Electrochemical modulation of surface plasmon wave to investigate DNA-based sensing platforms and detection of SARS-CoV2.

Anil Sharma
University of Louisville

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ELECTROCHEMICAL MODULATION OF SURFACE PLASMON WAVE TO
INVESTIGATE DNA-BASED SENSING PLATFORMS AND DETECTION OF SARS-
CoV2

By
Anil Sharma
B.S., Prithvi Narayan Campus, 2013
M.S., Tribhuvan University, 2017
M.S., University of Louisville, 2020

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences at the University of Louisville
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For the Degree of

Doctor of Philosophy in Physics

Department of Physics and Astronomy
University of Louisville
Louisville, Kentucky

May 2024
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A Dissertation Approved on

April 23, 2024

by the following Dissertation Committee:

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Dr. Sergio B. Mendes

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Dr. Gamini Sumanasekera

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Dr. Ming Yu

____________________
Dr. Martin G. O’Toole
DEDICATION

This dissertation holds a special dedication to my mother, who remained in my home country for all these years, away from me, just to witness my journey to a Ph.D. degree. Thank you, Mom, for your enduring patience and prayers that have guided me to where I am today. You have been my strength and inspiration throughout my journey. I am forever thankful for your love and dedication.

I am also immensely grateful to my dear friends and colleagues, whose support in moments of doubt and challenge, and readiness to help were invaluable during this journey.

Finally, my deepest appreciation is reserved for my beloved wife, Shrijana. She has been my constant companion, sharing each step of this arduous but rewarding path. Without her, this dream would have remained just that—a dream. Thank you, Shrijana, for making it all possible.
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ABSTRACT

ELECTROCHEMICAL MODULATION OF SURFACE PLASMON WAVE TO INVESTIGATE DNA-BASED SENSING PLATFORMS AND DETECTION OF SARS-CoV2

Anil Sharma

April 23, 2024

This dissertation investigates the influence of the placement of signal-generating molecules on the electrochemical response and efficiency of DNA hybridization sensors. The sensor design incorporates a methylene blue (MB)-modified single-stranded DNA (ssDNA) signaling probe alongside an unlabeled complementary ssDNA capture probe. Two distinct signaling probes were utilized: one with MB attached at the 3' end, bringing the redox marker closer to the electrode surface upon hybridization, and another with MB at the 5' end, positioning the marker further from the surface. The research involved comprehensive studies using electrochemical techniques such as cyclic voltammetry and electrochemical impedance spectroscopy, supplemented by optical impedance spectroscopy with electrochemical modulation of surface plasmon waves. A key focus was to assess the quantity of electrochemically active redox molecules at each redox label position and to determine the rate of electron transfer between the redox marker and the electrode.
The results, derived from both electrical and optical measurements, reveal that the proximity of the signal probe to the electrode surface, particularly with MB as the redox marker, significantly enhances sensor performance. A higher concentration of active redox markers and a substantially increased rate of electron transfer were observed in configurations where the marker was closer to the electrode surface.

Further, the potential mechanism of electron transfer when the redox marker is covalently bonded to the sugar-phosphate backbone of the signaling probe ssDNA was explored. The study indicates a collisional electron transfer process, meaning the transfer occurs when the redox molecule physically contacts the electrode surface.

Concluding this research, a novel, cost-effective electrochemical biosensing strategy is proposed for detecting synthetic genetic sequences that mimic specific zones of the SARS-CoV2 genome. By comparing different protocols and sensing strategies, pathways for further exploration and refinement of this sensing approach are opened, potentially offering significant advancements in the field of molecular diagnostics and bio-sensing technology. This work not only demonstrates the critical role of redox molecule positioning in sensor efficiency but also sets the stage for future innovations in DNA-based electrochemical sensing.
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CHAPTER 1 INTRODUCTION

1.1 Structure of a DNA

Deoxyribonucleic acid (DNA), constitutes a vital component within the cells of living organisms, serving as the carrier of genetic information. Its structure consists of four distinct components known as bases, which are attached to a phosphate-sugar backbone. Each base, when combined with the phosphate-sugar backbone, forms a structural unit within DNA referred to as a nucleotide. These four bases are Adenine (A), Guanine (G), Thymine (T), and Cytosine (C). A long chain of sequential arrangement of these bases attached to the pentose sugar or the phosphate-sugar backbone as shown in Figure 1-1 (a) is responsible for the formation of a single-stranded DNA molecule [2].

In 1953, James Watson and Francis Crick proposed the groundbreaking model for double-stranded DNA [5]. This model explained the formation of a double-stranded helical structure through hydrogen bonding interactions, with adenine pairing with thymine and cytosine pairing with guanine as depicted in Figure 1-2. Consequently, two individual DNA strands unite to create the distinctive double-stranded DNA helix as shown in Figure 1-1 (b).
Figure 1-1: (a) Chemical formula of a strand of DNA [2]. (b) A schematic representation of a DNA double helix structure [5].

Adenine-Thymine

Cytosine-Guanine

Figure 1-2: A schematic representation of the formation of hydrogen bonds between complementary DNA nucleobases [1].
1.2 DNA-based Biosensors

A biosensor is a specialized device that pairs a biological sensing component with a transducer to create a signal that is proportional to the amount of a specific target analyte being measured [6]. In the context of DNA biosensors, the sensitive element typically consists of single-stranded DNA (ssDNA) molecules that facilitate the hybridization of complementary single-stranded counterparts. There are several methods for transducing these hybridization signals, including: a) Optical transduction techniques such as surface plasmon resonance (SPR) [7-9], surface-enhanced Raman spectroscopy (SERS) [10, 11], optical fibers [12, 13], optical waveguides [14], and reflection interference contrast microscopy (RICM) [15, 16], b) Electrochemical transduction [17, 18], and c) Piezoelectric transduction [19-21]. Potential applications of DNA biosensors include diagnostic medicine [22], drug discovery [23], food industry [24], and environmental monitoring [25].

