: Low-cost, easy-to-assemble flow cell developed in this work

(a) The commercial thin-film electrode platform used in these experiments features 8 individual working electrodes (WEs) and a common Ag/AgCl pseudo reference (RE) and Au counter electrode (CE). (b) The typical experimental set-up with the electrode platform placed inside a 3D printed connector made in-house and the reagents drop-cast on top of the electrodes. (c) Proposed flow cell design consisting of a laser cut inlet/outlet layer made from PMMA, channel layer made from double sided polyester tape cut to size using a Cricut® electronic cutter and off-the-shelf mini luer fluidic adaptors attached to the PMMA using circular adhesive rings. (d) Before and (e) after flow cell attachment to the electrode platform.

Without flow cell, the minimum volume needed to cover all the electrodes is 400 μL (Figure 3.2 d); with the flow cell this volume can be reduced to 40 μL , or a 10-fold reduction (Figure 3.2 e). To encourage user adoption, we investigated here addition of the flow cell only at the final step of the preparation procedure, where the enzymatic reaction to detect IL-6 gives rise to a colorimetric signal measurable by chronoamperometry. This decision is motivated by the fact that preparation procedures for electrochemical sensing can be lengthy (typical sandwich ELISA biosensor preparation and measurement protocols can take around 7 hours from start to finish) and addition of the flow cell earlier can be seen as disruptive for established workflows. Once the individual components of the flow cell are fabricated, assembly only takes ~ 1 minute per flow cell (Video S1 - available at <https://www.nature.com/articles/s41598-024-64852-5Sec26>).

Spontaneous reaction between the pseudo-reference and TMB can be observed in micro-environments

3,3',5,5'-tetramethylbenzidine (TMB) is a chromogenic substrate typically used in ELISA assays for signal amplification following binding of the target analyte. In the presence of enzymes such as horseradish peroxidase (HRP) and hydrogen peroxide, the reduced form of TMB (clear) becomes oxidized, which changes its colour to blue. The more target that is bound by the capture antibody, the more enzymes will be present at the end of sensor functionalization process (before addition of TMB), and consequently a stronger color change will be developed in the medium. However, this reaction can in some cases be modulated through the presence of certain catalysts *e.g.*, metallic silver (Ag^+), which can either enhance [242, 243], inhibit [244], or even reverse [245] this reaction in a pH-dependent manner.

During initial experiments, the silver pseudo-reference electrode was observed to spontaneously reduce the TMB substrate, changing its color back to clear, acting in a peroxidase-like fashion (Figure 3.3 a inset). This reaction took place quickly (<1 min), without any external voltage, and the volume affected accounted for approx. 10% of the total volume within the flow cell. Consequently, the amount of oxidized TMB available for detection was decreased, which could lead to an underestimation of target presence. To resolve this issue, 2 possible solutions were investigated: exposure of the Ag/AgCl pseudo-reference to a solution of 10% v/v sodium hypochlorite (NaClO , more commonly known as bleach) and anodization in a 1 M solution of sodium chloride (NaCl) (Figures 3.3 b,c insets). Both methods aim to increase the amount of AgCl (non-reactive) relative to Ag on the surface of the reference electrode through chlorination, which was indeed the case as shown in Figure 3.3 d where the percentage of elemental Cl is substantially increased following both treatments. Anodization appeared to deposit more Cl on the electrode surface and resulted in a more uniform surface coverage compared to bleaching (Figures 3.3 b and c). In fact, with bleaching, the original Ag/AgCl crystals became difficult to distinguish on the scanning electron microscope scan compared to the out of the box (OOTB) condition

(Figure 3.3 a main figure) hinting that this technique may damage the surface of the reference electrode. It was found that, out of all the conditions investigated, anodization was the only modification capable of stopping the pseudo reference interaction, whereas bleaching only delayed it for a short amount of time (~ 5 -10 minutes) – the later could be due to the presence of large gaps present in the surface following treatment leaving a large portion of Ag still exposed and able to react with the substrate. None of the modifications statistically significantly affected the electrochemical charge transfer properties of the system (Figure 3.3 e), but as shown in Figure 3.3 f, anodizing after acid cleaning seemed to alter the variability of the sensors to a lesser extent (compared to doing it the other way around) and therefore this method was taken forward.

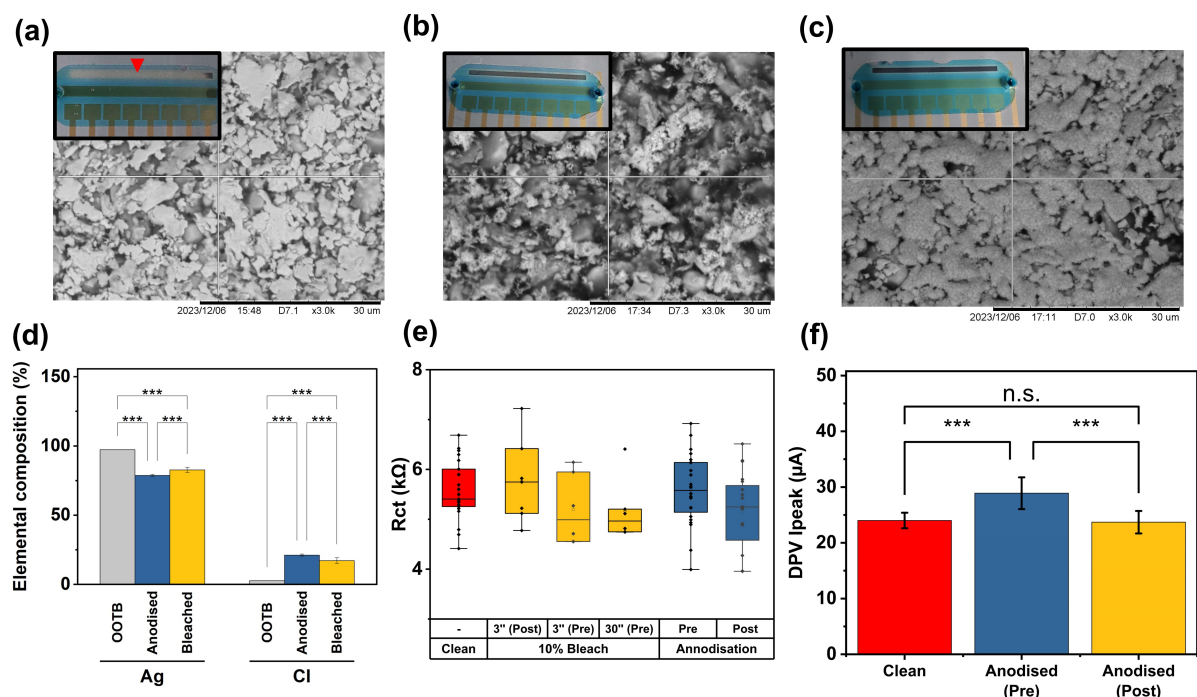


Figure 3.3: Spontaneous reaction between the detection substrate TMB and the reference electrode and protocol developed to minimise it

(a) Spontaneous reaction taking place between the Ag/AgCl pseudo-reference electrode and 3,3',5,5'-tetramethylbenzidine (TMB) locally reducing the oxTMB produced by the HRP-TMB reaction taking place on the detection surface superimposed on SEM scan of the reference electrode surface. (b) The Ag/AgCl reference after being submerged in 10% v/v bleach for 30 minutes exposed to a solution of oxTMB - the cross-reactivity is reduced but not eliminated and the surface appears damaged by the bleach. (c) The Ag/AgCl reference after anodization in 1 M NaCl exposed to a solution of oxTMB – the cross reactivity with TMB is minimized and the integrity of the Ag crystals appears to be maintained. (d) Elemental composition analysis of Ag/AgCl reference electrode surface and the impact of anodization and bleaching treatments by energy dispersive X-ray Spectroscopy (bars and SD are based on 10 different measurements taken in different locations across the reference electrode surface). (e) The effect of bleaching and anodization treatment and interplay with sulfuric acid cleaning (pre or post Ag/AgCl treatment) on the charge transfer resistance of the Au surface. Each box delineates the interquartile range (IQR), the straight line denotes the median, the hollow square represents the mean and whiskers denote the range within 1.5xIQR. (f) The difference between the peak current of the differential pulse voltammogram when the Ag/AgCl electrode is anodized before (pre) as opposed to after (post) sulfuric acid cleaning highlighting that for best results cleaning must be carried out before anodization. Statistical significance is indicated by * where *** denotes $p \leq 0.001$. n.s. = non-significant.

The flow cell significantly enhances electrochemical detection of IL-6 in samples

Next, we isolated the effects of anodizing the silver pseudo-reference and adding the flow cell at the end of the sensor preparation procedure on the performance of the IL-6 electrochemical sandwich immunosensor in a non-complex sample (PBS buffer). It was noticed that when the substrate was drop cast onto the electrodes, anodizing the silver electrode shifted the current dose response curve downwards by around 19.7 nA (Figure 3.4 a). This can be attributed to a potential shift of 50 mV in the TMB oxidation peak registered by the electrode (Figure S4 - Appendix E), which was not corrected in the step voltage applied during the chronoamperometry measurement. Since the overall dose-response in the case when the substrate was dropcast onto the electrode (as opposed to being introduced to the sensing surface via the flow cell) seemed to be unaffected by anodisation except for the 19.7 nA current offset, for further experiments this modification was only applied when working with the flow cell.

Adding the flow cell not only lowered the limit of detection (LoD) from 1875 pg/ml to 468 pg/ml (Figure 3.4 b), but it also improved the sensitivity of the sensor around 5.5-fold over the higher concentration range (468-3750 pg/ml). This increase in sensitivity is also evident in the time dependent current transients showing a much wider response range compared to before (Figure 3.4 c). This boost in performance was attributed to the spatial confinement of the oxidized TMB in the region directly above the detection surface, which limits diffusion of electroactive species into the bulk solution where they are harder to detect.

A dramatic increase in sensitivity (x5.8) was noted with the flow cell for IL-6 spiked in 10% human serum (0.2 μ m filtered – Figure 3.5 a). An additional control experiment was also carried out at confirming the importance of the primary antibody within the detection scheme and assay specificity to IL-6 (Figure S5 - Appendix E). However, in our previous work we have demonstrated more extensively the specificity and selectivity of these reagents for their target and their suitability for this type of application [105, 111].

Unprocessed human serum and neat human plasma (Figures 3.5 b and c) had a very poor response when the flow cell was not present, with only high concentrations (>3 ng/ml) eliciting a response. Upon addition of the flow cell, the LoD was considerably lowered, and the dose-dependent curve could be clearly distinguished. The LoDs of the biosensor in different media before and after the addition of the flow cell are summarized in Table 3.1 below. The dose-response behaviors of the sensor with the flow cell are also compared in Figure S6 - Appendix E. The lowest limit of detection was achieved when testing the sensor in neat human plasma, whereas the highest sensitivity over the higher working range (>1 ng/ml) was in PBS and filtered dilute serum. This improvement in the LoD in the most complex samples tested could be attributed to the presence of molecular factors that are critical to antibody function and stability (e.g. albumins, protease inhibitors) [246] that are absent in ‘cleaner’ media such as PBS. This result also showcases that the additional filtration step may actually remove some of these critical compounds leading to a slightly higher LoD. However, this mechanism may vary depending on the antibody-target combination. In future work, we plan on exploring the effect of the flow cell on different assays to further elucidate this. Another interesting effect was noted when comparing the behavior of the sensors modified with the flow cell over the ultrahigh working range with and without anodisation of the reference electrode – Figure S7 - Appendix E. It was hypothesised that more substantial effects will likely take place at these higher concentrations as more of the TMB will become oxidised and consequently diffuse towards the reference electrode. What we saw is that for non-anodised electrodes, a Hook effect appears to be taking place around 10 ng/ml whereas that appears to be damped in the anodized configuration.

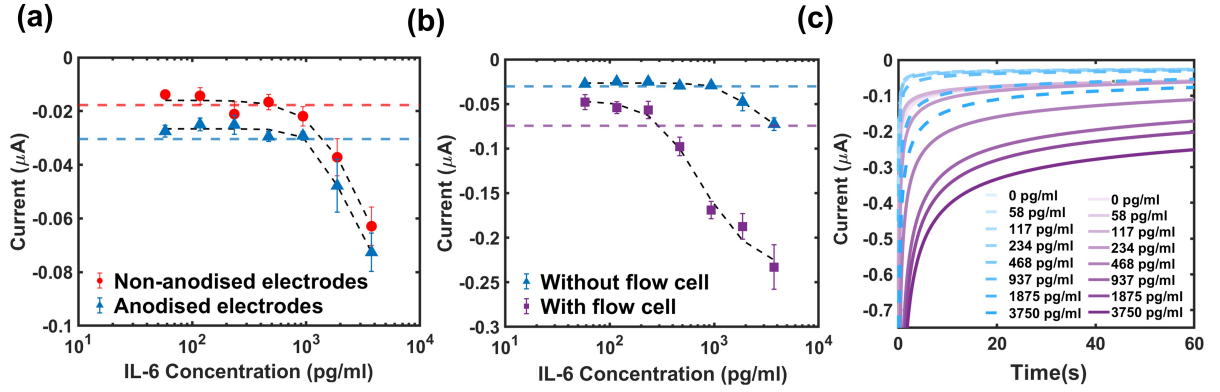


Figure 3.4: Dose response of electrochemical IL-6 immunosensor showcasing the effect of annodisation of the Ag/AgCl reference electrode and adding the flow cell at the final step of the sensor preparation procedure

(a) The difference between the dose-dependent responses of the electrochemical immunosensor to IL-6 spiked in PBS for unmodified (red) and anodized (blue) electrodes during fully manual preparation (no flow cell present). Top (red) and bottom (blue) dashed lines represent the average blank reading $\pm$ 3 SDs for unmodified ($n=8$) and anodized electrodes respectively ($n=6$). (b) The effect of adding the flow cell at the final step of the sensor preparation procedure compared to fully manual preparation and testing (IL-6 spiked in PBS). Both responses are with anodized electrodes. Each marker in (a) and (b) represents the average over all measurements $\pm$ 1 SD and the top (blue) and bottom (purple) dashed lines represent the average blank reading $\pm$ 3 SDs for the ‘without’ and ‘with flow cell’ conditions respectively ($n=6$). Further details on the blank means and $\pm$ 3 SD interval can be found in Table S3. (c) Dose-depended transient current response to different concentrations of IL-6 spiked in PBS without (dashed, blue lines) and with the flow cell (purple, solid lines) showing increase in sensitivity upon addition of the flow cell (single measurement per line). Both datasets were acquired with anodized electrodes.

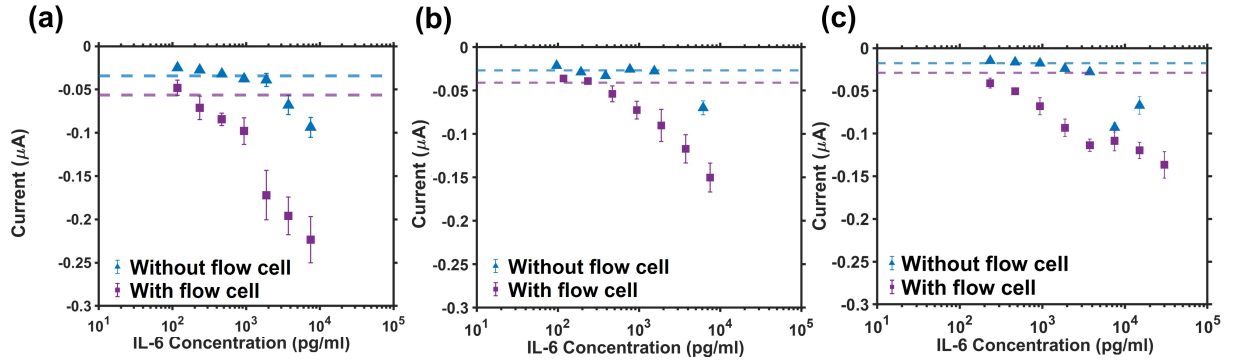


Figure 3.5: The effect of flow cell addition on the electrochemical IL-6 immunosensor challenged in 10% filtered and unfiltered serum and neat, unprocessed human serum

(a) Dose-dependent response of the electrochemical immunosensor to IL-6 spiked in 0.2 μm filtered human serum (10% v/v) with and without the flow cell present at the final detection step showing a large increase in sensitivity following the addition of the flow cell. (b) Dose-dependent response of the electrochemical immunosensor to IL-6 spiked in 10% unfiltered human serum with and without the flow cell present at the final detection step showing a substantial increase in sensitivity and the limit of detection following the addition of the flow cell. (c) Dose-dependent response of the electrochemical immunosensor to IL-6 spiked in neat unprocessed human plasma before and after integrating the flow cell into the preparation protocol. Each marker represents the average over 8 measurements $\pm$ 1 SD and the horizontal dashed lines represent the average blank reading (0 pg/ml) $\pm$ 3 SDs ($n=8$). Further details on the blank means and $\pm$ 3 SD interval can be found in Table S3.

Sample tested	LoD (pg/ml)	
	With flow cell	Without flow cell
PBS	468	937
10% (v/v) Human Serum (0.2 μm filtered)	234	468
10% (v/v) Human Serum (un-filtered)	468	3080
Neat Human Plasma	<234	1875

Table 3.1: Limits of detection (defined as the first data point falling outside of the 99% confidence interval around the blank reading) before and after flow cell addition - a comparison between different sample types.

3.5 Conclusion

This paper presents a low-cost flow cell design that can be easily fabricated using consumer grade materials (PMMA sheets, double sided adhesives, and off-the-shelf fluidic adaptors) and methods (laser and electronic cutting). The fabrication method proposed is accessible to researchers from a non-microfluidic or engineering background, allows for rapid and accurate prototyping cycles and, once a suitable geometry has been identified, the production of large numbers of fluidic cells in a short time frame which can be stored until ready for use. We validated this flow cell using a commercial gold electrode platform and a sandwich electrochemical immunoassay format for detecting IL-6, an inflammatory cytokine of interest across a variety of different pathologies. First, we determined the optimal electrode cleaning protocol to ensure that the Au detection surface was devoid of contaminants that could interfere with the electron transfer processes. We found that electrochemical voltage cycling in 0.1 M sulfuric acid for 10 cycles yielded sensors with substantially lower overall variability compared to O_2 plasma etching. Once we started using the flow cell with the immunoassay detection format, we observed a cross-reactivity between the Ag/AgCl pseudo reference and the ELISA substrate (TMB), which was decreasing the amount of oxidized TMB produced by the target-antibody immunocomplexes immobilized on the electrode surface. This effect was, in the absence of the flow cell, impossible to discern visually through the large volume of bulk fluid that is needed to cover all electrodes during testing. With the aid of the flow cell, we also found that this effect could be eliminated with minimal impact to the sensing quality by anodizing the reference electrode in a 1 M NaCl solution for 1 min at 1 V DC.

Finally, we tested the sensor in 4 different types of media: PBS, 10% v/v 0.2 micro filtered human serum, unfiltered 10% v/v human serum and neat human plasma. To facilitate user adoption and minimize disruption to already established workflows, the potential of the flow cell is demonstrated here at the final step of the electrochemical immunosensor preparation protocol, when the signal amplification reaction takes place. We showed that by confining the product of the enzymatic reaction to the vicinity of the

detection surface, a substantial increase in the sensitivity of the sensor and decrease in the limit of detection could be obtained in both spiked buffer and complex media such as human plasma. We envision that in the future, the flow cell will become an invaluable accessory in the electrochemical biosensor researcher's toolkit, allowing faster and more sensitive sensors to be developed at a low price point and eventually facilitate translation of these sensors into the clinical environment.

**WAFFLE - An automated platform
for enhancing the performance of
electrochemical biosensors**

Highlights

- A novel integrated platform for standardising the fabrication of electrochemical biosensors using the flow cell (termed the WAFFLE) was created and tested with the IL-6 and cortisol assays discussed in earlier chapters of this thesis.
- The WAFFLE was demonstrated to increase the antibody loading density for the IL-6 sensor which translated into a 4-fold increase in the detection sensitivity of this sensor in spiked undiluted human serum.
- The WAFFLE also significantly decreased the variability of the cortisol aptamer-based sensors and produced a clearer discrimination between positive and negative spiked samples.
- A new method for impedance data fitting based on the Markov chain Monte Carlo method was introduced and shown to improve the robustness of equivalent circuit data fitting and resolve the issue of local minima trapping.

Published work

Dobrea, A., Blake, R., Macdonald, D., McKenzie, C., Corrigan, D. K. & Jimenez, M. WAFFLE – an automated platform for enhancing the performance of electrochemical biosensors. *Lab Chip* 2026; 26 (6): 2035–2046.

4.1 Prologue

Although they hold promise in decreasing the costs and time associated with biomarker investigations, electrochemical biosensor for targets beyond blood glucose have not yet realized their full translational potential [60, 61]. One of the key contributing factors is their high variability which may be linked to a lack of automation and standardization tools available at research level.

This was a theme that was encountered throughout Chapters 2 and 3 of this thesis. For instance, Chapter 2 reported for the first time the use of the cortisol aptamer CSS.1 within an unmodified gold biosensor operating in impedance mode. However, towards the end of that work, the signal originating from aptamer target binding was difficult to isolate from the background noise, which was attributed primarily to a change in the underlying gold material on the supplier’s side. However, a significant contribution to that noise likely originates in the manual preparation of these sensors, which typically at research level involves drop casting reagent droplets onto the working electrodes followed by pipette washing, an inherently variable multi-step process exposing the sensor to evaporation, contamination and interoperator variability. This was also further emphasized by the addition of the flow cell at the final step of the sensor functionalization procedure (Chapter 3). While it provided a significant boost in assay sensitivity, the flow cell also acted akin to a magnifying glass, emphasizing the underlying variability between individual sensors prepared manually.

Furthermore, the lack of standardization also expands beyond the preparation of these sensors in the laboratory. Electrochemical impedance spectroscopy is a technique frequently utilized during sensor development for studying the assembly of detection surface monolayers [247–249] and biomarker quantification [114]. This technique is discussed in detail in Section 1.5.1 but typically involves the measurement of an impedance spectra of the biosensor, which is subsequently modelled and interpreted using electrical circuit analogues to understand the difference impedance contributors (*e.g.*, species diffusion, bioreceptor assembly on the detection surface) – essentially treating the biosensor as an

electrical ‘black box’. A very common approach used within literature for fitting electrical models to impedance data is by relying on proprietary software *e.g.*, PSTrace, ZView, Nova with impedance fitting functions. However, a common limitation of those is the tendency of the solver to become trapped in local minima due to sensitivity to the initialization parameters and thus land on solutions that may not represent the global optimum of the parameter space.

In this thesis Chapter, these challenges are tackled by introducing two new tools for electrochemical biosensing research.

First, the Wee Ally for Flow Functionalisation of Low-cost Electrodes (WAFFLE) is introduced - a fully automated platform designed for standardizing the preparation of electrochemical biosensors. The WAFFLE platform revolves around a fluidic reagent cartridge manufactured using a similar methodology as reported in Chapter 3, interfaced with normally closed memory-shaped alloy microvalves by memetis. The flow within the cartridge is actuated through a PID control system operating a mp-6 micropump by Bartels mikrotechnik. The action of the valves and micropump are controlled from a custom printed circuit board revolving around an ESP32 microcontroller. A graphical user interface was also created for remote device operation and can be accessed from any WiFi enabled device by simply inputting the microcontroller IP address into the preferred web browser. In this Chapter, it is demonstrated that WAFFLE preparation can lead to a 4-fold increase in the detection sensitivity of the electrochemical IL-6 immunosensor (first introduced in Chapter 3) in neat unprocessed human serum. This was attributed to an increase in the antibody loading density on the electrode surface which is likely due to the confinement effect observed in microfluidic channels [210] - please see Appendix F for further explanation. Although the preparation of these sensors was conducted under stop flow conditions (*i.e.*, reagents were flown into the electrode chamber, then flow was stopped during the duration of the incubation) it is likely that the initial flow used was sufficient to improve the rate of antibody-target binding. It is also equally possible that continuous flow conditions may further improve the sensitivity of this assay although this has not been tested in this work [210].

The cortisol aptamer sensor reported in Chapter 2 was also trialed with the WAFFLE platform and a substantial decrease in the variability of the sensors was observed, along-

side an increase in the discrimination between negative and positive spiked samples. Interestingly, unlike the case of the IL-6 antibodies, WAFFLE preparation led to a lower magnitude of the charge transfer resistance value of the sensors suggesting lower aptamer loading onto the detection platform. Given the improved target response, it is possible that the higher R_{ct} for the manually prepared sensors was due to non-specific contamination of the surface introduced during handling of the electrode, a contribution which was minimized in the WAFFLE implementation (as the detection medium and electrodes were enclosed and thus shielded from environmental contamination). While the benefits of the WAFFLE for biosensor preparation have been clearly shown, the novelty of this platform also consists in its use to automate the full assay preparation, whereas previous reports typically conduct some of the preparation steps manually by dipping or pipetting onto the electrodes before integration within fluidics. It is hoped that the WAFFLE will provide a new avenue for standardizing electrochemical biosensor fabrication and help accelerate their commercial deployment. Its flexibility is one of its key strengths – the design of the fluidic cartridge may be easily modified and quickly prototyped to fit any off-the-shelf or custom planar electrode design.

The second angle tackled in this Chapter was on the data processing side. As demonstrated in this Chapter, common tools used for fitting and interpreting impedance data are prone to becoming stuck in local optimization minima and thus landing on a sub-optimal solution. This issue may however be corrected by integrating the Markov chain Monte Carlo algorithm into the equivalent circuit fitting scheme. Since typical impedance fitting software is proprietary, this was achieved by modifying an open-source impedance fitting code – Zfit by Jean Luc Dellis [99]. In the final results section of this Chapter, it is demonstrated that this modification can provide a more consistent estimation of key circuit parameters for a variety of different circuit models and impedance spectra behaviours. The code for running the fitting was uploaded on GitHub and can be further adapted for wider use within the electrochemical sensing community.

While there was not sufficient time left in the project to explore their diagnostic po-

tential with samples from endometriosis patients, it is hoped that these two innovations, the WAFFLE and the new approach for impedance data fitting, will provide a new framework for increasing the reproducibility and robustness of electrochemical biosensors, and accelerate further research on these systems for endometriosis diagnostics and beyond.

This work has been published in RSC Lab on a Chip in 2026 and has been included in its entirety following this prologue. The supplementary information for this article can be found in Appendix G. My contribution included designing, manufacturing and validating the WAFFLE, conceptualisation, data curation, formal analysis, investigation, methodology, software, validation, writing and editing the original draft under the guidance of Dr Melanie Jimenez and Prof. Damion Corrigan. Permission to reproduce this work in this thesis has been granted by The Royal Society of Chemistry.

4.2 Introduction

Electrochemical biosensors are powerful tools that have had a profound impact on medical diagnostics and healthcare, particularly in diabetes care. With their commercial potential and scalability, there has been substantial interest in developing these sensors for a wider range of point-of-care applications, aiming to complement or replace more time consuming and expensive techniques such as ELISA or mass spectroscopy [250–254]. However, their wider adoption beyond glucose monitoring is still untapped – with challenges remaining pertaining to sensor stability, assay multiplexing, reproducible formation of homogeneous and well-defined surface assembled monolayers (SAMs) [255–257], and detection sensitivity in complex media (*e.g.*, blood) [60].

Microfluidics has long been identified as an ideal candidate to solve some of these challenges, offering clear signal enhancement and automation benefits [65, 66, 258]. Systems based on digital microfluidics [144, 259, 260], droplet microfluidics [149, 261], paper-based [262–264] or capillary-driven devices [265] for instance have all been proposed. Importantly however, most systems use microfluidics to enhance sample processing and/or automate some, but not all the biosensor preparation steps, therefore not fully harnessing their potential for full assay automation, a factor critical towards in-field deployment. In our previous work, we confirmed that fluidic confinement can improve the detection of a target molecule (~ 7 -fold improvement in human serum)[183], but fluidic integration can also enhance the sensor preparation reliability [211].

