

Development of Electrochemical Sensors for Nucleic Acid Biomarkers

Xiaotong Meng

Department of Bioengineering

Imperial College London

This thesis is submitted for the degree of

Doctor of Philosophy

August 2023

Declaration

I hereby declare that the work presented in this thesis are original and are the result of my own work. Where others have contributed, every effort has been made to reference and acknowledge their work. The thesis has not been submitted in whole or in part for consideration for any other degree or qualification at Imperial College London or any other University; however, parts of this thesis have been published (submitted) in peer-reviewed journals as indicated at the start of each chapter.

Xiaotong Meng

August 2023

Copyright Declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

I would like to express my sincere gratitude to my PhD supervisors, **Prof Danny O'Hare** and **Dr Sylvain Ladame**, for their exceptional supervision and guidance throughout this work. Especially to Sylvain, not only for his support on the professional and academic fronts but also for his encouragement and support on my personal development. His mentorship has been invaluable to me.

I would like to express my deepest gratitude to **my parents**, whose love and support have been my greatest source of strength throughout the ups and downs of my PhD journey. Their guidance, wisdom, kindness, and resilience have illuminated my path and fortified my resolve. Thanks for being by my side, especially during the challenging times of the pandemic. I am deeply appreciative of their pivotal contribution to the success of my research.

I would like to express my heartfelt appreciation and fond remembrance for **my grandfather**, who passed away on 5th October 2020. His support for my choices and his encouragement to embark on my PhD path have left an enduring impact on my academic journey. His steadfast belief in my capabilities has been a source of strength and confidence for me. While I feel a twinge of sadness that he is not here to witness my achievements and share in my joy, I hold dear the cherished memories we shared.

My journey would not have been the same without the wonderful colleagues I had the pleasure of working with. I extend my heartfelt thanks to **Philip Gillespie, Rob Channon, Nazmul Islam, Sally Gowers, Aldyla Raditya, Isobel Steer, Yu-Chih Chen, Loukia Petrou, Saylee Jangam, James McLeod, Daanyaal Khan, Daniels Connor, Oliver Teenan, Jenna Robinson, Ahmad Kenaan, Seigfred Prado, Victor Ubels, Marc Soler**, and many others. Their inspiring discussions, kind encouragements, and genuine friendship have made my time in the lab enjoyable and enriching.

I would like to thank **Dr Victoria Crossley** from the Graduate School for providing me the chance to participate as a Student Programme Partner in the Research Impact program. This experience not only heightened my enthusiasm for research but also enriched my insight into

translating academic research into tangible real-world impact. Furthermore, being able to contribute my student viewpoint to the programs has allowed me to become more deeply engaged within the Imperial Community.

A special thank you to the Bioengineering student office, particularly **Daze Osuide**, **Raleigh Mangsat** and **Jo Adam** (Finance office), for their support and responsiveness to all my queries and discussions. Their positive role has made navigating through my PhD studies smoother.

Lastly, I am grateful to **IC-CSC** for generously funding my PhD project.

Thank you all for being a part of this incredible journey. Your contributions have made a difference, and I am truly grateful for each and every one of you.



Abstract

Over the decades, numerous techniques have been developed for cancer diagnosis, based on high resolution imaging, body fluid analysis, and tissue biopsies. While these methods are effective at identifying late-stage carcinomas, they are either invasive or lack sensitivity for early diagnosis. Imaging techniques for instance often do not have the resolution required to detect small solid tumours in the early stages of cancer. Bodily fluids contain a broad range of clinically useful molecular biomarkers and the analysis of liquid biopsies (e.g. blood tests) have great potential as non- or minimally invasive diagnostic tools for many pathologies, including cancer. This study presents original nucleic acid-based electrochemical biosensors with potential future applications for the detection of cancer-associated biomarkers from bodily fluids, with a specific focus on circulating microRNA biomarkers for early diagnosis of prostate cancer, chosen as our model system. Sequence-specific capturing of nucleic acids was achieved by functionalizing electrode surfaces with bespoke molecular probes based on a Peptide Nucleic Acid (or PNA) scaffold using various immobilization chemistries and strategies, including gold-thiol bonds, silanization of hydroxyls on carbon followed by click chemistry, trans-esterification, carbodiimide chemistry, and polystreptavidin-biotin interaction. While all these immobilization techniques have shown some effectiveness, their performances towards the nucleic acid-based electrochemical sensor varied. A range of sensing strategies has been explored that included sandwich assays, electrochemical oligonucleotide templated reactions (EOTR), chronoamperometry, electrochemical impedance spectroscopy (EIS) and DNA intercalation. While all the sensing strategies worked to some degree, the best approach was the sandwich assay, offering a linear dynamic range from 77 nM to 180 nM and limit of detection (LoD) of 77 nM. With clinical endogenous concentrations of miRNA in bodily fluids ranging from atto-molar to nano-molar levels depending on the chosen sample treatment and detection methods, this proof-of-concept demonstration shows great promise, although improvement are required both in terms of sensitivity and specificity.

Table of Contents

Abstract	7
Table of Contents	9
List of Publications	13
List of Figures.....	15
List of Tables	21
List of Abbreviations	23
1 Introduction	25
1.1 Prostate Cancer and Early Detection	25
1.1.1 Overview of Cancer	25
1.1.2 Prostate Cancer Facts.....	26
1.1.3 Current Diagnostic Methods for Prostate Cancer.....	27
1.1.4 Diagnostic Evaluation.....	28
1.2 Diagnostic Biomarkers	33
1.2.1 Cancer Biomarkers	34
1.2.2 Human Biomarker Sources	42
1.3 Biosensors and Instrumentation.....	45
1.3.1 Biosensors	45
1.3.2 Biosensor Instrumentation with Three-electrode Systems	49
1.4 Electrochemical Techniques	60
1.4.1 Cyclic Voltammetry	60
1.4.2 Square Wave Voltammetry (SWV).....	68
1.4.3 Differential Pulse Voltammetry (DPV)	68
1.4.4 Chronoamperometry/Chronocoulometry	72
1.4.5 Electron Impedance Spectroscopy (EIS)	73
1.4.6 Summary	75
1.5 Aims and Objectives.....	76
2 Electrode Functionalisation Strategies: How to Best Immobilize Oligonucleotide Capture Probes .	79
2.1 Introduction of PNAs.....	80
2.1.1 Structures of DNA, RNA and PNA.....	80
2.1.2 Properties of DNA, RNA and PNA.....	81
2.2 Surface Functionalization Strategies.....	84
2.2.1 Immobilization on Gold Electrode	86

2.2.2	Immobilization on Carbon Electrode	91
2.2.3	Immobilization on 2D Material	94
2.2.4	Avidin-biotin Linking	96
2.3	Controlling Probe Density, Orientation and Accessibility	96
2.3.1	Backfilling for Controlling Probe Density and Orientation.....	98
2.3.2	Backfilling for Passivation	98
2.3.3	Controlling Probe Orientation and Density with DNA Nanostructures	98
2.3.4	Surface Coating for Antifouling.....	99
2.4	Materials	100
2.4.1	DNA and PNA Oligomers.....	101
2.4.2	PNA Synthesis.....	103
2.4.3	Electrochemical Setup.....	105
2.5	Surface Functionalization.....	106
2.5.1	Polystreptavidin-biotin Interaction on Carbon Electrodes	107
2.5.2	Silanization on Carbon Electrodes	118
2.5.3	Immobilization on Graphene Electrodes	127
2.5.4	Immobilization on Gold Electrodes.....	131
2.6	Conclusion.....	137
3	DNA Quantification	141
3.1	Materials	143
3.1.1	Oligomer Sequences	143
3.1.2	Electrochemical Apparatus	143
3.2	DNA Quantification of Polystreptavidin-biotin Interaction Immobilized Carbon Electrode.....	144
3.2.1	Methods.....	144
3.2.2	Results and Discussion	145
3.3	DNA Quantification on Silanized Carbon Electrode	148
3.3.1	Methods.....	148
3.3.2	Results and Discussion	149
3.4	DNA Quantification on Graphene Electrode.....	151
3.4.1	Methods.....	152
3.4.2	Results and Discussion	152
3.5	Conclusion.....	153
4	Sensing	157
4.1	Materials and Methods.....	158
4.1.1	Oligomer Sequence	158

4.1.2	Materials and Electrochemical Apparatus	161
4.1.3	Electrochemical Sensing Strategies.....	161
4.2	Results and Discussion	165
4.2.1	Sandwich Assay	165
4.2.2	Electrochemical Oligonucleotide Template Reaction (EOTR).....	168
4.2.3	Chronoamperometry	174
4.2.4	Electrochemical Impedance Spectroscopy (EIS)	185
4.2.5	DNA Intercalation.....	189
4.3	Conclusion.....	193
5	Summary and Outlook	195
5.1	Immobilization strategies and its optimization	195
5.2	Sensing Techniques.....	196
	Reference	200
	Appendix Supplementary Figures for Chapter 4.....	219
	EOTR.....	220
	Chronoamperometry on Polystreptavidin-biotin System.....	221
	Chronoamperometry on Gold-thiol System	223
	Appendix Copyright Permission	225

List of Publications

1. **Xiaotong Meng**, Danny O'Hare, Sylvain Ladame, "Surface immobilization strategies for the development of electrochemical nucleic acid sensors." *Biosensors and Bioelectronics*, Volume 237, 2023, 115440, <https://doi.org/10.1016/j.bios.2023.115440>.
2. Philip Gillespie, Robert B. Channon, **Xiaotong Meng**, Md Nazmul Islam, Sylvain Ladame, Danny O'Hare, "Nucleic acid sensing via electrochemical oligonucleotide-templated reactions." *Biosensors and Bioelectronics*, Volume 176, 2021, 112891, <https://doi.org/10.1016/j.bios.2020.112891>.
3. (Manuscript in preparation) On-chip Electrochemical Sensing Platform of miRNA Biomarkers for Early Detection of Prostate Cancer.
4. (Manuscript in preparation) Immobilization Strategies for DNA Sensor on Screen-printed Carbon Electrode

List of Figures

Figure 1-1 Correlation between tumour size (time) and metastasis. ⁵	26
Figure 1-2 Illustrative diagram for sensitivity and specificity.	30
Figure 1-3 Categories of cancer biomarkers.	34
Figure 1-4 Schematic representation of microRNA biogenesis in animal cells.	38
Figure 1-5 Schematic diagram of op-amps.	54
Figure 1-6 Voltage follower.	55
Figure 1-7 Current follower.	56
Figure 1-8 (a) Three electrode system connected with potentiostat. (b) A full potentiostatic system.	59
Figure 1-9 Voltammogram of the reversible reduction of a 1 mM [Fc] ⁺ solution to [Fc], at a scan rate of 100 mV/s.	62
Figure 1-10 SWV waveform and DPV waveform.	70
Figure 1-11 Randles circuit.....	74
Figure 1-12 Structure mapping of the experimental chapters.	77
Figure 2-1 Structure of DNA, RNA and PNA.	81
Figure 2-2 Schematic representation of the main key steps to produce an electrochemical nucleic acid biosensor working electrode. ¹⁵⁰	85
Figure 2-3 Physical and chemical immobilization strategies depending on electrode materials. ¹⁵⁰	86
Figure 2-4 Self-assembled monolayer with (a) thiolated nucleic acids and (b) thiol derivatives for further functionalization. ¹⁵⁰	88

Figure 2-5 Potential window of glassy carbon and metal electrodes. ¹⁵⁰	91
Figure 2-6 Silanization with (3-Aminopropyl)triethoxysilane (APTES). ¹⁵⁰	93
Figure 2-7 Carbodiimide (EDC/NHS) coupling chemistry mechanics.	94
Figure 2-8 Orientation control by backfilling. ¹⁵⁰	97
Figure 2-9 Representative PNA capture probe structures.	102
Figure 2-10 PNA synthesis schematic diagram.	103
Figure 2-11 Three-electrode system with bulk electrodes and disposable screen-printed carbon electrode (SPCE).	106
Figure 2-12 Immobilization strategies on three different electrodes.	107
Figure 2-13 Cyclic voltammetry of 1 mM freely diffusing aniline solution at a scan rate of 100 mV/s in 100 mM KCl electrolyte using bulk gold electrode.	110
Figure 2-14 Biotin-(PEG) ₆ -OH structure.	111
Figure 2-15 Biotinylated-PNA immobilization on SPCE.	113
Figure 2-16 Co-immobilization efficiency test by SWV in 100 μ M 4-methyl-catechol solution.	115
Figure 2-17 Co-immobilization stability test by DPV (baseline corrected).	116
Figure 2-18 (a) Silanization with 3- (trimethoxysilyl)propyl methacrylate on carbon electrode. (b) Click chemistry diagram between alkene and thiol.	118
Figure 2-19 Representative schemes for click chemistry.	121
Figure 2-20 Co-immobilization efficiency and stability SWV test of PNA immobilization on silanized carbon electrode via aqueous phase.	122
Figure 2-21 Comparison before and after the silanization of SPCE in 250 μ M RuHex (cyclic voltammetry scan rate 100 mV/s).	123

Figure 2-22 Co-immobilization efficiency SWV test of PNA immobilization on silanized carbon electrode via organic phase.	124
Figure 2-23 Adduct peak comparison from SWV by silanization in aqueous and organic phases.	125
Figure 2-24 Molecular structure of ferrocenecarboxylic acid.	126
Figure 2-25 DPV test with ferrocene -COOH immobilized bulk glassy carbon electrode (baseline corrected).	126
Figure 2-26 Schematic reactions for amine-terminated PNA probe on NHS-treated graphene electrode.	128
Figure 2-27 Capture probe immobilization characterization by adduct peak height from SWV.	130
Figure 2-28 Characterization of Thiol-PNA-Catechol-141 immobilization on bulk Au electrode.	133
Figure 2-29 SWV test in 1 mM aniline solution with Thiol-PNA-Catechol-141 immobilized bulk gold electrode.	134
Figure 2-30 EIS investigation of 20 μ M Thiol-PNA-Catechol-141 and 1 μ M Thiol-PNA-Aniline-141 for the immobilization on bulk gold electrode.	135
Figure 2-31 Bar plot comparison with charge transfer resistance of 20 μ M Thiol-PNA-Catechol-141 (PNA1) and 1 μ M Thiol-PNA-Aniline-141 (PNA2) for the immobilization on bulk gold electrode.	136
Figure 3-1 Hybridization efficiency test by MB-labelled DNA on different electrode surfaces.	142
Figure 3-2 SWV measurement of 1 μ M freely diffusing MB-DNA141 on polystyreneptavidin-functionalized screen-printed carbon electrode.	145

Figure 3-3 DNA quantification of MB-DNA with complementary DNA and non-complementary DNA on polystreptavidin treated SPCE by SWV.	146
Figure 3-4 DNA quantification of methylene blue labelled DNA with complementary DNA and non-complementary DNA on silanized (aqueous phase) SPCE by SWV.	150
Figure 3-5 DNA quantification of 100 nM methylene blue labelled DNA with complementary DNA and non-complementary DNA on silanized (organic solvent phase) SPCE by SWV.	151
Figure 3-6 DNA quantification using MB-DNA with complementary DNA and non-complementary DNA on NHS-graphene electrode by SWV.	153
Figure 3-7 Comparison of SWV for DNA quantification using 100 nM complementary MB-DNA (125 nM for NHS-graphene electrode) with four different immobilization methods.	155
Figure 4-1 Sensing strategies on polystreptavidin-biotin functionalized SPCEs.	158
Figure 4-2 Representative PNA sensing probe structures.	160
Figure 4-3 Sensing strategies based on polystreptavidin-biotin immobilization method. ...	163
Figure 4-4 Sandwich sensing with PNA-AQ-374a	166
Figure 4-5 Stability test of sandwich assay sensing using PNA-AQ-374a (n=3).	167
Figure 4-6 DPV investigation of EOTR on SPCE using polystreptavidin-biotin method.	170
Figure 4-7 Electrochemical oligonucleotide templated reaction (EOTR, 500 nM complementary and non-complementary DNA) sensing with PNA-Catechol-141.	171
Figure 4-8 Electrochemical oligonucleotide templated reaction (EOTR, 500 pM - 500 nM complementary and non-complementary DNA) sensing with PNA-Catechol-141.	173
Figure 4-9 Cyclic voltammetry characterization of 50 μ M RuHex in 10 mM Tris buffer with 100 mV/s scan rate.	176
Figure 4-10 Chronoamperometry data for charge fitting and calculation with complementary DNA.	178

Figure 4-11 Chronoamperometry data for charge fitting and calculation with non-complementary DNA.....	180
Figure 4-12 Charge associated with immobilized species.	181
Figure 4-13 Surface charge from RuHex with 500 nM complementary DNA (DNA141) after 0.5% TWEEN washing.	182
Figure 4-14 (a) Chronoamperometry with single measurement (after washing with 1X PBS) (b) Statistical test between 500 nM complementary DNA (DNA141) and non-complementary DNA (DNA150).	183
Figure 4-15 Chronoamperometry with single measurements (after washing with 1X PBS), one-to-one tracking line plot.	184
Figure 4-16 Chronoamperometry with multiple measurements (with multiple wash steps).	185
Figure 4-17 EIS measurements using SPCE.	186
Figure 4-18 EIS sensing with wide range complementary and non-complementary DNA. ..	187
Figure 4-19 Surface change with each step surface treatment by EIS. ST=streptavidin, PNA= Biotin-PNA-Aniline-141, DNA=DNA141, n=11, 38 and 7.	188
Figure 4-20 EIS measurements with 500 nM and 1 μ M complementary (DNA141) and non-complementary DNA (DNA150) followed by 0.5% TWEEN washing.	189
Figure 4-21 DNA intercalation sensing by SWV using 200 μ M MB for 10 minutes without wash.	190
Figure 4-22 Box plot of DNA intercalation sensing by SWV using (a) 100 nM and (b) 200 nM DNA with different MB concentration.....	191
Figure 4-23 Box plot of DNA intercalation sensing by SWV using 200 μ M MB for 5 minutes and washed with 1X PBS.....	192

Figure A-1 Matrix-assisted laser desorption/ionization (MALDI) results of HPLC purified PNA-AQ-374a.	219
Figure A-2 DPV investigation of EOTR on SPCE using polystreptavidin-biotin method.	220
Figure A-3 Chronoamperometry data for charge fitting and calculation (after 0.05% TWEEN washing, 0.25 s measurement).....	221
Figure A-4 Chronoamperometry data for charge fitting and calculation (after 0.05% TWEEN washing, 5 s measurement).....	222
Figure A-5 Charge associated with complementary DNA (DNA141) and control non-complementary DNA (DNA150) on bulk gold electrode.	223
Figure A-6 Chronoamperometry data for charge fitting and calculation with bulk gold electrode (0.25 s measurement).	224

List of Tables

Table 1-1 Types of disease prevention and their strategies. ¹³	27
Table 1-2 Confusion matrix.	29
Table 1-3 Formulas for probabilistic approaches.	30
Table 1-4 Sensitivity and specificity of PSA from different literatures.	31
Table 1-5 Relative merits of current available techniques.	39
Table 1-6 Pros and cons of different body fluid: blood, sweat, tears, saliva, and urine.	43
Table 1-7 Three-electrode system components and their properties.	51
Table 1-8 Basic properties of op-amps.	54
Table 1-9 Comparison of parameter difference between DPV and SWV in a practical measurement.	71
Table 2-1 Property comparison of PNA, DNA and RNA.	82
Table 2-2 Bond strength of typical immobilization methods on metal. ¹⁴⁶	89
Table 2-3 Oligomer nomenclature and sequences.	102
Table 2-4 Comparison of SWV data by different immobilization methods.	138
Table 3-1 Oligomer Nomenclature and Sequences.	143
Table 4-1 Oligomer Nomenclature and Sequences.	159

List of Abbreviations

AC	Alternating current
Ag/AgCl	Silver/silver chloride electrode
APTES	(3-Aminopropyl)triethoxysilane
AQ	Anthraquinone
Bhoc	Benzhydryloxycarbonyl group
BSA	Bovine serum albumin
CV	Cyclic Voltammetry
CTCs	Circulating tumour cells
DPV	Differential Potential Voltammetry
DIC	N,N'-Diisopropylcarbodiimide
DMF	Dimethylformamide
DIPEA	N,N-diisopropylethylamine
DRE	Digital rectal examination
DNA	Deoxyribonucleic acid
EOTR	Electrochemical oligonucleotide template reaction
EIS	Electrochemical Impedance Spectroscopy
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ERSPC	European Randomised study of Screening for Prostate Cancer
Fmoc	9-Fluorenylmethoxycarbonyl chloride
FDA	The US Food and Drug Administration
FN	False negative
FP	False positive
FET	Field-effect transistors
GP	General practitioner
HPLC	High Performance Liquid Chromatography
HATU	2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate

IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium chloride
LoD	Limit of Detection
LSV	Linear sweep voltammetry
MALDI	Matrix-assisted Laser Deposition Ionization
MRI	Magnetic Resonance Imaging
MB	Methylene blue
miRNA	MicroRNA
NMP	N-methylpyrrolidone
NHS	N-hydroxysuccinimide
OTR	Oligonucleotide Template Reaction
Op-Amps	Operational amplifiers
PNA	Peptide nucleic acid
PSA	Prostate specific antigen
PPV	Positive predictive value
RuHex	Hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$)
ROC	Receiver-operating characteristic
SCE	Saturated calomel electrode
SPCE	Screen-printed Carbon Electrode
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TP	True positive
TPR	True positive rate
TN	True negative
TNR	True negative rate
TFA	Trifluoroacetic acid
USPSTF	US Preventative Services Task Force
2D	Two-dimensional

1 Introduction

The main goal of this thesis is to explore and develop new strategies and platform technologies for the sensitive and sequence-specific electrochemical detection of short, single-stranded nucleic acid biomarkers. Such technologies have great potential for rapid, low-cost and non-invasive diagnosis of many pathologies, including many cancer types. Although the work described in this thesis focuses exclusively on technology development and no testing was carried out on clinical samples, a possible long-term application is for improving early detection of prostate cancer and prostate cancer was therefore chosen as a model system for future applications throughout this thesis. This chapter will introduce:

1. The importance of early detection in cancer diagnosis and current challenges, in particular for prostate cancer.
2. Molecular biomarkers in bodily fluids and the emergence of miRNAs as clinically useful biomarkers.
3. A general overview of statistical validation of diagnostic techniques and an overview of the design and operation of diagnostic tests.
4. Electrochemical system and voltammetry techniques.

1.1 Prostate Cancer and Early Detection

1.1.1 Overview of Cancer

Cancer is the second leading cause of mortality for human beings. Currently one in five people worldwide develop cancer during their lifetime¹ and cancer accounts for about 1 in every 6 deaths worldwide. In 2020, there were 19.3 million new cancer cases and almost 10.0 million cancer deaths worldwide according to the International Agency for Research on Cancer.² Early cancer diagnosis is a critical topic in the twenty-first century, especially given the estimated population of 1.5 billion people aged 65 and older by 2050. By 2040, the global incidence is expected to reach 27.5 million new cancer cases, with 16.2 million cancer deaths, solely due to the growth and aging of the population.³ Early diagnosis of cancer aims to identify symptomatic patients at the earliest possible stage, providing them with the optimal opportunity for successful treatment.⁴ As shown in Figure 1-1, tumors grow exponentially over time and the bigger size leads to higher probability of metastasis. The relationship of

metastasis probability to tumor size implies early detection can reduce cancer-specific mortality.

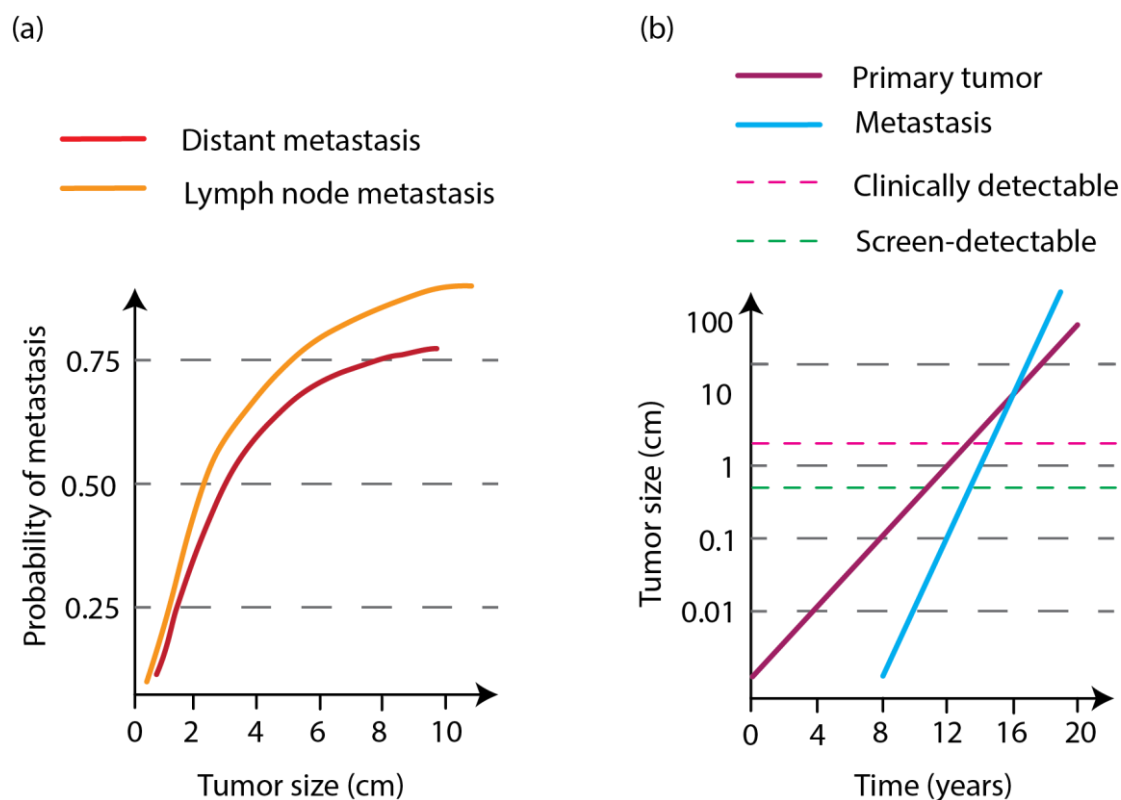


Figure 1-1 Correlation between tumor size (time) and metastasis. (a) Probability of metastasis by primary breast tumor size. (b) Log-linear growth of a primary tumor and metastasis over time (adapted and replotted from reference⁵).

1.1.2 Prostate Cancer Facts

In men, prostate cancer is the most frequently diagnosed cancer in 112 countries.² In 2020, globally, there were 1,414,259 new recorded cases and 375,304 new recorded deaths of prostate cancer.^{2,6} In the UK alone, more than 47,500 men are diagnosed with prostate cancer every year. Approximately 1 in 8 men will develop prostate cancer at some point in their lives, and about 1 man in 41 will die of prostate cancer.⁷ Prostate cancer is more likely to develop in older men, with about 6 out of 10 cases diagnosed in men who are 65 or older.⁷⁻¹⁰ Cancer is a complex and dynamic disease that can change quickly. Developing reliable and robust

non-invasive platforms for diagnosis, patient stratification, and treatment response monitoring is crucial in effectively managing cancer.¹¹ This research seeks approaches in the secondary prevention of prostate cancer, which involves screening and reducing the impact of the disease, as shown in Table 1-1. The early diagnosis of cancer, including prostate cancer, is crucial in reducing mortality rates. Survival rates are directly linked to the stage at which they are diagnosed.¹²

Table 1-1 Types of disease prevention and their strategies.¹³

Types of prevention	Prevention Strategies
Primordial prevention	Broad steps to prevent disease (not individual prevention). Government policies like increasing taxes on cigarettes; decreasing advertisement of tobacco are primordial prevention.
Primary prevention	Prevent the disease through education and immunization.
Secondary prevention	Screening for disease early and reduce the impact.
Tertiary prevention	Treat disease and softening the impact of an ongoing illness or injury that has lasting effects.
Quaternary prevention	Making sure not to repeat tests.

1.1.3 Current Diagnostic Methods for Prostate Cancer

1.1.3.1 Prostate specific antigen (PSA) and digital rectal examination (DRE)

The diagnosis of prostate cancer is a global challenge due to its complexity and heterogeneity, as well as the lack of symptoms in its early stages.¹⁴ Until the 1970s, the primary diagnostic method for prostate cancer was digital rectal examination (DRE), which involves checking the prostate gland for the presence of lumps or hardening. Consequently, most cases of prostate cancer were diagnosed at a later stage when the disease was already advanced and symptomatic.¹⁵ Another screening method for prostate cancer is prostate-specific antigen (PSA). PSA is a kallikrein-like serine protease secreted by epithelial cells in the prostate gland,

responsible for seminal fluid liquefaction and can be measured in the blood. The US Food and Drug Administration (FDA) agency approved serum PSA tests in conjunction with DRE for prostate cancer screening in 1994.¹² Since then, PSA and DRE have been considered as the gold standards for prostate cancer diagnosis and screening for decades. However, in recent years in the UK, PSA test is not recommended for large-scale screening as its sensitivity and specificity were too low, leading to over-diagnosis and over-treatment.¹⁶ Although DRE remains a key step in the pathway to prostate cancer diagnosis until now, studies have found that DRE has little effect in preventing metastatic prostate cancer or reducing mortality to any appreciable degree.¹⁴

1.1.3.2 Limitations of PSA

The serological PSA level is low in healthy men with an increased level observed after disruption of the basement membrane of the prostate gland, for example, as a result of prostate cancer and other conditions like an enlarged prostate inflammation (prostatitis) or urinary infection.^{12,17} The threshold for a normal PSA level is usually considered 4 ng/mL (approximately 153.3 nM). However, according to the European Randomised study of Screening for Prostate Cancer (ERSPC), 40–50% of screen-detected cases often results in overtreatment with subsequent side-effects.¹⁸ On the one hand, up to 15% of men with PSA levels within the healthy range (i.e., below 4 ng/mL) have prostate cancer.¹² In another study, men with PSA greater than 4 ng/mL have about 1 in 3 chances of having cancer and those with PSA more than 10 ng/mL have about 2 in 3 chances of having cancer.¹⁹ There is therefore an urgent need for a new diagnostic test for prostate cancer with significantly greater sensitivity and specificity than PSA.²⁰

1.1.4 Diagnostic Evaluation

In order to determine the optimal treatment for patients, doctors often require additional information beyond what patients can provide. This information is typically obtained through clinical tests. A good test should yield reliable results that can aid decision-making and guide doctors towards appropriate treatment options for their patients. There are three general methods used to quantitatively describe a test: the probabilistic approach, Bayesian analysis, and information theory.

1.1.4.1 Probabilistic Approaches

1.1.4.1.1 Confusion Matrix

A confusion matrix (Table 1-2), also known as an error matrix, is a table that visualizes the performance of a classification model by comparing its predicted results against the true values. Probabilistic approaches focus on the data obtained from the tests. Sensitivity is also called true positive rate (TPR): true positive (TP) (positive test results from individuals with the condition) divided by all the positive (TP + false negative (FN)). Specificity is also known as true negative rate (TNR): true negative (TN) over all the negative (TN + false positive (FP)).

Table 1-2 Confusion matrix.

Test \ Reality	Positive	Negative
Positive	TP	FP
Negative	FN	TN

As shown in Table 1-3 and Figure 1-2, sensitivity, also known as the true positive rate or the recall, quantifies the ability of a test to correctly identify positive cases. It is calculated as the ratio of true positive results to the total number of actual positive cases. Specificity, on the other hand, measures the true negative rate or the ability of a test to correctly identify negative cases. It is calculated as the ratio of true negative results to the total number of actual negative cases (true negatives plus false positives).

Positive predictive value (PPV) is dependent on the prevalence of the disease, while sensitivity and specificity are not, as can be noticed from the formulas in Table 1-3. Prevalence is the proportion of actual disease cases in a particular population at a given time. It is obtained from real data statistics, and it cannot be calculated before its given time period. The PPV represents clinical significance. It indicates the degree to which the results can be trusted and answers the question “if a subject has a positive test result, what is the probability that the

patient has the condition?”. These PPV values are functions of the disease prevalence as well as the quality of the test.

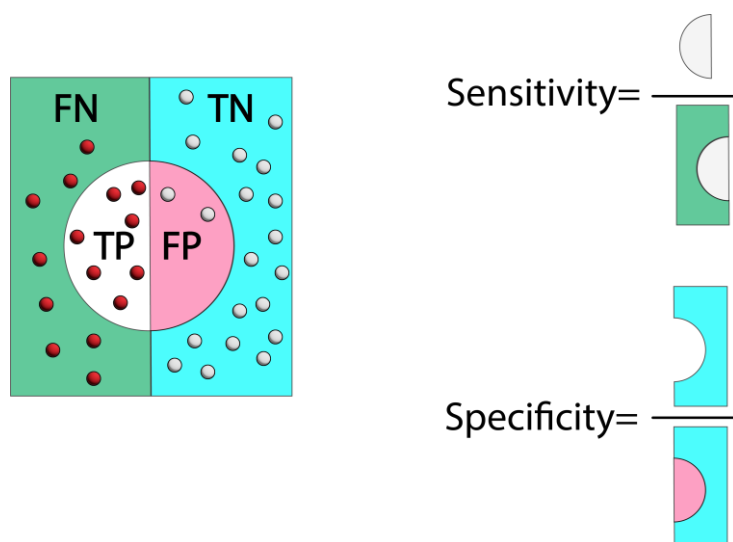


Figure 1-2 Illustrative diagram for sensitivity and specificity.

Table 1-3 Formulas for probabilistic approaches.

Parameters	Definition
Sensitivity (TPR)	$TPR = \frac{TP}{P} = \frac{TP}{TP + FN} = 1 - FNR$
Specificity (TNR)	$TNR = \frac{TN}{N} = \frac{TN}{TN + FP} = 1 - FPR$
Prevalence	$\frac{\text{Number of individuals with the disease}}{\text{Total population in the sample}} \times 100\%$
Accuracy	$\frac{TP + TN}{P + N}$

Positive predictive value (PPV)	$\frac{\text{Sensitivity} \times \text{prevalence}}{\text{Sensitivity} \times \text{Prevalence} + (1 - \text{Specificity})(1 - \text{Prevalence})}$
--	---

PSA testing frequently leads to overdiagnosis and overtreatment. This is because the PSA level can be affected by other conditions like hyperplasia and prostatitis. Thus, up to 75% of biopsies directed by PSA screening could have been avoided.¹² So, how reliable is really the PSA test? Table 1-4 reflects the sensitivity (TPR) and specificity (TNR) of the PSA tests from peer-reviewed studies. Setting different PSA levels as thresholds results in a trade-off between sensitivity and specificity, and none of them proves to be satisfactory as a diagnostic approach. The appropriate balance between sensitivity and specificity depends on the screening program's objectives, the condition being screened for, and the potential consequences of false results. For large population screening tests (no symptoms), specificity is more important than sensitivity because the number of healthy individuals outweighs the number of patients.²¹

Table 1-4 Sensitivity and specificity of PSA from different literatures.

	Sensitivity	Specificity	References
PSA in serum with a cut-off of 4.1 ng/mL	20%	94%	12
PSA in serum with a cut-off of 2.6 ng/mL	40%	81%	12
Clinical performance of PSA	80%	14%	12
Improved PSA test	89.29%	54.29%	22

1.1.4.1.2 Receiver-operating Characteristic (ROC) and Area Under the Curve (AUC)

The objective of any diagnostic test is to differentiate between individuals who are sick and those who are healthy, based on the biomarker being studied. However, there is often an

overlap between these two populations. Therefore, selecting an appropriate or optimal threshold for the diagnostic test is essential.²⁴

The evaluation of a diagnostic method can be achieved through the use of the receiver-operating characteristic (ROC) curve and Area Under the Curve (AUC), which aids in analysing whether the test can achieve a confidence level greater than 50% in differentiating between healthy and sick individuals using a combination of sensitivity and specificity. The ROC curve is a graphical representation of the performance of a diagnostic test or classifier. It illustrates the trade-off between the true positive rate (sensitivity) and the false positive rate (1-specificity) at different classification thresholds. The AUC quantifies the overall performance of a diagnostic test or classifier based on the ROC curve. It represents the area under the ROC curve and provides a single numerical value to measure the discriminatory power of the test. The AUC ranges from 0 to 1, where an AUC of 1 indicates a perfect classifier, and an AUC of 0.5 suggests a classifier with performance no better than random chance.²³

1.1.4.2 Bayesian analysis

Despite achieving high levels of sensitivity and specificity in clinical approaches, it is necessary to update the real probability of a positive test result indicating the presence of the disease based on additional evidence such as prevalence and symptoms. Bayesian analysis allows the result of the test to be connected to the prior probability, which, in this case, is the age-related prevalence of prostate cancer. In 2023, the overall prostate cancer prevalence in the UK is 1.489%.⁷ The prevalence can vary significantly based on age: 3.562% for men aged 70-79 years old, 0.412% for men aged 50-59 years old, and 0.030% for men aged 40-49 years old. It's very rare among men younger than 40 (data calculated based on male population and prostate cases in England).²⁴ Prevalence is sometimes easily confused with incidence. Incidence is a measure of new disease cases that develop in a population during a specified time period, whereas prevalence is the proportion of the population already affected by a specific disease within a given time period.²⁵ The mathematical statement of Bayesian theorem is:

$$P(A|B) = \frac{P(A \& B)}{P(B)} = \frac{P(A)P(B|A)}{P(B)} \quad 1.1$$

Where A and B represents for different events. $P(A)$ is the probability of A happening or A is true. $P(B)$ is the probability of B happening or B is true. $P(B)$, which is also called prior probability, is the prevalence in this topic. $P(A|B)$ means the probability of A happening or A is true given the condition that B has happened, or B is true. $P(A \& B)$ means the probability of both A and B are being true.

Consider the example of a 79-year-old man who takes a prostate cancer test with a sensitivity of 95% and receives a positive result. Does this imply that he has a high probability of having prostate cancer? In fact, the prevalence of prostate cancer at his age must also be taken into account. The prevalence of prostate cancer in this age group is 3.562%.²⁶ Let A represent the probability that the man has prostate cancer and B represent the probability that the test result is positive. Therefore:

$$P(A) = 3.562\%$$

$$P(B|A) = 95\%$$

$$P(B|\text{not}A) = 5\%$$

$$P(A|B) = \frac{P(A \& B)}{P(B)} = \frac{P(A)P(B|A)}{P(B|A)P(A) + P(B|\text{not}A)P(\text{not}A)} = \frac{0.03562 \times 0.95}{0.95 \times 0.03562 + 0.05 \times 0.96438} = 0.41 = 41\%$$

Despite the fact that the 79-year-old man received a positive test result for prostate cancer from a highly sensitive test of 95%, the probability of him having the disease is only 41% due to the prevalence of prostate cancer in his age group.

1.2 Diagnostic Biomarkers

The utilization of appropriate technologies for evaluating the quality of diagnostic methods is crucial, and the choice of biomarker also significantly influences the effectiveness of the diagnosis. Biomarkers can be cellular, biochemical, and molecular (proteomic, genetic, and epigenetic) alterations to recognize or monitor a normal, abnormal, or simply a biological process.²⁸

1.2.1 Cancer Biomarkers

Cancer biomarkers can be classified into different categories based on their chemical structure, including protein-based, DNA-based, RNA-based, small molecule-based, and enzyme or antibody-based biomarkers, as depicted in Figure 1-3. Additionally, in certain specific methods, cancer cells themselves can be considered as a type of biomarker. The different chemical structures of biomarkers determine the type of recognition and chemistry employed for their analysis. Detection of cancer biomarkers within complex biological media is a huge challenge because of severe biofouling and nonspecific adsorptions.²⁹ However, unremitting efforts of exploring different types of available cancer biomarkers have been undertaking in the last decades.

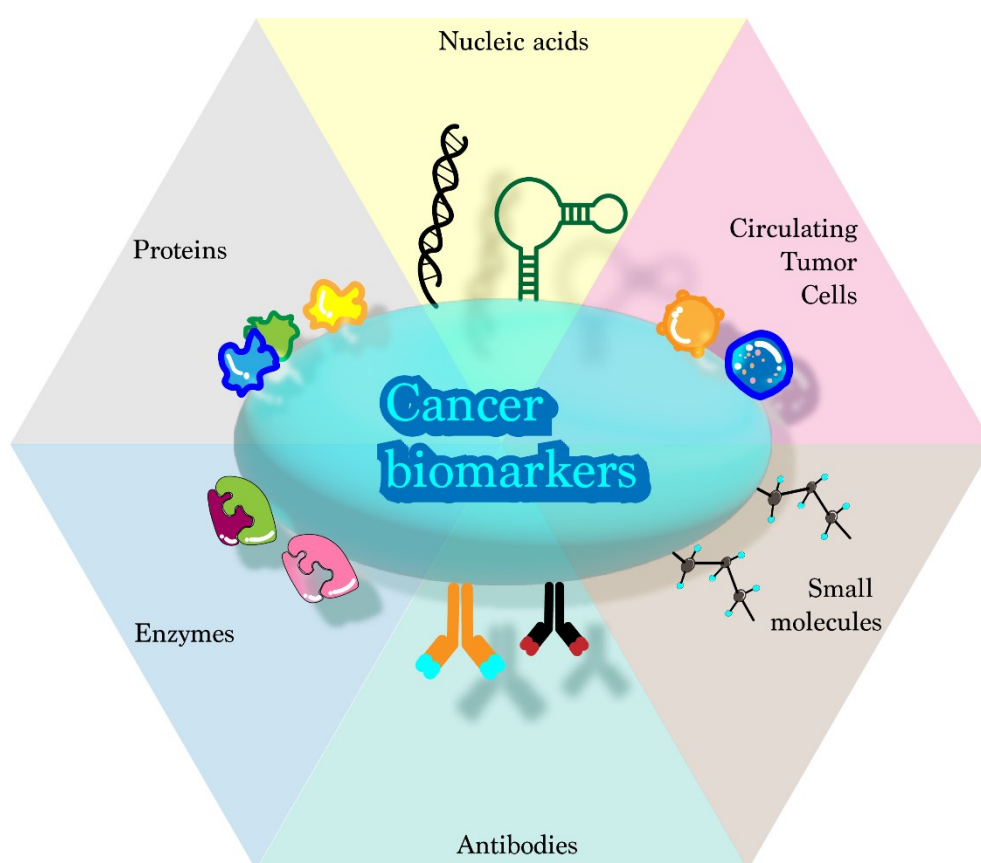


Figure 1-3 Categories of cancer biomarkers.

1.2.1.1 Protein-based Biomarkers

Proteins, comprising about 18% of the human body, play vital roles in various biological processes. They serve as catalysts, contribute to cell structure, and participate in molecular transport. Abnormal protein expression is often associated with diseases.³⁰ Protein biomarkers used for cancer diagnosis, include proteins produced by cancer cells themselves or in response to cancer.^{31–33} Using proteins as biomarkers has some limitations. Some proteins may not be specific to a particular disease or condition, leading to less satisfactory specificity. Additionally, protein levels in the body can vary due to factors such as age, sex, diet, and individual variability, making it challenging to establish a clear threshold for diagnosing a specific condition. Proteins are also sensitive to degradation, denaturation, or proteolytic activity, which can affect the integrity of the protein biomarker. Proper sample handling and storage are crucial to maintain the stability and accuracy of protein biomarker measurements.³⁴

1.2.1.2 Enzyme-based Biomarkers

The changes of enzyme activity in tissues, body fluids, and serum, as well as changes of the isoenzymes and enzyme variants in patients with malignancy suggest that these parameters may be used as biomarkers in the diagnosis of some types of cancer.³⁵ Deubiquitinating enzymes, a family of proteases that remove ubiquitin tags from proteins of interest undergoing proteasomal degradation, are expressed in different types of cancer.³⁶ Cyclooxygenase-2 is also a studied tumor-related enzyme as a cancer imaging biomarker because it is highly expressed in various tumors but barely in normal cells. However, cyclooxygenase-2 can be expressed at different levels in inflammatory lesions which is considered as a clinical limitation for the discrimination of tumors from inflammation.³⁷ The main challenge when working with enzyme-based biomarker is the decrease of the enzyme activity during the test.³⁸

1.2.1.3 Antibody-based Biomarkers

The coronavirus pandemic, which started in 2020, has spurred the development and use of fast antigen tests. Antibodies produced by the immune systems of the patients are responsible for removal of alien substances like viruses or cancer cells. These serum immuno-biomarkers are produced in large quantities despite the presence of very small amount of the

corresponding antigens. Therefore, antibodies can be treated as a class of disease biomarkers especially for cancer diagnosis. In the last few decades, the evidence of antibodies in human serum of cancer patients has created abundant opportunities by utilizing the immune system as a source of clinically useful cancer biomarker.³⁸ For example, cytokine interleukin-2 (IL-2) is used as a prognosis biomarker for immunotherapy of cancer.³⁹ Antigen tests can offer rapid and cost-effective testing, with the ability to produce quick results. However, their inaccuracy is a big challenge.⁴⁰

1.2.1.4 Small molecule-based Biomarkers

Small molecules, such as volatile organic compounds (VOCs), can serve as cancer biomarkers. VOCs collected from human breath can indicate lung cancer, with specific compounds absent in healthy individuals or differing in relative concentrations.⁴¹ These VOCs can also be detected in other body fluids, but breath analysis is preferable due to its non-invasive nature. Over 100 VOCs related to different types of cancer have been studied.⁴² Additionally, non-volatile small molecules like lysophosphatidic acid and sarcosine are associated with ovarian and prostate cancer, respectively. Detecting these molecules is challenging, but their identification holds potential for early cancer diagnosis.⁴³

1.2.1.5 Circulating Tumor Cells as Biomarker

Circulating tumor cells (CTCs) are cells shed from a primary tumor into the bloodstream, with the potential to form metastatic tumors. They are found in metastatic carcinomas like breast, prostate, lung, and colorectal cancer, but are rare in healthy individuals. CTCs are characterized by intact viable nuclei, cytokeratin and EpCAM positivity, and absence of the CD45 antigen. The number of detectable CTCs is low, typically 1-10 per milliliter of blood, and varies depending on the collection technique used.¹¹ Currently, most CTC isolation platforms require that the whole blood is processed soon after collection, which makes it hard to study using frozen samples. In addition, CTCs are fragile and tend to degrade when collected in standard blood collection tubes.

1.2.1.6 Nucleic acids-based Biomarker

Nucleic acids can serve as biomarkers for pathological processes. Their specific sequences and expression levels can be utilized for diagnosis, prognosis, and monitoring of various diseases and conditions.^{44–46} Circulating cell-free nucleic acids is a new family of biomarkers for a broad

range of cancers that attracted lots of interests in the past decade.^{45,47,48} The first trial of using circulating cell-free nucleic acids as biomarkers for cancer diagnosis was in 1977 by Leon et al.⁴⁹ Circulating cell-free nucleic acids include circulating cell-free DNA (cfDNA) and RNA (messenger RNA (or mRNA), long noncoding RNA (lncRNA) and microRNA (or miRNAs). Concentration of cfDNA was found significantly increased in serum of cancer patients when compared to healthy individuals and it was suggested that this correlated with malignancy.^{47,50} However, the total concentration of cfDNA in the blood of cancer patients varies considerably, which makes it difficult to determine a threshold for diagnosis.¹¹ Apart from DNA, some noncoding RNAs have been discovered and confirmed to be closely related to tumorigenesis and metastasis. The clinical potential of using noncoding RNAs in liquid biopsy as biomarkers with clinical value provides new approaches for cancer diagnosis, prognosis, and response to treatment.⁵¹ Among the families of noncoding RNAs, miRNAs hold great promise, and they will be discussed in more details in the sections below.

1.2.1.6.1 MiRNA as cancer biomarker

MiRNAs were first discovered in 1993 by Feinbaum et al in *C. elegans*.⁵² MiRNAs are short (20-25 nucleotides) non coding RNA molecules that play a pivotal role in the regulation of various developmental and physiological processes by exerting control over gene expression.^{53,54} Over the past decade, studies have shown that deregulated expression of miRNAs contribute to the pathogenesis of most, if not all, human malignancies.^{45,53–55}

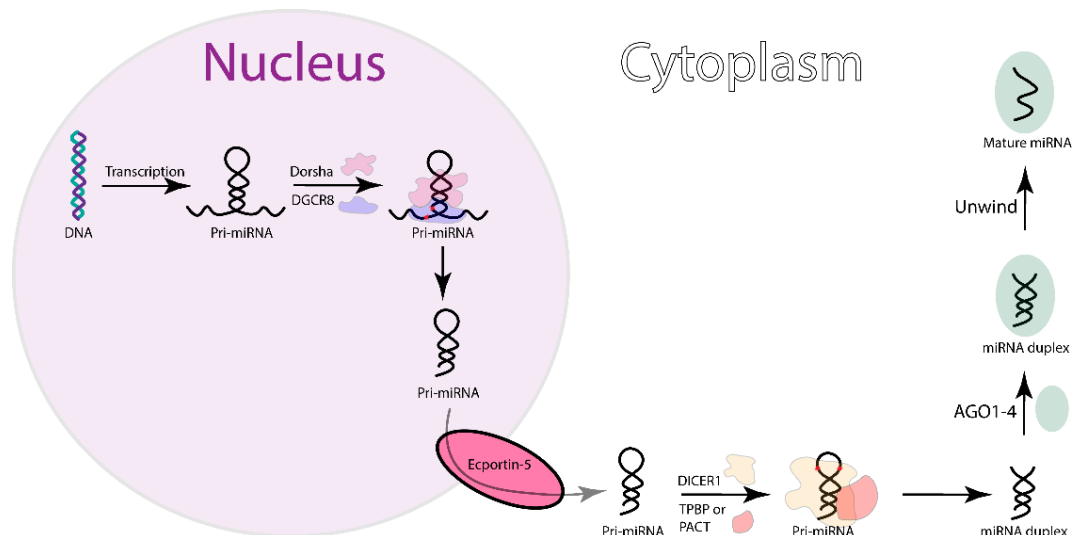


Figure 1-4 Schematic representation of microRNA biogenesis in animal cells.

As shown in Figure 1-4, miRNAs are produced in the nucleus of cells as pri-miRNA precursor characterized by a hairpin stem-loop structure. After processing by an enzyme called Drosha, the pri-miRNA becomes pre-miRNA, a shorter hairpin structure with a 3' overhang. The 3' overhang targets the pre-miRNA for export from the nucleus by Exp5 protein. In the cytoplasm, the pre-miRNA loop is cleaved by the dicer enzyme to leave a double-stranded duplex RNA. The active strand becomes complexed to Ago proteins and the inactive strand dissociates and is degraded. The single-stranded mature miRNA becomes available to bind to cytoplasmic messenger RNA (mRNA) and inhibit their translation.

MiRNAs are released in the bloodstream (and other bodily fluids including urine or saliva) as a result of pathological processes such as cell death (via either necrosis or apoptosis) and may originate from both healthy and malignant tissues. Changes in the level of circulating cell-free nucleic acids can reflect on tumor growth and cancer progression.⁴⁵ MiRNAs are significantly more stable than long mRNAs in many specimen types and are more resistant to chemical and enzymatic degradation.^{56,57} MiRNAs in bodily fluids are stable for 72 hours if kept refrigerated or frozen and are stable for at least 24 hours at room temperature,⁵⁸ which satisfies clinical requirements. They can also withstand large changes in temperature and pH, most likely because they are mainly present in these fluids either bound to proteins or trapped within exosomes.⁵⁹ Consistent changes in miRNA expression have been observed in

tumors without evident specific cytogenetic abnormalities, suggesting that pathways that are commonly deregulated in human cancer could directly affect and deregulate miRNA expression.⁵⁵ Multiple studies in the recent literature have demonstrated that miRNA deregulation in bodily fluids can be used to improve the diagnosis or prognosis of many cancer types. Dying tumor cells can indeed discharge miRNAs, which then circulate stably into the blood.^{60,61} Therefore, miRNAs have great potential as a new generation of blood-based fingerprints for the detection of human cancers, especially at an early stage, via non-invasive diagnostic tools.

1.2.1.6.2 Current Available miRNA Detection Methods

Current gold standards for miRNA detection include quantitative reverse transcription polymerase chain reaction (RT-qPCR),^{62–64} northern blot,⁶⁵ RNA sequencing and DNA microarrays.⁶⁶ Most of these techniques rely on multiple amplification steps to increase the number of target molecules and facilitate their detection. The main advantages and drawbacks of these techniques are summarized in Table 1-5.

Table 1-5 Relative merits of current available techniques.

Techniques	Advantages	Disadvantages
RT-qPCR	1.High sensitivity (with down to single molecule detection limit ⁶⁷ and 60–75% sensitivity ⁶⁸). 2.High specificity (81%). ⁶⁹ 3.Low demand on sample quantity. ⁷⁰	1.Amplification steps introduced errors. 2.High cost. 3.High levels of background fluorescence. 4.Non-isothermal. ⁷⁰ 5.Requiring careful primer design. 6.Requiring special equipment and expertise. ⁷¹

Northern blot	1.No requirement for any special equipment or expertise. ⁷¹ 2.Tissue specificity. ⁷²	1.Inaccuracy. 2.Time-consuming. 3.Low sensitivity. ^{73,74}
RNA sequencing	Higher coverage and greater resolution of the dynamic nature of the transcriptome. ⁷⁵	Time-consuming.
DNA microarrays	Specificity can be improved by elongating the length of the probes.	1.Requiring special tools and expertise. ⁷⁶ 2. Cross-hybridization artifacts. 3. Poor quantification of lowly and highly expressed genes. 4.Need to know the sequence in advance. ⁷⁷

In miRNA detection, RT-qPCR is widely recognized as the “gold standard”.⁷⁸ Despite the fact that RT-qPCR has high sensitivity, with minimum levels of bodily fluid required (typically less than 200 µl)⁷⁹ this technology holds some limitations:

- (1) risks of contamination and error during each amplification step (RT and then PCR);
- (2) high cost and lack of specificity of custom-made oligonucleotide probes;
- (3) high levels of background fluorescence due to incomplete fluorescence quenching or differences in quality and purity between commercial batches of probes;
- (4) RT-qPCR is a non-isothermal process involving multiple enzymes and is therefore not easily amenable to incorporation in miniaturized devices and remains exclusively a research tool.⁷⁰

Protocols mentioned in Table 1-5, including the gold standard RT-qPCR, use complex, multi-step and lengthy procedures that can only be carried out by trained professionals in fully

equipped laboratories.⁸⁰ This is incompatible with patients being tested and given the result of their test while at the GP's clinic, with potentially long delays between the initial and follow-up appointments.

1.2.1.6.3 Specific Challenges for Detecting miRNAs

Despite the promising aspect of miRNA as minimally invasive biomarker for cancer diagnosis, some technological challenges remain to be addressed. One of the main obstacles to the development of simple blood tests based on miRNA detection is the absence of standardized technologies to detect them directly from whole blood. Other challenges or concerns are also listed below:

(i) Issues related to miRNA endogenous concentration:

MiRNA absolute concentrations in blood are highly patient-dependent, as different patients naturally produce varying levels of miRNA, and are also influenced by the extraction and detection method used. It is commonly acknowledged that miRNAs are present at low levels and comprise less than 0.01% of the total RNA mass in a given sample.⁸¹ The true endogenous concentrations of circulating miRNAs are highly controversial. For example, Bosson et al. reported the endogenous miRNA are in the mid nano-molar range,⁸² while Shin et al. reported the endogenous miRNA are in the pico-molar range.⁸³ To account for this variation, it is preferable to measure multiple miRNA levels simultaneously and to assess relative concentrations rather than absolute concentrations.

To ensure reliable and reproducible measurement of miRNA levels, automated sample processing for miRNA extraction and purification from whole blood is required. However, the absence of standardized technologies for such processing has resulted in major inconsistencies in the recent literatures.^{58,84,85} Nguyen et al. reported serum concentrations of miRNA141 for control and prostate cancer patients ranging from 31.7 Ct to 30.2 Ct (or 10.6 Delta-Ct to 7.9 Delta-Ct, normalized by subtracting the Ct value of an endogenous U6-small nuclear RNA as reference),⁸⁶ while Bryant et al. reported serum concentrations of miRNA-141 for the same groups ranging from -0.37 Delta-Ct to 1.3 Delta-Ct using 25 fmol μl^{-1} of

C. elegans miR-39 as a normalizer in downstream analyses.⁸⁷ (The Ct value is the PCR cycle number at which a sample's reaction curve intersects the threshold line, and is inverse to the amount of target nucleic acid in the sample, meaning that a larger Ct number corresponds to a smaller concentration.) Automated sample processing for miRNA extraction and purification from whole blood is necessary to guarantee consistent and repeatable detection of miRNA levels.

(ii) Issues related to sensitivity:

Although the true concentration of miRNAs in blood is still being debated, it is commonly accepted to be very low (from femto-molar to nano-molar),^{82,83,88,89} which makes them challenging to detect without any pre-amplification step.⁹⁰ Enzymatic amplification steps, while effective, are however associated with risks of contamination and error.

(iii) Issues related to specificity:

Specificity is also a major issue for miRNA detection. Because of their short length (22-25 nucleotides), miRNAs often have very similar sequences, sometimes differing by no more than one nucleotide.⁹¹ This means that sensors need to be able to discriminate between very similar sequences.

(iv) Issues related to miRNA form:

The form under which miRNAs exist in the bloodstream remains subject to debate. Although a small fraction of miRNA is suspected to be freely circulating, most of them are complexed to proteins or trapped in exosomes.^{59,92} Detecting accurate levels of circulating miRNAs therefore requires all miRNAs to be efficiently extracted before analysis.

1.2.2 Human Biomarker Sources

Human biomarkers can be derived from either body fluids or tissues. Although biomarker levels are usually lower in body fluids compared in tissue, body fluids are typically considered less invasive compared to tissues.^{11,93,94} Different body fluids can provide different biomarkers and have different emphasis on various biological information. The choice of the matrix (or biofluids) and of the molecular biomarkers present in this matrix are therefore critical when designing a new diagnostic test. Blood, sweat, tears, saliva and urine are the most commonly

used bodily fluids from human body. The relative merits of the most commonly used bodily fluids are summarized in Table 1-6.

Sweat is a surprisingly biomarker-rich fluid, especially for applications of continuous monitoring where other biofluids prove more challenging.⁹⁵ Despite the abundant sources of sweat glands with densities of > 100 glands/cm² at many locations of the human body,⁹⁶ sweat as a biomarker suffers from many unresolved challenges like low volume or contamination.

Tears are critical body fluid from the surface of the eye. Tears contain a number of molecules including proteins/peptides, lipids, small molecule metabolites, and electrolytes. Tear fluid is a useful and an accessible source most applied for eye-related condition like evaluating ocular surface tissues (cornea and conjunctiva), inflammation, lacrimal gland function and a number of disease conditions, such as dry eye as well as response to treatment.⁹⁷

Saliva is an easily accessible biofluid with a greater non-stimulated volume compared with tears or sweat but suffers from challenges like fouling, contamination, and difficulty in filtration due to viscosity. In some situations, high viscosity can pose challenges in sample handling and processing, particularly in analytical techniques that require precise sample volumes or rapid fluid flow. High viscosity can affect the performance of certain instruments, such as pipettes, and may lead to difficulties in obtaining consistent and accurate results.^{96,98}

Table 1-6 Pros and cons of different body fluid: blood, sweat, tears, saliva, and urine.

Body fluid	Advantages	Disadvantages	Ref
Blood	Large sample volumes. More biomarkers compared to other body fluid. Related to whole body.	Painful. More complexed compared to other body fluid. Chemical reactions among biomarkers. Blood clots.	96,99
Sweat	Non-invasive.	Small sample volumes.	95,100

	No pain. Easy access.	Contamination with bacteria or other species because skin is exposed outside and the sample collection takes time. Composition varies with region of skin and temperature. Unknown concentration due to evaporation. Filtration and dilution of large analytes. Mixing of old and new sweat.	
Tears	Non-invasive. No pain.	Small sample volumes. Composition depends on reason for tear production.	97
Saliva	Non-invasive. No pain. Easy access.	Small sample volumes. Contamination (e.g. from food). Viscosity of the sample.	98
Urine	Non-invasive. No pain. Large sample volumes.	Low concentration of the target of interest compared to blood.	96,101

Urine is an easily accessible body fluid that has long been used for point-of-care tests and is available on a real-time basis in hospitals.⁹⁶ Urine specimens are uniquely valuable for epidemiologic research to assess various exposures, including dietary factors, environmental pollutants, endogenous metabolites, and other molecules that are excreted by the kidneys. Compared with blood samples or tissues, the collection of spot urine samples is noninvasive, easy to implement, and subject to smaller collection efforts.¹⁰¹ But the concentrations of interested biomarkers in urine are typically lower than in blood.⁹⁶

Blood is the one carrying the most abundant of information as the content of blood is the most affluent. Blood is recirculated throughout the body, delivering oxygen and nutrients to every cell and transporting products from and toward all different tissues.⁹⁹ While blood is pumped through all the major organ systems in human body, key biomarkers are delivered

or removed.⁹⁶ Blood comprises approximately 55% blood plasma and around 45% various types of blood cells.¹⁰² The protein concentration in plasma or serum typically ranges from 60 to 80 mg/mL and includes albumins (up to 60%) and globulins (up to 40%).¹⁰³ How to separate the biomarker of interest from the complex blood is the first challenge to deal with. Plenty of methods have been researched for the separation of the different components in the blood, including centrifugation, laminar flow, dielectrophoresis, magnetic interaction and biochemical interaction.

In summary, blood is the most complex body fluid of the commonly accessible bodily fluid shown in Table 1-6. The size distribution of blood components spans from <1 nm for small molecules and ions to approximately 15 μm for white blood cells.¹⁰³ Sweat, tear, saliva and urine, in contrast, have limited substances. The choice of the matrix (or biofluids) and of the molecular biomarkers present in this matrix are critical when designing a new diagnostic test.

1.3 Biosensors and Instrumentation

While biomarkers provide valuable information about specific biological processes or disease conditions, biosensors serve as essential tools for quantifying and detecting these biomarkers. A sensor is a device that measures physical parameters from the environment or biological systems and converts them into electronic, optical, colorimetric or other readable format, which can be interpreted by humans or machines alike. If a sensor is used to detect biological or physicochemical substances or states in an organism, it is referred to as a biosensor, regardless of the sensing mechanism. They typically consist of a biological or chemical element (e.g., enzymes, antibodies, or nucleic acids) that interacts with the target molecule (biomarker), and a transducer that converts the biological response into a signal that can be measured and analyzed.¹⁰⁴

In this section, the basic mechanism of biosensors will be introduced and the basic principles of the electrochemical setup including instrumentation and Faradic process will be revealed.