1.2.1 Principle Mechanism of DNA Sensors

Nucleic acid hybridization forms the foundation of DNA biosensors. DNA, the famous double helix structure proposed by Watson and Crick, consists of four repeating nucleotides: adenine, guanine, cytosine, and thymine [2]. This double helix structure comprises two complementary strands held together by hydrogen bonds. The magic happens when a single-stranded DNA (ssDNA) molecule seeks out and binds to its complementary strand in a sample. This binding is possible because specific nucleotide bases only form hydrogen bonds with their complements: adenine pairs with thymine, and cytosine pairs with guanine [2]. In RNA, uracil takes thymine's place and pairs with adenine. The stability of this binding depends on the nucleotide sequences of both
strands. An exact match results in a very stable double-stranded DNA (dsDNA), while one or more base mismatches lead to increasing instability and weaker binding [6]. DNA is especially well-suited for biosensing applications due to the highly specific and robust interactions between complementary sequences. Additionally, the two hybridized strands can be readily separated by adjusting temperature and pH, making it straightforward to regenerate the sensor platform in DNA sensors.

In a typical setup, a single-stranded probe sequence is anchored within the recognition layer, where base-pairing interactions attract the target DNA to the surface. DNA's consistent and repetitive structure makes its assembly on the recognition surface well-defined. This interface is where the critical process of capturing the target takes place to generate the recognition signal. Therefore, immobilizing nucleic acid probe sequences predictably while retaining their natural attraction for target DNA is crucial for the overall performance of the device [18].

The method used to report this recognition event ultimately depends on the chosen signal transduction approach as discussed in the previous section.

1.2.2 DNA Sensors Based on Electrochemical Transduction

The ability to confine immobilized DNA probe sequences to various electrode substrates is the heart of electrochemical detection modes for DNA sensors. Several techniques have been innovated to secure the DNA probe onto the solid surface of biosensors. These include the formation of self-assembling monolayers (SAMs) on gold electrodes [26], the attachment of biotinylated DNA probes via biotin-avidin interactions on the electrode surface [27], the use of electro-polymerization to generate probes of varying lengths [28], and the self-assembly of thiol-derivatized DNA strands [29, 30].
The signal transduction for electrochemical detection is done in two ways: label-free or direct and label-based or indirect DNA detection. DNA-based label-free electrochemical biosensors are designed using two main approaches. The first approach leverages the inherent electroactivity of target nucleic acid molecules, while the second detects changes in electrochemical signals related to the hybridization reaction, without labeling DNA probes or target analytes with electroactive molecules. Initially, label-free DNA biosensors primarily depended on the natural redox-active characteristics of DNA bases, particularly the electrochemical oxidation signal of guanine. For example, Palecek and the group's [31] pioneering work on the direct oxidation of guanine using a mercury drop electrode laid the groundwork for label-free DNA electrochemical biosensors.

Further, Wang and colleagues [32, 33] developed various label-free electrochemical methods centered on the intrinsic redox activity of target guanine for DNA hybridization detection. While this approach is highly sensitive, it faces challenges due to considerable background currents as high potential is needed for direct DNA oxidation. Advances have been made to enhance the signal-to-noise ratio, including numerical methods, and the use of physical separation techniques to mitigate background interference [34]. Although label-free DNA detection streamlines the analysis process by simplifying the readout and reducing both time and costs, it does not achieve the same level of sensitivity as indirect (label-based) DNA detection methods [16].

1.2.3 Label-based Electrochemical DNA Detection

Indirect methods use redox mediators like Co(phen)$_3^{3+}$, Ru(NH$_3$)$_6^{3+}$,
Ru(bpy)$_3$$^{3+/2+}$, Os(bpy)$_3$$^{3+/2+}$, K$_3$Fe([CN])$_6$$^{3-/4-}$, Ferrocene (Fc), Methylene Blue (MB), etc. to facilitate reversible electron exchange between the electrode and the mediator. Some approaches use Electrochemical Impedance Spectroscopy (EIS) technique to study interfacial properties like resistance and capacitance. This technique focuses on the changes in charge transfer resistance of a redox couple, enabling the collection of data about biorecognition events on electrode surfaces while some uses electroactive indicators as signal generating molecules. Detection, in the latter case, is achieved by comparing the electrochemical signals of electroactive indicators before and after the introduction of target analytes. The interaction between electroactive indicators (such as Ferrocene, Methylene Blue, Thionine, etc.) and DNA molecules generally falls into three categories: electrostatic binding [17, 35], surface binding [36], and intercalation [37, 38]. Electrostatic binding involves the interaction of indicator molecules with DNA's negatively charged deoxyribose-phosphate backbone through electrostatic forces. Surface binding occurs when indicators and DNA molecules bind via the hydrophobic interactions of the bases. Intercalation refers to the insertion of indicator molecules between DNA base pairs in its double-helix structure, facilitated by hydrogen bonds, van der Waals forces, or stacking interactions.