Electrochemical immunosensor preparation depends on the assay layout but typically comprises a series of steps for 1) Attachment of capture antibody to the electrode surface *e.g.*, through spontaneous absorption or chemical modification, 2) Backfilling the surface to prevent non-specific interactions, 3) Sample addition, 4) Detection antibody attachment which may be conjugated with an enzyme *e.g.*, horseradish peroxidase or alkaline phosphatase and 5) Substrate addition which is converted by the enzyme into a detectable signal (Figure 4.1). These steps are commonly performed manually, generating an inherent sensor-to-sensor variability [211]. To explore automated sensor preparation

(and thus minimise this variability) requires precise multiple-steps reagents control; this often relies in the literature on syringe pumps [266–269], pressure controllers [270, 271] and large multi-way valving systems [271–273], all providing excellent accuracy but often sacrificing on device size and/or reagent volumes. Some papers have targeted this issue, providing solutions where multiple fluid routing is possible with a small device footprint and low reagent volumes. For instance, Zhao *et al.* demonstrated a plug-and-play fluidic system that can be interfaced directly with off-the-shelf electrodes (eSIREN), for the DNA-based detection of SARS-CoV-2 from swab lysates. Their system uses a small off-the-shelf pressure pump and a solenoid valve manifold controlled via a smartphone [152]. In a similar vein, Uludag and colleagues presented an integrated system for reagent dispensing over an electrode made in-house, demonstrating feasibility for E.coli [155] and prostate specific antigen [153] detection. However, for both platforms, only the final steps of the functionalisation procedure are performed using fluidics (steps 2-8 or 5-8 in Figure 4.1). That is also the case for Dulay *et al.* which created an automated platform for electrochemical sensor preparation using multiple reagents reservoirs (each with their own in-line peristaltic pump) [156] and Kadimisetty *et al.* who produced an impressive platform based on a 3D printed fluidic chip with micropumps [157] (steps 5-8 in Figure 4.1).

In this work, we propose a system that can perform the full electrochemical sensor preparation pipeline automatically, along with readout. The system, named WAFFLE (Wee Ally for Flow Functionalisation of Low-cost Electrodes), has been designed to accommodate a variety of functionalisation procedures and electrode designs, relies on accessible fabrication methods and fully open-source documentation. The WAFFLE can be controlled by any Wi-Fi enabled device via its bespoke GUI, and has been optimised for miniaturisation, low power consumption, and low dead volumes. The robustness of our approach not only relies on the hardware; we also developed a new signal processing pipeline relying on the Markov chain Monte Carlo algorithm to significantly improve the readout of the sensor. We demonstrate the advantages of the WAFFLE using 3 model assays: 2 electrochemical immunoassays for IL-6 and cardiac troponin respectively, and one impedimetric aptamer-based sensor for cortisol. For all assays, a significant decrease in the coefficient of variation and increase in the detection sensitivity is reported thus

confirming the potential of the approach. The work provides a new avenue for biosensor fabrication standardisation that will ultimately facilitate technology translation.

We strongly believe that the WAFFLE, alongside this new approach to data fitting will become indispensable parts of the electrochemical biosensor researcher's toolkit.

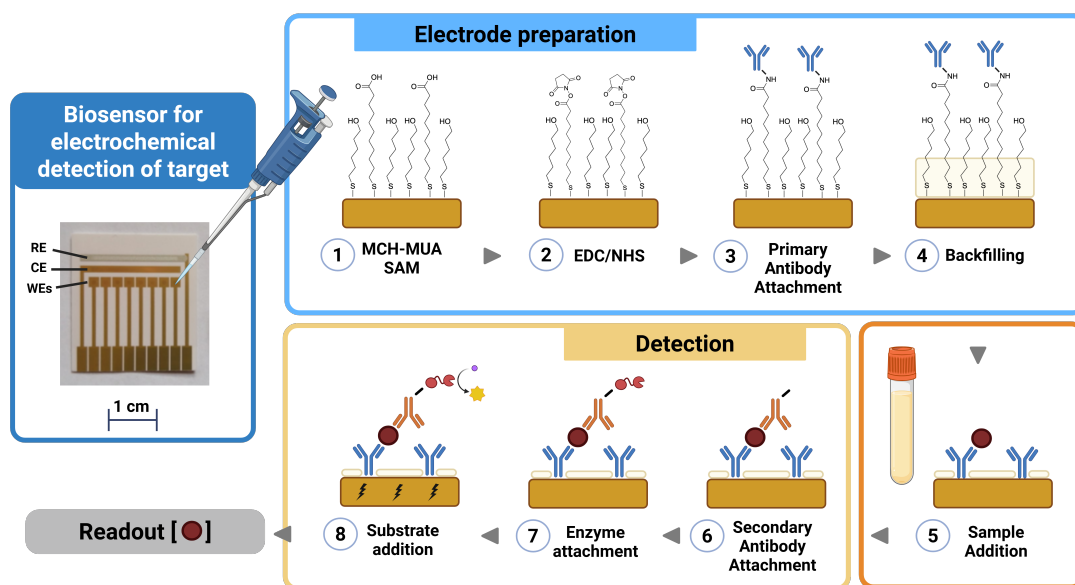


Figure 4.1: Typical steps involved in preparing a sandwich-type electrochemical immunoassay using the standard N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide/N-Hydroxysuccinimide chemisorption mechanism

The electrochemical biosensor used in this work with the Ag/AgCl pseudoreference (RE), gold counter (CE) and 8 working electrodes (WE) is shown on the left. The preparation of these sensors involves the deposition of a thiol layer, in the case of this work a mixture of 11-Mercaptoundecanoic acid (MUA) and 6-Mercapto-1-hexanol (MCH), onto which the capture antibody is attached via carbodiimide chemistry. The surface is then backfilled to prevent non-specific binding before introducing the sample (*e.g.*, human serum) containing the target of interest which is captured by the antibodies present on the surface. A biotinylated secondary (or detection) antibody is then added which forms an immunocomplex with the captured target and primary antibody. An enzyme (*e.g.*, horseradish peroxidase) is then attached to the secondary antibody which in the presence of a chromogenic substrate (*e.g.*, 3,3',5,5'-Tetramethylbenzidine) gives rise to a coloured product which can be directly detected at the electrode surface. These steps are typically performed manually using pipettes or by submerging the electrodes in the required reagents.

4.3 Materials and Methods

Chemicals and materials

Sulfuric acid (98%), N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide (EDC), sodium chloride (NaCl), magnesium chloride (MgCl), phosphate buffer (PBS) tablets, 6-Mercapto-1-hexanol (MCH), 11-mercaptoundecanoic acid (MUA), fluorescein, potassium ferricyanide (99%), potassium ferrocyanide trihydrate (99%), Tris(2-carboxyethyl)phosphine hydrochloride, hydrocortisone, 0.2 μ m Whatman filters and HPLC-purified aptamer sequences [134] (Table S1) were purchased from Merck. Gibco™ PBS, 0.5 M MES buffer (pH 5.5) and molecular grade water were purchased from Fisher Scientific. The ultrapure water (18.2 M Ω .cm) used in the experiments was from an Elga Purelab Chorus water purification system. IL-6 primary and secondary antibodies, target protein and streptavidin-linked horseradish peroxidase were part of the Human IL-6 DuoSet ELISA kit (DY206) and were purchased together with the reagent diluent (1% w/v BSA in PBS), pre-made TMB substrate with hydrogen peroxide (DY999B) and plate coating buffer (DY006) from Biotechne R&D Systems. Troponin primary (19C7) and HRP-labelled secondary antibodies (4C2) were purchased from Novusbio and Biotechne respectively. Recombinant human troponin I standards (8ICR2) and troponin-depleted human serum (8TFS2) were purchased from Hytest.

Electrode platform

The gold (Au) sensors used in this work are from FlexMedical Solutions (Livingston, UK) and they each contain 8 working electrodes and 1 common Au counter and silver/silver chloride (Ag/AgCl) reference electrode per chip (Figure 4.1).

Fluidics

Mini luer fluidic connectors (male and female), silicone tubing, 0.2 and 4.5 ml reservoirs, luer to mini luer fluidic adaptors were purchased from microfluidic ChipShop (Jena, Germany). Mp-6 micropumps, mp-damper, Takasago valves and the mp-lowdriver chip were purchased from Bartels Mikrotechnik (Dortmund, Germany), the normally closed memory shape alloy valves were from memetis GmbH (Karlsruhe, Germany) and the flow sensor from Sensirion AG (Switzerland). The mp-valveDriverM and mp-valveDriverT were purchased from Darwin microfluidics. 3 mm thick PMMA sheets were purchased from Stockline Plastics Ltd (Glasgow, UK), 0.1 mm thick PET spacer layers from ZHluja (Amazon UPC 766972224591) with adhesive transfer tape (3M™ 468MP). PMMA sheets are laser cut to the correct dimensions using an Epilog Mini 50 W laser cutter while PET sheets are cut using a Cricut Maker 3.

Instrumentation

Multiplexing for the valves is achieved using a TPIC6C595 shift register (Texas Instruments) with an SQ2364EES-T1 MOSFET (Vishay Intertech) on each valve control line acting as an electronic switch. The 3 drivers and flow sensor are controlled by an ESP32-S2-Mini development board (Espressif Systems) via the I2C communication protocol, while the shift register is controlled via the SPI interface. The graphical user interface takes the form of a website and is created using HTML. The Arduino PID control library by br3ttb (<https://github.com/br3ttb>) was used in the code to automatically adjust the voltage amplitude of the pump to return the desired flow rate. To minimise

the number of wires and size of the controller, the electrical circuit was converted into a PCBA format using the KiCad software and ordered from JLCPCB (Hong Kong, China). All the components were installed in a plastic enclosure designed in Autodesk Fusion360 and 3D printed using a Creality Ender 3 v3 ke printer in white hyper PLA filament.

WAFFLE assembly

The full protocol to build the WAFFLE step-by-step and all the design files have been uploaded to the following repository: <https://github.com/ADobrea797/WAFFLE>.

Flow characterisation

To characterise the flow rate generated by the mp-6 micropump, flow sensor data was displayed in real time in the ArduinoIDE serial monitor and automatically captured in an Excel worksheet using the Parallax Data Acquisition (PLX-DAQ) tool. The flowrate was characterised across all the inlets in triplicate. To assess washing efficiency, the system was filed with either concentrated fluorescent dye or 2 mM ferri-ferrocyanide solution and then flushed with clean filtered 1xPBS. The decrease in fluorescent signal intensity over time at varying flow rates was monitored via video recording using a Dinolite tabletop digital microscope, with images analysed using ImageJ. For the ferri-ferrocyanide solution washout, the decrease in the peak current of the differential pulse voltammogram data over time at varying flow rates was recorded using a PalmSens4 potentiostat and laptop running the PSTrace 5.10 software.

Electrochemical sensor preparation

Before use, all sensors were rinsed with deionised (DI) ultrapure water and dried under stream of Argon, before being cleaned by 10 cycles of voltage cycling (0 – 1.6 V) in 0.1 M sulfuric acid using a voltage step of 0.02 V and scan rate of 0.2 V/s. Following this, the sensors are rinsed again with DI water and dried under Argon before being placed in a clean storage box until ready to be used, which is always on the same day.

IL-6 and troponin detection

A 1:4 mixture of MUA and MCH (200 M MUA:800 M MCH) is prepared in 20% (v/v) ethanol and 80% (v/v) 1xPBS and incubated for 1 h at room temperature with TCEP in a 1:5 thiol to TCEP ratio to reduce the terminal disulphide bond and allow effective gold attachment. This solution is then pipetted to cover all 8 working electrodes (for manual preparation) or introduced using flow (for WAFFLEF preparation) to the sensing area and incubated overnight at 4°C in a humidity chamber (critical for the manually prepared sensors to prevent evaporation but not needed for the sensors prepared with fluidics). The next day, unreacted thiols are washed away using 3 ml of DI water dispensed using a pipette for the manually prepared electrodes or by flowing in DI water using the waffle set to dispense 3 ml/min for 1 minute. A solution containing 5 mM EDC and 15 mM NHS in 0.1 M MES buffer is then introduced to the sensing area (using a pipette or flowing in for 15 s at 2 ml/min) and incubated for 15 minutes at room temperature. Next, the EDC/NHS solution is taken off without washing and 12.5 µg/ml primary antibodies are placed on the sensing area. Alternatively, for the WAFFLE, the antibodies are flown in directly after the EDC/NHS solution at 2 ml/min for 15 s. Sensors are then incubated for 1 h at room temperature. Following this incubation step, as well as in-between all the remaining steps, the sensors are washed with 1xPBS (Gibco™) using 3 ml pipette-

dispensed for manual preparation or using a 2 ml/min flow rate for 1 minute. The sensors are then backfilled using reagent diluent (1% w/v BSA in PBS, pH 7.2-7.4, 0.2 μ M filtered) for 1 hour at room temperature. The samples (PBS or neat serum) containing the target protein (troponin or IL-6) are then introduced, followed by secondary antibody addition and incubation for 1 hour each at room temperature. For the IL-6 assay, this is followed by a 20-minute incubation with 1x Streptavidin-HRP in the dark at room temperature. Finally, the sensor is washed for one final time before introducing the substrate solution – a pre-made mix of Tetramethylbenzidine (TMB) and H_2O_2 and incubated for 20 minutes in the dark before measuring the solution by performing chronoamperometry at -0.2 V, 0.1 s time interval for 60 seconds. The value of the stabilised current for each measurement at 60 seconds is then extracted for data analysis and plotting. The limit of detection (LoD) was defined as the first experimental datapoint falling outside the ± 3 standard deviation zone around the average blank reading (0 pg/ml target).

Cortisol detection

For the cortisol electrochemical aptamer-based (E-AB) sensor, 2 μ l of 100 μ M anchor aptamer was incubated with 10 mM TCEP in a 1:1 volumetric ratio for 1 h at room temperature. The anchor aptamers were then diluted to the working concentration (200 nM) using aptamer buffer (1 M NaCl, 10 mM MgCl_2 , 5 mM KCl in molecular grade water, pH 7.5)[134] with 0.1% (v/v) Tween-20 surfactant. 2 μ l of aptamers were then pipetted on each working electrode and the sensors were placed inside a humidity chamber and incubated overnight at 4°C. For the WAFFLE, the aptamers were delivered to the sensing area using flow. The following day, excess unbound aptamers were washed off using 3 ml of aptamer buffer dispensed using a pipette for the manually prepared sensors or by flowing in buffer at 2 ml/min for 3 minutes. Next, the surface was backfilled using 1 mM MCH pipetted over the working electrodes or flown in at 2 ml/min for 15 seconds before another overnight incubation at 4°C. The third day the electrodes were again washed to remove unbound thiols. Cortisol specific aptamers were then diluted to 1 μ M using aptamer

buffer (1:5 ratio of anchor to detection aptamer) and folded by heating up to 95°C for 5 minutes using a miniPCR machine (miniPCR bio). Following this, the aptamers were allowed to reach room temperature before being introduced to the sensing surface and incubated for 2 h at room temperature. Finally, after a final washing step, cortisol spiked aptamer buffer was introduced to the sensing area and incubated for a further hour. An impedance spectroscopy measurement was taken after the last 2 steps, before and after the addition of cortisol, against the open circuit potential, using 61 frequencies between 0.1 Hz and 100 kHz. All electrochemical data in this study is acquired using a PalmSens4 potentiostat with a MUX8-R2 multiplexer unit from PalmSens (Houten, Netherlands).

Data analysis and plotting

Impedance data was fitted using Randles equivalent circuit model incorporating a constant phase element rather than an ideal capacitor to account for non-ideal sensor behaviour. Data fitting was performed either using the standard fitting tool of PStace 5.10 or the open-source impedance fitting code Zfit by Jean-Luc Dellis modified with the Markov chain Monte Carlo (MCMC) method (full code attached in the Supplementary Information – S6). All data was then exported, plotted and analysed using GraphPad Prism 10. Where only 2 distributions are compared, for instance in the Manual vs WAFFLE graphs, a student's t test was used to determine statistical significance. Where multiple comparisons were made, *e.g.*, a control group against multiple sequential treatments, a one-way ANOVA with a Tukey post hoc test was used.

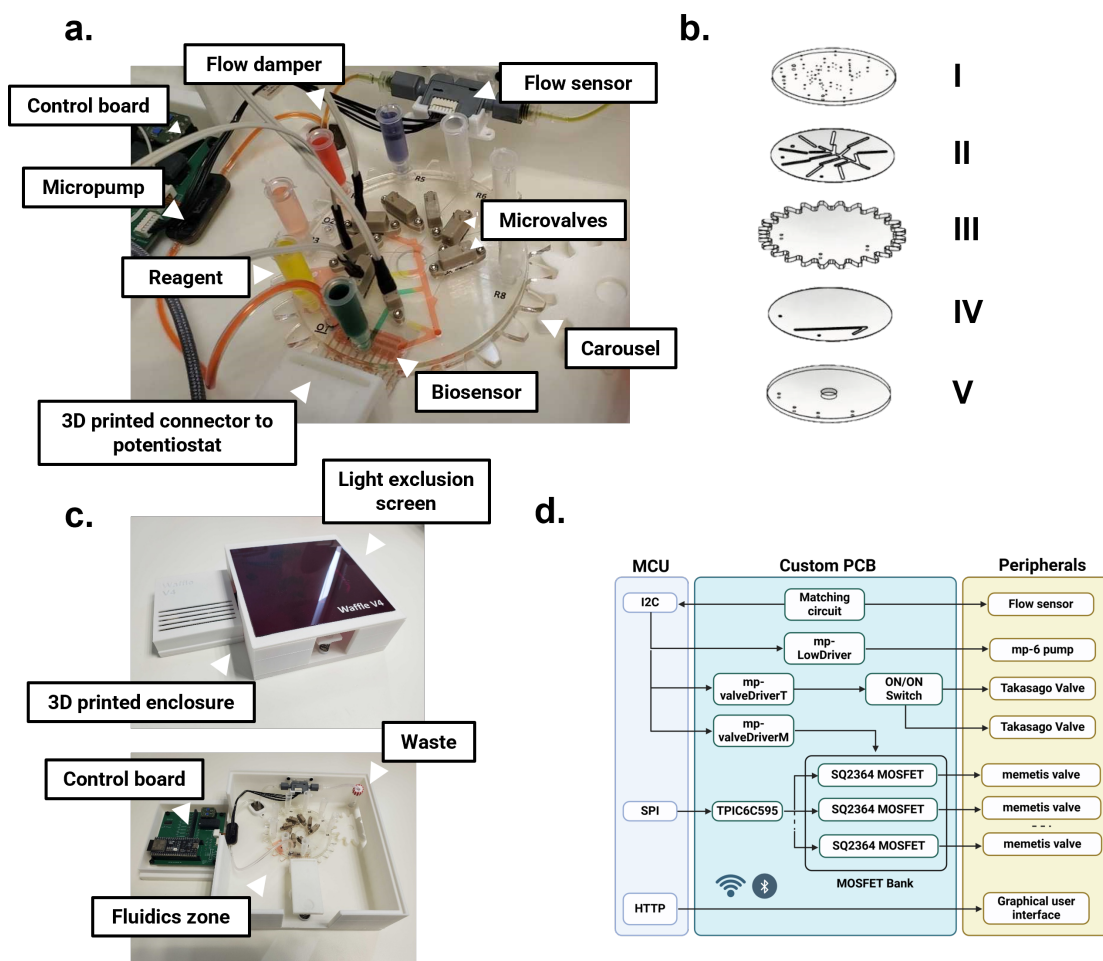


Figure 4.2: The Wee Ally for Flow Functionalisation of Low-Cost Electrodes (WAFLE) Platform

(a) Reagents cassette employing the memetis normally closed memory shape alloy valves, flow cell interfacing the electrochemical sensor, reagent reservoirs, tubing and fluidic adaptors. The flow is actuated using an mp-6 micropump, Sensirion flow sensor and electronic control board (b) Exploded assembly of the multilayer reagents cassette which includes (I) Inlets layer with holes for the reservoirs and in-line valves – 3 mm thick laser cut PMMA, (II) Channels layer connected to the memetis valves for reagent selection – 2 layers of double-sided adhesive with a PET spacer, (III) Middle layer with spur gear profile for allowing rotation to the next electrode under test and inlet holes for access to the fluidic channel on the bottom side – 3 mm thick laser cut PMMA, (IV) Flow cell splitter channels for selection of the desired flow cell to receive the reagent/buffer – double sided adhesive with PET spacer, (V) Flow cell inlet/outlet layer to which the electrode is attached via double sided tape delineating a channel on top of the electrodes. (c) 3D printed Waffle enclosure with semi-opaque lid for protecting light sensitive reagents. A two-chamber design with the electronics placed at a slightly higher elevation was employed to separate the electronics from the fluidics (top). The inside of the Waffle showing placement of components such as electrode connector, flow damper, flow sensor etc. (bottom) (d) High level diagram of the electronic control unit using a bank of SQ2364 MOSFETs with a TPIC6C595 open drain shift register for valve selection via the user interface and the drivers used for controlling the actuators.

4.4 Results and discussion

This work proposes to integrate all the commonly manual steps depicted in Figure 4.1 for electrochemical sensing in the automated, portable WAFFLE platform. The platform comprises 1) a flow cell to allow fluids to pass on top of the electrodes as described in our previous work [183], 2) a carousel to handle all the reagents and buffers required to perform the electrode preparation and assay of choice, 3) an electronic control board, and 4) a user interface. Full instructions for manufacturing the WAFFLE have been uploaded to GitHub and are available at <https://github.com/ADobrea797/WAFFLE>, with examples of processing in Video S1 (available at <https://doi.org/10.1039/d5lc00988j>).

WAFFLE features

The WAFFLE features reservoirs for all the reagents required for preparing a typical sandwich immunoassay: for instance, thiol molecules for SAM formation, EDC/NHS linker, primary and secondary antibodies, backfilling agent, enzyme label, sample and chromogenic substrate ($n = 8$ reagents in total). Each reservoir connects to a side channel that feeds into a larger main channel that directs the flow over the surface of the biosensor (Figure 4.2a). An exploded view of the fluidic cartridge and the final assembled device can also be seen in Figures 4.2b and c. Each side channel features a memetis valve, which allows the selection of the required reagent. An mp-6 micropump is located downstream of the sensor actuating the flow with the help of a Sensirion flow sensor and flow damper. The damper serves to smooth the pulsatile flow created by the pump allowing the flow sensor to record an accurate reading. The placement of the pump downstream of the electrodes is to minimise the dead volume introduced into the system. The signal required for driving the mp-6 pump is generated using the mp-lowdriver integrated circuit (IC) chip which can provide the required waveform for running the pump up to 150 V amplitude and 2000

Hz frequency. This IC was chosen due to its easy integration and low power requirements which make it suitable for battery powered devices. Although the applied frequency can also be adjusted to modulate the flow rate (Supplementary Figure S1 - Appendix G), this was locked in this study at 100 Hz as per the manufacturer's recommendation for water-based liquids and flowrate setting was driven by the voltage amplitude applied to the micropump. The voltage amplitude generated by the mp-lowdriver is adjusted in real time using a proportional-integral-derivative (PID) element. To minimise the risk to users as the electronics operate at high voltages, the electronics and fluidics were housed inside of a 3D printed plastic enclosure with the fluidic zone designed to be slightly lower than the electronics zone to prevent risks associated with leaks (Figure 4.2c). A dark tinted semi-transparent lid was also laser cut and installed into the 3D printed frame to protect light sensitive reagents (*e.g.*, TMB) while allowing the user to see the fluidic operations. As the ESP32 MCU controlling the system (Figure 4.2d) is WiFi enabled, the graphical user interface (GUI) created is a website which can be simply accessed by inputting the unique IP address of the microcontroller *e.g.*, 'http://192.XXX.XX/' into the preferred web browser. By controlling all the functions using a webpage interface, the need for additional peripherals (*e.g.* LCD screen, push buttons, LEDs) is removed which improves long term device reliability, and user accessibility is promoted through the use of free, open access software (*e.g.*, ArduinoIDE) for running the device (Video S1 - available at <https://doi.org/10.1039/d5lc00988j>).

The total cost for one WAFLLE, without valves is £400 (544 USD). This price can be further decreased to £150 (204 USD) by designing the drivers (AC voltage and constant current generators) from scratch rather than buying them off the shelf. Currently, the valves are the priciest element at £88.40 (120 USD) per unit. While enabling smooth automation, the valving system can be simplified, *e.g.*, by using manual valves.

Fluidic characterisation

Preliminary data demonstrated some flow fluctuations across different reagent inlets (Figure S2 - Appendix G), prompting the addition of a PID controller. After calibration (Figure S3 - Appendix G), $k_p = 5$, $k_i = 15$, $k_d = 1$ were the chosen parameters to produce a rapid response (< 20 s) with a steady state error of 0.5% for all the inlets (Figures 4.3 a and b). The system was found stable in the 0.1 – 2.5 ml/min range (Figure 4.3 c), the upper limit being imposed by the flow sensor.

Since the preparation of electrodes revolves around sequential addition of reagents with washing phases in between, the system then had to be calibrated for washing time (i.e., time needed to fully fill or empty the sensor with a specific reagent). Washing times were consequently characterised using a standard redox media consisting of 2 mM ferri-ferrocyanide or a fluorescent dye flushed by PBS. Figure 4.3d shows that it takes 15 seconds at 1 and 2 ml/min to remove the redox couple and return to the baseline current of clear PBS. These results were in agreement with data acquired using the dye, with the channel fully fluorescent after 15 s (Figure 4.3e, Video S2, available at <https://doi.org/10.1039/d5lc00988j>). All the assays presented hereafter are consequently done with a flowrate of 2 ml/min for 15 seconds for reagents loading/unloading.

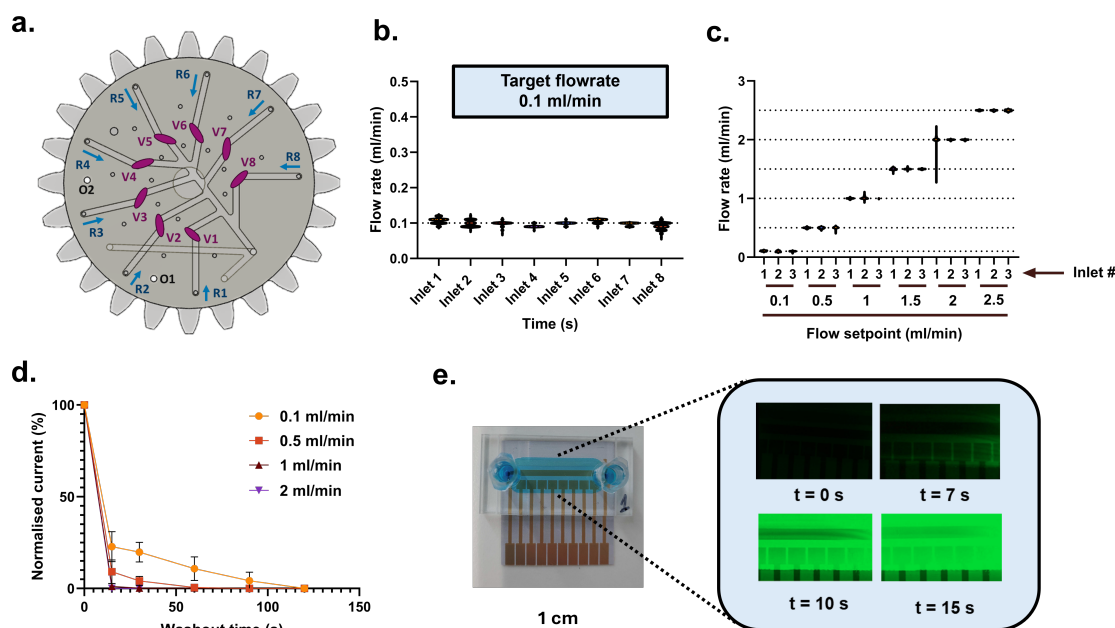


Figure 4.3: Waffle Fluidic Characterisation

(a) Schematic view of the reagent cartridge with individual reagent lines (R1-8), microvalves (V1-8) and outlets (O1 and 2) (b) The returned flow rates across the different inlets of the reagent cassette for a set point flow rate of 0.1 ml/min. Proportional-Integral-Derivative (PID) control integrated into the platform code allows automatic selection and real time adjustment of the voltage amplitude provided to the pump allowing automatic harmonization of the flow rate across the different branches of the fluidic cassette. (c) PID control allows the selection of a wide range of setpoint flowrates with a <20 s response time. (d) Washout times required by different flow rates to replace 1 mM ferri-ferrocyanide in 1xPBS with clean buffer over the biosensor surface– a proxy experiment for sequential delivery of different reagents to the biosensor surface. The signal was monitored using the peak current of the differential pulse voltammogram. (e) Fluorescence microscopy images acquired showing the middle of the flow channel on top of the electrodes while fluorescent dye is flown in at 1 ml/min flow rate (inlet is on the right side).