1.3.1 Biosensors

Biosensors are analytical devices that can detect the presence or concentration of a specific biological or chemical substance.^{105,106} In a biosensor system, the analyte (or biomarker in this

research) is the substance of interest that requires detection. The bioreceptor is a molecule that specifically recognizes the analyte. Bioreceptors can be enzymes, cells, oligonucleotides (or analogues such as peptide nucleic acids), and antibodies. The process of signal generation, in the form of light, heat, pH, charge, or mass change, results from the interaction between the bioreceptor and the analyte and is called bio-recognition. The transducer is an element that converts one form of energy into another. In a biosensor, the role of the transducer is to convert the bio-recognition event into a measurable signal. This process of energy conversion is known as signalization. Usually, the concentration of the analyte in the biological or chemical reactions measured by the biosensor is proportional to the signals generated by the biosensor.¹⁰⁴ Therefore, it is important to know the dynamic range of a biosensor, where the signals scale with analyte concentration.¹⁰³

1.3.1.1 Evaluation of Biosensors

Biosensors' performance can be characterized by some crucial properties including reproducibility, stability, linearity, sensitivity, specificity, and selectivity. **Reproducibility** is the ability of the biosensor to generate identical responses for a duplicated experimental set-up. Reproducible signals provide high reliability and robustness to the inference made on the response of a biosensor. **Stability** is the degree of susceptibility to ambient disturbances in and around the biosensing system. These disturbances can cause a drift in the output signals of a biosensor under measurement, which can cause an error in the readout and can affect the precision and accuracy of the biosensor. Stability is the most crucial feature in applications where a biosensor requires long incubation steps or continuous monitoring. Because most biosensor systems are non-linear, it is important to find the dynamic linear range of a biosensor to illustrate a good linearity between the analyte concentration and the generated signal. Therefore, **linearity** of a biosensor refers to its ability to produce a response that is directly proportional to the concentration or amount of the target analyte being measured. This is true for most of the biosensors but some biosensors' response scale with concentration in a log-linear format.¹⁰⁷ **Sensitivity** (analytical sensitivity) refers to the slope of the calibration curve, or the ability to detect small changes in analyte concentration. In this context, sensitivity of a biosensor is not to be confused with the statistical measure of sensitivity (true positive rate) used in diagnostic testing, as mentioned earlier in section 1.1.4. A steeper slope indicates a greater change in response for a given change in concentration. **LoD** is the

minimum concentration of an analyte that can be reliably distinguished from the blank control, and at which detection becomes feasible. Analytical sensitivity is occasionally employed interchangeably with the LoD. Nevertheless, it's important to note that the LoD might exist at a concentration lower than the linear range of an assay. In such cases, where the calibration curve loses validity, it's advisable to refrain from using these terms interchangeably.¹⁰⁸ In other words, a biosensor with high sensitivity can accurately discriminate between different concentrations or levels of the analyte, allowing for precise and reliable quantification. In many applications, both analyte detection and measurement of analyte concentrations across a wide range are necessary. Therefore, achieving a high sensitivity is crucial for successful biosensor performance.¹⁰⁴ Both sensitivity and specificity are very influential properties to characterize a biosensor. **Specificity** refers to the ability of a biosensor to exclusively measure and quantify a single component within a multi-component system with minimum interference from other components. **Selectivity**, on the other hand, refers to the capability of a biosensor to accurately determine multiple components of interest simultaneously and independently, with minimum interference or disturbance from other components within a complex mixture. This means that each component can be quantified without being influenced or affected by the presence of other components.¹⁰⁹

1.3.1.2 Selection of Biosensors

Biosensors exist that are based on different transduction methods, all characterised by their own advantages and limitations. **Mass-based biosensors**, also called gravimetric biosensors, can detect a small change in the mass of the binding material, such as proteins or antibodies on the surface, producing a measurable signal. **Thermal biosensors** measure the heat energy absorbed or released during the biological reaction. Biosensors that rely on field-effect transistors (FETs) are called **field-effect transistor biosensors (Bio-FET)**.¹¹⁰ FETs are three-terminal devices that regulate the current flowing through them using an electric field. The FETs can directly translate the interactions between the analyte and FET surface into electrical current. This is possible because the surface potential changes when a biomolecule binds to the bioreceptor surface. **Acoustic biosensors** are based on the change of an acoustic wave in response to the amount of analyte absorbed, and their transduction systems usually consist of piezoelectric materials (crystal). Acoustic biosensors could have more potential applications if the challenges of non-specific binding and poor sensitivity can be tackled.

Optical biosensors rely on an optical transduction system that incorporates biorecognition elements such as enzymes, antibodies, aptamers, nucleic acids, cells, or even tissues. These biosensors work by exploiting the optical properties of the analytes, which can either be intrinsic or functionalized, such as absorption, transmission, reflection, refraction, phase, amplitude, frequency, or light polarization. When exposed to stimulation, the optical properties of the analytes change, producing a measurable response. **Electrochemical biosensors** detect analytes by converting a biochemical reaction into an electrical signal.¹¹¹ They typically consist of an electrode modified with a biorecognition element that selectively binds to the analyte. The binding event results in a change in the electrode's electrical properties, which can be measured and correlated with the analyte concentration.

Overall, biosensors can be designed to detect a diverse range of biological targets, with biorecognition signals transformed into electrochemical, optical, gravimetric, thermal, electronic, or acoustic signals depending on the nature of the target and the method employed. However, most transducers generate either optical or electrical signals that are typically proportional to the number of analyte-bioreceptor interactions. This has led to the development of the two most extensively studied and applied categories of biosensors: optical biosensors and electrochemical biosensors.

1.3.1.3 Electrochemical and Optical Biosensors

Electrochemical biosensors are widely used due to their high sensitivity, selectivity, and portability, while optical sensors are favoured for their high accuracy and ability to detect a wide range of analytes. Electrochemical biosensors use electrochemical methods to measure the presence of an analyte of interest. They typically consist of three main components: the working electrode, the reference electrode, and the counter electrode. When a voltage is applied across the working and reference electrodes, an electrochemical reaction takes place at the surface of the working electrode, which generates a current that is proportional to the concentration of the target molecule. The Faradaic process is a key component of electrochemical biosensors. In the case of biosensors, the biological element on the working electrode acts as a catalyst to facilitate the redox reaction of the target molecule. The resulting electrical signal can be amplified and analysed to determine the concentration of the target molecule.

Electrochemical biosensors offer high sensitivity and specificity in terms of measurement capabilities, making them suitable for applications in medical diagnostics. They are relatively easy to fabricate and can be miniaturized, which makes them portable and suitable for point-of-care testing. Electrochemical sensors also have the advantage of being relatively inexpensive. Optical sensors also offer high sensitivity and specificity and are used in a variety of applications, including medical diagnostics and environmental monitoring. However, they are often more expensive than electrochemical sensors and can be more challenging to fabricate and miniaturize. Nevertheless, advances in nanotechnology and microfabrication techniques are enabling the development of miniaturized optical sensors that could be used in portable devices. Overall, the choice between electrochemical and optical sensors depends on the specific application and the desired performance characteristics. In this thesis, we will focus on using electrochemical biosensors detecting nucleic acid biomarker for the future application of early prostate cancer diagnosis.

1.3.2 Biosensor Instrumentation with Three-electrode Systems

A typical electrochemical system is composed of three electrodes and an electrolyte solution.

1.3.2.1 Three-Electrode System

A typical three-electrode electrochemical system is shown in Figure 1-8 (a). Three electrodes including a working electrode, a counter electrode, and a reference electrode. Each electrode serves a specific function in the electrochemical measurement:

- The working electrode is the electrode where the electrochemical reaction of interest takes place. It is typically the electrode that is modified or functionalized to facilitate the desired electrochemical process. The working electrode is where the analyte is detected or where the desired electrochemical reaction occurs.
- The counter electrode (or auxiliary electrode) is an electrode used to complete the electrical circuit in the system. It provides a path for the flow of current between the working electrode and counter electrode during the electrochemical reaction. The counter electrode is typically made of an inert material, platinum for example, which does not participate in the electrochemical reaction. Typically, the counter electrode's

total surface area exceeds that of the working electrode to ensure it does not impede the kinetics of the electrochemical processes being studied.^{112,113}

- The reference electrode is used as a reference point to measure and control the potential of the working electrode. It provides a stable and known reference potential against which the potential of the working electrode is measured. And this stable reference potential is independent of analyte concentration or current. Commonly used reference electrodes include the silver/silver chloride electrode (Ag/AgCl) and saturated calomel electrode (SCE).

In electrochemical measurements, the three-electrode system is preferred over the two-electrode system. Because in voltammetry, the current generated at the working electrode must flow somewhere and the counter electrode is used as a current path rather than reference electrode. The counter electrode carries the same magnitude of current as the working electrode but in the opposite direction when the working electrode undergoes a redox reaction. In certain cases, a two-electrode setup can be used with the reference electrode serving as both the reference electrode and counter electrode, but only if small currents are passed through the reference electrode to prevent significant potential changes across the reference electrode-electrolyte interface. This approach is commonly employed when working with micro- and smaller-sized working electrode.¹¹³

In a typical three-electrode electrochemical system, except from the three electrodes, an electrolyte solution is used to provide conductivity, ion source and electrochemical environment (the solution works as a matrix where the electrons can transfer at the heterogenous phase between electrode and electrolyte). The ions act as a medium, allowing for the flow of current between the electrodes. They provide a pathway for the movement of charged species involved in the electrochemical reactions. The composition of the electrolyte solution can influence the kinetics and thermodynamics of the electrochemical reactions. It can also affect factors such as the electrode surface charge, reaction rates, and the stability of reactive intermediates. The choice of electrolyte solution depends on the specific electrochemical system and the nature of the electrochemical reactions being monitored. The selection of an appropriate electrolyte solution is crucial for maintaining a stable and well-defined electrochemical environment, ensuring accurate and reproducible measurements.

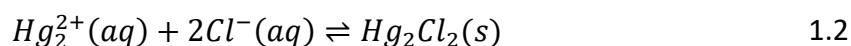
Table 1-7 summarizes the properties of each component in a typical three-electrode system. The three-electrode system allows for accurate control and measurement of the electrochemical processes happening at the working electrode by providing a stable reference potential and a path for current flow. This setup is widely used in various electrochemical techniques, such as cyclic voltammetry, pulsed voltammetry, chronoamperometry/chronocoulometry, and impedance spectroscopy. Detailed introduction of voltammetry techniques is presented in section 1.4.

Table 1-7 Three-electrode system components and their properties.

Typical components	Properties
Working electrode	• Cell current is limited by the working electrode reaction. • Provide location for electrochemical reaction.
Reference electrode	• Provide a stable and known reference potential against working electrode.
Counter electrode	• Provide current path between working electrode and counter electrode. • Must be large compared with working electrode. • Sometimes counter electrode reaction-solvent current electrolysis causes pH change, back reaction can be a problem. • Compliance of the control amplifier.
Electrolyte solution	• Ensures that the majority charge carriers in solution are not in the analyte, the only modes of mass transport are then diffusion and convection. • Populates the electric double layer with inert ions to minimize the effect of the electrode reaction. • Lower the resistance.

1.3.2.2 Why Reference Electrode Potential Is at A Constant Value

Reference electrodes are designed to maintain a constant potential (e.g., $E = +0.197\text{ V}$ for Ag/AgCl and $E = +0.241\text{ V}$ for calomel). This is realized by using constant concentration (sometimes saturating concentration) of chloride ions. Take the saturated calomel electrode for example:



$$K_{sp} = \alpha_{\text{Hg}_2^{2+}} \times (\alpha_{\text{Cl}^{-}})^2 \quad 1.3$$

$$\alpha_{\text{Hg}_2^{2+}} = \frac{K_{sp}}{(\alpha_{\text{Cl}^{-}})^2} \quad 1.4$$

For the half reaction:



$$E = E_0 - \frac{RT}{2F} \ln \frac{1}{\alpha_{\text{Hg}_2^{2+}}} \quad 1.6$$

Replacing $\alpha_{\text{Hg}_2^{2+}}$ with $\frac{K_{sp}}{(\alpha_{\text{Cl}^{-}})^2}$:

$$E = E_0 - \frac{RT}{2F} \ln \frac{(\alpha_{\text{Cl}^{-}})^2}{K_{sp}} \quad 1.7$$

$$E = E_0 + \frac{RT}{2F} \ln K_{sp} - \frac{RT}{2F} \ln (\alpha_{\text{Cl}^{-}})^2 \quad 1.8$$

E_0 and $\frac{RT}{2F} \ln K_{sp}$ are constants. In $\frac{RT}{2F} \ln (\alpha_{Cl^-})^2$, the saturated concentration of chloride ion makes activity of chloride ion α_{Cl^-} a constant. Therefore, the potential E of reference electrode is maintained. Usually the $[Cl^-]$ ranges from 1 M to saturating. For AgCl reference electrode, however, saturated conditions are avoided due to the formation of complexes such as $AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$.¹¹³

Open circuit gain means no feedback, which is about 10^6 , that's why in practice always disconnecting reference electrode as the last step protects the working electrode. A similar named concept is open circuit potential. Open circuit potential is the potential between the working electrode and the reference electrode when there's no current flowing. Open circuit potential is also a voltage measurement technique. Set up appropriate gain is necessary to obtain high quality data and avoid digitization noise in electrochemistry.

1.3.2.3 Potentiostat

Instrumentation is an important aspect of electrochemical biosensors. It includes the use of potentiostats, which are devices that control the voltage applied to the working electrode and measure the resulting current. Potentiostats serve two main purposes:

1. To control the potential difference between the reference electrode and the working electrode.
2. To convert the measured current into a voltage signal that can be displayed, stored, and analyzed.

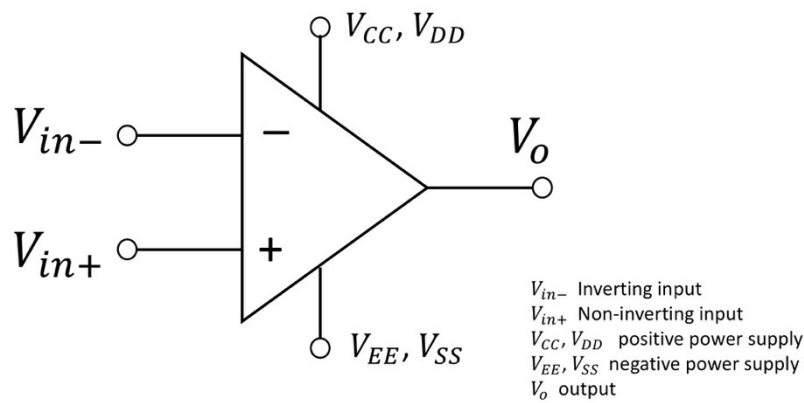


Figure 1-5 Schematic diagram of op-amps.

As an electronic hardware, the heart of the potentiostatic electronic circuits are operational amplifiers (op-amps). Op-amps (Figure 1-5) are fundamentally a voltage amplifying device designed to be used with external feedback components such as resistors and capacitors between its output and input terminals. These feedback components determine the resulting function or “operation” by virtue of the different feedback configurations whether resistive, capacitive or both. The op-amps can perform a variety of different operations such as add, subtract, integration and differentiation. The magnification, named gain A , can be greater than 10^6 , as shown in the equation 1.14. However, the voltage range that can be delivered by an op-amp output is limited by its own power supply range or that of the potentiostat, typically $\pm 10\text{ V}$ or $\pm 15\text{ V}$.¹¹³ In theory, the input impedance of an op-amp is infinitely big while the output impedance is infinitely small. And op-amp has very a broad bandwidth. In practice, those parameters all have their ranges, as shown in Table 1-8.¹³³

Table 1-8 Basic properties of op-amps.

	Theoretical parameter	Practical parameter
Input impedance	infinite	$10^{12}\ \Omega$

Output impedance	0	1 – 10 Ω
Bandwidth	infinite	5-20 MHz

The potentiostat circuit comprises two essential configurations: the voltage follower and the current follower, which collectively contribute to the circuit's functionality. These configurations play distinct roles in maintaining the desired operation of the potentiostat system.

1.3.2.3.1 Voltage follower

When part of the output voltage is fed back to the inverting input that creates a feedback loop, the circuit is called a voltage follower, as shown in Figure 1-6. When negative feedback is applied, the op-amp's output adjusts in such a way that it drives the input voltage difference to zero. As shown in the following equations:

$$V_{in-} = V_o \quad 1.9$$

$$V_{in-} = V_{in+} \quad 1.10$$

$$V_o = V_{in+} \quad 1.11$$

Therefore, in a voltage follower (feedback circuit) the input voltage is the same as the output voltage. As shown in Figure 1-6.

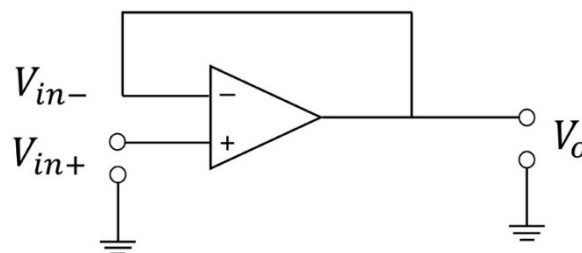


Figure 1-6 Voltage follower.

1.3.2.3.2 Current Follower

Another important circuit in potentiostat is current follower, or a current-to-voltage (i/E or i/V) converter, as shown in Figure 1-7. the output voltage is proportional to the input current by a scale factor determined by R_f . Therefore R_f is called the gain. Because the potential at two input terminals is the same, S is a virtual ground. Current follower allows currents to be converted to equivalent voltages while the current source is maintained at ground potential.

112

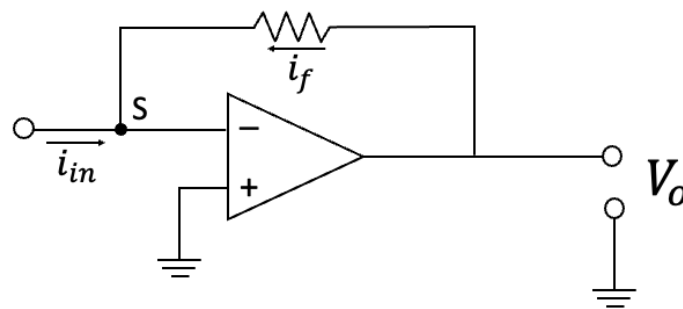


Figure 1-7 Current follower.

The calculation process to demonstrate the working principle of a current follower is shown as following:

According to Kirchhoff's law, current at the summing point S is zero:

$$i_{in} = -i_f \quad 1.12$$

From Ohm's law we know that:

$$i_f = \frac{V_o - V_s}{R_f} = -i_{in} \quad 1.13$$

Substituting V_s with an expression with V_o :

$$V_o = A V_s \quad 1.14$$

$$V_s = \frac{V_o}{A} \quad 1.15$$

$$\frac{V_o - \frac{V_o}{A}}{R_f} = \frac{V_o(1 - \frac{1}{A})}{R_f} = -i_{in} \quad 1.16$$

Considering:

$$A > 10^6 \quad 1.17$$

$$1 - \frac{1}{A} \cong 1 \quad 1.18$$

V_o can be expressed as:

$$V_o = -i_{in} R_f \quad 1.19$$

The output voltage V_o have a linear relationship with the input current i_{in} by a factor of $-R_f$. The negative sign is because of the inverting input.

The voltage follower circuit looks very similar to the current follower, however, there's a key difference that the current follower typically has a virtual ground at one of the input terminals. The voltage follower circuit has its output connected directly to the feedback element, while the current follower has its output connected to a resistor in series with the feedback element. As a result, the current follower requires a virtual ground at one of the input terminals to maintain the input voltage equal to the output voltage, which is necessary for the circuit to function properly.

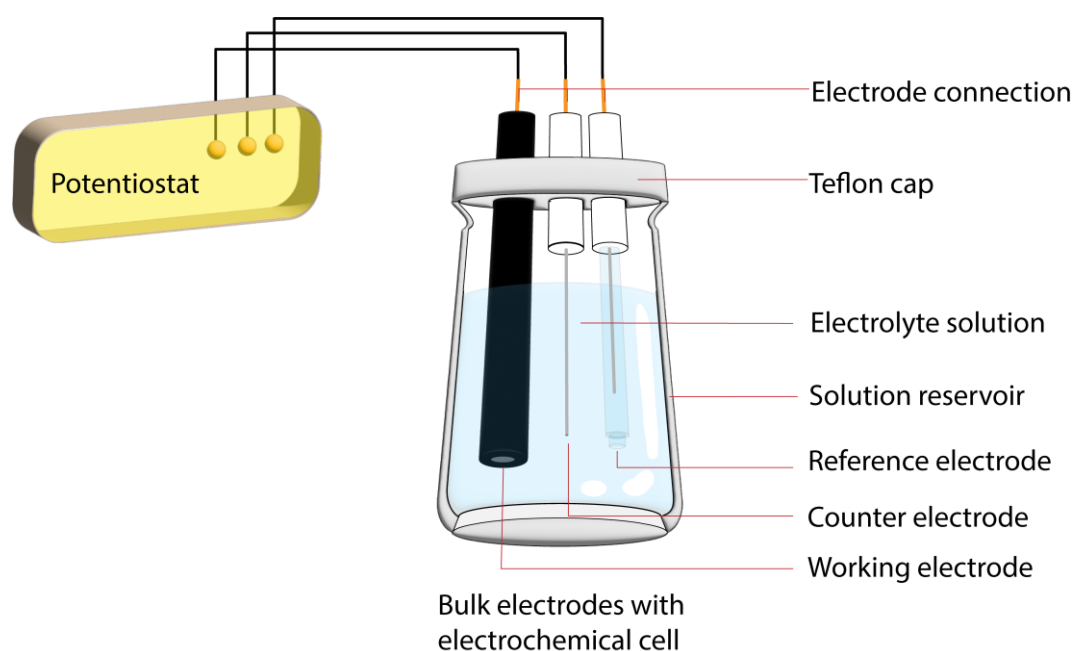
1.3.2.4 Summary

Together, the three-electrode configuration and potentiostat enable precise control and accurate measurement of electrochemical processes. The voltage follower maintains potential control between the reference electrode and the working electrode (typically at virtual ground), regardless of the cell current (provided the op-amp compliance is not

exceeded, and compliance refers to the maximum output voltage the op-amp can provide). The Op-amp's high input impedance prevents significant current flowing through the reference electrode, thus maintaining accurate potential differences between the reference terminal and the test solution. The current follower, on the other hand, monitors and controls the current flowing between the working and counter electrodes. It ensures that the desired current is maintained during the electrochemical process. By adjusting the potential at the working electrode, the current follower actively adjusts the potential at the counter electrode to maintain a steady current flow.

At the potentiostat level, as shown in Figure 1-8 (b), output from the voltage follower feeds back to the non-inverting input of the op-amp (at the top of the Figure). The current follower allows the working electrode to remain at virtual ground because of the feedback, which is an essential condition for the operation of the system.¹¹² In most potentiostat, the potential is applied to counter electrode until the desired potential is attained on the reference electrode.

(a)



(b)

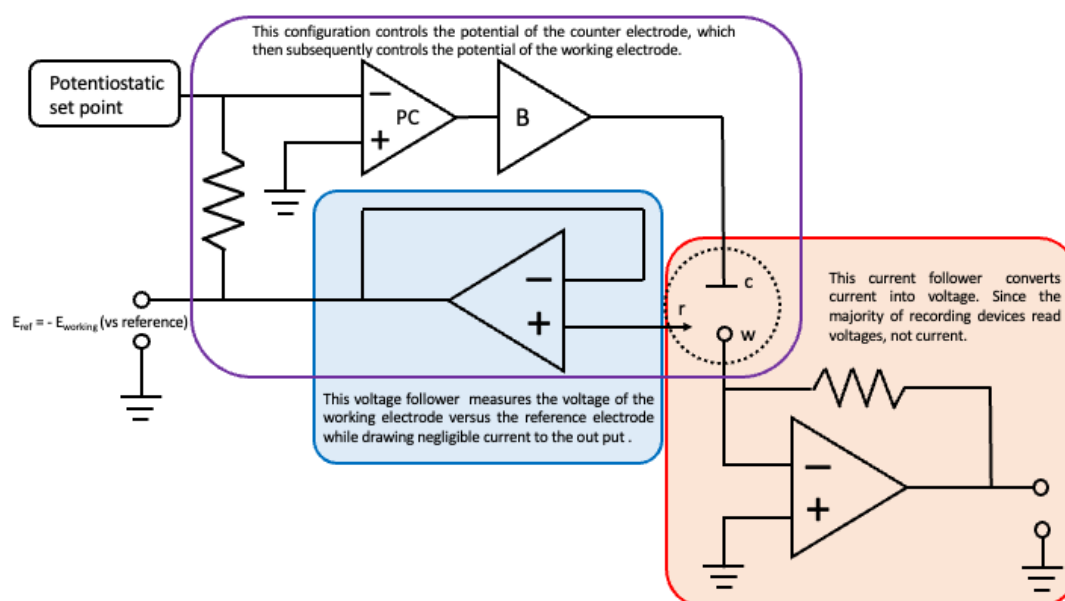


Figure 1-8 (a) Three electrode system connected with potentiostat. (b) A full potentiostatic system based on a potential control amplifier (purple circle) for controlling the potential of the electrochemical cell (on the counter electrode), a voltage follower (blue circuit) for measuring potential between the working electrode and the reference electrode, a current follower (orange circle) for measuring the current between the working electrode and the counter electrode (PC is potential control amplifier and B is booster for improving the available output voltage).

Potentiostats allow the user to set various control parameters for the three-electrode system, such as the applied potential or current, scan rate (for cyclic voltammetry), and measurement time intervals. Additionally, potentiostats have built-in data acquisition capabilities, enabling the collection and storage of current and potential data over time. This data can be further analyzed to study electrochemical processes, calculate kinetic parameters, and determine electrochemical properties of the system under investigation.

1.4 Electrochemical Techniques

Electrochemistry is a powerful tool for investigating reactions involving electron transfer, which correlates the flow of electrons with chemical changes. Voltammetry serves as the core methodology for studying and analyzing heterogeneous electrochemical processes and investigating electrolysis mechanisms. As discussed in section 1.3.2, the study of electrochemical processes often employs a three-electrode system. The potential is measured between the working and reference electrodes, while the current is measured between the working and counter electrodes. In voltammetry, information about an analyte is obtained by measuring the current while controlling the potential (varying or maintaining it constant), or vice versa. Based on the difference in the stimulation waveform, the most commonly employed voltammetry techniques include cyclic voltammetry, pulsed voltammetry, chronoamperometry or chronocoulometry. Electrochemical impedance spectroscopy, which measures the impedance of an electrochemical cell as a function of frequency, is also an important electrochemical technique used to study the electrochemical system.

1.4.1 Cyclic Voltammetry

Cyclic voltammetry is the most widely employed methodology in the field of voltammetry to study electrode kinetics and investigate the chemical reactivity of species. In cyclic voltammetry, the potential of a working electrode is ramped linearly versus time. In comparison with linear sweep voltammetry (LSV), where a fixed potential range is employed, the voltage is scanned from a lower limit to an upper limit and not going back. The slope in the waveform is called scan rate, ν , which can be positive or negative according to oxidation or reduction processes. Following the International Union of Pure and Applied Chemistry (IUPAC) convention, potential in cyclic voltammetry is plotted from low to high. However, the

US convention plots the potential from high to low. In a cyclic voltammetry experiment, the current flowing through the working electrode is recorded as a function of the applied potential. The voltammogram is plotted using current versus potential.

1.4.1.1 Initial Condition and Boundary Condition

Cyclic voltammetry typically but not necessarily start at current $i = 0$. $i = 0$ means there's no reaction. When $t = 0$, the concentration of the redox species in the electrochemical cell is the same everywhere, which is the initial condition, and is expressed mathematically as shown below, where the C is the concentration of the redox species and x is the distance from the working electrode.

At $t = 0$,

$$C_{x=0} = C_{x=\infty} \quad 1.20$$

$$C_{all\ x} = C_{x=\infty} \quad 1.21$$

When $t > 0$, the reaction is either kinetic limited or diffusion limited. Mathematically the boundary condition is expressed as follows:

At $t > 0$,

$$C_{x=0} = 0 \quad 1.22$$

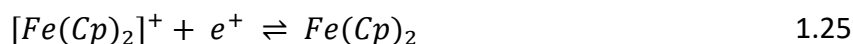
$$C_{x < \sqrt{2Dt}} < C_{x=\infty} \quad 1.23$$

$$C_{x > \sqrt{2Dt}} = C_{x=\infty} \quad 1.24$$

Where $\sqrt{2Dt}$ is the diffusion layer and D is the diffusion coefficient of the redox species.

1.4.1.2 Why There Are Peaks

For a redox species, a typical cyclic voltammogram is a duck-shaped curve in its redox window, as shown in Figure 1-9 (a). The concentration profiles at the electrode and electrolyte interface are helpful to understand the voltammogram of cyclic voltammetry. Take ferrocenium for example (Cp=cyclopentadienyl, C_5H_5):



$Fe(Cp)_2$ can be abbreviated as Fc , therefore the reaction can be rewritten as:

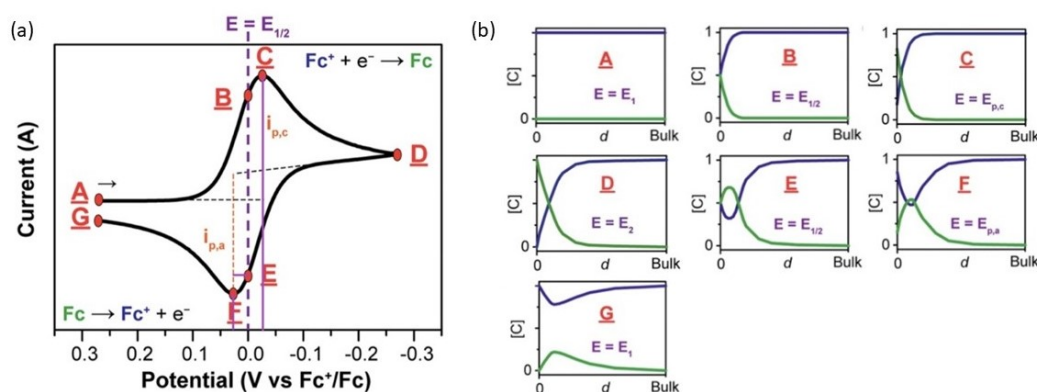


Figure 1-9 (a) Voltammogram of the reversible reduction of a 1 mM $[Fc]^+$ solution to $[Fc]$, at a scan rate of 100 mV/s. (b) Concentration profile of $[Fc]^+$ (blue line) and $[Fc]$ (green line).¹¹⁴ Adapted with permission from reference¹¹⁴. Copyright © 2022 American Chemical Society.

In Figure 1-9 (a), current starts at 0 A, which we discussed in initial condition. It shows that at $t = 0$ s, concentration of Fc^+ is the same as in the solution everywhere. At $t > 0$ s, current rises as the voltage is swept from its initial value and the equilibrium position keeps shifting. Please note in Figure 1-9 (a) the current is plotted with potential from high to low, that is, US convention.

Concentration of Fc^+ at the surface of the electrode decreases as Fc^+ (reactant) at the working electrode surface are reduced to Fc (product) and this local concentration difference drives Fc^+ in bulk solution diffuse towards the electrode surface. More reactants are being converted into product at the surface of the electrode until at some point the diffusion layer has grown sufficiently above the electrode so that the flux of reactant to the electrode is not fast enough to satisfy that required by the Nernst equation, a peak occurs. At the peak, concentration of the reactant at the electrode surface becomes zero as all Fc^+ arrives at the working electrode surface are immediately converted to Fc . As shown at point C in Figure 1-9 (a) and (b).¹¹⁵ Before point C, the current is controlled by the electron transfer kinetics, after point C, the current is controlled by diffusion as diffusion is the slowest step after the peak. Electrochemical experiments simply reflect the rate of the slowest step in the process and provides information. After the peak, current keeps dropping because the increasing diffusion layer provides less and less reactant to the electrode surface. Diffusion layer, also called the Nernst diffusion layer, is characterized by $\delta = \sqrt{2Dt}$, where D is diffusion coefficient of the redox species, and it is a constant. So, the diffusion layer thickness δ is only related to the time of reaction. After point D, the same process happens again but with the reactant and product changing roles. Electrode starts to draw electrons from Fc in the liquid towards itself and causing oxidation. Again, the oxidation peak occurs at point F, with the same reason as the reduction peak. The decreasing amount of Fc in front of the electrode, which also results in a lack of electrons, until eventually no electrons are left to draw close. The curve is at its highest point. And then current is controlled by diffusion. The oxidation peak and reduction peak are separated due to the diffusion of the analyte to and from the electrode.¹¹⁴ For adsorbed species, there's no diffusion, so in theory there's no peak-to-peak separation.

For a reversible case (see section 1.4.1.6), if the scan rate is increased, current will also increase because faster scan rates lead to a decrease in the size of the diffusion layer and thus leading to higher currents. Although the peak current is increased by faster scan rate, peak position should not shift, which is a characteristic of that specific redox species' electrode reactions. In practice, the peak position would have changed slightly because of Ohmic drop for diffusion species.

1.4.1.3 Ohmic Drop

Ohmic drop is mainly produced due to the resistance of electrolyte solution. However, there are other reasons that can contribute to the uncompensated resistance, which in turn can result in Ohmic drop. These reasons are shown in the list below.

1. Low electrolyte solution conductivity.
2. Poor electrode conductivity.
3. High electrode contact resistance (corrosion or insulating film on the electrode surface).
4. Blocked reference electrode frit.¹¹³

This resistance between the reference electrode and the working electrode is not compensated by the potentiostat. The Ohmic drop primarily affects the shape and magnitude of the current response during the cyclic voltammetry scan. It can lead to changes in the current baseline and affect the observed current values. Practical ways of reducing Ohmic drop include:

1. Decreasing resistance of the electrolyte solution by increasing the electrolyte concentrations.
2. Decreasing the distance between working electrode and reference electrode to reduce the uncompensated resistance.
3. Using slow scan rates.
4. Diminishing current by reducing the size of the working electrode.¹¹⁴
5. Reducing analyte concentration to reduce the current.¹¹³

By analyzing the variation of peak position as a function of scan rate it is possible to gain an estimate for the electron transfer rate constants. This leads to the important relationship between peak current and scan rate that is described by Randles-Sevcik equation.

1.4.1.4 Randles-Sevcik Equation

In reversible cyclic voltammetry, the peak current is featured by Randles-Sevcik equation. For diffusion-controlled species, the peak current i_p is:

$$i_p = 0.4436nFAC \sqrt{\frac{nFvD}{RT}} \quad 1.27$$

While for adsorbed species, the peak current i_p is:

$$i_p = \frac{n^2 F^2}{4RT} v A \Gamma^* \quad 1.28$$

n is the number of electrons transferred in the reaction or half-reaction.

F is the Faraday constant, 96485 C mol^{-1} .

R is the universal gas constant, $8.314 \text{ JK}^{-1}\text{mol}^{-1}$.

T is the temperature in Kelvin, K .

A is the effective area of the working electrode, m^2 . In many cases can be in cm^2 .

C is the concentration of the redox species, mol m^{-3} or mol cm^{-3} .

D is the diffusion coefficient, m^2s^{-1} or cm^2s^{-1} .

Γ^* is the surface coverage of the adsorbed species, mol m^{-2} or mol cm^{-2} .

v is scan rate, in V/s .

Faster scan rates lead to a decrease in the size of the diffusion layer; as a consequence, higher currents are observed. In a potential controlled measurement, when the scan rate is fast, thin-layer effect is dominant; when the scan rate is slow, diffusion is dominant.

1.4.1.5 Double Layer Capacitance Calculation Method

In cyclic voltammetry from background electrolyte, the double layer capacitance can be calculated from the hysteresis:

$$Q = CU \quad 1.29$$

$$\frac{dQ}{dt} = C \frac{dU}{dt} \quad 1.30$$

$$\frac{dQ}{dt} = I = 2 \times \text{hysteresis} \quad 1.31$$

$$\frac{dU}{dt} = v \quad 1.32$$

Therefore, the double layer capacitance can be estimated as:

$$C = \frac{I}{v} \quad 1.33$$

Q is charge, in C .

C is capacitance, in F .

U is voltage, in V .

t is time, in s .

v is scan rate, in V/s .

I is current, in A .

The double layer capacitance, as shown in equation 1.33, is independent of potential. It should be noted that equation 1.33 provides an approximation that is often convenient and practical to use. The more precise double layer capacitance can be measured by electrochemical impedance spectroscopy (section 1.4.5) by analyzing the Nyquist plot. The

double layer capacitance is primarily influenced by two factors: the surface area of the electrode and the properties of the electrolyte solution.

1.4.1.6 Criteria for Judging Electrochemical Reversibility

Reversibility characterizes the electron transfer kinetics between the electrode and the analyte, where we can investigate if the Nernstian equilibrium is established at the heterogeneous interface or not. Standards and rules to judge reversibility by cyclic voltammetry include peak separation, peak current ratio, and peak width at half height.

1. Peak separation ΔE_p .

The two peaks are separated due to the diffusion of the analyte to and from the electrode.¹¹⁴ The peak separation reflects the electron transfer kinetics at the heterogeneous interface.

$$\Delta E_p \approx |E_{pa} - E_{pc}| = 2.3 \frac{RT}{nF} = \frac{59.2}{n} \text{ mV} \quad 1.34$$

Where:

E_{pa} is the anodic peak potential, E_{pc} is the cathodic peak potential.

R is the universal gas constant, $8.314 \text{ J K}^{-1} \text{ mol}^{-1}$.

T is the temperature in Kelvin.

n is the number of electrons transferred in the cell reaction or half-reaction.

F is the Faraday constant, 96485 C mol^{-1}

2. Peak current ratio.

$$\frac{i_p^a}{i_p^c} = 1 \quad 1.35$$

Where i_p^a is the anodic peak current and i_p^c is the cathodic peak current in a cyclic voltammetry.

If the peak current ratio deviates very far from the value 1, this means one of the redox reactions is favored thermodynamically and thus the electrochemical equilibrium is difficult to establish. The other case that can cause the peak current ratio far from 1 is the homogenous reaction taking place and this current ratio change will be even more obvious when increasing the scan rates.

3. Peak width at half height.

$$E_{i_p} \approx \frac{57.5}{n} \text{ mV} \quad 1.36$$

Because the peak in cyclic voltammetry is usually broad, the peak potential might be difficult to determine. Therefore, the peak width at half height E_{i_p} is introduced to evaluate and analyse peaks.¹¹²

1.4.2 Square Wave Voltammetry (SWV)

Square Wave Voltammetry (SWV) is a voltammetric technique that is more sensitive than cyclic voltammetry. In SWV, the waveform is a series of pulses superimposed on a staircase with the same duration of pulse and staircase. Current is measured in a forward pulse and reverse pulse (Figure 1-10). SWV data are typically presented as the current difference between sampled current for the forward (I_1 in Figure 1-10) and reverse (I_2 in Figure 1-10) pulses scans, though forward and backward sampled currents can also be useful in isolation. As with many other electrochemical techniques, direction of measurement can initially sweep positively (towards an anodic limit) or negatively (toward a cathodic limit). The way in which the current is measured at each pulse aids in minimizing the measurement of double layer charging (background) current.^{116,117} Typical minimum pulse period for SWV is 0.002 s (maximum frequency 500 Hz).¹¹⁸

1.4.3 Differential Pulse Voltammetry (DPV)

Differential Pulse Voltammetry (DPV) is also a pulse technique that is designed to minimize background charging currents from double layer capacitance. The waveform in DPV is similar to SWV but essentially different, as shown in Figure 1-10. In its digital implementation, the

linear ramp is approximated with a staircase whose amplitude must be small compared with the modulating pulse and the underlying potential change can be presumed not to change during the duration of the pulse. Current is sampled just before the application of the potential pulse and sampled again at the end of the pulse.¹¹⁹ The potential of the working electrode is then stepped back by a lesser value than during the forward pulse such that baseline potential of each pulse is incremented throughout the sequence. Typical pulse period is between 0.5 s to 5 s and typical pulse width is between 0.005 s to 0.05 s, as shown in Figure 1-10.^{112,118}

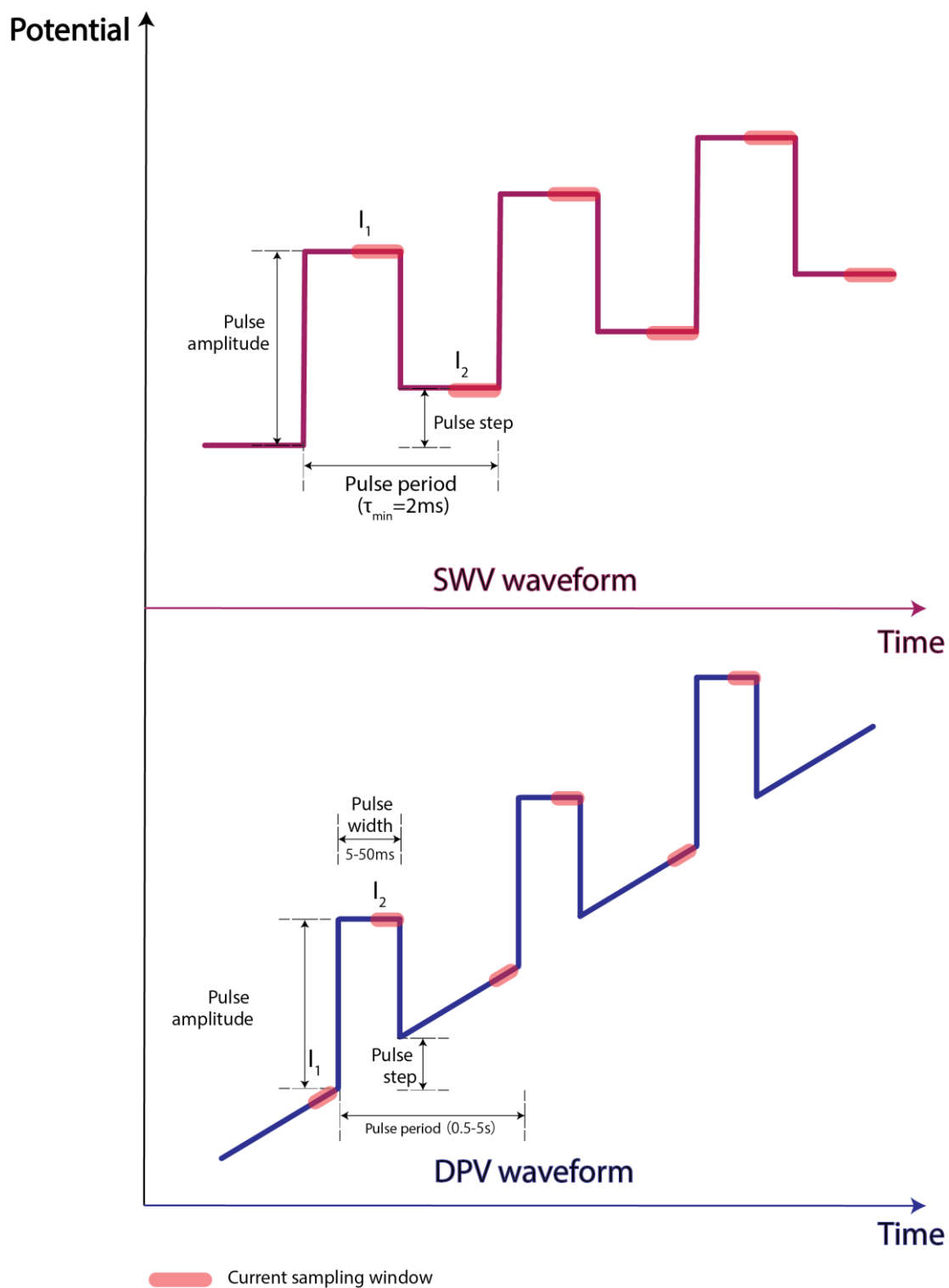


Figure 1-10 SWV waveform and DPV waveform.

As illustrated in Figure 1-10 (a) and (b), the waveforms of the SWV have equal time intervals for both forward and backward pluses, while DPV does not. That's why, in practice, the pulse

parameter can be set using frequency for SWV while for DPV three parameters are needed: Pulse Width (seconds), Sample Width (seconds) and Pulse Period (seconds), as shown in Table 1-9.

Table 1-9 Comparison of parameter difference between DPV and SWV in a practical measurement.

DPV	SWV
Initial $E(V)$	Initial $E(V)$
Final $E(V)$	Final $E(V)$
Increment $E(V)$	Increment $E(V)$
Amplitude (V)	Amplitude (V)
Pulse Width (seconds)	Frequency (Hz)
Sample Width (seconds)	
Pulse Period (seconds)	

Difference between SWV and DPV:

1. Data process methods are different.
2. DPV typically takes longer than SWV.
3. Difference in potential waveform: SWV uses a continuous linear ramp with superimposed square wave pulses, whereas DPV uses discrete potential pulses with a reset between each pulse. This difference in potential waveform shapes results in different sensitivities and advantages for each technique in terms of peak resolution, sensitivity, and selectivity in electrochemical measurements.

Common features between SWV and DPV:

1. Both are designed to minimize the background charging current.
2. Capacitive charging current and immobilized species induced current both decay as a function of time. But the time constant of decaying for the two distinct processes varies. Which helps to study the immobilized species.

1.4.4 Chronoamperometry/Chronocoulometry

Chronoamperometry is an electrochemical technique in which the potential waveform is stepped, usually from a region where no electrode reaction occurs to one where the reaction is diffusion limited. The resulting current consists of both Faradaic current and capacitive charging current. In general, the time constant associated with the decay of the double layer capacitance is relatively fast, typically on the order of microseconds to milliseconds. This time constant represents the charging or discharging of the double layer capacitance due to the movement of ions at the electrode-electrolyte interface. On the other hand, the timescale of faradaic processes, such as electrochemical reactions involving the transfer of charge and redox reactions, can vary significantly. Faradaic processes often exhibit slower kinetics compared to the charging or discharging of the double layer capacitance. The timescale for faradaic processes can range from milliseconds to seconds or even longer, depending on factors like reaction rates, electrode kinetics, and diffusion limitations.¹¹²

The capacitive charging current decays exponentially with time, The Faradaic process occurring at the working electrode is monitored as a function of time and decays as $t^{\frac{1}{2}}$ for freely diffusing species. For adsorbed species, faradaic current decays as e^{-kt} . When a constant potential is applied to the working electrode with respect to the reference electrode. The current produced on the surface of the working electrode from amperometry is proportional to the concentration of the analyte present in the solution. As illustrated in Cottrell equation (1.37).

1.4.4.1 Cottrell Equation

In a controlled potential experiment, the change in electric current with respect to time is expressed mathematically as follows:

$$i = \frac{nFAC_j^0 \sqrt{D_j}}{\sqrt{\pi t}} \quad 1.37$$

Equation 1.37 is known as Cottrell equation. For a simple redox event, the measured current is contingent on the speed at which the analyte undergoes diffusion towards the electrode. Consequently, this current is referred to as "diffusion-controlled". The integrated Cottrell expression represents the charge from diffusion process. Together with double layer charge Q_{dl} (integrated from charging current) and surface-bound redox species charge $nF\Gamma^*$ (integrated from faradaic current), the overall charge from a chronoamperometry/chronocoulometry test is expressed as per equation 1.38.¹²⁰

$$Q = \frac{2nFAC_j^0 \sqrt{D_j}}{\sqrt{\pi}} \sqrt{t} + Q_{dl} + nF\Gamma^* \quad 1.38$$

n is the number of electrons transferred in the reaction or half-reaction.

F is the Faraday constant, 96485 C mol^{-1}

A is the effective area of the working electrode, m^2 . In many cases can be in cm^2 . (section 1.5.5)

C_j^0 is the initial concentration of the redox species j , $mol \text{ m}^{-3}$ or $mol \text{ cm}^{-3}$.

D_j is the diffusion coefficient of the redox species j , $m^2 s^{-1}$ or $cm^2 s^{-1}$.

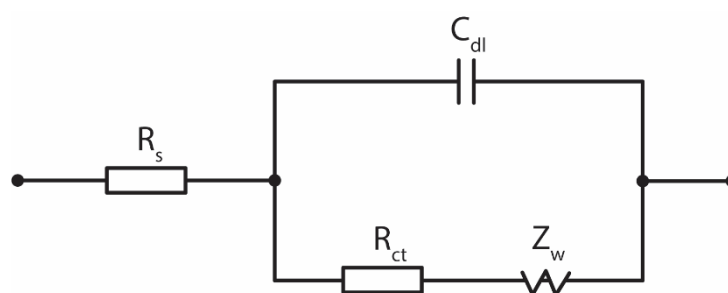
Γ^* is the surface coverage of the adsorbed species, $mol \text{ m}^{-2}$ or $mol \text{ cm}^{-2}$.

Q_{dl} is the double layer capacitive charge, C .

1.4.5 Electron Impedance Spectroscopy (EIS)

In electrochemistry, electrochemical impedance spectroscopy (EIS) characterizes the time response of electrochemical systems using low amplitude alternating current (AC) voltages over a range of frequencies. It is often used to understand the processes that occur at the

interface of an electrode. EIS uses redox mediators (usually ferricyanide and ferrocyanide) to indirectly identify the capture of a specific target analyte on the functionalized working electrode. Upon capture of the target analyte, the available surface area for the redox mediator reduces, which in turn results in a decrease of the electrochemical current or an increase of the interface resistance.³⁰ EIS can be used to provide information about film thickness, conductivity, reaction at the interface and the impedance of the electrode surface. EIS can also be used to estimate physical parameters such as surface roughness and porosity. The electrochemical cell can be simulated as a Randles circuit, as shown in Figure 1-11. Charge transfer resistance R_{ct} is dependent on the ease of electron transfer of a redox analyte; the higher R_{ct} the slower the electron transfer kinetics.¹¹³



C_{dl} = Double layer capacitance

R_s = Resistance of the electrolyte solution

R_{ct} = Charge transfer resistance

Z_w = Warburg impedance (diffusional resistance element)

Figure 1-11 Randles circuit.

The non-linear nature of the electrochemical systems makes the stimulation signal limited to a small region. When performing an EIS measurement, by applying a small periodic voltage (typically 5-10 mV¹²¹⁻¹²³) or current at several frequencies, a small current or voltage can be detected as response. The stimulation is voltage in most cases.

For resistors, the impedance is a real number, but capacitors have both real and imaginary part in its impedance. The EIS result can be organized as Bode Plot and Nyquist Plot. Bode plot is actually two plots in one. The x axis is a logarithmic scale of the frequency $\lg f$ and one ordinate is the logarithmic of the impedance $\lg Z$ while the second ordinate is the phase shift Φ . The advantage of this plot is that all information is included. For Nyquist Plot, the negative imaginary impedance $-Z''$ is plotted versus the real part of the impedance Z' . The choice between Bode Plot and Nyquist Plot depends on the specific goal of the measurement.

1.4.6 Summary

Cyclic voltammetry, pulsed voltammetry, chronoamperometry or chronocoulometry, and electrochemical impedance spectroscopy are all electrochemical techniques that can provide different types of information about the studied electrochemical systems.

Cyclic voltammetry provides information about the redox behavior, reversibility, and kinetics of electrochemical reactions. Cyclic voltammetry is useful for studying redox processes, determining redox potentials, identifying electrode processes, and investigating electrochemical mechanisms. It can also provide insights into the diffusion and transport properties of species in solution.

Pulsed voltammetry, including SWV and DPV, both are designed to minimize the background charging current. For practical analysis, SWV is commonly regarded as the preferred pulse method in electrochemical studies due to its several advantages: SWV offers effective background suppression, comparable to that of DPV; SWV exhibits slightly higher sensitivity than DPV (DPV is sometimes more sensitive than SWV depending on the type of analyte to be investigated and the medium of study, moreover, the sensitivity of SWV is most of the time better than that of DPV especially in the reversible or quasi-reversible systems);^{112,124} significantly faster scan times; and wider applicability to a diverse range of electrode materials and systems. However, DPV offers advantages over SWV in certain aspects. DPV provides better peak separation, making it suitable for complex systems with overlapping peaks. It is commonly used for quantitative analysis due to accurate peak current measurements. DPV is well-suited for studying irreversible processes and offers slightly lower background currents. It is also simpler to implement and analyse.¹¹² The selection between DPV and SWV depends on specific experimental requirements and research objectives.

Chronoamperometry and chronocoulometry are similar techniques with the main difference being the mathematical integration of the current signal in chronocoulometry to determine the accumulated charge. Chronoamperometry is often used for studying electrode kinetics, determining rate constants, and investigating diffusion-controlled processes.

EIS provides information about the electrical properties and behavior of the electrochemical system, such as double layer capacitance, charge transfer resistance, and diffusion processes. EIS is widely used for characterizing electrode surfaces, studying interfacial phenomena, determining kinetic parameters, and assessing the impedance response of various electrochemical systems.

1.5 Aims and Objectives

The overarching aim of this thesis is to develop and validate innovative, user-friendly, and cost-effective electrochemical sensing platforms for the detection of circulating cell-free nucleic acid biomarkers. Each experimental chapter (Chapters 2-4) focuses on the design, engineering, and technical evaluation of a unique platform technology specifically developed for the electrochemical nucleic acid sensor.

The thesis encompasses three main objectives, as follows:

1. Investigation and comparison of probe immobilization methods for various electrode materials, such as carbon, graphene, and gold (Chapter 2).
2. Study of the hybridization efficiency of the capture probe immobilized onto the electrode (Chapter 3).
3. Design, validation, and comparison of sensing strategies based on the platform established in Chapter 2 (Chapter 4).

The structure mapping of this thesis is shown in Figure 1-12.

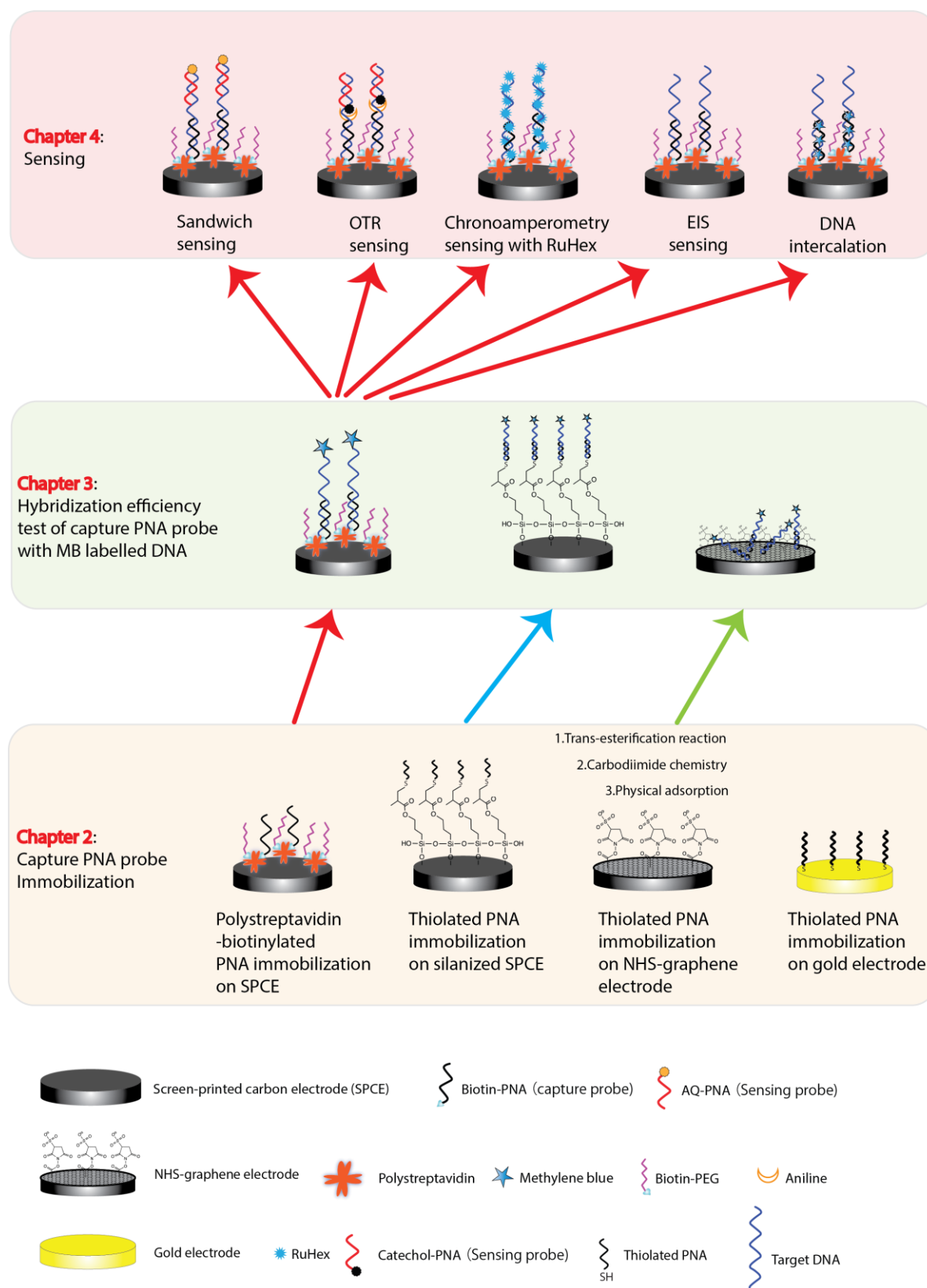


Figure 1-12 Structure mapping of the experimental chapters.

2 Electrode Functionalisation Strategies: How to Best Immobilize Oligonucleotide Capture Probes

Part of this chapter has been published in the following manuscript:

Xiaotong Meng, Danny O'Hare, Sylvain Ladame, "Surface immobilization strategies for the development of electrochemical nucleic acid sensors." *Biosensors and Bioelectronics*, Volume 237, 2023, 115440, <https://doi.org/10.1016/j.bios.2023.115440>.

Part of this chapter is due to be published in the following manuscripts:

(Manuscript in preparation) Immobilization Strategies for DNA Sensor on Screen-printed Carbon Electrode.

(Manuscript in preparation) On-chip Electrochemical Sensing Platform of miRNA Biomarkers for Early Detection of Prostate Cancer.

One of the most typical approaches for electrochemical detection of nucleic acids involves the efficient and sequence-specific capturing of these DNA or RNA targets onto the electrode surface. This is most commonly achieved by the use of oligonucleotides (or oligonucleotide analogues) chemically immobilized (or physically adsorbed) onto the electrode surface and acting here as hybridization or capture probes. In this thesis, capture probes were designed that were based on a Peptide Nucleic Acid (or PNA) backbone and various strategies were investigated to successfully immobilize PNA probes onto various electrode materials. In this chapter, the advantages of using PNAs rather than DNA as capture probes will be discussed and an overview of the different chemistries available to immobilize them onto electrodes will also be introduced. Finally, different chemistries were tested to immobilize PNAs onto carbon-based and metal-based electrodes and the results from these experiments are presented and discussed in the sections below.

2.1 Introduction of PNAs

PNA is a synthetic pseudo-peptide oligomer analogous to DNA or RNA,¹²⁵ with initial applications of gene-targeting antisense drug that inhibiting gene expression inside cells.^{126–128} In this work, miRNA and its corresponding DNA sequence serve as the target biomarkers. PNA was chosen as the capture and sensing probe to hybridize with the target miRNA or DNA. The neutral nature of the PNA backbone enhances its affinity towards RNA, surpassing that of DNA or RNA themselves. Moreover, PNA demonstrates increased sensitivity to mutations, a critical factor to consider given the high similarity among miRNA sequences.

2.1.1 Structures of DNA, RNA and PNA

Compared with DNA and RNA, in the structure of PNA, the sugar-phosphate backbone has been replaced with an achiral polyamide backbone composed of repeating N-(2-aminoethyl)glycine units, as shown in Figure 2-1. The nucleobases in PNA are linked to the polyamide backbone via methylene carbonyl linkages. The N-(2-aminoethyl) glycine backbone of PNA contributes to its high thermal stability and resistance to enzymatic degradation. Unlike RNA, which contains a hydroxyl (-OH) group on the nucleoside sugar, DNA and PNA exhibit greater stability despite the structural similarities between DNA and RNA.^{129–133} The stability order for nucleic acids is PNA > DNA > RNA (Table 2-1).

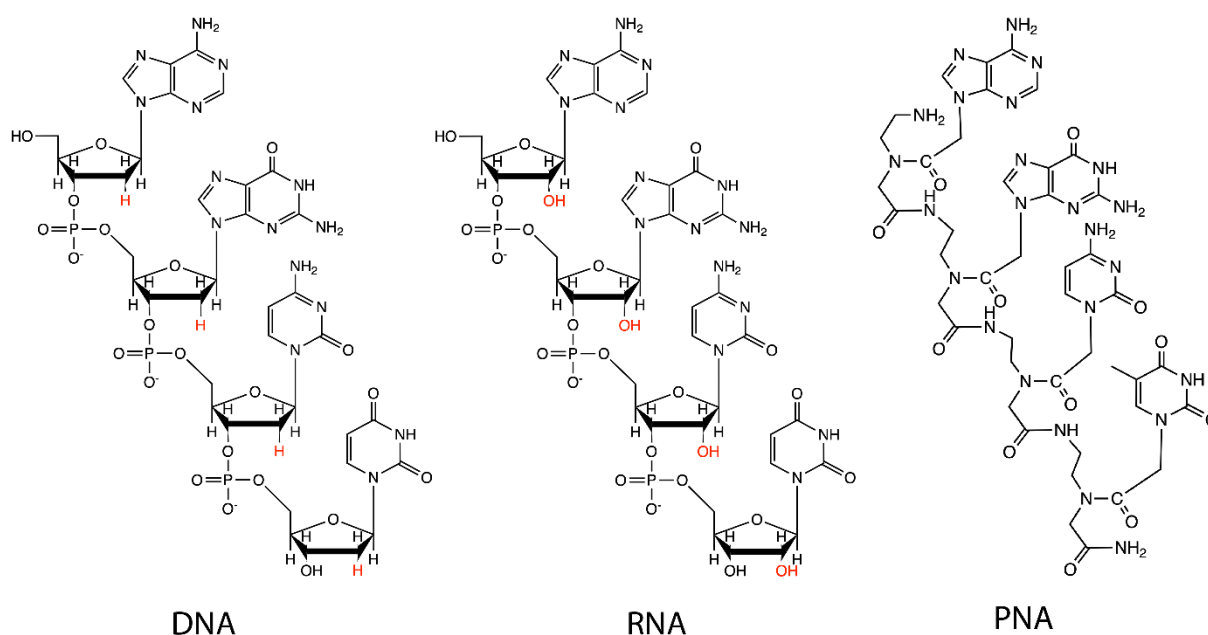


Figure 2-1 Structure of DNA, RNA and PNA.