Electrochemical detection methods, including DNA-based electrochemical sensors (E-DNA), electrochemical aptamer-based sensors (E-AB), electrochemical sandwich assays, and competition-type assays employing oligonucleotide probes attached to a redox-active indicator, are pivotal in biosensor technology [39-45]. Predominantly, these types of sensors incorporate MB [40, 45-49] or Fc [39, 50-53] as signaling elements due to their reversible redox behaviors, suitable redox potentials, and easy conjugation
with synthesized oligonucleotides. However, E-DNA sensors constructed using methylene blue (MB)-labelled oligonucleotides exhibit superiority over those labelled with ferrocene (Fc) in aspects of electrochemical signal stability, durability during long-term storage, resilience to repeated signal interrogations, and reproducibility throughout multiple sensor regenerations [54].

1.3 Surface Plasmon Resonance (SPR)

SPR is an opto-electrical phenomenon that occurs when light interacts with a metal surface. This phenomenon enables the detection of biomolecules on a chemically modified gold surface. The fundamental concept at play is the alteration in the local index of refraction when biomolecules are adsorbed on the metal surface. The optical behavior observed is directly proportional to the concentration of analyte that has adhered to the metal film [55]. SPR technique is a scalable, high-throughput approach that functions without labels and is suitable for array formats [56].

1.4 Electrochemical Modulation of Surface Plasmon Wave (ECM-SPW)

SPR can be combined with electrochemistry, a technique known as EC-SPR. In this approach, the thin metal film on the substrate serves a dual purpose: it excites surface plasmons and functions as a working electrode for electrochemical detection or control. The EC-SPR configuration offers the advantage of simultaneously acquiring data on both the electrochemical and optical characteristics of films with nanometer-scale thicknesses. The electrochemically modulated surface plasmon wave (ECM-SPW) technique has proven effective in characterizing thin films, exploring redox reactions occurring at metal/liquid interfaces, and advancing biosensing applications [57-62].

To establish a transduction principle that primarily detects electroactive faradaic
currents through an optical signal, the ECM-SPW technique incorporates redox mediators like Methylene Blue (MB), known for its robust and reversible spectral changes under electrochemical modulation. Thanks to its high selectivity for faradaic redox reactions and minimal susceptibility to interference from other electroactive substances, MB has been employed as a redox mediator and electrochemical indicator in oligonucleotide hybridization sensors. By integrating electrochemistry with plasmonic waves, ECM-SPW's sensitivity and versatility have been further augmented, allowing for the precise observation of subtle changes in optical properties resulting from electrochemical phenomena.

1.5 Outline of the Dissertation

Chapter 2 begins with a description of the design and construction of a DNA-based sensing platform. The core principles of DNA hybridization sensors, encompassing the immobilization of DNA on the sensor's surface, the hybridization process of DNA strands within an electrochemical cell, and the methods for enhancing the signal from the attached redox marker on the DNA strand is explored. This chapter also provides an in-depth examination of two distinct systems: one in which the redox marker is positioned near the electrode surface, and another where it is situated further away.

Various electrochemical and optical techniques, such as Cyclic Voltammetry, AC Voltammetry, Electrochemical Impedance Spectroscopy (EIS), and optical methods, are utilized for analysis. The chapter concludes with a comparative evaluation of these two systems, both electrochemically and optically, focusing on the density of electroactive species and the electron transfer rate between the electrode and the redox mediator.
Chapter 3 is dedicated to exploring the potential mechanisms of electron transfer between the electrode surface and a redox mediator that is covalently bound to the DNA's sugar-phosphate group. The purpose of this investigation is to assess whether the negatively charged sugar-phosphate backbone could facilitate electron transfer.

In Chapter 4, the application of this sensing strategy is detailed. It includes a comparative study of various protocols employing the sensor for detecting genetic analogues from the SARS-CoV-2 genome, proposing a cost-effective detection method. Finally, Chapter 5 concludes with suggestions for enhancing the sensor's performance and an outlook on future possibilities for this sensing platform is provided.
CHAPTER 2 EFFECT OF THE REDOX MARKER LOCATION IN THE 
ELECTROCHEMICAL RESPONSE OF DNA HYBRIDIZATION SENSORS

2.1 Introduction

Electrochemical DNA hybridization sensors have significantly advanced; however, the impact of the position of redox label placement on DNA probes and its consequential effect on charge transfer processes continues to be a subject for ongoing discussion due to the multiplicity of electron-transfer routes present at DNA interfaces [54]. Various explanatory models have been posited to elucidate the charge transfer mechanisms between redox labels such as methylene blue (MB) and the electrode interface. These models encompass the mediation of MB reduction through DNA base-pair stacking, the direct interaction of the redox molecule with the electrode enabled by the DNA's flexible bending, and the propagation of charge across the counterions aligned with the sugar-phosphate structure of DNA [63-66]. The length of the hydrocarbon chain linking the redox label to the DNA sequence is also critical in delineating the nature of electron transfer routes. Short chain lengths, which are strongly coupled to DNA base stack, tend to support DNA-mediated charge transfer [67], whereas extended chains impart greater mobility to the redox tag, thus facilitating direct electron transfer to the electrode through DNA duplexes' flexible bending [65]. Furthermore, the specific positioning of the redox labels along the DNA sequence not only influences hybridization efficacy but also the resultant electrochemical signals [68, 69].
Research by Silva et al. in 2018 highlighted that electrochemical signals are more intense when redox markers are positioned at the terminal end of a DNA monolayer, with diminished signals when markers are located closer to the base. Proximal positioning of labels often results in restricted ion movement due to DNA's perm-selective coating, leading to slower charge transfer kinetics and weaker signals. Conversely, labels placed atop the DNA layer enhance ion movement, thus expediting electron transfer kinetics and amplifying the electrochemical response [70]. However, the nanoswitch-based sensing approach detailed by the Meunier group indicates a decline in electron transfer efficiency between the redox entity and electrode with the redox active species positioned at a distance from the electrode [57]. Similarly, the elastic bending diffusion model of DNA duplexes outlined by Anne and Demaille suggests enhanced electron transfer efficiency when redox-tagged DNA duplexes undergo elastic bending, promoting closer contact between the redox tag and the electrode [71].