Sensing advantages of the WAFFLE

The full automation of the WAFFLE was first tested for the detection of inflammation marker IL-6. To evaluate the antibody loading density on the electrode surface, impedance data of the functionalised surface was probed with a standard ferri-ferrocyanide couple, and the response was fitted with Randles equivalent circuit. The R_{ct} value increased from $246.8 \pm 148.5 \text{ k}\Omega$ to $579.1 \pm 71.7 \text{ k}\Omega$ and the coefficient of variability was reduced

from 0.60 to 0.12 when the sensors were prepared with the WAFFLE platform compared to manual preparation (Figure 4.4a). This suggested a higher antibody loading density and more uniform and reproducible SAM formation across the different electrodes. Furthermore, compared to the standard preparation of electrodes (fully manual and without any microfluidic component), the WAFFLE displays a significant boost in signal for the IL-6 dose response in PBS (Figure 4.4b). The LoD decreased from 400 to 211 pg/ml with the WAFFLE preparation, and a sensitivity increase of 2.6-fold was observed over the linear range of the sensor. A similar signal enhancement was found in human serum, with a 4-fold increase in sensitivity when the sensor is prepared fully automatically (Figure 4.4c). This signal enhancement might be linked to the creation of an antibody layer on the surface of the electrodes that is less variable (perhaps due to the minimisation of environmental contamination and evaporation) compared to manual preparation, and with a higher antibody loading. The signal enhancement could be translated to other assays. For troponin for instance, a cardiac biomarker, a signal enhancement of x1.67 was recorded in serum (Figure S4 - Appendix G). This enhancement could probably be further increased by further optimising the flow regime or antibody attachment chemistry to the gold surface (Figure S4c - Appendix G).

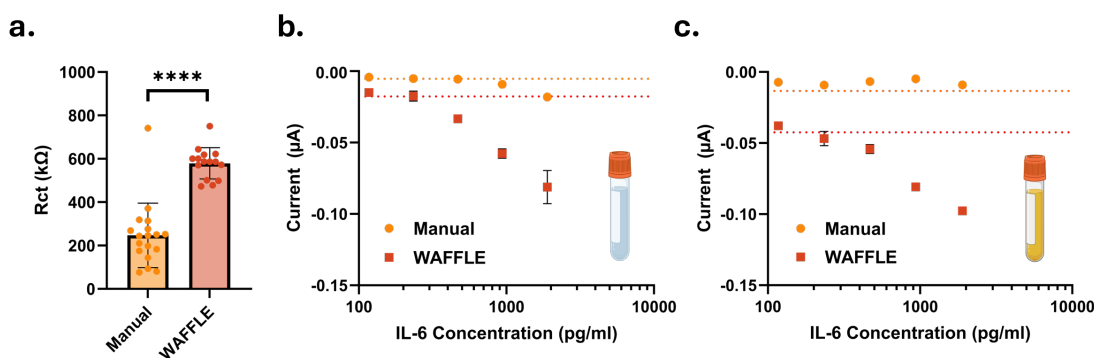


Figure 4.4: Comparison between manual and WAFFLE-made electrochemical sensors with antibodies attached via EDC/NHS coupling

(a) The difference between an IL-6 antibody layer deposited on the electrode surface manually via pipetting or using the WAFFLE showing that performing all the functionalization steps automatically increases the amount of antibodies attached to the surface ($n = 16$). R_{ct} values were determined by fitting the impedance response with Randles equivalent circuit. (b) Dose responses of WAFFLE and manually made sensors to increasing concentrations of IL-6 spiked in PBS and (c) neat human serum. Each marker represents the average over 8 electrodes $\pm$ 1 SD. Dashed lines represent the average blank reading - 3SDs ($n = 8$) and are used to determine the experimental limit of detection. **** - $p < 0.0001$. Blank averages with standard deviations for b and c can be found in Table S2.

For cortisol sensing, the mechanism of attachment to the electrode and detection principle is different. As opposed to the previous assays, the sequence of automation of the WAFFLE needs to include 1) anchor aptamer attachment, 2) backfilling the surface to prevent non-specific binding, 3) immobilisation of the cortisol specific aptamer, 4) sample introduction and 5) readout of the impedance signal through the introduction of a redox mediator (Figure 4.5a). The aptamer loading density to the electrode surface was lower when preparing the sensors with the WAFFLE compared to manually; however, the variability of these sensors was drastically reduced for both the anchor attachment (Figure 4.5b) and the duplex formation (Figure 4.5c). Interestingly, the response recorded with WAFFLE-prepared sensors did not match the expected behaviour from a standard displacement impedimetric system, where the charge transfer resistance would be expected to decrease in the presence of the target. It appears that in our system the presence of cortisol has an anchoring effect; this might be due to the increase dwelling time in the buffer containing MgCl, preventing the removal of the cortisol aptamers by flow. While more work would be needed to understand this phenomenon, the data still demonstrate an increased sensitivity for cortisol detection in our system (Figure 4.5d).

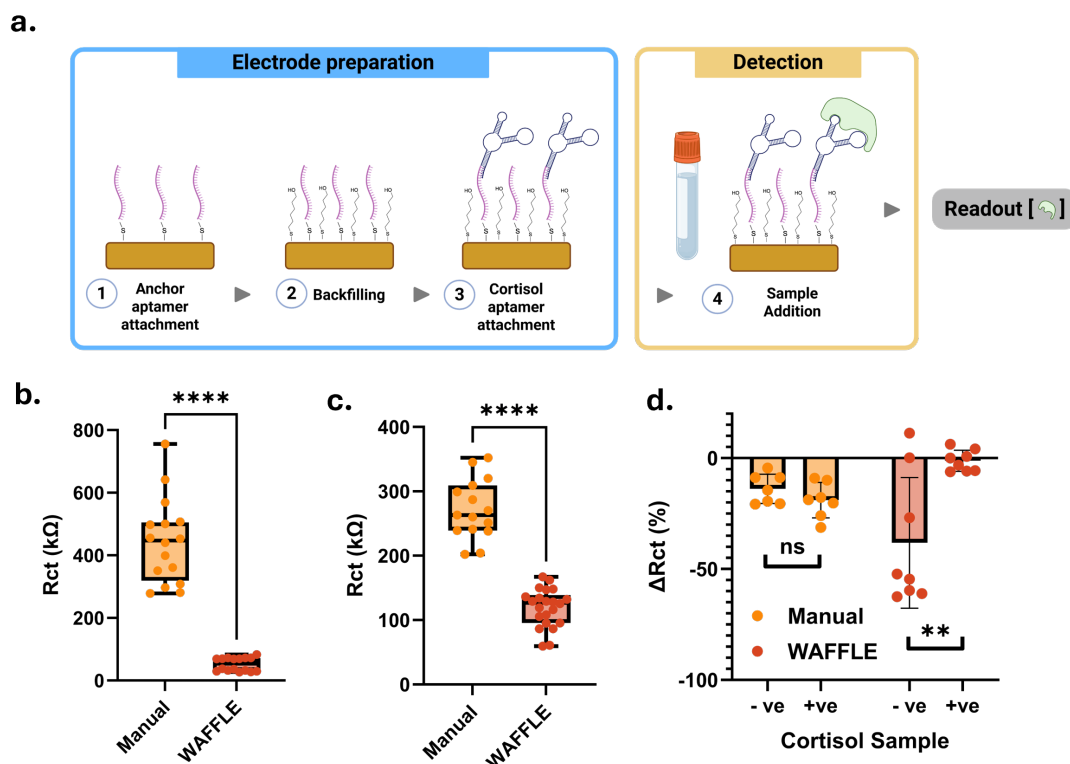


Figure 4.5: Schematic representation of the steps involved in preparing the cortisol aptasensor and the effect of WAFFLE preparation

(a) The process involved attaching the anchor aptamer to the gold electrode surface via a thiol termination at the 5' end. The surface is then backfilled using 6-Mercapto-1-hexanol (MCH) before attaching the cortisol specific aptamer. Upon addition of the sample containing the target, the interaction between cortisol and its specific aptamer gives rise to a measurable change in the impedance characteristics of the bioactive surface. (b) Comparison between aptamer deposition (direct aptamer attachment via thiol termination) using the WAFFLE versus manual preparation. (c) Comparison between aptamer deposition (full duplex assembly with mercaptohexanol backfill) using the WAFFLE versus manual preparation. (d) Cortisol spiked in aptamer buffer detection using aptasensors prepared manually versus with the WAFFLE platform. Data was fitted using Randles equivalent circuit in PsTrace 5.10. -ve represents a blank sample i.e. 0 pg/ml cortisol and +ve is a high cortisol concentration i.e. 23 ng/ml. ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$. Box plots display the interquartile range (IQR), the straight line denotes the median and the whiskers represent the minimum and maximum values recorded.

Markov chain Monte Carlo impedance data fitting

We have demonstrated so far that the WAFFLE is a hardware solution capable of improving the sensitivity and reliability of electrochemical sensors for a range of detection assays. As previously mentioned, beyond the hardware, data fitting is a key component to extrapolate an analyte concentration. This is typically performed using black box proprietary software also used for running the potentiostat (*e.g.*, PsTrace) or equivalents [85, 105, 192, 274–277], but these tend to be strongly sensitive to initialisation values, frequently leading to sub-optimal fits due to local minima. To promote robustness throughout the whole sensing process, we propose a new fitting approach using the Markov chain Monte Carlo (MCMC) method. MCMC has been widely employed for quantifying uncertainties of data fitting, by cycling through the parameter space within defined upper and lower bounds. By using this statistical approach, the most likely fitting parameters can be extrapolated. To the best of our knowledge, we propose the first open-source code to tap into the unique capabilities of MCMCs to solve data fitting challenges for impedance spectroscopy (Supplementary Information – Section S6). While these capabilities are demonstrated here with the WAFFLE, the code can be used on its own for any assay relying on model fitting. Figure 4.6 compares side by side impedance data fitted using PsTrace 5.10 (PalmSens proprietary software) and the proposed MCMC approach for the 3 assays tested here (IL-6, first row; troponin, second row; cortisol, third row). The electrical model for each assay was estimated using the observed impedance behaviour (raw Nyquist plots of the impedance spectra in Supplementary Figure S5 - Appendix G): Randles equivalent circuit with and without a Warburg element and a double parallel RC grouping in series with a resistor. These datasets were specifically selected for this exercise as they are more challenging to fit and/or assess visually (*e.g.*, by not having a clearly defined inflexion point at the end of the semicircle, or between a double semicircle response). The initialisation value of R_{ct} was varied from 1 to 100000 Ω to compare performances, while all other parameters were locked in at unit value. As demonstrated in Figures 4.6 a,b and c, optimisation using PsTrace can lead to significant variation in R_{ct} estimation (average CoV 0.92) depending on the initialisation. By contrast, the MCMC

approach shows significantly less sensitivity, providing a consistent estimation for all the tested initialisation values, and for all the models tested. This approach consequently adds an extra layer of robustness, complementing the WAFFLE for a complete electrochemical process. The full code with 3 example circuits is available in Section S6 of the Supplementary Information and can be adapted for wider use within the community.

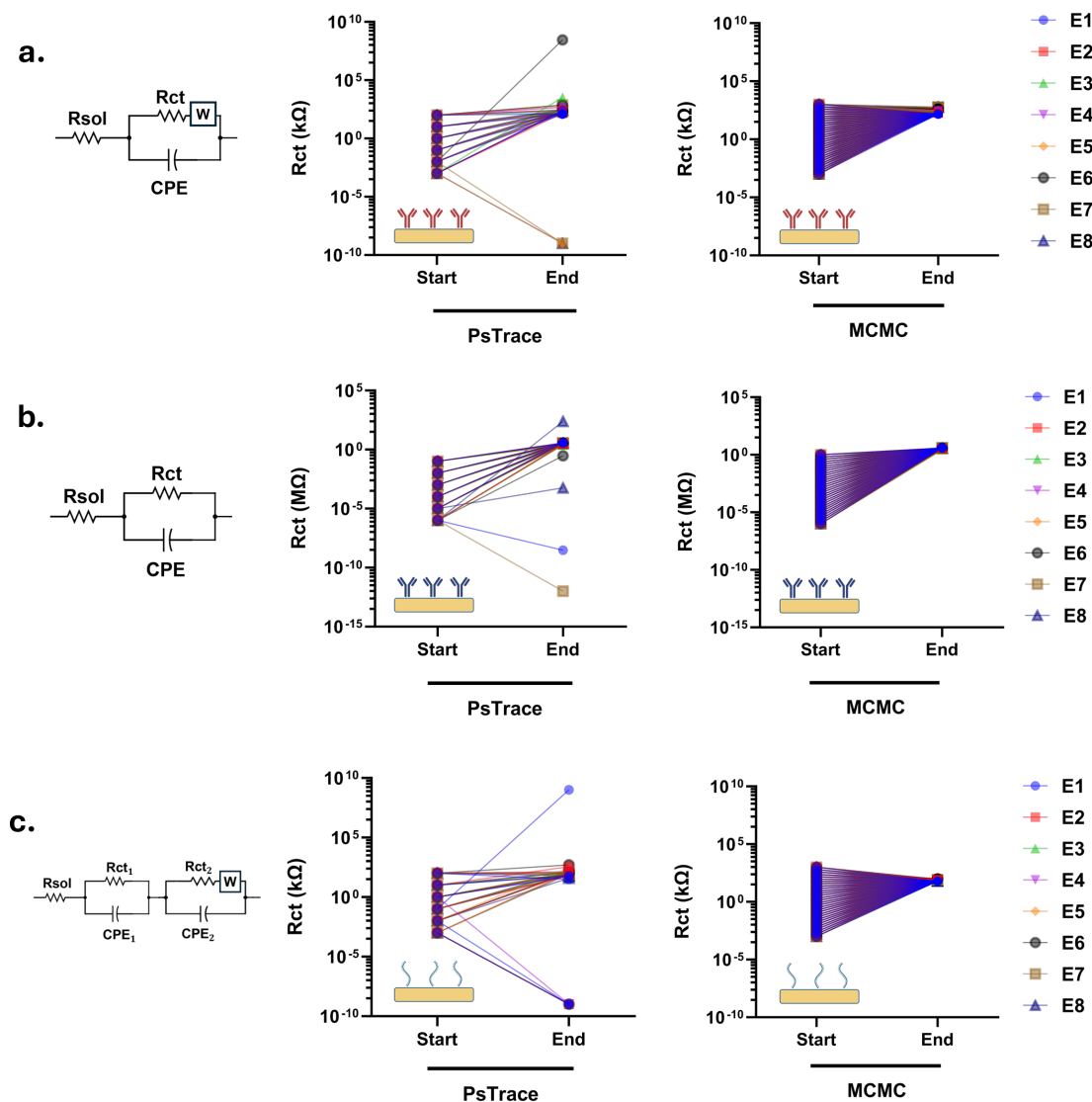


Figure 4.6: Comparison between different impedance responses fitted using a standard ‘black box’ proprietary software (PSTrace 5.10; middle column) and a modified version of Zfit using the Markov chain Monte Carlo (MCMC) algorithm (right column)

(a) Standard Randles equivalent circuit featuring the solution resistance (R_{sol}) in series with a parallel arrangement of the charge transfer resistance (R_{ct}), Warburg element (W) and a constant phase element (CPE). R_{ct} values represent 8 different working electrodes functionalized with IL-6 primary antibodies immobilized by physisorption. (b) Randles equivalent circuit without the Warburg element. R_{ct} values represent 8 different working electrodes functionalized with troponin I primary antibodies immobilized by EDC/NHS coupling. (c) Double RC grouping modelling a double semicircle impedance response. R_{ct} values refer to 8 different working electrodes functionalized with thiolated capture aptamers. Data fitted in PSTrace 5.10 uses different initialization (‘Start’) values for R_{ct} ($1 - 100000 \Omega$) while all other parameters were initialized as unit value and shows the values on which the algorithm converges (‘End’). Data fitted in the MCMC-modified Zfit algorithm uses a larger (104) input sampling pool between predefined parameter bounds which was illustrated by the larger line density in the right column.

4.5 Conclusion

Electrochemical biosensors have already transformed medical diagnostics and are likely to continue to do so in the coming decades. While significant research efforts are directed towards the development of these sensors and their underlying chemistries, microfluidic integration remains an underrepresented topic in the electrochemical biosensing community and its potential for full assay automation has not been fully explored. We hope to help address this by providing two new tools that we believe will become indispensable in the biosensor developer’s toolkit: the WAFFLE, an automated fluidic platform that replaces the manual steps associated with fabricating these sensors (pipette dispensing of reagents and washing) and a novel framework for analysing impedance data – one of the fundamental steps employed in electrochemical biosensor development. With the WAFFLE, we demonstrate a notable decrease in the variability of the bioactive surface monolayer and enhancement of the signal generated compared to manual preparation. We also uncovered some interesting insights into the assembly of the active surface detection layer under flow – a topic of much interest in the electrochemical biosensing community. We also demonstrated that the robustness of impedance fitting can be substantially improved by using the Markov chain Monte Carlo method. Together, we believe that these two innovations could represent a fundamental shift in the way electrochemical biosensors are developed and provide a springboard for accelerating their development and deployment to the point of need.

Chapter 5

Conclusions and future outlook

As discussed in Chapter 1, electrochemical biosensors hold immense promise in decreasing the time and costs associated with biomarker investigations, two important bottlenecks currently limiting further research in the community into complex, systemic diseases such as endometriosis. Yet despite having revolutionized diabetes management, electrochemical biosensors have had limited translational success for targets beyond glucose. Two key contributing factors to this are poor sensitivity in complex media and high variability, issues arising in no small part due to a lack of automation and standardization tools available. This thesis set out to tackle these challenges by producing a plug and play biomarker interrogation platform based on low-cost electrochemical biosensors for 2 key biomarkers of interest in endometriosis, cortisol, a small steroidal hormone, and IL-6, an inflammatory cytokine. In doing so, advancements to the field of electrochemical sensing were made, from deepening our understanding of the integration of a novel cortisol bioreceptor into a low-cost impedimetric detection format, to reporting on an easy-to-manufacture fluidic accessory aimed at promoting microfluidic use within the electrochemistry community which eventually culminated with the creation of a fully integrated platform for automating the manual steps involved in electrochemical biosensor development, along with the readout.

Biosensor development

The first objective of this thesis was to develop a low-cost electrochemical biosensor for cortisol. Initial work with the cortisol aptasensor highlighted some of the important factors responsible for sensor performance and a set of optimal conditions to maximise the target response. For instance, it was found that the biosensor performed best when cleaned using oxygen plasma and used with the original selection buffer supplemented with 0.1% (v/v) Tween-20 surfactant. However, a change in the gold substrate material yielded sensors with significant batch-to-batch variability. Different factors were investigated to correct for this including the aptamer-gold attachment procedure (thiol reduction

agent, sequence of immobilization steps, deposition concentration), but the signal remained largely obscured by the high background noise.

At the end of Chapter 2, different avenues for future work were suggested for improving the sensing performance such as co-immobilisation, different backfilling agents, sequence intercalators, electrode surface modifications and the use of an internal redox probe. However, it was cautioned that with increased assay preparation complexity, the final cost will also rise and thus the balance of cost-benefit must be kept in check to produce a commercially attractive system and not negate the cost advantages of an aptamer-based system.

As a significant amount of time was dedicated to the development and troubleshooting of the cortisol aptamer-based assay, it was decided for the IL-6 assay to use a well-established enzyme-linked protocol for the detection scheme as the overarching goal of the work was to create a reliable test bed for validating the function of the automated tool developed in Chapter 3, rather than create a novel detection strategy for this marker.

Although the detection range demonstrated for IL-6 in Chapters 3 and 4 were above the clinically relevant concentration ranges for this biomarker in serum, extrapolation of unknown sample concentrations could still potentially be achieved using for instance standard addition methods [278] – however this was not tested in this thesis.

Development of low-cost, easy-to-assemble fluidic devices for enhancing the sensitivity of electrochemical assays

As discussed in the Conclusion of Chapter 2 and Prologue to Chapter 3, microfluidics provides a very attractive alternative for enhancing the performance of electrochemical sensors without sacrificing on device cost. The benefits have been previously demonstrated in literature and include increased sensitivity while retaining assay selectivity, improved consistency and assay reliability, automation, lowering of reagent volumes required and multiplexing capabilities. To explore how this may help with the cortisol assay, an easy-to-manufacture and assemble flow cell was created (Chapter 3) and validated using a more

well established, low-cost electrochemical immunoassay for IL-6 based on physisorbed antibodies to establish proof of principle.

Through this work it became apparent that although the benefits of fluidic integration are known within the electrochemical biosensing community, only a small fraction of research outputs in this field (less than 6%) feature a fluidic component. The reasons for this are multifaceted and will vary between different laboratories, but 3 significant factors which may be at play are 1) a very limited range of off-the-shelf fluidic solutions available to researchers, 2) the high entry barrier to microfluidics (setting up a facility and training users) and 3) the recurring time cost *i.e.*, how much time would adopting fluidics add to a typical day in an electrochemistry lab. This reframes the question of ‘Why aren’t more research groups using fluidics?’ to ‘What are the logistical and user-experience barriers to fluidic adoption?’.

The low-cost flow cell reported in Chapter 3 was designed specifically for addressing these barriers by allowing rapid assembly (< 1 min) and integration within experimental workflows with minimal disruption, and ease of manufacturing in bulk quantities without a prior requirement for engineering or microfluidics expertise. The flow cell was demonstrated to allow an up to 7-fold increase in the detection sensitivity of a low-cost electrochemical immunoassay for IL-6 and additionally highlighted a spontaneous reaction taking place between the Ag/AgCl pseudoelectrode and the detection substrate TMB affecting sensing reliability, and allowed the investigation of different methods for minimizing it. As this approach can be generalised and applied to any electrochemical immunoassay based on surface-immobilised antibodies it is hoped that those findings would encourage and inspire wider adoption of fluidics within the community and that the flow cell may become a part of the electrochemical biosensor researcher’s toolkit.

Development of a fully automated assay for standardizing the fabrication of electrochemical biosensors

To further demonstrate the advantages of the flow cell, it was used in Chapter 4 as a building block to create a fully automatic system for standardizing the fabrication of electrochemical biosensors at research level.

As highlighted in Prologue to Chapter 4, one key factor currently limiting the translation of electrochemical biosensors is their variability which stems from a lack of automation and standardization tools available at research level. This issue extends not only to the hardware, but also the way in which data (*e.g.*, electrochemical impedance spectra) is analyzed. This thesis tackled these issues through a two-pronged approach: the development of a fully automated platform for standardizing the fabrication process of electrochemical assays and the proposal of a new method for analyzing impedance spectroscopy data (an important technique in biosensor development) based on the Markov chain Monte Carlo algorithm. The WAFFLE was designed using a modular approach so that any of its constituent subsystems (*e.g.*, pumping system, valving system, fluidics) can be repurposed and adapted to suit the needs of electrochemical assays based on planar electrodes. The user interface is intuitive and may be accessed from any WiFi enabled device, making it easy to integrate within larger Internet-of-Things systems and requires no previous software development experience to operate.

WAFFLE was used to automate the full fabrication pipeline of the IL-6 and cortisol sensors and was demonstrated to produce a 4-fold increase in the sensitivity of the IL-6 sensor in neat unprocessed human serum, a greater discrimination between positive and negative spiked cortisol samples and a decrease in the coefficient of variability compared to sensors prepared manually. This increase in performance was attributed to a mixture between the microfluidic confinement effect and a minimization of environmental contamination and evaporation of surface deposited reagents. Although in this work the reagents were introduced using flow, followed by incubation under stop flow conditions, it is likely that the confinement effect as described by Kang *et al.* is partly responsible for the sens-

itivity increase seen [210]. It would be interesting to explore whether incubation under flow conditions and in smaller channels (*e.g.*, $< 50\ \mu\text{m}$ height) than those described here ($\sim 150\ \mu\text{m}$) would help further enhance this effect.

The IL-6 assay presented in Chapter 3 was conducted using physisorbed primary antibodies, whereas in Chapter 4 the antibodies were immobilised to the surface *via* the carbodiimide attachment mechanism EDC/NHS – which could explain why the LoDs are different between the two Chapters. This change was necessary as it was found during developmental work that antibodies attached by physisorption would be removed from the surface during the automated washing cycle performed by the WAFFLE, and thus a stronger surface attachment mechanism was necessary to hold them into place. The worse LoD for the EDC/NHS protocol could be linked to the more limited assay development and optimisation work conducted to push the sensitivity of this assay compared to the physisorption.

The PBS and serum results shown in Figure 4.4 follow a similar absolute current magnitude increase with increasing IL-6 concentration, but the PBS trace appears to have a slightly steeper incline in the LoD region and an overall lower but consistent background current across all the measured datapoints. This is likely a matrix-dependent offset, caused by serum proteins or other nonspecific interactions with the sensing surface, and a similar effect has been seen previously with IL-6 detected using an interdigitated electrode array [279]. Furthermore, it is worth mentioning that the serum used in this work was purchased off-the-shelf and pooled from a wide donor base. It is likely that if testing was done with samples from individual patients, there will be a higher degree of variability compared to PBS, however this was not tested in this thesis.

The impedance fitting algorithm, built upon the open-source MATLAB code Zfit, was demonstrated in this work to eliminate the issue of local minima trapping commonly encountered with impedance software (*e.g.*, PsTrace, Nova), and thus provides a more robust estimate of equivalent circuit model parameters. This approach is highly versatile and may be applied to any data fitting problem by simply modifying the fitted equation and parameter bounds.

Both the WAFFLE design and the MCMC code were uploaded on GitHub and made freely available to promote wider use within the electrochemical biosensing community.

Finally, an important limitation of this study is that all assays were performed by the same individual (the author of this thesis), and thus to remove the potential of confirmation bias, future work should include assays prepared by different operators to account for this.

Suggested avenues for further development

As discussed in Chapter 1, endometriosis is a disease with a systemic manifestation and the literature suggests that a multiplex biomarker panel presents the best opportunity for replacing the invasive laparoscopy diagnostic test [280–282]. While this thesis introduced the WAFFLE platform and demonstrated its sensing benefits for 2 biomarkers of interest for endometriosis independently, further work could be directed at detection multiplexing, introduction of a sample processing unit or improvements in the general usability of the device.