PNAs can hybridize via Watson-Crick base-pairing with complementary DNA or RNA because their configuration and intermolecular distances are similar to that of natural nucleic acids.^{125,133} PNAs can form both parallel and antiparallel duplexes, in contrast to the antiparallel strand formation in the natural nucleic acids DNA and RNA.¹³³

PNA itself can be engineered for different applications. The nucleobase monomers can be designed and synthesized for specific purposes.¹³⁴ For example, high-performance pyrrolidiny peptide nucleic acid (also known as acpcPNA) have a stronger binding affinity toward complementary DNA compared to conventional DNA and PNA probes.¹³⁵

2.1.2 Properties of DNA, RNA and PNA

PNAs are widely used in research because they can hybridize to complementary DNA or RNA oligonucleotides with greater affinity and specificity than standard oligonucleotides.^{129–131,136}

Schwarz determined the binding constants (K_b) for PNA/DNA and DNA/DNA hybridization reactions using isothermal titration calorimetry. K_b for PNA(TT)/DNA is $1.8 \times 10^6 \text{ M}^{-1}$ and K_b for DNA(TT)/DNA is $0.37 \times 10^6 \text{ M}^{-1}$. K_b for PNA(AG)/DNA is $28.3 \times 10^6 \text{ M}^{-1}$ and K_b for DNA(AG)/DNA is $11.7 \times 10^6 \text{ M}^{-1}$. The higher the binding constant, the stronger the

interaction.¹³⁷ This is due to the neutral charge of the PNA backbone, in contrast to the negative backbone of DNA and RNA, which can lead to electrostatic repulsion between the two strands. Also, PNA/DNA duplexes can be easily formed at low ionic strength conditions because they have higher thermal stability.¹³² In addition to the greater chemical stability conferred by the PNA backbone, its synthetic nature also allows for easier production and functionalization compared to traditional oligonucleotides. Detailed properties comparison among PNA, DNA and RNA is shown in Table 2-1.

Table 2-1 Property comparison of PNA, DNA and RNA.

	PNA	DNA	RNA	References
pH	Wide range	5-9	4-5	138-140
Charge	Neutral	Negative	Negative	125,126,133
Aqueous solubility	Low solubility	Soluble	Soluble	141
Mismatch sensitivity	PNA-DNA > DNA-DNA			142
Melting temperature	PNA-DNA > DNA-DNA			136,139,143
Stability	PNA > DNA > RNA			129-133

2.1.2.1 pH

In terms of pH, DNA has both acidic and alkaline components. It consists of an acidic phosphate group, an alkaline nitrogenous base, and a sugar group. However, because the nitrogenous bases of DNA are oriented inward on the ladder and the sugar-phosphate backbones of each chain is on the outside, the acidic components of the molecule dominate.

While the exact pH value of DNA is not explicitly reported anywhere, there is substantial evidence to support that the DNA duplex is quite stable from pH 5 to 9. If the pH becomes too acidic or alkaline, DNA molecules are susceptible to DNA melting, depurination and denaturation.¹³⁸ Montazersaheb et al. prove that at acidic pH (pH 4.5~6.5), DNA started to depurinate while PNA showed excellent stability.¹³⁹ RNA is most stable at pH 4-5 and is unstable at alkaline pH.¹⁴⁰

2.1.2.2 Stability

The stability order for nucleic acid is PNA>DNA>RNA. The N-(2-aminoethyl) glycine backbone of PNA contributed to its outstanding stability. PNA not only exhibits high thermal stability, but also is resistant to enzymatic degradation. The -OH group on the RNA nucleoside sugar is the reason why RNA is less stable than DNA despite the highly similarity of DNA and RNA structure.^{129–133}

2.1.2.3 Solubility

DNA and RNA, as essential biomolecules, exhibit nice aqueous solubility. The artificial PNA molecules, on the other hand, have poor solubility in aqueous solutions. This is because PNAs are charge-neutral compounds. Neutral PNA molecules have a tendency to aggregate, a phenomenon dependent on the sequence and length of the PNA oligomer. PNA solubility is also related to the length of the oligomer and purine: pyrimidine ratio.¹⁴¹ Generally, the solubility of PNAs decreases as the polymer length. To improve the solubility, some strategies have been developed including terminal modification with charged amino acids such as L-lysine, arginine as well as modification of aminoethyl glycine backbone. L-lysine and arginine can increase the solubility of PNA, but it brings positive charge to PNA, which can cause non-specific binding between negatively charged DNA or RNA and PNA. It is important to find the trade-off balance between solubility and influence of positive charge for specific research or application.

2.1.2.4 Charge

DNA and RNA are negatively charged because of the phosphate backbone while PNA is overall neutral in charge. Therefore, PNA can hybridize with DNA not only with less repulsion but also with higher stability. That's why PNA is preferred in many applications than DNA and RNA if replaceable. PNA sequence design can influence the charge transfer constant on an

electrode.¹⁴⁴ On the other hand, charge property of DNA can be modified by lowering pH to protonate adenine and cytosine^{145–147} or forming covalent bonding between metal ions and nucleobases, including Hg^{2+} and thymine, Ag^+ and cytosine, Au^{3+} and adenine or cytosine.¹⁴⁸

2.1.2.5 Melting temperature

The duplex melting temperature is a threshold where the hydrogen bonds between nucleobases start to break.¹⁴³ PNAs have higher thermal melting temperature compared to corresponding DNA–DNA or DNA–RNA duplexes. The melting temperature can vary depending on the specific DNA and PNA sequences, experimental conditions, and the measurement techniques used. But generally melting temperature of a PNA–DNA duplex for per base pair is 1° C higher than DNA–DNA duplex.¹³⁹ In another specific study, Wang et al. found the melting temperature of a normal dA10–dT10 DNA hybrid is 23°C while the melting temperature of a similar dA10–dT10 DNA/PNA hybrid is 86°C.¹³⁶

2.2 Surface Functionalization Strategies

A schematic representation of a typical electrochemical nucleic acid biosensor and the key steps to prepare it are shown in Figure 2-2. The surface functionalization of the working electrode is typically the first and crucial step in sensor development for nucleic acid electrochemical sensors, because the success and reliability of subsequent steps heavily rely on the robustness of this initial surface functionalization. The process involves chemical or physical functionalisation of conductive materials or electrodes with sequence-specific hybridization probes made of standard oligonucleotides (DNA or RNA) or synthetic analogues (PNA). The robustness of such sensors is mostly influenced by the ability to control the density and orientation of the probe at the surface of the electrode, making the chemistry used for this immobilisation a key parameter. The selection of an appropriate immobilization technique is one of the key aspects of sensor preparation by ensuring effective capture of the analyte of interest whilst preventing nonspecific binding of non-target analytes, including other nucleic acids with different primary sequences and/or structures.¹⁴⁹

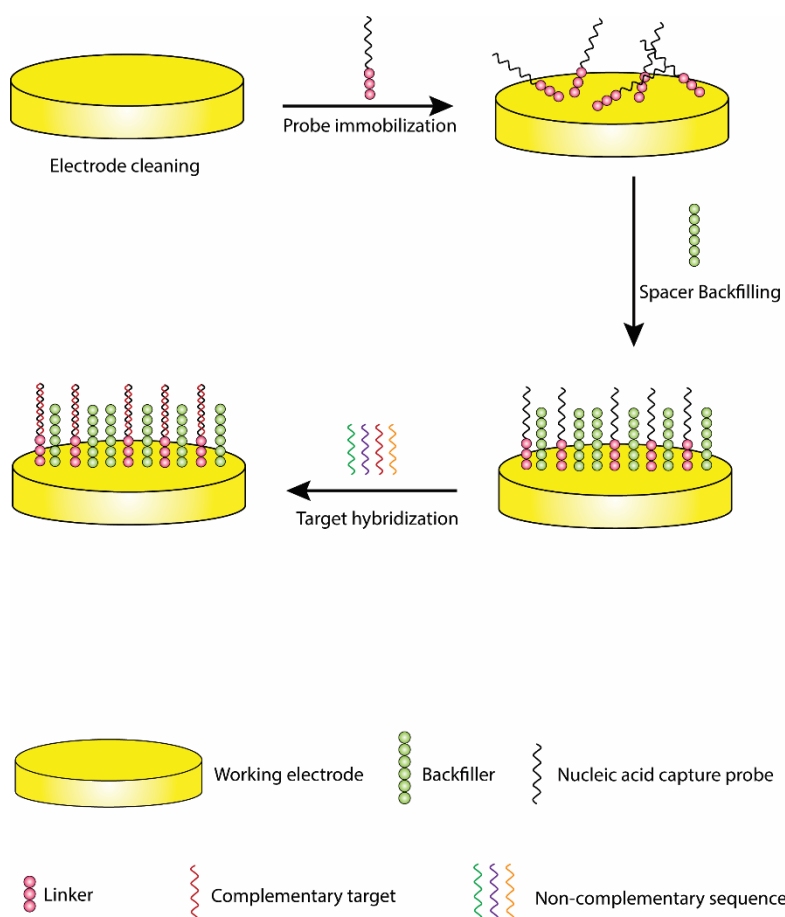


Figure 2-2 Schematic representation of the main key steps to produce an electrochemical nucleic acid biosensor working electrode.¹⁵⁰

A good electrode needs to meet two important criteria: being conductive and being inert enough so that they do not participate in any chemical reactions. The solvent window which is used to describe the region where they don't react also differs from different electrode materials. The most widely used and studied electrode materials are gold and carbon. Silicon-related materials and indium tin oxide (ITO) are also popular due to their comparatively high conductivity and cost-effectiveness. Polymers, hydrogels, nanoparticles and two-dimensional materials are commonly used to modify electrode materials like glassy carbon or gold for the purpose of introducing more surface area or new functional groups. As shown in Figure 2-3, whilst some surface immobilization strategies can be universally applied to any electrode regardless of the material (double arrows in Figure 2-3), most methods are highly material-specific (single arrows in Figure 2-3).¹⁵⁰

Based on the nature of the transduction platform and that of the probe to be attached, immobilization can be either physical or chemical. The former includes physical adsorption,¹⁵¹ electrostatic interaction,^{152–154} physical entrapment,^{155,156} hydrophilic and hydrophobic interactions.¹⁵⁷ Chemical immobilization strategies include covalent bonding,^{158,159} covalent cross-linking¹⁶⁰ and affinity binding.¹⁶¹ Physical immobilization processes are generally simpler and faster than chemical immobilization, but they often result in functionalized surfaces being less robust and less stable. In contrast, chemical immobilizations are more complex (often being a multistep process) but typically result in functionalized surfaces exhibiting stronger resistance to environment changes. Overall, a successful immobilization strategy will generate, in a highly reproducible fashion, functionalized surfaces that are stable for at least six months, a commonly accepted threshold for nucleic acid biosensors with commercial applications.¹⁰⁴

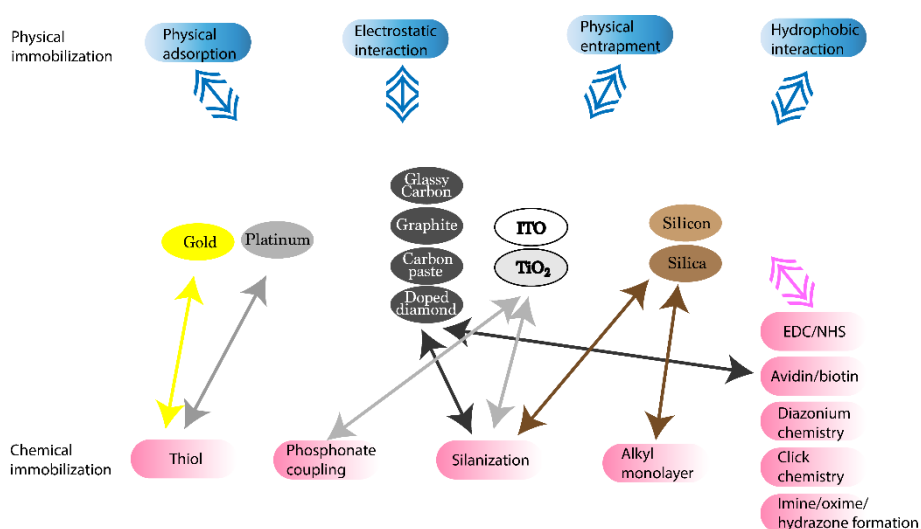


Figure 2-3 Physical and chemical immobilization strategies depending on electrode materials.¹⁵⁰

2.2.1 Immobilization on Gold Electrode

Gold is by far the most extensively studied metal electrode material, with formation of a self-assembled monolayer (SAM) using thiolated molecules the most widely used immobilization strategy.^{162–165} For gold electrode, other successful strategies include the use of poly adenine

(polyA) motifs as anchoring tags^{166–168} or physical immobilization through electrostatic adsorption and hydrophobic interactions.¹⁵⁷

2.2.1.1 *Gold-thiol Self-assembled Monolayer*

Direct immobilization of thiol-functionalized DNA or PNA probes is one of the most important strategies for functionalizing gold electrodes^{145,169} because the formation of gold-thiol SAM is a spontaneous reaction.¹⁶² A typical procedure for this process is illustrated in Figure 2-4(a). Reaction with thiol derivatives enable the introduction of a vast range of functional groups (Figure 2-4 (B)) onto otherwise chemically inert gold surfaces for follow-up immobilization via alternative biocompatible and biorthogonal chemistries including carbodiimide-mediated peptide coupling or click chemistry.¹⁷⁰ Gold-thiol bonds can remain stable in the presence of extremely large electric field at the electrode interface, and the immobilized probe can be reused for subsequent hybridization reactions without loss of efficiency,¹⁷¹ which suits well with electrochemical interrogations of the proposed sensor.

The chemistry of thiol on gold self-assembly is often followed by electrode surface passivation via insertion of short-chain alkyl thiols within the SAM¹⁷² in order to improve the stability of the sensor. The spontaneous self-assembly of ω -hydroxy thiols from aqueous solution on Au electrodes produces blocking monolayers which decrease electron transfer rates at the interface.

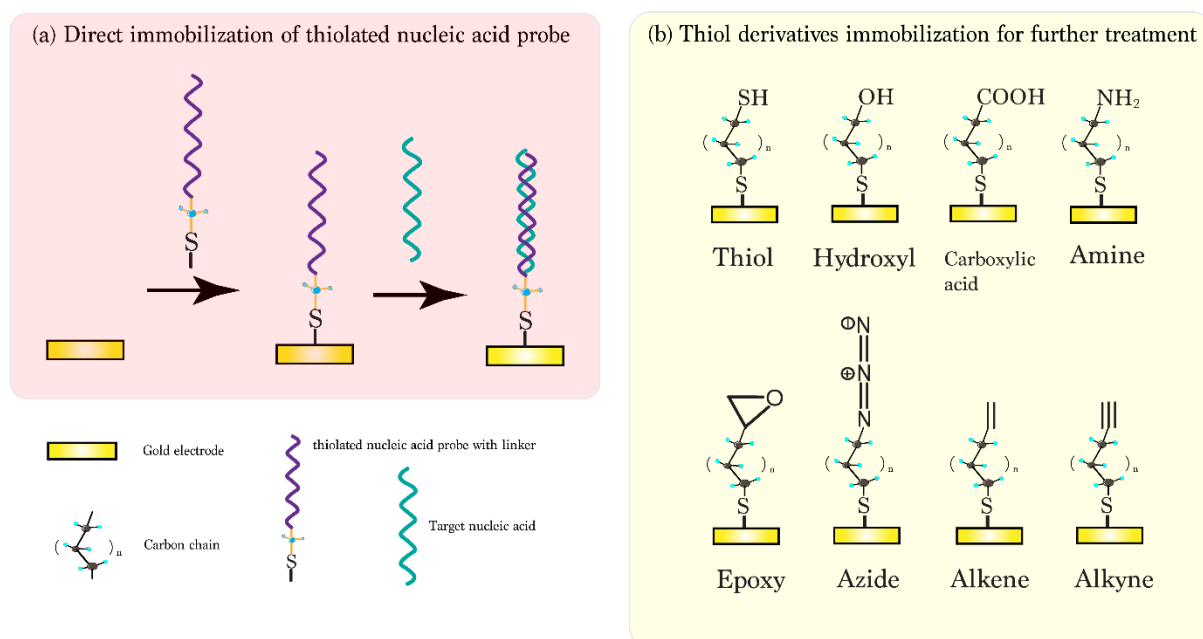


Figure 2-4 Self-assembled monolayer with (a) thiolated nucleic acids and (b) thiol derivatives for further functionalization.¹⁵⁰

2.2.1.2 PolyA anchoring

Non-covalent functionalization of gold surfaces with oligonucleotides is also possible via direct interactions between gold and the nitrogen atoms of nucleobases. With purines (adenine and guanine) exhibiting stronger chemisorption properties on gold than pyrimidines (cytosine and thymine), poly adenine (PolyA) tails are most commonly used for effective immobilization via synergistic binding. This is because PolyA chemisorbs on the gold surface with most robustness. For example, Demers and colleagues showed that polyT chemisorbs much weakly than polyA on both gold thin films and gold nanoparticle substrates.^{167,173} PolyA chemisorbing provides an effective anchoring site with an upright conformation which favors downstream analyte capturing through hybridization. Zhu et al. found that lateral spacing and surface density of DNA on gold surface can be systematically modulated by adjusting the length of the polyA block, with increasing polyA length leading to enhanced stability of the surface-bound DNA duplex.¹⁶⁶ This strategy was successfully applied to the capture of messenger RNAs (mRNAs) as well as for the functionalization of gold with DNA aptamers that contained a nine adenine 5'-end tail.¹⁷⁴ However, it is noteworthy that high chemisorption between purines and gold can also compete with sequence-specific hybridization, thus

leading to high levels of non-specific off-target binding. Demers et al. used temperature programmed desorption and reflection absorption Fourier-transform infrared spectroscopy, the strength of a covalent gold-thiol bond is stronger than that of a nucleobase chemisorbed onto gold,¹⁶⁷ as shown in Table 2-2. However, this can change when multiple nucleobases (or multiple thiols) are involved, which can lead to polyA chemisorption competing with Au–S covalent bonding.¹⁶⁸

Table 2-2 Bond strength of typical immobilization methods on metal.¹⁵⁰

	Binding strength (kJ/mol)	References
Thymine-Au	111	167,175
Cytosine-Au	128	
Adenine-Au	131	
Guanine-Au	146	
Thiol-Au	167	163
	209	
	126	176
	188	177
	253.6±14.6	178
Thiol-Ag	216.7±14.6	

2.2.1.3 Challenges and Solutions

A critical limitation of electrochemical sensors built on gold electrodes is their lack of monolayer stability over time caused by the weakness of the Au-S bond.^{179,180} With a binding

energy of 126-253 kJ/mol according to different studies,^{163,175–178} the Au-S bond is significantly weaker than the average covalent bond binding energy in the range of 200 to 800 kJ/mol,¹⁸¹ as shown in Table 2-2. In most cases, the formation of a SAM is disrupted or weakened by reduction effect of thiols and further weakening effect of the self-assembled thiols on the Au-Au interactions,¹⁶⁵ thus losing some of the energetic gain from multiple Au-S bonds.¹⁰⁵

Reductive desorption of alkanethiol with different terminal groups was investigated by Munakata et al.¹⁸² For $-OH$ terminated thiols and $-CH_3$ terminated thiols, the kinetics of the desorption reaction initiated by the proton permeation is mainly determined not by the blocking effect of intermediate alkyl groups but by that of the ω -terminal group. For the $-COOH$ terminated and $-NH_2$ terminated thiols, the intermolecular transfer of H^+ is slightly influenced by the blocking effect of the intermediate alkyl chains.

In order to enhance the stability of thiol SAM, different factors have been investigated.¹⁶⁴ Surface cleanliness, pH¹⁸³ and solvent¹⁶³ have been shown to play important roles in enhancing the stability of thiol SAM on gold electrodes. The choice of thiolated ligand is also important, multidentate di- or tri-thiols were shown to provide enhanced stability.^{184–186} Multidentate interactions not only increase the number of Au-S bonds but also increase binding energy due to the chelate effect.¹⁷² The self-assembly process of dithiols is considered to take place following a pathway similar to that of monothiols, in which a disulfide bond is reduced, forming two Au-S surface bonds.¹⁸⁰ Compared with monothiols, dithiols are reported to have shorter deposition times¹⁶⁴ from as little as 30 min although there is no consensus on methodologies.

Phares et al. demonstrated that DNA sensors anchored with a flexible tri-thiol on gold electrode exhibited enhanced stability, retaining 75% of their original signal after 50 days storage in buffer.¹⁸⁶ In contrast, sensors fabricated using a rigid monothiol anchor retained only 35-40% of the original signal. After 50 scans of alternating current voltammetry, sensors using DNA immobilized via tri-thiols retained 94% of their original signal while sensors using monothiols only retained only 75%. Despite the merits of using multi-dentate thiols compared with monothiol, their broader use is hampered by synthetic challenges.¹⁸⁴

Hydrophobic thiols have long been known to impart higher monolayer packing density compared to hydrophilic thiols. However, this strategy can only be applied in buffered solutions, as deployment of the hydrophobic surface in biological fluids can lead to immediate fouling and loss of sensor signal.¹⁷²

2.2.2 Immobilization on Carbon Electrode

Carbon is another commonly used electrode material with high versatility and easily tuneable physical and chemical properties.^{170,187} Carbon materials have significantly more complex surface chemistry than metals, at least in part because carbon can form a wider variety of chemical bonds. As shown in Figure 2-5, commonly used carbon materials such as glassy carbon have a wider working potential window compared with metal electrodes such as gold and platinum.^{188–190} However, the practical utility of the wider potential window will depend on specific target electrochemistry. Solvent oxidation and reduction potentials also contribute to the anodic and cathodic potential limits and are largely electrocatalytic in nature. The desired electrochemistry may also be shifted to more extreme potentials, as is found with boron-doped diamond for example. Different carbon structures will affect electrodes' properties and performance. Glassy carbon, boron doped diamond, graphite and carbon paste are all conductive enough to work as electrodes. Graphene and carbon nanotubes are special cases, being two-dimensional (2D) materials. Carbon surfaces are prone to react with oxygen and water. The thermodynamic stability of carbon-oxygen bonds makes most carbon material susceptible to oxidation processes, such as treatment with oxygen plasma.

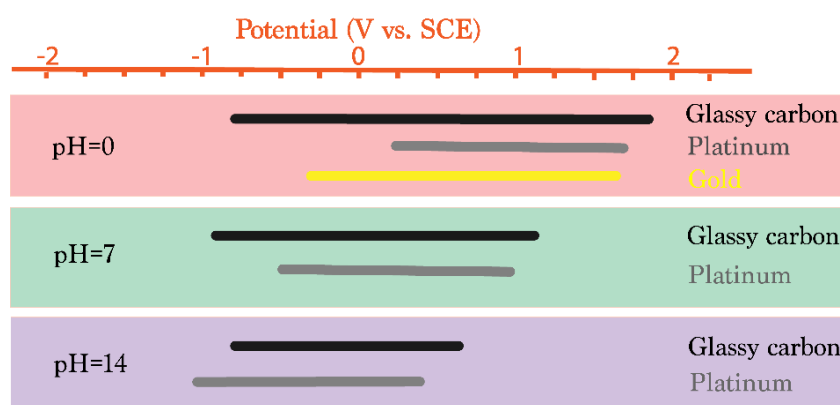


Figure 2-5 Potential window of glassy carbon and metal electrodes.¹⁵⁰

Due to structural and processing difference, carbon electrodes can exhibit a broad range of physical and chemical properties and surface natural functional groups. The most commonly used carbon electrode material is glassy carbon. Glassy carbon is a pyrolyzed material that contains mainly sp^2 hybridized carbon chains. The structure of glassy carbon is generally presented as randomly intertwined ribbons of graphitic planes. Glassy carbon can be thought of as a mixture of graphite and a glassy ceramic. The disordered nature of glassy carbon makes atomic level structural characterization difficult and the randomness results in significant uncertainty about the detailed microstructure.¹⁸⁷

Graphite exhibits both metallic and nonmetallic properties.¹⁹¹ In industry, graphite is typically made using premium petroleum needle coke, coal tar pitch, and some additives.¹⁹² This graphite needs further treatment before application as an electrode. Graphite paste electrodes are made by mixing natural graphite (65-80%), an organic binder such as wax, resin or a polymer (~13%) and clay or spindle oil (8-30%).¹⁹³ Graphite reinforcement carbon is used in pencil lead, which is the same material used as graphite electrode for sensing.¹⁹⁴ Pencil graphite leads used as working electrodes are currently known as pencil graphite electrodes. Cost effectiveness of pencil graphite electrodes can be reflected by comparing \$190 per glassy carbon electrode with \$0.13 for pencil graphite electrode.¹⁹⁵

Carbon paste electrodes were introduced in 1958 by Ralph Norman Adams.¹⁹⁶ They can be conveniently modified with various functional molecules.¹⁹⁷ Carbon paste is made of graphite or other carbon powder mixed with a liquid paste, typically mineral oils or silicone oils¹⁸⁰ and has a wide anodic potential range (+1.7V vs, saturated calomel electrode (SCE)).¹⁹⁸ The biggest challenge of working with carbon paste electrodes is the lack of a standard for calibration. Basic electrochemical characteristics for most commercially available solid electrodes from each manufacturer are comparable, with carbon paste electrode being an exception. Each carbon paste unit is different from another, where the physical, chemical and electrochemical properties may differ from one preparation to the other, making calibration with each individual carbon paste electrode almost impossible. For these reasons, working with carbon paste electrode is strongly experience-dependent.¹⁹⁹

Diamond has a natural unstrained state with an ultrawide electronic bandgap of 5.6 eV, meaning that it is a strong electrical insulator inside which electrons do not move through readily.²⁰⁰ Doping can improve the conductivity of diamond, with boron doped diamond a common alternative that can reduce the resistivity from $10^{12} \Omega m$ to $1 \sim 10^{-4} \Omega m$.²⁰¹

2.2.2.1 Silanization

Chemical immobilization via silanization is compatible with any electrode surface that can generate hydroxyl groups.²⁰² Although silanization is believed to be most efficient with silica and glass surfaces,²⁰³ it can also be successfully applied to including carbon electrodes.^{204,205} Formation of an alkoxy silane polymer on the electrode surface is achieved through $-Si-O-Si-$ bond formation.¹⁸⁰ Typical silanization steps include cleaning, surface activation and reaction with an alkoxy silane. Surface activation involves generation of hydroxyl ($-OH$) groups using KOH solution, oxygen plasma or ozone treatment, either alone or in combination. A broad range of silanes are now readily available that enable the introduction of diverse functional groups for attachment of oligonucleotides (or oligonucleotide analogues), making silanization a popular and versatile functionalization method. In Figure 2-6, APTES was used to functionalize the electrode surface with amine groups available for further treatment.

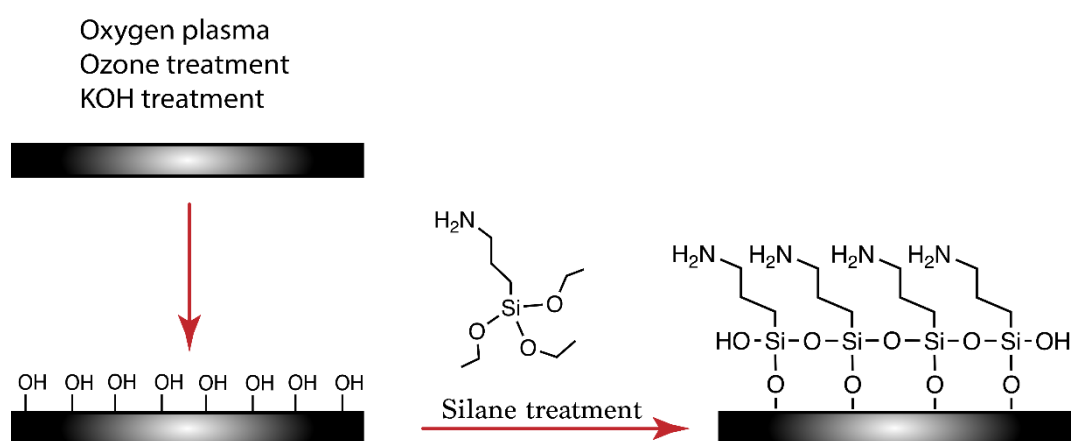


Figure 2-6 Silanization with (3-Aminopropyl)triethoxysilane (APTES).¹⁵⁰

2.2.2.2 Carbodiimide Chemistry

Another commonly used approach for functionalizing carbon electrodes is to utilize carboxylate groups for amide bond formation through carbodiimide coupling chemistry, taking advantage of the abundant carbon-based functional groups present on the carbon surface. EDC and NHS coupling chemistry, which gained popularity in the late 1990s,²⁰⁶ is commonly employed for this purpose. EDC activates the free carboxyl groups, while NHS (or its water-soluble analogue, sulpho-NHS) reacts with the carboxyl groups, forming a stable amine-reactive NHS ester intermediate that can be coupled with primary amines under physiological pH conditions.¹⁷⁰ The reaction scheme is illustrated in Figure 2-7. For other electrode material, with the presence of either carboxylic acid or amine groups, carbodiimide chemistry can also be applied. A range of chemicals and treatments can be used to introduce these desired functional groups. For example, KOH and oxygen plasma can be used to generate hydroxyl, amino and carboxyl-terminated moieties on carbon surfaces.

For PNA specifically, its intrinsic structure contains both amine and carboxylic acid groups, as depicted in Figure 2-7. This inherent composition makes it convenient to immobilize PNA on carbon-based electrode substrates.

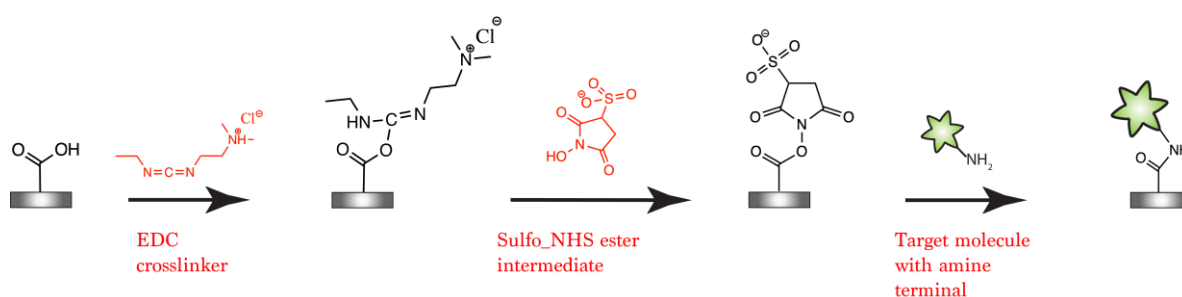


Figure 2-7 Carbodiimide (EDC/NHS) coupling chemistry mechanics.

2.2.3 Immobilization on 2D Material

Dimensions and surface areas are two parameters that can greatly influence the performance of an electrochemical sensor.²⁰⁷ The high density of active surface sites over a large area³⁰ and their ability to form different shapes and sizes with distinct properties²⁰⁸ make 2D

materials highly valuable for electrochemical biosensing applications. The high surface-to-volume ratio of nano-objects makes their electrical properties increasingly susceptible to external influences²⁰⁷. The atomic thickness of 2D materials enable high sensitivity as the nanoscale thinness yields a strong response to surface adsorption events compared with its bulk material. The unique size-dependent properties of 2D materials can be rationally manipulated to assemble on top of bulk electrode surfaces to ensure high effective surface concentration of immobilized bioreceptor, easy electrocatalytic transformation of analytes and fast electron transfer processes at the sensing interface.²⁰⁹ 2D materials can be physically deposited or electrodeposited onto a broad range of substrate electrode.^{210,211}

Carbon-based nanomaterials including carbon nanotubes, fullerene, graphene, and nano-diamonds have gained increasing interest owing to their biocompatibility and their unique structural, mechanical, thermal, and electrical properties.²⁰⁹

Other 2D materials such as MoS_2 , WS_2 , phosphorene (black phosphorus) or antimonene (antimony) are also of great interest. MoS_2 and WS_2 are typical representatives of transition metal dichalcogenides. MoS_2 and WS_2 with one to three layers, which are considered as 2D material, are of particular interest because they have direct band gap while the bulk MoS_2 and WS_2 have indirect band gap. 2D MoS_2 and WS_2 also exhibit rapid heterogeneous electron transfer and high density of electronic states. Bulk antimony is typically semi-metal. But can be transformed into indirect semi-conductors with band gaps of 2.49 and 2.28 eV when thinned into one atomic layer.²¹²

Fortunati et al. reported single-walled carbon nanotubes screen printed electrode for DNA sensing¹⁵⁹. In this amperometric genosensor, PNA probes were immobilized on the single walled carbon nanotubes (SWCNTs) via carbodiimide chemistry. SWCNTs were selected in this research because of the native carboxylic acid moieties present on the edges of the nanotubes, onto which the amine-terminated PNA could be covalently attached. This genosensor was able to detect trace levels of non-amplified DNA in genetically modified soy DNA with a limit of detection via DPV of 64 pM and a dynamic linear range between 250 pM to 2.5 nM. Pyrene was used here as backfillers to prevent non-specific interaction with the electrode substrates, ultimately improving signal-to-background ratio.

2.2.4 Avidin-biotin Linking

Avidin, polystreptavidin or neutravidin proteins can bind up to four biotin molecules to form an avidin-biotin complex with high specificity and strong affinity. The polystreptavidin-biotin complex is among the strongest known non-covalent interactions between a protein and a ligand²¹³ via multiple hydrogen bonds formation ($Ka = 1 \times 10^{15} M^{-1}$). The binding enthalpy of biotin to polystreptavidin is from -97.9 to -149.85 kJ/mol^{214–216} at room temperature and pH = 7.4. The level of stability of this interaction is almost irreversible and comparable to that of a covalent bond.³⁸ Moreover, the interaction between biotin and (strept)avidin is very rapid and specific. Once the (strept)avidin-biotin complex is formed, it is unaffected by extreme variations in pH, temperature, organic solvents and other denaturing agents.²¹⁷

Whilst it is more common to immobilize (strept)avidin on the electrode surface for capturing of a biotin-terminated target, the reverse strategy is also possible.²¹⁸ Although proteins from the avidin family can physically adsorb to most electrode surfaces, some pretreatment of the electrode can improve immobilization efficiency. Like all other physical immobilization methods, physical adsorption of (strept)avidin can potentially be challenged by multiple rinse cycles. Covalent immobilization of the protein to the electrode surface provides a more robust coating and can proceed through either carbodiimide chemistry and amide bond formation²¹⁹ or through glutaraldehyde-mediated crosslinking on glass slides.¹⁴⁹

2.3 Controlling Probe Density, Orientation and Accessibility

Probe density, orientation and distance from the electrode surface are key parameters that can strongly influence the sensor performance. Probe density is related to the probe concentration immobilized and backfilling treatment.¹⁶⁹ Overcrowded surface coverage of the immobilized probe can lead to steric hindrance.^{220–222} Therefore, probe density not only influences the efficiency of hybridization but also the kinetics of capturing the target molecule.^{223,224}

Oligonucleotide immobilization is usually constructed on the basis of a two-step assembly including probe immobilization and backfilling, or passivation in some cases, as illustrated in Figure 2-8. Backfilling forms a close-packed film that can passivate the gold surface and reduce the background signal via disrupting non-specific adsorption. Carefully selected backfilling

molecules also play an important role for controlling the orientation of the surface-immobilized probes,²²⁵ and facilitating their accessibility.

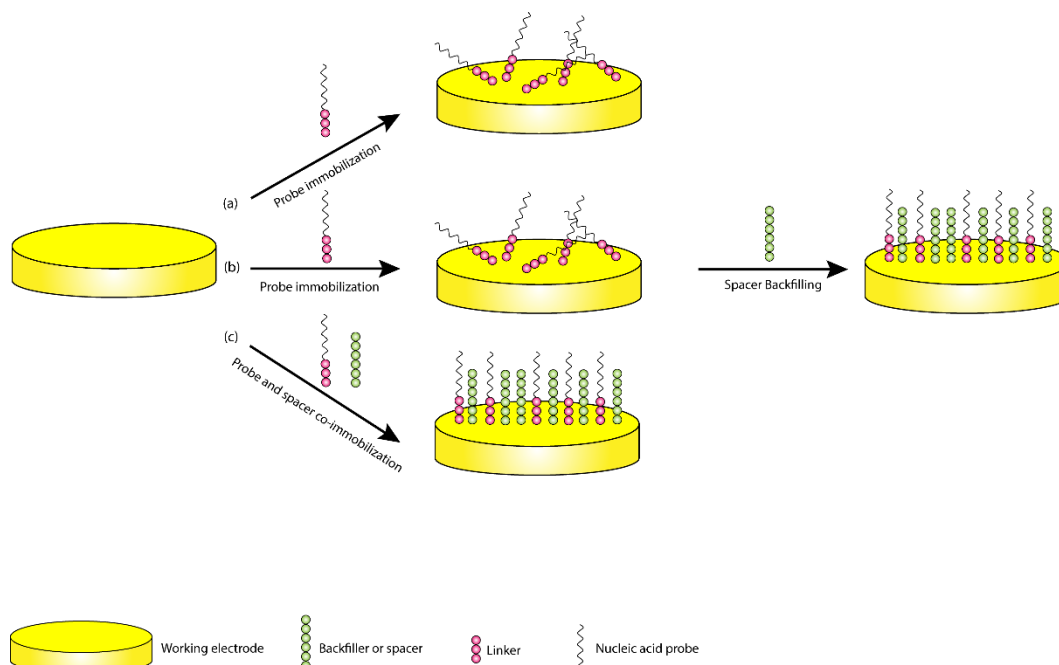


Figure 2-8 Orientation control by backfilling.¹⁵⁰

The orientation and density of oligonucleotide probes immobilized on electrode surfaces are mostly controlled through backfilling,²²⁶ probe nanostructure design²²⁷ or these two methods combined.²²⁸ Alternatively, the orientation of thiolated DNA probes on gold surface can also be controlled by applying a negative potential at the electrode surface in order to repel the immobilized oligonucleotide, which is also negatively charged.^{229,230} However, this method is limited by the need to change the potential applied on the electrode since the orientation controlling condition may conflict with sensing requirement. And the orientated probe can be influenced by high ionic strengths and interactions with the ionic double layer.²³¹

2.3.1 Backfilling for Controlling Probe Density and Orientation

Backfilling of the electrode surface can help control the probe density as well as their spacing. But backfilling is also a useful way to control the orientation of these probes on the electrode surface which is key to most sensing applications.^{231,232}

For the purpose of orientation and density control, the most intensively studied backfilling strategies are immobilization of mercaptohexanol or other thiol derivatives on gold electrodes. The orientation, density and regularity of the thiol derivative backfiller coverage depends on the type of thiols and also on the immobilization process.²³²

2.3.2 Backfilling for Passivation

For the purpose of decreasing non-specific interaction, backfillers can be categorized as physical blockers and chemical linkers.²³³ Physical blockers are usually proteins such as bovine serum albumin (BSA)^{234–238} and casein.²³⁹ Casein works similarly to BSA, but primarily blocks the enzyme from binding to the functionalized surface.²³⁹ Polyethylene glycol (PEG) SAMs are extensively used chemical linkers to modify electrodes to prevent aggregation,²⁴⁰ nonspecific protein adsorption and to increase hydrophilicity.²⁴¹ On gold electrode, mercaptohexanol or other thiol derivatives play a significant role in displacing non-specifically adsorbed probes.^{169,242}

2.3.3 Controlling Probe Orientation and Density with DNA Nanostructures

Probe orientation and density of electrode surfaces can also be controlled by using rigid two- or three-dimensional nanostructures as linkers between the solid surface and the oligonucleotide probe. Notable progress in this research area has been achieved by exploring multiple creative designs including but not limited to tetrameric junction,²⁴³ three-dimensional cube,²⁴⁴ two-dimensional DNA lattices,²⁴⁵ DNA triple crossover complex,²⁴⁶ DNA origami²⁴⁷ and DNA tetrahedron,^{248–251} octahedron.²⁵²

DNA origami is a promising branch of DNA nanotechnology that involves molecular self-folding, which relies on the nanoscale folding of a long, single-stranded DNA known as scaffold into a two-dimensional or three-dimensional shape through hybridization of oligonucleotides known as staple strands.²⁵³

DNA tetrahedrons are pyramidal three-dimensional nanostructures formed by the complementary pairing of four single-stranded DNA.²⁵⁰ They are characterized by high stability, excellent biocompatibility, rich functional modification sites, simplicity of synthesis and modification.²⁵⁴ DNA tetrahedrons can be used for DNA immobilization in electrochemical sensors with controlled probe direction and distance. The surface of the DNA tetrahedron can be modified with functional groups, which can then be used for the chemical immobilization on electrodes. Once the DNA tetrahedron is immobilized on the electrode, single-stranded DNA probes can be attached to the tetrahedron. These probes can then be used to capture specific target molecules in the sample. The use of tetrahedral DNA as a capture probe immobilized on electrode offers several advantages for electrochemical sensors. First, the rigid structure allows for a precise control of probe direction and distance, thereby avoiding molecular entanglements and improving the sensitivity of electrochemical sensors. Second, within a certain range, larger DNA tetrahedrons can lead to longer distances between probes and higher hybridization efficiencies. As a result, DNA tetrahedron enables controllable steric hindrance.^{255–257}

DNA nanostructures exhibit stronger nuclease resistance compared with single-stranded DNA and double-stranded DNA.^{258,259} Because of these unique properties, these organized structures have been successfully used as linkers for controlled immobilization of oligonucleotides on electrode surfaces.

2.3.4 Surface Coating for Antifouling

Biofouling is the process of nonspecific protein adsorption and/or microbial adsorption on biomedical materials^{260,261} which is induced by the strong tendency of proteins that can physically adsorb onto the surfaces.²⁶² This is a persistent challenge for any interfaces that are exposed to complex biological media. Antifouling is effective at both reducing background interference and maintaining enough target binding ability. Commonly applied strategies for mitigating or preventing fouling include nanoporous coating,^{263,264} zwitterionic molecule treatment,^{172,265–268} conducting polymer,^{260,263,268} PEG^{260,261} and hydrogel coating.¹⁷²

Nanoporous coating can effectively stop protein adsorption on the electrode surface if the size of the pores is appropriately designed.^{263,264} Short oligonucleotides can diffuse into pores

and then hybridize with immobilized capture strands while access of serum protein to the electrode surface is restricted. Zwitterionic thiols (or other polymers including peptides²⁶⁸ in the blocking monolayer can mitigate sensor fouling.²⁶⁷ But zwitterionic monolayers are still limited by long-term stability issue. A more ubiquitous method to reduce fouling is through the deposition of hydrogels or membranes on top of a sensor surface. Hydrogel composition, extent of crosslinking, and membrane thickness can be customized according to different demands and hydrogels are stable over longer periods.²⁶⁹

Integrating both the recognition elements and the antifouling materials on the same layer is a commonly used method which however suffers from relatively low target loading due to limited penetration of biomacromolecules in the highly packed brush film formed by poly-sulfobetaine and poly-carboxybetaine.^{270–272} It is difficult for the protein molecules to penetrate through the brushes due to steric hindrance, and they are most likely conjugated on the outermost poly-carboxybetaine chains.²⁷² Another popular approach involves the direct grafting of the recognition probes to the antifouling materials which can bring high target binding, but can also cause large nonspecific adsorption, due to the very loose polymeric structure.

Self-polymerization of dopamine can form thin, surface-adherent polydopamine films on the surface of various inorganic and organic materials. This kind of chemistry was first discovered in mussels, hence given the name of mussels-inspired chemistry.^{266,273} Cui et al. reported strategies for preparing low-fouling surfaces on different substrates via this mussel-inspired immobilisation of zwitterionic peptides.²⁶⁵ They used polydopamine coatings, zwitterionic glutamic acid- and lysine-based peptide immobilization on different substrates, including noble metals, metal oxides, polymers and semiconductors. The peptide-functionalized surfaces showed resistance to human blood serum adsorption and effectively prevented the adhesion of gram-negative and gram-positive bacteria as well as mammalian cells.

2.4 Materials

Bulk gold electrodes and bulk glassy carbon electrodes were purchased from CH Instruments, (USA). Screen-printed carbon electrodes (SPCEs) were purchased from Metrohm (UK). NHS-functionalized graphene electrodes were purchased from Integrated Graphene LTD (UK).

Unless stated otherwise, bespoke PNA oligomers were synthesised in-house by standard manual solid-phase peptide synthesis (SPPS) together with Dr Philip Gillespie, Dr Md Nazmul Islam and Dr Rob Channon. Biotin-PNA-Aniline-141 was kindly provided by Dr Md Nazmul Islam. PNA monomers were purchased from Link Technologies (UK). HPLC purified single-stranded DNA oligonucleotide was purchased from Integrated DNA Technologies (UK). 3-(trimethoxysilyl)propyl methacrylate was purchased from Sigma Aldrich. Phosphate buffered saline (PBS, 1X) was purchased from Thermo Fisher Scientific Inc. and used as received. Polystreptavidin was purchased from BioTeZ Berlin-Buch GmbH (Berlin, Germany). Biotin-(PEG)₆-OH was purchased from PurePEG LLC (San Diego, USA). Unless otherwise noted, all other reagents as well as chemical and reactive groups were purchased from Sigma Aldrich.

2.4.1 DNA and PNA Oligomers

There are multiple clinically validated miRNAs which are deregulated in the blood of patients with prostate cancer when compared to healthy controls. They are (not limited to) miRNA141,^{87,274} miRNA374a,^{275,276} miRNA375,^{87,277} miRNA21,²⁷⁸ miRNA221⁶¹ and miRNA20a.²⁷⁹ In this research, we have used miRNA141 (DNA141) and miRNA374a (DNA374a) as target sequences for testing (miRNA127, DNA127, miRNA150, DNA150 as controls). In this chapter specifically, PNA capture probes are complementary to miRNA141(DNA141).

The PNA oligomer and DNA oligonucleotide sequences used in this chapter are presented in Table 2-3. It is noteworthy that DNA sequences were used throughout as a proxy for miRNAs due to their greater stability and lower cost (e.g. DNA141 as a proxy for miRNA141). The 7-mer and 8-mer PNA oligomers were designed that also contained 2 arginine residues for increased solubility in water and also contained either an aniline or catechol moiety to help monitor attachment onto electrodes. The sequence of miRNA141 and DNA141 can be found in Table 2-3. Figure 2-9 illustrates the structures of the PNA capture probes used in this Chapter. All PNA oligomers were purified by high-performance liquid chromatography (HPLC) and characterised by matrix-assisted laser desorption/ionization (MALDI). Purified PNAs were then freeze-dried and stock solutions prepared in deionized water (typically at 1mM), aliquoted and stored at -20C.

Table 2-3 Oligomer nomenclature and sequences.

Category	Name	Sequence
PNA capture probes	Biotin-PNA-Aniline-141	C _{term} -Aniline-CAT TTC T-Arg-Arg-Biotin-N _{term}
	Thiol-PNA-Aniline-141	C _{term} -Aniline-CAT TTC TA-Arg-Arg-Thiol-N _{term}
	Thiol-PNA-Catechol-141	C _{term} -Thiol-Arg-Arg-T GTG ACA-Catechol-N _{term}
Complementary sequence	DNA141	5'-TAA CAC TGT CTG GTA AAG ATGG-3'
	miRNA141 (hsa-miR-141-3p)	5'-UAA CAC UGU CUG GUA AAG AUGG-3'

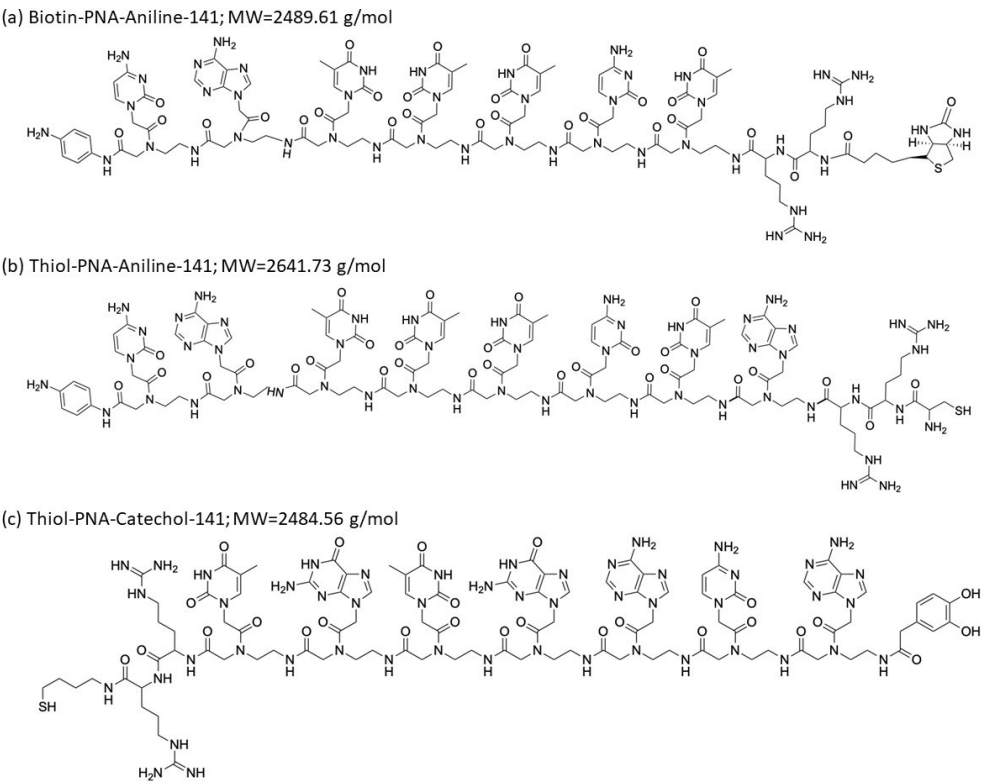


Figure 2-9 Representative PNA capture probe structures. (a) Biotin-PNA-Aniline-141. (b) Thiol-PNA-Aniline-141. (c) Thiol-PNA-Catechol-141.

2.4.2 PNA Synthesis

PNAs were synthesised by standard manual SPPS using 2-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) as a coupling agent.^{301–303} The overall SPPS strategy is summarised in Figure 2-10.

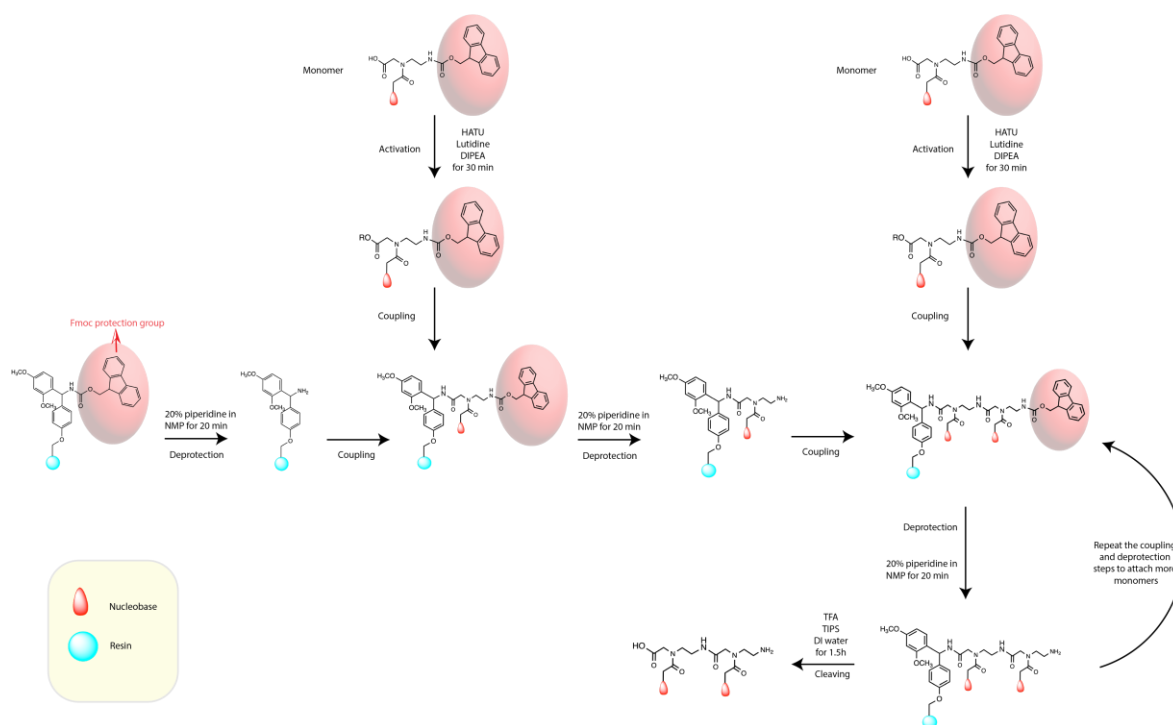


Figure 2-10 PNA synthesis schematic diagram.

2.4.2.1 PNA Synthesis Protocol

With the key steps of the protocol followed for PNA synthesis are detailed below:

1. Resin Swelling:

Resin was swelled in dimethylformamide (DMF) for 30 minutes and washed with DMF for next step monomer coupling.

2. Deprotection:

If the resin has a Fmoc protection group. Deprotection is needed before coupling with monomers. Leave the resin in piperidine solution (20% piperidine in NMP) and place it on a rotator for 20 minutes. Then wash three times with DMF before next step.

3. Coupling:

- Monomers (thymine, cytosine, adenine, guanine, arginine and electrochemical reporters) are dissolved in n-methylpyrrolidone (NMP) with a concentration of 0.1M.
- HATU is dissolved in NMP with a concentration of 0.5M.
- Base mix is prepared by mixing 2,6-lutidine, DIPEA and NMP with a volume ratio of 1 : 1 : 3.

Mix the monomer solution, HATU solution and base mix with a volume ratio of 80% : 14% : 6% and allow 3 minutes for mixing and reaction. Then pour the mixed monomer, HATU and base mix into the cartridge with the deprotected resin and place it on a rotator for 30 minutes for coupling reaction.

4. Double coupling:

Repeat the last coupling step to increase the coupling successful rate.

5. For each monomer, repeat the coupling procedure twice.

6. PNA cleaving:

Once all of the monomers have been attached to the resin in the designed order, wash the resin thoroughly with DMF and DIC in the last wash for the purpose of drying the resin quickly. Apply cleavage cocktail solution (trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and DI water mixed with a ratio of 95% : 2.5% : 2.5%) to the resin and allow to react for 90 minutes.

7. PNA extraction:

After the PNA cleavage, PNA have been detached from the resin and dissolved in the cleavage cocktail solution. Mix the PNA containing cleavage cocktail solution and diethyl ether with the ratio of around 1 : 19. The PNA is not soluble in diethyl ether and should precipitate quickly. Use centrifuge to collect the solid PNA from diethyl ether and wash multiple times with diethyl ether if needed.

2.4.3 Electrochemical Setup

Both the bulk three-electrode electrochemical system and disposable electrodes are used in this research. The schematic illustrations of these two setups are shown in Figure 2-11. In the bulk three-electrode system, bulk glassy carbon electrode with 3.0 mm diameter was used for preliminary electrochemical investigations. For the immobilization of thiolated PNA capture probe (Thiol-PNA-Catechol-141) on gold electrode, a 2.0 mm diameter bulk gold electrode was used. In this bulk electrode system, a platinum wire worked as the counter electrode and a silver/silver chloride electrode worked as the reference electrode.

SPCE has working and auxiliary electrodes made of carbon. The diameter of the working electrode is 4.0 mm. The reference electrode is made of silver, which in theory should be considered as pseudo-reference electrode (right, in Figure 2-11). A pseudo-reference electrode, also known as a quasi-reference electrode, does not have a stable and well-defined potential under all conditions. The key difference between a reference electrode and a pseudo-reference electrode lies in the stability and reproducibility of the potential. A reference electrode is carefully designed and calibrated to maintain a stable potential, while a pseudo-reference electrode may exhibit some changes in potential due to the difference of the ionic strength of the electrolyte solution that could lead to potential drift or variability. However, both types of electrodes serve the purpose of providing a reference potential for electrochemical measurements, albeit with varying degrees of accuracy and stability. In this research, the same ionic strength of the background electrolyte solution is used to help minimize the potential drift or variation from pseudo-reference electrode. Except from SPCE, another disposable electrode was used in this research: NHS-functionalized graphene electrode (Integrated Graphene LTD, UK). The working electrode is 4 mm in diameter and is made of N-hydroxysuccinimide (NHS)-functionalised 3-dimensional graphene (3-dimensional super-porous scaffold of carbon layers displaying the desired properties of graphene). The reference electrode is made of screen printed Ag/AgCl and the counter electrode is made of 3-dimensional graphene.

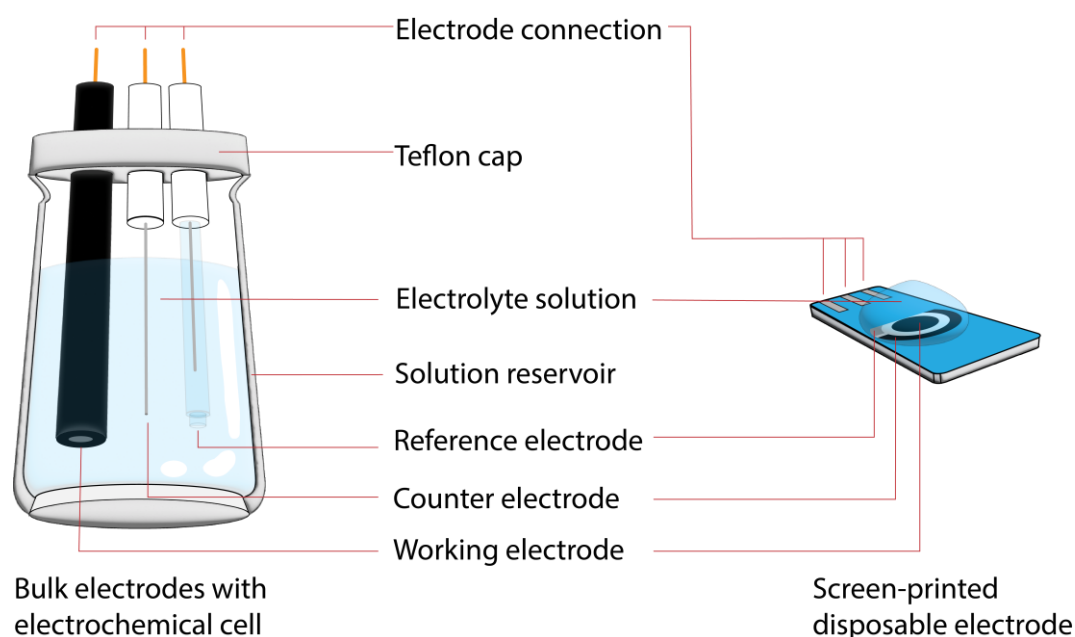


Figure 2-11 Three-electrode system with bulk electrodes and disposable screen-printed carbon electrode (SPCE).

The electrochemical measurement techniques used in this chapter include cyclic voltammetry (CV), square wave voltammetry (SWV) and differential potential voltammetry (DPV). These electrochemical measurements were performed by using a CHI 660E electrochemical workstation (CH Instruments, USA). Data visualization was built by using Python Libraries (Matplotlib and Seaborn).

2.5 Surface Functionalization

In this chapter, four different PNA immobilization methods have been trialled and compared on three different electrode materials. As shown in Figure 2-12, these methods include polystreptavidin-biotin interaction and silanization (two approaches) on SPCE, immobilization on NHS-graphene electrode and Au-thiol linkage on bulk gold electrode.

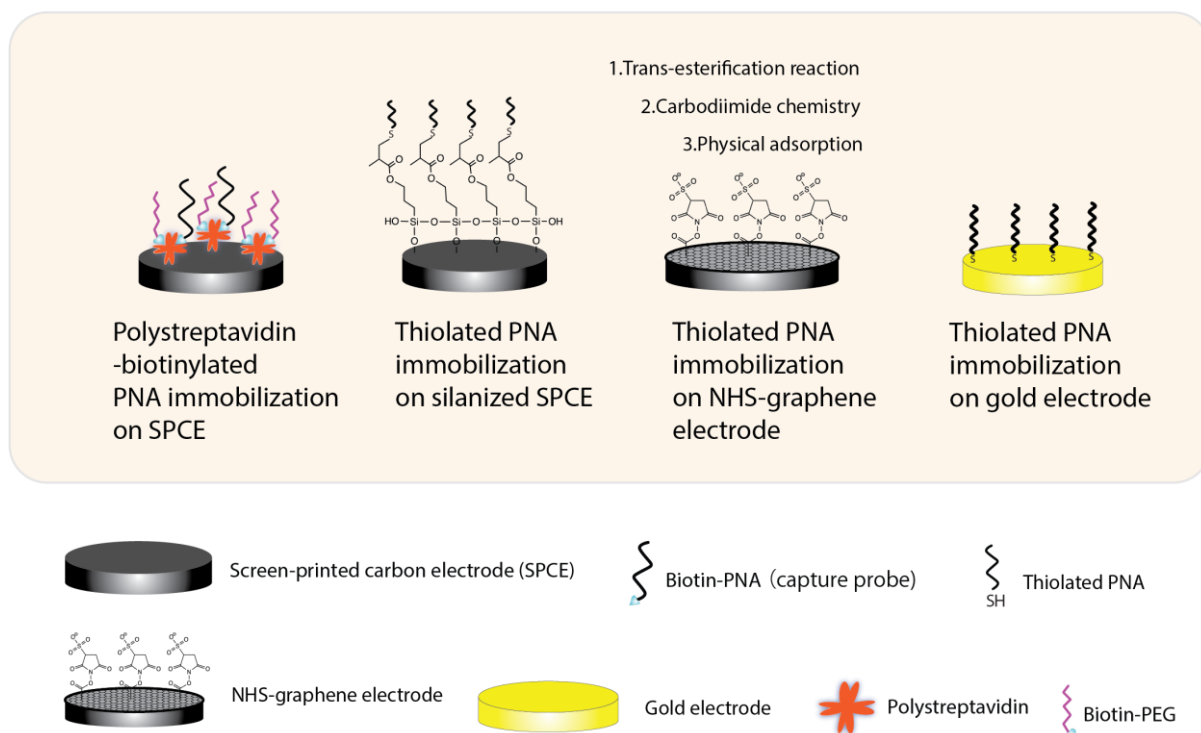


Figure 2-12 Immobilization strategies on three different electrodes.