This chapter is dedicated to the effects of redox marker positioning in ssDNA on the efficiency of DNA hybridization and the subsequent electrochemical outputs. We utilized ssDNA probes with methylene blue (MB) positioned at two distinct spots along the oligonucleotides: one near the electrode surface and another further away. We tracked the MB signal changes following hybridization with a complementary capture ssDNA affixed to an electrode, employing various electrical and optical measurement methods such as cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and electrochemically modulated surface plasmon resonance (ECM-SPR).

The increasing use of Electrochemical Impedance Spectroscopy (EIS) in DNA
hybridization sensors is due to its effectiveness in identifying the resistive and capacitive properties of various biomolecules, such as DNA, RNA, and proteins, when attached to electrodes. EIS introduces small amplitude sinusoidal signals to perturb a stable system slightly and monitors the impedance over a wide range of alternating current (AC) frequencies [72]. This technique excels in measuring the electron transfer resistance at the interface where nucleic acids are bonded to the electrode. The resistance typically increases with the addition of any insulating material, which is a concept widely utilized in the design of impedimetric sensors [72]. Often, a redox probe is used to measure changes in charge transfer resistance when the electrode surface hybridizes with the target DNA.

2.2 Basic Framework of Sensing Device

2.2.1 Experimental Setup

The experimental setup was engineered to accommodate both electrochemical and optical analyses of the targeted system. Figure 2-1 shows a schematic representation of the experimental setup. A gold-coated glass slide was attached to an equilateral BK-7 glass prism (sourced from Edmund Optics) using index-matching gel with a refractive index of 1.52 (Cargille). This assembly was integrated with a custom-designed electrochemical flow cell featuring a three-electrode setup. Here, the gold film acted as the working electrode (WE), while a pair of gold-coated pins (with a cross-sectional area of 1.52 mm², provided by Mouser Electronics) served as the reference electrode (RE) and counter electrode (CE). These electrodes were interfaced with a CHI660D potentiostat (CH Instruments, USA) for precise potential control.
Optically, the setup employed the well-established Kretschmann configuration for the Surface Plasmon Resonance (SPR) setup and utilized a continuous-wave (CW) laser (Obis from Coherent) with a 685 nm wavelength operating at 5 mW. As illustrated in Figure 2-1, linearly polarized light featuring transverse-magnetic (TM) polarization was produced using a half-wave plate and a linear polarizer to stimulate the surface plasmon wave. To meticulously regulate the angle of incidence, the electrochemical flow cell was positioned on a rotational stage. The reflected optical intensity was monitored through a Photomultiplier Tube (PMT) and an Agilent DSO8104A Infiniium oscilloscope.

For Electrochemical Impedance Spectroscopy (EIS) measurements, the potentiostat was linked to a DS345 function generator (Stanford Research Systems), which provided sinusoidal potential modulation to induce redox oscillations of the Methylene Blue (MB). The modulated optical signals at a predetermined incident angle were captured by the PMT and then relayed to an SR830 DSP lock-in amplifier (Stanford Research Systems). Both the magnitude and phase of these signals were subsequently recorded using the oscilloscope.
Figure 2-1: A schematic representation of EC-SPR setup for DNA hybridization sensor. A potentiostat was for electrical control, CW laser, a retarder, and a linear polarizer for TM polarized light. PMT, lock-in amplifier, voltage amplifier, and oscilloscope were used for data acquisition. A function generator was coupled with the potentiostat to apply potential modulation. The sensing surface consists of a gold-coated glass slide.

2.2.2 Selection of Molecule of Interest

The primary purpose of this work was to study the effect of the placement of the redox label MB at different sites of oligonucleotides in the electrochemical response of the hybridized DNA duplex in an aqueous medium. On the other hand, the secondary purpose was to use the electrochemical response to design a biosensor that could detect the genetic material of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2). Therefore, the first thing was to select the base sequence and length of the oligonucleotides. Altogether three types of oligos were used: anchor, competitor, and target. The anchor was a 30-base oligonucleotide ssDNA with the following sequence: 5'-/5ThioMC6-D/AAG ATC TCA ATG GTA ACT GGT ATG ATT TCG-3', which had a C6 S-S (disulfide) modification at the 5'-end. The thiol modification was done to attach the anchor primer onto the gold surface using thiol gold chemistry [30] which will be
discussed in the following chapters. The target was a 60-base oligonucleotide with the following sequence: 5’-CAC TAC CTG GCG TGG TTT GTA TGA AAT CAC CGA AAT CAT ACC AGT TAC CAT TGA GAT CTT-3’, which mimicked a specific region known as RdRP (RNA-dependent RNA polymerase) of the Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) genome. Figure 2-2 shows the structure of SARS-CoV2 and Figure 2-3 shows a schematic diagram to show the RdRP region in 30 kb long SARS-CoV2 genome. The target is a synthetic mimic of the base sequence from 14010-14069 from the RdRP region.