For multiplexing the detection and allowing more flexibility in the number of assays that can be performed on-chip, more reagent reservoirs and channels can be added. To allow the addition of more reagents without drastically increasing the device footprint, the valves could be re-located off-the-platform, upstream of the reagent reservoirs which could instead consist of chambers inside the fluidic chip similar to the design described by Kadimisetty and colleagues [157]. In such an implementation, the valves would serve as on-demand air openings, allowing the selection of the required reagent without introducing any dead volume into the liquid path. Alternatively, the multiplexed detection could be tackled from an assay development angle, where multiple antibodies for different targets (*e.g.*, cortisol, IL-6 or others) are introduced simultaneously but each is labelled with a different detection enzyme (*e.g.*, alkaline phosphatase, horseradish peroxidase, glucose oxidase), each producing its own distinct electrochemical signature.

From a full integration perspective, introducing a subsystem into the WAFFLE that could automate the sample processing step (*e.g.*, extracting serum from whole blood)

and/or sensor calibration would also be beneficial and further decrease variability and operator errors. The sample processing could for instance be achieved by embedding a plasma separation membrane in the outlet of the sample reservoir or within the sample channel to obtain serum from whole blood, rather than performing this step off-the-platform *via* centrifugation [283, 284]. To account for samples with a high haematocrit and a higher risk of clogging, several approaches have been reported in literature for instance using filtration *via* lateral displacement [285] or cell margination [286]. In a similar vein, a concentration gradient generator design [245] could be integrated immediately upstream of the sensor detection area to automatically perform sensor calibration prior to sample analysis [287].

On the electronics side, the pump driver used in the WAFFLE was chosen to deliver the required flow rates based on the geometry of the channels used in this work and to minimise power consumption and thus ensure portability. However, smaller channels with higher flow resistances may require a more powerful version of the driver (*e.g.*, mp-driver or mp-highdriver rather than the mp-lowdriver) and/or multiple pumps in series to generate the required flow rates. Similarly, some applications may require bi-directional flow (*e.g.*, to promote mixing) which can be achieved by using two micropumps in parallel. Additional modules such as a piezoelectric or acoustic mixing or a heating module may also be integrated underneath the detection zone to further enhance sensing performance [288, 289]. Furthermore, the platform was designed to allow easy integration within Internet-of-Things systems, so perhaps further work could focus on developing an in-the-cloud version of the software which could be linked to other lab automation architectures [290].

While the WAFFLE was demonstrated in this work to benefit electrochemical assays, it is not solely confined to this application. For instance, it could provide the basis of a low-cost, automatic discrete droplet dispensing system similar to the BioDot instruments [291] or an antibody dispenser for small-scale production of lateral flow strips [292]. It is likely that each new application of the WAFFLE may require small tweaks to the original design, however it is hoped that the work presented here will provide a jumping off point for further development.

Clinical application to endometriosis

Endometriosis is a complex, systemic disease with a current time to diagnosis of around 9 years and 4 months [293]. Digital tools, such as apps (*e.g.*, Phendo) where patients log their daily symptoms, are an important medium through which the people affected can translate their experience of pain into clinical language [25]. This serves to increase the visibility of endometriosis-related pain, and recent work has indicated that digital tool usage can potentially decrease the current time to diagnosis by up to 4.36 years [37]. Additionally, data generated through user activity and wearable systems, such as fitness trackers, can help advance research into non-invasive phenotype classifications [34], the prevalence of certain symptoms [35] and the interpersonal variability in the experience of endometriosis-related pain [39]. Complementing these digital tools with biomarker tracking (*e.g.*, inflammation markers play a central role in endometriosis [294]) could provide an additional dimension to the generated data and encourage patients to seek a diagnosis sooner, however no such platform currently exists that can allow at-home use.

The tools presented in this thesis, with further development on sample processing (*e.g.*, by enabling finger-prick blood collection, filtration and delivery to the sensing surface), assay sensitivity, and further miniaturisation, could form the basis of a complement to symptom tracking apps that patients can use at home given the portable, low-cost, easy-to-multiplex nature of electrochemical biosensors and accompanying hardware. The modular design of the WAFFLE platform also allows it to be adapted to different biomarkers of interest by changing the reagents used for functionalising the sensors.

The WAFFLE could also be used to accelerate and standardise the development of electrochemical sensors for newly discovered biomarkers of endometriosis. For instance, the National Institute for Health and Care Excellence announced its endorsement of a new diagnostic test for endometriosis based on salivary miRNA which can be used in the National Health System (NHS) [295]. Similarly, a distinct hormonal fingerprint has been identified including 11-oxygenated androgen and 11-ketotestosterone which successfully detected >95% of endometriosis patients in a blinded set of samples [296]. Other work us-

ing omics techniques have uncovered new specific biomarkers and biomarker panels [280, 297, 298], some previously unexplored for endometriosis such as afamin (liver protein), coagulation factor XII (plasma protein) and proteoglycan 4 (glycoprotein) [280]. A low-cost test targeting these markers could decrease barriers to accessibility in low-income and resource-constrained environments.

Appendices

A The CSS.1 aptamer secondary structure

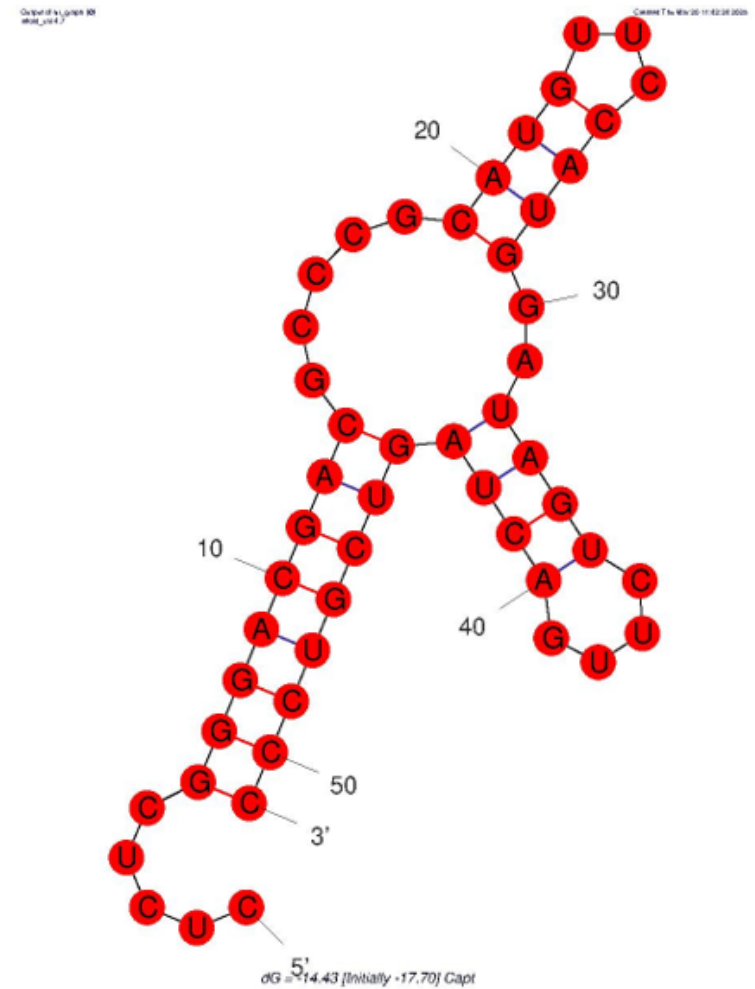


Figure 1: Secondary aptamer structure as predicted by mfold [182]

B Validation of cortisol aptamer binding

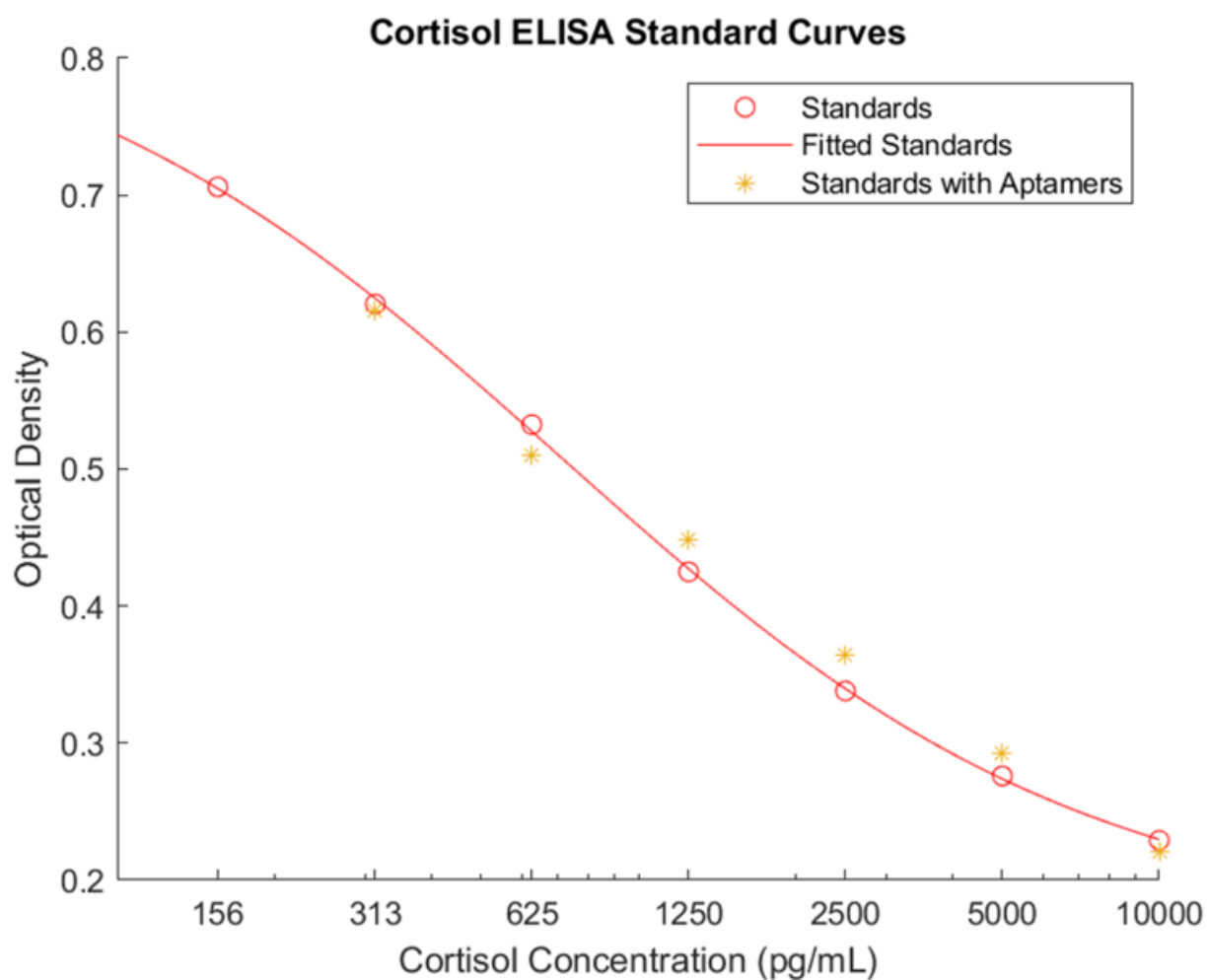


Figure 2: Calibration curve generated using an optical competitive cortisol ELISA assay, and equivalent calibrant wells with 200 nM cortisol aptamers added (gold stars). Cortisol addition resulted in an average 20% decrease in the optical signal output out of those wells demonstrating competitive binding against the cortisol antibodies present in the ELISA kit.

C Further information on cortisol aptasensor

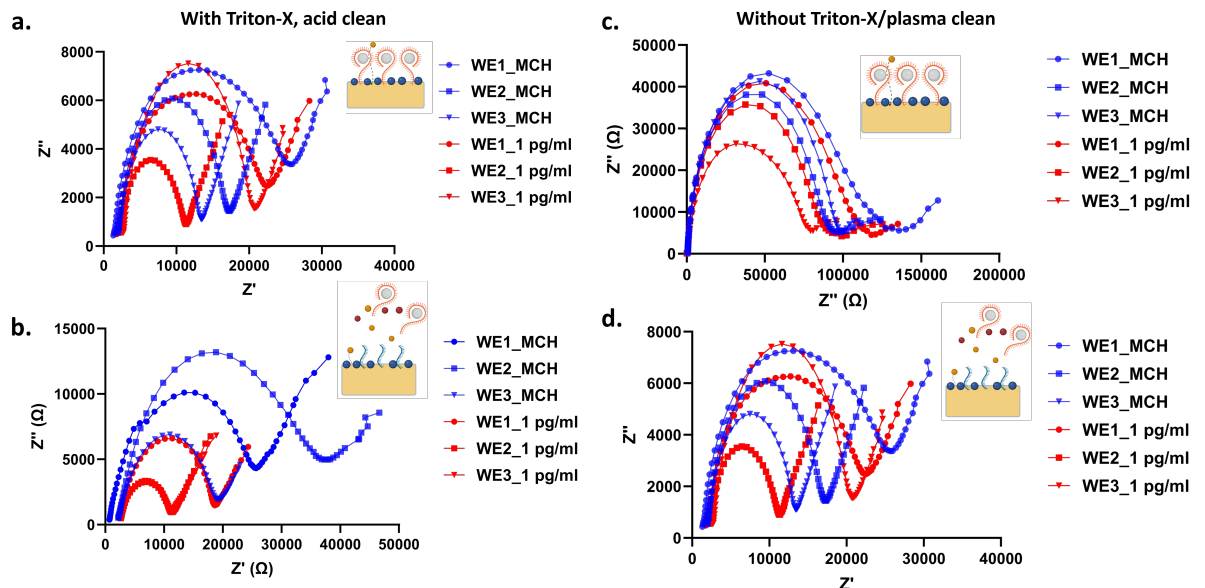


Figure 3: The effect of cortisol addition on the Nyquist plot of individual working electrodes for different experimental conditions discussed in Section 2.3. Data represents 3 working electrodes before (at the MCH backfilling step) and after cortisol addition (1 pg/ml) and demonstrated a decrease in charge transfer resistance upon target binding suggesting removal of receptor off the electrode surface.

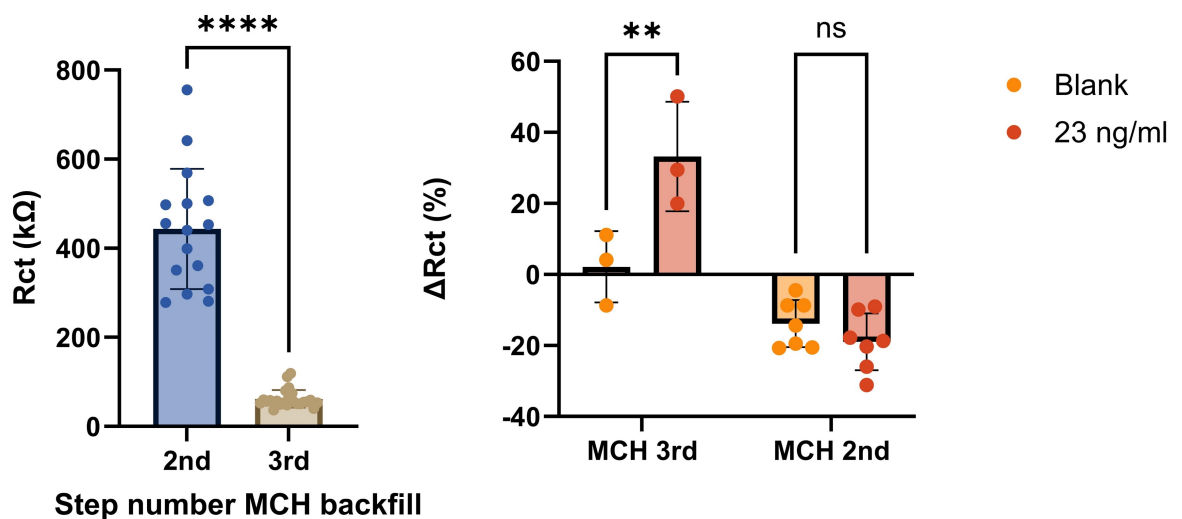
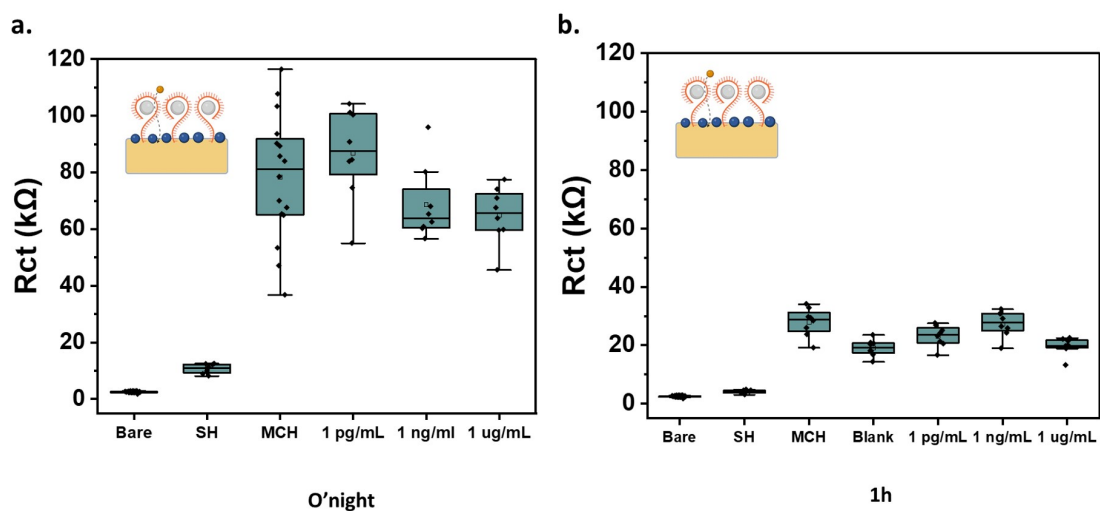


Figure 4: Signal to noise ratios of cortisol aptasensors backfilled after the 3rd or 2nd step in the functionalisation procedure respectively i.e. after the anchor deposition or after the duplex assembly



8

Figure 5: Effect of 1 h incubation vs o'night for the SH configuration. Results in lower coverage and sensitivity not significantly improved

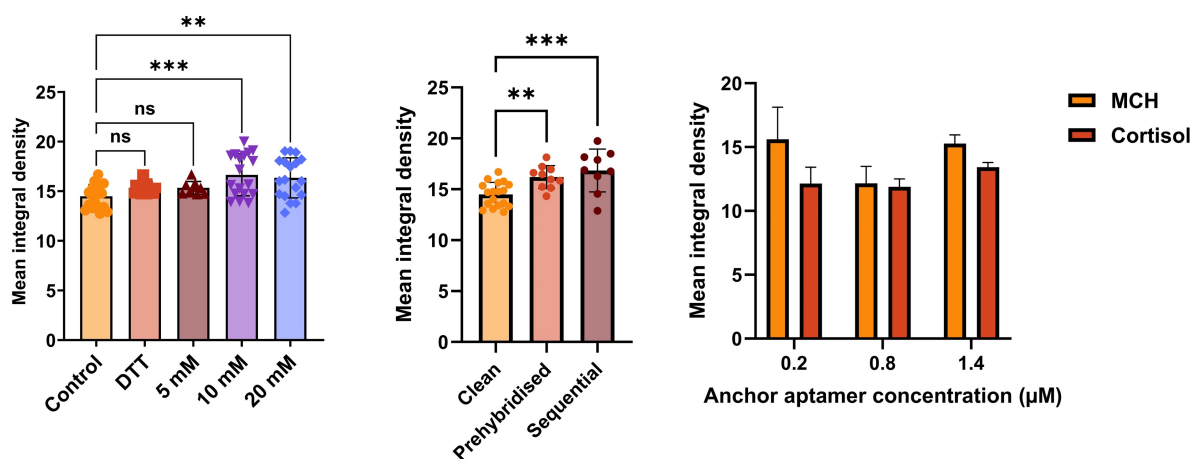


Figure 6: Fluorescence equivalent of data shown in Figure 2.7

D Commentary of dose response shape and mechanism of enhancement seen in Chapter 3

When plotted on a log scale, immunoassays like the one demonstrated in Chapter 3 typically display a sigmoidal response as demonstrated in Figure 3.4 and 3.5 of this thesis (without the lower asymptote in the case of these figures as the experimental range was too narrow to capture this; Figure S6 in the supplementary information of that paper, shown in Appendix E, displays the lower plateau more clearly). This behaviour results from the fact that antigen-antibody interactions are governed by the law of mass action and are limited by the finite number of available binding sites [299], which progressively saturate with increasing target concentration. The resulting curve is often described using a 4-parameter logistic regression model [300]:

$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B}$$

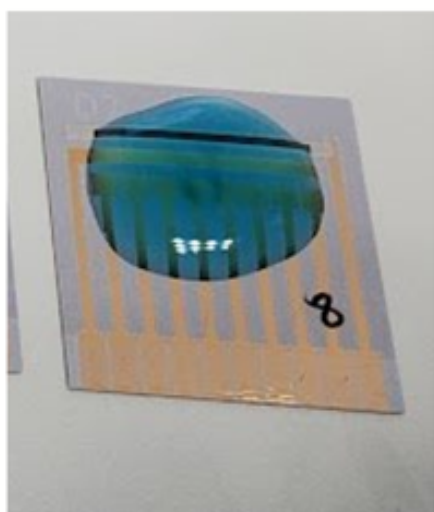
where:

- y is the measured assay response, such as absorbance or current.
- x is the analyte concentration.
- A is the response at the lower asymptote (lower plateau).
- D is the response at the upper asymptote (maximum plateau).
- C is the analyte concentration at the inflection point, corresponding to the midpoint between the lower and upper response plateaus.

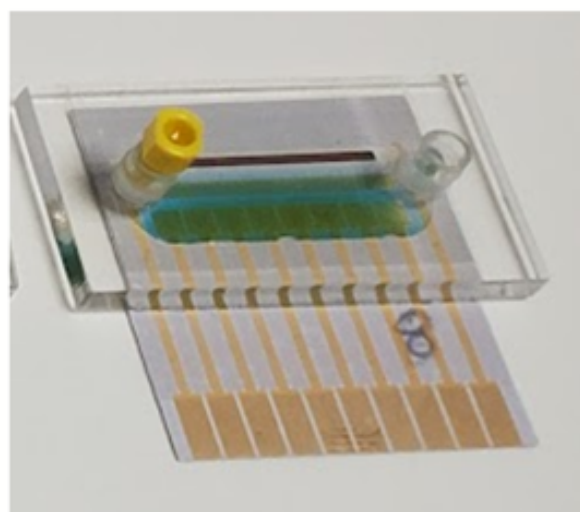
- B is the slope (Hill slope) parameter, which determines the steepness of the concentration–response curve.

At low analyte concentrations, there is insufficient target capture to produce a detectable signal, displaying as a lower asymptote. As the concentration increases towards the inflection point, signal generation increases exponentially, which in log space translates to a linear response. At high concentrations, the finite number of binding sites leads to saturation, resulting in the upper asymptote.

In terms of the driver for the signal enhancement seen with the flow cell compared to manual preparation in Chapter 3, liquid confinement ensures that there is minimal liquid movement (and thus solution mixing) when the sensors are carried by hand from the dark incubation chamber (pipette box) and into the electrode connector for measurement (see Figure below). This reduces the convection of the oxidised (blue) TMB away from the detection surface where it is being measured by chronoamperometry and translates into a higher current magnitude.



Without Flow Cell



With Flow Cell

E Supplementary information - Flow cell publication

Supporting Information

A plug-and-play, easy-to-manufacture fluidic accessory to
significantly enhance the sensitivity of electrochemical
immunoassays

*Alexandra Dobrea^{*1,2}, Nicole Hall¹, Stuart Milne^{1,3}, Damion K Corrigan³, Melanie Jimenez¹*

¹Department of Biomedical Engineering, University of Strathclyde, Glasgow, UK.

²James Watt School of Engineering, University of Glasgow, Glasgow, UK.

³Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, UK.

*Corresponding author: alexandra.dobrea@strath.ac.uk

Table S1. Coefficients of variability across the different conditions investigated for plasma etching (different power and exposure time settings) and sulfuric acid voltage cycling in sulfuric acids of various molarities (0.1-0.5 M) compared against out of the box (OOTB) electrodes. Values were calculated across 3 different electrode platforms for an n=24 for each condition. NT=not tested

		Rct CoVs (%)							
		Power (% of 200 W)				H2SO4 (10 CV Scans)			OOTB
		20	50	80	95	0.5 M	0.2 M	0.1 M	
Time (s)	21	34.95681	NT	28.79783	37.93176				
	62	60.54746	43.37948	49.21802	20.67388	11.5664	14.85953	10.76492	33.24095
	99	36.76136	NT	22.65838	NT				
		DPV Ipeak CoVs (%)							
Time (s)	21	10.97095	NT	13.35211	16.0161				
	62	21.60761	13.94275	8.078009	10.23699	5.820916	3.239721	5.820916	19.77644
	99	10.53344	NT	10.1379	NT				
		CV Ipeak CoVs (%)							
Time (s)	21	6.188177	NT	1.352927	2.015955				
	62	2.794081	3.106236	1.785074	2.071971	3.502296	2.852956	2.969417	8.671622
	99	2.715874	NT	3.498075	NT				

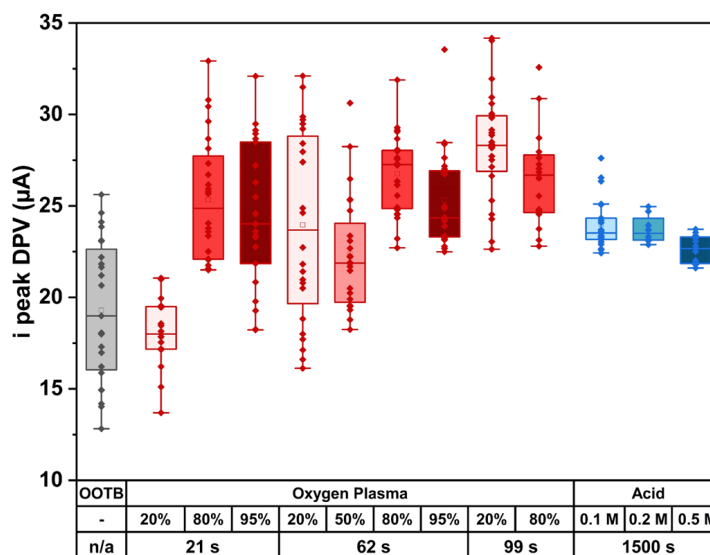


Figure S2. Comparison of the effect of oxygen plasma and sulfuric acid voltage cycling on the peak current of the differential pulse voltammogram. Datapoints represent 3 different screen-printed electrode platforms with 8 working electrodes each for an n=24 per tested condition. Each box delineates the interquartile range (IQR), the straight line denotes the median, the hollow square represents the mean and whiskers denote the range within 1.5xIQR. OOTB=out of the box.

Table S2. Material costs for fabricating one flow cell. Prices are from Stockline Plastics Ltd. (Glasgow, UK), RS Components (Glasgow, UK) and Microfluidic ChipShop (Germany).

Component	Cost (£)	No. of flow cells	Cost/flow cell (£)
Clear acrylic sheet (3 mm thick) 750x500mm	11.64	717.70	0.02
Double Sided Polyester Tape (0.23mm thick, 50mx38mm)	29.26	3703.70	0.01
Stand alone mini female fluidic adaptor (pack of 10)	6.49	5.00	1.30
Mini female adhesive ring (pack of 10)	6.91	5.00	1.38
		Total	2.70

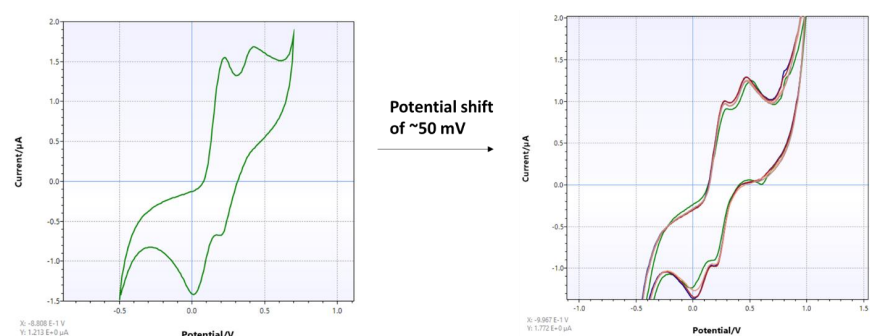


Figure S4. Potential shift observed in the characteristic TMB cyclic voltammogram induced by the anodization of the Ag/AgCl reference electrode. (Left) Non modified reference electrode (Right) Anodised reference electrode. Data acquired in PSTrace 5.9.