2.5.1 Polystreptavidin-biotin Interaction on Carbon Electrodes

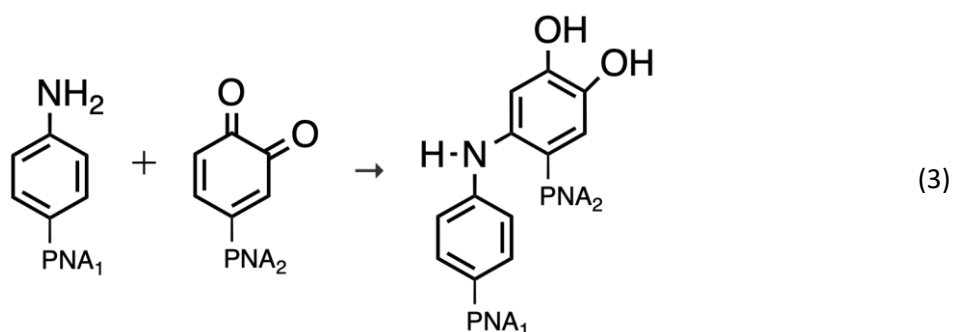
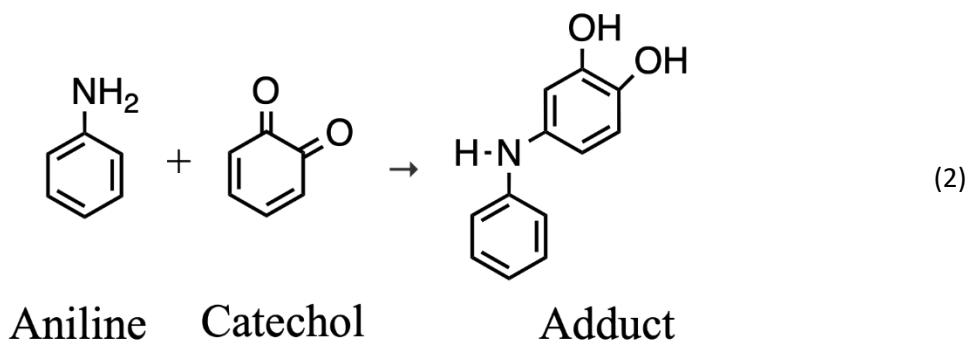
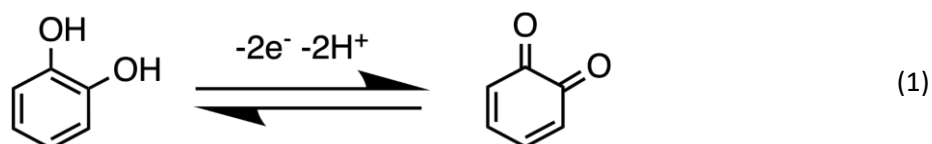
In this section, the immobilization of the PNA capture probe (Biotin-PNA-Aniline-141) on the SPCE was performed. Biotin-PNA-Aniline-141 contained a biotin and an aniline at its N- and C-terminus, respectively. The biotin was used to form a very strong, near irreversible complex with polystreptavidin, which can then be physically adsorbed onto the SPCE. The aniline was used to confirm electrochemically the successful immobilization of the PNA capture probe. This was achieved via reacting the immobilized aniline with freely diffusing 4-methyl-catechol and monitoring the formation of the covalent adduct. The effect of the addition of biotinylated PEG, used here as a back-filler, on PNA density and orientation was also investigated.

2.5.1.1 Immobilization of Biotinylated PNA Capture Probe

Polystreptavidin and Biotin-PNA-Aniline-141 complex was physically adsorbed on SPCE as shown in Figure 2-15 (a) by depositing a mixture (no pre-incubation) of polystreptavidin and biotinylated PNA. Briefly, 20 μL of solution containing 500 nM polystreptavidin and 5 μM

biotinylated PNA in 1X PBS was dropped on the electrode. After 1 h at room temperature, the applied solution was dried. A gentle wash (avoid high pressure washing and keep the washing time within 2 seconds) with 1X PBS was applied to remove no-adsorbed polystreptavidin and the excess of PNA.

In order to determine if the PNA immobilization was successful, the chemical properties of the aniline tag were exploited. Although aniline itself does not exhibit specific electrochemical peaks in voltammetry, it can react with catechol to form an adduct,^{197,280–282} which produces distinctive electrochemical peaks that can be quantified. Quinones derived from catechol participate in Michael addition reactions with aniline and form the corresponding catechol-aniline adducts according to an ECE (Electron-Transfer-Complex-Exchange) mechanism, as shown in Scheme 2-1. Except from the desired adducts, there are other possible side reaction including direct electron transfer to the quinoid form, auto-addition and dimerization. Both catechol and adduct have signature peaks on voltammetry. The chemical irreversibility²⁸² of this reaction (Scheme 2-1 (2)) facilitates the verification of the immobilization by observing the emerging signal from adduct (Figure 2-15 (c) and (d)). This reaction is not only used for the electrochemical investigation of the capture PNA probe, but also used for electrochemical oligonucleotide-templated reaction (EOTR) sensing in Chapter 4.



Scheme 2-1 The reaction mechanism for the electrochemical oxidation of catechol in the presence of aniline.

A solution of 100 μL freely diffusing 4-methyl-catechol solution (100 μM in 1X PBS) was deposited on the functionalized electrode and formation of the product(s) of the 1,4-addition catechol-aniline reaction was monitored by SWV, scanning from -0.3 V to 0.4 V. The scan terminated at 0.4 V because more positive potential can cause aniline polymerization,²⁸³ which is an irreversible reaction that was not desired in this electrochemical investigation. As shown in Figure 2-13, 1 mM aniline solution in 100 mM potassium chloride (KCl) electrolyte (pH=7.4) was tested electrochemically with cyclic voltammetry. During the initial scan, no oxidation peak was observed within the range of 0.2-0.3 V. However, upon applying a potential higher than 0.4 V, subsequent scans revealed emerging peaks within the 0.2-0.3 V

range. This emergence of peaks is attributed to the polymerization of aniline. The progressive increase in peak separation observed from the second to the fourth scan can be attributed to the accumulation of polymerized aniline on the electrode surface, leading to a reduction in electron transfer kinetics. Consequently, all experiments involving aniline will be conducted while avoiding potentials exceeding 0.4 V to prevent aniline polymerization. The potential increment step was 0.01 V, amplitude was 0.05 V and frequency was 50 Hz. The gain was set at 10^{-5} A/V range. 5 seconds quiet time was set prior to the scanning to realize a proper initial condition for all scans.

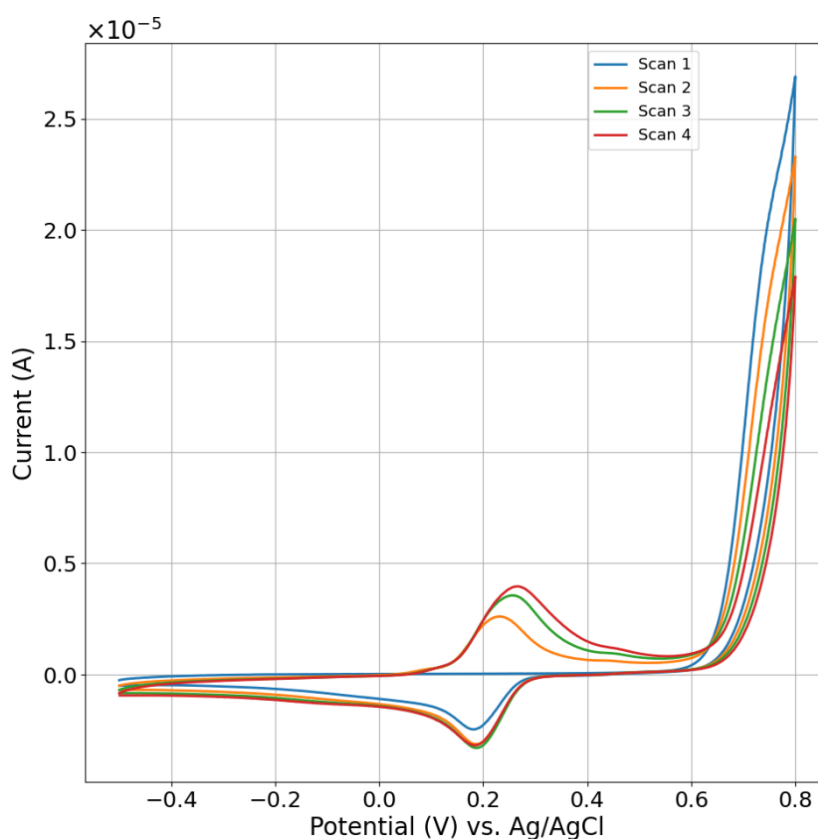


Figure 2-13 Cyclic voltammetry of 1 mM freely diffusing aniline solution at a scan rate of 100 mV/s in 100 mM KCl electrolyte using bulk gold electrode.

2.5.1.2 Surface Optimization with PEG

The effect of introducing a biotinylated PEG (Biotin-(PEG)₆-OH, structure shown in Figure 2-14) as a backfiller was then explored. As previously mentioned in section 2.3, PEG is widely used in the modification of electrodes to prevent aggregation, nonspecific adsorption, and to increase hydrophilicity. Biotin-(PEG)₆-OH was co-immobilized with Biotin-PNA-Aniline-141 to space-out the immobilized PNA, thereby reducing their surface density while also potentially increasing their availability by reducing steric hindrance (Figure 2-15 (a)). Different concentrations of Biotin-PNA-Aniline-141 and Biotin-PEG were investigated in order to find the optimum ratio. The surface stability was also tested by comparing the signal decrease over time. DPV was used for the stability test from -0.3 V to 0.4 V. Potential increment was 0.01 V. Pulse amplitude was 0.05 V. The pulse period was 0.1 s, pulse width was 0.05 s and sample width was 0.02 s. 2 s quiet time was set prior to the DPV scanning to realize a proper initial condition for all scans. The gain was set at 10^{-5} A/V range.

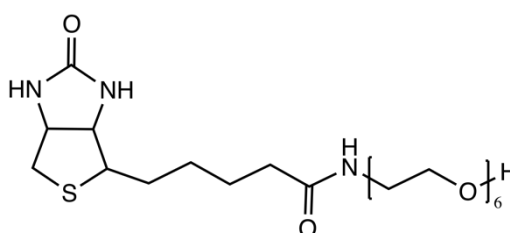


Figure 2-14 Biotin-(PEG)₆-OH structure.

2.5.1.3 Results and Discussion

2.5.1.3.1 Capture Probe Immobilization

As shown in Figure 2-15 (c) and (d), by testing the Biotin-PNA-Aniline-141 capture probe immobilized electrode with SWV addition of 100 μ M freely diffusing 4-methyl-catechol solution onto PNA-functionalised electrodes resulted in the appearance of a new adduct peak at -0.15 V position that increased when increasing the number of SWV scan cycles. In theory, the peaks of 4-methyl-catechol located at approximately 0.2V should decrease as the adduct

peak increases with subsequent scan cycles. Some possible explanations for why this was not observed are proposed below. Firstly, to avoid the aniline polymerization (as shown in Figure 2-13), the scans were terminated at 0.4 V before the peaks were complete. This made it challenging to compare peak heights using baseline correction. Secondly, even if the peaks of 4-methyl-catechol were complete for baseline correction, the bulk concentration of 100 μM 4-methyl-catechol was significantly higher compared to the Biotin-PNA-Aniline-141 capture probes immobilized on the electrode surface (potentially in the nanomolar range). The small amount of generated adducts on the electrode surface therefore only had a negligible impact on the bulk concentration of freely diffusing 4-methyl-catechol. In contrast, no adduct peak was observed on the control electrodes lacking the PNA probes (Figure 2-15 (b)), but otherwise subjected to the same treatment with polystreptavidin. In this control group, only the catechol signature peak at the 0.25 V position was observed. There's a slight shift of the catechol peak location from the experimental group (0.18 V) to the control group (0.25 V). The observed peak shift may stem from surface-induced thermodynamic, kinetic alterations, or a combination thereof. It is also plausible that the modification led to a change in the local electrode-electrolyte pH, contributing to this shift.

The emerging adduct peaks on the experimental group indicated the successful immobilization of the polystreptavidin/Biotin-PNA-Aniline-141 complex on SPCE through physical adsorption on the electrode surface. The advantage of this strategy includes the easy preparation of the surface at room temperature without the need for complicated or delicate chemical reactions and the excellent binding efficiency between polystreptavidin and biotin, characterized by high specificity. The potential drawback of this strategy is the fact that physical adsorption is typically less robust/stable than covalent (and irreversible) immobilization strategies. While it simplifies the surface preparation process, care should be taken to avoid multiple washes. Nevertheless, only the necessary washing may lead to the partial removal of the adsorbed polystreptavidin, as shown in Figure 4-19, which will be discussed in detail in section 4.2.4.

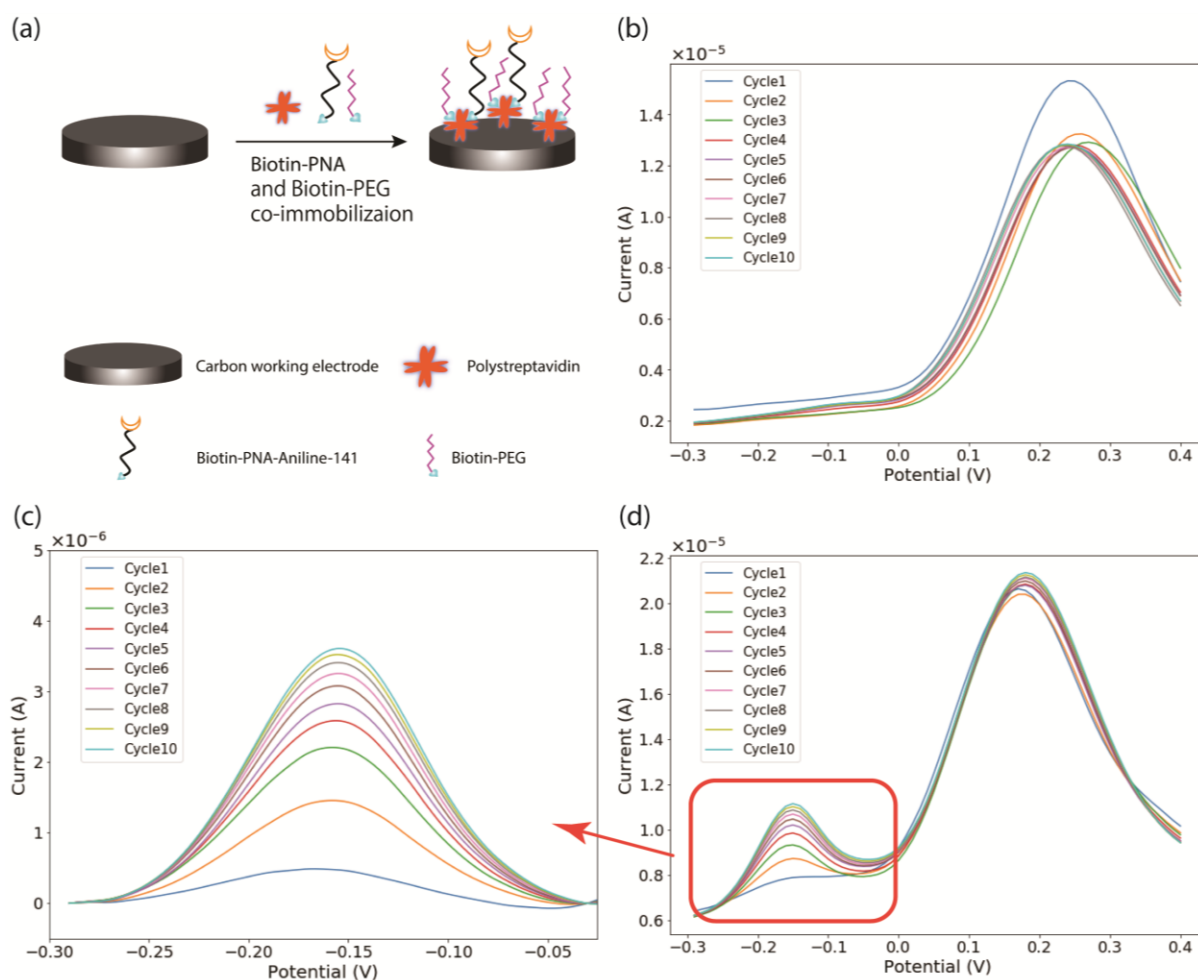


Figure 2-15 (a) Schematic diagram of biotinylated-PNA immobilization on SPCE. Capture PNA probe Biotin-PNA-Aniline-141 immobilization characterization: (b) Control group with bare SPCE tested in 100 μ M 4-methyl-catechol solution by SWV. (c) and (d) Experimental group with Biotin-PNA-Aniline-141 immobilized on polystreptavidin treated SPCE, tested in 100 μ M 4-methyl-catechol solution by SWV, (c) is baseline corrected and (d) is original SWV data.

2.5.1.3.2 Co-immobilization Efficiency

Once successful immobilization of biotinylated PNA on SPCE, the addition of Biotin-(PEG)₆-OH was explored. Whilst maintaining a fixed concentration of 500 nM polystreptavidin, Biotin-PNA-Aniline-141 concentration was varied (250 nM, 2.5 μ M, 5 μ M, 10 μ M, 25 μ M) with and without increasing concentrations of Biotin-PEG (250 nM, 2.5 μ M, 5 μ M, 10 μ M, 50 μ M, 250

μM and $500\ \mu\text{M}$). Different ratios between Biotin-PNA-Aniline-141 and Biotin-PEG have been tested. As shown in Figure 2-16 (a) and (b), the adduct peak (near $-0.1\ \text{V}$) increased when the concentration of the capture PNA probe was increased from $250\ \text{nM}$ to $10\ \mu\text{M}$. However, the adduct peak decreased when the PNA concentration was further increased to $25\ \mu\text{M}$. This indicates that the optimum PNA concentration for immobilization for this system is between 5 to $10\ \mu\text{M}$. The adduct peak height with different PNA:PEG ratios is shown in Figure 2-16 (c). To minimize the steric hindrance effects, an optimum concentration of $5\ \mu\text{M}$ Biotin-PNA-Aniline-141 was chosen for further use in the subsequent research. The corresponding SWV data is shown in Figure 2-16 (a). For the $5\ \mu\text{M}$ Biotin PNA capture probe, multiple ratios of PNA:PEG have been tested to find the optimum ratio. As shown in Figure 2-16 (b) and Figure 2-16 (c, red bars), the optimum group that observed with the strongest signal is the 1:50 (Biotin-PNA-Aniline-141 : Biotin-PEG) by using $5\ \mu\text{M}$ Biotin-PNA co-immobilized with $250\ \mu\text{M}$ Biotin-PEG.

Overall, lower concentration Biotin-PNA ($250\ \text{nM}$ and $2.5\ \mu\text{M}$) has similar adduct generating proficiency as the higher concentration ($25\ \mu\text{M}$). This is because when the Biotin-PNA concentration is very low, there are sparse capture probe available to the aniline-catechol $1'4$ addition reaction. When the Biotin-PNA concentration is very high, the surface is potentially blocked by capture probes and the probes are stacking upon each other. This condition can impede the efficiency of the $1'4$ addition reaction between aniline and catechol. The other potential explanation is that the immobilized polystreptavidin are already saturate with $250\ \text{nM}$ of Biotin-PNA-Aniline-141.

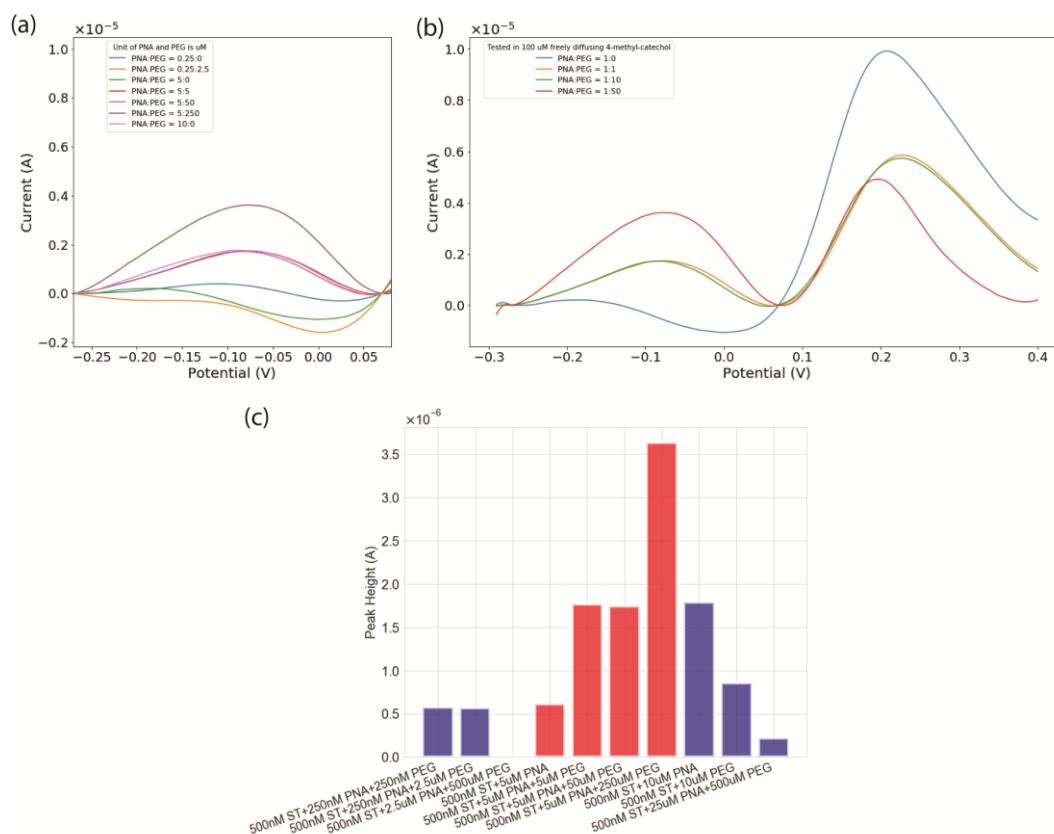


Figure 2-16 Co-immobilization efficiency test by SWV in 100 μ M 4-methyl-catechol solution.

(a) SWV test with different ratios of Biotin-PNA-Aniline-141 : Biotin-PEG co-immobilized carbon surfaces (baseline corrected). (b) SWV test of 5 μ M Biotin-PNA-Aniline-141 with different Biotin-PEG concentrations co-immobilized carbon surfaces (part data from (a), baseline corrected). (c) Co-immobilization efficiency test by checking adduct peak height from SWV with different ratios of Biotin-PNA-Aniline-141 : Biotin-PEG co-immobilized carbon surfaces in 100 μ M 4-methyl-catechol solution (bar chart of (a), some concentrations were not shown in (a) because the peak location was not in the -0.25 to 0.05 V region). All concentrations treatment was conducted with 500 nM polystreptavidin.

2.5.1.3.3 Co-immobilization Stability

Further characterization about the stability of co-immobilized capture probes and PEG spacer were investigated by checking the adduct peak at 0-day and 4-day time points after surface

functionalization. The functionalized electrodes were stored in a dry condition in the refrigerator for up to 4 days. Again, this test was carried out in 100 μM 4-methyl-catechol solution (in 1X PBS). As mentioned in last section (2.5.1.3.2), 500nM polystreptavidin and 5 μM Biotin-PNA-Aniline-141 was chosen as the optimum concentration. Therefore, in the stability test, only the concentration of Biotin-PEG changed to investigate the functionalized electrode surface. As shown in Figure 2-17 (a), 1 : 10 ratio of Biotin-PNA-Aniline-141 : Biotin-PEG with 5 μM Biotin-PNA-Aniline-141 and 50 μM Biotin-PEG was the ratio with the best stability by investigating the adduct decrease over time. The 1 : 50 ratio of Biotin-PNA-Aniline-141 : Biotin-PEG with 5 μM Biotin-PNA-Aniline-141 and 250 μM Biotin-PEG was not able to produce a detectable peak via DPV in the stability test. Despite the 1 : 50 (PNA : PEG) ratio representing the strongest signal when applying the same concentration of 5 μM Biotin-PNA-Aniline-141, it was not able to maintain a stable performance over time when compared with 1 : 10 (PNA : PEG) ratio. 1 : 0, 1 : 1, 1 : 5 and 1 : 10 DPV peak height remain in similar range, but the peak shifted from ~ -0.1 V to ~ -0.06 V. This peak shift could result from surface-induced thermodynamic, kinetic changes, or a combination thereof. Alternatively, it could be attributed to a shift in local electrode-electrolyte pH resulting from the modification.

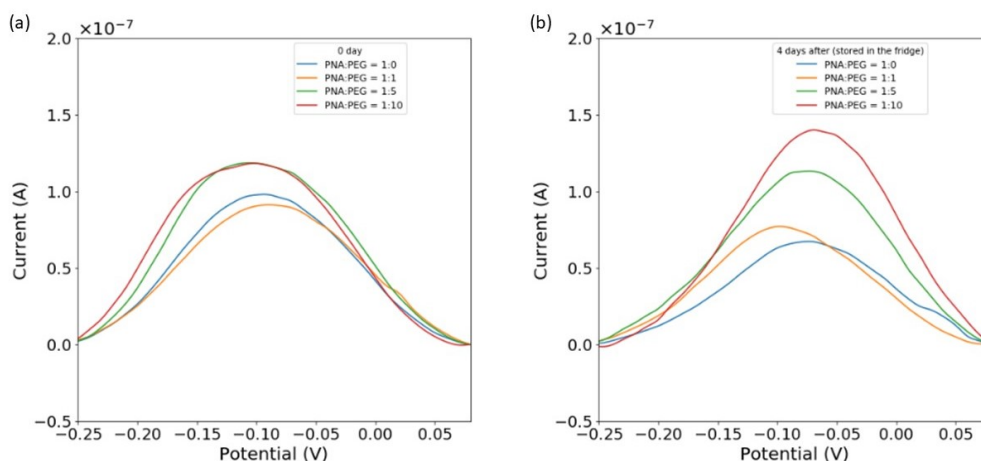


Figure 2-17 Co-immobilization stability test by DPV (baseline corrected). DPV test in 100 μM 4-methyl-catechol solution with different ratios Biotin-PNA-Aniline-141 : Biotin-PEG co-immobilized carbon surfaces (a) immediately after the surface functionalization and (b) after 4 days storage in the fridge.

Both SWV and DPV are considered as sensitive voltammetry techniques by separating the faradaic current (related to the analyte of interest) from the charging current (related to the double-layer capacitance), as mentioned in section 1.4.3. With the immobilized species, the induced current decays exponentially over time, resembling the decay of charging current. However, the time constant of decaying difference distinguishes the charging current from the immobilized species induced current. Hence, both SWV and DPV remain valuable techniques for sensitive detection with immobilized species. The decision to use SWV for the capture probe immobilization and PNA : PEG co-immobilization efficiency test was based on its sensitivity and rapid response. On the other hand, DPV was chosen for the PNA : PEG co-immobilization stability test due to its superior ability to differentiate the signal from the noise, which becomes crucial when the signal decreases.

2.5.1.4 Conclusion

The immobilization of the biotinylated capture PNA probe on the SPCE through the polystreptavidin-biotin interaction demonstrated ease in the preparation and delivered satisfactory performance. The functionalized surface can be optimized by co-immobilizing biotinylated PNA capture probe (Biotin-PNA-Aniline-141) with biotinylated PEG (Biotin-(PEG)₆-OH). The optimal concentration for achieving both a strong signal and minimizing steric hindrance effects was found to be 500 nM polystreptavidin and 5 μ M Biotin-PNA-Aniline-141. To further increase the signal to noise ratio, different ratios of PNA : PEG were investigated and a stability test carried out by monitoring the signal decrease over time. 1 : 50 (PNA : PEG) ratio resulted in the strongest signal when applying the same concentration of 5 μ M Biotin-PNA-Aniline-141. However, 1 : 10 ratio of Biotin-PNA-Aniline-141 : Biotin-PEG with 5 μ M Biotin-PNA-Aniline-141 and 50 μ M Biotin-PEG was the ratio with the best stability by investigating the adduct decrease over time. Overall, the immobilization with 5 μ M Biotin-PNA-Aniline-141 and 50 μ M Biotin-PEG (1 : 10 ratio of Biotin-PNA-Aniline-141 : Biotin-PEG with) with 500 nM polystreptavidin was considered as the optimum ratio for this immobilization strategy which demonstrated the highest efficiency and stability.

2.5.2 Silanization on Carbon Electrodes

Silanization is a commonly used and versatile method for surface modification and functionalization as it is compatible with any electrode surface that can generate hydroxyl groups.^{150,202,284} Silanization refers to the process of modifying the electrode surface by attaching or coating it with a layer of silane molecules. Silanes are chemical compounds containing a silicon atom bonded to organic groups. While silanization is known to be highly effective for treating silica and glass surfaces,²⁰³ it can also be successfully applied to carbon surfaces.^{285,286} Silanization on a solid substrate involves the formation of an alkoxy silane polymer through the -Si-O-Si- bond formation process.¹⁸⁰ In this section, electrode silanization was used to introduce a methacrylate (shown in Figure 2-18 (a)) functional group onto the electrode surface for subsequent covalent immobilization of a thiolated PNA (Thiol-PNA-Aniline-141) via click chemistry (Figure 2-18 (b) and Figure 2-19).

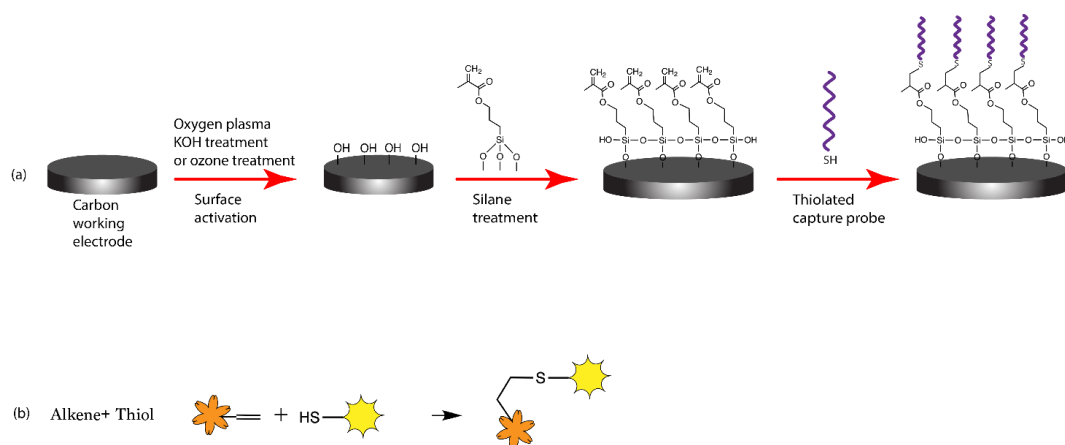


Figure 2-18 (a) Silanization with 3- (trimethoxysilyl)propyl methacrylate on carbon electrode. (b) Click chemistry diagram between alkene and thiol.

Two protocols of silanization were investigated in this chapter: in aqueous vapor phase and in organic solvent phase with isopropanol. Both protocols involve a two-step process as illustrated in Figure 2-18: surface activation and silane treatment. The purpose of the surface activation step is to generate hydroxyl groups on otherwise mostly chemically inert surfaces. The surface functionalization involves chemical activation using a mild oxidation agent (1%

w/v KOH) followed by a 3-minute oxygen plasma treatment, enabling the utilization of the combined effects of both wet and plasma treatment. The mild oxidation agent KOH was used to avoid acid-based activation whereas harsh chemical activation can generate a non-homogeneous functional layer²⁸⁷ on the surface with associated non-homogeneous PNA distribution.

The detailed protocol for the surface activation is:

1. Working electrode area of the SPCEs was washed with a cleaning solution composed of 30% H₂O₂, 25% Ammonia and DI water (10 µL in total) with the ratio of 1 : 1 : 5 for 1 minute followed by extensive wash with DI water.
2. The SPCEs were then incubated with 100 µL of 1% (wt/vol) KOH in DIW for 10 min and washed extensively with DIW.
3. The electrodes need to be dried under high pressure nitrogen blow for next step oxygen plasma treatment.
4. The KOH-activated surface was treated with O₂ plasma in the plasma cleaner (ATTO PLASMA SYSTEM (Diener electronic GmbH & Co. KG, Germany)) set at a plasma strength of 100 W for 3 minutes. The maximum input power of the instrument is 200 W. Vacuum pump with a pumping down pressure of 0.04 mbar (1 bar = 750.062 Torr) was set. Oxygen supply period was 5 minutes with oxygen pressure of 0.4 mbar.

Silane treatment protocol varies with aqueous vapor phase and organic solvent phase.

2.5.2.1 Silanization in Aqueous Vapor Phase

After the surface activation, the SPCEs were immediately placed in a vacuum desiccator for silane treatment.

5. 100 µL of 2% 3-(trimethoxysilyl)propyl methacrylate in DI water was mixed well before applying on the plasma-treated electrodes. Place the 3-(trimethoxysilyl)propyl methacrylate-added electrodes in a desiccator and create vacuum with a chemical resistant vacuum pump. Incubate the vacuum desiccator at 80 °C for 1 h.
6. Place the desiccator under fume hood let it cool down. Then the electrodes should be ready for capture PNA probe immobilization.

While silanization in aqueous vapor phase generates the desired silane layer on top of the electrode, this technique often results in polymerization, irreproducibility and instability of the silane films.^{288,289} Therefore, another protocol in anhydrous conditions was carried out.

2.5.2.2 Silanization in Organic Solvent Phase with Isopropanol

The first 4 steps of the protocol are the same with aqueous vapor phase. 3-(trimethoxysilyl)propyl methacrylate was then mixed with isopropanol (IPA) with a volume ratio of 5% : 95% in a clean container. The electrodes were immersed in the 3-(trimethoxysilyl)propyl methacrylate and IPA mixture for 6 hours. The container should be sealed to avoid contact with oxygen and water in the air. The silanized electrodes were then washed thoroughly with IPA and dried at ambient temperature under the fume hood.

2.5.2.3 Immobilization via Click Chemistry on Silanized Electrode

The PNA used for the immobilization on silanized carbon electrode is Thiol-PNA-Aniline-141 (Table 2-3 and Figure 2-9). The methacrylate group introduced by silanization on the SPCEs using 3-(trimethoxysilyl)propyl methacrylate can react with the thiol group from the capture PNA probe via click chemistry, as shown in the scheme (3) in Figure 2-19. Click chemistry is characterized by a strong thermodynamic driving force that propels the reaction rapidly and irreversibly towards a single reaction product with high yield, and remarkable reaction specificity.¹⁵⁰ The most commonly representative click chemistries examples are shown in Figure 2-19.

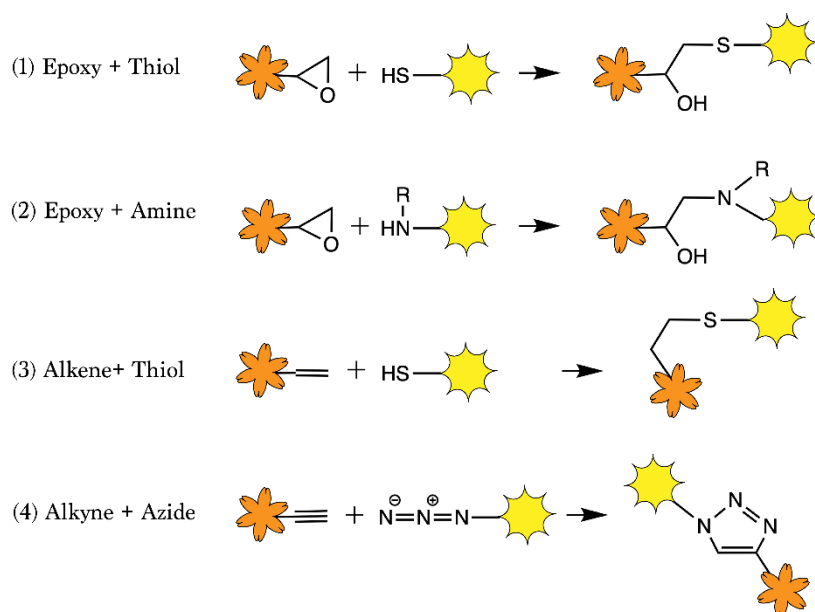


Figure 2-19 Representative schemes for click chemistry.

On silanized SPCE, by using click chemistry between alkene (from methacrylate group) and thiol (from PNA capture probe Thiol-PNA-Aniline-141), 100 μL of 10 μM Thiol-PNA-Aniline-141 (in 1X PBS) was deposited on the working electrode for immobilization. The immobilization process lasted for 1 h at room temperature, in a clean sealed dark box to slow down the evaporation and avoid the sample from drying.

2.5.2.4 Results and Discussion

2.5.2.4.1 Aqueous Phase Immobilization

Similar to polystreptavidin-biotin immobilization, the immobilization efficiency on the silanized electrodes was investigated by checking the appearance of the adduct peak between the immobilized aniline PNA and freely diffusing catechol (previously described in Scheme 2-1). 100 μM 4-methylcatechol solution (in 1X PBS) was used as electrolyte. A PEG spacer (Thiol-(PEG)₆-OH) was co-immobilized with Thiol-PNA-Aniline-141 capture probe for the purpose of probe orientation and density control, as shown in Figure 2-20.

When testing in 100 μM 4-methylcatechol in PBS, adduct SWV peaks were plotted in bars for comparison among different PNA with PEG concentrations. With freshly silanized carbon electrodes, the PNA to PEG ratio did not affect the immobilization efficiency too much (yellow

bars in Figure 2-20). For the long-term stored silanized carbon electrodes, 5uM Thiol-PNA-Aniline-141 with 25 μM Thiol-(PEG)₆-OH was the best ratio as it gave the strongest adduct peak (blue bars in Figure 2-20). However, due to limited repeats, the above conclusion is not absolute. The reason why we didn't proceed with more groups with the freshly prepared silanized electrode (orange bars in Figure 2-20) was the limitation of this method using aqueous phase: the blocked surface, as illustrated in Figure 2-21.

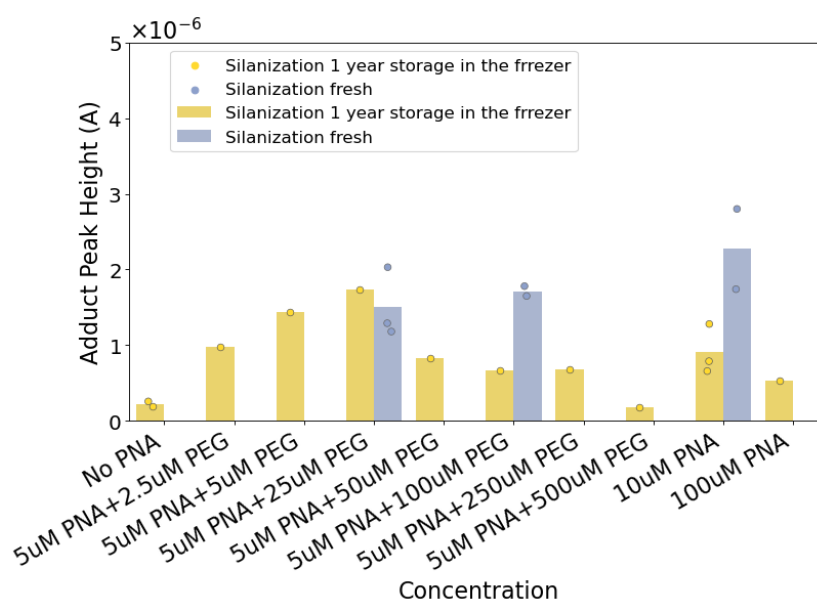


Figure 2-20 Co-immobilization efficiency and stability SWV test of PNA immobilization on silanized carbon electrode via aqueous phase (PNA is Thiol-PNA-Aniline-141, PEG is Thiol-(PEG)₆-OH).

CV tests was conducted in 250 μM Hexaammineruthenium(III) chloride ($[\text{Ru}(\text{NH}_3)_6]^{3+}$ (RuHex), dissolved in 10 mM Tris buffer and purged with Nitrogen for 20 minutes prior to electrochemical test. An external reference electrode (Ag/AgCl) was used with the SPCE for the consideration of better-quality data. However, as we have discussed in section 2.4.3, if the same ionic strength of the background electrolyte solution is used for the experiments where comparisons are carried out, the potential drift or variability from the original built-in pseudo-reference electrode on the screen-printed electrode can be avoided to the most

extent. Despite the 250 μM RuHex in Tris buffer have been purged with nitrogen, the micro liter range (100 μL) solution on electrode with large surface area for testing can be saturated with oxygen quickly, which can be seen from the tendency of oxygen reduction at the -0.4 V in the CV graph. Figure 2-21 demonstrated a significant electrode kinetic change of the working electrode before and after the silane treatment. This is potentially because the electrode surface is blocked by the silane polymerization after silanization in aqueous phase. The polymerized silane layer, which is insulating, slowed down the kinetics of the electron transfer at the electrode/electrolyte interface.

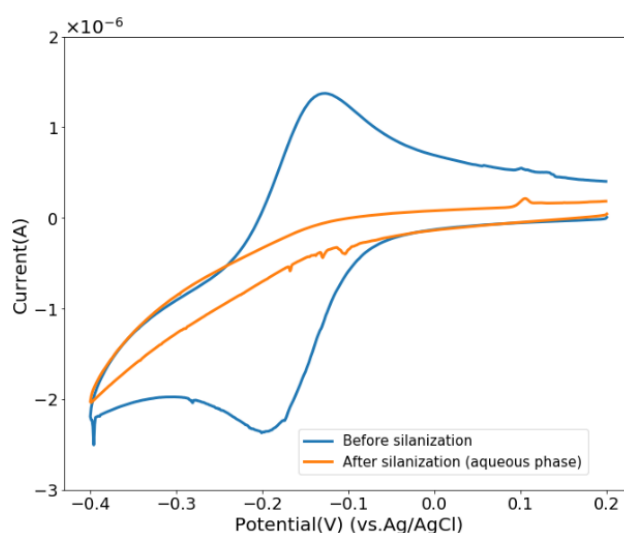


Figure 2-21 Comparison before and after the silanization of SPCE in 250 μM RuHex (cyclic voltammetry scan rate 100 mV/s).

2.5.2.4.2 Organic Phase Immobilization

To reduce the polymerization effect from the silane vapor deposition protocol, another protocol using organic solvent phase silanization was carried out. Again, 5 μM Thiol-PNA-Aniline-141 + 25 μM Thiol-(PEG)₆-OH was the best ratio as it gave the strongest adduct peak, as shown in Figure 2-22. However, all the PNA and PEG ratio gave adduct peaks with very similar range. Therefore, PEG is not making a significant difference in improving the immobilization performance on organic solvent phase silanized carbon electrode. All the error bars in the bar plots in this thesis represent the standard deviation (SD).

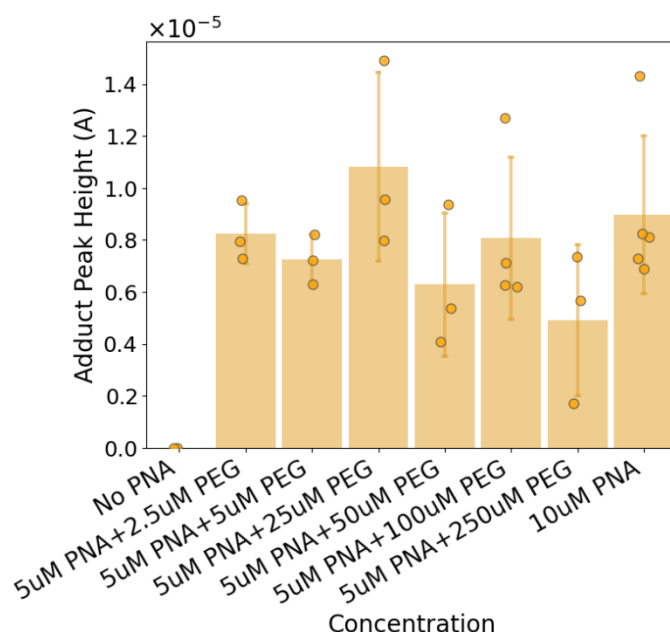


Figure 2-22 Co-immobilization efficiency SWV test of PNA immobilization on silanized carbon electrode via organic phase (PNA is Thiol-PNA-Aniline-141, PEG is Thiol-(PEG)₆-OH).

Thiol-PNA-Aniline-141 immobilization efficiency was tested on both aqueous and organic phase silanized SPCEs. As shown in Figure 2-23 (a), adduct peak is significantly stronger from the organic solvent phase silanization group compared with the aqueous phase silanization group, when tested in 100 mM 4-methylcatechol solution (in 1X PBS). Representative SWV (two repeats on two different SPCEs) with 10 μ M Thiol-PNA-Aniline-141 immobilization on organic phase and aqueous phase silanized carbon electrodes are displayed in Figure 2-23 (b) and (c), respectively.

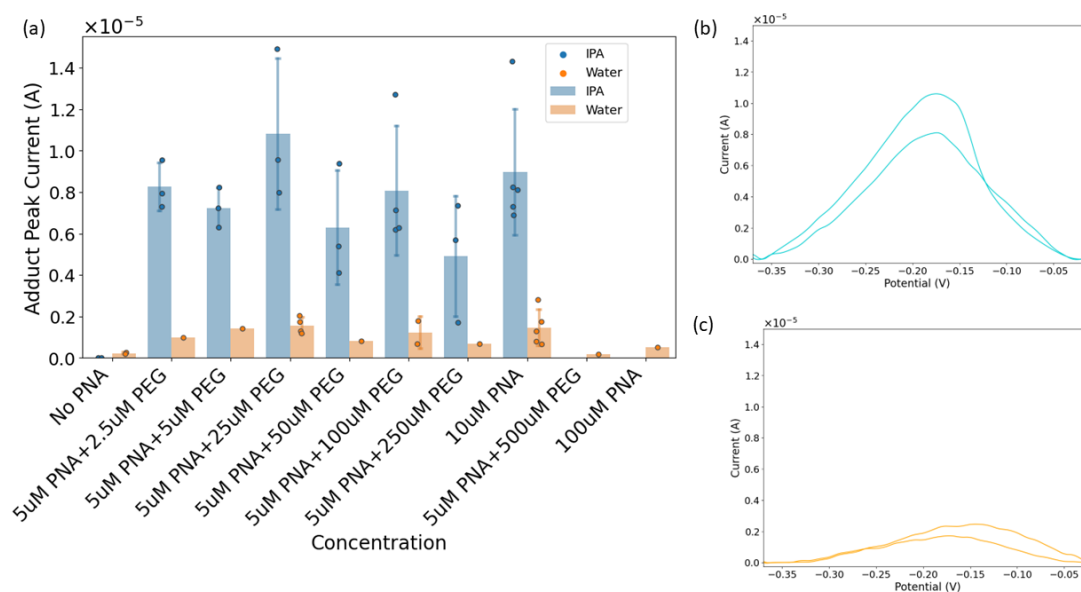


Figure 2-23 (a) Adduct peak comparison from SWV by silanization in aqueous and organic phases. Original SWV data of the bars in red rectangular is shown in (b) and (c). Two repeat SWV investigation of 10 μ M Thiol-PNA-Aniline-141 immobilization on two difference electrodes silanized in organic phase (b) and aqueous phase (c) (baseline corrected).

Except from 3-(trimethoxysilyl)propyl methacrylate, (3-Aminopropyl)triethoxysilane (APTES) have been used in aqueous vapor silanization, which was followed by carbodiimide chemistry using EDC/NHS to form an amide bond between the amine group from APTES and the carboxylic acid group on the ferrocenecarboxylic acid (Figure 2-24). The ferrocenecarboxylic acid was used as a preliminary electrochemical reporter to test the immobilization efficiency. This selection was based on its structurally defined attributes, featuring a carboxylic acid functional group suitable for the carbodiimide reaction and the inclusion of a ferrocene moiety, which serves as a reporter molecule. The structure of ferrocenecarboxylic acid is shown in Figure 2-25. Although the immobilization of two-step silanization and carbodiimide chemistry worked, as can be spotted from the ferrocene signature peak, further exploration of this method was limited by the low reproducibility, which was indicated by the difference between the two experimental group repeats (blue and orange curve in Figure 2-25).

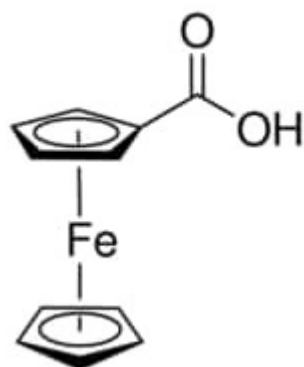


Figure 2-24 Molecular structure of ferrocenecarboxylic acid.

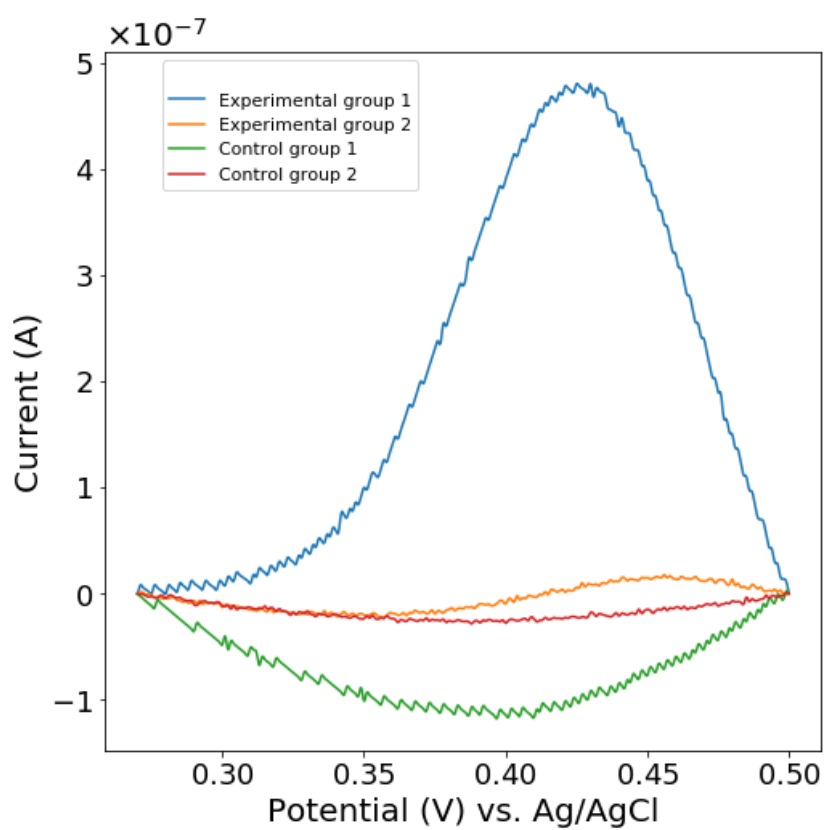


Figure 2-25 DPV test with ferrocene -COOH immobilized bulk glassy carbon electrode (baseline corrected).

2.5.2.5 Conclusion

The PNA immobilization works effectively on both aqueous and organic phase silanized SPCEs via click chemistry. Despite the fact that it is hard to conclude an optimum PNA to PEG ratio for Thiol-PNA-Aniline-141 immobilization, the overall immobilization efficiency using organic phase silanization is much higher than using aqueous phase. This is most likely because the presence of water causes the polymerization of silane on the electrode surface, which reduces the electron transfer rate due to the increased thickness of the less conductive silane layer. On the other hand, with organic phase silanization, a monolayer of 3-(trimethoxysilyl)propyl methacrylate was silanized on the electrode surface and the electron transfer rate can be more efficient than the former.

2.5.3 Immobilization on Graphene Electrodes

Different from the carbon ink used for SPCE, graphene is a two-dimensional (2D) material that characterized by its unique hexagonal structural, exceptionally strong while flexible mechanical properties and remarkable electron mobility.²⁰⁹ The high surface-to-volume ratio of nano-objects makes graphene and other 2D material's electrical properties increasingly susceptible to external influences.²⁰⁷ The atomic thickness of graphene enables high sensitivity which is due to the strong response to surface adsorption events compared with its bulk material. Like most 2D materials, graphene can be physically deposited or electrodeposited onto a broad range of substrate electrode.^{210,211} In this research, we used commercially sourced NHS-functionalized graphene electrodes for PNA immobilization.

2.5.3.1 Potential Chemistries and Approaches

The same thiolated PNA (Thiol-PNA-Aniline-141) was immobilized onto NHS-functionalized graphene electrodes. However, this time, the PNA could potentially react with the electrode surface in at least three different ways, as explained below:

- Trans-esterification reaction between the NHS ester and thiol on the Thiol-PNA-Aniline-141 (Scheme 1 in Figure 2-26), so that the capture probe can be covalently immobilized on the graphene electrode.

- Carbodiimide chemistry between NHS ester and the aniline group on the Thiol-PNA-Aniline-141 capture probe (Scheme 2 in Figure 2-26), so that the capture probe can be covalently immobilized on the graphene electrode.
- Physical adsorption of the Thiol-PNA-Aniline-141 capture probe on the graphene electrode (Scheme 3 in Figure 2-26), so that the capture probe is physically immobilized on the graphene electrode.

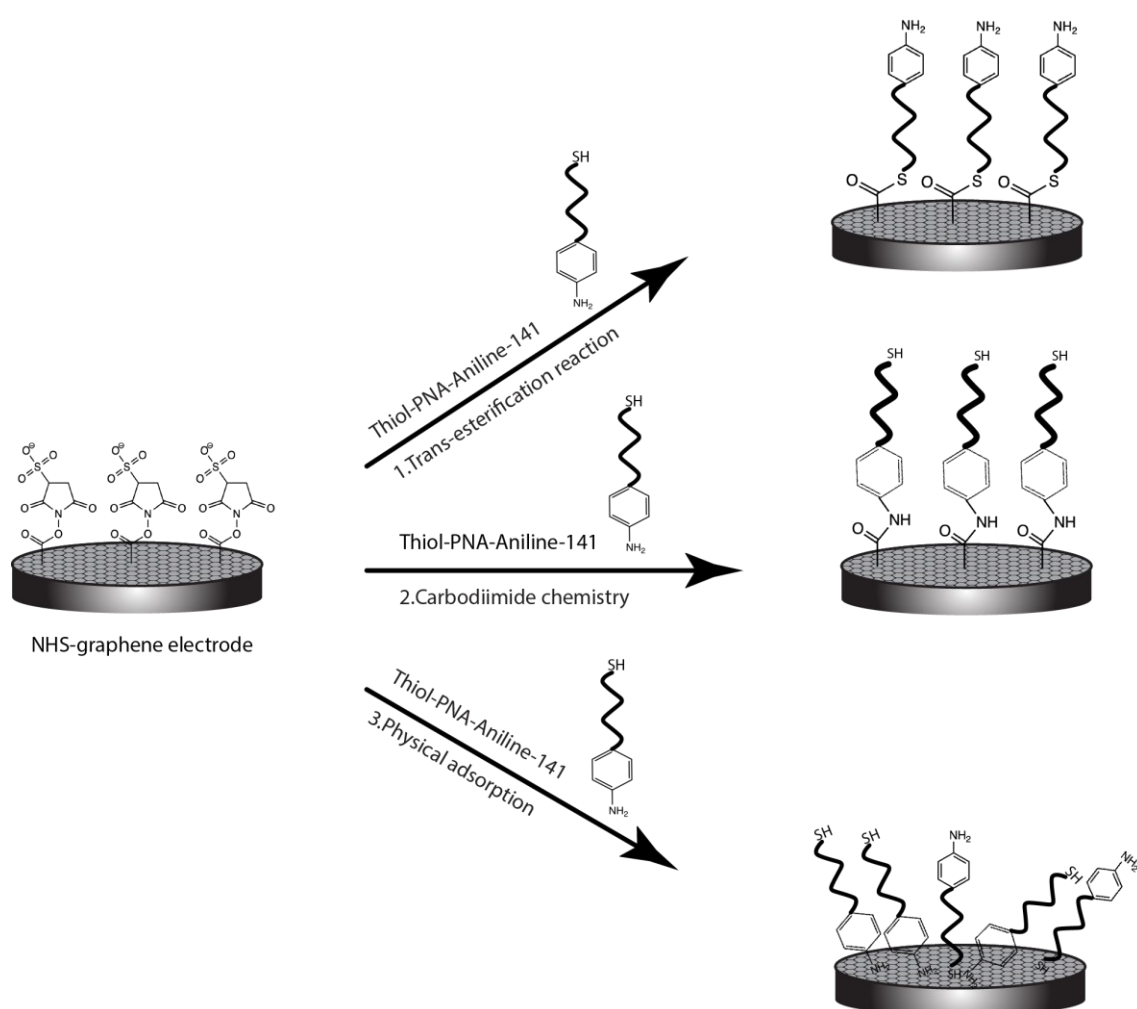


Figure 2-26 Schematic reactions for amine-terminated PNA probe on NHS-treated graphene electrode: trans-esterification reaction, EDC/NHS coupling (carbodiimide chemistry) and physical adsorption.

100 μL of 5 μM or 10 μM Thiol-PNA-Aniline-141 solution in 1X PBS was applied on the NHS-graphene electrode for immobilization overnight in a sealed petri dish in the fridge to prevent evaporation. Then the electrodes were washed gently and carefully to remove any un-immobilized species before electrochemical investigation.

2.5.3.2 Results and Discussion

Similar to silanization immobilization efficiency investigation, 100 μM 4-methyl-catechol solution in 1X PBS was used to confirm the presence of aniline from the Thiol-PNA-Aniline-141 via monitoring adduct formation. As shown in Figure 2-27, 5 μM and 10 μM of Thiol-PNA-Aniline-141 immobilization on NHS-graphene electrode was investigated respectively. In Figure 2-27 (a), three repeat SWV curve from cycle 6 of the same experimental group were plotted. The corresponding bar chart was plotted in Figure 2-27 (b).

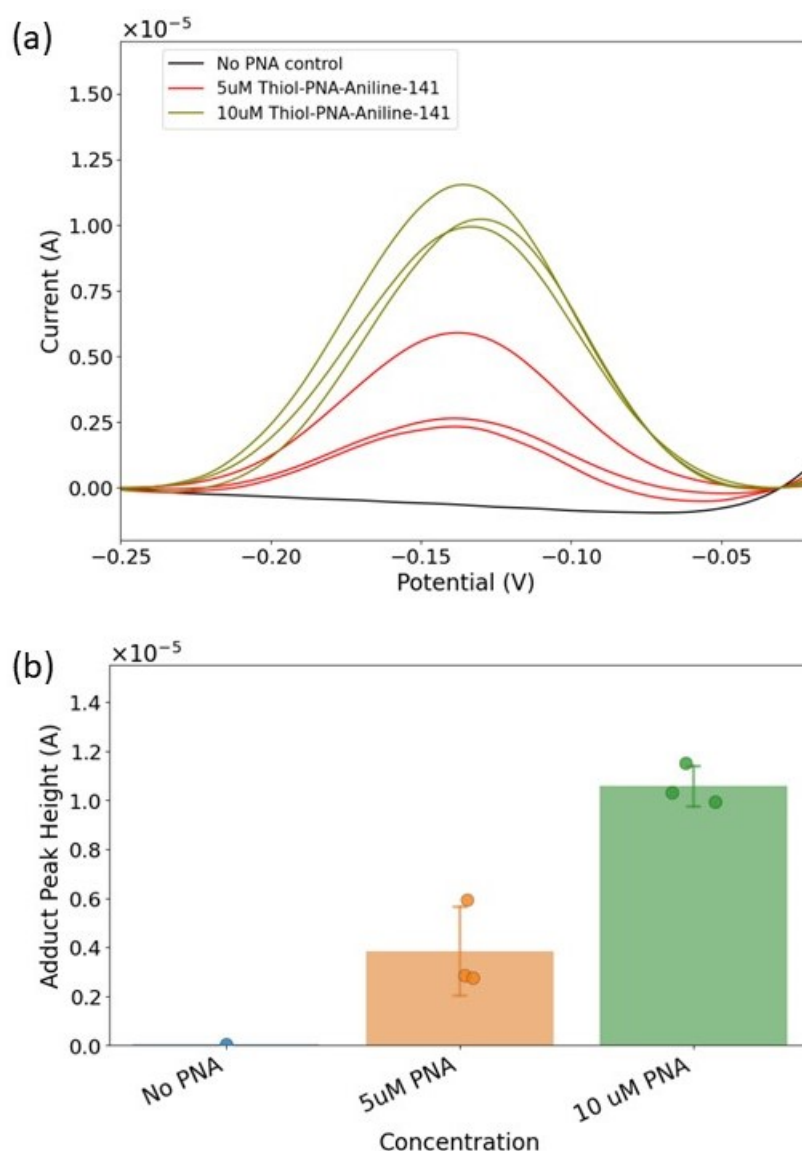


Figure 2-27 Capture probe immobilization characterization by adduct peak height from SWV. (a) SWV of adduct on the graphene electrode surface using 5 and 10 μ M Thiol-PNA-Aniline-141 with three repeats respectively (control have one repeat, all SWV in (a) are baseline corrected). (b) Adduct peak comparison between 5 and 10 μ M Thiol-PNA-Aniline-141 immobilization (error bars in all graphs represent standard deviation (n = 3), PNA is Thiol-PNA-Aniline-141).

The data in Figure 2-27 indicates a successful immobilization of Thiol-PNA-Aniline-141 capture probe on NHS-functionalised graphene electrode. As illustrated in Figure 2-26, when a thiol group (-SH) reacts with an NHS ester, a thioester bond is formed between the thiol group and

the NHS ester. This trans-esterification reaction (or thiolation) is commonly used for conjugation of biomolecules in bioconjugation chemistry. The reaction conditions are typically mild and involve incubation of the thiol-containing biomolecule with an excess of the NHS ester in a buffered aqueous solution at neutral pH and room temperature. The reaction is usually complete within a few hours.