![Diagram of SARS-CoV2 structure](image1)

Figure 2-2: The structure of SARS-CoV2 with its constituent protein [3].

![Schematic diagram of 30 kb genome](image2)

Figure 2-3: Schematic diagram of 30 kb genome of SARS-CoV2, RdRP region is shown in sky blue [3].
The first 30 bases from the 3'-end of the target oligo had a complementary base sequence to the anchor oligo. Two types of oligos were used as competitors. The first had 26 bases with the sequence: 5'-CGA AAT CAT ACC AGT TAC CAT TGA GA-3', and the second had 30 bases with the sequence: 5'-CGA AAT CAT ACC AGT TAC CAT TGA GAT CTT-3'. The former had an electro-active redox-marker methylene blue covalently bound to the 3'-end, and the latter had the redox-marker attached to the 5'-end. All ssDNA strands were purified by HPLC and were purchased from Integrated DNA Technologies (IDT), USA, and Biomers, Germany.

2.2.3 Methylene Blue (MB) as Redox Marker

Methylene blue is a widely used redox-active marker in electrochemical studies involving DNA, RNA, and proteins. This organic compound has the capacity to either intercalate between the base pairs of nucleic acids, or can be covalently bound to the phosphate group, making it particularly suitable for studies related to DNA hybridization sensors, electron transfer mechanisms, and electron transfer pathways in DNA. The redox behavior of methylene blue allows it to cycle between its reduced and oxidized forms, facilitating the study of electron transfer processes. Figure 2-4 shows the chemical structure of methylene blue and leucomethylene blue in chloride form. Methylene blue is blue in its oxidized form, and in its reduced form, so-called leucomethylene blue, is colorless. This reversible redox behavior is frequently exploited in electrochemical experiments to probe the kinetics and mechanisms of electron transfer in DNA systems.
On the other hand, the refractive index of MB undergoes a reversible change during its redox transition, making it a valuable property for sensing applications. This fluctuation in refractive index can be transduced into a detectable optical signal, which can be quantified using a variety of spectroscopic techniques. Such optical changes offer direct insight into analyte concentration as well as electron transfer kinetics. Additionally, MB exhibits a distinct absorption peak at approximately 664 nm, situated in the red segment of the visible spectrum as shown in Figure 2-5. This specific peak is sensitive to the redox status of MB, providing a reliable metric for tracking redox-based transitions. This is particularly advantageous for investigating electron transfer routes in molecules tagged with MB. The strong absorption at this wavelength also facilitates the electrochemical modulation of MB's optical characteristics, making it exceptionally suited for sensors that integrate both optical and electrochemical detection methods [73].
2.2.4 Thiol Gold Chemistry

Thiol-gold chemistry refers to the strong affinity between sulfur atoms (usually in the form of thiol groups, -SH) and gold surfaces. This chemical interaction is often exploited in fabricating self-assembled monolayers (SAMs) on gold surfaces. In these systems, molecules with terminal thiol groups form a single, well-ordered layer on a gold surface through chemisorption. This layer can be used for various applications, ranging from biosensors to electronic devices. The thiol-gold bond is a result of the sharing of electrons between the sulfur atom and the gold atoms on the surface, forming a quasi-covalent bond that is relatively strong and stable. The bond strength typically ranges from 40 to 50 kcal/mol \[74\]. The resulting SAMs can be highly ordered and packed, offering excellent surface coverage and functionalization capabilities \[30\].

![Figure 2-5: UV-Vis absorption spectra of MB solution \[4\].](image)
This chemistry is particularly relevant for DNA hybridization sensors in which Thiol-modified DNA strands can be immobilized on gold electrodes, creating a highly ordered and stable interface for subsequent hybridization and electron transfer studies [75]. In this study, the anchor (or the capture probe) oligo was modified with the thiol group to facilitate the adsorption of the capture probe on the gold surface. Figure 2-6 shows an anchor oligonucleotide modified with Thiol at the 5’ end.

![C6 Thiol modified oligo](image)

**Figure 2-6: C6 Thiol modified oligo.**

2.3 Sensor Surface Fabrication

2.3.1 Gold Film Deposition Using Sputtering Technique

Gold-coated substrates were fabricated using a Nano-Master DC magnetron sputtering system. Upon achieving a base pressure of $6 \times 10^{-6}$ Torr in the deposition chamber, atomically cleaned microscope glass slides (sourced from VWR, USA) were introduced into the chamber via the Load Lock Chamber (LLC). Utilizing a Radio Frequency (RF) power of 100 watts and Argon flow rate of 150 sccm deposition was initiated. After elevating the chamber pressure to $8 \times 10^{-3}$ Torr by the introduction of Argon gas, a 5 nm layer of chromium was first sputtered onto the glass slides to serve as an adhesion layer. This was followed by the deposition of a 50 nm gold layer to complete the substrate preparation.
2.3.2 Electrochemical Cleaning of Gold Surface

Among various techniques for cleaning gold surfaces such as ozone cleaning, potassium hydroxide and hydrogen peroxide baths, potassium hydroxide potential sweeps, piranha cleaning, sulfuric acid potential cycling, hydrochloric acid potential cycling, reducing agent solution baths, and Aqua Regia etching. Sulfuric acid potential cycling is often regarded as one of the most straightforward and effective methods for preparing gold surfaces for sensing applications [76]. Cyclic voltammetry in sulfuric acid cleans a gold surface through a series of electrochemical processes. During the anodic sweep, the gold oxidizes and forms a gold oxide layer that removes weakly bound contaminants. The cathodic sweep reduces this layer back to gold, displacing strongly adsorbed impurities. Sulfate ions from the acid form a monolayer on the gold surface, which is stripped away in subsequent cycles along with any loose contaminants. This cycle of oxidation and reduction also leads to structural rearrangements of the gold atoms, exposing a fresher, less contaminated surface.