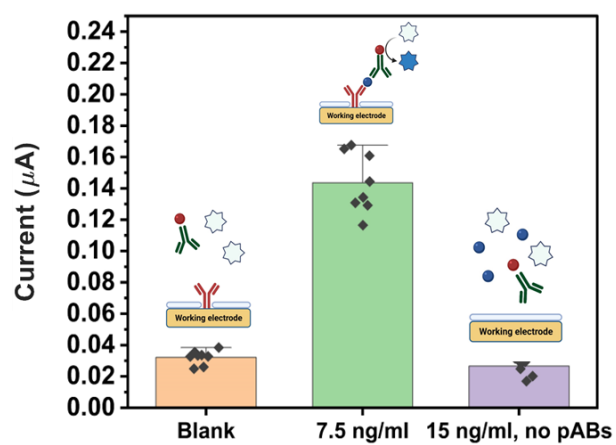


Figure S5. Control experiment carried out in 10% v/v unfiltered serum spiked with IL-6 confirming assay specificity to IL-6 (n=8)

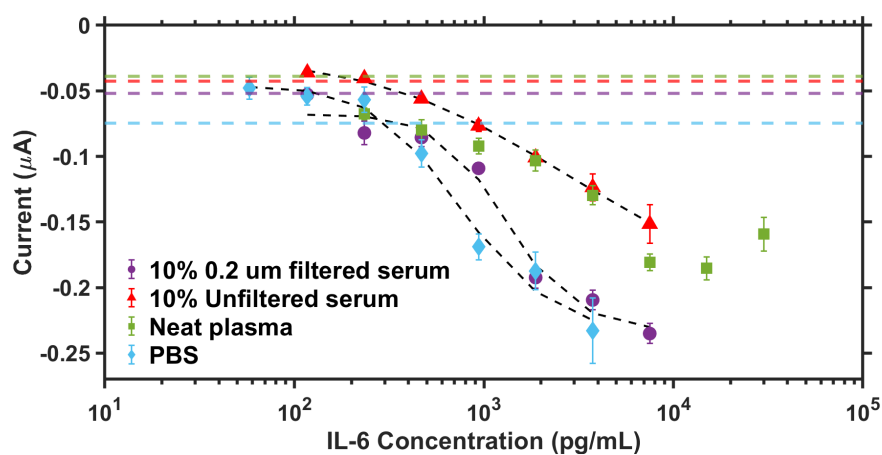


Figure S6. Dose response curves of the biosensor to different testing media: 10% 0.2 um filtered and unfiltered human serum, neat human plasma and PBS (n=8 per concentration point). Horizontal dashed lines represent the blank reading (0 pg/ml) - 3 SDs (n=8). Black dashed lines are the lines of best fit for each dataset (4 parameter logistic regression model).

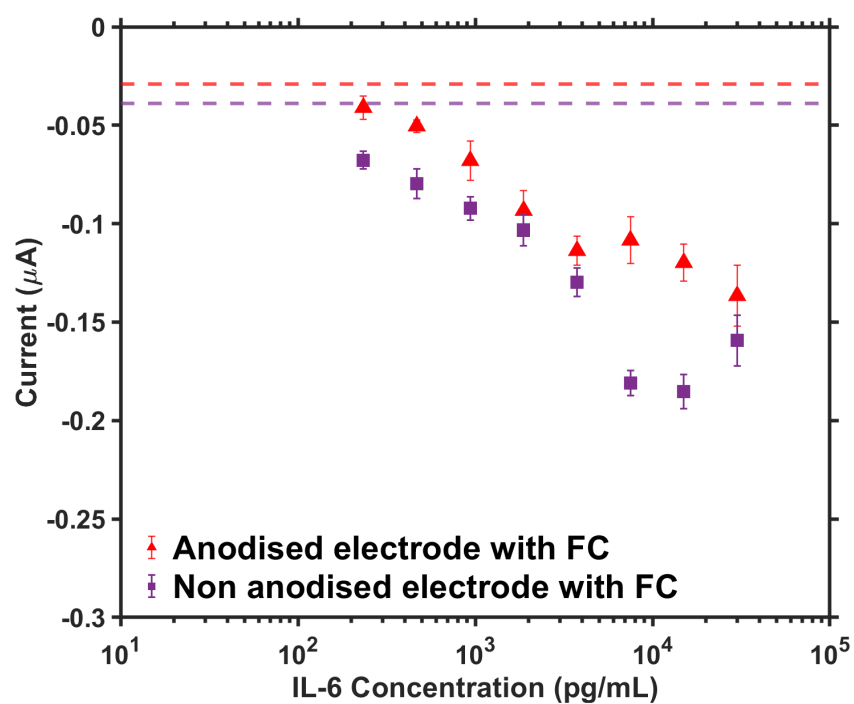


Figure S7. Side by side comparison between anodized and non-anodized electrodes with the flow cell attached and the effect on the dose-response to IL-6 suggesting a dampening of the Hook effect over the ultrahigh concentration range. Datapoints are average over 8 measurements (1 electrode platform/concentration point). Dashed lines represent the blank reading (0 pg/ml) -3 SDs ($n=8$).

Table S3. Blank (0 pg/ml IL-6) readings displayed in the main paper Figures

Condition/Sample tested	Figure	Blank current value $\pm$ 3 SD (nA)
Non-anodised electrodes dose-response/PBS	4a – red (top) line	-15.5 $\pm$ 2.2
Anodised electrodes dose-response/PBS	4a – bottom (blue) line	-26.3 $\pm$ 6.8
Anodised electrodes dose-response (without flow cell)/PBS	4b – top (blue) line (same as 4a bottom/blue line)	-26.3 $\pm$ 6.8
Anodised electrodes dose-response (with flow cell)/PBS	4b – bottom (purple) line	-62.0 $\pm$ 12.6
Non-anodised electrodes without flow cell /10% (v/v) 0.2 μ m filtered serum	5a – top (blue) line	-26.1 $\pm$ 8.0
Anodised electrodes with flow cell /10% (v/v) 0.2 μ m filtered serum	5a – bottom (purple) line	-36.3 $\pm$ 19.0
Non-anodised electrodes without flow cell /10% (v/v) un-filtered serum	5b – top (blue) line	-21.2 $\pm$ 5.7
Anodised electrodes with flow cell /10% (v/v) un-filtered serum	5b – bottom (purple) line	-32.2 $\pm$ 13.6
Non-anodised electrodes without flow cell /neat plasma	5c – top (blue) line	-14.7 $\pm$ 2.9
Anodised electrodes with flow cell /neat plasma	5c – bottom (purple) line	-20.8 $\pm$ 8.2

F Commentary on the mechanism of enhancement seen in Chapter 4

Microfluidic confinement has been suggested to enhance the sensitivity of electrochemical immunoassays by leveraging the channel height and flow rate to increase the mass transfer of target molecules from the bulk solution to the antibody-functionalised electrode surface [210]. This relationship is described by the Levich mass-transfer coefficient (Eqn. 1), k_{Lev} :

$$k_{\text{Lev}} = 0.925 \left(\frac{D^2 V_f}{4h^2} \right)^{1/3} \quad (1)$$

where k_{Lev} is the mass-transfer coefficient, D is the diffusion coefficient of the target analyte, V_f is the volumetric flow rate, and h is the half-height of the microfluidic channel. Equation 1 therefore shows that:

$$k_{\text{Lev}} \propto V_f^{1/3} h^{-2/3}$$

Thus, increasing the flow rate (V_f) or decreasing the channel height (h) increases the mass-transfer coefficient, meaning that target molecules are delivered more efficiently to the sensing surface. The effect of mass transport on antibody–antigen binding is subsequently described by Equation 2 [210]:

$$\frac{\partial c_{A-B_s}}{\partial t} = k_{\text{on,obs}} c_{A,b} c_{B,s} = (k_{\text{on}}^{-1} + k_{\text{Lev}}^{-1})^{-1} c_{A,b} c_{B,s} \quad (2)$$

where c_{A-B_s} is the surface concentration of the antigen–antibody complex; t is time; $k_{\text{on,obs}}$ is the observed association rate constant; $c_{A,b}$ is the concentration of target analyte in the bulk solution; $c_{B,s}$ is the concentration of available receptor or antibody binding sites at the sensor surface; and k_{on} is the intrinsic association rate constant of the target–receptor interaction. The subscript **b** denotes species in the bulk solution, while **s** denotes those at the sensor surface.

As the electrochemical response of an immunosensor is dependent on the amount of target captured at the electrode surface through the formation of immunocomplexes, this enhanced target accumulation produces a larger detectable signal within a given assay time, which may account for some of the sensitivity enhancements seen in this thesis. Additionally, similar to the case of the flow cell used without any flow (Chapter 3) liquid confinement ensures that there is minimal liquid movement (and thus solution mixing) when the sensors are carried by hand from the dark incubation chamber (pipette box) and into the electrode connector for measurement. This reduces the convection of the oxidised (blue) TMB away from the detection surface where it is being measured by chronoamperometry and translates into a higher current magnitude.

G Supplementary information - Waffle publication

Supporting Information

WAFFLE - An automated platform for enhancing the performance of electrochemical biosensors

*Alexandra Dobrea,^{*a,b} Rowan Blake,^c Daniel Macdonald,^c Cormack McKenzie^a, Yoann Altmann,^d Damion K. Corrigan^c and Melanie Jimenez^b*

^a*Biomedical Engineering Department, University of Strathclyde, Glasgow, G4 0NW, United Kingdom.*

^b*James Watt School of Engineering, University of Glasgow, Glasgow, G12 8QQ, United Kingdom.*

^c*Pure and Applied Chemistry Department, University of Strathclyde, Glasgow, G4 0NW, United Kingdom.*

^d*James Watt School of Engineering, Heriot-Watt University, EH14 4AS Edinburgh, UK*

^{*}Corresponding author: alexandra.dobrea@strath.ac.uk

TABLE S1 APTAMER SEQUENCES USED IN THIS STUDY		
Component	5'-3' Sequence	No. of bases
<i>Capture Aptamer</i> (C)	(thiC6) - TTT TTT TTT TTT TTT TTG TCG TCC CGA GAG	33
<i>Cortisol Aptamer</i> (A)	CTC TCG GGA CGA CGC CCG CAT GTT CCA TGG ATA GTC TTG ACT AGT CGT CCC	51

ss = single strand; thiC6 = thiol group

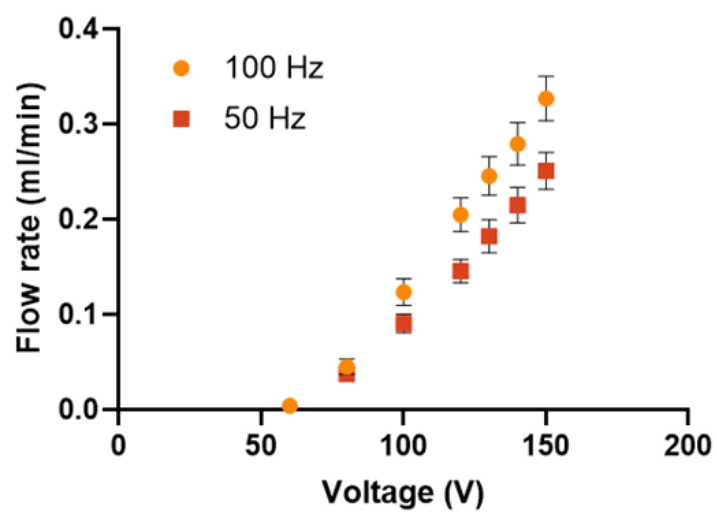


Figure S1. The effect of varying the pump frequency on the resultant flow rate ($n=3$ repeats for each condition). Each datapoint represents the average reading ± 1 standard deviation

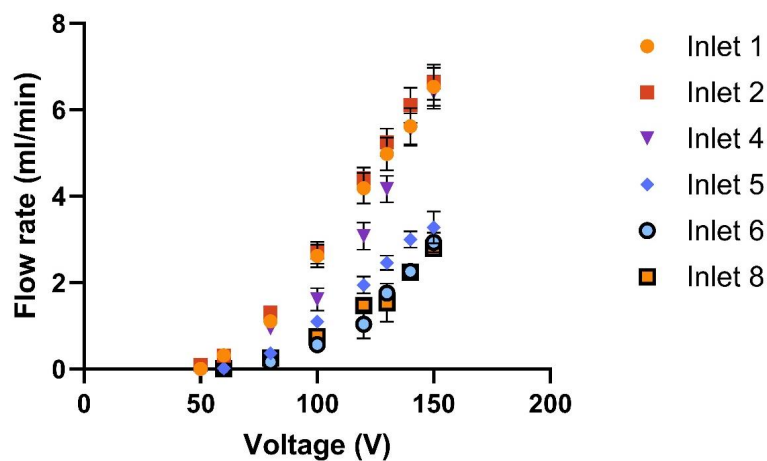


Figure S2. Differences in the resultant flow rates across the different inlets of the reagent cartridge as a function of the sine voltage amplitude applied to the Bartels micropump. Each datapoint represents the average reading ± 1 standard deviation

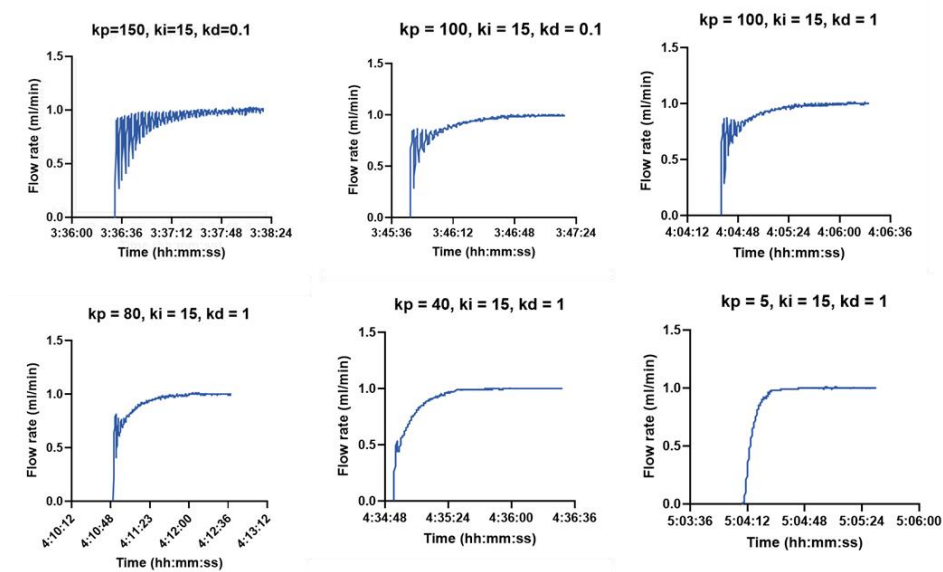


Figure S3. PID algorithm tuning. Flow rate setpoint = 1 ml/min

Table S2. Blank (0 pg/ml target) readings displayed in the main paper Figure 3

Condition/Sample tested	Figure	Blank current value $\pm$ 3 SD (nA)
Manual dose-response IL-6/PBS	3b – yellow (top) line	-4.0 ± 1.2
WAFFLE dose-response IL-6/PBS	3b – red (bottom) line	-10.6 ± 2.5
Manual dose-response IL-6/neat serum	3c – yellow (top) line	-9.7 ± 1.8
WAFFLE dose-response IL-6/neat serum	3c – red (bottom) line	-32.5 ± 3.3
Manual dose-response Tnl/neat serum	3e – yellow (top) line	-4.6 ± 1.5
WAFFLE dose-response Tnl/neat serum	3e – red (bottom) line	-8.9 ± 1.2

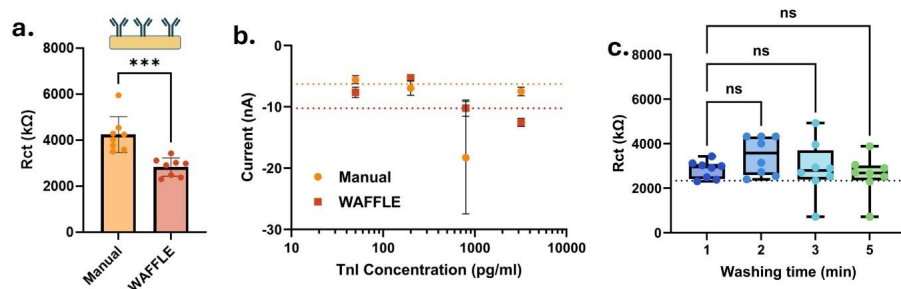


Figure S4. (a) The difference between a troponin antibody layer deposited on the electrode surface manually via pipetting or using the WAFFLE. (b) Dose responses of WAFFLE and manually made sensors to increasing concentrations of troponin (Tnl) spiked in neat troponin-depleted human serum. Each marker represents the average over 8 electrodes $\pm$ 1 SD. Dashed lines represent the blank reading $\pm$ 1SDs (n=8). (c) The effect of additional washing time on the troponin antibody layer (flow rate = 2 ml/min). Dashed line represents the impedance of the underlying EDC/NHS layer $\pm$ 1 SD (n=8), demonstrating effective attachment to the carbodiimide SAM.

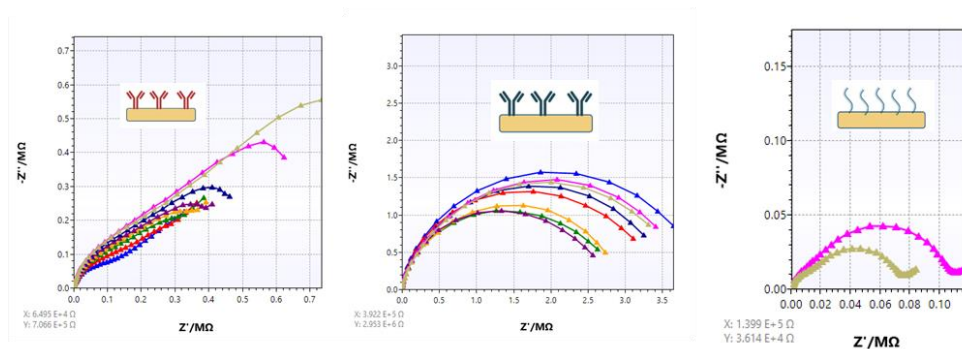


Figure S5. Different Nyquist plots acquired for electrodes ($n=8$ WEs per pannel) functionalised with (left) physisorbed IL-6 antibodies, (centre) TnI antibodies attached by EDC/NHS chemisorbtion on MUA SAM and (right) CSS.1 anchor aptamers attached to the surface via a thiol modification at the 5' end. Redox couple used for acquiring this data was 1 mM ferri-ferrocyanide in 1xPBS.

Bibliography

- [1] Khaleel I. Al-Obaidy and Muhammad T. Idrees. ‘Endometriosis With Cystic Degeneration: A Rare Disease of Males’. In: *International Journal of Surgical Pathology* 27.3 (2019), pp. 311–314. ISSN: 19402465. DOI: 10.1177/1066896918797438.
- [2] World Health Organization. *Endometriosis - fact sheets*. 2023. URL: <https://www.who.int/news-room/fact-sheets/detail/endometriosis>.
- [3] Caterina Morassutto et al. ‘Incidence and estimated prevalence of endometriosis and adenomyosis in Northeast Italy: A data linkage study’. In: *PLoS ONE* 11.4 (2016), pp. 1–11. ISSN: 19326203. DOI: 10.1371/journal.pone.0154227.
- [4] Christel Meuleman et al. ‘High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners’. In: *Fertility and Sterility* 92.1 (2009), pp. 68–74. ISSN: 00150282. DOI: 10.1016/j.fertnstert.2008.04.056. URL: <http://dx.doi.org/10.1016/j.fertnstert.2008.04.056>.
- [5] Stacey A. Missmer et al. ‘Impact of endometriosis on life-course potential: A narrative review’. In: *International Journal of General Medicine* 14 (2021), pp. 9–25. ISSN: 11787074. DOI: 10.2147/IJGM.S261139.
- [6] Steven Simoens et al. ‘The burden of endometriosis: Costs and quality of life of women with endometriosis and treated in referral centres’. In: *Human Reproduction* 27.5 (2012), pp. 1292–1299. ISSN: 14602350. DOI: 10.1093/humrep/des073.
- [7] Endometriosis UK. “‘Dismissed, ignored and belittled’ The long road to endometriosis diagnosis in the UK’. In: March (2024), pp. 1–23.

- [8] Krina T. Zondervan, Christian M. Becker and Stacey A. Missmer. ‘Endometriosis’. In: *The New England Journal of Medicine* 382.13 (2020), pp. 1244–1256. ISSN: 0028-4793. DOI: 10.1056/NEJMra1810764.
- [9] National Institute for Health and Care Excellence. *Endometriosis: diagnosis and management*. 2024. URL: <https://www.nice.org.uk/guidance/ng73>.
- [10] K. C. Schliep et al. ‘Endometriosis diagnosis and staging by operating surgeon and expert review using multiple diagnostic tools: an inter-rater agreement study’. In: *BJOG: An International Journal of Obstetrics and Gynaecology* 124.2 (2017), pp. 220–229. ISSN: 14710528. DOI: 10.1111/1471-0528.13711.
- [11] Linda C. Giudice, Andrew W. Horne and Stacey A. Missmer. ‘Time for global health policy and research leaders to prioritize endometriosis’. In: *Nature Communications* 14.1 (2023), pp. 1–4. ISSN: 20411723. DOI: 10.1038/s41467-023-43913-9.
- [12] Andrew W Horne and Stacey A Missmer. ‘Pathophysiology, diagnosis, and management of endometriosis’. In: *BMJ* 379 (2022). DOI: 10.1136/bmj-2022-070750. eprint: <https://www.bmj.com/content/379/bmj-2022-070750.full.pdf>. URL: <https://www.bmj.com/content/379/bmj-2022-070750>.
- [13] Philippa T K Saunders and Andrew W Horne. ‘Endometriosis : Etiology , pathobiology , and therapeutic prospects’. In: *Cell* 184.11 (2021), pp. 2807–2824. ISSN: 0092-8674. DOI: 10.1016/j.cell.2021.04.041. URL: <https://doi.org/10.1016/j.cell.2021.04.041>.
- [14] Sanjay K. Agarwal et al. ‘Clinical diagnosis of endometriosis: a call to action’. In: *American Journal of Obstetrics and Gynecology* 220.4 (2019), 354.e1–354.e12. ISSN: 10976868. DOI: 10.1016/j.ajog.2018.12.039. URL: <https://doi.org/10.1016/j.ajog.2018.12.039>.
- [15] Peter A.W. Rogers et al. ‘Research Priorities for Endometriosis: Recommendations from a Global Consortium of Investigators in Endometriosis’. In: *Reproductive Sciences* 24.2 (2017), pp. 202–226. ISSN: 19337205. DOI: 10.1177/1933719116654991.

- [16] Andrew W. Horne et al. ‘Top ten endometriosis research priorities in the UK and Ireland’. In: *The Lancet* 389.10085 (2017), pp. 2191–2192. ISSN: 1474547X. DOI: 10.1016/S0140-6736(17)31344-2.
- [17] Jodie Fryer et al. ‘Health Care for Women International Understanding diagnostic delay for endometriosis : A scoping review using the social- ecological framework Understanding diagnostic delay for endometriosis : A scoping review using the social-ecological framework’. In: *Health Care for Women International* 46.3 (2025), pp. 335–351. DOI: 10.1080/07399332.2024.2413056. URL: <https://doi.org/10.1080/07399332.2024.2413056>.
- [18] Vicki Nisenblat et al. ‘Blood biomarkers for the non-invasive diagnosis of endometriosis’. In: *Cochrane Database of Systematic Reviews* 2016.5 (2016). ISSN: 13616137. DOI: 10.1002/14651858.CD012179.
- [19] Zhao Tian et al. ‘Current biomarkers for the detection of endometriosis’. In: *Chinese Medical Journal* 133.19 (2020), pp. 2346–2352. ISSN: 25425641. DOI: 10.1097/CM9.0000000000001063.
- [20] Quanah J Hudson et al. ‘Challenges in uncovering non-invasive biomarkers of endometriosis’. In: *Experimental Biology and Medicine* (2020), pp. 437–447. DOI: 10.1177/1535370220903270.
- [21] Vicki Nisenblat et al. ‘Combination of the non-invasive tests for the diagnosis of endometriosis’. In: *Cochrane Database of Systematic Reviews* 2016.7 (2016). ISSN: 1469493X. DOI: 10.1002/14651858.CD012281.
- [22] Axelle Brulport et al. ‘An integrated multi-tissue approach for endometriosis candidate biomarkers: a systematic review’. In: *Reproductive Biology and Endocrinology* 22.1 (2024), pp. 1–26. ISSN: 14777827. DOI: 10.1186/s12958-023-01181-8. URL: <https://doi.org/10.1186/s12958-023-01181-8>.
- [23] Hugh S. Taylor, Alexander M. Kotlyar and Valerie A. Flores. ‘Endometriosis is a chronic systemic disease: clinical challenges and novel innovations’. In: *The Lancet* 397.10276 (2021), pp. 839–852. ISSN: 1474547X. DOI: 10.1016/S0140-6736(21)00389-5. URL: [http://dx.doi.org/10.1016/S0140-6736\(21\)00389-5](http://dx.doi.org/10.1016/S0140-6736(21)00389-5).

- [24] Matteo Morotti, Katy Vincent and Christian M Becker. ‘Mechanisms of pain in endometriosis’. In: *European Journal of Obstetrics and Gynecology* 209 (2017), pp. 8–13. ISSN: 0301-2115. DOI: 10.1016/j.ejogrb.2016.07.497. URL: <http://dx.doi.org/10.1016/j.ejogrb.2016.07.497>.
- [25] Elina Helosvuori. ‘Sensing pain : Embodied knowledge in endometriosis’. In: *Health* (2024). DOI: 10.1177/13634593231214938.
- [26] Xinrui Wang and Hongyan Wang. ‘Digital health technologies in pain management for patients with endometriosis : A scoping review’. In: *PLoS ONE* (2026), pp. 1–14. DOI: 10.1371/journal.pone.0345756. URL: <https://doi.org/10.1371/journal.pone.0345756>.
- [27] Tivizio Pavic, K Nadarajah and Alain Somat. ‘Endometriosis Support and Development of Digital Technology – Based Interventions : Systematic Review Corresponding Author :’ in: *JMIR HUMAN FACTORS Review* 12 (2025). DOI: 10.2196/71859.
- [28] CitizenEndo. *Research Projects at Citizen Endo*. 2026. URL: <https://citizenendo.org/research/>.
- [29] EndoCare Tech. *Endocare : Endo/Period Tracker*. URL: <https://play.google.com/store/apps/details?id=com.mmtech.endocare%7B%5C%7Dhl=en%7B%5C%7DGB%7B%5C%7Dpli=1>.
- [30] ENdi. *Endi App*. URL: <https://www.endiapp.com/> (visited on 29/07/2026).
- [31] Frendo. *Frendo*. URL: <https://www.frendo.co.uk/frendoapp> (visited on 29/07/2026).
- [32] Christian M Becker et al. ‘ESHRE guideline: endometriosis’. In: *Hum Reprod Open*. (2022), pp. 1–26. DOI: doi:10.1093/hropen/hoac009.
- [33] Wenwei Li, Huiyan Feng and Qingjian Ye. ‘Factors contributing to the delayed diagnosis of endometriosis — a systematic review and meta-analysis’. In: *Frontiers in Medicine* July (2025). DOI: 10.3389/fmed.2025.1576490.