However, considering the strong absorbent nature of graphene, there's a possibility that Thiol-PNA-Aniline-141 could be physically adsorbed on the graphene electrode. The occurrence of carbodiimide chemistry between the NHS ester and the amine group on the aniline terminal of Thiol-PNA-Aniline-141 (Scheme 2 in Figure 2-26) is less likely to happen because the amine group at that position is less reactive.

2.5.3.3 Conclusion

Thiolated PNA (Thiol-PNA-Aniline-141) have been successfully immobilized on NHS-functionalised graphene electrode, confirmed by data from Figure 2-27. In theory, there are three potential approaches for the immobilization. The observation of adduct peaks in Figure 2-27 suggests that some of the aniline is still free to react, which would rule out aniline reacting with NHS ester to form amide bond. Adsorption would also leave the aniline free to react. Current data cannot confirm whether they occur simultaneously or with different ratios.

From the chemistry perspective, the reaction between the NHS ester and the amine group on the aniline terminal of Thiol-PNA-Aniline-141 via carbodiimide chemistry is less likely to happen because the amine group at that position is less reactive. Between the remaining two approaches, the covalent linkage via trans-esterification between the NHS ester and thiol on the Thiol-PNA-Aniline-141 capture probe should be more robust compared to physical adsorption of the capture probe on graphene electrode. However, due to the difficulty in determining the leading process in this immobilization method, it was not pursued further.

2.5.4 Immobilization on Gold Electrodes

As discussed in section 2.2.1, gold is a commonly used and most extensively studied metal electrode material by far.²⁹⁰ In this section, thiolated capture probe Thiol-PNA-Catechol-141 was immobilized on bulk gold electrodes via thiol-gold interaction in a standard electrochemical cell as illustrated in Figure 2-11. The benefit of using catechol-labelled PNA

was the direct monitoring of the immobilization by using the catechol's electrochemical property. The immobilization efficiency was tested by investigating the oxidation and reduction peaks of catechol group from the capture probe PNA.

2.5.4.1 Methods

The detailed parameters about bulk gold electrode, Ag/AgCl reference electrode, Pt counter electrode and bulk electrochemical cell have been introduced in section 2.4.3. The PNA probe was immobilized on a rod gold electrode via gold thiol interaction. 20 μ M Thiol-PNA-Catechol-141 prepared in 1X PBS was applied on the bulk gold electrode for 1 h, followed by washing with 1X PBS to remove any unbound PNA probe. Another approach using 1 μ M Thiol-PNA-Aniline-141 prepared in 1X PBS for immobilization on the bulk gold electrode for 1 h was used for immobilization performance improvement and comparison, which was also followed by washing with 1X PBS to remove any unbound PNA capture probe. CV, SWV and EIS was used to investigate the immobilization efficiency of capture probe on gold electrode.

2.5.4.2 Results and Discussion

As shown in Figure 2-28 (a), oxidation and reduction peaks of catechol from CV of PNA immobilized on bulk gold electrodes was detected in the range of 0.2 to 0.4 V, with hysteresis increasing with the accelerating scan rate. Correlation between the scan rate and the reduction peak current from Figure 2-28 (a) is illustrated in Figure 2-28 (b). This indicates that the Thiol-PNA-Catechol-141 probes were immobilized on the electrode surface because the peak current is proportional to scan rate rather than the square root of scan rate (Randles-Sevcik equation, 1.28, in section 1.4.1.4).

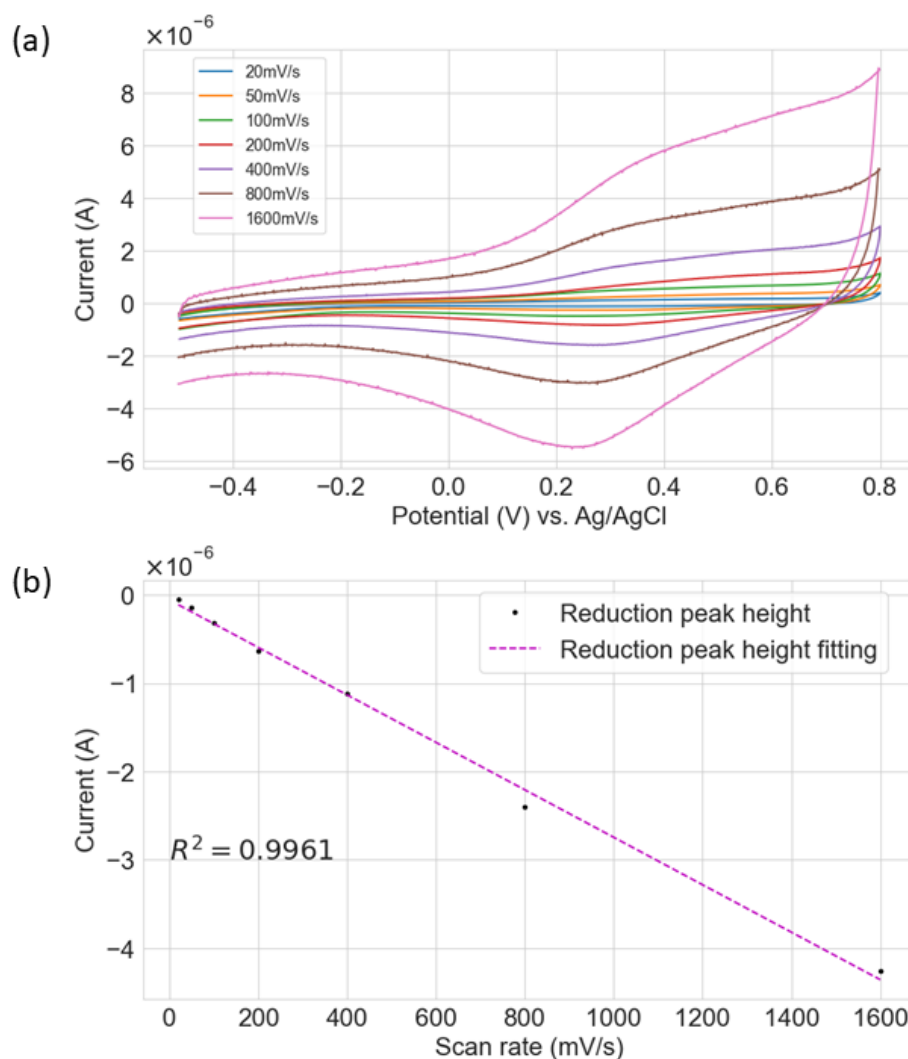


Figure 2-28 Characterization of Thiol-PNA-Catechol-141 immobilization on bulk Au electrode. (a) Cyclic voltammetry of rod gold electrode after catechol-PNA immobilization and (b) Correlation between the scan rate and peak current of cyclic voltammetry after catechol-PNA immobilization on rod Au electrode.

The linear relationship between scan rate and CV peak verified the successful immobilization of Thiol-PNA-Catechol-141 probes on gold electrode. To further test the accessibility of the catechol moiety once the PNA is immobilized onto the electrode surface, the functionalized electrodes were further treated with 1 mM aniline solution (prepared in 100 mM potassium chloride (KCl) background electrolyte). As shown in Figure 2-29 (a), after multiple SWV scans, an adduct peak emerged with similar characteristics to that of the adduct formed between

immobilized aniline PNA and freely diffusing catechol (reaction scheme shown in Scheme 2-1). The un-zoomed complete data is shown in Figure 2-29 (b).

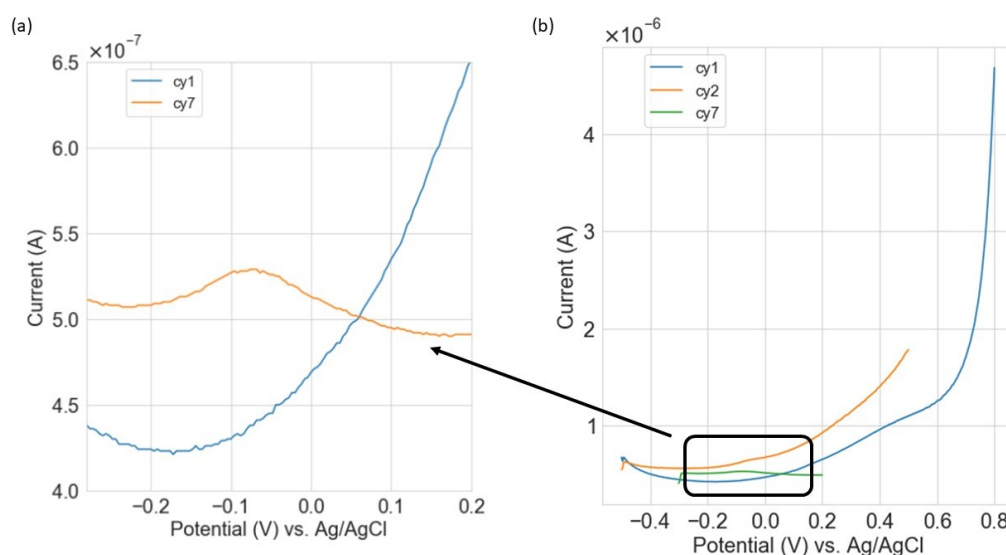


Figure 2-29 SWV test in 1 mM aniline solution with Thiol-PNA-Catechol-141 immobilized bulk gold electrode (a) zoomed, (b) original data.

Although the data in Figure 2-28 and Figure 2-29 confirmed the successful immobilization of Thiol-PNA-Catechol-141 probes on bulk gold electrode, this result is not very reproducible. Potentially due to the highly active catechol group on the Thiol-PNA-Catechol-141. Considering the active reaction property of the catechol group on the Thiol-PNA-Catechol-141, another approach using Thiol-PNA-Aniline-141 for the immobilization on the bulk gold electrode was carried out. The bulk gold electrode was immersed in 1 μ M Thiol-PNA-Aniline-141 in 1X PBS for an hour at room temperature, after which the electrode was washed by 1X PBS buffer to remove any un-immobilized species. As shown in Figure 2-30 and Figure 2-31, 1 μ M Thiol-PNA-Aniline-141 immobilization on bulk gold electrode represented more charge transfer resistance increase that due to surface change compared with the 20 μ M Thiol-PNA-Catechol-141, as shown in Figure 2-30 and Figure 2-31. The results demonstrated the Thiol-PNA-Aniline-141 is more efficient than Thiol-PNA-Catechol-141 in terms of immobilizing on

bulk gold electrode via Au-thiol linkage. This could be true because Thiol-PNA-Catechol-141 is more reactive than Thiol-PNA-Aniline-141. However, the different performance between Thiol-PNA-Catechol-141 and Thiol-PNA-Aniline-141 immobilization efficiency could simply be because of the less reproducibility of thiolated PNA immobilization on gold electrode and the not-enough valid repeats (many repeats show no detectable peaks) for comparison, which has been a challenge across the entire research.

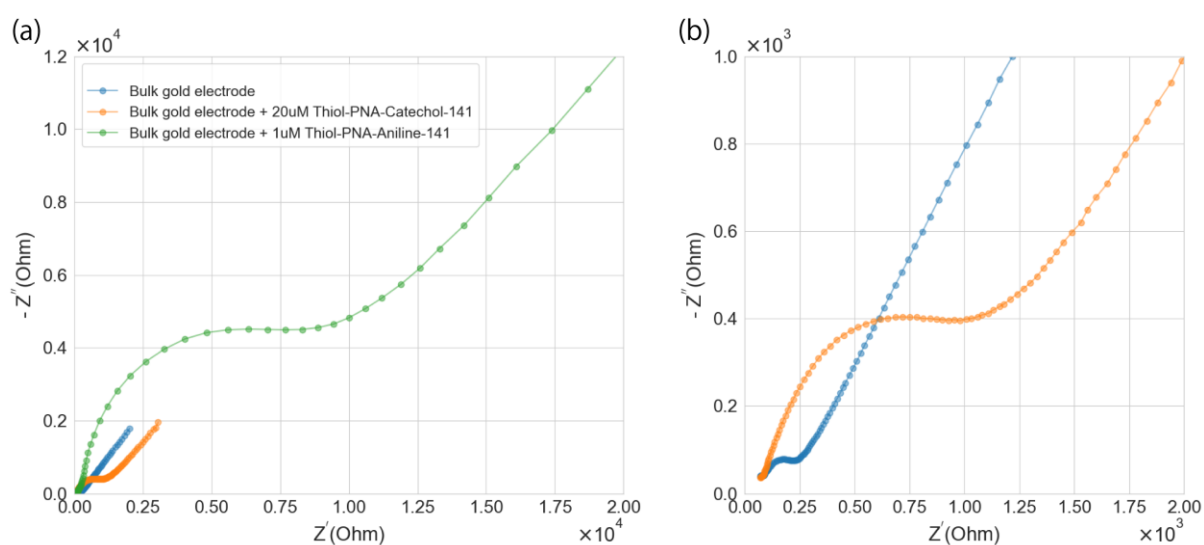


Figure 2-30 EIS investigation of 20 μ M Thiol-PNA-Catechol-141 and 1 μ M Thiol-PNA-Aniline-141 for the immobilization on bulk gold electrode. (a) Original data. (b) Zoom of (a).

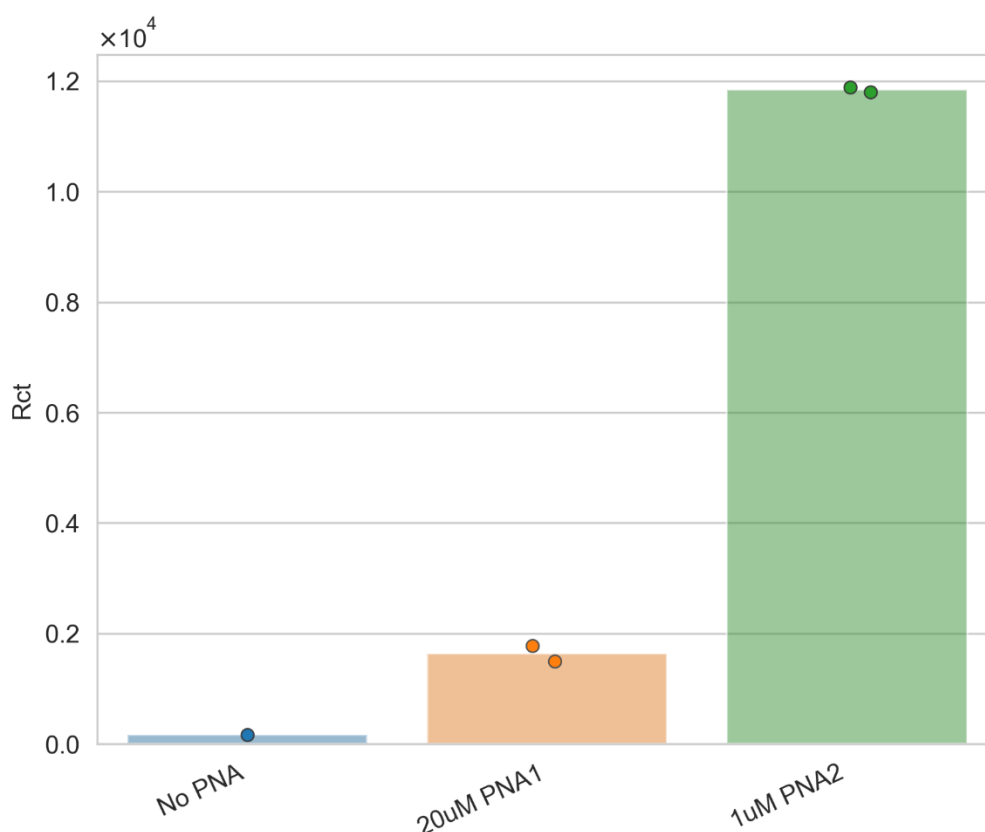


Figure 2-31 Bar plot comparison with charge transfer resistance of 20 μ M Thiol-PNA-Catechol-141 (PNA1) and 1 μ M Thiol-PNA-Aniline-141 (PNA2) for the immobilization on bulk gold electrode.

2.5.4.3 Conclusion

By using bulk gold electrode as working electrode, two different PNA capture probes has been tried: Thiol-PNA-Catechol-141 and Thiol-PNA-Aniline-141. The choice of modifying the capture probe by catechol (Thiol-PNA-Catechol-141) benefit from the direct monitoring of immobilization by investigating the catechol peak. However, due to the reactive nature of catechol, Thiol-PNA-Catechol-141 can be unstable and can degrade in the process of room temperature immobilization. This could potentially result in low reproducibility of the immobilization. To overcome this challenge, Thiol-PNA-Aniline-141 was used as an alternative capture probe for immobilization. The more stable property of Thiol-PNA-Aniline-141 could contributed to its more robust immobilization efficiency than Thiol-PNA-Catechol-141. But

overall, the expensive cost of gold and the weak covalent bond strength of Au-thiol¹⁵⁰ are the main challenges for cost-effective sensor applications using this immobilization method.

2.6 Conclusion

Four distinct PNA immobilization strategies have been carried out in this chapter on three different electrode materials.

- Polystreptavidin-biotin interaction on SPCEs.
- Silanization on SPCEs followed by click chemistry between the silanized surface and PNA capture probe.
- Trans-esterification on graphene electrode.
- Gold-thiol SAM on bulk gold electrode.

On SPCEs, polystreptavidin-biotin interaction and silanization have been implemented. Both of them presented high efficiency for immobilization on the cost-effective electrode. Silanization is considered as a more robust surface functionalization method compare with polystreptavidin-biotin method, where polystreptavidin was physically adsorbed on the electrode and silanization involves covalent bonding. However, the high immobilization efficiency and facile preparation process makes polystreptavidin-biotin method the most extensively used approach in the following research of DNA quantification and sensing.

On graphene electrode, multiple potential immobilization processes could happen simultaneously, including trans-esterification, carbodiimide chemistry and physical adsorption. Similar to gold electrode, further exploration and optimization with graphene electrode were hindered by the high-cost nature of the electrode material.

Hook et al compared the robustness of gold-thiol immobilization directly with polystreptavidin-biotin immobilization method with both PNA and DNA strands by simultaneously measuring changes in the induced energy dissipation.²⁹¹ In that research, biotinylated PNA and DNA coupled onto a polystreptavidin coated substrate have high efficiency to hybridize with both single-mismatch and fully complementary DNA. While both thiolated PNA and DNA immobilized directly to a bare-gold surface forming a rigid structure

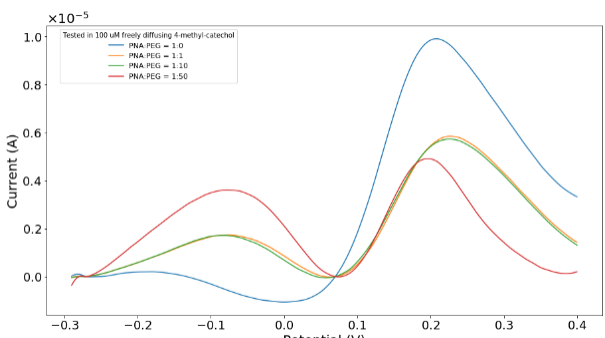
is not very efficient in hybridizing towards fully complementary DNA, probably due to multiple contact points to the surface as nucleobases can chemisorb¹⁶⁸ on Au surface spontaneously.

As summarized in Table 2-4, all of the above-mentioned methods worked for PNA probe immobilization. Nonetheless, the quality of the resulting surfaces showed variation, which influenced the performance of the functionalized surfaces as indicated by the SWV data included in the table. Due to the surface differences, the adduct signal from the immobilized PNA probe and the catechol signal from the freely diffusing 4-methyl-catechol exhibited distinct characteristics, such as variations in peak width, noise degree, and slightly different peak locations.

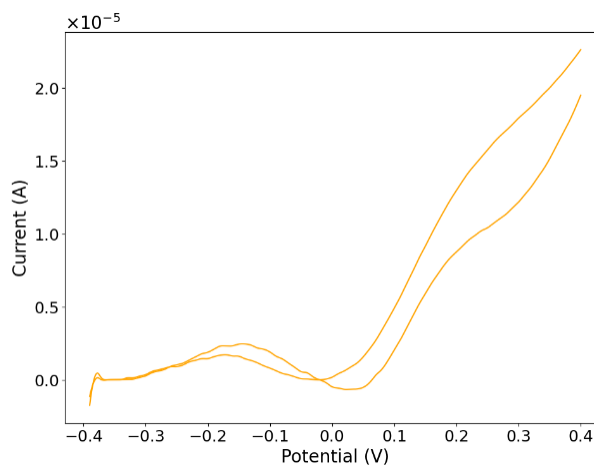
Please note that on the bulk gold electrode, 20 μ M Thiol-PNA-Catechol-141 immobilized, and the surface was test in 1 mM aniline solution (1X PBS). That's why there's no obvious catechol peak observed. Because there's a subtle adduct peak, it is possible most of the Thiol-PNA-Catechol-141 have reacted with the freely diffusing aniline forming adduct.

Except for the Au-thiol method, which exhibited unsatisfactory reproducibility and lower immobilization efficiency compared to other methods, the polystreptavidin-biotin, silanization, and NHS-graphene immobilization methods were tested in the next chapter for DNA hybridization.

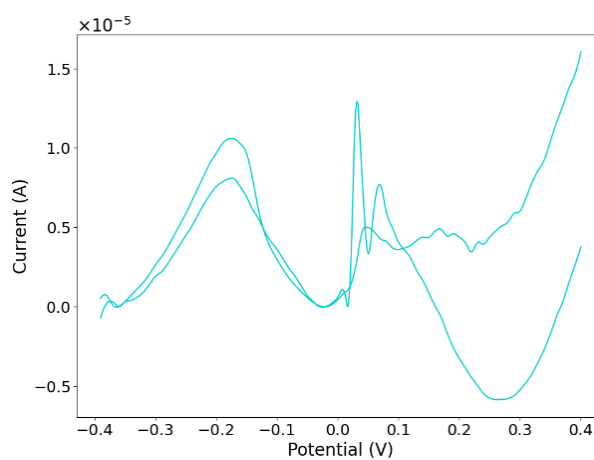
Table 2-4 Comparison of SWV data by different immobilization methods.

Immobilization Protocol	SWV tested in 100 μ M 4-methyl-catechol solution (1X PBS), baseline corrected for comparison.
500 nM Polystreptavidin + 5 μ M Biotin-PNA-Aniline-141 + Varying Biotin-PEG on SPCE for 1 h.	 Tested in 100 μM freely diffusing 4-methyl-catechol - PNA-PEG = 1:0.0 - PNA-PEG = 1:1.0 - PNA-PEG = 1:1.0 - PNA-PEG = 1:1.50 Current (A) $\times 10^{-5}$ Potential (V)

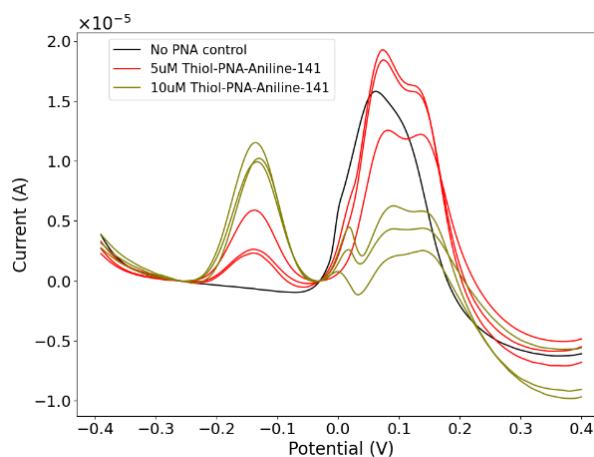
Silanization (aqueous phase) on SPCE followed by 10 μM Thiol-PNA-Aniline-141 treatment for 1 h.



Silanization (organic phase) on SPCE followed by 10 μM Thiol-PNA-Aniline-141 treatment for 1 h.

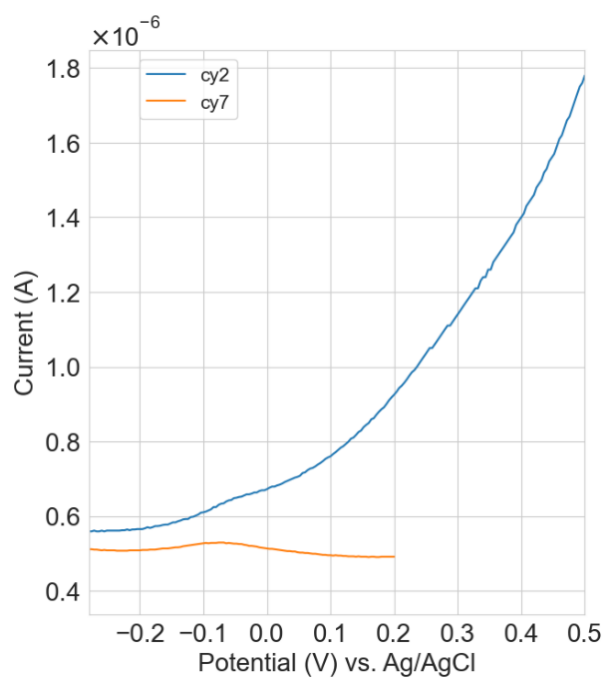


5 μM or 10 μM Thiol-PNA-Aniline-141 on NHS-graphene electrode overnight.



20 μM Thiol-PNA-Catechol-141
immobilized bulk gold electrode.

Test in 1 mM aniline solution (1X PBS, no baseline
correction).



3 DNA Quantification

Part of this chapter is due to be published in the following manuscript:

(Manuscript in preparation) On-chip Electrochemical Sensing Platform of miRNA Biomarkers for Early Detection of Prostate Cancer.

Having demonstrated the successful immobilization of electrode surfaces with PNA oligomers via a range of different chemistries, this chapter explores the ability of surface-bound PNAs to efficiently capture freely diffusing complementary DNA oligonucleotides. DNA141 was used as our target of choice as a proxy for miR141 RNA because of its greater stability. As shown in Figure 3-1, in order to directly test the capture efficiency, a DNA141 oligonucleotide labelled with an electrochemical reporter methylene blue (MB) was chosen as our fully complementary target. This allowed us to quantify the amount of hybridized target DNA by directly measuring the electrochemical signal from the MB reporter. MB is a widely used and well-studied redox reporter in electrochemistry featured with its favorable safety profile and cost-effectiveness. Please note that labelling the target sequence with an electrochemical reporter in this chapter is for the purpose of characterising the functionalised surface only. Sensing strategies will be discussed in Chapter 4.

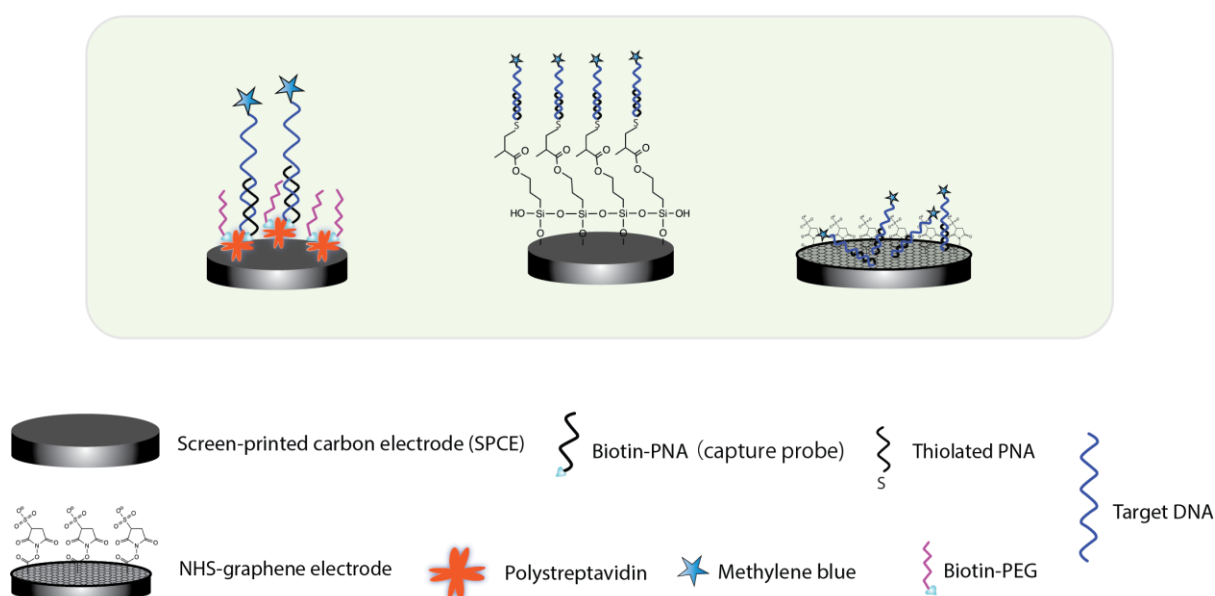


Figure 3-1 Hybridization efficiency test by MB-labelled DNA on different electrode surfaces.

3.1 Materials

3.1.1 Oligomer Sequences

The oligonucleotide sequences used in this chapter are displayed in Table 3.1. Methylene blue is noted as “MB” and arginine is noted as “Arg” in the sequence. PNA capture probe structures were previously shown in Figure 2-9. DNA141 was used as a preliminary target sequence for sensing rather than miRNA141. Because DNA presents stronger stability than miRNA141. DNA127 was used as a non-complementary sequence for control. DNA and methylene blue (MB)-labelled DNA were purchased from IDT (Integrated DNA Technologies, Inc., USA).

Table 3-1 Oligomer Nomenclature and Sequences.

Category	Name	Sequence
PNA capture probes	Biotin-PNA-Aniline-141	C _{term} -Aniline-CAT TTC T-Arg-Arg-Biotin-N _{term}
	Thiol-PNA-Aniline-141	C _{term} -Aniline-CAT TTC TA-Arg-Arg-Thiol-N _{term}
DNA capture probe		5'-Biotin-CCAT CTT TAC CAG ACA GTG TT-3'
Complementary DNA	DNA141	5'-TAA CAC TGT CTG GTA AAG ATGG-3'
	MB-DNA141	5'-MB-TAA CAC TGT CTG GTA AAG ATGG-3'
Non-complementary DNA	DNA127	5'-CTG AAG CTC AGA GGG CTC TGAT-3'
	MB-DNA127	5'-MB-CTG AAG CTC AGA GGG CTC TGAT-3'

3.1.2 Electrochemical Apparatus

The electrochemical interrogation in this chapter was mainly based on SWV detection of the MB reporter labelled on DNA sequences. All SWV in this chapter are carried out in 1X PBS with amplitude being 0.05 V, increment potential being 0.01 V, frequency being 45 Hz, quiet time

being 5 s and gain was set as 10^{-5} A/V. As can be seen from Figure 3-2, the peak of MB labelled DNA is at -0.27 V vs Ag pseudo reference electrode on SPCE, when tested in 1X PBS. SWV peaks are fitted by CHI 660E electrochemical workstation (CH Instruments, USA). Data visualization was built by using Python Libraries (Matplotlib and Seaborn).

3.2 DNA Quantification of Polystreptavidin-biotin Interaction Immobilized Carbon Electrode

In this section, DNA quantification was carried out on the polystreptavidin-biotin method functionalized screen-printed electrode to test the hybridization efficiency between the PNA capture probe and the target DNA.

3.2.1 Methods

Based on the optimum ratio among polystreptavidin, capture probe (Biotin-PNA-Aniline-141) and spacer Biotin-PEG (Biotin-(PEG)₆-OH) discussed in section 2.5.1, 500 nM polystreptavidin, 5 μ M Biotin-PNA-Aniline-141 and 50 μ M Biotin-PEG were mixed together in a volume of 20 μ L. The sample (20 μ L) was deposited on the SPCE (working electrode area) and left to dry under fume hood because the ventilation accelerates the drying process (could also leave to dry on bench). This process usually takes 1 hour under ambient condition. Before moving onto the next steps, the polystreptavidin treated surface was not washed. Washing will be conducted after the DNA hybridization. Because multiple washing is avoided, which may potentially deprive some of the immobilized polystreptavidin on the electrode surface that was physically adsorbed.

Different concentrations of methylene blue labelled target DNA (MB-DNA141) in the range of 10 nM to 500 nM were prepared with 1X PBS. Then, 100 μ L prepared aliquot MB-DNA141 was left on the treated SPCE. MB-DNA141 was left on the electrode for 20 minutes in order to give enough time for PNA-DNA hybridization (hybridization between Biotin-PNA-Aniline-141 and MB-DNA141). A non-complementary DNA sequence labelled with methylene blue (MB-DNA127) was selected as a control group. The non-DNA group was treated by 1X PBS for the same time length. After hybridization, MB-DNA141 was removed, and the treated electrode surface was thoroughly washed by 1X PBS. Finally, the fully functionalized electrode was tested in 1X PBS by SWV.

3.2.2 Results and Discussion

A signature SWV of methylene blue labelled DNA is shown in Figure 3-2. On polystreptavidin treated SPCE, 1 μM freely diffusing MB-DNA141 in 1X PBS was tested by SWV. The SWV interrogation in this section are carried out in 1X PBS with initial potential being -0.5 V, final potential being 0 V. This indicates the peak location of methylene blue labelled DNA is at $\sim$ -0.27 V (vs Ag pseudo reference electrode).

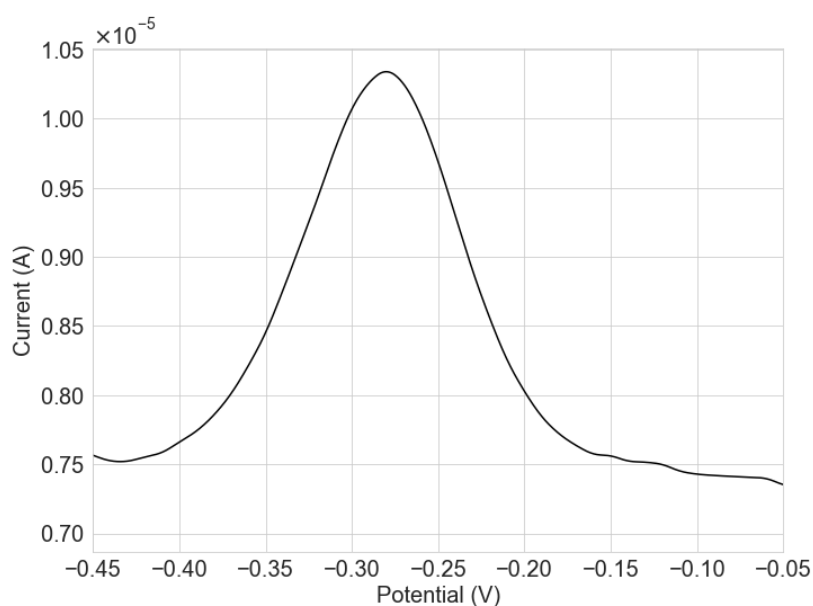


Figure 3-2 SWV measurement of 1 μM freely diffusing MB-DNA141 (methylene blue labelled DNA141, in 1X PBS) on polystreptavidin-functionalized screen-printed carbon electrode.

As shown in Figure 3-3, peak current from SWV were displayed as box plots with each concentration have three repeats. As known from Figure 3-2, MB labelled DNA have a signature peak at around -0.27 V (vs Ag pseudo reference electrode) on polystreptavidin treated SPCE. Complementary DNA (MB-DNA141) hybridize with the capture PNA probe Biotin-PNA-Aniline-141 immobilized on the electrode surface having a dynamic linear range from 10 nM to 400 nM, as illustrated in Figure 3-3 (a) (blue boxes and dots). With concentrations higher than 400 nM, a plateau has been reached gradually. With the non-complementary DNA (MB-DNA127), obvious non-specific adsorption can be observed (orange

box and dots in Figure 3-3 (a)) ranging between 10 nM to 500 nM. However, difference between target (complementary DNA) and non-target (non-complementary DNA) is statistically significant. The kinetics of hybridization are extremely sensitive to the degree of mismatch. This non-specific adsorption can be alleviated by further treatment with BSA,^{234–238} casein,²³⁹ TWEEN20²⁹² or sodium dodecyl sulfate (SDS).^{155,184,219,293–296}

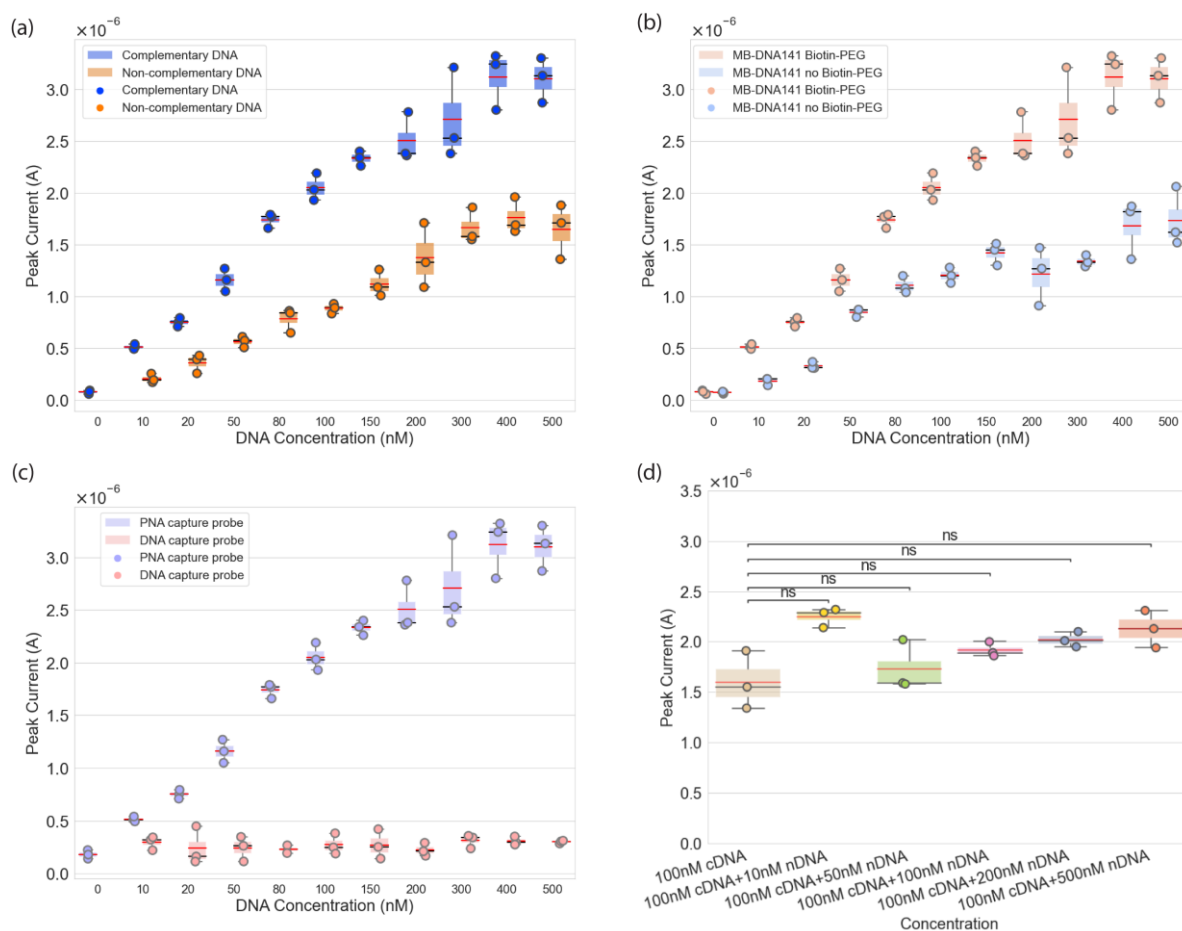


Figure 3-3 (a) DNA quantification of MB-DNA with complementary DNA (MB-DNA141) and non-complementary DNA (MB-DNA127) on polystyrene-treated SPCE by SWV. (b) DNA quantification of MB-DNA141 with and without Biotin-PEG on polystyrene-treated SPCE by SWV. (c) DNA quantification of MB-DNA141 with PNA capture probe (Biotin-PNA-Aniline-141) and DNA capture probe on polystyrene-treated SPCE by SWV. (d) Specificity test of the hybridization efficiency using mixed cDNA (complementary DNA, MB-DNA141) and nDNA (non-complementary DNA, DNA127) by SWV (n = 3).

As mentioned in 2.5.1.2, biotin-PEG plays an important role in increasing the signal while using the same amount of analyte. As shown in Figure 3-3 (b), complementary DNA hybridization and detection have been carried out with and without biotin-PEG. The cyan and orange boxes and dots in Figure 3-3 (b) both used the complementary DNA (MB-DNA141) as hybridization target. The difference was in the immobilization process. For the biotin-PEG group, 10 μL , 5 μL and 5 μL of 500 nM polystreptavidin, 5 μM Biotin-PNA-Aniline-141 and 50 μM Biotin-PEG were used for immobilization while the no biotin-PEG group used 1X PBS replacing the 50 μM Biotin-PEG for immobilization. As can be observed from Figure 3-3 (b), the dynamic range upper limit has shifted from 150 nM to 400 nM before and after the Biotin-PEG treatment. And the signals have been amplified after applying biotin-PEG co-immobilization with Biotin-PNA-Aniline-141. This improved performance after the Biotin-PEG treatment is due to the surface probe density and orientation control, as have been discussed in section 2.5.1.3.2.

In order to compare the capacity of probes based on a PNA or DNA scaffold to efficiently capture freely diffusing DNA oligonucleotides through hybridization, a biotinylated PNA and a biotinylated DNA were immobilized onto otherwise identical electrodes (SPCE). Please note that the biotinylated DNA probe is 21-mer while the biotinylated PNA probe is 7-mer, as shown in Table 3-1. In Figure 3-3 (c), the pink boxes and dots are SWV peaks obtained by using DNA capture probe. Surprisingly, the signals were very weak compared with those obtained from electrodes functionalised with Biotin-PNA-Aniline-141 (purple boxes and dots in Figure 3-3 (c)). There are multiple potential reasons to explain the low hybridization efficiency between DNA capture probe and MB-DNA:

- The first reason potentially contributing to this difference is the electrostatic repulsion between DNA strands as DNA is a negatively charged molecule.
- Secondly, this could be explained by the fact that surface bound DNA-DNA duplexes are generally less stable than the same species in homogeneous solution.^{297,298}
- The third potential reason for lower hybridization efficiency between the DNA capture probe and MB-DNA141 is related to probe orientation. 21-mer DNA capture probe is threefold longer than the 7-mer PNA probe, the longer DNA probe is more likely to cause steric hindrance,²²² which can decrease the efficiency of hybridization and reduce the signal to noise ratio.

- Finally, DNA-DNA hybridization is highly dependent on ionic strength and buffer composition,²⁹⁹ unlike PNA-DNA hybridization, which is less affected by these factors. Therefore, the hybridization efficiency between DNA capture probe and MB-DNA can potentially be improved by changing a different buffer solution. However, further detail is beyond the discussion of this research.

The specificity of DNA hybridization on the polystyrene-avidin functionalized electrode were tested by using a mixture of target DNA (MB-DNA141) and non-complementary DNA (DNA127) with different concentrations. As shown in Figure 3-3 (d), the complementary DNA concentration remained constant at 100 nM, while the non-complementary DNA concentrations are 0 nM, 10 nM, 50 nM, 100 nM, 200 nM and 500 nM. As can be concluded from the data and figure, the presence and different concentrations of the non-complementary DNA does not affect the recognition of target complementary DNA with the designed sensor.

Kruskal-Wallis test was used for assessing whether there are any significant differences between the groups. This test is an extension of the Mann-Whitney U test and is used to compare the medians of three or more independent groups. It is a non-parametric alternative to one-way ANOVA.

3.3 DNA Quantification on Silanized Carbon Electrode

In this section, DNA quantification was carried out on the silanized SPCE to test the hybridization efficiency between the PNA capture probe and the target DNA.

3.3.1 Methods

Using silanization method, PNA-functionalized electrodes, in terms of both aqueous phase and organic phase silanization, were produced as described in section 2.5.2. After the immobilization, the functionalized electrodes were washed thoroughly using 1X PBS. Then the electrodes were ready for DNA quantification.

Different concentrations of methylene blue labelled target DNA (MB-DNA141) in the range of 50 nM to 1 μ M were prepared in 1X PBS. Then, 100 mL of MB-DNA141 solution was left on the functionalized electrodes for 20 minutes in order to give enough time for PNA-DNA

hybridization (hybridization between Thiol-PNA-Aniline-141 and MB-DNA141). A non-complementary DNA sequence labelled with methylene blue (MB-DNA127) was selected as a control group. The non-DNA group was treated by 1X PBS for the same time length. After hybridization, MB-DNA141 was removed, and the treated electrode surface was thoroughly washed by 1X PBS. Finally, the fully functionalized electrode was tested in 1X PBS by SWV.

3.3.2 Results and Discussion

With the aqueous phase immobilization method, signal from the electrochemical reporter on the methylene blue labelled DNA (MB-DNA141) after the hybridization with the capture probe Thiol-PNA-Aniline-141 by SWV is similar to that of the control using a non-complementary DNA (MB-DNA127), except for the lower-nano-molar range (50 nM and 100 nM), as shown in Figure 3-4 (c). The original SWV data of both complementary DNA hybridization (Figure 3-4 (a)) and non-complementary DNA (Figure 3-4 (c)) have strong background noise and the peaks are broad over 0.4 V range. Compared with the peak range of methylene labelled DNA on polystreptavidin functionalized SPCE from -0.45 V to -0.15 V (Figure 3-2), the peak range of silanization in aqueous phase is from -0.6 V to -0.2V. This broader peak range may be due to the distinct electron transfer kinetics contributed by factors including immobilized film thickness, probe orientation and probe density. The significant variation or spread in 750 nM and 1000 nM groups is likely attributed to the difference of the silanized film thickness and MB-DNA adsorption on the functionalized electrode surface.

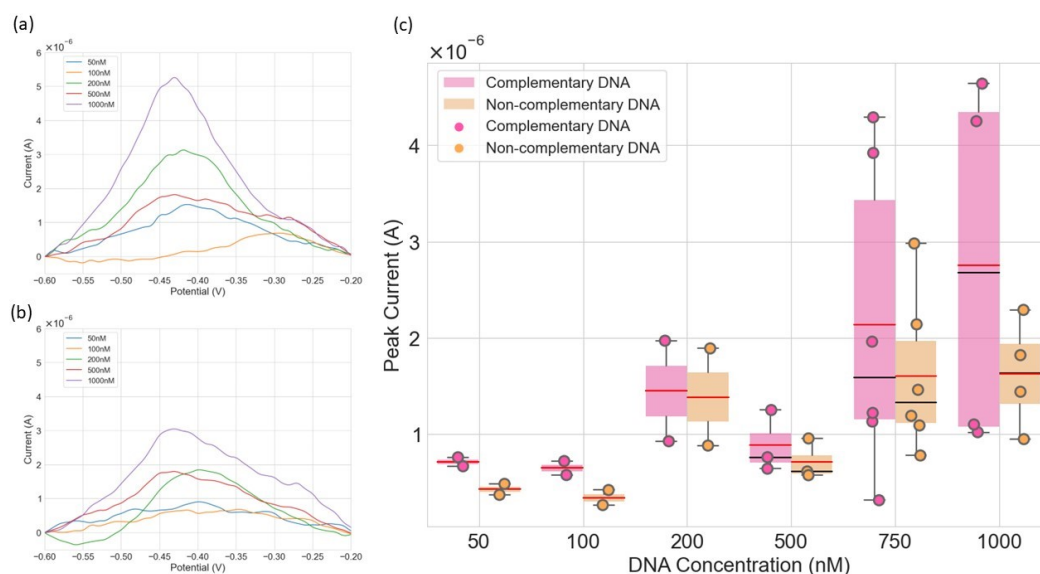


Figure 3-4 DNA quantification of methylene blue labelled DNA with complementary DNA (MB-DNA141) (a) and non-complementary DNA (MB-DNA127) (b) on silanized (aqueous phase, prepared from the same bench silanization) SPCE by SWV (baseline corrected). (c) Box plot of multiple repeats (including (a) and (b)).

Data displayed in Figure 2-23 (a) confirm the better performance of organic phase silanization compared with the aqueous phase silanization possibly due to reduced silane polymerization. However, the DNA hybridization quantification with organic phase silanization followed by click chemistry immobilization of the Thiol-PNA-Aniline-141 have ultra-broad signals rather than “peaks” from SWV interrogation. As shown in Figure 3-5, the SWV of complementary DNA (MB-DNA141 Figure 3-5 (a) and (c)) and non-complementary DNA (MB-DNA127 Figure 3-5 (b) and (d)) after hybridization with organic phase silanization are over the range from -0.7 V to 0 V and could not be analysed further. The signal heights are of similar level for both complementary DNA (MB-DNA141, Figure 3-5 (a)) and non-complementary DNA (MB-DNA127, Figure 3-5 (b)). Interestingly, after 1% BSA treating for 2 hours, the signal height of the complementary DNA group was amplified while the non-complementary DNA group was decreased. This could be explained by kinetic changes caused by BSA treatment.

BSA is a protein that can form a passivating layer on the electrode surface. This layer may alter the accessibility of electroactive sites on the electrode, affecting the rate of electron

transfer and potentially impeding or enhancing specific electrochemical reactions. Additionally, the BSA layer can modify the surface charge and topography of the electrode, influencing the adsorption behavior of analytes or reaction intermediates. The steric hindrance and charge effects caused by the BSA layer can also influence the kinetics of molecular interactions at the electrode surface.³⁰⁰

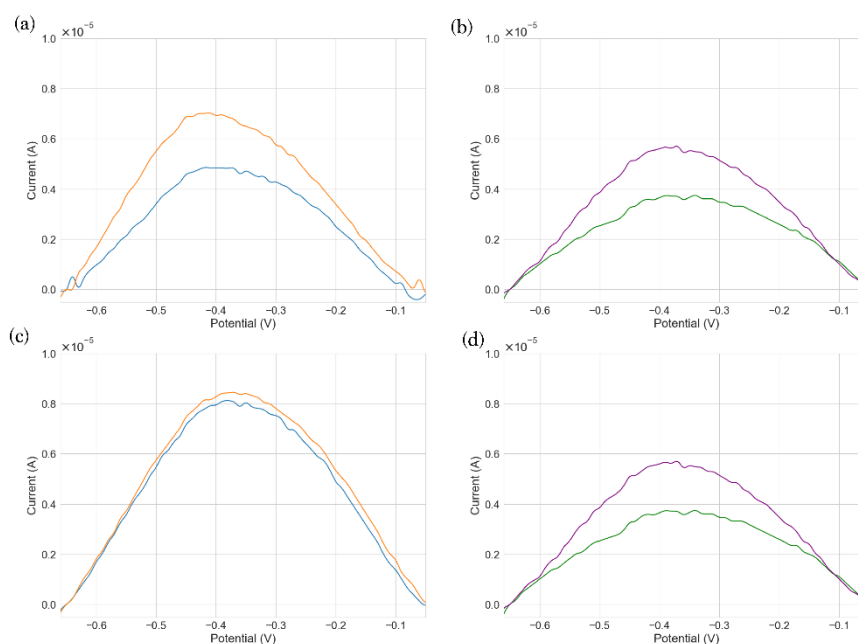


Figure 3-5 DNA quantification of 100 nM methylene blue labelled DNA with complementary DNA (MB-DNA141) (a) and non-complementary DNA (MB-DNA127) (b) on silanized (organic solvent phase) SPCE by SWV (two repeats each). SWV of methylene blue labelled DNA with complementary DNA (MB-DNA141) (c) and non-complementary DNA (MB-DNA127) (d) after treating with 1% BSA for 2 hours (data of (c) and (d) are from the same electrodes as those used in (a) and (b). (Baseline corrected).

3.4 DNA Quantification on Graphene Electrode

In this section, DNA quantification was carried out on the functionalized graphene electrode to test the hybridization efficiency between the PNA capture probe and the target DNA.

3.4.1 Methods

The immobilization method and protocol were produced as described in section 2.5.3. After the immobilization, the functionalized electrodes were washed thoroughly using 1X PBS. Then the electrodes were ready for DNA quantification.

Solutions of 125 nM, 250 nM and 500 nM methylene blue-labelled target DNA (MB-DNA141) were prepared in 1X PBS and deposited on the functionalized electrodes for 20 minutes for PNA-DNA hybridization to occur (hybridization between Thiol-PNA-Aniline-141 and MB-DNA141). A non-complementary DNA sequence labelled with methylene blue (MB-DNA127) was selected as a control group. The non-DNA group was treated by 1X PBS for the same time length. After hybridization, MB-DNA141 was removed, and the treated electrode surface was thoroughly washed by 1X PBS. Finally, the fully functionalized electrode was tested in 1X PBS by SWV.

3.4.2 Results and Discussion

As shown in Figure 3-6, 125 nM DNA groups with both complementary and non-complementary are at similar levels as the no DNA control group. The mean value signal of 125 nM complementary DNA group is slightly higher than the non-complementary DNA. However, this difference is not statistically significant (with p-value from t-test between 125 nM complementary DNA group and 125 nM non-complementary DNA group being 0.3026). The standard deviation of 125 nM complementary DNA group is 1.82×10^{-7} and the standard deviation of 125 nM non-complementary DNA group is 7.34×10^{-8} . Similar situation applies for the 250 nM and 500 nM of methylene blue labelled target DNA.

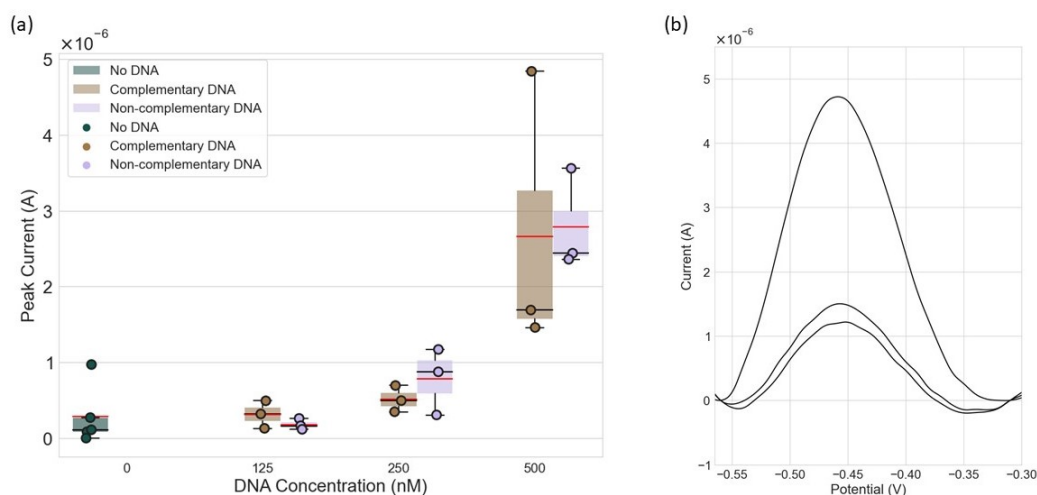


Figure 3-6 (a) Box plot of DNA quantification using MB-DNA with complementary DNA (MB-DNA141) and non-complementary DNA (MB-DNA127) on NHS-graphene electrode by SWV ($n \geq 3$). (b) SWV data of 500 nM complementary DNA (MB-DNA141), baseline corrected.

On the graphene electrode, even though the immobilization of capture PNA probe Thiol-PNA-Aniline-141 was demonstrated, DNA hybridization test did not show statistically significant differences between the complementary and non-complementary DNA. This could be explained by the way PNAs are attached and/or oriented on the electrode surface, making them unavailable to engage in Watson-Crick hybridization with complementary DNA oligonucleotides. For example, adsorption of the PNA bases onto graphene could explain this result. At higher DNA concentrations, as shown in Figure 3-6 (b), the signal originating from MB demonstrates reduced reproducibility. This phenomenon may also be attributed to the predominant influence of MB-DNA adsorption compared to the PNA/DNA hybridization process. The inherent complexity and limited control of this adsorption process contribute to the diminished reproducibility observed.

3.5 Conclusion

This chapter investigated the hybridization efficiency between the PNA capture probe and the target DNA on the functionalized electrode. By labelling the target DNA with methylene blue, an intensively studied electrochemical reporter, target DNA can be quantified directly. Thus, this section provided meaningful guidance for further sensor development.

Quantification of DNA capturing has been tested on various PNA-functionalized electrodes: polystreptavidin-treated SPCE, silanized SPCE and NHS-terminated graphene electrode. Surprisingly, only the polystreptavidin treated SPCE was able to distinguish the complementary target sequence from the non-complementary control sequence. With the silanized electrodes, the main challenge for DNA quantification is the detected signal being a very broad peak (Figure 3-4 (a and b), Figure 3-5 and Figure 3-7), which applied for both aqueous vapor phase and organic solvent phase silanization. And broad peak is usually very difficult to define the peak height for comparison. This is because the silanized monolayer may lead to slower charge transfer kinetics, increased electrode capacitance, and altered mass transport properties, all of which contribute to the observed change in peak shape.

With the NHS-graphene electrodes, the inability of discriminating between the complementary DNA to non-complementary DNA is probably due to strong PNA adsorption on graphene which resulted in strong non-specific binding. Nonspecific physical/mechanical interactions could cause the DNA sequence to collapse or deform and cause electron transfer between the collapsed DNA-MB and electrode surface, which induce false positive signals⁸⁹.

It is worth noting that on polystreptavidin-treated SPCE, a DNA capture probe was tested in contrast to the PNA capture probe used in this research. The better performance of PNA capture probe compared with DNA capture probe is because of less electrostatic repulsion, less stable DNA-DNA duplex hybridization at heterogeneous condition and the difference in binding kinetics with longer sequence and the difference in probe orientation. The kinetics of hybridization are sensitive to the position at which hybridization occurs along the immobilized strand.

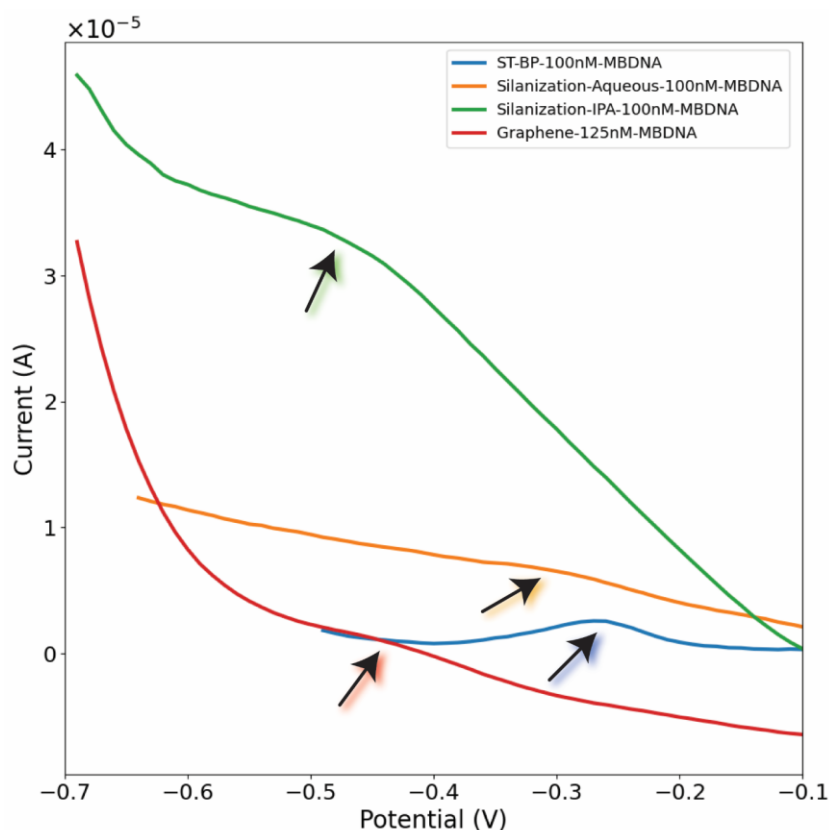


Figure 3-7 Comparison of SWV for DNA quantification using 100 nM complementary MB-DNA (125 nM for NHS-graphene electrode) with four different immobilization methods: Blue line: 500 nM Polystreptavidin, 5 μ M Biotin-PNA-Aniline-141, 50 μ M Biotin-PEG on SPCE for 1 h + 100 μ L 100 nM MB-DNA141 for 20 min + wash; Orange line: Silanization in aqueous phase + click chemistry immobilization of PNA capture probe + on SPCE for 1 h + wash + 100 μ L 100 nM MB-DNA141 for 20 min + wash; Green line: Silanization in organic phase + click chemistry immobilization of PNA capture probe + on SPCE for 1 h + wash + 100 μ L 100 nM MB-DNA141 for 20 min + wash; Red line: 100 μ L 10 μ M PNA capture probe immobilization on NHS-graphene electrode overnight (sealed) + wash + 100 μ L 100 nM MB-DNA141 for 20 min + wash, baseline corrected.

In summary, the polystreptavidin and biotin immobilization method demonstrated the best performance in terms of both complementary DNA hybridization and non-complementary discrimination (Figure 3-3). Furthermore, the signal obtained from MB exhibited a distinct and well-defined peak, when comparing with other immobilization methods using the same (or similar) amount of analyte, as shown in Figure 3-7. When employing 100 nM complementary

MB-DNA, the MB signal obtained through aqueous phase silanization exhibits a weak and broad peak. On the other hand, the MB peak achieved through organic phase silanization using the same concentration MB-DNA is more pronounced, but the peak remains broad. The peak on NHS-graphene is not readily apparent at 125 nM MB-DNA concentration. With higher concentrations (500 nM), the MB peak becomes clear and well-defined. However, the performance is not consistently reproducible, and NHS-graphene is unable to effectively discriminate between complementary and non-complementary target sequences. As a result, the upcoming chapter will delve into further enhancements and sensing strategies based on the polystreptavidin/Biotin-PNA immobilization method.

4 Sensing

Part of this chapter is due to be published in the following manuscript:

(Manuscript in preparation) On-chip Electrochemical Sensing Platform of miRNA Biomarkers for Early Detection of Prostate Cancer.

Having demonstrated in Chapter 2 that it was possible to successfully immobilize PNA oligomers onto electrode surfaces and demonstrated in Chapter 3 that these PNAs were able to capture freely diffusing complementary DNA oligonucleotides, chapter 4 explores the possibility of using PNA-functionalised electrode for label-free DNA detection. As shown in Figure 4-1, five different sensing strategies were investigated that are based one (1) a sandwich assay, (2) an electrochemical oligonucleotide template reaction (EOTR) sensing, (3) chronoamperometry sensing a electrochemical impedance spectroscopy (EIS) sensing by investigating surface charge transfer resistance and (5) DNA intercalation sensing by using freely diffusing MB. All the above-mentioned sensing strategies have been carried out for sensing applications in this research.