The freshly prepared gold film was coupled with the flow cell and electrochemically treated in 0.05 M H2SO4 by taking a cyclic voltammetric scan from 0.4 to 1.5 V at 200 mV/s until reproducible voltammograms were obtained as shown in Figure 2-7.
Figure 2-7: CV scan of a gold surface with 0.05 M sulfuric acid solution from 0.4 to 1.5 Volts at a scan rate of 200 mV/s. The measured potential is with reference to the Au electrode.

2.4 Mechanism of DNA Hybridization Sensors

2.4.1 Immobilization of Oligonucleotides on Gold Surface

Following the cleaning of the gold surface, the flow cell was flushed with Milli-Q water. Subsequently, 300 μL of a 650 nM solution of anchor ssDNA in 1 M KH$_2$PO$_4$ buffer (pH 3.8) was introduced into the flow cell and allowed to incubate at room temperature in a dark environment for four hours. The high ionic concentration of the buffer mitigates electrostatic repulsion between adjacent DNA strands by providing improved electrostatic shielding for the negatively charged molecules, facilitating greater surface coverage of thiolated ssDNA [30]. The flow cell was then washed with 1X PBS (10 mM phosphate buffer saline) and treated with a 2 mM 6-mercapto-hexanol (MCH) solution, prepared in the same PBS buffer, for a one-hour incubation at room temperature. MCH acts as a spacer thiol, reducing nonspecific ssDNA adsorption on the
gold surface and optimizing ssDNA exposure for effective hybridization [75]. The successful immobilization of anchor ssDNA was verified through a shift towards a higher value in the effective refractive index as observed in the minimum reflectance position of the SPR curves, compared to the curves generated with unmodified gold or bare gold in PBS buffer solution. Figure 2-8 shows the SPR curve for unmodified gold surface in PBS buffer and after immobilization of anchor at different concentrations. The reflectance (R) is plotted along Y-axis and the effective refractive index ($n\sin\theta$) is plotted along X-axis, where $n$ is the refractive index of prism and $\theta$ is the angle of incidence at the prism. The diagram clearly shows an increasing trend in the shift of minimum reflectance towards a higher refractive index as the concentration increases from 163 nM to 970 nM. Notably, further increasing the concentration beyond 970 nM results in a decreased shift and an upward movement of the SPR curve. This upward shift for higher anchor concentrations is likely due to the excessive accumulation of anchor ssDNA on the sensor's surface, impacting both the real and imaginary parts of the refractive index or the extinction coefficient nearby.

Additionally, the more pronounced shift with 970 nM anchor compared to 650 nM indicates that ssDNA's population density peaks at 970 nM during immobilization. Given the need for optimal anchor density that allows room for the complementary strand to hybridize effectively, 650 nM is considered the most suitable concentration for anchor immobilization, despite the maximum capture probe density observed at 970 nM.
2.4.2 Hybridization of Complementary DNA Strands

The hybridization process was carried out at room temperature in the electrochemical flow cell. Following the immobilization of the anchor ssDNA, a 300 μL solution containing 650 nM of competitor ssDNA in 1 M KH₂PO₄ buffer (pH 3.8) was introduced into the flow cell. This mixture was incubated in a dark environment for 1.5 hours to facilitate hybridization. Subsequently, the flow cell was flushed with 1X PBS to eliminate any unhybridized ssDNA. A schematic diagram to show the hybridization of two complementary strands to form a DNA duplex and the two distinct positions of redox

Figure 2-8: SPR curve for bare gold in PBS buffer (in yellow), after immobilization of anchor of different concentrations: 163 nM (brown), 325 nM (green), 650 nM (black), 970 nM (gray), 1300 nM (violet), and 2600 nM (blue)
probe MB upon hybridization is shown in Figure 2-9.

Figure 2-9: Hybridization of complementary competitor ssDNA with gold surface bound anchor ssDNA to position MB at two different locations, one at the distal end and the other at the proximal end of the duplex.

First SPR measurements were taken for several experiments to see if the SPR curves would suffice to confirm hybridization of two DNA strands. Figure 2-10 shows the SPR curves for several experiments where a shift towards higher refractive index in the SPR curve can be seen after incubating the flow cell with competitor primer having MB closer to the electrode surface.
Figure 2-10: SPR curves for bare gold in PBS (yellow), after immobilization of anchor (black), and after injecting competitor primer with MB closer to the electrode surface (blue)

Similarly, Figure 2-11 shows the SPR curves for experiments where a shift towards a higher refractive index in the SPR curve can be seen after incubating the flow cell with
competitor primer having MB far from the electrode surface upon hybridization with anchor.

Figure 2-11: SPR curves for bare gold in PBS (yellow), after immobilization of anchor (black), and after injecting competitor primer with MB far from the electrode surface (blue).

The refractive index properties of oligonucleotides are often characterized by considering the real and imaginary parts. In this context, the imaginary part of the refractive index or the extinction coefficient is typically assumed to be zero [77], while the real part of the refractive index for a 27 nt oligonucleotide is measured at 1.46 [78].