- [34] Iñigo Urteaga. ‘Learning endometriosis phenotypes from patient-generated data’. In: *npj Digital Medicine* (2020). ISSN: 2398-6352. DOI: 10.1038/s41746-020-0292-9. URL: <http://dx.doi.org/10.1038/s41746-020-0292-9>.
- [35] Adrienne Pichon et al. ‘Augmenting the Clinical Data Sources for Enigmatic Diseases : A Cross-Sectional Study of Self-Tracking Data and Clinical Documentation in Endometriosis’. In: *Online resource* (2020).
- [36] Ipek Ensari et al. ‘Associations between physical exercise patterns and pain symptoms in individuals with endometriosis : a cross- sectional mHealth- based investigation’. In: *BMJ Open* (2022). DOI: 10.1136/bmjopen-2021-059280.
- [37] Yihan Xu et al. ‘Economic evaluation of a digital symptom checker for endometriosis using a Markov decision process model’. In: *npj / digital medicine* (2026), pp. 1–13.
- [38] Endometriosis Society. *How Wearable Tech Is Changing Endometriosis Tracking*. 2025. URL: <https://endometriosisassn.org/wearable-technology-for-endometriosis/> (visited on 16/07/2026).
- [39] Katherine Edgley et al. ‘Symptom tracking in endometriosis using digital technologies : Knowns , unknowns , and future prospects’. In: *Cell Reports Medicine* 4.9 (2023), p. 101192. ISSN: 2666-3791. DOI: 10.1016/j.xcrm.2023.101192. URL: <https://doi.org/10.1016/j.xcrm.2023.101192>.
- [40] Katherine Edgley et al. ‘Insights into endometriosis symptom trajectories and assessment of surgical intervention outcomes using longitudinal actigraphy’. In: *npj / digital medicine Published* (2025). DOI: 10.1038/s41746-025-01629-8.
- [41] Katja M Schmalenberger et al. ‘How to study the menstrual cycle: Practical tools and recommendations’. In: *Psychoneuroendocrinology* (2021). DOI: 10.31219/osf.io/94jua..
- [42] K. E. May et al. ‘Peripheral biomarkers of endometriosis: A systematic review’. In: *Human Reproduction Update* 16.6 (2010), pp. 651–674. ISSN: 13554786. DOI: 10.1093/humupd/dmq009.

- [43] Paloma Yáñez-Sedeño, Susana Campuzano and José M. Pingarrón. ‘Multiplexed electrochemical immunosensors for clinical biomarkers’. In: *Sensors (Switzerland)* 17.5 (2017). ISSN: 14248220. DOI: 10.3390/s17050965.
- [44] Jinsol Kim, Juho Jeong and Seung Hwan Ko. ‘Electrochemical biosensors for point-of-care testing’. In: *Bio-Design and Manufacturing* 7.4 (2024), pp. 548–565. ISSN: 25228552. DOI: 10.1007/s42242-024-00301-6. URL: <https://doi.org/10.1007/s42242-024-00301-6>.
- [45] Zhijun Li and Aiqin Zhong. ‘Electrochemical detection strategies for cardiovascular disease biomarkers: Applications in troponin I and troponin T assays’. In: *International Journal of Electrochemical Science* 19.8 (2024), p. 100675. ISSN: 14523981. DOI: 10.1016/j.ijoes.2024.100675. URL: <https://doi.org/10.1016/j.ijoes.2024.100675>.
- [46] Li Zhang et al. ‘Electrochemical biosensors represent promising detection tools in medical field’. In: *Advanced Sensor and Energy Materials* 2.4 (2023), p. 100081. ISSN: 2773045X. DOI: 10.1016/j.asems.2023.100081. URL: <https://doi.org/10.1016/j.asems.2023.100081>.
- [47] Lanpeng Guo et al. ‘Electrochemical protein biosensors for disease marker detection: progress and opportunities’. In: *Microsystems and Nanoengineering* 10.1 (2024). ISSN: 20557434. DOI: 10.1038/s41378-024-00700-w.
- [48] Ahmed Barhoum et al. ‘Modern designs of electrochemical sensor platforms for environmental analyses: Principles, nanofabrication opportunities, and challenges’. In: *Trends in Environmental Analytical Chemistry* 38.October 2022 (2023), e00199. ISSN: 22141588. DOI: 10.1016/j.teac.2023.e00199. URL: <https://doi.org/10.1016/j.teac.2023.e00199>.
- [49] Akbar Khanmohammadi et al. ‘An overview to electrochemical biosensors and sensors for the detection of environmental contaminants’. In: *Journal of the Iranian Chemical Society* 17.10 (2020), pp. 2429–2447. ISSN: 17352428. DOI: 10.1007/s13738-020-01940-z. URL: <https://doi.org/10.1007/s13738-020-01940-z>.

- [50] Md Saiful Islam et al. ‘Electrochemical Biosensor for Evaluation of Environmental Pollutants Toxicity’. In: *Environments - MDPI* 10.4 (2023), pp. 1–22. ISSN: 20763298. DOI: 10.3390/environments10040063.
- [51] Ali Sahragard, Pakorn Varanusupakul and Manuel Miró. ‘Nanomaterial decorated electrodes in flow-through electrochemical sensing of environmental pollutants: A critical review’. In: *Trends in Environmental Analytical Chemistry* 39.July (2023). ISSN: 22141588. DOI: 10.1016/j.teac.2023.e00208.
- [52] M. R. Hasan et al. ‘Recent development in electrochemical biosensors for cancer biomarkers detection’. In: *Biosensors and Bioelectronics: X* 8.July (2021), p. 100075. ISSN: 25901370. DOI: 10.1016/j.biosx.2021.100075. URL: <https://doi.org/10.1016/j.biosx.2021.100075>.
- [53] Jae Hyun Kim et al. ‘Technological advances in electrochemical biosensors for the detection of disease biomarkers’. In: *Biomedical Engineering Letters* 11.4 (2021), pp. 309–334. ISSN: 2093985X. DOI: 10.1007/s13534-021-00204-w. URL: <https://doi.org/10.1007/s13534-021-00204-w>.
- [54] Shipeng Gao, José M. Guisán and Javier Rocha-Martin. ‘Oriented immobilization of antibodies onto sensing platforms - A critical review’. In: *Analytica Chimica Acta* 1189 (2022). ISSN: 18734324. DOI: 10.1016/j.aca.2021.338907.
- [55] Mitkumar Patel, Mayuri Agrawal and Akshay Srivastava. ‘Signal amplification strategies in electrochemical biosensors via antibody immobilization and nanomaterial-based transducers’. In: *Materials Advances* 17 (2022), pp. 8864–8885. ISSN: 26335409. DOI: 10.1039/d2ma00427e.
- [56] M. Jalal Uddin, Nabil H. Bhuiyan and Joon S. Shim. ‘Fully integrated rapid microfluidic device translated from conventional 96-well ELISA kit’. In: *Scientific Reports* 11.1 (2021), pp. 1–9. ISSN: 20452322. DOI: 10.1038/s41598-021-81433-y. URL: <https://doi.org/10.1038/s41598-021-81433-y>.
- [57] Talha Jamshaid et al. ‘Magnetic particles: From preparation to lab-on-a-chip, biosensors, microsystems and microfluidics applications’. In: *TrAC - Trends in Analytical Chemistry* 79 (2016), pp. 344–362. ISSN: 18793142. DOI: 10.1016/j.trac.2015.10.022. URL: <http://dx.doi.org/10.1016/j.trac.2015.10.022>.

- [58] Monica Mattarozzi et al. ‘Aptamer-based assays: strategies in the use of aptamers conjugated to magnetic micro- and nanobeads as recognition elements in food control’. In: *Analytical and Bioanalytical Chemistry* 414.1 (2022), pp. 63–74. ISSN: 16182650. DOI: 10.1007/s00216-021-03501-6.
- [59] Suchi Mercy George, Saloni Tandon and Balasubramanian Kandasubramanian. ‘Advancements in Hydrogel-Functionalized Immunosensing Platforms’. In: *ACS Omega* 5.5 (2020), pp. 2060–2068. ISSN: 24701343. DOI: 10.1021/acsomega.9b03816.
- [60] Jie Wu et al. ‘Device integration of electrochemical biosensors’. In: *Nature Reviews Bioengineering* 1.5 (2023), pp. 346–360. ISSN: 2731-6092. DOI: 10.1038/s44222-023-00032-w. URL: <http://dx.doi.org/10.1038/s44222-023-00032-w>.
- [61] Niamh Docherty et al. ‘Maximising the translation potential of electrochemical biosensors’. In: *Chem. Commun.* 61 (71 2025), pp. 13359–13377. DOI: 10.1039/D5CC02322J. URL: <http://dx.doi.org/10.1039/D5CC02322J>.
- [62] Avinash V. Police Patil et al. ‘Recent Advances in Electrochemical Immunosensors with Nanomaterial Assistance for Signal Amplification’. In: *Biosensors* 13.1 (2023). ISSN: 20796374. DOI: 10.3390/bios13010125.
- [63] Ewen O. Blair and Damion K. Corrigan. ‘A review of microfabricated electrochemical biosensors for DNA detection’. In: *Biosensors and Bioelectronics* 134.March (2019), pp. 57–67. ISSN: 18734235. DOI: 10.1016/j.bios.2019.03.055. URL: <https://doi.org/10.1016/j.bios.2019.03.055>.
- [64] Jaligam Murali Mohan et al. ‘Emerging trends in miniaturized and microfluidic electrochemical sensing platforms’. In: *Current Opinion in Electrochemistry* 33 (2022), p. 100930. ISSN: 24519111. DOI: 10.1016/j.coelec.2021.100930. URL: <https://doi.org/10.1016/j.coelec.2021.100930>.
- [65] Ana Fernández-la-Villa, Diego F. Pozo-Ayuso and M. Castaño-Álvarez. ‘Microfluidics and electrochemistry: an emerging tandem for next-generation analytical microsystems’. In: *Current Opinion in Electrochemistry* 15 (2019), pp. 175–185. ISSN: 24519111. DOI: 10.1016/j.coelec.2019.05.014. URL: <https://doi.org/10.1016/j.coelec.2019.05.014>.

- [66] Darius G. Rackus, Mohtashim H. Shamsi and Aaron R. Wheeler. ‘Electrochemistry, biosensors and microfluidics: a convergence of fields’. In: *Chemical Society Reviews* 44.15 (2015), pp. 5320–5340. ISSN: 14604744. DOI: 10.1039/c4cs00369a.
- [67] Catherine B Wykes, Justin Clark and Khalid S Khan. ‘Accuracy of laparoscopy in the diagnosis of endometriosis: a systematic quantitative review’. In: *BJOG: An International Journal of Obstetrics & Gynaecology* 111.November (2004), pp. 1204–1212. DOI: 10.1111/j.1471-0528.2004.00433.x.
- [68] Emily Liu et al. ‘Urinary biomarkers for the non-invasive diagnosis of endometriosis’. In: *Cochrane Database of Systematic Reviews* 2015.12 (2015). ISSN: 1469493X. DOI: 10.1002/14651858.CD012019.
- [69] Lauren E. McCrae, Wei Ting Ting and Matiar M.R. Howlader. ‘Advancing electrochemical biosensors for interleukin-6 detection’. In: *Biosensors and Bioelectronics: X* 13.October 2022 (2023). ISSN: 25901370. DOI: 10.1016/j.biosx.2022.100288.
- [70] Caroline B. Appleyard, Idhaliz Flores and Annelyn Torres-Reverón. ‘The Link Between Stress and Endometriosis: from Animal Models to the Clinical Scenario’. In: *Reproductive Sciences* 27.9 (2020), pp. 1675–1686. ISSN: 19337205. DOI: 10.1007/s43032-020-00205-7.
- [71] Mieke van Aken et al. ‘Hair cortisol and the relationship with chronic pain and quality of life in endometriosis patients’. In: *Psychoneuroendocrinology* 89.December 2017 (2018), pp. 216–222. ISSN: 18733360. DOI: 10.1016/j.psyneuen.2018.01.001. URL: <https://doi.org/10.1016/j.psyneuen.2018.01.001>.
- [72] Fernando M. Reis et al. ‘Is Stress a Cause or a Consequence of Endometriosis?’ In: *Reproductive Sciences* 27.1 (2020), pp. 39–45. ISSN: 19337205. DOI: 10.1007/s43032-019-00053-0.
- [73] Miguel Zea et al. ‘Electrochemical sensors for cortisol detections: Almost there’. In: *TrAC - Trends in Analytical Chemistry* 132 (2020), p. 116058. ISSN: 18793142. DOI: 10.1016/j.trac.2020.116058. URL: <https://doi.org/10.1016/j.trac.2020.116058>.

- [74] John J. Coté et al. ‘Serum Interleukin-6 Levels in Endometriosis and Endometriosis-Associated Ovarian Cancer: A Systematic Review and Meta-Analysis Demonstrating a Biological Gradient’. In: (2026).
- [75] S Martínez et al. ‘Serum interleukin-6 levels are elevated in women with minimal – mild endometriosis’. In: *Human Reproduction* 22.3 (2007), pp. 836–842. DOI: 10.1093/humrep/de1419.
- [76] K. F.S. Petrelluzzi et al. ‘Salivary cortisol concentrations, stress and quality of life in women with endometriosis and chronic pelvic pain’. In: *Stress* 11.5 (2008), pp. 390–397. ISSN: 10253890. DOI: 10.1080/10253890701840610.
- [77] Maria Quiñones et al. ‘Anxiety, coping skills and hypothalamus-pituitary-adrenal (HPA) axis in patients with endometriosis’. In: *J Reprod Biol Health* (2015). DOI: 10.7243/2054-0841-3-2.
- [78] Minal Pandey et al. ‘Advanced Materials for Biological Field-Effect Transistors (Bio-FETs) in Precision Healthcare and Biosensing’. In: *Advanced Healthcare Materials* 2500400 (2025), pp. 1–34. DOI: 10.1002/adhm.202500400.
- [79] Daeun Sung and Jahyun Koo. ‘A review of BioFET ’ s basic principles and materials for biomedical applications’. In: *Biomedical Engineering Letters* 11.2 (2021), pp. 85–96. ISSN: 2093-985X. DOI: 10.1007/s13534-021-00187-8. URL: <https://doi.org/10.1007/s13534-021-00187-8>.
- [80] Ie Mei Bhattacharyya and Gil Shalev. ‘Electrostatically Governed Debye Screening Length at the Solution- Solid Interface for Biosensing Applications’. In: *ACS Sensors* (2020). DOI: 10.1021/acssensors.9b01939.
- [81] Aleksandar Vacic et al. ‘Determination of Molecular Configuration by Debye Length Modulation’. In: *Journal of American Chemical Society* (2011), pp. 13886–13889.
- [82] I B O Connor and J G Wall. *Immobilization of antibodies on cardiovascular stents. Section 16.2.1 Immunoglobulin structure and target binding*. Elsevier Ltd., 2018, pp. 319–341. ISBN: 9780081004968. DOI: 10.1016/B978-0-08-100496-8.00017-2. URL: <https://doi.org/10.1016/B978-0-08-100496-8.00017-2>.

- [83] Allen J. Bard and Larry R. Faulkner. *Electrochemical Methods: Fundamentals and Applications*. Ed. by David Harris et al. 2nd. John Wiley & Sons, 2001.
- [84] Satish K. Tuteja, Connor Ormsby and Suresh Neethirajan. ‘Noninvasive Label-Free Detection of Cortisol and Lactate Using Graphene Embedded Screen-Printed Electrode’. In: *Nano-Micro Letters* 10.3 (2018), pp. 1–10. ISSN: 21505551. DOI: 10.1007/s40820-018-0193-5. URL: <https://doi.org/10.1007/s40820-018-0193-5>.
- [85] Stuart Hannah et al. ‘Rapid antibiotic susceptibility testing using low-cost, commercially available screen-printed electrodes’. In: *Biosensors and Bioelectronics* 145 (2019). ISSN: 18734235. DOI: 10.1016/j.bios.2019.111696.
- [86] Andrea Cruz et al. ‘Non-invasive diagnosis and monitoring tool of children ’ s mental health : A point-of-care immunosensor for IL-6 quantification in saliva samples’. In: September (2022), pp. 1–10. DOI: 10.3389/fnins.2022.919551.
- [87] Cameron L Bentley, David Perry and Patrick R Unwin. ‘Stability and Placement of Ag/AgCl Quasi-Reference Counter Electrodes in Con fi ned Electrochemical Cells’. In: *Analytical Chemistry* (2018). DOI: 10.1021/acs.analchem.8b01588.
- [88] Iman Khosravi Bigdeli et al. *Electrochemical impedance spectroscopy (EIS) for biosensing*. Elsevier Inc., 2021, pp. 533–554. ISBN: 9780128233580. DOI: 10.1016/B978-0-12-823358-0.00025-3. URL: <http://dx.doi.org/10.1016/B978-0-12-823358-0.00025-3>.
- [89] Xinke Liu et al. ‘Salivary Electrochemical Cortisol Biosensor Based on Tin Disulfide Nanoflakes’. In: *Nanoscale Research Letters* 14 (2019), pp. 1–9. ISSN: 1556276X. DOI: 10.1186/s11671-019-3012-0.
- [90] Nidhi Dhull et al. ‘Highly sensitive and non-invasive electrochemical immunosensor for salivary cortisol detection’. In: *Sensors and Actuators, B: Chemical* 293.December 2018 (2019), pp. 281–288. ISSN: 09254005. DOI: 10.1016/j.snb.2019.05.020. URL: <https://doi.org/10.1016/j.snb.2019.05.020>.

- [91] Yi Fang, Yogeswaran Umasankar and Ramaraja P. Ramasamy. ‘A novel bi-enzyme electrochemical biosensor for selective and sensitive determination of methyl salicylate’. In: *Biosensors and Bioelectronics* 81 (2016), pp. 39–45. ISSN: 18734235. DOI: 10.1016/j.bios.2016.01.095. URL: <http://dx.doi.org/10.1016/j.bios.2016.01.095>.
- [92] Sofia G. Meirinho et al. ‘Development of an electrochemical RNA-aptasensor to detect human osteopontin’. In: *Biosensors and Bioelectronics* 71 (2015), pp. 332–341. ISSN: 18734235. DOI: 10.1016/j.bios.2015.04.050.
- [93] Luiz Otávio Orzari et al. ‘A novel disposable self-adhesive inked paper device for electrochemical sensing of dopamine and serotonin neurotransmitters and biosensing of glucose’. In: *Biosensors and Bioelectronics* 138. February (2019), p. 111310. ISSN: 18734235. DOI: 10.1016/j.bios.2019.05.015. URL: <https://doi.org/10.1016/j.bios.2019.05.015>.
- [94] Noémie Elgrishi et al. ‘A Practical Beginner’s Guide to Cyclic Voltammetry’. In: *Journal of Chemical Education* 95.2 (2018), pp. 197–206. ISSN: 19381328. DOI: 10.1021/acs.jchemed.7b00361.
- [95] Pablo Fanjul-Bolado, María Begoña González-García and Agustín Costa-García. ‘Amperometric detection in TMB/HRP-based assays’. In: *Analytical and Bioanalytical Chemistry* 382.2 (2005), pp. 297–302. ISSN: 16182642. DOI: 10.1007/s00216-005-3084-9.
- [96] Xiaochun Li, Iveta Triskova and Libuse Trnkova. ‘A new experimental approach to understanding microscopic details of the electrode/electrolyte interface’. In: *Electrochimica Acta* 442. January (2023), p. 141921. ISSN: 00134686. DOI: 10.1016/j.electacta.2023.141921. URL: <https://doi.org/10.1016/j.electacta.2023.141921>.
- [97] Seyed Saeed Madani, Erik Schaltz and Søren Knudsen Kær. ‘A Review of Different Electric Equivalent Circuit Models and Parameter Identification Methods of Lithium-Ion Batteries’. In: *ECS Transactions* 87.1 (2018), pp. 23–37. ISSN: 1938-6737. DOI: 10.1149/08701.0023ecst.

- [98] Mark E. Orazem and Bernard Tribollet. *Electrochemical Impedance Spectroscopy*. John Wiley & Sons, Ltd, 2008. ISBN: 9780470381588.
- [99] Jean-Luc Dellis. *Zfit*. 2025. URL: <https://uk.mathworks.com/matlabcentral/fileexchange/19460-zfit>.
- [100] Ankush Medhi and D. Mohanta. ‘Deciphering Highly Sensitive Non-Enzymatic Glucose Sensor Based on Nanoscale CuO/PEDOT-MoS₂ Electrodes in Chronoamperometry’. In: *ECS Advances* 1.4 (2022). ISSN: 27542734. DOI: 10.1149/2754-2734/ac9324.
- [101] K. Yugender Goud et al. ‘Wearable Electrochemical Microneedle Sensor for Continuous Monitoring of Levodopa: Toward Parkinson Management’. In: *ACS Sensors* 4.8 (2019), pp. 2196–2204. ISSN: 23793694. DOI: 10.1021/acssensors.9b01127.
- [102] Katesara Phasuksom and Anuvat Sirivat. ‘Chronoamperometric detection of enzymatic glucose sensor based on doped polyindole/MWCNT composites modified onto screen-printed carbon electrode as portable sensing device for diabetes’. In: *RSC Advances* 12.44 (2022), pp. 28505–28518. ISSN: 20462069. DOI: 10.1039/d2ra04947c.
- [103] Sylwia Baluta et al. ‘Differential pulse voltammetry and chronoamperometry as analytical tools for epinephrine detection using a tyrosinase-based electrochemical biosensor’. In: *RSC Advances* 12.39 (2022), pp. 25342–25353. ISSN: 20462069. DOI: 10.1039/d2ra04045j.
- [104] Theodora W. von Zuben, Airtton G. Salles and Juliano A. Bonacin. ‘Low-cost open-source potentiostats: A comprehensive review of DIY solutions and fundamental concepts of electronics and its integration with electrochemistry’. In: *Electrochimica Acta* 498.June (2024), p. 144619. ISSN: 00134686. DOI: 10.1016/j.electacta.2024.144619. URL: <https://doi.org/10.1016/j.electacta.2024.144619>.
- [105] Raquel Sánchez-Salcedo et al. ‘Comparing nanobody and aptamer-based capacitive sensing for detection of interleukin-6 (IL-6) at physiologically relevant levels’. In: *Analytical and Bioanalytical Chemistry* 6.0123456789 (2023). ISSN: 16182650. DOI: 10.1007/s00216-023-04973-4. URL: <https://doi.org/10.1007/s00216-023-04973-4>.

- [106] L. S.Selva Kumar et al. ‘Label free nano-aptasensor for interleukin-6 in protein-dilute bio fluids such as sweat’. In: *Analytical Methods* 8.17 (2016), pp. 3440–3444. ISSN: 17599679. DOI: 10.1039/c6ay00331a.
- [107] Yuji Gao et al. ‘A flexible multiplexed immunosensor for point-of-care in situ wound monitoring’. In: *Science Advances* 7.21 (2021), pp. 1–15. ISSN: 23752548. DOI: 10.1126/sciadv.abg9614.
- [108] Yesim Tugce Yaman et al. ‘Peptide nanotubes/self-assembled polydopamine molecularly imprinted biochip for the impedimetric detection of human Interleukin-6’. In: *Bioelectrochemistry* 145 (2022), p. 108053. ISSN: 1878562X. DOI: 10.1016/j.bioelechem.2022.108053. URL: <https://doi.org/10.1016/j.bioelechem.2022.108053>.
- [109] Daniela Oliveira et al. ‘Molecular Imprinted Polymers on Microneedle Arrays for Point of Care Transdermal Sampling and Sensing of Inflammatory Biomarkers’. In: *ACS Omega* 7.43 (2022), pp. 39039–39044. ISSN: 24701343. DOI: 10.1021/acsomega.2c04789.
- [110] Elif Burcu Aydın, Muhammet Aydın and Mustafa Kemal Sezgintürk. ‘A novel electrochemical immunosensor based on acetylene black/epoxy-substituted-polypyrrole polymer composite for the highly sensitive and selective detection of interleukin 6’. In: *Talanta* 222.June 2020 (2021), p. 121596. ISSN: 00399140. DOI: 10.1016/j.talanta.2020.121596. URL: <https://doi.org/10.1016/j.talanta.2020.121596>.
- [111] Christopher Russell et al. ‘Development of a needle shaped microelectrode for electrochemical detection of the sepsis biomarker interleukin-6 (IL-6) in real time’. In: *Biosensors and Bioelectronics* 126.December 2018 (2019), pp. 806–814. ISSN: 18734235. DOI: 10.1016/j.bios.2018.11.053. URL: <https://doi.org/10.1016/j.bios.2018.11.053>.

- [112] Chiye Zhang et al. ‘Microfluidic electrochemical magnetoimmunosensor for ultra-sensitive detection of interleukin-6 based on hybrid of AuNPs and graphene’. In: *Talanta* 240.December 2021 (2022), p. 123173. ISSN: 00399140. DOI: 10.1016/j.talanta.2021.123173. URL: <https://doi.org/10.1016/j.talanta.2021.123173>.
- [113] Meng Qi et al. ‘Graphene Oxide Thin Film with Dual Function Integrated into a Nanosandwich Device for in Vivo Monitoring of Interleukin-6’. In: *ACS Applied Materials and Interfaces* 9.48 (2017), pp. 41659–41668. ISSN: 19448252. DOI: 10.1021/acsami.7b10753.
- [114] Huinan Chen et al. ‘The Applications of Electrochemical Immunosensors in the Detection of Disease Biomarkers: A Review’. In: *Molecules* 28.8 (2023). ISSN: 14203049. DOI: 10.3390/molecules28083605.
- [115] Ulf Andreasson et al. ‘A practical guide to immunoassay method validation’. In: *Frontiers in Neurology* 6.Aug (2015), pp. 1–8. ISSN: 16642295. DOI: 10.3389/fneur.2015.00179.
- [116] Karen L. Cox et al. ‘Immunoassay methods’. In: *Assay Guidance Manual* 1.Md (2020), pp. 404–408. URL: https://www.ncbi.nlm.nih.gov/books/NBK53196/%20pdf/Bookshelf%7B%5C_%7DNBK53196.pdf.
- [117] John R. Crowther. *Elisa Theory and Practice*. Vol. 42. Humana Press, 1995. URL: http://scioteca.caf.com/bitstream/handle/123456789/1091/RED2017-Eng-8ene.pdf?sequence=12%7B%5C_%7DisAllowed=y%7B%5C_%7D0Ahttp://dx.doi.org/10.1016/j.regsciurbeco.2008.06.005%7B%5C_%7D0Ahttps://www.researchgate.net/publication/305320484%7B%5C_%7DSISTEM%7B%5C_%7DPEMBETUNGAN%7B%5C_%7DTERPUSAT%7B%5C_%7DSTRATEGI%7B%5C_%7DMELESTARI.
- [118] Mirian Hervás, Miguel A. López and Alberto Escarpa. ‘Electrochemical immunosensing on board microfluidic chip platforms’. In: *TrAC - Trends in Analytical Chemistry* 31 (2012), pp. 109–128. ISSN: 01659936. DOI: 10.1016/j.trac.2011.06.020.