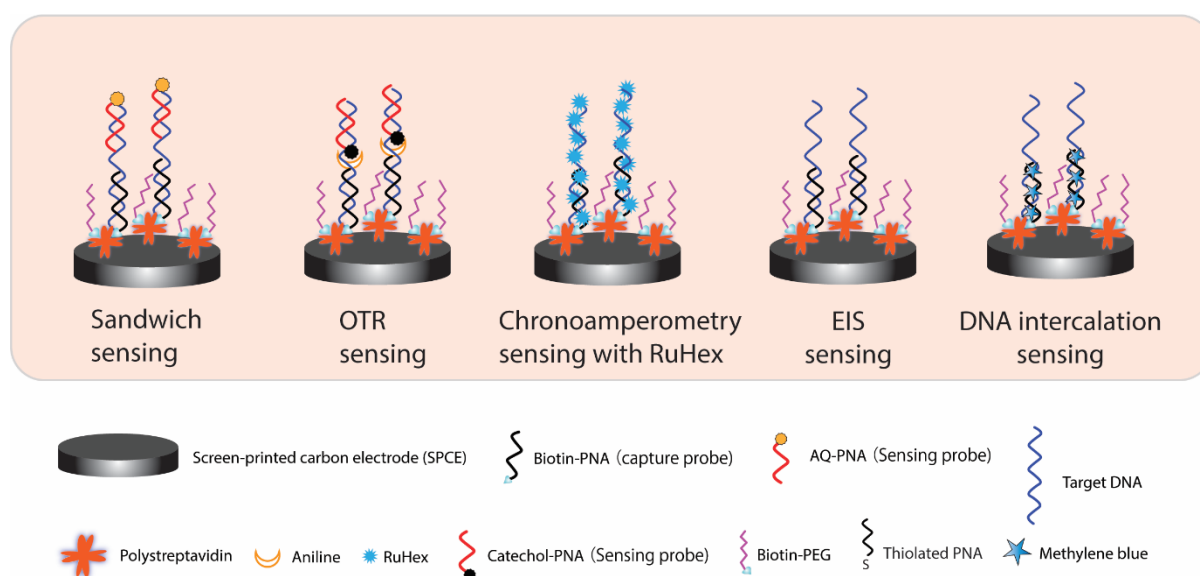


Figure 4-1 Sensing strategies on polystreptavidin-biotin functionalized SPCEs.

4.1 Materials and Methods

4.1.1 Oligomer Sequence

In this chapter, instead of using miRNA141 and DNA141 as the target sequence, another sequence that is correlated with prostate cancer has been used: miRNA374a and DNA374a.^{275,276} Although the shift in the sequence is not ideal, it was carried out this way due

to the availability of parts of the stock PNA probes, which only required functional group attachment, saving time in the process. Despite the fact that a different sequence was used, this sequence is still correlated with prostate cancer. And most importantly, the working principle behind the immobilization and DNA hybridization discussed in last two chapters are the same.

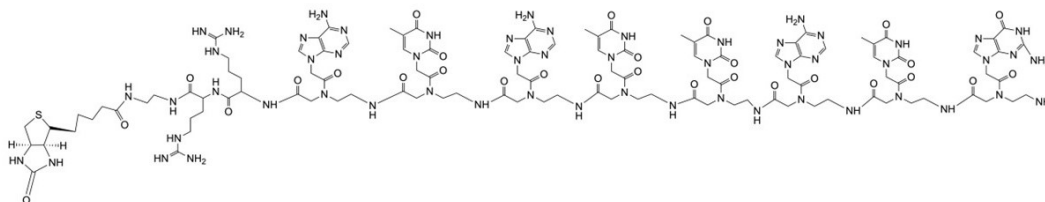
The oligonucleotide sequences used in this chapter are displayed in Table 4-1. Anthraquinone is noted as “AQ” and arginine is noted as “Arg”. AQ was chosen as the electrochemical reporter for sandwich sensing due to its peak location at around -0.65 V (vs Ag pseudo reference electrode, in 1X PBS). This location is notably distinct from the inherent peak of SPCE near -0.1 V, which will be discussed in detail in 4.2.2. The sequences and chemical structures of the PNA capture probes and sensing probes used in this chapter are shown in Table 4-1 and Figure 2-9 and 4-2. Biotin-PNA-374a and part of sensing probes (PNA-AQ-374a before the attachment of AQ) were kindly provided by Miss Loukia Petrou, where the attachment of AQ and PNA cleavage were conducted by myself. Sensing probe PNA-Catechol-141 was synthesized together with Dr Philip Gillespie, Dr Md Nazmul Islam and Dr Rob Channon. Structure and contribution of Biotin-PNA-Aniline-141 and Thiol-PNA-Aniline-141 can be found in Chapter 2. All PNA probes used in this research have been purified by HPLC (with the help from Mr Daanyaal Khan) and tested by MALDI (with the help from Miss Loukia Petrou) to confirm the correct structure. MALDI results of sensing probe PNA-AQ-374a is displayed in Figure A-1 in Appendix.

Table 4-1 Oligomer Nomenclature and Sequences.

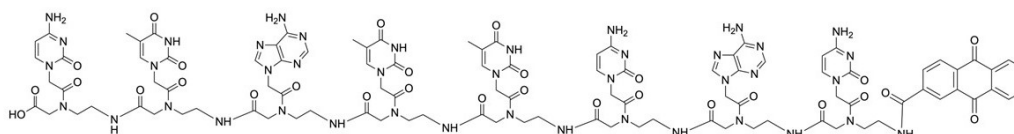
Category	Name	Sequence
PNA capture probes	Biotin-PNA-374a	C _{term} -Biotin-Arg-Arg- ATA TTA TG -N _{term}
	Biotin-PNA-Aniline-141	C _{term} -Aniline- CAT TTC T -Arg-Arg-Biotin-N _{term}
	Thiol-PNA-Aniline-141	C _{term} -Aniline- CAT TTC TA -Arg-Arg-Thiol-N _{term}
PNA sensing probes	PNA-AQ-374a	C _{term} - CTA TTC AC -AQ-N _{term}

	PNA-Catechol-141	C _{term} -Arg-Arg-T GTG ACA -Catechol-N _{term}
DNA sequences	DNA374a	5'-TTA TAA TAC AAC CTG ATA AGTG-3'
	DNA141	5'-TAA CAC TGT CTG GTA AAG ATGG-3'
	DNA127	5'-CTG AAG CTC AGA GGG CTC TGAT-3'
	DNA150	5'-TCT CCC AAC CCT TGT ACC AGTG-3'
miRNA sequences	miRNA374a (hsa-miR-374a-5p)	5'-UUA UAA UAC AAC CUG AUA AGUG-3'
	miRNA141 (hsa-miR-141-3p)	5'-UAA CAC UGU CUG GUA AAG AUGG-3'
	miRNA127 (hsa-miR-127-5p)	5'-CUG AAG CUC AGA GGG CUC UGAU-3'
	miRNA150 (hsa-miR-150-5p)	5'-UCU CCC AAC CCU UGU ACC AGUG-3'

(a) Biotin-PNA-374a; MW = 2780.89 g/mol (capture PNA probe)



(b) PNA-AQ-374a; MW = 2355.28 g/mol



(c) PNA-Catechol-141; MW = 2396.39 g/mol

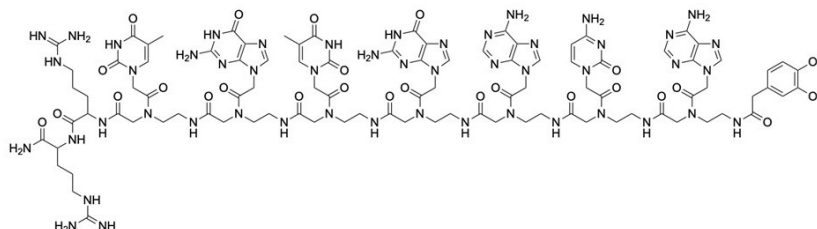


Figure 4-2 Representative PNA sensing probe structures. (a) Biotin-PNA-374a. (b) PNA-AQ-374a. (c) PNA-Catechol-141.

4.1.2 Materials and Electrochemical Apparatus

The electrochemical measurements including cyclic voltammetry (CV), square wave voltammetry (SWV), differential potential voltammetry (DPV), chronoamperometry and electrochemical impedance spectroscopy (EIS) were all performed on a CHI 660E electrochemical workstation (CH Instruments, USA).

SPCE (Dropsens110) was bought from Metrohm Dropsens (Metrohm AG, Switzerland). Working and counter electrodes of SPCE are made of carbon, while reference electrode is made of silver (pseudo reference electrode). The working electrode diameter is 4 mm. NHS-functionalized graphene electrode was purchased from Integrated Graphene (Scotland, UK).

Anthraquinone-2-carboxylic acid was purchased from Thermo Scientific (UK). N-succinimidyl ferrocenecarboxylate was bought from Tokyo Chemical Industry Co., LTD (Tokyo, Japan). Methylene blue intercalator was purchased from Sigma Aldrich (St. Louis, MO United States). Biotin-PEG was purchased from PurePEG LLC (San Diego, CA USA). DNA was purchased from IDT (Integrated DNA Technologies, Inc., USA). All the molecules were dissolved in 1X PBS (Thermo Fisher Scientific Inc., USA) unless otherwise specified. Unless otherwise noted, all other reagents as well as chemical and reactive groups were purchased from Sigma Aldrich.

4.1.3 Electrochemical Sensing Strategies

As shown in Figure 4-3, 5 different sensing strategies were explored, all using SPCEs functionalized with PNA capture probes via polystreptavidin-biotin coupling optimized in chapter 2. The principle of each of these strategies is briefly described below:

In a sandwich assay, the DNA target simultaneously hybridizes to two PNA probes complementary to either its 3' or 5' ends (Figure 4-3 sensing strategy 1). Here, one of the PNA probes is biotinylated and immobilized onto the electrode surface (and acts as a capture probe) and the second PNA is freely diffusing and labelled with an electrochemical reporter anthraquinone (AQ) (and acts as a sensing probe). The double-probe sandwich assay requires the simultaneous binding of two probes for the reporter (AQ) to be brought to the electrode

surface and be detected. It is therefore highly specific. This property makes sandwich assay suitable for use directly in complex materials, such as blood serum.³⁰¹

EOTR sensing (Figure 4-3 sensing strategy 2), similarly to a sandwich assay, is based on the simultaneous hybridization of two PNA probes to the same DNA (or RNA) template. However, unlike in sandwich assays where the signal is generated by an electrochemically active reports, the measured signal in EOTR is generated by the product of a chemical reactions between two probe heads attached to either end of the PNA probes, as shown in reaction Scheme 2-1 (3). Hence the DNA target acts as a template for a chemical reaction that would otherwise be highly unfavourable due to the low concentration of probes.^{325,326}

Whilst many examples of oligonucleotide template reactions (OTRs) have been reported that allow the detection of short DNA or RNA oligonucleotides based on a fluorescence readout,^{88,302} Ladame and O'Hare have recently reported on the first example of EOTR in solution.³⁰³ In this peer-reviewed preliminary test, two complementary PNA oligomers were designed and synthesized to initiate a controlled 1,4 addition reaction between an aniline and a quinone upon hybridization to a target miRNA in solution. The result confirmed that the EOTR strategy provides greater control to the operator on when to initiate or halt the reaction, i.e., only upon anodic oxidation of the catechol probe into its orthoquinone will the reaction begin. This proof-of-concept study provides evidence that EOTR has great potential for sequence-specific sensing of nucleic acid biomarkers (DNA and miRNA) under near physiological pH and salt concentrations.

Here, we explored the first example of EOTR on electrode surfaces.

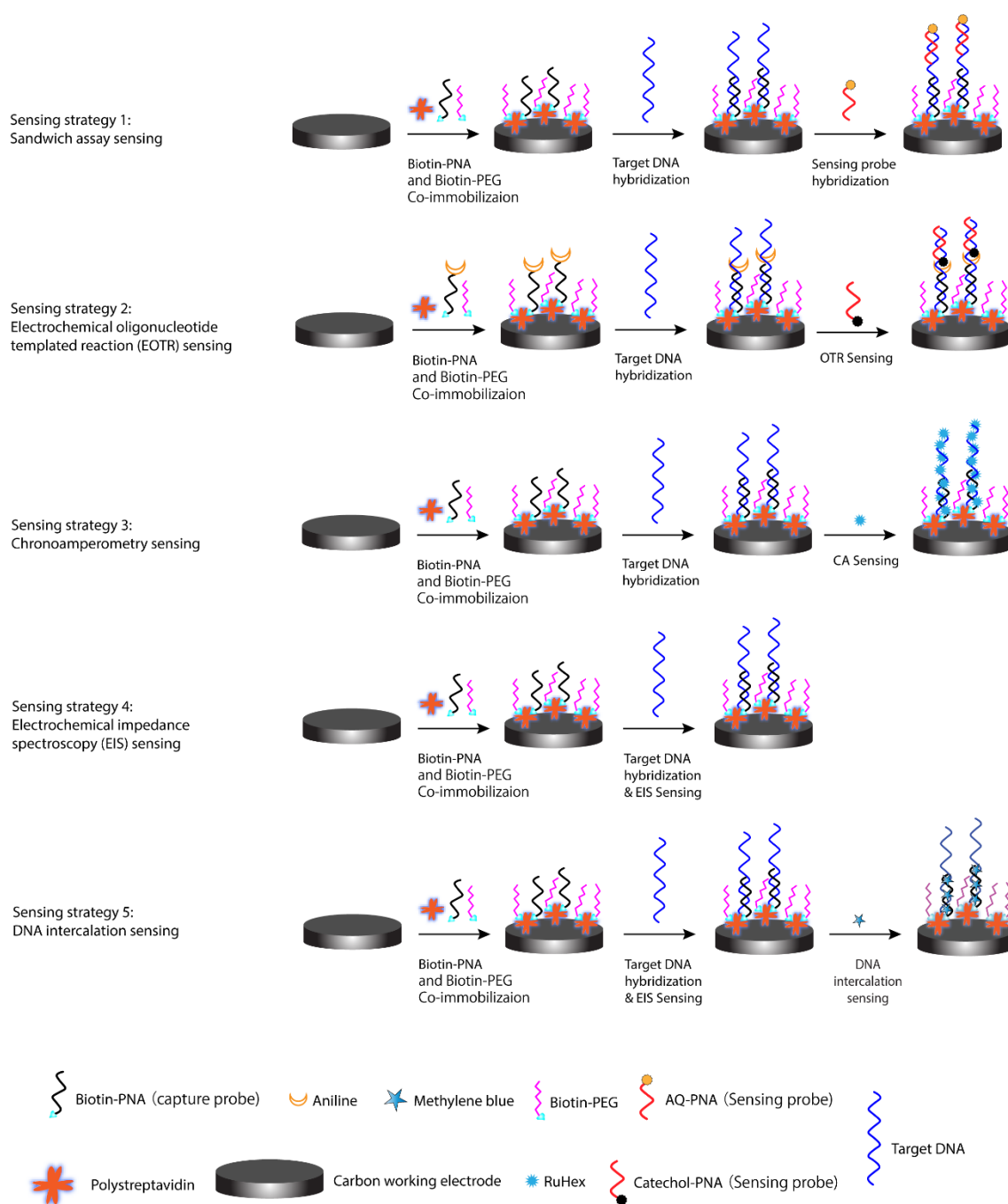


Figure 4-3 Sensing strategies based on polystreptavidin-biotin immobilization method.

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is stepped, and the resulting current from faradic process occurring at the electrode is monitored as a function of time. In CA sensing, hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, noted as RuHex), a redox cation, is used to exchange for native counterions associated with the nucleotide phosphate residues of the probe¹²⁰. As shown in Figure 4-3

sensing strategy 3, RuHex serves as the electroactive indicator, which binds to anionic phosphate of DNA strands in a stoichiometric approach. One RuHex cation can bind to three nucleotide phosphate residues due to electrostatic interaction. And the number of target sequence can be calculated from RuHex signal via chronoamperometry.

EIS is a technique used to investigate properties of electrode reactions in electrochemical systems, which can provide information on the system's electrical and chemical properties³⁰⁴. EIS measures the electrical impedance of an electrochemical system as a function of frequency. An EIS interrogation involves applying a small alternating current (AC) waveform (usually in millivolt range) to an electrochemical system and measuring the resulting AC current response. The stimulation is kept small to ensure that the cell's response is pseudo-linear. By analyzing the impedance of the system as a function of frequency, it is possible to extract information about the system's resistance, capacitance, and inductance. The impedance of an electrochemical system can be analysed as the combination of two components: the resistive component (related to the system's electrical properties) and the capacitive component (related to the system's chemical properties). The capacitive component arises from the charging and discharging of the electrical double layer (EDL) at the electrode-electrolyte interface, which is formed due to the accumulation of charged species (ions or molecules) at the electrode surface.¹¹² As shown in Figure 4-3 sensing strategy 4, EIS measurement should be carried out between each step functionalization to monitor the electrode surface changes. Applying EIS as a sensing method presents a key advantage in that it is a non-label technique.

DNA intercalation is another label-free sensing technique explored in this research. DNA intercalation refers to the insertion of a molecule between the stacked base pairs of double-stranded nucleic acid. As shown in Figure 4-3 sensing strategy 5. The key characteristic of an intercalator is its ability to insert itself between the base pairs of double-stranded nucleic acid, which is facilitated by the planar aromatic structure of the molecule. Methylene blue as a widely used electrochemical reporter is used as a DNA intercalator. Sensing is realized by detecting the methylene blue signal, which is proportional to the concentration of target sequence captured on the electrode surface.

4.2 Results and Discussion

4.2.1 Sandwich Assay

The sandwich assay-based sensing strategy was performed on the polystreptavidin-biotin immobilized SPCEs. Based on the optimum ratio among polystreptavidin, capture probe (Biotin-PNA-374a) and spacer Biotin-PEG (Biotin-(PEG)₆-OH) discussed in section 2.5.1, 500 nM polystreptavidin, 5 μ M Biotin-PNA-374a and 50 μ M Biotin-PEG were used in this section for capture PNA probe immobilization. Different concentrations of DNA mixed with 500 nM sensing PNA (PNA-AQ-374a) in 1X PBS was left at room temperature for 10 minutes to allow hybridization before applying on the sensor. The DNA-PNA mixture (DNA374a and PNA-AQ-374a) was then applied on the capture probe (Biotin-PNA-374a) functionalized electrode for 5 minutes, followed by a thorough wash with 1X PBS. SWV was conducted to test the fully functionalized electrode. DNA141 is used here as a non-complementary control because of the sequence difference between DNA374a and DNA141.

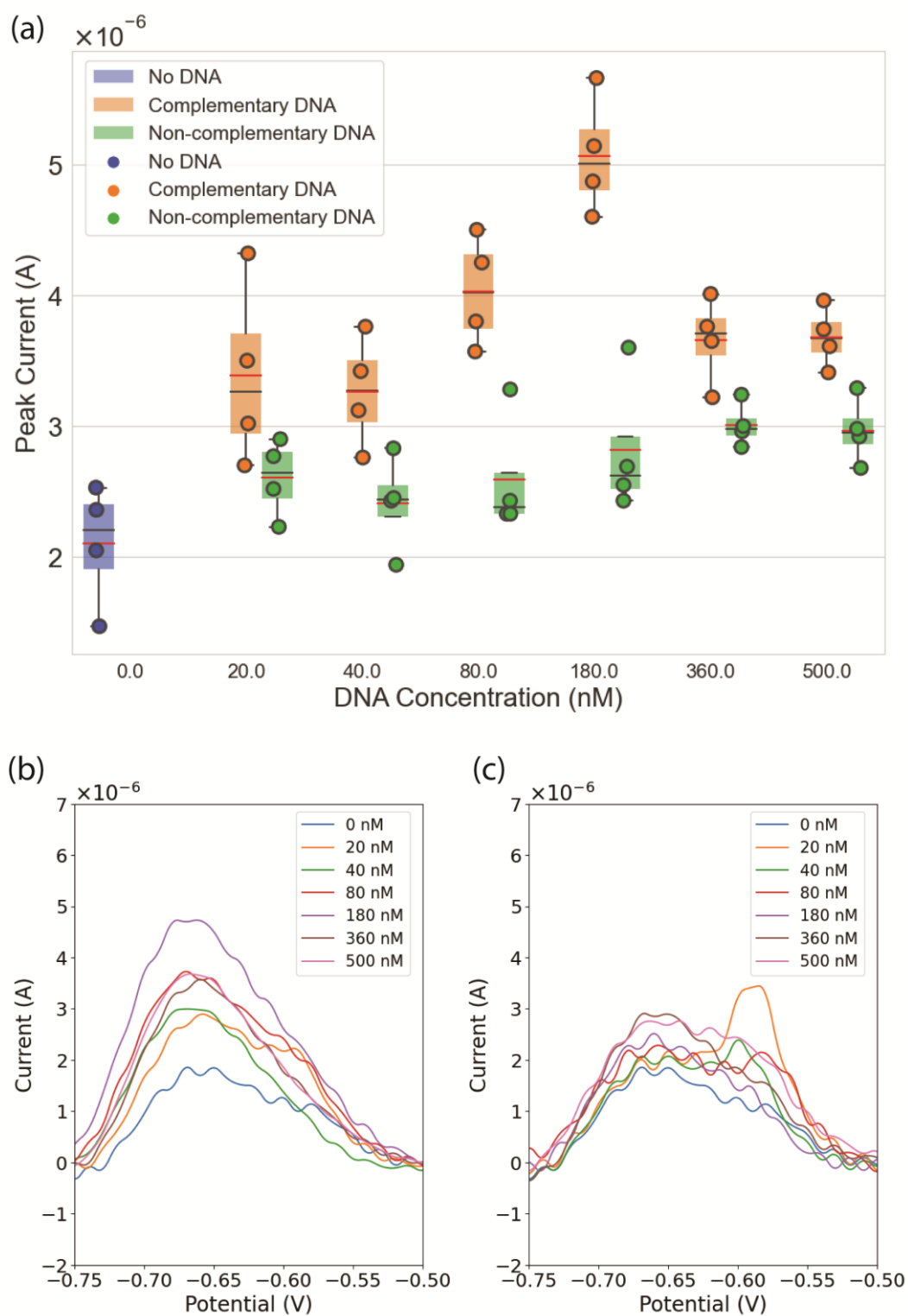


Figure 4-4 Sandwich sensing with PNA-AQ-374a. (a) Box plot of complementary (DNA374a) and non-complementary DNA (DNA141) sensing with PNA-AQ-374a ($n = 4$). (b) SWV data of complementary DNA and (c) SWV data of non-complementary DNA (baseline corrected).

As shown in Figure 4-4, sandwich sensing on polystyrene-avidin treated SPCEs works for nano range sample detection. As illustrated in Figure 4-4 (a), the dynamic range is from 77 nM to 180 nM and limit of detection (LoD) is 77 nM. Observable signal was spotted even without DNA adding, as shown in the blue box and dots in Figure 4-4 (a). This is background noise caused by non-specific interaction of the sensing probe with the electrode surface, most likely because of hydrophobic interactions between the PNA probes. As shown in Figure 4-4 (b) and (c), the original SWV data confirmed the ability of this detection system in discriminating the complementary DNA to non-complementary DNA in the range 77 nM to 180 nM. This range is consistent with the DNA quantification test in Figure 3-3 (a).

The decrease of 360 nM and 500 nM in Figure 4-4 (a) is because of the short 10 minutes hybridization for sensing probe PNA (PNA-AQ-374a) and target sequence. Single stranded DNA diffuse faster than DNA-PNA. This could be improved by allowing longer hybridization time. However, there's a risk of PNA-AQ-374a stick on the tube wall at room temperature.

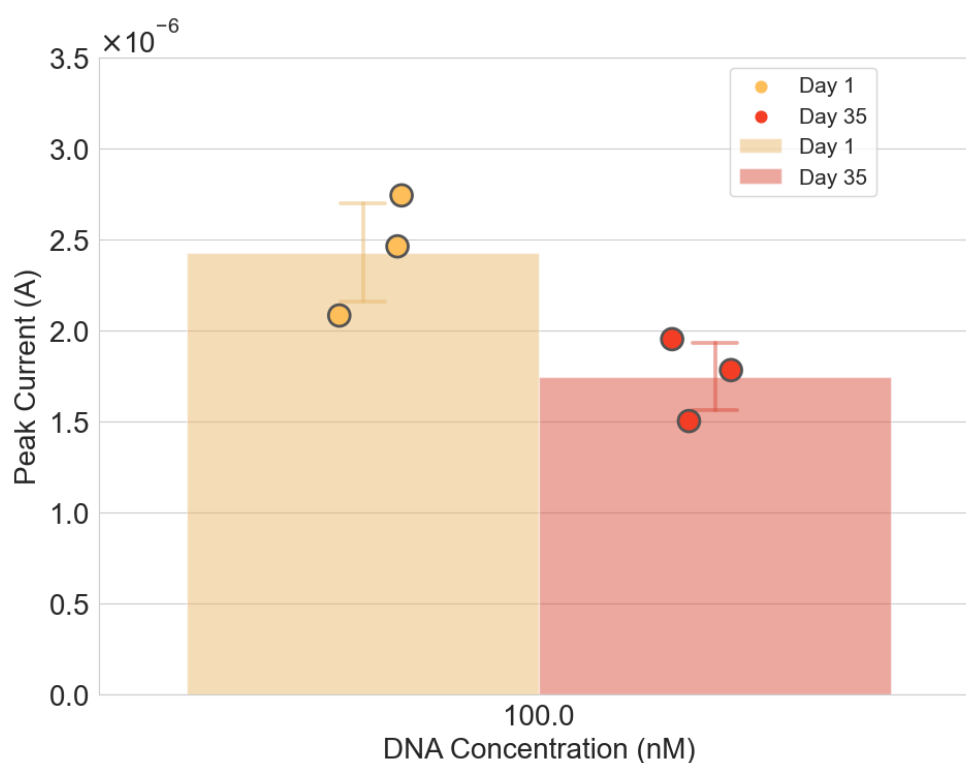


Figure 4-5 Stability test of sandwich assay sensing using PNA-AQ-374a (n=3).

The synthesized sensor by using polystreptavidin-biotin method demonstrated stability at room temperature. As shown in Figure 4-5, by using 100 nM target sequence (DNA374a), the sensor maintained 71.8% signal intensity after 35 days storage at ambient condition.

4.2.2 Electrochemical Oligonucleotide Template Reaction (EOTR)

With similar schematics, EOTR sensing is considered more specific than sandwich assay sensing because EOTR's signal is controlled by an electrochemical reaction, which could result in a decreased background signal from non-specific binding compared to sandwich assay sensing. Because the two essential EOTR PNA probes (capture probe and sensing probe) was designed and synthesized for DNA141, the PNA capture probe used for this experiment is thus Biotin-PNA-Aniline-141 and the PNA sensing probe is PNA-Catechol-141. Based on the optimum ratio among polystreptavidin, capture probe (Biotin-PNA-Aniline-141) and spacer Biotin-PEG (Biotin-(PEG)₆-OH) discussed in section 2.5.1, 500 nM polystreptavidin, 5 mM Biotin-PNA-Aniline-141 and 50 mM Biotin-PEG were used in this section for PNA capture probe immobilization.

As shown in Figure 4-3 sensing strategy 2. A potential at 0.3 V was applied to the electrode for 200 s before DPV test to oxidize the catechol (Scheme 2-1 (1)) so that it is ready to react with aniline for the EOTR. The potential was kept smaller than 0.4 V to avoid aniline polymerization. In the DPV, the potential was swept from -0.3 V to 0.3 V. Potential increment was 0.01 V. Pulse amplitude was 0.05 V. The pulse period was 0.5 s, pulse width was 0.05 s and sample width was 0.02 s. 2 s quiet time was set prior to the DPV scanning to realize a proper initial condition for all scans. The gain was set at 10^{-4} A/V range.

The initial trial with EOTR sensing was carried out by using 100 nM DNA, where the experimental group utilized the complementary sequence DNA141 and the control group employed the non-complementary DNA127. Following full electrode functionalization, which included Biotin-PNA-Aniline-141 immobilization, 20 μ L 100 nM DNA (in 1X PBS) was applied on the working electrode for 20 min followed by a thorough wash. As original raw data of DPV shown in Figure 4-6, two repeats with 100 nM complementary DNA (blue and orange curve) and single measurement with 100 nM non-complementary DNA (green curve) all shown

catechol peaks near 0.2 V and adduct peaks in the range of -0.1 V and 0 V. This indicates that EOTR occurred, but the system was unable to distinguish between the complementary and non-complementary sequences. As shown in Figure 4-6, three more control groups were carried out. Based on the full EOTR system, the red curve represents the DPV test without the presence of the sensing probe (PNA-Catechol-141), the purple curve represents the DPV test without the capture probe (Biotin-PNA-Aniline-141 and spacer Biotin-PEG), and the brown curve represents the DPV test without the DNA target. Significantly, the peak located around 0 V originates from the SPCE itself, potentially arising from gradual silver leakage or other manufacturing-related impurities. This peak overlaps with and hinders the EOTR signal, posing a substantial challenge for employing EOTR in lower concentration sensing applications. The control experiments conducted without the capture PNA probe (purple), without the DNA target (brown), and with the non-complementary DNA (green) all exhibited "adduct" peaks within the range of -0.1 V to 0 V. This phenomenon could stem from non-specific adsorption on the electrode surface or possibly be inherent to the original peak of the SPCE. Consequently, a higher concentration of the target DNA, 500 nM, was employed for testing. The rationale behind this was that if the peak resulting from the 500 nM target sequence is more pronounced than the intrinsic peak near 0 V on the SPCE, then the system might achieve discrimination between the complementary and non-complementary sequences.

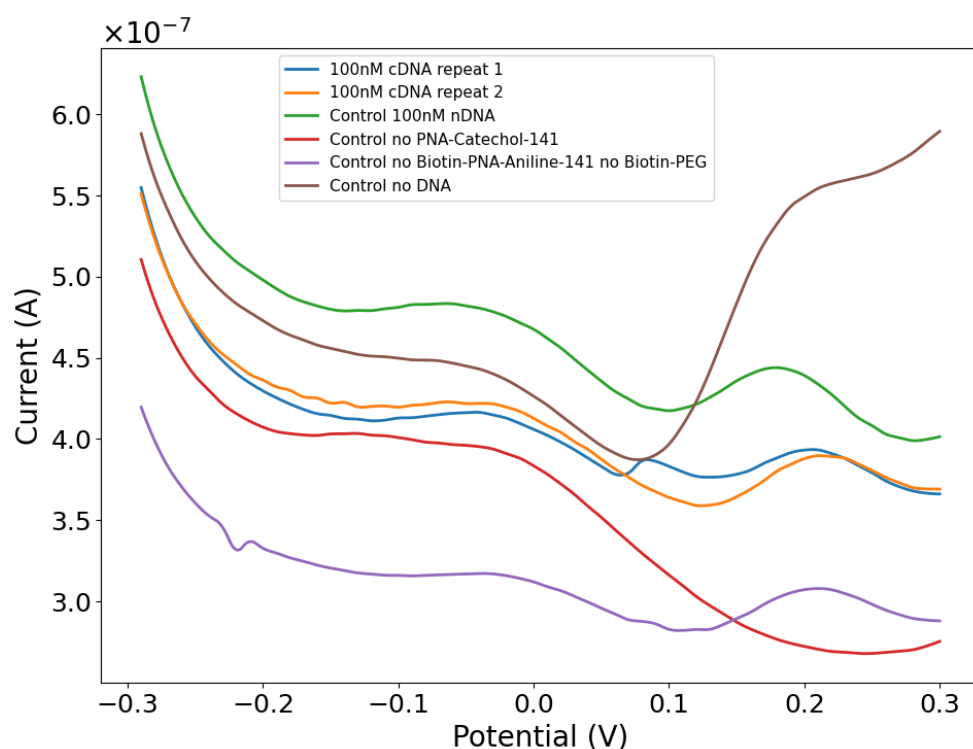


Figure 4-6 DPV investigation of EOTR on SPCE using polystreptavidin-biotin method (cDNA is complementary DNA141 and nDNA is non-complementary DNA127, no base line correction.

The baseline corrected graph see appendix Figure A-2).

Different from experiment in Figure 4-6, target DNA in this test was pre-mixed with PNA-Catechol-141 before applying on the capture probe immobilized SPCE. 2.5 μM of PNA-Catechol-141 sensing probe was mixed with 500 nM complementary and non-complementary DNA, respectively for 1 hour to allow for hybridization before applying on the capture PNA probe immobilized electrode. On the capture probe functionalized electrode, 100 μL of the sensing probe and target DNA duplex was deposited for 30 minutes to allow the target sequence to hybridize to the PNA capture probe. The fully functionalized electrode was then washed thoroughly by 1X PBS prior to electrochemical investigation.

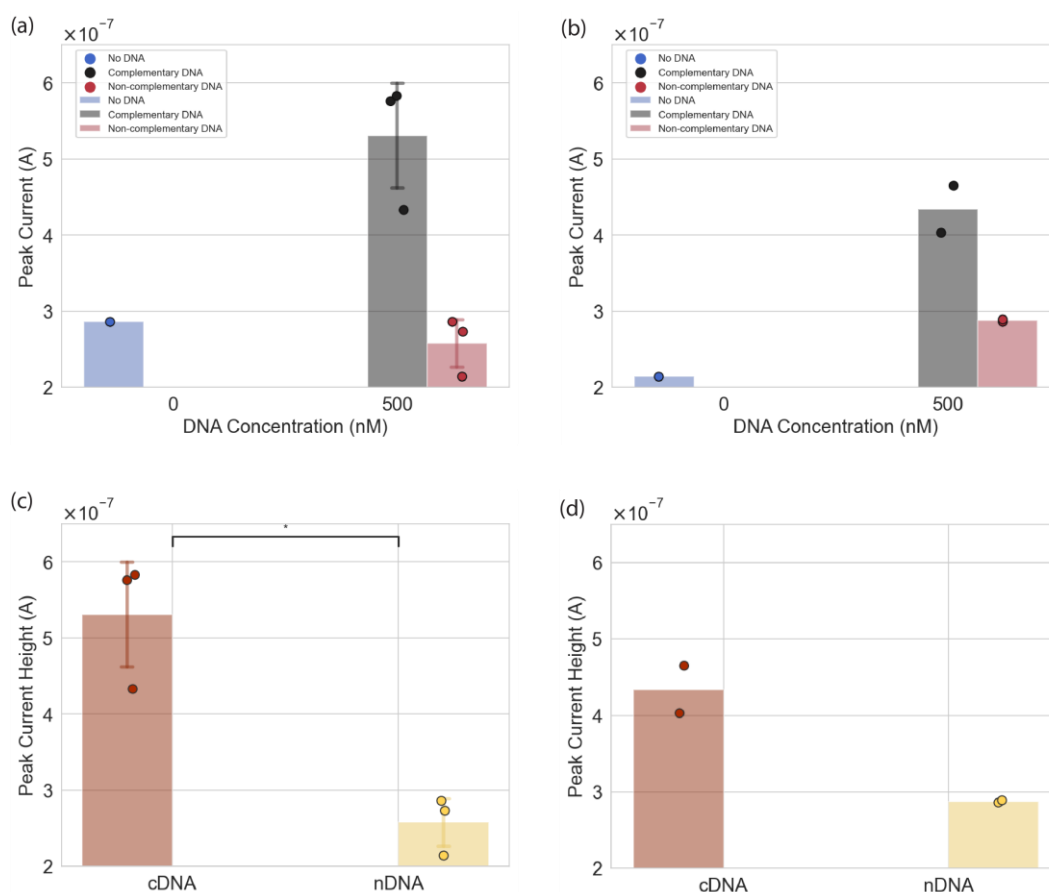


Figure 4-7 Electrochemical oligonucleotide templated reaction (EOTR) sensing with PNA-Catechol-141. (a) SWV adduct peak bar plot of complementary (DNA141) and non-complementary DNA (DNA127) EOTR sensing with PNA-Catechol-141 (b) SWV adduct peak of (a) after treated with 1% BSA for 40 min and washed by 1X PBS. (c) Significance test of 500 nM complementary DNA (cDNA) and non-complementary DNA (nDNA) in (a). (d) Bar plot comparing 500 nM complementary DNA (cDNA) and non-complementary DNA (nDNA) in (b).

The sensing data with 500 nM complementary and non-complementary DNA by investigating the EOTR adduct peak by using SWV is shown in Figure 4-7. The adduct peak locates in the range of -0.1 to 0 V. The system can distinguish the complementary sequence to the non-complementary sequence with statistical significance (Figure 4-7 (a) and (c)). Importantly, the no DNA control and non-complementary DNA group are in similar level, which validated the hypothesis of the less non-specific binding of EOTR compared with sandwich assay. As illustrated in Figure 4-7 (b) and (d), the background signal can be further reduced by treating

the fully functionalized electrode surface with 1% BSA solution for 1 hour and rinse gently with 1X PBS. However, 1% BSA not only decreased the non-specific binding but also weakened the target signal. Comparing Figure 4-7 (c) and (d), the p-value between complementary and non-complementary sequence increased from 0.01749 to 0.1320 before and after BSA treatment by using Welch's t-test, implying that the significant difference decreased. As shown in Figure 4-7 (c) and (d), some peak disappeared after BAS treatment, resulting in only two repeats for Figure 4-7 (d). The limited number of repeats raises concerns about the statistical power of the analysis. Given the small sample size of two repeats for the test, the statistical significance was not displayed on the graph.

Inspired by the promising results in Figure 4-7, more target DNA concentrations using EOTR sensing method on the fully functionalized SPCE was tested by SWV. When varying the DNA target concentration from 500 pM to 500 nM, as shown in Figure 4-8 (a), no clear trend was observed despite the fact that the complementary DNA groups always have stronger signal than the non-complementary DNA groups. To delve deeper into this experiment, surface optimization was conducted by implementing a 1% BSA passivation step on all the electrodes analyzed in Figure 4-8 (a). The purpose of this passivation was to mitigate non-specific binding and suppress background peaks originating from the manufacturing process. The results of this optimization are depicted in Figure 4-8 (b).

Different from the data shown in Figure 4-7 (b) and (d), where the electrode with target sequence underwent a 40-minute treatment by 1% BSA, electrodes in Figure 4-8 (b) were subjected to a 1-hour treatment with 1% BSA. Following this treatment, the electrodes were thoroughly washed with 1X PBS before the testing with SWV again. Interestingly, after BSA treatment, there was an intriguing increase in the adduct signal originating from complementary DNA. Conversely, all of the adduct signals from the control groups, utilizing non-complementary DNA, exhibited a certain degree of reduction. This comparison is evident when comparing Figure 4-8 (a, before BSA treatment) and (b, after BSA treatment). The increase of adduct peak after BSA treatment is potentially because of the reduced probe density and improved probe orientation. As shown in Figure 4-8 (c) and (d), adduct peak distribution before and after BSA treatment have shifted from -0.03 V to 0.05 V. A possible explanation for this peak shift is the probe density change induced surface change.

However, overall the sensor was not able to distinguish between the complementary and non-complementary DNA in the range of 500 pM to 500 nM with a clear trend. It is reasonable to speculate the dynamic linear range may exist in lower pico molar range. Although it is impossible to rule out that the sensor may perform better at lower DNA concentrations (in the lower pM range), investigations with EOTR sensing were stopped because of recurrent issues caused by the electrode manufacturing defect generating a variable peak within the range of -0.1 to 0 V (as shown in Figure 4-6 red curve).

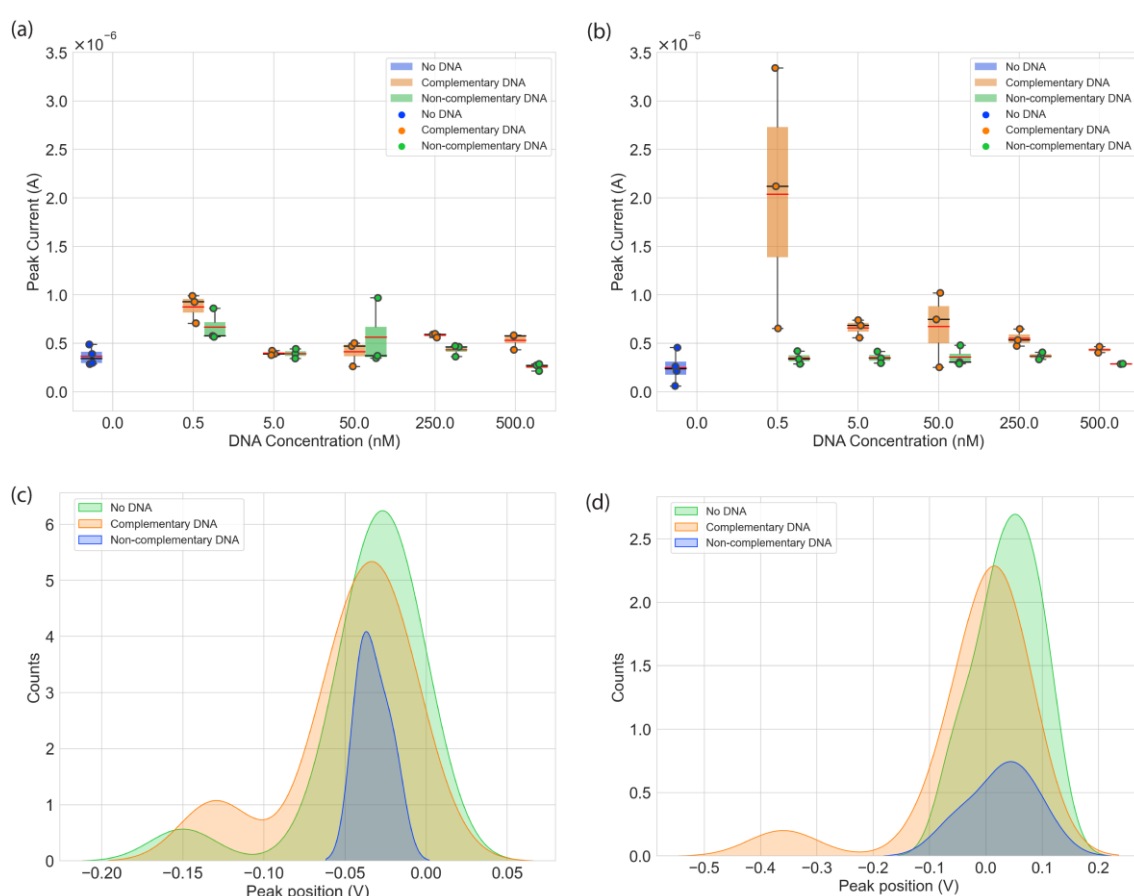


Figure 4-8 Electrochemical oligonucleotide templated reaction (EOTR) sensing with PNA-Catechol-141. (a) Adduct peak box plot of complementary (DNA141) and non-complementary DNA (DNA127) sensing with PNA-Catechol-141 by SWV. (b) Adduct peak box plot of complementary (DNA141) and non-complementary DNA (DNA127) sensing with PNA-Catechol-141 by SWV after 1 h treatment with 1% BSA followed by PBS washing. (c) Adduct peak distribution of (a). (d) Adduct peak distribution of (b).

4.2.3 Chronoamperometry

Chronoamperometry provides valuable information about the kinetics and mechanisms of electrochemical reactions occurring at the electrode surface. As shown in equation 4.4, current recorded during a chronoamperometry test is composed of three parts: diffusion current, double-layer current and current from immobilized redox species at the surface of the working electrode. After integration with time, charge Q is expressed by three parts as well: diffusion charge, double-layer charge and charge from immobilized redox species, as displayed in equation 4.5.

Diffusion current:

$$i_{diff} = \frac{nFA\sqrt{D}C_{\infty}}{\sqrt{\pi t}} \quad 4.1$$

Double layer current:

$$i_{dl} = \frac{\Delta E}{R} \exp\left(-\frac{t}{RC}\right) \quad 4.2$$

Current from immobilized redox species:

$$i_{ads} = i_{\Gamma^*} \quad 4.3$$

Total current:

$$i = \frac{nFA\sqrt{D}C_{\infty}}{\sqrt{\pi t}} + \frac{\Delta E}{R} \exp\left(-\frac{t}{RC}\right) + i_{\Gamma^*} \quad 4.4$$

Total current integrated with time t :

$$Q = \frac{2nFA\sqrt{D}C_{\infty}}{\sqrt{\pi}}\sqrt{t} - C\Delta E \exp\left(-\frac{t}{RC}\right) + nFA\Gamma^* \quad 4.5$$

i_{diff} is diffusion current.

i_{dl} is double layer charging.

i_{ads} is current from immobilized redox species.

n is the number of electrons per molecule for reduction.

F is the Faraday constant (96485.3329 C/mol).

A is the real electrode surface area.

D is the diffusion coefficient in m^2/s .

π is the ratio of a circle's circumference to its diameter (3.1415926).

t is time in seconds.

ΔE is the potential step applied during the experiment in volts.

R is the resistor value in ohms.

C is the capacitor value in Farads.

i_{Γ^*} is Faradaic current generated by the redox reporter adsorbed on the electrode surface in amperes.

Q is charge in coulombs.

As can be seen from equation 4.5, diffusion and double layer resulted charge are related to time, while adsorbed redox species charge is not. This means when $t = 0$, $Q = Q_{ads} + Q_{dl} = nFA\Gamma^* + C\Delta E$. Therefore, $Q_{t=0}$ eliminated diffusion charge $\frac{2nFA\sqrt{D}C_{\infty}}{\sqrt{\pi}}\sqrt{t}$. And Q_{dl} is a constant that no longer changes with time t . Based on this, the immobilized redox species is determined from the difference between chronocoulometric intercepts for the identical chronoamperometry experiments in the presence and absence of the target sequence.

The saturated surface excess of redox marker is converted to DNA probe surface density with the relationship:

$$\Gamma_{DNA} = \Gamma^* \left(\frac{z}{m} \right) N_A \quad 4.6$$

Where Γ_{DNA} is the probe surface density in molecules/ cm^2 , m is the number of bases in the probe DNA, which refers to 22 nucleotides (nt) for both the complementary sequence (DNA141) and non-complementary control sequence (DNA150). N_A is Avogadro's number (6.02×10^{23}) and z is the charge of the redox molecule, which is 3 for the case of RuHex.

4.2.3.1 Chronoamperometry with polystreptavidin-biotin system

On the PNA immobilized surface, different concentrations of complementary DNA141 and non-complementary DNA150 was applied on the functionalized working electrode from low to high (1 aM, 1 fM, 1 pM, 1 nM and 1 μ M in 1X PBS) with a total volume of 20 μ L for 10 min to allow hybridization, respectively. Chronoamperometry was carried out after probe immobilization and each step DNA hybridization with different concentrations.

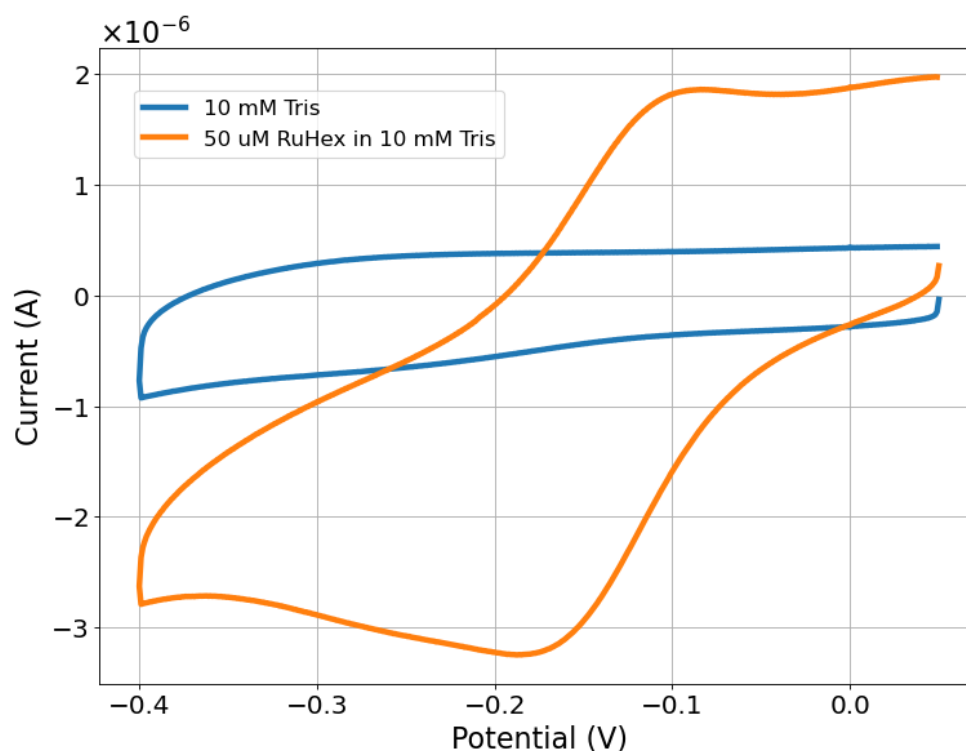


Figure 4-9 Cyclic voltammetry characterization of 50 μ M RuHex in 10 mM Tris buffer with 100 mV/s scan rate.

The CV graph in Figure 4-9 shown the oxidation peak of RuHex is at -0.084 V and the reduction peak is at -0.185 V. Which gave guidance on chronoamperometry experiment settings. In the chronoamperometry experiment, chronoamperometry on SPCE was conducted by using the following parameters:^{120,305} The initial potential was set at 0 V. The high potential was set to 0.2 V, while the low potential was set to -0.5 V. The chronoamperometry steps initiated from positive to negative, totalling 2 steps with each step lasting for 0.25 s. The pulse width was 0.25 s and the sample Interval was 0.002 s. Quiet time was set as 2 s and the sensitivity was set as 10^{-4} .

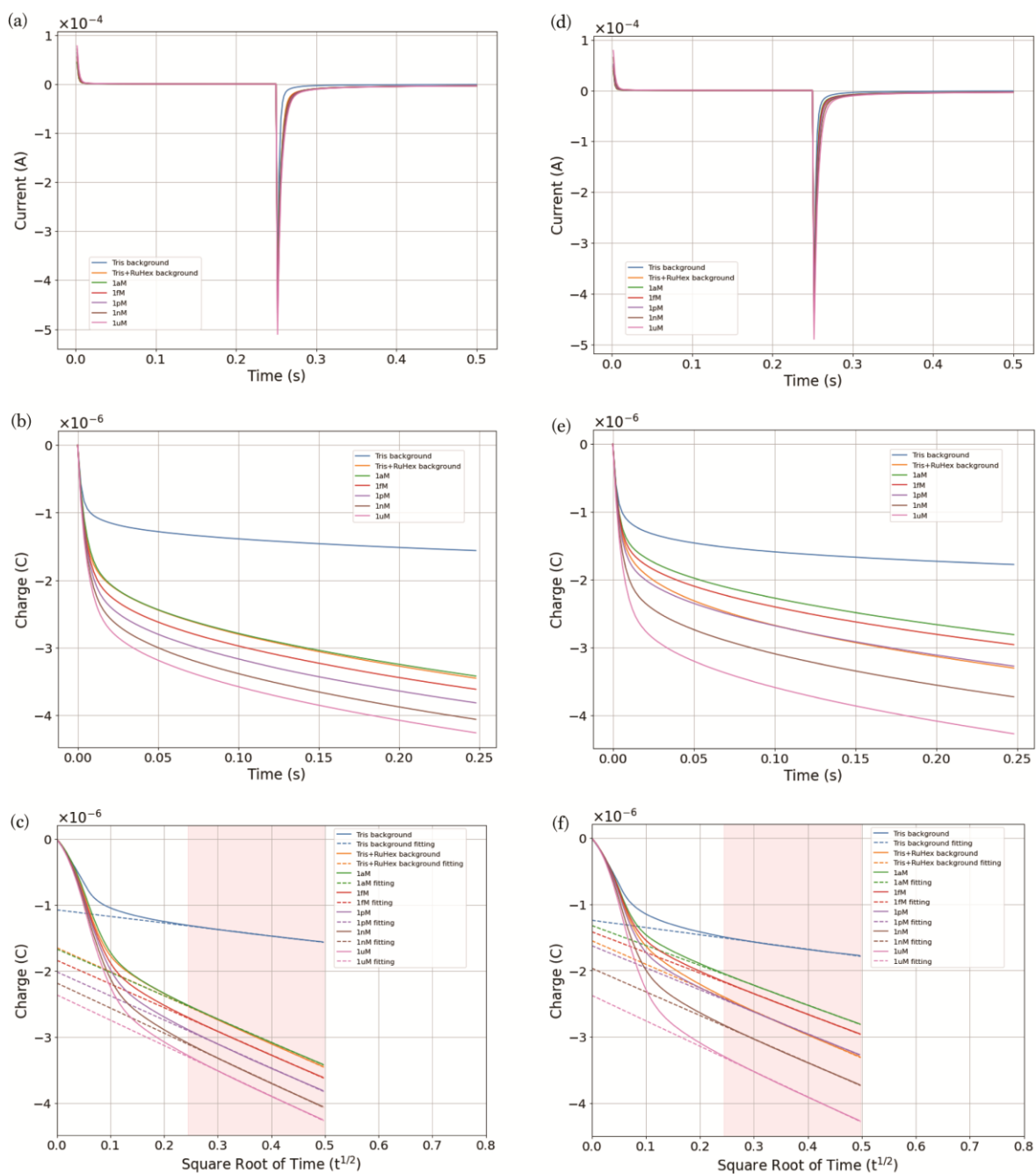


Figure 4-10 Chronoamperometry data for charge fitting and calculation with complementary DNA. (a) Original chronoamperometry data. (b) Charge calculated from the reduction part by integrating current with time. (c) Charge fitting by excluding the diffusion and double layer effect. (d) (e) (f) are another group repeating (a) (b) (c).

As shown in the original chronoamperometry data in Figure 4-10 (a), the working electrode was kept at a positive potential for the purpose of holding the $[Ru(NH_3)_6]^{3+}$ cation in the 3+

state to realize a consensus initial state for the boundary condition. The value is speculated from the cyclic voltammetry of 50 μM $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in Tris buffer, as shown in Figure 4-9, where 0.2 V is a reasonable value for this purpose. Then the electrode surface was reduced by applying a -0.5 V potential for 0.25s. The current gathered in this state includes diffusing current, double layer current and current from $[\text{Ru}(\text{NH}_3)_6]^{3+}$ reporters that electrostatically bind to the immobilized redox species. This reduction current was integrated with time. The resulted charge is shown in Figure 4-10 (b). The linear fitting of reduction charge is shown in Figure 4-10 (c). The purpose of fitting is to eliminate the charge from double layer and diffusion by operating a deduction with the fitted intercept, according to equation 4.5. The same data processing was used for the control group where a non-complementary DNA was used (Figure 4-11).

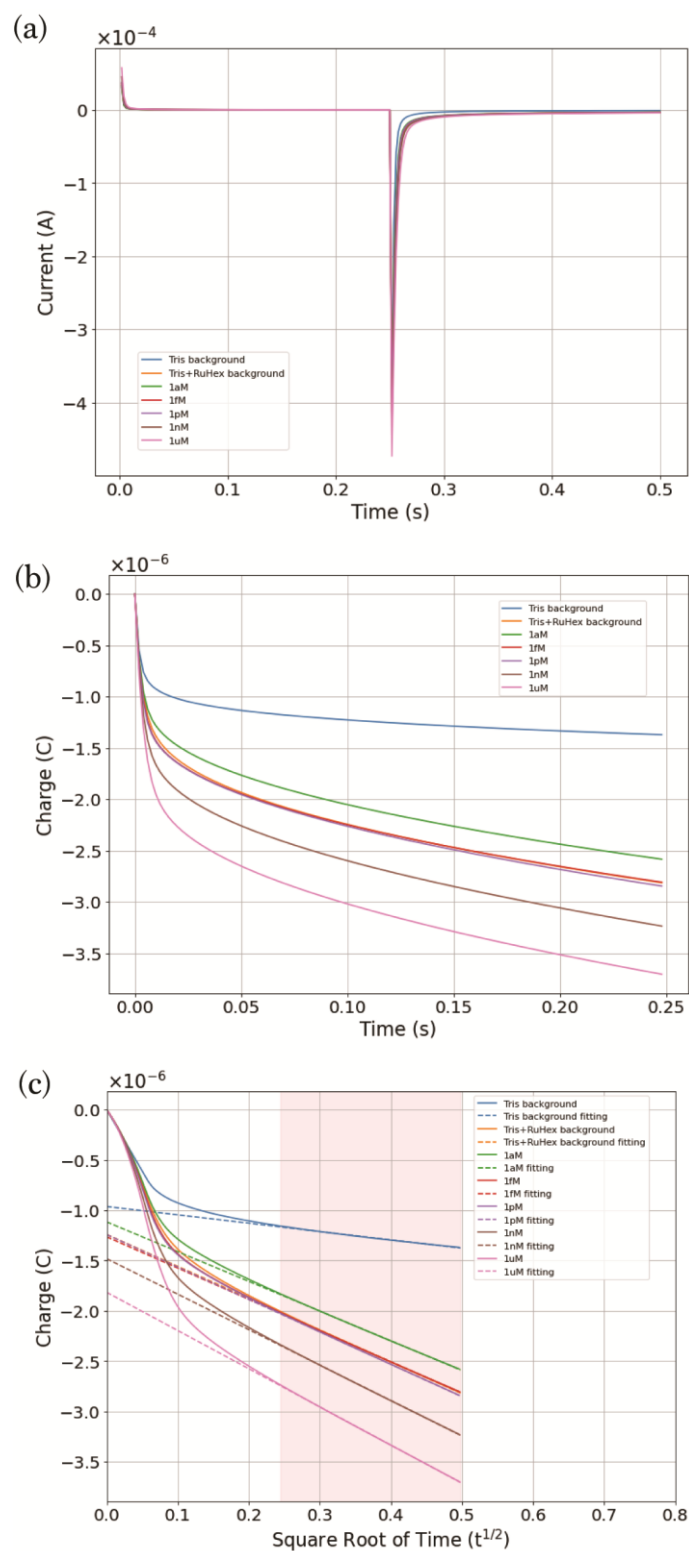


Figure 4-11 Chronoamperometry data for charge fitting and calculation with non-complementary DNA. (a) Original chronoamperometry data. (b) Charge calculated from the reduction part by integrating current with time. (c) Charge fitting by excluding the diffusion and double layer effect.

The charge from surface bound RuHex that electrostatically bind to target DNA was calculated and results are shown on Figure 4-12. Despite the trend in the increase of charge with increasing DNA concentration and the distinct difference between the complementary and non-complementary sequence, the calculated total maximum charge on the surfaces indicated that the background signal was much stronger than the real signal of interest, preventing an accurate measurement in the atto-molar to pico-molar range. Another potential explanation is that the system's dynamic range is not within the atto-molar to pico-molar. With only the nano-molar range data making sensing, TWEEN was applied on the system to reduce the effect of non-specific binding.

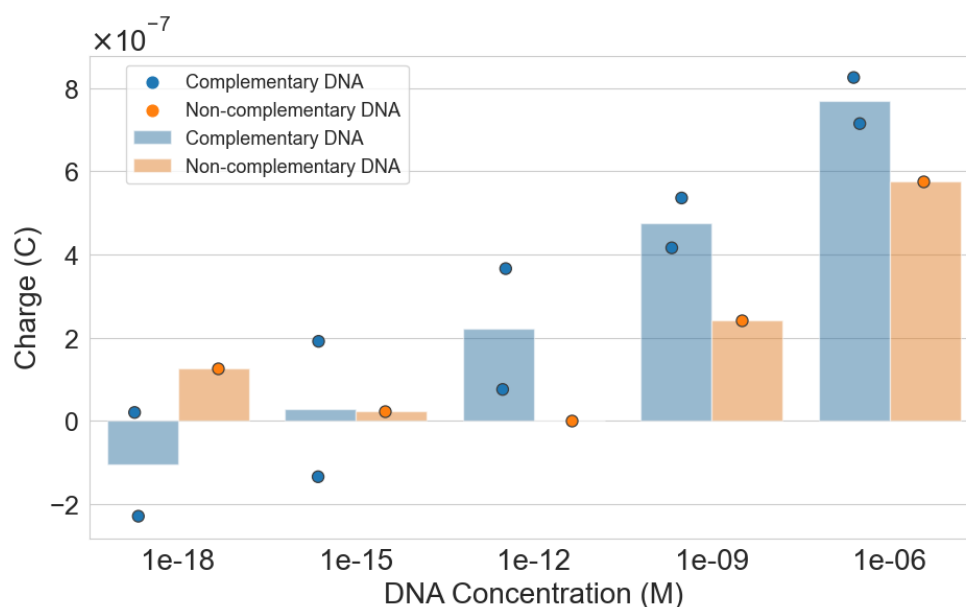


Figure 4-12 Charge associated with immobilized species.

4.2.3.2 TWEEN Effect on Chronoamperometry with polystreptavidin-biotin system

In the previous section, chronoamperometry results were affected by the strong background signal potentially from the non-specific binding of non-complementary sequence or accumulation of RuHex at the electrode surface. Therefore, TWEEN was applied to reduce the background signal. 0.5% TWEEN in PBS was used to wash the fully functionalized working electrode. A preliminary result with 500 nM complementary DNA and a no DNA control (two

repeats each) was carried out. With such small sample size, it becomes difficult to draw reliable conclusions from the test results, and the statistical power of the test may be low. Nevertheless, a Wilcoxon test with Bonferroni correction has been conducted. And the p value of the statistical test is 0.5, not significant. Further analysis about the results are limited to the changed shape of the integrated charge (Figure A-3 and Figure A-4).