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When investigating the hybridization of a competitor primer strand with an anchor primer strand using Surface Plasmon Resonance (SPR), it is expected that the SPR curve would shift towards a higher refractive index or to the right. However, we saw a negative shift or the shift towards lower refractive index in the SPR curve in some instances as shown in figure 2-12. In such cases the DNA duplex might have undergone a conformational change which would bring a decrease in the dielectric at the vicinity of the sensor surface.
These observations highlight a key point: relying solely on SPR as a tool to confirm the hybridization of competitor primers with the anchor primer may not always be reliable. Therefore, it is proposed that an electrochemical method such as cyclic voltammetry, capable of detecting the presence of the redox probe MB attached to the competitor primer, could be a more dependable approach for confirming hybridization in such cases.

Confirmation of the successful hybridization between the surface-anchored ssDNA and its complementary strand was obtained through a cyclic voltammetry scan, which revealed cathodic and anodic peaks for the redox label (MB). Figure 2-12 is the cyclic voltammogram anchor, anchor hybridized with competitor primer with MB close to the electrode surface, and anchor competitor duplex with MB positioned far from the electrode surface.
Figure 2-13: Cyclic Voltammograms for anchor (in black), anchor hybridized with the competitor with MB at 5' end (in red), and anchor hybridized with the competitor with MB at 3' end (in blue).

The significance of no peak-to-peak separation between the reduction and oxidation peak potentials for MB positioned closer to the electrode surface and the presence of hysteresis in the peak potentials when MB is positioned farther away from the electrode surface is explained in the following section.

To confirm that the signal from methylene blue (MB) isn't due to nonspecific competitor primer binding or MB adsorption on the electrode surface, cyclic voltammetry (CV) scans were performed under two conditions: (a) with a 650 nM solution of the competitor primer, and (b) with a 650 nM solution of free MB following identical protocols. Figure 2-13 shows the cyclic voltammogram for these two conditions. In both instances, no distinct redox peaks were detected within the CV's sensitivity range, suggesting that the noted redox peaks are likely due to the hybridization between the
competitor and anchor primers.

Figure 2-14: Cyclic voltammograms for 650 nM of competitor primer solution (A) and 650 nM of free MB solution (B). CV scans were taken in the range -0.25 V to 0.25 V at a scan rate of 20 mV/s.

2.4.3 Optimization of Redox Signal from MB

In the electrochemically modulated surface plasmon wave (ECM-SPW) sensing approach, utilizing the contrast in the optical signals between the oxidized and reduced states of the probe is crucial. Therefore, refining the surface plasmon resonance (SPR) setup is essential to enhance the optical disparity of the methylene blue (MB) molecule during its redox transitions [61]. Initially, competitor single-stranded DNA (ssDNA) is coupled with anchor ssDNA, verified by cyclic voltammetry (CV) to ascertain successful hybridization. Subsequent standard SPR assessments were carried out at two distinct electric potentials (-0.2 V and 0.2 V), corresponding to the reduced and oxidized states of MB, respectively. The variation in reflectance (ΔR) across these states was evaluated in
Figure 2-15: SPR curve for competitor primer hybridized with anchor primer when the potential applied at the working electrode is +0.2 V (blue) and -0.2 V (orange). The difference in reflectance, $\Delta R$ (green) is maximum at the left of SPR angle.

relation to the effective refractive index. A graph plotting reflectance versus effective index as shown in Figure 2-14 revealed that the left side of the resonance angle (the angle where reflectance is minimal) exhibited the greatest $\Delta R$ magnitude. These observations are linked to modifications in both the real and imaginary components of MB's refractive index throughout the redox process, pinpointing the optimal angle (or effective refractive index) for ECM-SPW measurements to maximize the optical signal strength when electrochemical control is applied in a fixed-angle setting.
2.5 Results and Discussion

The investigation focused on assessing the impact of the redox probe (MB) attachment at both the 3' and 5' ends of complementary ssDNA on the performance of label-based DNA hybridization sensors. To accomplish this, a combination of electrochemical and spectroscopic methods was employed to examine the efficiency of electron transfer with the two different label positions. Specifically, Electrochemical Impedance Spectroscopy (EIS) measurements were conducted, enabling the construction of an equivalent circuit for the estimation of parameters such as charge transfer resistance, capacitance, and electron transfer rate constant. Additionally, Optical Impedance Spectroscopy (OIS) was utilized to assess sensor performance, with a subsequent comparative analysis conducted with the insights obtained through EIS analysis.

2.5.1 CV Analysis

2.5.1.1 Cyclic Voltammetry and its Optical Analogy

Voltammetric experiments were conducted within a micro-electrochemical cell with a volume of 250 μL, comprising a gold film as the working electrode and two gold-coated pins serving as the reference and counter electrodes. These measurements were performed under the conditions of 1X PBS buffer (pH 7.4) at room temperature. A cyclic voltammetry (CV) scan ranging from -0.25 V to 0.15 V (vs. the gold electrode) was executed, employing a scan rate of 20 mV/s. Figure 2-15 illustrates the cyclic voltammogram and the corresponding variations in reflected optical intensity in relation
to the applied potential for three distinct systems.