- [119] David I. Walsh et al. 'Enabling Microfluidics: from Clean Rooms to Makerspaces'. In: *Trends in Biotechnology* 35.5 (2017), pp. 383–392. ISSN: 18793096. DOI: 10.1016/j.tibtech.2017.01.001.
- [120] R Cancelliere et al. 'Label-free electrochemical immunosensors : A practical guide'. In: *Trends in Analytical Chemistry* 180. February (2024), p. 117949. ISSN: 0165-9936. DOI: 10.1016/j.trac.2024.117949. URL: <https://doi.org/10.1016/j.trac.2024.117949>.
- [121] Gorachand Dutta and Peter B. Lillehoj. 'An ultrasensitive enzyme-free electrochemical immunosensor based on redox cycling amplification using methylene blue'. In: *Analyst* 142. August (2017), pp. 3492–3499. ISSN: 0003-2654. DOI: 10.1039/c7an00789b.
- [122] Bolu Sun et al. 'Electrochemical immunosensor based on ferrocene derivatives amplified signal for detection of acute myocardial infarction warning biomarker-cTnI'. In: *Microchemical Journal* 199. January (2024), p. 110057. ISSN: 0026-265X. DOI: 10.1016/j.microc.2024.110057. URL: <https://doi.org/10.1016/j.microc.2024.110057>.
- [123] Zihua Wang et al. 'A novel oriented immunosensor based on AuNPs-thionine-CMWCNTs and staphylococcal protein A for interleukin-6 analysis in complicated biological samples'. In: *Analytica Chimica Acta* 1140 (2020), pp. 145–152. ISSN: 0003-2670. DOI: 10.1016/j.aca.2020.10.025. URL: <https://doi.org/10.1016/j.aca.2020.10.025>.
- [124] Parvin Samadi et al. 'A novel electrochemical immunosensor for ultrasensitive detection of CA125 in ovarian cancer'. In: *Biosensors and Bioelectronics* 153. January (2020), p. 112029. ISSN: 0956-5663. DOI: 10.1016/j.bios.2020.112029. URL: <https://doi.org/10.1016/j.bios.2020.112029>.
- [125] Luciana De Souza Freire et al. 'An Electrochemical Immunosensor Based on Carboxylated Graphene / SPCE for IgG-SARS-CoV-2'. In: *MDPI Biosensors* M (2022).
- [126] Seo-eun Lee et al. 'Gold-Nanoparticle-Coated Magnetic Beads for ALP-Enzyme-Based Electrochemical Immunosensing in Human Plasma'. In: *MDPI Materials* (2022).

- [127] Bhargav D Mansuriya and Zeynep Altintas. ‘Enzyme-Free Electrochemical Nano-Immunosensor Based on Graphene Quantum Dots and Gold Nanoparticles for Cardiac Biomarker Determination’. In: *MDPI Nanomaterials* (2021).
- [128] Lixin Zhao et al. ‘A nanozyme - based competitive electrochemical immunosensor for the determination of E - selectin’. In: *Microchimica Acta* (2022). ISSN: 1436-5073. DOI: 10.1007/s00604-022-05495-z. URL: <https://doi.org/10.1007/s00604-022-05495-z>.
- [129] Anton Popov et al. ‘Electrochemistry Ultra-sensitive electrochemical immunosensors for clinically important biomarker detection : Prospects , opportunities , and global trends’. In: *Current Opinion in Electrochemistry* 46 (2024), p. 101524. ISSN: 2451-9103. DOI: 10.1016/j.coelec.2024.101524. URL: <https://doi.org/10.1016/j.coelec.2024.101524>.
- [130] Andrew D. Ellington and Jack W. Szostak. ‘In vitro selection of RNA molecules that bind specific ligands’. In: *Nature* 346.6287 (1990), pp. 818–822. ISSN: 00280836. DOI: 10.1038/346818a0.
- [131] Craig Tuerk and Larry Gold. ‘Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase’. In: 9 (), pp. 505–510.
- [132] Sina Ardalan and Anna Ignaszak. ‘Can Electrochemical Aptasensors Achieve the Commercial Success of Glucose Biosensors?’ In: *Advanced Sensor Research* 3.4 (2024), pp. 1–7. ISSN: 2751-1219. DOI: 10.1002/adsr.202300132.
- [133] Maureen McKeague et al. ‘The minimum aptamer publication standards (MAPS guidelines) for de novo aptamer selection’. In: *Aptamers* 6.2514-3247 (2022), pp. 10–18. ISSN: 2514-3247.
- [134] Kyung Ae Yang et al. ‘High-Affinity Nucleic-Acid-Based Receptors for Steroids’. In: *ACS Chemical Biology* 12.12 (2017), pp. 3103–3112. ISSN: 15548937. DOI: 10.1021/acscchembio.7b00634.

- [135] Shima Dalirirad, Daewoo Han and Andrew J. Steckl. ‘Aptamer-Based Lateral Flow Biosensor for Rapid Detection of Salivary Cortisol’. In: *ACS Omega* 5.51 (2020), pp. 32890–32898. ISSN: 24701343. DOI: 10.1021/acsomega.0c03223.
- [136] Yanan Luan et al. ‘Wearable Sensing Device Integrated with Prestored Reagents for Cortisol Detection in Sweat’. In: *ACS Sensors* (2024). ISSN: 23793694. DOI: 10.1021/acssensors.4c00112.
- [137] Azrulsyafiqzainol Abidin et al. ‘Current and potential developments of cortisol aptasensing towards point-of-care diagnostics (POTC)’. In: *Sensors (Switzerland)* 17.5 (2017). ISSN: 14248220. DOI: 10.3390/s17051180.
- [138] Ajeet Kaushik et al. ‘Recent advances in cortisol sensing technologies for point-of-care application’. In: *Biosensors and Bioelectronics* 53 (2014), pp. 499–512. ISSN: 09565663. DOI: 10.1016/j.bios.2013.09.060. URL: <http://dx.doi.org/10.1016/j.bios.2013.09.060>.
- [139] Chenqi Niu et al. ‘Comparing two cortisol aptamers for label-free fluorescent and colorimetric biosensors’. In: *Sensors and Diagnostics* 1.3 (2022), pp. 541–549. ISSN: 26350998. DOI: 10.1039/d2sd00042c.
- [140] Fang Zhang and Juewen Liu. ‘Label-Free Colorimetric Biosensors Based on Aptamers and Gold Nanoparticles: A Critical Review’. In: *Analysis and Sensing* 1.1 (2021), pp. 30–43. ISSN: 26292742. DOI: 10.1002/anse.202000023.
- [141] Sheng Wang et al. ‘A Handheld Plug-and-Play Microfluidic Liquid Handling Automation Platform for Immunoassays’. In: *SSRN Electronic Journal* (2022). DOI: 10.2139/ssrn.4141268.
- [142] Sheng Wang, You Zhou and Zhenyu Li. ‘A microfluidic cover converts a standard 96-well plate into a mass-transport-controlled immunoassay system’. In: *Biomicrofluidics* 18.1 (2024). ISSN: 19321058. DOI: 10.1063/5.0183651.
- [143] Inês E. Silva et al. ‘A Versatile Platform for Point-of-Care Detection of Molecular Biomarkers’. In: *IEEE Sensors Journal* 24.16 (2024), pp. 26388–26396. ISSN: 15581748. DOI: 10.1109/JSEN.2024.3423678.

- [144] Richard P.S. De Campos et al. ‘“plug-n-Play” Sensing with Digital Microfluidics’. In: *Analytical Chemistry* 91.3 (2019), pp. 2506–2515. ISSN: 15206882. DOI: 10.1021/acs.analchem.8b05375.
- [145] Subramanian Nellaiappan et al. ‘Electrochemical flow injection analysis platforms for biomedical applications – Progress and prospects’. In: *Analytical Biochemistry* 700.November 2024 (2025). ISSN: 10960309. DOI: 10.1016/j.ab.2025.115795.
- [146] Orawon Chailapakul et al. ‘Recent electrochemical and optical sensors in flow-based analysis’. In: *Sensors* 6.10 (2006), pp. 1383–1410. ISSN: 14248220. DOI: 10.3390/s6101383.
- [147] Yuxing Shi et al. ‘Application of centrifugal microfluidics in immunoassay, biochemical analysis and molecular diagnosis’. In: *Analyst* 146.19 (2021), pp. 5800–5821. ISSN: 13645528. DOI: 10.1039/d1an00629k.
- [148] Jingwei Yi et al. ‘Multiplexed digital ELISA in picoliter droplets based on enzyme signal amplification block and precisely decoding strategy: A universal and practical biodetection platform’. In: *Sensors and Actuators B: Chemical* 369.May (2022), p. 132214. ISSN: 09254005. DOI: 10.1016/j.snb.2022.132214. URL: <https://doi.org/10.1016/j.snb.2022.132214>.
- [149] Gareth W.H. Evans et al. ‘A portable droplet microfluidic device for cortisol measurements using a competitive heterogeneous assay’. In: *Analyst* 146.14 (2021), pp. 4535–4544. ISSN: 13645528. DOI: 10.1039/d1an00671a.
- [150] Madhurima Borah and Hemant Sankar Dutta. ‘Advances in paper-based ELISA techniques: From innovations in devices to emerging applications’. In: *TrAC - Trends in Analytical Chemistry* 184.October 2024 (2025), p. 118123. ISSN: 18793142. DOI: 10.1016/j.trac.2024.118123. URL: <https://doi.org/10.1016/j.trac.2024.118123>.
- [151] Cody Carrell et al. ‘Capillary driven microfluidic sequential flow device for point-of-need ELISA: COVID-19 serology testing’. In: *Analytical Methods* 15.22 (2023), pp. 2721–2728. ISSN: 17599679. DOI: 10.1039/d3ay00225j.

- [152] Haitao Zhao et al. ‘Accessible detection of SARS-CoV-2 through molecular nanostructures and automated microfluidics’. In: *Biosensors and Bioelectronics* 194. August (2021), p. 113629. ISSN: 18734235. DOI: 10.1016/j.bios.2021.113629. URL: <https://doi.org/10.1016/j.bios.2021.113629>.
- [153] Yildiz Uludag et al. ‘An integrated lab-on-a-chip-based electrochemical biosensor for rapid and sensitive detection of cancer biomarkers’. In: *Analytical and Bioanalytical Chemistry* 408.27 (2016), pp. 7775–7783. ISSN: 16182650. DOI: 10.1007/s00216-016-9879-z.
- [154] Zeynep Altintas et al. ‘A fully automated microfluidic-based electrochemical sensor for real-time bacteria detection’. In: *Biosensors and Bioelectronics* 100. July 2017 (2018), pp. 541–548. ISSN: 18734235. DOI: 10.1016/j.bios.2017.09.046. URL: <http://dx.doi.org/10.1016/j.bios.2017.09.046>.
- [155] Yıldız Uludag et al. ‘Lab-on-a-chip based biosensor for the real-time detection of aflatoxin’. In: *Talanta* 160 (2016), pp. 381–388. ISSN: 00399140. DOI: 10.1016/j.talanta.2016.07.060.
- [156] Samuel B. Dulay et al. ‘Automated microfluidically controlled electrochemical biosensor for the rapid and highly sensitive detection of *Francisella tularensis*’. In: *Biosensors and Bioelectronics* 59 (2014), pp. 342–349. ISSN: 18734235. DOI: 10.1016/j.bios.2014.03.024. URL: <http://dx.doi.org/10.1016/j.bios.2014.03.024>.
- [157] Karteek Kadimisetty et al. ‘Automated 3D-Printed Microfluidic Array for Rapid Nanomaterial-Enhanced Detection of Multiple Proteins’. In: *Analytical Chemistry* 90.12 (2018), pp. 7569–7577. ISSN: 15206882. DOI: 10.1021/acs.analchem.8b01198.
- [158] Lizhe Chen et al. ‘Enhanced Sensitivity in Acute Myocardial Infarction Diagnosis Through Optimized Multiplex Biomarker Detection on a Paper-Based Microfluidic Platform with Individual Control Capabilities’. In: *Sensors and Actuators B: Chemical* 422. September 2024 (2024), p. 136616. ISSN: 09254005. DOI: 10.1016/j.snb.2024.136616. URL: <https://doi.org/10.1016/j.snb.2024.136616>.

- [159] Sotirios Papamatthaiou et al. ‘LoCKAmp: lab-on-PCB technology for <3 minute virus genetic detection’. In: *Lab Chip* 23 (20 2023), pp. 4400–4412. DOI: 10.1039/D3LC00441D. URL: <http://dx.doi.org/10.1039/D3LC00441D>.
- [160] Michael Vogeser et al. ‘Multicenter performance evaluation of a second generation cortisol assay’. In: *Clin Chem Lab Med* 55.6 (2017), pp. 826–835. DOI: 10.1515/cclm-2016-0400.
- [161] Yu Jeong et al. ‘Comparison of IL- 6 measurement methods with a special emphasis on COVID- 19 patients according to equipment and sample type’. In: *Journal of Clinical Laboratory Analysis* December 2021 (2022), pp. 1–8. DOI: 10.1002/jcla.24182.
- [162] F G Bellagambi et al. ‘Determination of salivary α -amylase and cortisol in psoriatic subjects undergoing the Trier Social Stress Test’. In: *Microchemical Journal* 136 (2018), pp. 177–184. ISSN: 0026-265X. DOI: 10.1016/j.microc.2017.04.033. URL: <https://doi.org/10.1016/j.microc.2017.04.033>.
- [163] Talha Iqbal et al. ‘Cortisol detection methods for stress monitoring in connected health’. In: *Health Sciences Review* 6.November 2022 (2023), p. 100079. ISSN: 27726320. DOI: 10.1016/j.hsr.2023.100079. URL: <https://doi.org/10.1016/j.hsr.2023.100079>.
- [164] Hanan Awad Alkozi, Hanin Abdullah Alhudhayf and Norah Mohammed Abdulrahman Alawad. ‘Association Between Dry Eye Disease with Anxiety and Depression Among Medical Sciences Students in Qassim Region: Cortisol Levels in Tears as a Stress Biomarker’. In: *Journal of Multidisciplinary Healthcare* 17.September (2024), pp. 4549–4557. ISSN: 11782390. DOI: 10.2147/JMDH.S488956.
- [165] Nora A. O’Byrne et al. ‘Sleep and circadian regulation of cortisol: A short review’. In: *Current Opinion in Endocrine and Metabolic Research* 18 (2021), pp. 178–186. ISSN: 24519650. DOI: 10.1016/j.coemr.2021.03.011.
- [166] Mahita Kadmiel and John A. Cidlowski. ‘Glucocorticoid receptor signaling in health and disease’. In: *Trends in Pharmacological Sciences* 34.9 (2013), pp. 518–530. ISSN: 01656147. DOI: 10.1016/j.tips.2013.07.003. URL: <http://dx.doi.org/10.1016/j.tips.2013.07.003>.

- [167] J. González-Cabrera et al. ‘Acute and chronic stress increase salivary cortisol: A study in the real-life setting of a national examination undertaken by medical graduates’. In: *Stress* 17.2 (2014), pp. 149–156. ISSN: 10253890. DOI: 10.3109/10253890.2013.876405.
- [168] Patrice Boucher and Pierrich Plusquellec. ‘Acute Stress Assessment From Excess Cortisol Secretion: Fundamentals and Perspectives’. In: *Frontiers in Endocrinology* 10.November (2019), pp. 1–12. ISSN: 16642392. DOI: 10.3389/fendo.2019.00749.
- [169] Lisa J. Weckesser et al. ‘Do venepuncture procedures induce cortisol responses? A review, study, and synthesis for stress research’. In: *Psychoneuroendocrinology* 46 (2014), pp. 88–99. ISSN: 18733360. DOI: 10.1016/j.psyneuen.2014.04.012. URL: <http://dx.doi.org/10.1016/j.psyneuen.2014.04.012>.
- [170] Jai Eun An et al. ‘Wearable Cortisol Aptasensor for Simple and Rapid Real-Time Monitoring’. In: *ACS Sensors* 7.1 (2022), pp. 99–108. ISSN: 23793694. DOI: 10.1021/acssensors.1c01734.
- [171] Lory Cantelli et al. ‘DNA super-lattice-based aptasensor for highly sensitive and selective detection of cortisol’. In: *Biosensors and Bioelectronics: X* 12.August (2022), p. 100228. ISSN: 25901370. DOI: 10.1016/j.biosx.2022.100228. URL: <https://doi.org/10.1016/j.biosx.2022.100228>.
- [172] Madhavi Pali et al. ‘CATCH (Cortisol Apta WATCH): ‘Bio-mimic alarm’ to track Anxiety, Stress, Immunity in human sweat’. In: *Electrochimica Acta* 390 (2021), p. 138834. ISSN: 00134686. DOI: 10.1016/j.electacta.2021.138834. URL: <https://doi.org/10.1016/j.electacta.2021.138834>.
- [173] Ziyu Huang et al. ‘An ultrasensitive aptamer-antibody sandwich cortisol sensor for the noninvasive monitoring of stress state’. In: *Biosensors and Bioelectronics* 190.April (2021), p. 113451. ISSN: 18734235. DOI: 10.1016/j.bios.2021.113451. URL: <https://doi.org/10.1016/j.bios.2021.113451>.

- [174] Zahra Rezapoor-Fashtali, Mohammad Reza Ganjali and Farnoush Faridbod. ‘A Novel Electrochemical Aptasensor Based on a New Platform of Samarium Molybdate Flower-like Nanoparticles@Poly(pyrrole) for Non-Invasive Determination of Saliva CORTISOL’. In: *Biosensors* 12.9 (2022). ISSN: 20796374. DOI: 10.3390/bios12090720.
- [175] Fatemeh Mohammadi et al. ‘Recent advances in aptamer-based platforms for cortisol hormone monitoring’. In: *Talanta* 266.P1 (2024), p. 125010. ISSN: 00399140. DOI: 10.1016/j.talanta.2023.125010. URL: <https://doi.org/10.1016/j.talanta.2023.125010>.
- [176] Bo Wang et al. ‘Wearable aptamer-field-effect transistor sensing system for noninvasive cortisol monitoring’. In: 0967.January (2022), pp. 1–16.
- [177] Mohammad Janghorban et al. ‘Redox-Concatenated Aptamer Integrated Skin Mimicking Electrochemical Patch for Noninvasive Detection of Cortisol’. In: *ACS Sensors* 9.2 (2024), pp. 799–809. ISSN: 23793694. DOI: 10.1021/acssensors.3c02110.
- [178] Antra Ganguly et al. ‘Autonomous, Real-Time Monitoring Electrochemical Aptasensor for Circadian Tracking of Cortisol Hormone in Sub-microliter Volumes of Passively Eluted Human Sweat’. In: *ACS Sensors* 6.1 (2021), pp. 63–72. ISSN: 23793694. DOI: 10.1021/acssensors.0c01754.
- [179] Naveen K. Singh et al. ‘Cortisol Detection in Undiluted Human Serum Using a Sensitive Electrochemical Structure-Switching Aptamer over an Antifouling Nanocomposite Layer’. In: *ACS Omega* 6.42 (2021), pp. 27888–27897. ISSN: 2470-1343. DOI: 10.1021/acsomega.1c03552.
- [180] Alex M. Downs et al. ‘Improved calibration of electrochemical aptamer-based sensors’. In: *Scientific Reports* 12.1 (2022), pp. 1–10. ISSN: 20452322. DOI: 10.1038/s41598-022-09070-7. URL: <https://doi.org/10.1038/s41598-022-09070-7>.
- [181] Yasmin Liu et al. ‘Electrochemical Aptamer-Based Biosensors for Cocaine Detection in Human Saliva: Exploring Matrix Interference’. In: *Analytical Chemistry* (2025). ISSN: 15206882. DOI: 10.1021/acs.analchem.4c03423.

- [182] Michael Zuker. ‘Mfold web server for nucleic acid folding and hybridization prediction’. In: *Nucleic Acids Research* 31.13 (2003), pp. 3406–3415. ISSN: 03051048. DOI: 10.1093/nar/gkg595.
- [183] Alexandra Dobrea et al. ‘A plug-and-play, easy-to-manufacture fluidic accessory to significantly enhance the sensitivity of electrochemical immunoassays’. In: *Scientific Reports* 14.1 (2024), pp. 1–11. ISSN: 20452322. DOI: 10.1038/s41598-024-64852-5. URL: <https://doi.org/10.1038/s41598-024-64852-5>.
- [184] Dejian Zhang and Gyoko Nagayama. ‘Effective Wetting Area Based on Electrochemical Impedance Analysis: Hydrophilic Structured Surface’. In: *Langmuir* 35.50 (2019). DOI: 10.1021/acs.langmuir.9b03349.
- [185] Perrine Lasserre et al. ‘SARS-CoV-2 Aptasensors Based on Electrochemical Impedance Spectroscopy and Low-Cost Gold Electrode Substrates’. In: *Analytical Chemistry* 94.4 (2022), pp. 2126–2133. ISSN: 15206882. DOI: 10.1021/acs.analchem.1c04456.
- [186] Kunjie Lu et al. ‘Detecting electrolyte wetting defects in lithium-ion batteries based on electrochemical impedance spectroscopy II Detecting electrolyte wetting defects in lithium-ion batteries based on electrochemical impedance spectroscopy’. In: *Cell Reports Physical Science* 7.4 (2026), p. 103224. ISSN: 2666-3864. DOI: 10.1016/j.xcrp.2026.103224. URL: <https://doi.org/10.1016/j.xcrp.2026.103224>.
- [187] Kevin Raiber et al. ‘Removal of self-assembled monolayers of alkanethiolates on gold by plasma cleaning’. In: *Surface Science* 595.1-3 (2005), pp. 56–63. ISSN: 00396028. DOI: 10.1016/j.susc.2005.07.038.
- [188] Asta Makaraviciute et al. ‘Systematic Approach to the Development of Micro-fabricated Biosensors: Relationship between Gold Surface Pretreatment and Thiolated Molecule Binding’. In: *ACS Applied Materials and Interfaces* 9.31 (2017), pp. 26610–26621. ISSN: 19448252. DOI: 10.1021/acsami.7b08581.
- [189] Hua Ma et al. ‘A novel label-free electrochemical aptamer sensor based on base mismatch and strand displacement for detection of kanamycin’. In: *Bioelectrochemistry* 170.December 2025 (2026).

- [190] Bingling Li, Yan Du and Shaojun Dong. ‘Reusable , label-free electrochemical aptasensor for sensitive detection of small molecules’. In: *RSC Chem Comm* (2007), pp. 3780–3782. DOI: 10.1039/b707057h.
- [191] Justin Henri et al. ‘A guide to using nucleic acid aptamers in cell based assays’. In: *Aptamers* 3.November (2019), pp. 4–9. ISSN: 2514-3247.
- [192] Perrine Lasserre. ‘Electrochemical detection of antibiotics and antibiotic susceptibility’. PhD thesis. University of Strathclyde, 2024. URL: https://stax.strath.ac.uk/concern/file%7B%5C_%7Dsets/6t053g71x?locale=en.
- [193] Merck. *Protocol for Thiol-Modified Oligonucleotide DDT Reduction*. 2025. URL: https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/materials-science-and-engineering/drug-delivery/oligo-reduction?srsltid=AfmB0oojnA6TUQ85HqMGYxsGt7aVD4m-Bt06%7B%5C_%7DLjahEIdhZI9nQxvUQSt.
- [194] Harsh Sharma and Raj Mutharasan. ‘Half antibody fragments improve biosensor sensitivity without loss of selectivity’. In: *Analytical Chemistry* 85.4 (2013), pp. 2472–2477. ISSN: 00032700. DOI: 10.1021/ac3035426.
- [195] Elise Burmeister Getz et al. ‘A comparison between the sulfhydryl reductants tris(2- carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry’. In: *Analytical Biochemistry* 273.1 (1999), pp. 73–80. ISSN: 00032697. DOI: 10.1006/abio.1999.4203.
- [196] A. Hauke et al. ‘Superwetting and aptamer functionalized shrink-induced high surface area electrochemical sensors’. In: *Biosensors and Bioelectronics* 94.November 2016 (2017), pp. 438–442. ISSN: 18734235. DOI: 10.1016/j.bios.2017.03.024.
- [197] Simon D. Keighley et al. ‘Optimization of DNA immobilization on gold electrodes for label-free detection by electrochemical impedance spectroscopy’. In: *Biosensors and Bioelectronics* 23.8 (2008), pp. 1291–1297. ISSN: 09565663. DOI: 10.1016/j.bios.2007.11.012.

- [198] Franziska V. Oberhaus, Dieter Frense and Dieter Beckmann. ‘Immobilization techniques for aptamers on gold electrodes for the electrochemical detection of proteins: A review’. In: *Biosensors* 10.5 (2020). ISSN: 20796374. DOI: 10.3390/BIOS10050045.
- [199] Vanessa Biagiotti et al. ‘Probe accessibility effects on the performance of electrochemical biosensors employing DNA monolayers’. In: *Analytical and Bioanalytical Chemistry* 402.1 (2012), pp. 413–421. ISSN: 16182642. DOI: 10.1007/s00216-011-5361-0.
- [200] Mengying Deng et al. ‘Programming Accessibility of DNA Monolayers for Degradation-Free Whole-Blood Biosensors’. In: *ACS Materials Letters* 1.6 (2019), pp. 671–676. ISSN: 26394979. DOI: 10.1021/acsmaterialslett.9b00404.
- [201] Netzahualcoyotl Arroyo-Currás et al. ‘From the beaker to the body: Translational challenges for electrochemical, aptamer-based sensors’. In: *Analytical Methods* 12.10 (2020), pp. 1288–1310. ISSN: 17599679. DOI: 10.1039/d0ay00026d.
- [202] Hao Wan et al. ‘Screen-printed gold electrode with gold nanoparticles modification for simultaneous electrochemical determination of lead and copper’. In: *Sensors and Actuators, B: Chemical* 209 (2015), pp. 336–342. ISSN: 09254005. DOI: 10.1016/j.snb.2014.11.127. URL: <http://dx.doi.org/10.1016/j.snb.2014.11.127>.
- [203] Magdalena R. Raykova et al. ‘Towards direct detection of tetracycline residues in milk with a gold nanostructured electrode’. In: *PLoS ONE* 18.6 June (2023), pp. 1–18. ISSN: 19326203. DOI: 10.1371/journal.pone.0287824. URL: <http://dx.doi.org/10.1371/journal.pone.0287824>.
- [204] Peggy Reich, Regina Stoltenburg and Dieter Beckmann. ‘Development of An Impedimetric Aptasensor for the Detection of Staphylococcus aureus’. In: (2017). DOI: 10.3390/ijms18112484.
- [205] Ana Maria Titoiu et al. ‘Detection of Allergenic Lysozyme during Winemaking with an Electrochemical Aptasensor’. In: *Electroanalysis* (2019), pp. 2262–2273. DOI: 10.1002/elan.201900333.

- [206] Guoqing Ying et al. ‘Construction and application of an electrochemical biosensor based on an endotoxin aptamer’. In: *Biotechnology and Applied Biochemistry* (1990), pp. 323–327. DOI: 10.1002/bab.1610.
- [207] Alexander Shaver et al. ‘Optimization of Vancomycin Aptamer Sequence Length Increases the Sensitivity of Electrochemical, Aptamer-Based Sensors In Vivo’. In: *ACS Sensors* (2022). DOI: 10.1021/acssensors.2c01910.
- [208] Seyed Mohammad et al. ‘Biosensors and Bioelectronics A novel electrochemical aptasensor based on nontarget-induced high accumulation of methylene blue on the surface of electrode for sensing of α - synuclein oligomer’. In: *Biosensors and Bioelectronic* 123.September 2018 (2019), pp. 14–18. ISSN: 0956-5663. DOI: 10.1016/j.bios.2018.09.081. URL: <https://doi.org/10.1016/j.bios.2018.09.081>.
- [209] Alan J. Heeger, Chunhai Fan and Kevin Plaxco. *Reagentless, reusable, bioelectronic detectors*. 2011.
- [210] Shaoyu Kang and Jason J. Davis. ‘Leveraging microfluidic confinement to boost assay sensitivity and selectivity’. In: *Chemical Science* 16.16 (2025), pp. 6965–6974. ISSN: 20416539. DOI: 10.1039/d5sc00199d.
- [211] Karuna Chandnani et al. ‘Technological advancement and current standing of microfluidic chip based devices for targeted analysis of biomarkers’. In: *Microchemical Journal* 195.July (2023), p. 109532. ISSN: 0026265X. DOI: 10.1016/j.microc.2023.109532. URL: <https://doi.org/10.1016/j.microc.2023.109532>.
- [212] Ergun Alperay Tarim, Michael G Mauk and Mohamed El-tholoth. ‘Emerging Microfluidic Plasma Separation Technologies for Point-of-Care Diagnostics : Moving Beyond Conventional Centrifugation’. In: *MDPI Biosensors* (2026), pp. 1–34.
- [213] NICE. *Lyme disease - diagnosis and management guidelines*. <https://www.nice.org.uk/guidance/ng95/resources/lyme-disease-pdf-1837756839877>. 2018.
- [214] Hai Jiang, Lan Feng and Jinxing Lu. ‘Updated Guidelines for the Diagnosis of Human Brucellosis — China, 2019’. In: *China CDC Weekly* 2.26 (2020), pp. 487–489. ISSN: 20967071. DOI: 10.46234/ccdcw2020.129.