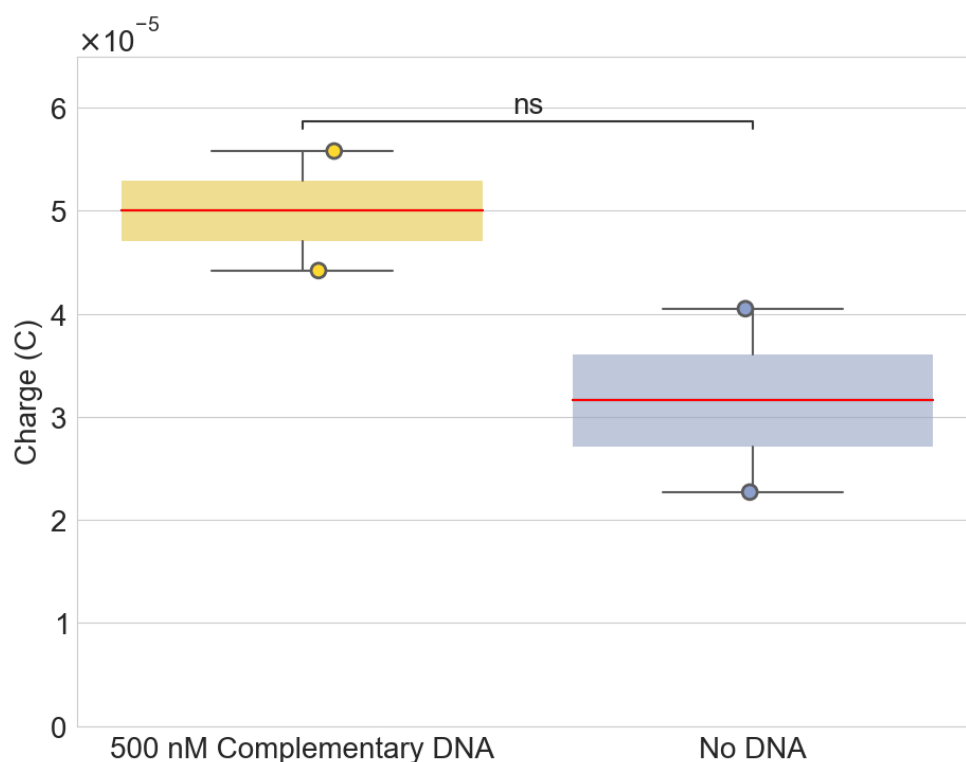


Figure 4-13 Surface charge from RuHex with 500 nM complementary DNA (DNA141) after 0.5% TWEEN washing.

4.2.3.3 Comparing Single Measurement and Multiple Measurements on Dropsens Electrode

To investigate if the multiple washing steps and measurements on the same electrode degrades the functionalized surface, comparison was conducted between two groups where single measurement (with single wash step) and multiple measurement (with multiple wash steps) were carried out, respectively.

4.2.3.3.1 Single Measurement

In this experiment, all electrodes were considered identical, where in reality there will always be differences because of the manufacturing process. Figure 4-14 (a) shows that comparing chronoamperometry based on different electrodes was not possible for the polystreptavidin-biotin system as the system was not able to distinguish the experimental group from the control. By doing a Wilcoxon test with Bonferroni correction between the 500 nM complementary and non-complementary DNA after subtracting the background charge (no DNA control), as shown in Figure 4-14 (b), the p value is 0.625, no significant. This is because:

1. The difference among the electrodes posted significant effect on the result. And this electrode difference was more pronounced than the differences observed in our results based on multiple target concentrations from the chronoamperometry measurements.
2. RuHex adsorb on both the carbon electrode³⁰⁶ and the PNA-bounded electrode.

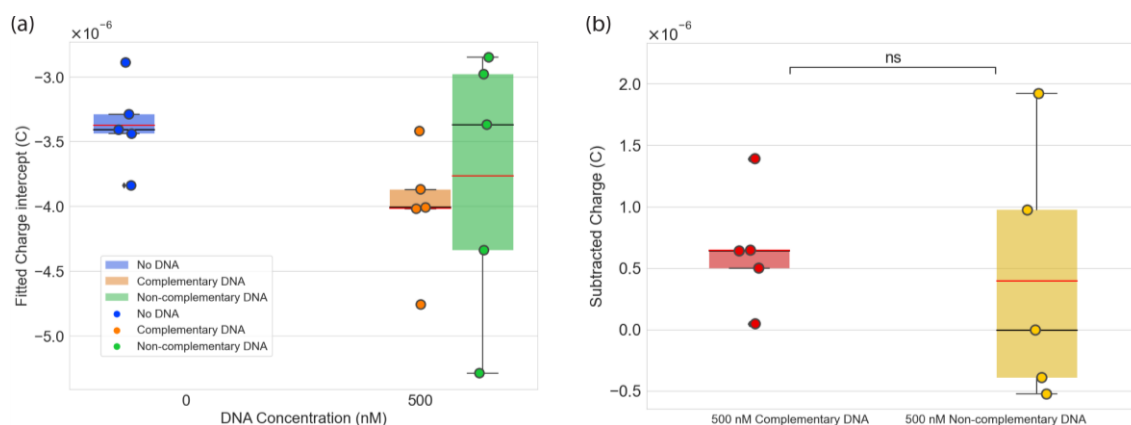


Figure 4-14 (a) Chronoamperometry with single measurement (after washing with 1X PBS)
 (b) Statistical test between 500 nM complementary DNA (DNA141) and non-complementary DNA (DNA150).

4.2.3.3.2 Multiple Measurements on the Same Electrode

Here, multiple measurements were all made on the same electrode for background measurement and charge calculation after DNA hybridization. As shown in Figure 4-15 and Figure 4-16 (a), there is an observable increase for the complementary DNA from 100 nM to 500 nM, where the control remains in the same level of signal intensity with a non-complementary DNA. However, the statistical analysis by using a Wilcoxon test with Bonferroni correction between the 100 nM and 500 nM complementary DNA presented a p value of 0.125. Which means the difference is not statistically significant. The reproducibility of this sensing strategy needs improvement before it can be considered as a reliable method.

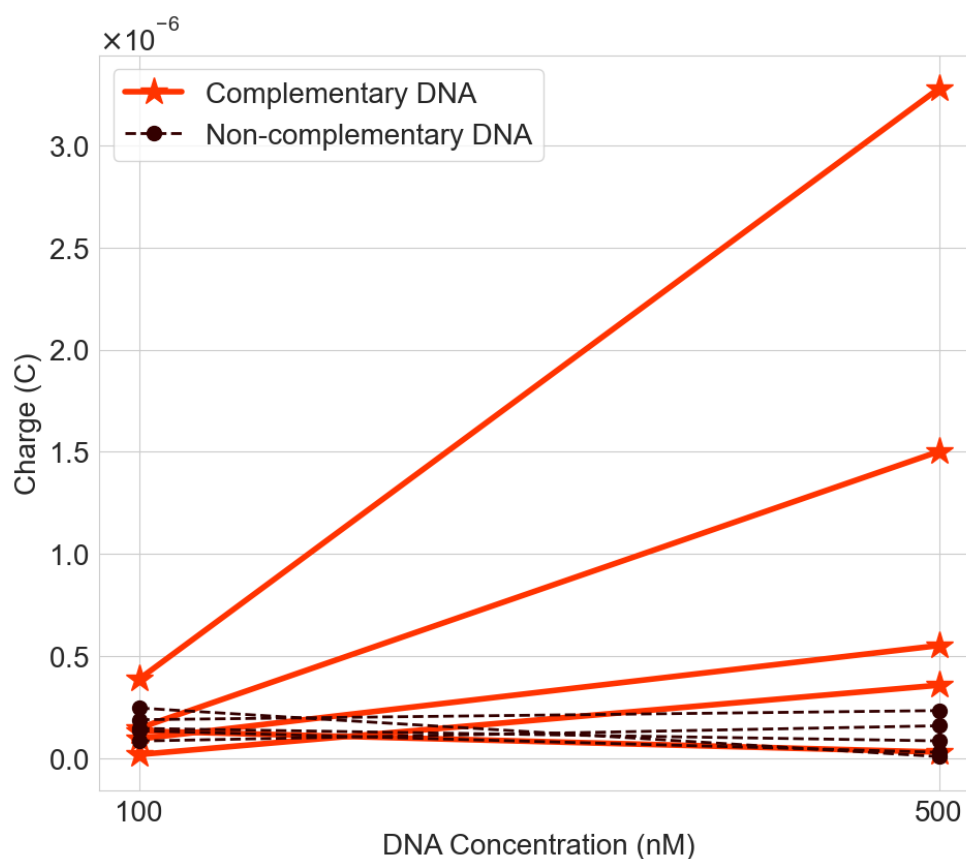


Figure 4-15 Chronoamperometry with single measurements (after washing with 1X PBS), one-to-one tracking line plot.

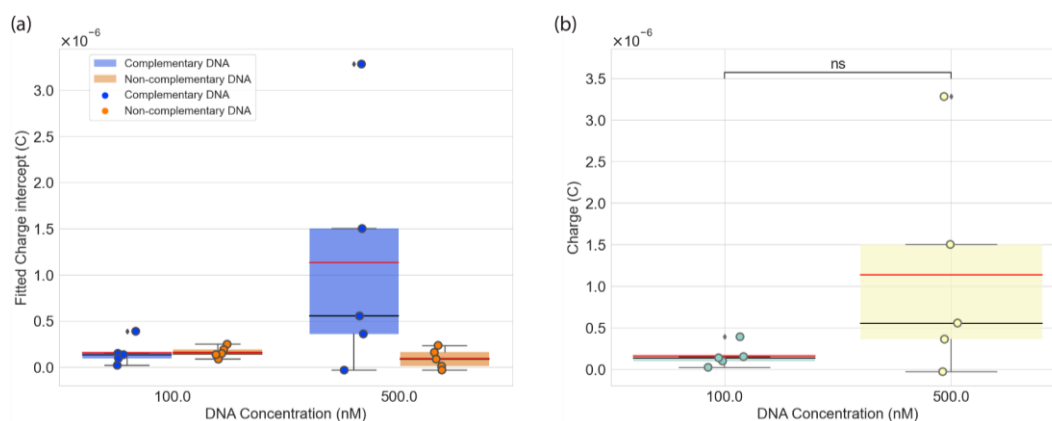


Figure 4-16 Chronoamperometry with multiple measurements (with multiple wash steps). (a) Box plot with 100 nM and 500 nM complementary (DNA141) and non-complementary DNA (DNA150). (b) Statistical test between 100 nM and 500 nM complementary DNA (n=5).

4.2.4 Electrochemical Impedance Spectroscopy (EIS)

EIS sensing was conducted by using Biotin-PNA-Aniline-141 as capture PNA probe immobilized on SPCE and DNA141 as target sequence. DNA150 was using as a non-complementary control sequence. EIS measurements were made in the presence of 1.25 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 100 mM KCl, where the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ works as a redox probe. A consistent response current was obtained after the incubation with complementary and non-complementary DNA for 20 minutes and washing, respectively.

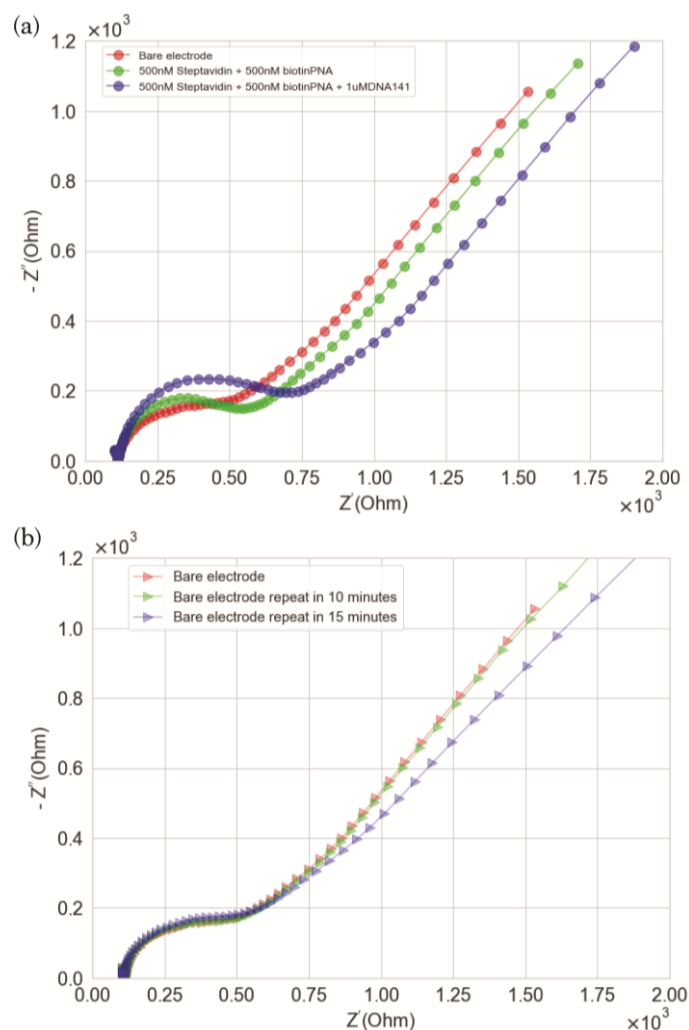


Figure 4-17 EIS measurements using SPCE. (a) Experimental group with three EIS interrogations on 1. bare SPCE, 2. polystreptavidin and Biotin-PNA-Aniline-141 treated SPCE, and 3. 1 μM complementary DNA (DNA141) treated SPCE with polystreptavidin and Biotin-PNA-Aniline-141. (b) Control group with three EIS interrogations on bare SPCE treated with 1X PBS, repeated for three time by different time intervals.

An initial EIS measurement was made on polystreptavidin and Biotin-PNA-Aniline-141 treated SPCE. On the same electrode, after 20 minutes of DNA incubation, a second EIS test was performed in the same buffer solution after washing the surface gently with 1.25 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 100 mM KCl buffer solution to maintain a constant ionic strength.

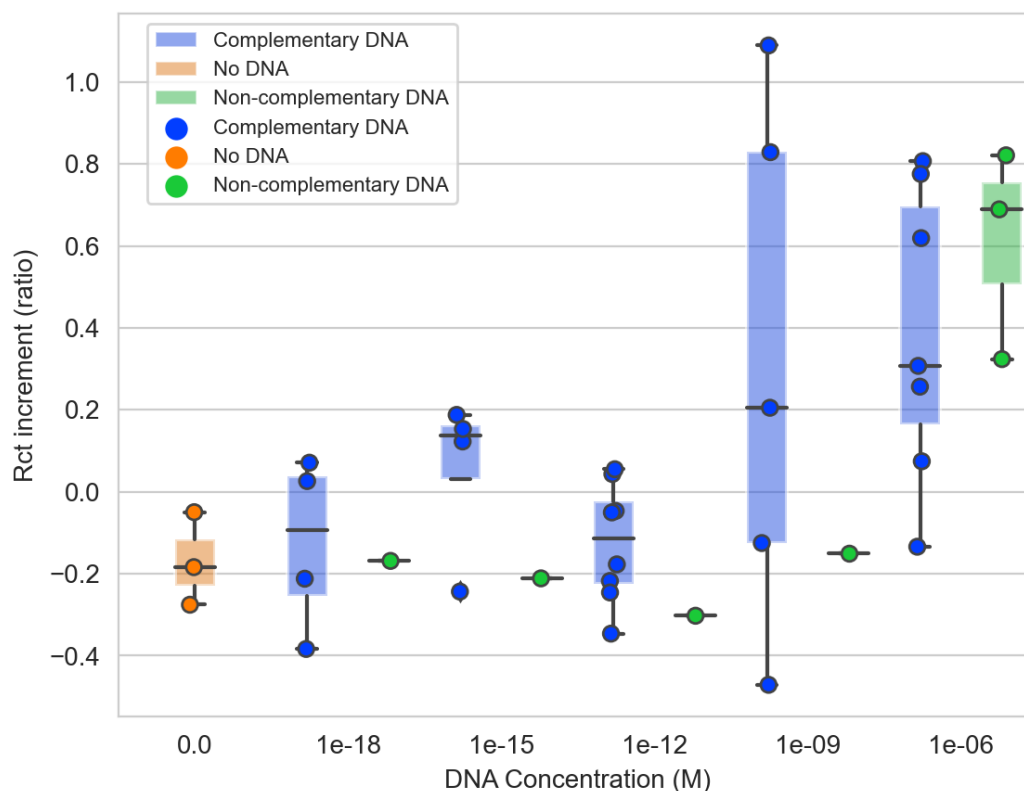


Figure 4-18 EIS sensing with wide range complementary and non-complementary DNA.

As shown in Figure 4-18, both complementary (DNA141) and non-complementary DNA (DNA150) were detected by EIS. DNA150 is used as a control for the complementary sequence DNA141 according to the Biotin-PNA-Aniline-141 (Table 4-1). In the atto-molar to pico-molar range, negative charge transfer resistance values were observed that are possibly due to the immobilized polystreptavidin film partially detaching from the electrode surface during the 4-minute-long EIS measurement with AC potential applied consistently. In the nM range, the positive values shown the capability of detecting the target sequence, although with a significant lack of sequence specificity as the system was unable to distinguish between the complementary DNA and non-complementary DNA.

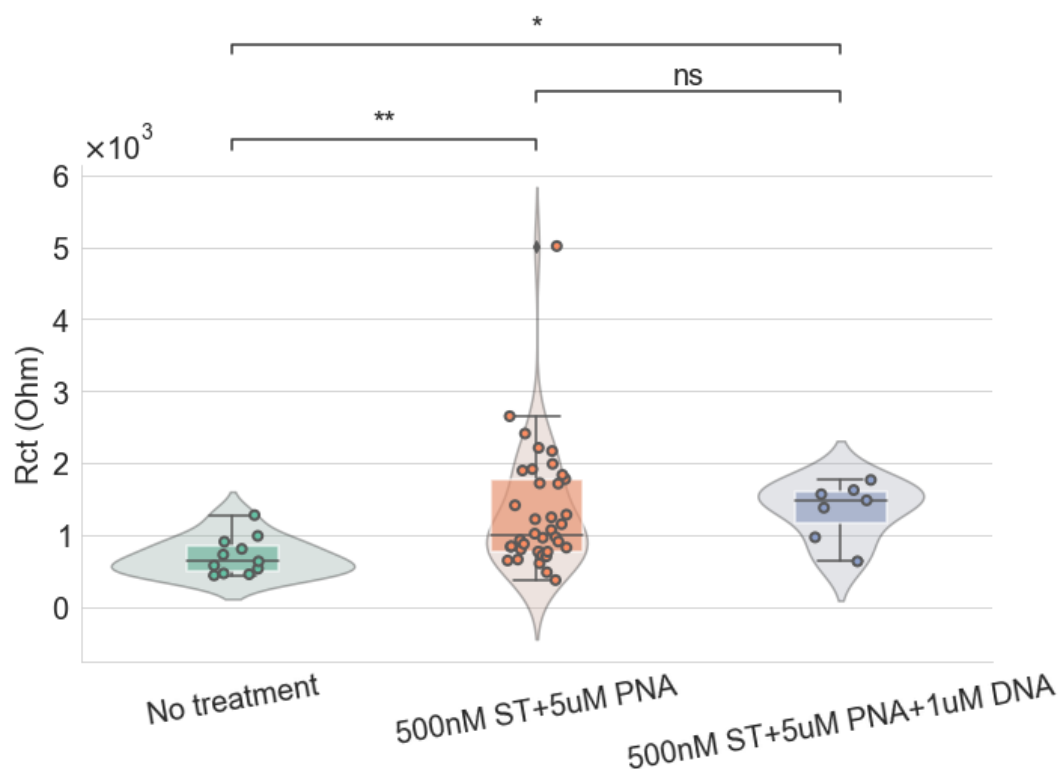


Figure 4-19 Surface change with each step surface treatment by EIS. ST=streptavidin, PNA= Biotin-PNA-Aniline-141, DNA=DNA141, n=11, 38 and 7.

Figure 4-19 shows that charge transfer resistance R_{ct} changes between each step after washing. With multiple repeats, there's a statistical significance exist between no functionalized electrode (bare electrode) and PNA-immobilized electrode. Significant tests among multiple groups were analyzed by using the Kruskal-Wallis test with Bonferroni correction, followed by the Dunn's test for post hoc pairwise comparisons. Kruskal-Wallis test with Bonferroni correction, rather than ANOVA, was use for stronger statistical power. Because data collected in Figure 4-19 did not fit normal distribution (this does not mean the data in theory is not normally distributed), according to QQ plot. P value between the bare electrode (no treatment control) and PNA probe immobilized electrode (500 nM polystreptavidin+ 5 μ M capture PNA) is 0.009617. P value between the bare electrode and fully factionalized electrode (500 nM polystreptavidin+ 5 μ M capture PNA + 1 μ M complementary DNA) is 0.013. And there's no significant difference between PNA probe

immobilized electrode and fully factionalized electrode. However, the 4-minute-long EIS measurement with AC potential applied consistently and multiple washing steps between each step most likely contributed to partial degradation of the functionalized electrode. As shown in Figure 4-20, no significant improvement was observed after addition of 0.5% TWEEN during the washing steps.

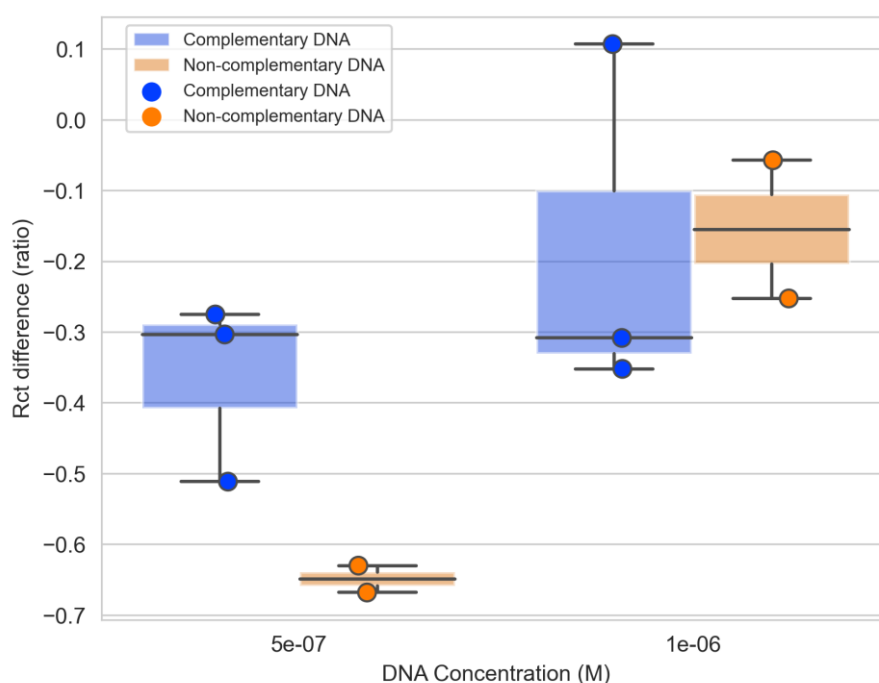


Figure 4-20 EIS measurements with 500 nM and 1 μ M complementary (DNA141) and non-complementary DNA (DNA150) followed by 0.5% TWEEN washing.

4.2.5 DNA Intercalation

Like EIS, DNA intercalation is another label-free sensing technique tested in this project. After capture PNA (Biotin-PNA-374a) immobilization via polystreptavidin-biotin method, 20 μ L DNA sample dispersed in 1X PBS was dropped on the functionalized electrode for 20 minutes to allow hybridization with the complementary sequence (DNA374a in this case). A non-complementary DNA141 (non-complementary to Biotin-PNA-374a) was used as a control. After the DNA hybridization, different concentrations of freely diffusing MB (from 5 μ M to

200 μM) in 1X PBS was added. After 10-minute incubation, SWV scans were recorded before and after washing the electrode with 1X PBS.

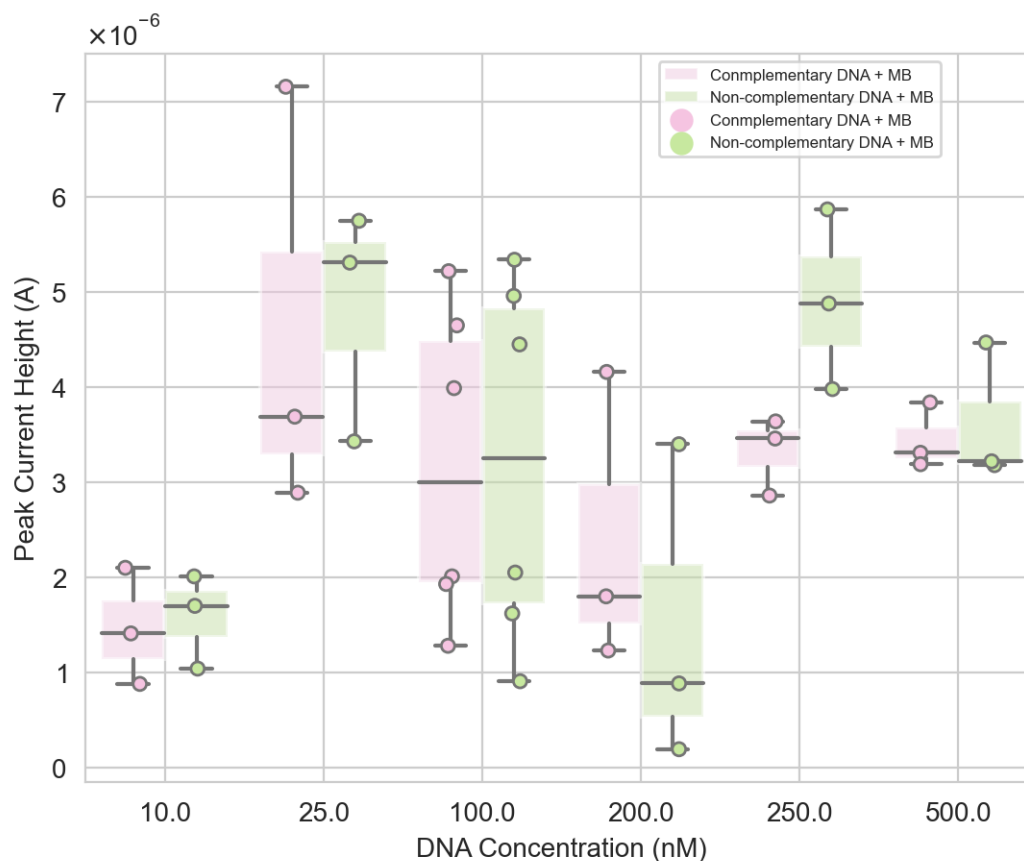


Figure 4-21 DNA intercalation sensing by SWV using 200 μM MB for 10 minutes without wash.

As shown in Figure 4-21, incubation with 200 μM MB for 10 minutes was first tested without washing. This assumes that surface bound MB signal is stronger than the MB signal remaining freely diffusing in solution. However, no clear trend was observed at such high MB concentration and lower concentrations of 20 μM and 5 μM MB were tested together with the addition of washing steps to remove unbound MB and reduce background noise, as shown in Figure 4-22. Unfortunately, this led to no significant improvement.

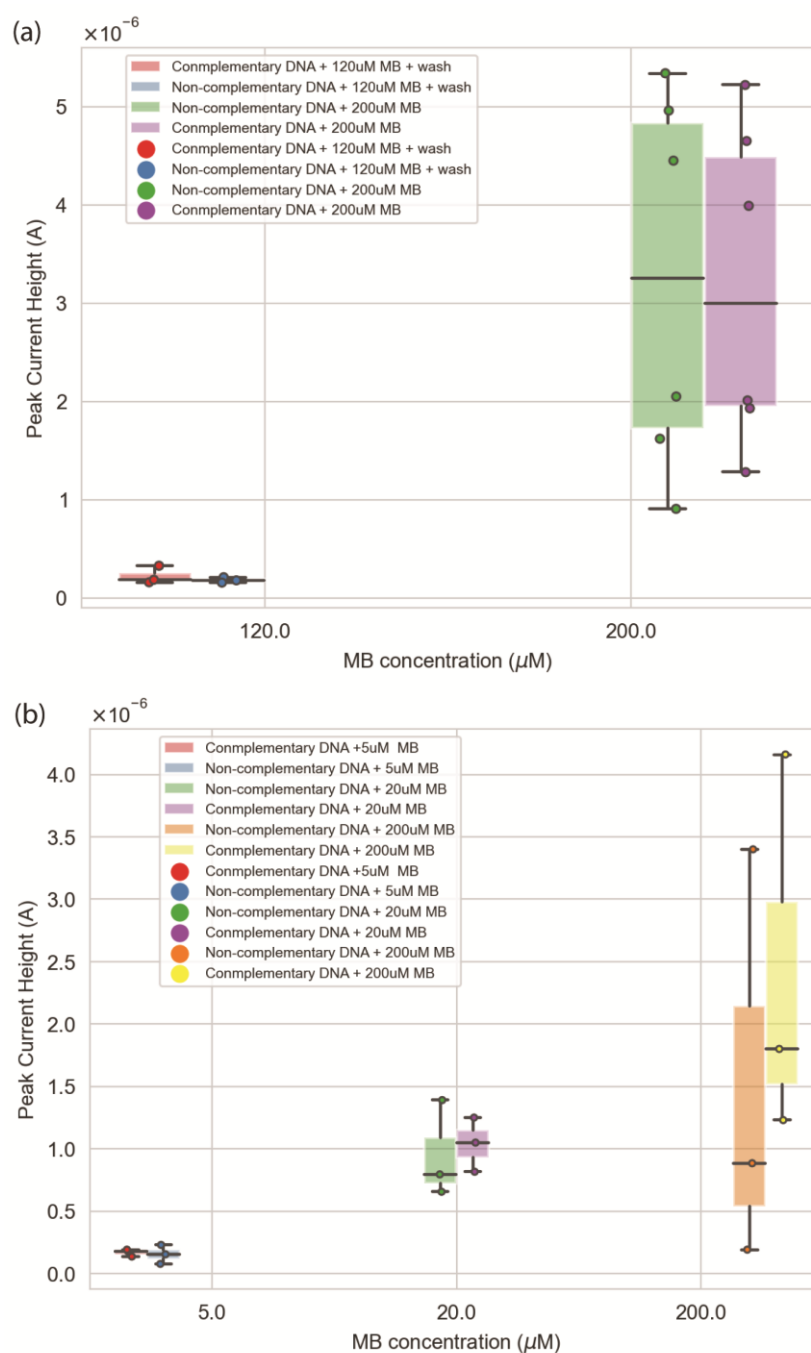


Figure 4-22 Box plot of DNA intercalation sensing by SWV using (a) 100 nM and (b) 200 nM DNA with different MB concentration.

Figure 4-21 and Figure 4-22 both demonstrated that the resulting signal enhanced along with the MB concentration increasing. With the unwashed group, this is probably due to the signal

from diffusion override the signal from the surface bound intercalated MB. But the same problem existed when the surface has been washed with PBS. Therefore, these results led to suggest that MB adsorbs non-specifically on the electrode surface, despite the fact that Biotin-PEG was used already to try and minimize non-specific binding. In further tests, the effect of decreasing MB incubation time and of adding washing steps on non-specific binding was investigated.

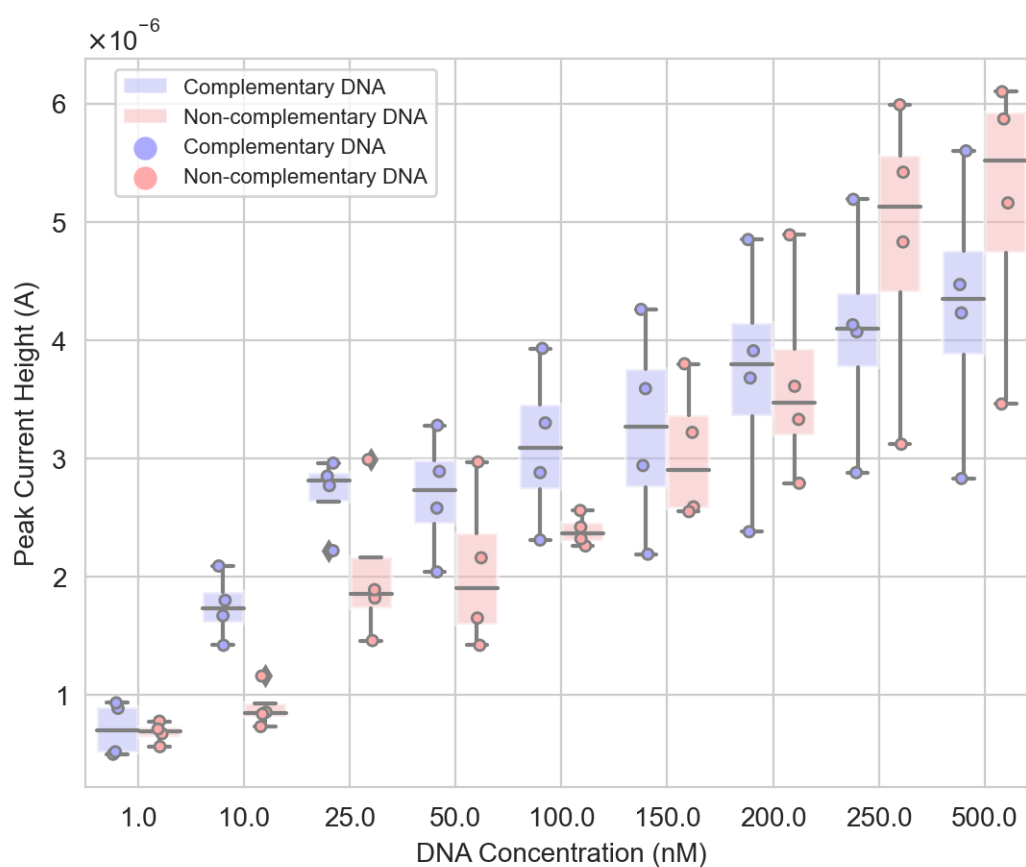


Figure 4-23 Box plot of DNA intercalation sensing by SWV using 200 μ M MB for 5 minutes and washed with 1X PBS.

As shown in Figure 4-23, 200 μ M MB was applied on the DNA-treated surface for 5 minutes. Washing helped with the removing of non-specifically adsorbed MB on carbon surface. This protocol revealed the narrow linear range to be between 10 nM to 25 nM. Further

optimization is therefore needed before this strategy can be used for the detection of endogenous circulating cell-free nucleic acids.

4.3 Conclusion

Five different sensing strategies have been carried out based on the polystreptavidin-biotin immobilized electrode.

Sandwich assay using anthraquinone as a reporter probe exhibited the most promising results in terms of sensitivity and specificity. The current dynamic linear range is 77 nM to 180 nM with a LoD is 77 nM. Further improvement of the sensitivity can be implemented before the next step multiplexed detection and miniaturization by optimizing the surface orientation and density.

EOTR can be a good alternative to replace sandwich assay as it provides better sensitivity and less background noise due to its controlled electrochemical switch for detection. However, catechol is not as stable as aniline. The adduct formation is only possible when the catechol moiety is in its quinoid form, giving the potential for explicit control of the EOTR.³⁰³ The reaction can only happen when the electrode potential allows the quinoid to form while avoiding aniline polymerization. Furthermore, the original designed EOTR sensing in this platform was using aniline and 4-methyl-catechol as two reacting probes, whose adduct peak is at between -0.1 V and 0 V (vs. pseudo reference electrode). The SPCE has an original peak at the same region (vs. Ag pseudo reference electrode) probably due to the gradually sliver leaking after multiple pulsed voltammetry scanning. Therefore, EOTR is not the optimal sensing technique for this platform using SPCE.

Chronoamperometry as a non-label sensing method displayed great convenience. No functional groups or electrochemical reporter need to be attached on the PNA probes. This technique utilizes the RuHex, which binds to DNA through electrostatic intercalation. Despite its convenience, its specificity in this application needs further investigation. On the other hand, EIS is another non-label method for sensing. EIS is essentially a technique for investigating surface changes; but the specificity between complementary and non-complementary sequence needs improvement. Although the data shown the possibility for further exploration in the nano molar range sensing for target sequence, it is challenging to

do so because the potentially unstable functionalized surface under EIS interrogation. Study has demonstrated that electrochemical sensor can degraded faster under AC potential and pulsed stimulations ¹⁷². The fact that EIS takes longer time to perform (3 to 7 minutes) compare with SWV or CV (usually less than 1 minute) makes the problem even bigger. Another challenge is that EIS background varies a lot for both bare SPCE and polystreptavidin and Biotin-PNA-Aniline-141 treated SPCE. This is probably because of the unsmooth surface of the SPCE. A brief trail has been conducted on bulk gold electrodes, but no improvement was found (Figure A-5 and Figure A-6).

5 Summary and Outlook

In this thesis, different surface functionalization and sensing strategies were thoroughly explored and evaluated to develop a biosensor capable of detecting short nucleic acid-based biomarker, as those found in circulation in a range of bodily fluids.

5.1 Immobilization strategies and its optimization

About surface functionalization, immobilization methods have been tried on SPCE, graphene electrode and bulk gold electrode. polystreptavidin-biotin, silanization, trans-esterification reaction, carbodiimide reaction (EDC/NHS) and gold-thiol interaction based immobilization of the capture PNA probe on the electrode surface have been explored. Among them, gold-thiol, silanization, trans-esterification reaction and carbodiimide reaction are covalent immobilization while polystreptavidin-biotin immobilization was based on the physical adsorption of polystreptavidin on the electrode.

Gold-thiol possessed less reproducibility due to the weak Au-thiol bond. Considering both the reproducibility and the cost-effectiveness regarding sensing applications, carbon was selected as an alternative to gold.

Silanization in aqueous phase was mainly restricted by the polymerization effect which can block the electrode surface due to the less conductive nature of the silanized film. Although an organic phase of silanization have been characterized with better signal to noise ratio, the further validation of this approach was limited by the weak signal from DNA quantification test.

Polystreptavidin and biotinylated PNA capture probe was proved to be the most effective immobilization method among all of the approaches studied in this research, as shown in Table 2-4. With the convenience of physical adsorption of polystreptavidin on the electrode surface, risk of less robust immobilization is the main concern of the strategy. To optimize the immobilization, several approached can be implemented for improvement:

1. Polystreptavidin can be covalently attached to the electrode surface by peptide bond formation between amine groups naturally existing on the polystreptavidin protein and NHS ester groups on the electrode surface.

2. Polystreptavidin can be crosslinked in hydrogel or mixed with nafion to perform extra structure assisted immobilization. By taking advantage of the functional groups on the polymer, which can be designed or selected or engineered, multiple choices of immobilization methods can be introduced.

DNA quantification was an intermediate step between the immobilization and sensing to double check the ability of the capture probe to hybridize with the target sequence. The probe performance using a DNA counterpart probe has been compared with the PNA-based probe. The results confirmed the advantages of using PNA probe in the hypothesis of having better affinity to DNA (and RNA due to the similar backbone to DNA). The target DNA sequence showed much higher efficiency in hybridizing to the PNA capture probe compared to the DNA capture probe. Additionally, the PNA capture probe exhibited satisfactory specificity by discriminating between complementary target DNA sequences and non-complementary DNA sequences.

With PNA probe immobilization on the electrode surface, reports either did not characterize¹⁵⁹ or characterized with very vague approaches such as CV²¹⁹ or EIS³⁰⁷ by monitoring the ferric/ferrocyanide redox change. Many examples were listed from our review paper.¹⁵⁰ The characterization with CV or EIS can only demonstrate there's change with the electrode surface but cannot specify which change. In this thesis, the characterization of PNA capture probe immobilization was mainly performed by functionalizing the PNA capture probe with an electrochemical reporter. The electrochemical reporter can be investigated directly or after reacting with another molecule, allowing for a comprehensive evaluation of the immobilization process.

5.2 Sensing Techniques

Five sensing strategies have been systematically investigated based on the polystreptavidin-biotin functionalized electrode.

EOTR and sandwich assay have similar structure designs. They all rely on the sensing probe signal from a PNA probe that can hybridize with the target sequence. In sandwich assay, the selection of anthraquinone as sensing probe was due to both its desired peak location in electrochemical investigation and great solubility in aqueous solution. The designed sensor demonstrated a linear range between 77 nM to 180 nM and a limit of detection of 77 nM.

Sensing performance could be further improved by limiting non-specific adsorption more effectively, a common issue with PNA-based sensors. These optimization goals can be realized by several potential approaches:

- Use another spacer that works better than Biotin-PEG (Biotin-(PEG)₆-OH) or use a combination of more than one spacer (or backfiller) to both remove the non-specific adsorption and stop steric hindrance.
- Using rigid two- or three-dimensional nanostructures as linkers between the solid surface and the PNA probe. There are many creative designs that can be tried: including but not limited to tetrameric junction,²⁴³ three-dimensional cube,²⁴⁴ two-dimensional DNA lattices,²⁴⁵ DNA triple crossover complex,²⁴⁶ DNA origami²⁴⁷ and DNA tetrahedron,^{248–251} octahedron.²⁵²
- Wash the proposed sensing platform by detergent (TWEEN 20³⁰⁸) or protein (BSA,^{234–238} casein²³⁹) to expedite the removal of non-specific adsorption while attaining the sensing efficiency can be an approach.

When applying these solutions, there are several factors to consider. To compete with the non-specific binding between a non-complementary sequence and the electrode surface, the spacers or backfillers' Gibbs free energy need to be smaller (larger absolute value) than the non-specific binding but larger (small absolute value) to not replace or compete with the capture probe. For example, surface Gibbs free energy of the thiol-modified DNA-oligo adsorption on gold is found to be -32.4 kJ/mol.³⁰⁹ In contrast, the hybridization Gibbs free energy between PNA and DNA, with PNA probes of 7-mer or 8-mer length used in this research, falls in the range of -40 to -50 kJ/mol.³¹⁰ Blocking the accessible surface with a molecule that exhibits no interaction with the target in the sample (especially nucleic acids and any other molecules with H-bonding, ion-dipole possibilities) is the only rational approach. This intermediary molecule, or spacer, should demonstrate inert properties while possessing sufficient reactivity to achieve a binding energy of approximately 100 kJ/mol with the surface. Such a level of binding energy typically necessitates the involvement of covalent or ionic bonding. It's also crucial that this molecule doesn't impede electron transfer from any potential reporter group employed. Balancing these requirements, however, presents a challenge due to their inherent contradictions.

In theory, EOTR is a good alternative to improve sandwich assay as it provides better sensitivity and less background noise due to its controlled electrochemical switch for detection. However, in this study, the effectiveness of EOTR has been constrained by an inherent peak at approximately -0.1 V to 0 V (vs. Ag pseudo reference electrode), as shown in Figure 4-6 red curve, potentially caused by gradual silver leakage or other manufactural contamination, which tends to overlap with and obstruct the EOTR signal. Therefore, EOTR is not the optimal sensing technique for this platform using SPCE. This can be solved by either change the electrode or use other EOTR reactions designs or changing the peak location to avoid this intrinsic background peak. It is indeed feasible to finely adjust the redox characteristics, encompassing both thermodynamic and kinetic aspects, of quinoid molecules.³¹¹ This deliberate modulation retains the capacity for the occurrence of the 1,4 addition reaction within the oxidized state.

Except the EOTR and sandwich assay, three non-label sensing strategies have been tried: EIS, chronoamperometry and DNA intercalation.

EIS is a convenient label-free technique. Obtaining an EIS spectrum is a time-consuming process that can take hours. To obtain accurate results, the system under measurement must remain at a steady state throughout the measurement period. In electrochemistry, a steady state refers to a condition where the system being studied is in a stable and unchanging state over a period of time, which can be expressed $\frac{dC(x)}{dt} = 0$ mathematically. The concentration anywhere $C(x)$ is not changing with time t . One of the most common challenges in EIS measurements and analysis is the occurrence of drift in the system being measured. In practical achieving a steady state can be challenging due to various factors that can affect the cell's stability. For instance, the cell can be influenced by the adsorption of solution impurities, formation of an oxide layer, accumulation of reaction products in solution, degradation of the coating, or temperature fluctuations.

Chronoamperometry and DNA intercalation are based on the interaction between an electrochemical reporter in the electrolyte and the functionalized electrode surface. Their narrow dynamic linear range in this application limited their further development. This can be improved by proper surface probe orientation and density control.

In summary, this thesis has contributed to the advancement of electrochemical biosensors for the purpose of detecting nucleic acid biomarkers, with potential future applications for early cancer diagnosis. The sensor development involves the strategic design of two PNA probes, each being fully complementary to half of the target sequence and being functionalized with different electrochemical probes. The proposed sensor realized a linear dynamic range from 77 nM to 180 nM and a LoD of 77 nM. This proof-of-concept marks a promising beginning. Yet, given clinical endogenous miRNA concentrations spanning from atto-molar to nano-molar levels, enhancing clinical suitability warrants achieving a lower LoD within the femto- or atto-molar range. This could be achieved by further optimising the surface probe density and orientation; designing new EOTR reactions; or improving the electrode quality.

Reference

1. World Health Organization's International Agency for Research on Cancer (IARC). Cancer Topics. Retrieved April 20, 2023, from <https://www.iarc.who.int/cancer-topics/>. (2023).
2. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209–249 (2021).
3. American Cancer Society. What Causes Cancer? Retrieved April 20, 2023, from <https://www.cancer.org/healthy/cancer-causes.html>. (2023).
4. World Health Organization. Promoting cancer early diagnosis (Retrieved June 28, 2023, from <https://www.who.int/activities/promoting-cancer-early-diagnosis>).
5. Pashayan, N. & Pharoah, P. D. P. The challenge of early detection in cancer. *Science* (1979) **368**, 589–590 (2020).
6. World Health Organization, I. A. for R. on C. Cancer Today. Retrieved April 20, 2023, from <https://gco.iarc.fr/today/home>. (2020).
7. Prostate Cancer UK. About prostate cancer. Retrieved April 20, 2023, from <https://prostatecanceruk.org/prostate-information-and-support/risk-and-symptoms/about-prostate-cancer>. (2023).
8. PERRIN, P., MAQUET, J. H., BRINGEON, G. & DEVONEC, M. Screening for Prostate Cancer. Comparison of Transrectal Ultrasound, Prostate Specific Antigen and Rectal Examination. *Br J Urol* **68**, 263–265 (1991).
9. Bell, K. J. L., Del Mar, C., Wright, G., Dickinson, J. & Glasziou, P. Prevalence of incidental prostate cancer: A systematic review of autopsy studies. *Int J Cancer* **137**, 1749–1757 (2015).
10. Pinsky, P. F., Prorok, P. C. & Kramer, B. S. Prostate Cancer Screening — A Perspective on the Current State of the Evidence. *New England Journal of Medicine* vol. 376 1285–1289 Preprint at <https://doi.org/10.1056/nejmsb1616281> (2017).
11. Brock, G., Castellanos-Rizaldos, E., Hu, L., Coticchia, C. & Skog, J. Liquid biopsy for cancer screening, patient stratification and monitoring. *Transl Cancer Res* **4**, 280–290 (2015).
12. Tkac, J. *et al.* Prostate-specific antigen glycoprofiling as diagnostic and prognostic biomarker of prostate cancer. *Interface Focus* **9**, (2019).
13. Kisling LA, M. D. J. Prevention Strategies. [Updated 2023 May 1]. 2023 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK537222/>. *StatPearls [Internet]. Treasure Island (FL) StatPearls Publishing*.
14. O'Reilly, J.-A. Prostate Cancer Detection: Complexities and Strategies. *J Cancer Treatment Diagn* **2**, 18–25 (2017).
15. Harvey, C. J., Pilcher, J., Richenberg, J., Patel, U. & Frauscher, F. Applications of transrectal ultrasound in prostate cancer. *Br J Radiol* **85**, S3–S17 (2012).
16. <https://www.nhs.uk/conditions/prostate-cancer/should-i-have-psa-test/>.
17. <https://www.nhs.uk/conditions/prostate-cancer/should-i-have-psa-test/>.

18. Schröder, F. H. *et al.* Screening and prostate cancer mortality: Results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. *The Lancet* **384**, 2027–2035 (2014).
19. Moul, J. W. Comparison of DRE and PSA in the Detection of Prostate Cancer. *Journal of Urology* **197**, (2017).
20. O'Reilly, J.-A. Prostate Cancer Detection: Complexities and Strategies. *J Cancer Treatment Diagn* **2**, 18–25 (2017).
21. J. André Knottnerus (Editor), F. B. (Editor). *The Evidence Base of Clinical Diagnosis: Theory and Methods of Diagnostic Research, 2nd Edition*. (Wiley, 2008).
22. Pérez-Lanzac-Lorca, A. *et al.* Correlation between the complex PSA/total PSA ratio and the free PSA/total PSA ratio, sensitivity and specificity of both markers for the diagnosis of prostate cancer. *Actas Urológicas Españolas (English Edition)* **37**, 498–503 (2013).
23. Nahm, F. S. Receiver operating characteristic curve: overview and practical use for clinicians. *Korean J Anesthesiol* **75**, 25–36 (2022).
24. Retrieved Sept 26, 2023, from <https://www.cancerdata.nhs.uk/prevalence>. (2023).
25. What is Prevalence? Retrieved Sept 26, 2023, from https://www.nimh.nih.gov/health/statistics/what-is-prevalence#part_2623.
26. Thompson, I. M. *et al.* Prevalence of prostate cancer among men with a prostate-specific antigen level ≤ 4.0 ng per milliliter. *New England Journal of Medicine* **350**, 2239–2246+2321 (2004).
27. Cancer Research UK. Prostate cancer incidence statistics. Retrieved April 21, 2023, from <https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence#collapseZero>.
28. Kumar, S., Mohan, A. & Guleria, R. Biomarkers in cancer screening, research and detection: Present and future: A review. *Biomarkers* vol. 11 385–405 Preprint at <https://doi.org/10.1080/13547500600775011> (2006).
29. Wang, G. *et al.* Mixed Self-Assembly of Polyethylene Glycol and Aptamer on Polydopamine Surface for Highly Sensitive and Low-Fouling Detection of Adenosine Triphosphate in Complex Media. *ACS Appl Mater Interfaces* **9**, 31153–31160 (2017).
30. Bolotsky, A. *et al.* Two-Dimensional Materials in Biosensing and Healthcare: From in Vitro Diagnostics to Optogenetics and beyond. *ACS Nano* **13**, 9781–9810 (2019).
31. di Martile, M., del Bufalo, D. & Trisciuglio, D. The multifaceted role of lysine acetylation in cancer: Prognostic biomarker and therapeutic target. *Oncotarget* **7**, 55789–55810 (2016).
32. Wu, L. & Qu, X. Cancer biomarker detection: Recent achievements and challenges. *Chem Soc Rev* **44**, 2963–2997 (2015).
33. Sjakste, N., Sjakste, T. & Vikmanis, U. Role of the nuclear matrix proteins in malignant transformation and cancer diagnosis. *Exp Oncol* **26**, 170–178 (2004).
34. Barbosa, E. B. *et al.* Proteomics: methodologies and applications to the study of human diseases. *Rev Assoc Med Bras (1992)* **58**, 366–75 (2012).

35. Liang, S.-L. & Chan, D. W. Enzymes and related proteins as cancer biomarkers: A proteomic approach. *Clinica Chimica Acta* **381**, 93–97 (2007).
36. Poondla, N., Chandrasekaran, A. P., Kim, K. S. & Ramakrishna, S. Deubiquitinating enzymes as cancer biomarkers: New therapeutic opportunities? *BMB Rep* **52**, 181–189 (2019).
37. Simmons, D. L., Botting, R. M. & Hla, T. Cyclooxygenase isozymes: The biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* **56**, 387–437 (2004).
38. Hoshi, T., Anzai, J. ichi & Osa, T. Electrochemical deposition of avidin on the surface of a platinum electrode for enzyme sensor applications. *Anal Chim Acta* **289**, 321–327 (1994).
39. Raeber, M. E., Sahin, D., Karakus, U. & Boyman, O. A systematic review of interleukin-2-based immunotherapies in clinical trials for cancer and autoimmune diseases. *EBioMedicine* **90**, 104539 (2023).
40. Guglielmi, G. Fast coronavirus tests: what they can and can't do. *Nature* **585**, 496–498 (2020).
41. Zilberman, Y. *et al.* Carbon nanotube/hexa-peri-hexabenzocoronene bilayers for discrimination between nonpolar volatile organic compounds of cancer and humid atmospheres. *Advanced Materials* **22**, 4317–4320 (2010).
42. Haick, H., Broza, Y. Y., Mochalski, P., Ruzsanyi, V. & Amann, A. Assessment, origin, and implementation of breath volatile cancer markers. *Chem Soc Rev* **43**, 1423–1449 (2014).
43. Biavardi, E. *et al.* Exclusive recognition of sarcosine in water and urine by a cavitand-functionalized silicon surface. *Proc Natl Acad Sci U S A* **109**, 2263–2268 (2012).
44. Qi, L. & Du, Y. Diagnosis of disease relevant nucleic acid biomarkers with off-the-shelf devices. *J Mater Chem B* **10**, 3959–3973 (2022).
45. Schwarzenbach, H., Hoon, D. S. B. & Pantel, K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* **11**, 426 (2011).
46. Gilboa, T., Garden, P. M. & Cohen, L. Single-molecule analysis of nucleic acid biomarkers – A review. *Anal Chim Acta* **1115**, 61–85 (2020).
47. Swarup, V. & Rajeswari, M. R. Circulating (cell-free) nucleic acids - A promising, non-invasive tool for early detection of several human diseases. *FEBS Letters* vol. 581 795–799 Preprint at <https://doi.org/10.1016/j.febslet.2007.01.051> (2007).
48. Tong, Y.-K. & Lo, Y. M. D. Diagnostic developments involving cell-free (circulating) nucleic acids. *Clinica Chimica Acta* **363**, 187–196 (2006).
49. Leon, S. A., Shapiro, B., Sklaroff, D. M. & Yaros, M. J. Free DNA in the Serum of Cancer Patients and the Effect of Therapy. *Cancer Res* **37**, 646–650 (1977).
50. BERA, A. *et al.* Circulating Cell-free DNA in Serum as a Marker for the Early Detection of Tumor Recurrence in Breast Cancer Patients. *Cancer Diagnosis & Prognosis* **2**, 285–292 (2022).
51. Jiang, N. *et al.* Liquid biopsy: Circulating exosomal long noncoding RNAs in cancer. *Clinica Chimica Acta* **495**, 331–337 (2019).
52. Feinbaum, R., Ambros, V. & Lee, R. The *C. elegans* Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*. *Cell* **116**, 843–854 (1993).

53. Wijesinghe, K. M., Kanak, M. A., Harrell, J. C. & Dhakal, S. Single-Molecule Sensor for High-Confidence Detection of miRNA. *ACS Sens* **7**, 1086–1094 (2022).
54. Wang, J., Chen, J. & Sen, S. MicroRNA as Biomarkers and Diagnostics. *J Cell Physiol* **231**, 25–30 (2016).
55. Croce, C. M. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* **10**, 704–714 (2009).
56. Gillespie, P., Ladame, S. & O'Hare, D. Molecular methods in electrochemical microRNA detection. *Analyst* **144**, 114–129 (2018).
57. Pritchard, C. C., Cheng, H. H. & Tewari, M. MicroRNA profiling: approaches and considerations. *Nat Rev Genet* **13**, 358–369 (2012).
58. McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. & Algeciras-Schimmich, A. Analysis of circulating microRNA: Preanalytical and analytical challenges. *Clin Chem* **57**, 833–840 (2011).
59. Chevillet, J. R. *et al.* Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proceedings of the National Academy of Sciences* vol. 111 14888–14893 Preprint at <https://doi.org/10.1073/pnas.1408301111> (2014).
60. Condrat, C. E. *et al.* miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells* **9**, 276 (2020).
61. Kotb, S. *et al.* Circulating miRNAs 21 and 221 as biomarkers for early diagnosis of prostate cancer. *Tumor Markers, Tumor Targeting and Translational Cancer Research* **35**, 12613–12617 (2014).
62. Joyce, C. Quantitative RT-PCR BT - RT-PCR Protocols. in (ed. O'Connell, J.) 83–92 (Humana Press, 2002). doi:10.1385/1-59259-283-X:083.
63. Oldach, D. "Real-time" polymerase chain reaction. *Gastroenterology* **116**, 763–765 (1999).
64. Pfaffl, M. W. Quantification Strategies in Real-time Polymerase Chain Reaction. *A-Z of Quantitative PCR* 87–112 (2004).
65. CHAFFEY, N. *Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. Molecular biology of the cell. 4th edn. Annals of Botany* vol. 91 (2003).
66. Pollack, J. R. *et al.* Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* **23**, 41 (1999).
67. Marx, V. PCR: paths to sensitivity. *Nat Methods* **11**, 241 (2014).
68. Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F. & Hellens, R. P. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods* **3**, 12 (2007).
69. van den Brand, M. *et al.* Evaluation of a real-time PCR assay for detection and quantification of bacterial DNA directly in blood of preterm neonates with suspected late-onset sepsis. *Crit Care* **22**, (2018).
70. Metcalf, G. A. D. *et al.* Amplification-free detection of circulating microRNA biomarkers from body fluids based on fluorogenic oligonucleotide-templated reaction between engineered

- peptide nucleic acid probes: Application to prostate cancer diagnosis. *Anal Chem* **88**, 8091–8098 (2016).
71. Várallyay, É., Burgyán, J. & Havelda, Z. MicroRNA detection by northern blotting using locked nucleic acid probes. *Nat Protoc* **3**, 190 (2008).
 72. Trayhurn, P. Northern blotting. *Proc. Nutr. Soc.* **55**, 583–589 (1996).
 73. Alwine, J. C., Kemp, D. J. & Stark, G. R. Method for Detection of Specific RNAs in Agarose Gels by Transfer to Diazobenzyloxymethyl-Paper and Hybridization with DNA Probes. *Proc Natl Acad Sci U S A* **74**, 5350–5354 (1977).
 74. Streit, S., Michalski, C. W., Erkan, M., Kleeff, J. & Friess, H. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. *Nat Protoc* **4**, 37 (2008).
 75. Kukurba, K. R. & Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb Protoc* **2015**, 951–69 (2015).
 76. Várallyay, É., Burgyán, J. & Havelda, Z. MicroRNA detection by northern blotting using locked nucleic acid probes. *Nat Protoc* **3**, 190 (2008).
 77. Kukurba, K. R. & Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb Protoc* **2015**, 951–69 (2015).
 78. Hattori, S. *et al.* Detection of Bladder Cancer by Measuring CD44v6 Expression in Urine With Real-time Quantitative Reverse Transcription Polymerase Chain Reaction. *Urology* **83**, 1443.e9-1443.e15 (2014).
 79. Almheiri, R. & Alghafri, R. Reduced volume for direct PCR amplification of blood reference samples using Identifiler® Direct and Globalfiler™ Express assays. *Forensic Sci Int Genet Suppl Ser* **6**, e340–e341 (2017).
 80. Graybill, R. M. & Bailey, R. C. Emerging Biosensing Approaches for microRNA Analysis. *Anal Chem* **88**, 431 (2016).
 81. Xue, T. *et al.* Ultrasensitive detection of miRNA with an antimonene-based surface plasmon resonance sensor. *Nat Commun* **10**, 28 (2019).
 82. Bosson, A. D., Zamudio, J. R. & Sharp, P. A. Endogenous miRNA and Target Concentrations Determine Susceptibility to Potential ceRNA Competition. *Mol Cell* **56**, 347–359 (2014).
 83. Shin, S. *et al.* Quantification of purified endogenous miRNAs with high sensitivity and specificity. *Nat Commun* **11**, 6033 (2020).
 84. Kim, D. J. *et al.* Plasma components affect accuracy of circulating cancer-related microRNA quantitation. *Journal of Molecular Diagnostics* vol. 14 71–80 Preprint at <https://doi.org/10.1016/j.jmoldx.2011.09.002> (2012).
 85. Cheng, H. *et al.* Plasma Processing Conditions Substantially Influence Circulating microRNA Biomarker Levels. *PLoS One* **8**, e64795 (2013).
 86. Nguyen, H. C. N. *et al.* Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. *Prostate* **73**, 346–354 (2013).

87. Bryant, R. J. *et al.* Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer* **106**, 768–774 (2012).
88. Petrou, L. *et al.* Lateral Flow Test (LFT) Detects Cell-Free MicroRNAs Predictive of Preterm Birth Directly from Human Plasma. *Adv Nanobiomed Res* **2**, 2200026 (2022).
89. Wang, T., Viennois, E., Merlin, D. & Wang, G. Microelectrode miRNA Sensors Enabled by Enzymeless Electrochemical Signal Amplification. *Anal Chem* **87**, 8173–8180 (2015).
90. Moldovan, L. *et al.* Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med* **18**, 371–390 (2014).
91. Wang, X. *et al.* MicroRNA identification based on sequence and structure alignment. *Bioinformatics* **21**, 3610–3614 (2005).
92. Arroyo, J. D. *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences* vol. 108 5003–5008 Preprint at <https://doi.org/10.1073/pnas.1019055108> (2011).
93. Kumar, S., Mohan, A. & Guleria, R. Biomarkers in cancer screening, research and detection: Present and future: A review. *Biomarkers* vol. 11 385–405 Preprint at <https://doi.org/10.1080/13547500600775011> (2006).
94. Booth, M. *et al.* Chemical Monitoring in Clinical Settings: Recent Developments toward Real-Time Chemical Monitoring of Patients. *Analytical Chemistry* vol. 90 Preprint at <https://doi.org/10.1021/acs.analchem.7b04224> (2017).
95. Sonner, Z. *et al.* The microfluidics of the eccrine sweat gland, including biomarker partitioning, transport, and biosensing implications. *Biomicrofluidics* **9**, 1–19 (2015).
96. Rogers, M. L. & Boutelle, M. G. Real-Time Clinical Monitoring of Biomolecules. *Annual Review of Analytical Chemistry* **6**, 427–453 (2013).
97. Zhou, L. *et al.* In-depth analysis of the human tear proteome. *J Proteomics* **75**, 3877–3885 (2012).
98. Helton, K. L., Nelson, K. E., Fu, E. & Yager, P. Conditioning saliva for use in a microfluidic biosensor. *Lab Chip* **8**, 1847–1851 (2008).
99. Toner, M. & Daniel, I. BLOOD-ON-A-CHIP. *Annu. Rev. Biomed. Eng.* **7**, 77–103 (2005).
100. Heikenfeld, J. Non-invasive Analyte Access and Sensing through Eccrine Sweat: Challenges and Outlook circa 2016. *Electroanalysis* **28**, 1242–1249 (2016).
101. Sun, Q. *et al.* Reproducibility of urinary biomarkers in multiple 24-h urine samples. *American Journal of Clinical Nutrition* **105**, 159–168 (2017).
102. Kim Barrett, Susan Barman, Scott Boitano & Heddwen Brooks. *Review of Medical Physiology*. (McGraw-Hill Education / Medical, 2012).
103. Leeman, M., Choi, J., Hansson, S., Storm, M. U. & Nilsson, L. Proteins and antibodies in serum, plasma, and whole blood—size characterization using asymmetrical flow field-flow fractionation (AF4). *Anal Bioanal Chem* **410**, 4867–4873 (2018).

104. Bhalla, N., Jolly, P., Formisano, N. & Estrela, P. Introduction to biosensors. *Essays Biochem* **60**, 1–8 (2016).
105. Naresh, V. & Lee, N. A review on biosensors and recent development of nanostructured materials-enabled biosensors. *Sensors (Switzerland)* **21**, 1–35 (2021).
106. White, R. M. A Sensor Classification Scheme. *IEEE Trans Ultrason Ferroelectr Freq Control* **34**, 124–126 (1987).
107. Ding, J. & Qin, W. Recent advances in potentiometric biosensors. *TrAC Trends in Analytical Chemistry* **124**, 115803 (2020).
108. Armbruster, D. A. & Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* **29 Suppl 1**, S49-52 (2008).
109. Danzer, K. Selectivity and specificity in analytical chemistry. General considerations and attempt of a definition and quantification. *Fresenius J Anal Chem* **369**, 397–402 (2001).
110. Vu & Chen. Field-Effect Transistor Biosensors for Biomedical Applications: Recent Advances and Future Prospects. *Sensors* **19**, 4214 (2019).
111. Simões, F. R. & Xavier, M. G. Electrochemical Sensors. in *Nanoscience and its Applications* 155–178 (Elsevier, 2017). doi:10.1016/B978-0-323-49780-0.00006-5.
112. Allen J. Bard, L. R. F. *Electrochemical Methods: Fundamentals and Applications, 2nd Edition*. (2001).
113. Colburn, A. W., Levey, K. J., O'Hare, D. & Macpherson, J. V. Lifting the lid on the potentiostat: a beginner's guide to understanding electrochemical circuitry and practical operation. *Physical Chemistry Chemical Physics* **23**, 8100–8117 (2021).
114. Elgrishi, N. *et al.* A Practical Beginner's Guide to Cyclic Voltammetry. *J Chem Educ* **95**, 197–206 (2018).
115. [https://en.wikipedia.org/wiki/Standard_electrode_potential_\(data_page\)](https://en.wikipedia.org/wiki/Standard_electrode_potential_(data_page)).
116. Osteryoung, J. G. & Osteryoung, R. A. Square wave voltammetry. *Anal Chem* **57**, 101–110 (1985).
117. Mirceski, V. *et al.* Square-Wave Voltammetry: A Review on the Recent Progress. *Electroanalysis* **25**, 2411–2422 (2013).
118. Christopher M. A. Brett & Ana Maria Oliveira Brett. *Electrochemistry: Principles, Methods, and Applications*. (Oxford University Press, 1993).
119. Liu, J. *et al.* Application and Progress of Chemometrics in Voltammetric Biosensing. *Biosensors (Basel)* **12**, 494 (2022).
120. Steel, A. B., Herne, T. M. & Tarlov, M. J. Electrochemical Quantitation of DNA Immobilized on Gold. *Anal Chem* **70**, 4670–4677 (1998).
121. Magar, H. S., Hassan, R. Y. A. & Mulchandani, A. Electrochemical Impedance Spectroscopy (EIS): Principles, Construction, and Biosensing Applications. *Sensors* **21**, 6578 (2021).
122. Park, S.-M. & Yoo, J.-S. Peer Reviewed: Electrochemical Impedance Spectroscopy for Better Electrochemical Measurements. *Anal Chem* **75**, 455 A-461 A (2003).

123. Oldham, K. B., Myland, J. C. & Bond, A. M. *Electrochemical Science and Technology*. (John Wiley & Sons, Ltd, 2011). doi:10.1002/9781119965992.
124. Deffo, G., Nde Tene, T. F., Medonbou Dongmo, L., Zambou Jiokeng, S. L. & Tonleu Temgoua, R. C. Differential pulse and square-wave voltammetry as sensitive methods for electroanalysis applications. in *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering* (Elsevier, 2023). doi:10.1016/B978-0-323-85669-0.00040-4.
125. Egholm, M., Buchardt, O., Nielsen, P. E. & Berg, R. H. Peptide nucleic acids (PNA). Oligonucleotide analogs with an achiral peptide backbone. *J Am Chem Soc* **114**, 1895–1897 (1992).
126. Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. Peptide nucleic acids (PNAs): potential antisense and anti-gene agents. *Anticancer Drug Des* **8**, 53–63 (1993).
127. Lee, R., Kaushik, N., Modak, M. J., Vinayak, R. & Pandey, V. N. Polyamide Nucleic Acid Targeted to the Primer Binding Site of the HIV-1 RNA Genome Blocks *in Vitro* HIV-1 Reverse Transcription. *Biochemistry* **37**, 900–910 (1998).
128. Lewis, M. R. *et al.* Radiometal-Labeled Peptide–PNA Conjugates for Targeting *bcl-2* Expression: Preparation, Characterization, and *in Vitro* mRNA Binding. *Bioconjug Chem* **13**, 1176–1180 (2002).
129. Jensen, K. K., Ørum, H., Nielsen, P. E. & Nordén, B. Kinetics for Hybridization of Peptide Nucleic Acids (PNA) with DNA and RNA Studied with the BIAcore Technique. *Biochemistry* **36**, 5072–5077 (1997).
130. Uhlmann, E. Peptide nucleic acids (PNA) and PNA-DNA chimeras: from high binding affinity towards biological function. *Biol Chem* **379**, 1045–52 (1998).
131. Egholm, M. *et al.* PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules. *Nature* **365**, 566 (1993).
132. Tomac, S. *et al.* Ionic Effects on the Stability and Conformation of Peptide Nucleic Acid Complexes. *J Am Chem Soc* **118**, 5544–5552 (1996).
133. Bakhtiar, R. Peptide nucleic acids: deoxyribonucleic acid mimics with a peptide backbone. *Biochem Educ* **26**, 277–280 (1998).
134. Li, F., Tang, J., Geng, J., Luo, D. & Yang, D. Polymeric DNA hydrogel: Design, synthesis and applications. *Prog Polym Sci* **98**, 101163 (2019).
135. Srisomwat, C. *et al.* Amplification-free DNA Sensor for the One-Step Detection of the Hepatitis B Virus Using an Automated Paper-Based Lateral Flow Electrochemical Device. *Anal Chem* **93**, 2879–2887 (2021).
136. WANG, G. & XU, X. S. Peptide nucleic acid (PNA) binding-mediated gene regulation. *Cell Res* **14**, 111–116 (2004).
137. Schwarz, F. Thermodynamic comparison of PNA/DNA and DNA/DNA hybridization reactions at ambient temperature. *Nucleic Acids Res* **27**, 4792–4800 (1999).
138. What is the pH of DNA. <https://www.aatbio.com/> (2021).

139. Montazersaheb, S., Hejazi, M. S. & Charoudeh, H. N. Potential of peptide nucleic acids in future therapeutic applications. *Adv Pharm Bull* **8**, 551–563 (2018).
140. Bernhardt, H. S. & Tate, W. P. Primordial soup or vinaigrette: Did the RNA world evolve at acidic pH? *Biol Direct* **7**, 4 (2012).
141. Ray, A. & Nordén, B. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *The FASEB Journal* **14**, 1041–1060 (2000).
142. Igloi, G. Variability in the stability of DNA-peptide nucleic acid (PNA) single-base mismatched duplexes: Real-time hybridization during affinity electrophoresis in PNA-containing gels. *Proc Natl Acad Sci U S A* **95**, 8562–8567 (1998).
143. SantaLucia, J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proceedings of the National Academy of Sciences* **95**, 1460–1465 (1998).
144. Paul, A. *et al.* Role of nucleobase energetics and nucleobase interactions in single-stranded peptide nucleic acid charge transfer. *J Am Chem Soc* **131**, 6498–6507 (2009).
145. Zhang, X., Servos, M. R. & Liu, J. Instantaneous and Quantitative Functionalization of Gold Nanoparticles with Thiolated DNA Using a pH-Assisted and Surfactant-Free Route. *J Am Chem Soc* **134**, 7266–7269 (2012).
146. Zhang, X., Liu, B., Dave, N., Servos, M. R. & Liu, J. Instantaneous Attachment of an Ultrahigh Density of Nonthiolated DNA to Gold Nanoparticles and Its Applications. *Langmuir* **28**, 17053–17060 (2012).
147. Zhang, X., Servos, M. R. & Liu, J. Fast pH-assisted functionalization of silver nanoparticles with monothiolated DNA. *Chemical Communications* **48**, 10114 (2012).
148. Wang, F. & Liu, J. Platinated DNA oligonucleotides: New probes forming ultrastable conjugates with graphene oxide. *Nanoscale* **6**, 7079–7084 (2014).
149. Ducker, R. E., Montague, M. T. & Leggett, G. J. A comparative investigation of methods for protein immobilization on self-assembled monolayers using glutaraldehyde, carbodiimide, and anhydride reagents. *Biointerphases* **3**, 59–65 (2008).
150. Meng, X., O'Hare, D. & Ladame, S. Surface immobilization strategies for the development of electrochemical nucleic acid sensors. *Biosens Bioelectron* **237**, 115440 (2023).
151. Cai, X. *et al.* Evaluation of Different Carbon Electrodes for Adsorptive Stripping Analysis of Nucleic Acids. *Electroanalysis* vol. 8 753–758 Preprint at <https://doi.org/10.1002/elan.1140080809> (1996).
152. Jose M. Guisan. *Immobilization of Enzymes and Cells*. (Humana Press Inc., 2006).
153. Mashhadizadeh, M. H. & Talemi, R. P. A new methodology for electrostatic immobilization of a non-labeled single strand DNA onto a self-assembled diazonium modified gold electrode and detection of its hybridization by differential pulse voltammetry. *Talanta* **103**, 344–348 (2013).
154. Ford, R., Devereux, S. J., Quinn, S. J. & O'Neill, R. D. Carbon nanohorn modified platinum electrodes for improved immobilisation of enzyme in the design of glutamate biosensors. *Analyst* **144**, 5299–5307 (2019).

155. Dong, X., Lu, X., Zhang, K. & Zhang, Y. Chronocoulometric DNA biosensor based on a glassy carbon electrode modified with gold nanoparticles, poly(dopamine) and carbon nanotubes. *Microchimica Acta* **180**, 101–108 (2013).
156. Lim, J. *et al.* Microfluidic device for one-step detection of breast cancer-derived exosomal mRNA in blood using signal-amplifiable 3D nanostructure. *Biosens Bioelectron* **197**, 113753 (2022).
157. Ma, C. D., Wang, C., Acevedo-Vélez, C., Gellman, S. H. & Abbott, N. L. Modulation of hydrophobic interactions by proximally immobilized ions. *Nature* **517**, 347–350 (2015).
158. Kang, D. *et al.* DNA biomolecular-electronic encoder and decoder devices constructed by multiplex biosensors. *NPG Asia Mater* **4**, 1–6 (2012).
159. Fortunati, S. *et al.* Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM) soy. *Biosens Bioelectron* **129**, 7–14 (2019).
160. Yuk, H., Zhang, T., Lin, S., Parada, G. A. & Zhao, X. Tough bonding of hydrogels to diverse non-porous surfaces. *Nat Mater* **15**, 190–196 (2016).
161. Liu, Z. *et al.* Stable single-walled carbon nanotube-streptavidin complex for biorecognition. *Journal of Physical Chemistry C* **114**, 4345–4352 (2010).
162. Bain, C. D. *et al.* Formation of Monolayer Films by the Spontaneous Assembly of Organic Thiols from Solution onto Gold. *J Am Chem Soc* **111**, 321–335 (1989).
163. Love, J. C., Estroff, L. A., Kriebel, J. K., Nuzzo, R. G. & Whitesides, G. M. Self-assembled monolayers of thiolates on metals as a form of nanotechnology. *Chem Rev* **105**, 1103–1169 (2005).
164. Vericat, C., Vela, M. E., Benitez, G., Carro, P. & Salvarezza, R. C. Self-assembled monolayers of thiols and dithiols on gold: new challenges for a well-known system. *Chem Soc Rev* **39**, 1805–1834 (2010).
165. Xue, Y., Li, X., Li, H. & Zhang, W. Quantifying thiol–gold interactions towards the efficient strength control. *Nat Commun* **5**, 4348 (2014).
166. Zhu, D. *et al.* PolyA-Mediated DNA Assembly on Gold Nanoparticles for Thermodynamically Favorable and Rapid Hybridization Analysis. *Anal Chem* **88**, 4949–4954 (2016).
167. Demers, L. M. *et al.* Thermal desorption behavior and binding properties of DNA bases and nucleosides on gold. *J Am Chem Soc* **124**, 11248–11249 (2002).
168. Kimura-Suda, H., Petrovykh, D. Y., Tarlov, M. J. & Whitman, L. J. Base-Dependent Competitive Adsorption of Single-Stranded DNA on Gold. *J Am Chem Soc* **125**, 9014–9015 (2003).
169. Herne, T. M. & Tarlov, M. J. Characterization of DNA Probes Immobilized on Gold Surfaces. *J Am Chem Soc* **119**, 8916–8920 (1997).
170. Yüce, M. & Kurt, H. How to make nanobiosensors: Surface modification and characterisation of nanomaterials for biosensing applications. *RSC Adv* **7**, 49386–49403 (2017).

171. Heaton, R. J., Peterson, A. W. & Georgiadis, R. M. Electrostatic surface plasmon resonance: Direct electric field-induced hybridization and denaturation in monolayer nucleic acid films and label-free discrimination of base mismatches. *Proceedings of the National Academy of Sciences* **98**, 3701–3704 (2001).
172. Shaver, A. & Arroyo-Currás, N. The challenge of long-term stability for nucleic acid-based electrochemical sensors. *Curr Opin Electrochem* **32**, 100902 (2022).
173. Petrovykh, D. Y. *et al.* Nucleobase Orientation and Ordering in Films of Single-Stranded DNA on Gold. *J Am Chem Soc* **128**, 2–3 (2006).
174. Fan, T. *et al.* Rolling circle amplification triggered poly adenine-gold nanoparticles production for label-free electrochemical detection of thrombin. *Sens Actuators B Chem* **266**, 9–18 (2018).
175. Ulman, A. Formation and Structure of Self-Assembled Monolayers. *Chem Rev* **96**, 1533–1554 (1996).
176. Lavrich, D. J., Wetterer, S. M., Bernasek, S. L. & Scoles, G. Physisorption and chemisorption of alkanethiols and alkyl sulfides on Au(111). *Journal of Physical Chemistry B* **102**, 3456–3465 (1998).
177. Nuzzo, R. G., Zegarski, B. R. & Dubois, L. H. Fundamental studies of the chemisorption of organosulfur compounds on gold(111). Implications for molecular self-assembly on gold surfaces. *J Am Chem Soc* **109**, 733–740 (1987).
178. Luo, Y.-R. *Handbook of Bond Dissociation Energies in Organic Compounds*. (CRC Press, 2002). doi:10.1201/9781420039863.
179. Pacchioni, G. A not-so-strong bond. *Nat Rev Mater* (2019) doi:10.1038/s41578-019-0094-3.
180. Suni, I. I. Substrate materials for biomolecular immobilization within electrochemical biosensors. *Biosensors (Basel)* **11**, (2021).
181. Luo, Y.-Ran. *Comprehensive handbook of chemical bond energies*. (Boca Raton, FL : Taylor & Francis, 2007).
182. Munakata, H., Oyamatsu, D. & Kuwabata, S. Effects of ω -functional groups on pH-dependent reductive desorption of alkanethiol self-assembled monolayers. *Langmuir* **20**, 10123–10128 (2004).
183. Yang, D.-F., Wilde, C. P. & Morin, M. Electrochemical Desorption and Adsorption of Nonyl Mercaptan at Gold Single Crystal Electrode Surfaces. *Langmuir* **12**, 6570–6577 (1996).
184. Wang, L. *et al.* Development of an electrochemical DNA biosensor with the DNA immobilization based on in situ generation of dithiocarbamate ligands. *Bioelectrochemistry* **88**, 30–35 (2012).
185. Henderson, J. I., Feng, S., Ferrence, G. M., Bein, T. & Kubiak, C. P. Self-assembled monolayers of dithiols, diisocyanides, and isocyanothiols on gold: ‘chemically sticky’ surfaces for covalent attachment of metal clusters and studies of interfacial electron transfer. *Inorganica Chim Acta* **242**, 115–124 (1996).
186. Phares, N., White, R. J. & Plaxco, K. W. Improving the stability and sensing of electrochemical biosensors by employing trithiol-anchoring groups in a six-carbon self-assembled monolayer. *Anal Chem* **81**, 1095–1100 (2009).

187. McCreery, R. L. Advanced Carbon Electrode Materials for Molecular Electrochemistry. *Chem Rev* **108**, 2646–2687 (2008).
188. Barón-Jaimez, J., Joya, M. R. & Barba-Ortega, J. Bismuth electrodes, an alternative in stripping voltammetry. *J Phys Conf Ser* **466**, 012025 (2013).
189. Menegazzo, N., Jin, C., Narayan, R. J. & Mizaikoff, B. Compositional and Electrochemical Characterization of Noble Metal–Diamondlike Carbon Nanocomposite Thin Films. *Langmuir* **23**, 6812–6818 (2007).
190. Koo, S.-M., Edelstein, M. D., Li, Q., Richter, C. A. & Vogel, E. M. Silicon nanowires as enhancement-mode Schottky barrier field-effect transistors. *Nanotechnology* **16**, 1482–1485 (2005).
191. David, I. G., Popa, D.-E. & Buleandra, M. Pencil Graphite Electrodes: A Versatile Tool in Electroanalysis. *J Anal Methods Chem* **2017**, 1–22 (2017).
192. Babich, A. & Senk, D. Coal use in iron and steel metallurgy. in *The Coal Handbook: Towards Cleaner Production* 267–311 (Elsevier, 2013). doi:10.1533/9781782421177.3.267.
193. Annu, Sharma, S., Jain, R. & Raja, A. N. Review—Pencil Graphite Electrode: An Emerging Sensing Material. *J Electrochem Soc* **167**, 037501 (2020).
194. Sengupta, R., Bhattacharya, M., Bandyopadhyay, S. & Bhowmick, A. K. A review on the mechanical and electrical properties of graphite and modified graphite reinforced polymer composites. *Prog Polym Sci* **36**, 638–670 (2011).
195. David, I. G., Popa, D.-E. & Buleandra, M. Pencil Graphite Electrodes: A Versatile Tool in Electroanalysis. *J Anal Methods Chem* **2017**, 1–22 (2017).
196. Adams, R. N. Carbon Paste Electrodes. *Anal Chem* **30**, 1576–1576 (1958).
197. Sugawara, K., Yugami, A., Terui, N. & Kuramitz, H. Electrochemical study of functionalization on the surface of a chitin/platinum-modified glassy carbon paste electrode. *Analytical Sciences* **25**, 1365–1368 (2009).
198. Sanghavi, B. J. & Srivastava, A. K. Simultaneous voltammetric determination of acetaminophen, aspirin and caffeine using an in situ surfactant-modified multiwalled carbon nanotube paste electrode. *Electrochim Acta* **55**, 8638–8648 (2010).
199. Carbon paste electrode. <https://www.wikipedia.org> (2020).
200. Shi, Z. *et al.* Metallization of diamond. *Proc Natl Acad Sci U S A* **117**, 24634–24639 (2020).
201. Manca, J. V. *et al.* High electrical resistivity of CVD-diamond. *Microelectronics Reliability* **39**, 269–273 (1999).
202. Brzoska, J. B., Azouz, I. Ben & Rondelez, F. Silanization of Solid Substrates: A Step toward Reproducibility. *Langmuir* **10**, 4367–4373 (1994).
203. Queffélec, C., Petit, M., Janvier, P., Knight, D. A. & Bujoli, B. Surface modification using phosphonic acids and esters. *Chem Rev* **112**, 3777–3807 (2012).

204. Sousa, M. de F. B. & Bertazzoli, R. Preconcentration and Voltammetric Determination of Mercury(II) at a Chemically Modified Glassy Carbon Electrode. *Anal Chem* **68**, 1258–1261 (1996).
205. Hou, S., Kasner, M. L., Su, S., Patel, K. & Cuellari, R. Highly Sensitive and Selective Dopamine Biosensor Fabricated with Silanized Graphene. *The Journal of Physical Chemistry C* **114**, 14915–14921 (2010).
206. Nakajima, N. & Ikada, Y. Mechanism of Amide Formation by Carbodiimide for Bioconjugation in Aqueous Media. *Bioconjugate Chem.* **6**, 123–130 (1995).
207. Grieshaber, D., MacKenzie, R., Vörös, J. & Reimhult, E. Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors* **8**, 1400–1458 (2008).
208. Kerativitayanan, P., Carrow, J. K. & Gaharwar, A. K. Nanomaterials for Engineering Stem Cell Responses. *Adv Healthc Mater* **4**, 1600–1627 (2015).
209. Cincotto, F. H. *et al.* Reduced graphene oxide-Sb₂O₅ hybrid nanomaterial for the design of a laccase-based amperometric biosensor for estriol. *Electrochim Acta* **174**, 332–339 (2015).
210. Wang, Q. *et al.* Electrophoretic Approach for the Simultaneous Deposition and Functionalization of Reduced Graphene Oxide Nanosheets with Diazonium Compounds: Application for Lysozyme Sensing in Serum. *ACS Appl Mater Interfaces* **9**, 12823–12831 (2017).
211. Zhang, Y. *et al.* Ultrasensitive detection of microRNA-21 by using specific interaction of antimonene with RNA as electrochemical biosensor. *Bioelectrochemistry* **142**, (2021).
212. Zhang, S., Yan, Z., Li, Y., Chen, Z. & Zeng, H. Atomically thin arsenene and antimonene: Semimetal-semiconductor and indirect-direct band-gap transitions. *Angewandte Chemie - International Edition* **54**, 3112–3115 (2015).
213. McConnell, D. B. Biotin's Lessons in Drug Design. *J Med Chem* **64**, 16319–16327 (2021).
214. Swamy, M. J. Thermodynamic analysis of biotin binding to avidin. A high sensitivity titration calorimetric study. *Biochem Mol Biol Int* **36**, 219–25 (1995).
215. Sun, S. *et al.* Systematic evaluation of avidin–biotin interaction by fluorescence spectrophotometry. *Spectrochim Acta A Mol Biomol Spectrosc* **89**, 99–104 (2012).
216. L. Mpye, K., Gildenhuys, S. & Mosebi, S. The effects of temperature on streptavidin-biotin binding using affinity isothermal titration calorimetry. *AIMS Biophys* **7**, 236–247 (2020).
217. González, M., Argaraña, C. E. & Fidelio, G. D. Extremely high thermal stability of streptavidin and avidin upon biotin binding. *Biomol Eng* **16**, 67–72 (1999).
218. Carrasquilla, C., Li, Y. & Brennan, J. D. Surface immobilization of structure-switching DNA aptamers on macroporous sol-gel-derived films for solid-phase biosensing applications. *Anal Chem* **83**, 957–965 (2011).
219. Chung, D. J. *et al.* One-step modification of various electrode surfaces using diazonium salt compounds and the application of this technology to electrochemical DNA (E-DNA) sensors. *Electrochim Acta* **76**, 394–403 (2012).

220. Mahshid, S. S., Camiré, S., Ricci, F. & Vallée-Bélisle, A. A Highly Selective Electrochemical DNA-Based Sensor That Employs Steric Hindrance Effects to Detect Proteins Directly in Whole Blood. *J Am Chem Soc* **137**, 15596–15599 (2015).
221. Ricci, F., Lai, R. Y., Heeger, A. J., Plaxco, K. W. & Sumner, J. J. Effect of Molecular Crowding on the Response of an Electrochemical DNA Sensor. *Langmuir* **23**, 6827–6834 (2007).
222. Shchepinov, M. Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. *Nucleic Acids Res* **25**, 1155–1161 (1997).
223. Peterson, A. W., Wolf, L. K. & Georgiadis, R. M. Hybridization of Mismatched or Partially Matched DNA at Surfaces. *J Am Chem Soc* **124**, 14601–14607 (2002).
224. Peterson, A. W. The effect of surface probe density on DNA hybridization. *Nucleic Acids Res* **29**, 5163–5168 (2001).
225. Daems, D. *et al.* Three-Dimensional DNA Origami as Programmable Anchoring Points for Bioreceptors in Fiber Optic Surface Plasmon Resonance Biosensing. *ACS Appl Mater Interfaces* **10**, 23539–23547 (2018).
226. Lee, C.-Y. *et al.* Surface Coverage and Structure of Mixed DNA/Alkylthiol Monolayers on Gold: Characterization by XPS, NEXAFS, and Fluorescence Intensity Measurements. *Anal Chem* **78**, 3316–3325 (2006).
227. Li, M. *et al.* Ultrasensitive Photoelectrochemical Biosensor Based on DNA Tetrahedron as Nanocarrier for Efficient Immobilization of CdTe QDs-Methylene Blue as Signal Probe with Near-Zero Background Noise. *Anal Chem* **90**, 8211–8216 (2018).
228. Liu, S., Su, W., Li, Z. & Ding, X. Electrochemical detection of lung cancer specific microRNAs using 3D DNA origami nanostructures. *Biosens Bioelectron* **71**, 57–61 (2015).
229. Zhang, Z.-L. *et al.* Investigation of DNA Orientation on Gold by EC-STM. *Bioconjug Chem* **13**, 104–109 (2002).
230. Kelley, S. O. *et al.* Orienting DNA Helices on Gold Using Applied Electric Fields. *Langmuir* **14**, 6781–6784 (1998).
231. Gębala, M. & Schuhmann, W. Controlled Orientation of DNA in a Binary SAM as a Key for the Successful Determination of DNA Hybridization by Means of Electrochemical Impedance Spectroscopy. *ChemPhysChem* **11**, 2887–2895 (2010).
232. Zhang, X. & Yadavalli, V. K. Surface immobilization of DNA aptamers for biosensing and protein interaction analysis. *Biosens Bioelectron* **26**, 3142–3147 (2011).
233. Lichtenberg, J. Y., Ling, Y. & Kim, S. Non-Specific Adsorption Reduction Methods in Biosensing. *Sensors* **19**, 2488 (2019).
234. Sultana, A. & Lee, J. E. Measuring Protein-Protein and Protein-Nucleic Acid Interactions by Biolayer Interferometry. *Curr Protoc Protein Sci* **79**, (2015).
235. Park, J. H. *et al.* Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Physical Chemistry Chemical Physics* **19**, 8854–8865 (2017).

236. Yu, L., Zhang, L. & Sun, Y. Protein behavior at surfaces: Orientation, conformational transitions and transport. *J Chromatogr A* **1382**, 118–134 (2015).
237. Zhdanov, V. P. & Kasemo, B. Monte Carlo Simulations of the Kinetics of Protein Adsorption. *Surface Review and Letters* **05**, 615–634 (1998).
238. Rabe, M., Verdes, D. & Seeger, S. Understanding protein adsorption phenomena at solid surfaces. *Adv Colloid Interface Sci* **162**, 87–106 (2011).
239. Gau, V. *et al.* Electrochemical molecular analysis without nucleic acid amplification. *Methods* **37**, 73–83 (2005).
240. Huang, J. *et al.* Femtomolar detection of nucleic acid based on functionalized gold nanoparticles. *Nanophotonics* **8**, 1495–1503 (2019).
241. Cerruti, M. *et al.* Poly(ethylene glycol) Monolayer Formation and Stability on Gold and Silicon Nitride Substrates. *Langmuir* **24**, 10646–10653 (2008).
242. Steel, A. B., Levicky, R. L., Herne, T. M. & Tarlov, M. J. Immobilization of Nucleic Acids at Solid Surfaces: Effect of Oligonucleotide Length on Layer Assembly. *Biophys J* **79**, 975–981 (2000).
243. Kallenbach, N. R., Ma, R.-I. & Seeman, N. C. An immobile nucleic acid junction constructed from oligonucleotides. *Nature* **305**, 829–831 (1983).
244. Chen, J. & Seeman, N. C. Synthesis from DNA of a molecule with the connectivity of a cube. *Nature* **350**, 631–633 (1991).
245. Winfree, E., Liu, F., Wenzler, L. A. & Seeman, N. C. Design and self-assembly of two-dimensional DNA crystals. *Nature* **394**, 539–544 (1998).
246. LaBean, T. H. *et al.* Construction, Analysis, Ligation, and Self-Assembly of DNA Triple Crossover Complexes. *J Am Chem Soc* **122**, 1848–1860 (2000).
247. Rothemund, P. W. K. Folding DNA to create nanoscale shapes and patterns. *Nature* **440**, 297–302 (2006).
248. Goodman, R. P. *et al.* Reconfigurable, braced, three-dimensional DNA nanostructures. *Nat Nanotechnol* **3**, 93–96 (2008).
249. Erben, C. M., Goodman, R. P. & Turberfield, A. J. Single-Molecule Protein Encapsulation in a Rigid DNA Cage. *Angewandte Chemie International Edition* **45**, 7414–7417 (2006).
250. Goodman, R. P. *et al.* Rapid Chiral Assembly of Rigid DNA Building Blocks for Molecular Nanofabrication. *Science (1979)* **310**, 1661–1665 (2005).
251. Goodman, R. P., Berry, R. M. & Turberfield, A. J. The single-step synthesis of a DNA tetrahedron. Electronic supplementary information (ESI) available: stoichiometry control. See <http://www.rsc.org/suppdata/cc/b4/b402293a/>. *Chemical Communications* 1372 (2004) doi:10.1039/b402293a.
252. Shih, W. M., Quispe, J. D. & Joyce, G. F. A 1.7-kilobase single-stranded DNA that folds into a nanoscale octahedron. *Nature* **427**, 618–621 (2004).
253. Ke, Y. *et al.* Scaffolded DNA Origami of a DNA Tetrahedron Molecular Container. *Nano Lett* **9**, 2445–2447 (2009).

254. Yan, J. *et al.* Tetrahedral DNA nanostructures for effective treatment of cancer: advances and prospects. *J Nanobiotechnology* **19**, 412 (2021).
255. Wang, C. *et al.* Tetrahedral DNA Framework-Programmed Electrochemical Biosensors with Gold Nanoparticles for Ultrasensitive Cell-Free DNA Detection. *Nanomaterials* **12**, 666 (2022).
256. Zhang, X.-L. *et al.* High-Efficiency 3D DNA Walker Immobilized by a DNA Tetrahedral Nanostructure for Fast and Ultrasensitive Electrochemical Detection of MiRNA. *Anal Chem* **95**, 4077–4085 (2023).
257. Bu, N.-N., Tang, C.-X., He, X.-W. & Yin, X.-B. Tetrahedron-structured DNA and functional oligonucleotide for construction of an electrochemical DNA-based biosensor. *Chemical Communications* **47**, 7689 (2011).
258. Huang, R., He, N. & Li, Z. Recent progresses in DNA nanostructure-based biosensors for detection of tumor markers. *Biosens Bioelectron* **109**, 27–34 (2018).
259. Mathur, D. & Medintz, I. L. The Growing Development of DNA Nanostructures for Potential Healthcare-Related Applications. *Adv Healthc Mater* **8**, 1801546 (2019).
260. Hui, N., Sun, X., Niu, S. & Luo, X. PEGylated Polyaniline Nanofibers: Antifouling and Conducting Biomaterial for Electrochemical DNA Sensing. *ACS Appl Mater Interfaces* **9**, 2914–2923 (2017).
261. Lowe, S., O'Brien-Simpson, N. M. & Connal, L. A. Antibiofouling polymer interfaces: poly(ethylene glycol) and other promising candidates. *Polym Chem* **6**, 198–212 (2015).
262. Sharma, S., Johnson, R. W. & Desai, T. A. XPS and AFM analysis of antifouling PEG interfaces for microfabricated silicon biosensors. *Biosens Bioelectron* **20**, 227–239 (2004).
263. Feng, T. *et al.* Low-Fouling Nanoporous Conductive Polymer-Coated Microelectrode for In Vivo Monitoring of Dopamine in the Rat Brain. *Anal Chem* **91**, 10786–10791 (2019).
264. Zhou, L. *et al.* In Vivo Monitoring of Oxygen in Rat Brain by Carbon Fiber Microelectrode Modified with Antifouling Nanoporous Membrane. *Anal Chem* **91**, 3645–3651 (2019).
265. Cui, J. *et al.* Nanoscale engineering of low-fouling surfaces through polydopamine immobilisation of zwitterionic peptides. *Soft Matter* **10**, 2656–2663 (2014).
266. Liu, F., Liu, X., Chen, F. & Fu, Q. Mussel-inspired chemistry: A promising strategy for natural polysaccharides in biomedical applications. *Prog Polym Sci* **123**, 101472 (2021).
267. Cui, M. *et al.* Mixed Self-Assembled Aptamer and Newly Designed Zwitterionic Peptide as Antifouling Biosensing Interface for Electrochemical Detection of alpha-Fetoprotein. *ACS Sens* **2**, 490–494 (2017).
268. Wang, G. *et al.* Zwitterionic peptide anchored to conducting polymer PEDOT for the development of antifouling and ultrasensitive electrochemical DNA sensor. *Biosens Bioelectron* **92**, 396–401 (2017).
269. Uludağ, İ. & Sezgintürk, M. K. A direct and simple immobilization route for immunosensors by CNBr activation for covalent attachment of anti-leptin: obesity diagnosis point of view. *3 Biotech* **12**, 33 (2022).

270. Vaisocherová, H. *et al.* Ultralow Fouling and Functionalizable Surface Chemistry Based on a Zwitterionic Polymer Enabling Sensitive and Specific Protein Detection in Undiluted Blood Plasma. *Anal Chem* **80**, 7894–7901 (2008).
271. Jiang, S. & Cao, Z. Ultralow-Fouling, Functionalizable, and Hydrolyzable Zwitterionic Materials and Their Derivatives for Biological Applications. *Advanced Materials* **22**, 920–932 (2010).
272. Huang, C.-J., Li, Y. & Jiang, S. Zwitterionic Polymer-Based Platform with Two-Layer Architecture for Ultra Low Fouling and High Protein Loading. *Anal Chem* **84**, 3440–3445 (2012).
273. Verlee, A., Mincke, S. & Stevens, C. v. Recent developments in antibacterial and antifungal chitosan and its derivatives. *Carbohydr Polym* **164**, 268–283 (2017).
274. Gezer, U., Tiryakioglu, D., Bilgin, E., Dalay, N. & Holdenrieder, S. Androgen Stimulation of PCA3 and miR-141 and Their Release from Prostate Cancer Cells. *Cell J* **16**, 488 (2015).
275. Rana, S., Valbuena, G. N., Curry, E., Bevan, C. L. & Keun, H. C. MicroRNAs as biomarkers for prostate cancer prognosis: a systematic review and a systematic reanalysis of public data. *Br J Cancer* **126**, 502–513 (2022).
276. Erdmann, K. *et al.* Elevated expression of prostate cancer-associated genes is linked to down-regulation of microRNAs. *BMC Cancer* **14**, 82 (2014).
277. Selth, L. A. *et al.* Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease. *Int J Cancer* **131**, 652–661 (2012).
278. Yaman Agaoglu, F. *et al.* Investigation of miR-21, miR-141, and miR-221 in blood circulation of patients with prostate cancer. *Tumor Markers, Tumor Targeting and Translational Cancer Research* **32**, 583–588 (2011).
279. Shen, J. *et al.* Dysregulation of circulating microRNAs and prediction of aggressive prostate cancer. *Prostate* **72**, 1469–1477 (2012).
280. Nematollahi, D. & Golabi, S. M. Electrochemical study of catechol and 4-methylcatechol in methanol. Application to the electro-organic synthesis of 4,5-dimethoxy- and 4-methoxy-5-methyl-o-benzoquinone. *Journal of Electroanalytical Chemistry* **405**, 133–140 (1996).
281. Adams, R. N., Hawley, M. D. & Feldberg, S. W. Nuances of the E.C.E. mechanism. II. Addition of hydrochloric acid and amines to electrochemically generated o-benzoquinones. *J Phys Chem* **71**, 851–855 (1967).
282. NEMATOLLAHI, D., GHASEMI, F., KHAZALPOUR, S. & VARMAGHANI, F. Kinetic study on electrochemical oxidation of catechols in the presence of cycloheptylamine and aniline: Experiments and digital simulation. *Journal of Chemical Sciences* **128**, 1887–1894 (2016).
283. Sapurina, I. & Stejskal, J. The mechanism of the oxidative polymerization of aniline and the formation of supramolecular polyaniline structures. *Polym Int* **57**, 1295–1325 (2008).
284. Boccafoschi, F., Fusaro, L. & Cannas, M. Immobilization of peptides on cardiovascular stent. in *Functionalised Cardiovascular Stents* 305–318 (Elsevier, 2018). doi:10.1016/B978-0-08-100496-8.00016-0.

285. Vennerberg, D., Hall, R. & Kessler, M. R. Supercritical carbon dioxide-assisted silanization of multi-walled carbon nanotubes and their effect on the thermo-mechanical properties of epoxy nanocomposites. *Polymer (Guildf)* **55**, 4156–4163 (2014).
286. Zhao, Y. *et al.* Silanized carbon dot-based thermo-sensitive molecularly imprinted fluorescent sensor for bovine hemoglobin detection. *Anal Bioanal Chem* **412**, 5811–5817 (2020).
287. Dixit, C. K., Vashist, S. K., MacCraith, B. D. & O’Kennedy, R. Multisubstrate-compatible ELISA procedures for rapid and high-sensitivity immunoassays. *Nat Protoc* **6**, 439–445 (2011).
288. Jönsson, U., Olofsson, G., Malmqvist, M. & Rönnerberg, I. Chemical vapour deposition of silanes. *Thin Solid Films* **124**, 117–123 (1985).
289. Fadeev, A. Y. & McCarthy, T. J. Self-Assembly Is Not the Only Reaction Possible between Alkyltrichlorosilanes and Surfaces: Monomolecular and Oligomeric Covalently Attached Layers of Dichloro- and Trichloroalkylsilanes on Silicon. *Langmuir* **16**, 7268–7274 (2000).
290. Lucarelli, F., Marrazza, G., Turner, A. P. F. & Mascini, M. Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors. *Biosens Bioelectron* **19**, 515–530 (2004).
291. Höök, F., Ray, A., Nordén, B. & Kasemo, B. Characterization of PNA and DNA Immobilization and Subsequent Hybridization with DNA Using Acoustic-Shear-Wave Attenuation Measurements. *Langmuir* **17**, 8305–8312 (2001).
292. Qi, H., Li, M., Zhang, R., Dong, M. & Ling, C. Double electrochemical covalent coupling method based on click chemistry and diazonium chemistry for the fabrication of sensitive amperometric immunosensor. *Anal Chim Acta* **792**, 28–34 (2013).
293. Zhu, N., Chang, Z., He, P. & Fang, Y. Electrochemically fabricated polyaniline nanowire-modified electrode for voltammetric detection of DNA hybridization. *Electrochim Acta* **51**, 3758–3762 (2006).
294. Hua, H., Liu, Y., Guan, X. & Li, Y. DNA nanosensors based on the use of single gold nanowire electrodes and Methylene Blue as an intercalator. *Microchimica Acta* **185**, (2018).
295. Donmez, S., Arslan, F. & Arslan, H. Sequence-specific label-free nucleic acid biosensor for the detection of the hepatitis C virus genotype 1a using a disposable pencil graphite electrode. *Artif Cells Nanomed Biotechnol* 1–6 (2015) doi:10.3109/21691401.2014.998831.
296. Kerman, K., Matsubara, Y., Morita, Y., Takamura, Y. & Tamiya, E. Peptide nucleic acid modified magnetic beads for intercalator based electrochemical detection of DNA hybridization. *Sci Technol Adv Mater* **5**, 351–357 (2004).
297. Watkins, H. M., Simon, A. J., Ricci, F. & Plaxco, K. W. Effects of Crowding on the Stability of a Surface-Tethered Biopolymer: An Experimental Study of Folding in a Highly Crowded Regime. *J Am Chem Soc* **136**, 8923–8927 (2014).
298. Peterson, A. W., Heaton, R. J. & Georgiadis, R. Kinetic Control of Hybridization in Surface Immobilized DNA Monolayer Films. *J Am Chem Soc* **122**, 7837–7838 (2000).
299. Lang, B. E. & Schwarz, F. P. Thermodynamic dependence of DNA/DNA and DNA/RNA hybridization reactions on temperature and ionic strength. *Biophys Chem* **131**, 96–104 (2007).

300. Bonaldo, S. *et al.* Influence of BSA Protein on Electrochemical Response of Genosensors. *IEEE Sens J* **23**, 1786–1794 (2023).
301. Zuo, X., Xiao, Y. & Plaxco, K. W. High Specificity, Electrochemical Sandwich Assays Based on Single Aptamer Sequences and Suitable for the Direct Detection of Small-Molecule Targets in Blood and Other Complex Matrices. *J Am Chem Soc* **131**, 6944–6945 (2009).
302. Al Sulaiman, D., Chang, J. Y. H. & Ladame, S. Subnanomolar Detection of Oligonucleotides through Templated Fluorogenic Reaction in Hydrogels: Controlling Diffusion to Improve Sensitivity. *Angewandte Chemie International Edition* **56**, 5247–5251 (2017).
303. Gillespie, P. *et al.* Nucleic acid sensing via electrochemical oligonucleotide-templated reactions. *Biosens Bioelectron* **176**, 112891 (2021).
304. Wang, S. *et al.* Electrochemical impedance spectroscopy. *Nature Reviews Methods Primers* **1**, 41 (2021).
305. Wang, J., Zhang, S. & Zhang, Y. Fabrication of chronocoulometric DNA sensor based on gold nanoparticles/poly(L-lysine) modified glassy carbon electrode. *Anal Biochem* **396**, 304–309 (2010).
306. Ganguly, A., Benson, J. & Papakonstantinou, P. Sensitive Chronocoulometric Detection of miRNA at Screen-Printed Electrodes Modified by Gold-Decorated MoS₂ Nanosheets. *ACS Appl Bio Mater* **1**, 1184–1194 (2018).
307. Raoof, J. B., Ojani, R., Golabi, S. M., Hamidi-Asl, E. & Hejazi, M. S. Preparation of an electrochemical PNA biosensor for detection of target DNA sequence and single nucleotide mutation on p53 tumor suppressor gene corresponding oligonucleotide. *Sens Actuators B Chem* **157**, 195–201 (2011).
308. Steinitz, M. Quantitation of the Blocking Effect of Tween 20 and Bovine Serum Albumin in ELISA Microwells. *Anal Biochem* **282**, 232–238 (2000).
309. Marie, R., Jensenius, H., Thaysen, J., Christensen, C. B. & Boisen, A. Adsorption kinetics and mechanical properties of thiol-modified DNA-oligos on gold investigated by microcantilever sensors. *Ultramicroscopy* **91**, 29–36 (2002).
310. Ratilainen, T., Holmén, A., Tuite, E., Nielsen, P. E. & Nordén, B. Thermodynamics of Sequence-Specific Binding of PNA to DNA. *Biochemistry* **39**, 7781–7791 (2000).
311. Chambers, J. Q. Electrochemistry of quinones. in *Quinonoid Compounds: Vol. 2 (1974)* 737–791 (John Wiley & Sons, Ltd.). doi:10.1002/9780470771303.ch3.

Appendix Supplementary Figures for Chapter 4

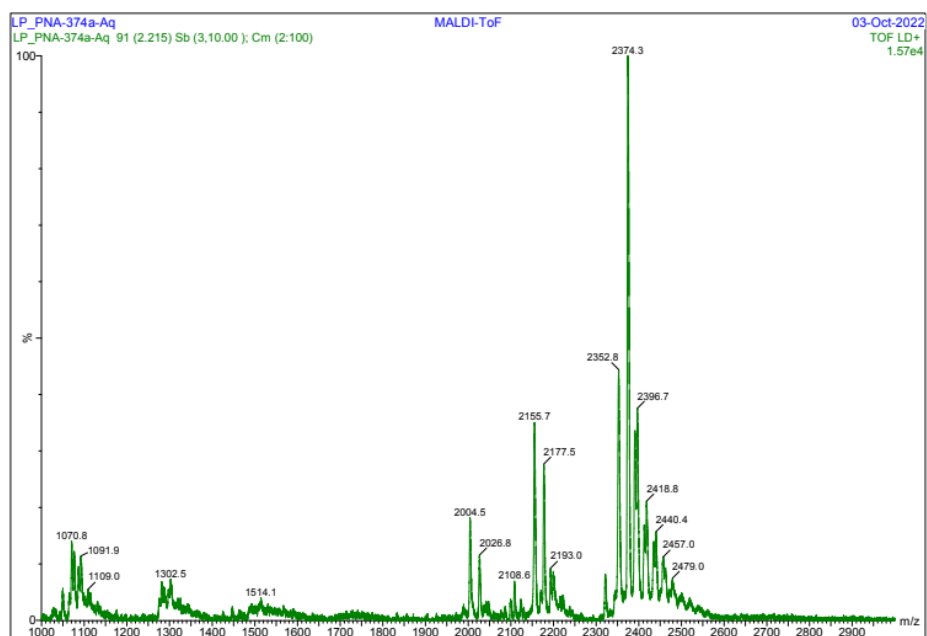


Figure A-1 Matrix-assisted laser desorption/ionization (MALDI) results of HPLC purified PNA-AQ-374a.

EOTR

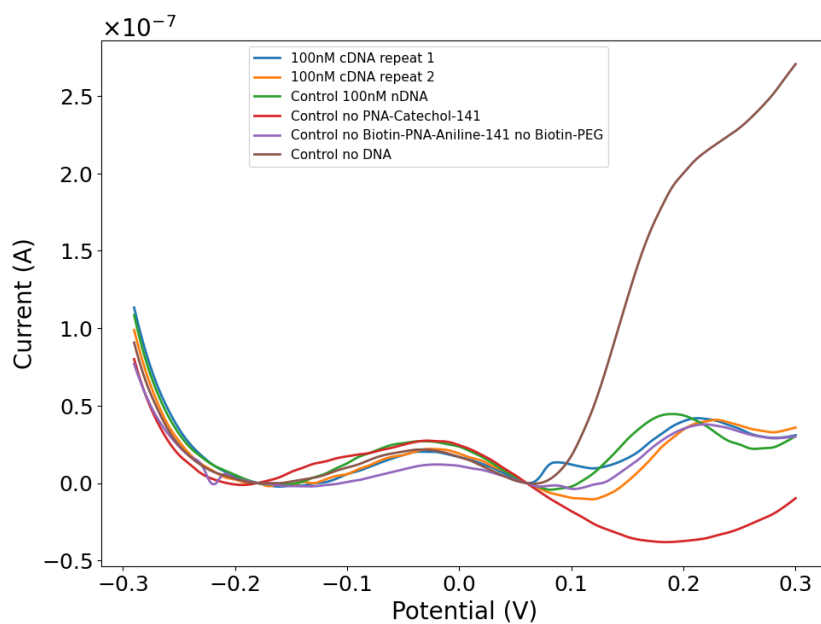


Figure A-2 DPV investigation of EOTR on SPCE using polystreptavidin-biotin method (cDNA is complementary DNA141 and nDNA is non-complementary DNA127, baseline corrected).

Chronoamperometry on Polystreptavidin-biotin System

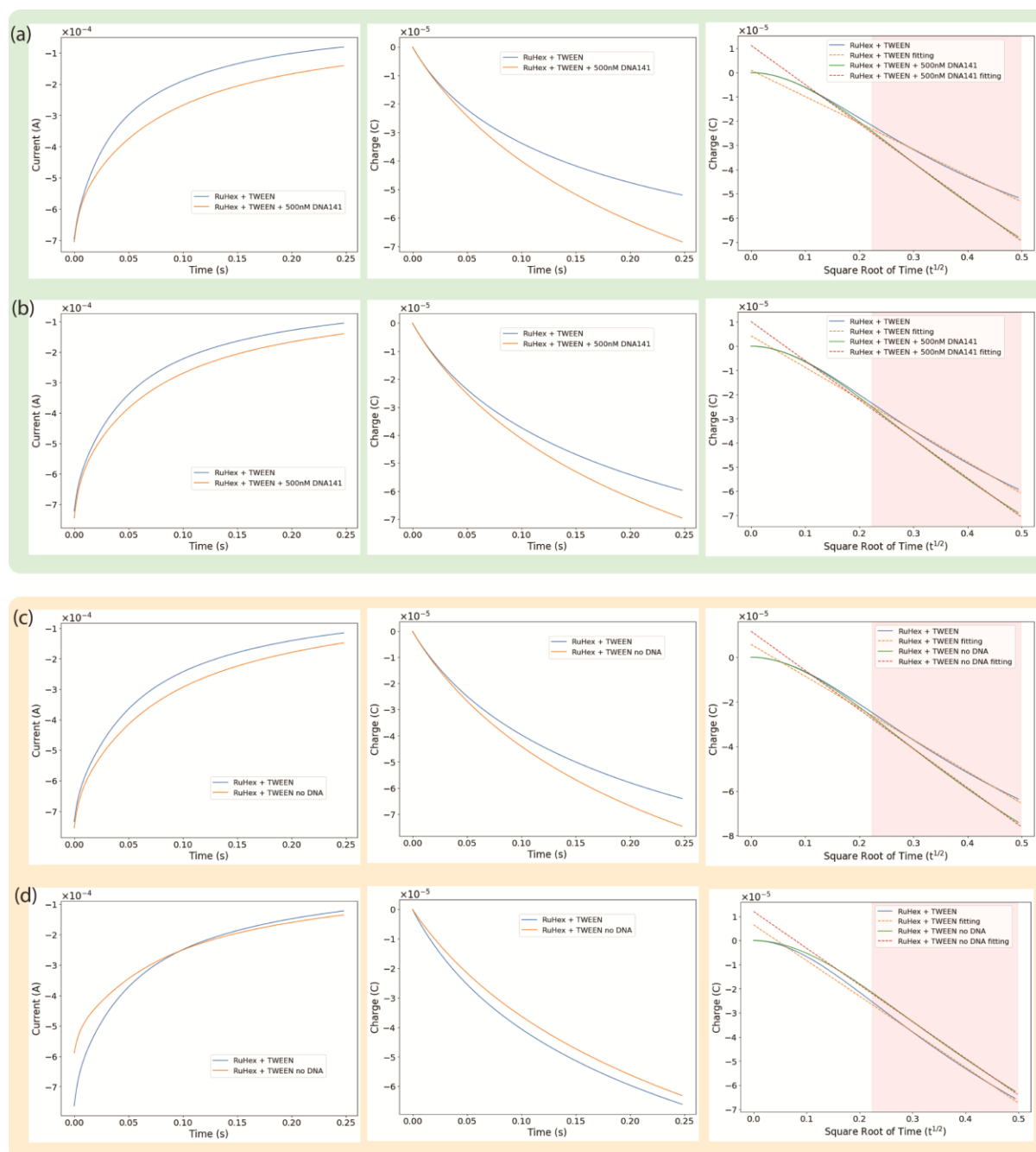


Figure A-3 Chronoamperometry data for charge fitting and calculation (after 0.05% TWEEN washing, 0.25 s measurement). (a) and (b) Two repeats of experimental groups using complementary DNA141. (c) and (d) Two repeats of control groups without DNA. Each panel contains three graphs from left to right: original chronoamperometry data, integrated charge plotted against time, and charge fitting with the double layer capacitance part omitted.

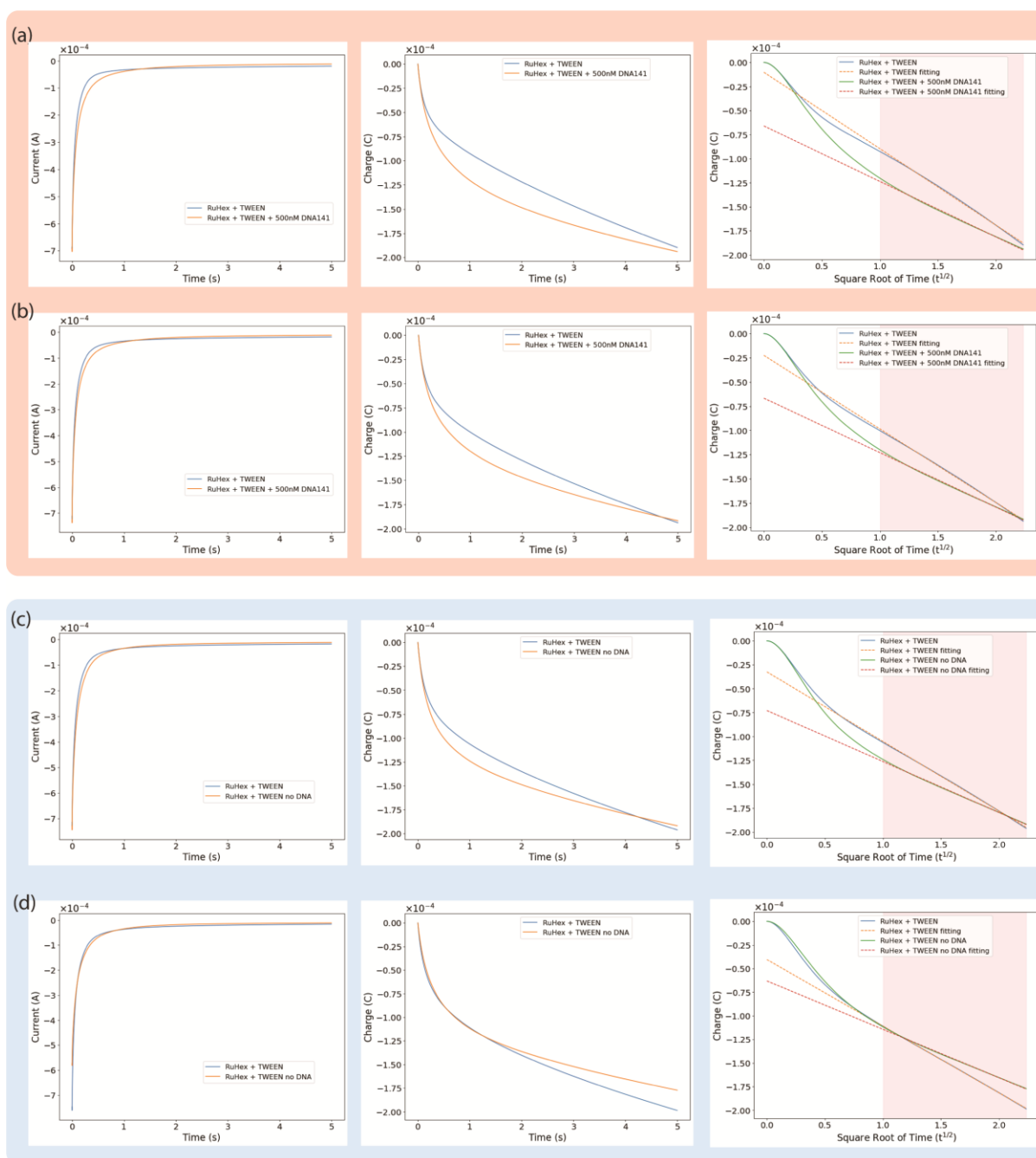


Figure A-4 Chronoamperometry data for charge fitting and calculation (after 0.05% TWEEN washing, 5 s measurement). (a) and (b) Two repeats of experimental groups using complementary DNA141. (c) and (d) Two repeats of control groups without DNA. Each panel contains three graphs from left to right: original chronoamperometry data, integrated charge plotted against time, and charge fitting with the double layer capacitance part omitted.

Chronoamperometry on Gold-thiol System

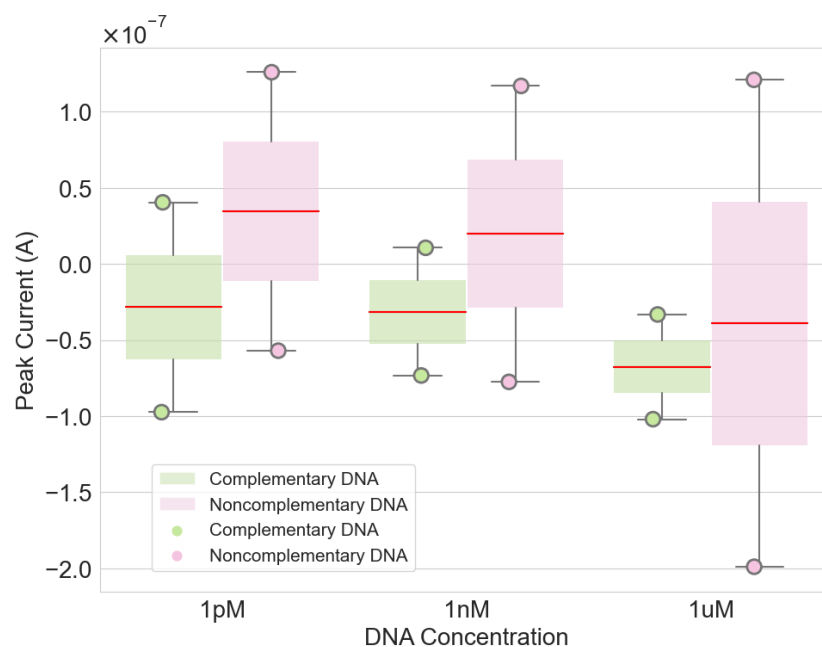


Figure A-5 Charge associated with complementary DNA (DNA141) and control non-complementary DNA (DNA150) on bulk gold electrode.

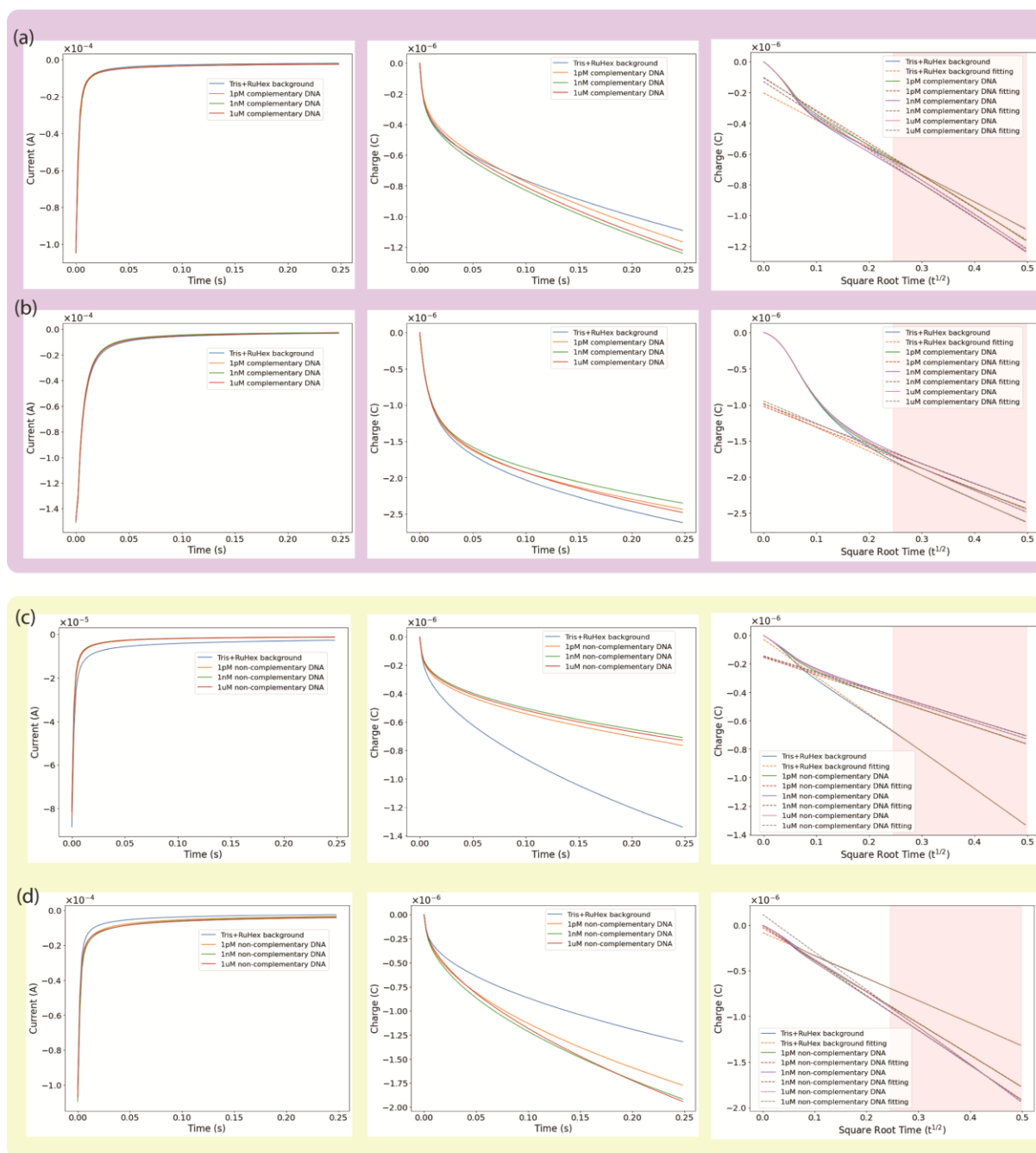


Figure A-6 Chronoamperometry data for charge fitting and calculation with bulk gold electrode (0.25 s measurement). (a) and (b) Two repeats of experimental groups using complementary DNA (DNA141). (c) and (d) Two repeats of control groups with non-complementary DNA (DNA150). Each panel contains three graphs from left to right: original chronoamperometry data, integrated charge plotted against time, and charge fitting with the double layer capacitance part omitted.

Appendix Copyright Permission



ACS Publications <acs@service-now.com>
To: Meng, Xiaotong

😊 ↶ ↷ ↲ ...
Tue 10/3/2023 12:24 PM

This email from acs@service-now.com originates from outside Imperial. Do not click on links and attachments unless you recognise the sender. If you trust the sender, add them to your [safe senders list](#) to disable email stamping for this address.

Dear Dr. Meng,

Thank you for contacting ACS Publications Support.

Your permission request is granted and there is no fee for this reuse.

In your planned reuse, you must cite the ACS article as the source, add this direct link: <https://pubs.acs.org/doi/10.1021/acs.jchemed.7b00361>, and include a notice to readers that further permissions related to the material excerpted should be directed to the ACS.

Please do not hesitate to contact us if you need any further assistance.

Sincerely,

Vojin Vucic
ACS Publications Support
Customer Services & Information
Website: <https://acs.service-now.com/acs>
Email: support@services.acs.org
Phone: 800-227-9919 | 202-872-(HELP) 4357