- [215] Bernard M. Branson et al. ‘Laboratory testing for the diagnosis of HIV infection : updated recommendations - Guidelines and Recommendations’. In: *Centers for disease control and prevention* (2014), pp. 4–7. URL: <http://stacks.cdc.gov/view/cdc/23447?links=false>.
- [216] World Health Organisation. *Guidelines on hepatitis B and C testing*. Vol. 66. WHO, 2017, pp. 1–170. ISBN: 9789241549981. URL: <https://apps.who.int/iris/bitstream/handle/10665/254621/9789241549981-eng.pdf?sequence=1%7B%5C%%7D0Ahttp://apps.who.int/iris/bitstream/10665/254621/1/9789241549981-eng.pdf?ua=1%7B%5C%%7D0Ahttp://www.ncbi.nlm.nih.gov/pubmed/28742301%7B%5C%%7D0Ahttp://www.ncbi.nlm.nih.gov/pubmed/2874>.
- [217] Daniel A. Leffler and Detlef Schuppan. ‘Update on serologic testing in celiac disease’. In: *American Journal of Gastroenterology* 105.12 (2010), pp. 2520–2524. ISSN: 00029270. DOI: 10.1038/ajg.2010.276. URL: <http://dx.doi.org/10.1038/ajg.2010.276>.
- [218] Catharine M. Sturgeon et al. ‘National academy of clinical biochemistry laboratory medicine practice guidelines for use of tumor markers in liver, bladder, cervical, and gastric cancers’. In: *Clinical Chemistry* 54.12 (2010). ISSN: 00099147. DOI: 10.1373/clinchem.2009.133124.
- [219] Samira Hosseini et al. *Advantages, Disadvantages and Modifications of Conventional ELISA*. In: *Enzyme-linked Immunosorbent Assay (ELISA): From A to Z*. 9789811067655. Springer, 2018, pp. 67–115. ISBN: 978-981-10-6765-5. URL: <http://link.springer.com/10.1007/978-981-10-6766-2>.
- [220] Meenakshi Choudhary and Kavita Arora. *Electrochemical biosensors for early detection of cancer*. INC, 2021, pp. 123–151. ISBN: 9780128234242. DOI: 10.1016/B978-0-12-823424-2.00024-7. URL: <http://dx.doi.org/10.1016/B978-0-12-823424-2.00024-7>.
- [221] Uros Zupancic et al. ‘Strategies for Multiplexed Electrochemical Sensor Development’. English. In: *Studies in Systems, Decision and Control*. Studies in Systems, Decision and Control. USA United States: Springer Nature, Jan. 2021, pp. 63–93. ISBN: 9789811596117. DOI: 10.1007/978-981-15-9612-4_3.

- [222] Veerappan Mani et al. ‘Multiplexed sensing techniques for cardiovascular disease biomarkers - A review’. In: *Biosensors and Bioelectronics* 216.August (2022), p. 114680. ISSN: 18734235. DOI: 10.1016/j.bios.2022.114680. URL: <https://doi.org/10.1016/j.bios.2022.114680>.
- [223] Mohanraj Sadasivam et al. ‘Magnetic bead-amplified voltammetric detection for carbohydrate antigen 125 with enzyme labels using aptamer-antigen-antibody sandwiched assay’. In: *Sensors and Actuators, B: Chemical* 312.February (2020), p. 127985. ISSN: 09254005. DOI: 10.1016/j.snb.2020.127985. URL: <https://doi.org/10.1016/j.snb.2020.127985>.
- [224] Pravanjan Malla et al. ‘Electrochemical immunoassay for serum parathyroid hormone using screen-printed carbon electrode and magnetic beads’. In: *Journal of Electroanalytical Chemistry* 895.May (2021), p. 115463. ISSN: 15726657. DOI: 10.1016/j.jelechem.2021.115463. URL: <https://doi.org/10.1016/j.jelechem.2021.115463>.
- [225] Madhusudan B. Kulkarni, Narasimha H. Ayachit and Tejraj M. Aminabhavi. ‘Recent Advances in Microfluidics-Based Electrochemical Sensors for Foodborne Pathogen Detection’. In: *Biosensors* 13.2 (2023). ISSN: 20796374. DOI: 10.3390/bios13020246.
- [226] Kai Jin et al. ‘A digital microfluidic system with integrated electrochemical impedance detection arrays’. In: *Journal of Physics: Conference Series* 2196.1 (2022). ISSN: 17426596. DOI: 10.1088/1742-6596/2196/1/012005.
- [227] Xing Xu et al. ‘Digital microfluidics for biological analysis and applications’. In: *Lab on a Chip* 23.5 (2023), pp. 1169–1191. ISSN: 14730189. DOI: 10.1039/d21c00756h.
- [228] Shambhulinga Aralekallu, Rajamouli Boddula and Vijay Singh. ‘Development of glass-based microfluidic devices: A review on its fabrication and biologic applications’. In: *Materials and Design* 225 (2023), p. 111517. ISSN: 18734197. DOI: 10.1016/j.matdes.2022.111517. URL: <https://doi.org/10.1016/j.matdes.2022.111517>.

- [229] Bruce K. Gale et al. ‘A review of current methods in microfluidic device fabrication and future commercialization prospects’. In: *Inventions* 3.3 (2018). ISSN: 24115134. DOI: 10.3390/inventions3030060.
- [230] Inês Miranda et al. ‘Properties and applications of PDMS for biomedical engineering: A review’. In: *Journal of Functional Biomaterials* 13.1 (2022). ISSN: 20794983. DOI: 10.3390/jfb13010002.
- [231] Ana Luisa Silva et al. ‘A 3D Printer Guide for the Development and Application of Electrochemical Cells and Devices’. In: *Frontiers in Chemistry* 9.July (2021). ISSN: 22962646. DOI: 10.3389/fchem.2021.684256.
- [232] Simon M. Scott and Zulfiqur Ali. ‘Fabrication methods for microfluidic devices: An overview’. In: *Micromachines* 12.3 (2021). ISSN: 2072666X. DOI: 10.3390/mi12030319.
- [233] Mohd Afiq Mohd Asri et al. ‘Low-cost and rapid prototyping of integrated electrochemical microfluidic platforms using consumer-grade off-the-shelf tools and materials’. In: *Lab on a Chip* 22.9 (2022), pp. 1779–1792. ISSN: 14730189. DOI: 10.1039/d1lc01100f.
- [234] Mahan Hosseinzadeh Fakhr et al. ‘Cleaning of LTCC, PEN, and PCB Au electrodes towards reliable electrochemical measurements’. In: *Scientific Reports* 12.1 (2022), pp. 1–11. ISSN: 20452322. DOI: 10.1038/s41598-022-23395-3. URL: <https://doi.org/10.1038/s41598-022-23395-3>.
- [235] Lee M. Fischer et al. ‘Gold cleaning methods for electrochemical detection applications’. In: *Microelectronic Engineering* 86.4-6 (2009), pp. 1282–1285. ISSN: 01679317. DOI: 10.1016/j.mee.2008.11.045. URL: <http://dx.doi.org/10.1016/j.mee.2008.11.045>.
- [236] Camille Colin et al. ‘Easy cleaning plus stable activation of glassy carbon electrode surface by oxygen plasma’. In: *Bioelectrochemistry* 154 (2023), p. 108551. ISSN: 1567-5394. DOI: <https://doi.org/10.1016/j.bioelechem.2023.108551>. URL: <https://www.sciencedirect.com/science/article/pii/S1567539423001883>.

- [237] Felix Widdascheck et al. ‘The effect of oxygen plasma treatment of gold electrodes on the molecular orientation of CuPc films’. In: *Applied Surface Science* 507 (2020), p. 145039. ISSN: 0169-4332. DOI: <https://doi.org/10.1016/j.apsusc.2019.145039>. URL: <https://www.sciencedirect.com/science/article/pii/S0169433219338565>.
- [238] Tomasz Wasilewski et al. ‘Development and Assessment of Regeneration Methods for Peptide-Based QCM Biosensors in VOCs Analysis Applications’. In: *Biosensors* 12.5 (2022), pp. 1–14. ISSN: 20796374. DOI: 10.3390/bios12050309.
- [239] Jean Michel Savéant. *Elements of Molecular and Biomolecular Electrochemistry: An Electrochemical Approach to Electron Transfer Chemistry*. John Wiley and Sons, Inc, 2006, pp. 1–485. ISBN: 0471445738. DOI: 10.1002/0471758078.
- [240] Julia B. Sun et al. ‘Doping density, not valency, influences catalytic metal-assisted plasma etching of silicon’. In: *Materials Horizons* 10.9 (2023), pp. 3393–3403. ISSN: 20516355. DOI: 10.1039/d3mh00649b.
- [241] I. Jane Chen and Ian M. White. ‘High-sensitivity electrochemical enzyme-linked assay on a microfluidic interdigitated microelectrode’. In: *Biosensors and Bioelectronics* 26.11 (2011), pp. 4375–4381. ISSN: 09565663. DOI: 10.1016/j.bios.2011.04.044. URL: <http://dx.doi.org/10.1016/j.bios.2011.04.044>.
- [242] Polina Kotelnikova et al. ‘Peroxidase-like activity of silver nanowires and its application for colorimetric detection of the antibiotic chloramphenicol’. In: *Talanta Open* 6 (Dec. 2022), p. 100164. DOI: 10.1016/j.talo.2022.100164.
- [243] Feng Zhang et al. ‘Test Paper for Colorimetric Inspection of Fatty Acids and Edible Oils’. In: *Sensors* 18.10 (2018). ISSN: 1424-8220. DOI: 10.3390/s18103252. URL: <https://www.mdpi.com/1424-8220/18/10/3252>.
- [244] Fatemeh Farshchi et al. ‘Optimization of a silver-nanoprism conjugated with 3,3',5,5'-tetramethylbenzidine towards easy-to-make colorimetric analysis of acetaldehyde: a new platform towards rapid analysis of carcinogenic agents and environmental technology’. In: *RSC Advances* 13.9 (2023), pp. 6225–6238. ISSN: 20462069. DOI: 10.1039/d3ra00355h.

- [245] Yi-Wei Wang et al. ‘A Simple Assay for Ultrasensitive Colorimetric Detection of Ag⁺ at Picomolar Levels Using Platinum Nanoparticles’. In: *Sensors* 17.11 (2017). ISSN: 1424-8220. DOI: 10.3390/s17112521. URL: <https://www.mdpi.com/1424-8220/17/11/2521>.
- [246] Hui Ma, Ciarán Ó’Fágáin and Richard O’Kennedy. ‘Antibody stability: A key to performance - Analysis, influences and improvement’. In: *Biochimie* 177 (2020), pp. 213–225. ISSN: 61831638. DOI: 10.1016/j.biochi.2020.08.019.
- [247] Alex Frago et al. ‘Electron Permeable Self-Assembled Monolayers of Dithiolated Aromatic Scaffolds on Gold for Biosensor Applications’. In: *Analytical Chemistry* 80.7 (2008), pp. 2556–2563.
- [248] Soraia Oliveira et al. ‘Real-Time Monitoring of Chemisorption of Antibodies onto Self- Assembled Monolayers Deposited on Gold Electrodes Using Electrochemical Impedance Spectroscopy’. In: *Langmuir* (2025). DOI: 10.1021/acs.langmuir.5c01062.
- [249] Shinn-jyh Ding, Bin-wha Chang and Ching-chou Wu. ‘Impedance spectral studies of self-assembly of alkanethiols with different chain lengths using different immobilization strategies on Au electrodes’. In: *Analytica Chimica Acta* 554 (2005), pp. 43–51. DOI: 10.1016/j.aca.2005.08.046.
- [250] Feiyun Cui, Zhiru Zhou and H. Susan Zhou. ‘Review—Measurement and Analysis of Cancer Biomarkers Based on Electrochemical Biosensors’. In: *Journal of The Electrochemical Society* 167.3 (2020), p. 037525. ISSN: 0013-4651. DOI: 10.1149/2.0252003jes.
- [251] Md Abdul Khaleque et al. ‘Bioreceptor modified electrochemical biosensors for the detection of life threatening pathogenic bacteria: a review’. In: *RSC Advances* 14.39 (2024), pp. 28487–28515. ISSN: 20462069. DOI: 10.1039/d4ra04038d.
- [252] Anjana Sajeevan et al. ‘Trends in ready-to-use portable electrochemical sensing devices for healthcare diagnosis’. In: *Mikrochimica acta* (2025). DOI: 10.1007/s00604-024-06916-x.

- [253] Zeus Saldaña-Ahuactzi et al. *Advancing foodborne pathogen detection: a review of traditional and innovative optical and electrochemical biosensing approaches*. Vol. 192. 2. Springer Vienna, 2025, p. 102. ISBN: 0123456789. DOI: 10.1007/s00604-024-06924-x. URL: <https://doi.org/10.1007/s00604-024-06924-x>.
- [254] Harsh Sable et al. ‘Review—Nanosystems-Enhanced Electrochemical Biosensors for Precision in One Health Management’. In: *Journal of The Electrochemical Society* 171.3 (2024), p. 037527. ISSN: 0013-4651. DOI: 10.1149/1945-7111/ad32a1.
- [255] Damion K. Corrigan et al. ‘A microelectrode array with reproducible performance shows loss of consistency following functionalization with a self-assembled 6-mercapto-1-hexanol layer’. In: *Sensors (Switzerland)* 18.6 (2018). ISSN: 14248220. DOI: 10.3390/s18061891.
- [256] Adrian Butterworth et al. ‘SAM composition and electrode roughness affect performance of a DNA biosensor for antibiotic resistance’. In: *Biosensors* 9.1 (2019). ISSN: 20796374. DOI: 10.3390/bios9010022.
- [257] Dan Bizzotto et al. ‘Beyond Simple Cartoons: Challenges in Characterizing Electrochemical Biosensor Interfaces’. In: *ACS Sensors* 3.1 (2018), pp. 5–12. ISSN: 23793694. DOI: 10.1021/acssensors.7b00840.
- [258] Jie Hu et al. ‘Portable microfluidic and smartphone-based devices for monitoring of cardiovascular diseases at the point of care’. In: *Biotechnology Advances* 34.3 (2016), pp. 305–320. ISSN: 0734-9750. DOI: 10.1016/j.biotechadv.2016.02.008. URL: <http://dx.doi.org/10.1016/j.biotechadv.2016.02.008>.
- [259] Kangning Cheng et al. ‘Automated and rapid chemiluminescence immunoassay for cardiac troponin I based on digital microfluidics’. In: *Microfluidics and Nanofluidics* 27.6 (2023), pp. 1–10. ISSN: 16134990. DOI: 10.1007/s10404-023-02652-5. URL: <https://doi.org/10.1007/s10404-023-02652-5>.
- [260] Alphonsus H.C. Ng et al. ‘A digital microfluidic system for serological immunoassays in remote settings’. In: *Science Translational Medicine* 10.438 (2018), pp. 1–12. ISSN: 19466242. DOI: 10.1126/scitranslmed.aar6076.

- [261] Matthew Y.H. Tang and Ho Cheung Shum. ‘One-step immunoassay of C-reactive protein using droplet microfluidics’. In: *Lab on a Chip* 16.22 (2016), pp. 4359–4365. ISSN: 14730189. DOI: 10.1039/c61c01121g.
- [262] Chung An Chen et al. ‘An electricity- And instrument-free infectious disease sensor based on a 3D origami paper-based analytical device’. In: *Lab on a Chip* 21.10 (2021), pp. 1908–1915. ISSN: 14730189. DOI: 10.1039/d11c00079a.
- [263] Fatih Ozefer and Ahu Arslan Yildiz. ‘Fabrication and development of a microfluidic paper-based immunosorbent assay platform (μ PISA) for colorimetric detection of hepatitis C’. In: *Analyst* 148.4 (2023), pp. 898–905. ISSN: 13645528. DOI: 10.1039/d2an01761j.
- [264] Hao Fu et al. ‘Paper-Based All-in-One Origami Nanobiosensor for Point-of-Care Detection of Cardiac Protein Markers in Whole Blood’. In: *ACS Sensors* 8.9 (2023), pp. 3574–3584. ISSN: 23793694. DOI: 10.1021/acssensors.3c01221.
- [265] Phuritat Kaewarsa et al. ‘Improving design features and air bubble manipulation techniques for a single-step sandwich electrochemical ELISA incorporating commercial electrodes into capillary-flow driven immunoassay devices’. In: *Analyst* (2023). DOI: 10.1039/d3an01704d.
- [266] Josef Horak et al. ‘Polymer-modified microfluidic immunochip for enhanced electrochemical detection of troponin i’. In: *Sensors and Actuators, B: Chemical* 209 (2015), pp. 478–485. ISSN: 09254005. DOI: 10.1016/j.snb.2014.12.006. URL: <http://dx.doi.org/10.1016/j.snb.2014.12.006>.
- [267] Yang Li et al. ‘Sensitive immunoassay of cardiac troponin I using an optimized microelectrode array in a novel integrated microfluidic electrochemical device’. In: *Analytical and Bioanalytical Chemistry* 412.30 (2020), pp. 8325–8338. ISSN: 16182650. DOI: 10.1007/s00216-020-02968-z.
- [268] Horn Jiunn Sheen et al. ‘Electrochemical biosensor with electrokinetics-assisted molecular trapping for enhancing C-reactive protein detection’. In: *Biosensors and Bioelectronics* 210.November 2021 (2022), p. 114338. ISSN: 18734235. DOI: 10.1016/j.bios.2022.114338. URL: <https://doi.org/10.1016/j.bios.2022.114338>.

- [269] Niranjana Haridas Menon, Yassa Beshai and Sagnik Basuray. ‘Fully Automated Microfluidic Electrochemical Platform as a Versatile Biosensor’. In: *IEEE Sensors Letters* 2.3 (2017), pp. 194–307. DOI: 10.1109/LSENS.2025.3588757. URL: <http://www.ieee.org/publications>.
- [270] Anirban Sinha et al. ‘An integrated microfluidic system with field-effect-transistor sensor arrays for detecting multiple cardiovascular biomarkers from clinical samples’. In: *Biosensors and Bioelectronics* 129. January (2019), pp. 155–163. ISSN: 18734235. DOI: 10.1016/j.bios.2019.01.001. URL: <https://doi.org/10.1016/j.bios.2019.01.001>.
- [271] Alexander Ecke, J  r  my Bell and Rudolf J. Schneider. ‘A three-dimensional microfluidic flow cell and system integration for improved electrochemical substrate detection in HRP/TMB-based immunoassays†’. In: *Sensors and Diagnostics* 2.4 (2023), pp. 887–892. ISSN: 26350998. DOI: 10.1039/d3sd00095h. URL: <http://dx.doi.org/10.1039/D3SD00095H>.
- [272] Jie Wu et al. ‘A disposable multianalyte electrochemical immunosensor array for automated simultaneous determination of tumor markers’. In: *Clinical Chemistry* 53.8 (2007), pp. 1495–1502. ISSN: 00099147. DOI: 10.1373/clinchem.2007.086975.
- [273] Jie Wu et al. ‘A disposable electrochemical immunosensor for flow injection immunoassay of carcinoembryonic antigen’. In: *Biosensors and Bioelectronics* 22.1 (2006), pp. 102–108. ISSN: 09565663. DOI: 10.1016/j.bios.2005.12.008.
- [274] Vincent J. Vezza et al. ‘An electrochemical SARS-CoV-2 biosensor inspired by glucose test strip manufacturing processes’. In: *Chemical Communications* 57.30 (2021), pp. 3704–3707. ISSN: 1364548X. DOI: 10.1039/d1cc00936b.
- [275] Hamed Ghorbanpoor et al. ‘Sensors and Actuators : A . Physical A fully integrated rapid on-chip antibiotic susceptibility test – A case study for Mycobacterium smegmatis’. In: 339. February (2022).

- [276] Dagwin Wachholz Junior et al. ‘Exploring a CRISPR/Cas12a-powered impedimetric biosensor for amplification-free detection of a pathogenic bacterial DNA’. In: *Biosensors and Bioelectronics* 285.May (2025), p. 117607. ISSN: 18734235. DOI: 10.1016/j.bios.2025.117607. URL: <https://doi.org/10.1016/j.bios.2025.117607>.
- [277] Alexandros Ch Lazanas and Mamas I Prodromidis. ‘Electrochemical Impedance Spectroscopy A Tutorial’. In: (2023). DOI: 10.1021/acsmeasuresciau.2c00070.
- [278] Susan Pang and Simon Cowen. ‘A generic standard additions based method to determine endogenous analyte concentrations by immunoassays to overcome complex biological matrix interference’. In: *Scientific Reports* November (2017), pp. 1–10. ISSN: 2045-2322. DOI: 10.1038/s41598-017-17823-y. URL: <http://dx.doi.org/10.1038/s41598-017-17823-y>.
- [279] Christiana Oh et al. ‘Electrochemical Immunosensing of Interleukin-6 in Human Cerebrospinal Fluid and Human Serum as an Early Biomarker for Traumatic Brain Injury’. In: *ACS Measurement Science Au* (2021). ISSN: 2694-250X. DOI: 10.1021/acsmeasuresciau.1c00013.
- [280] E M Schoeman et al. ‘Identification of plasma protein biomarkers for endometriosis and the development of statistical models for disease diagnosis’. In: *Human Reproduction* 40.2 (2025), pp. 270–279.
- [281] Michalina Kliber et al. ‘Potential biomarkers for early detection of endometriosis : current state of art (what we know so far)’. In: *Journal of Applied Genetics* (2025). DOI: 10.1007/s13353-025-01021-y.
- [282] Daniel Rodriguez Gutierrez et al. ‘Enhanced analysis of endometriosis patients ’ plasma using # Enzian annotation highlights potential biomarkers for early- stages of disease’. In: *npj Women’s Health* (2025).
- [283] Fatemeh Keyvani et al. ‘An integrated microfluidic electrochemical assay for cervical cancer detection at point-of-care testing’. In: *Nanoscale* 14.18 (2022). DOI: 10.1039/d1nr08252c.

- [284] Zhi-qiang Zuo, Jian-zhang Pan and Qun Fang. ‘An integrated microfluidic system for multi-target biochemical analysis of a single drop of blood’. In: *Talanta* 249.May (2022), p. 123585. ISSN: 0039-9140. DOI: 10.1016/j.talanta.2022.123585. URL: <https://doi.org/10.1016/j.talanta.2022.123585>.
- [285] Qingxue Gao et al. ‘Analytical Methods A simple and rapid method for blood plasma separation driven by capillary force with an application in protein detection †’. In: (2020), pp. 2560–2570. DOI: 10.1039/d0ay00240b.
- [286] Shadi Karimi et al. ‘Numerical and experimental analysis of a high-throughput blood plasma separator for point-of-care applications’. In: (2021), pp. 2867–2878.
- [287] Xinrui Pan et al. ‘Rapid Heavy Metals Detection Using LIBS with Concentration Gradient Generators’. In: *Analytical Chemistry* (2025). DOI: 10.1021/acs.analchem.5c02125.
- [288] Iremnur Akcakoca et al. ‘An electrochemical biosensor with integrated microheater to improve the sensitivity of electrochemical nucleic acid biosensors’. In: *Journal of Micromechanics and Microengineering* ().
- [289] F Kardous et al. ‘Biosensors and Bioelectronics Improving immunosensor performances using an acoustic mixer on droplet microarray’. In: *Biosensors and Bioelectronics* 26.4 (2010), pp. 1666–1671. ISSN: 0956-5663. DOI: 10.1016/j.bios.2010.09.007. URL: <http://dx.doi.org/10.1016/j.bios.2010.09.007>.
- [290] David F. Parks et al. ‘IoT cloud laboratory: Internet of Things architecture for cellular biology’. In: *Internet of Things* 20 (2022), p. 100618. ISSN: 2542-6605. DOI: <https://doi.org/10.1016/j.iot.2022.100618>. URL: <https://www.sciencedirect.com/science/article/pii/S2542660522001007>.
- [291] BioDot. *BioDot home page*. URL: <https://www.biodot.com/>.
- [292] Won Han and Joong Ho. ‘HardwareX Low-cost , open-source 3D printed antibody dispenser for development and small-scale production of lateral flow assay strips’. In: *HardwareX* 9 (2021), e00188. ISSN: 2468-0672. DOI: 10.1016/j.ohx.2021.e00188. URL: <https://doi.org/10.1016/j.ohx.2021.e00188>.

- [293] Endometriosis UK. ‘THE STATE OF ENDOMETRIOSIS CARE IN THE UK : A ROADMAP FOR DRIVING DOWN DIAGNOSIS TIMES AND FOREWORD’. In: (2026). URL: <https://www.endometriosis-uk.org/sites/default/files/2026-02/Endometriosis%20-%20The%20State%20of%20Endometriosis%20Care%20Report.pdf>.
- [294] Alaa Mosbah, Yasmin Nabel and Eman Khashaba. ‘Interleukin-6 , intracellular adhesion molecule-1 , and glycodelin A levels in serum and peritoneal fluid as biomarkers for endometriosis’. In: *International Journal of Gynecology and Obstetrics* 134 (2016), pp. 247–251. DOI: <https://doi.org/10.1016/j.ijgo.2016.01.018>.
- [295] National Institute for Health and Care Excellence. *New technologies could help cut years-long wait for endometriosis diagnosis*. 2026. URL: <https://www.nice.org.uk/news/articles/new-technologies-for-endometriosis-diagnosis-in-primary-care>.
- [296] Ioannis Simitsidellis et al. ‘Steroid hormone profiling reveals altered adrenal androgen production in endometriosis’. In: July (2026), pp. 22–34.
- [297] Cezary Wojtyła et al. ‘Metabolome analysis as a potential source of endometriosis biomarkers with the use of multiomics approach in its diagnosis’. In: (2025), pp. 1–9.
- [298] Leila Adamyan et al. ‘Journal of Endometriosis and Uterine Disorders Metabolomic biomarkers of endometriosis : A systematic review’. In: *Journal of Endometriosis and Uterine Disorders* 7.February (2024), p. 100077. ISSN: 2949-8384. DOI: 10.1016/j.jeud.2024.100077. URL: <https://doi.org/10.1016/j.jeud.2024.100077>.
- [299] Christine F Woolley et al. ‘Theoretical Limitations of Quantification for Noncompetitive Sandwich Immunoassays’. In: *Anal Bioanal Chem* 407.28 (2015), pp. 8605–8615. DOI: 10.1007/s00216-015-9018-2.Theoretical.
- [300] John W A Findlay and Robert F Dillard. ‘Appropriate Calibration Curve Fitting in Ligand Binding Assays’. In: *AAPS Journal* 9.2 (2007), pp. 260–267. DOI: <https://doi.org/10.1208/aapsj0902029>.