In Figure 2-15 (A), Curve (a) illustrates the cyclic voltammograms corresponding to the immobilized anchor on a gold surface immersed in 1X PBS buffer. Conversely, Curves (b) and (c) depict the voltammograms for the anchor hybridized with competitor...
ssDNA, where the redox label MB is positioned at the distal end (5') and proximal end (3') of the competitor ssDNA, respectively. Likewise, Figure 2-15 (B) represents the change in reflected optical signal (maintaining a fixed angle of incidence) during the CV scans for the afore-mentioned three different scenarios (a), (b), and (c). Figure 2-15 (C) is the reconstructed faradaic signal which is derived by taking the derivative of the reflected optical signal presented in Figure 2-15(B) with respect to the potential, as explained in literature [79]. The potential corresponding to the peak of the curve (b) and (c) is the redox potential associated with the covalently bound redox MB with DNA and this redox potential aligns with the value obtained through electrochemical CV analysis.

2.5.1.2 Formal Potential of Covalently Bound MB with DNA

In the case of Figure 2-15 (A), curve (c), where the anodic and cathodic peak potentials coincide at -87 mV, the formal potential of MB in this instance can be considered as -87 mV (vs gold electrode). Conversely, under the assumption that the reduced and oxidized states of MB molecules share the same diffusion coefficient within the solution being used, the formal potential should align precisely with the midpoint between the two peak potentials of reduction and oxidation [80] for curve (b), which is -106 mV. The slight shift of the formal potential towards a more negative potential in the latter case signifies an increased activation energy necessary for the redox transition. This observation indicates that electron transfer becomes less favorable when the redox species is positioned farther away from the electrode surface.

2.5.1.3 Density of Electro-active Species

The strength of the faradaic current is unambiguously superior for the redox probe
located near the electro-active surface. Moreover, unlike trace (c), trace (b) displays a strong hysteresis with a significant peak potential separation for the cathodic $E_{pc}$ and anodic $E_{pa}$ peak currents: $\Delta E_p = E_{pc} - E_{pa}$. The observed rise in hysteresis following the binding of the complementary ssDNA having the redox probe at the distal end suggests a decrease in the kinetics of electron transfer within the molecular binding system. In other words, the double-stranded DNA has the potential to interact with the electrode surface, albeit at a diminished rate due to its increased rigidity. To precisely determine these properties, the quantification of the density of electroactive DNA probes on the electrode surface, $\Gamma_{MB}$, was calculated using equation (1) [81]

$$\Gamma_{MB} = \frac{Q}{nFA} \quad \ldots \ (1)$$

Where $Q$ is the total charge (C) obtained by integrating the cathodic peak of voltammograms over potential corrected for background current, $n$ is the number of electrons in the redox reaction ($n = 1$), $F$ is the Faraday constant (96485 C/mol), and $A$ is the electro-active area (cm$^2$) of the working electrode.

$\Gamma_{MB}$ for the curve (b) is 1.17 pmol/cm$^2$, while that for the curve (c) is 2.19 pmol/cm$^2$. The density of electroactive species when compared with the surface coverage of a monolayer of 30-mer oligonucleotide indicates that the system is not densely packed [82]. It is worth noting that this relatively low probe density has been utilized in numerous recent applications that take advantage of DNA hybridization [35, 83].

2.5.1.4 Possible Mode of Electron Transfer

Notably, curve (b) in Figure 1(A) demonstrates a distinct feature of split cathodic and anodic peaks, suggesting that the energy required for electron transfer between MB
and the gold surface is elevated when MB is positioned farther from the gold surface, specifically at the distal end of ssDNA. In contrast, curve (c) does not exhibit this splitting phenomenon, indicating greater efficiency in electron transfer when the MB molecule is located at the proximal end of the primer.

We hypothesize that the electron transfer occurring in this context is likely to be collisional electron transfer rather than DNA-mediated electron transfer. Consequently, the enhanced density or accessibility of electroactive species when methylene blue (MB) is situated closer to the surface post-hybridization can possibly be attributed to the positioning of the MB monolayer either directly on the electrode surface or in very close proximity to it. It is important to note that the MB molecule is covalently attached to the oligonucleotide through a chain of six carbon atoms. Hence, it is highly probable that following the hybridization of the competitor primer with the anchor, the molecule may either undergo elastic collisions with the electrode or make direct contact with the electrode surface. In contrast, when the MB molecule is situated farther away from the electrode surface after the hybridization of the competitor with the anchor, as illustrated in Figure 2-9, the increased rigidity of the DNA duplex following hybridization restricts the availability of the MB molecule for collision with the electrode surface.

Consequently, this results in a reduction in the density of electroactive species when MB is positioned further away from the electrode surface upon the hybridization of the competitor with the anchor. The quantitative analysis of electron transfer is discussed in the following sections and the pathways of electron transfer is further elaborated in chapter 2.
2.5.2 EIS Analysis

Electrochemical Impedance Spectroscopy (EIS) measurements were conducted in the same electrochemical cell and with the same potentiostat used for voltammetric measurements. A direct current (dc) bias potential of -0.09 V, which corresponds to the formal potential of MB attached to DNA duplex, along with an alternating current (ac) amplitude of 10 mV was applied. The impedance spectra were recorded across a frequency range from 0.1 Hz to 10 kHz. An in-house Python program was employed to ensure Kramers-Kronig compliance of the impedance spectra and for fitting purposes.

2.5.2.1 Kramers-Kronig Compliance of Impedance Spectra

In the context of electrochemical impedance spectroscopy (EIS), it is expected that the real and imaginary impedances of a system, provided it adheres to causality, linearity, and stability, should adhere to the following Kramers-Kronig relations:

\[
Z''(\omega) = -\frac{2\omega}{\pi} \int_{0}^{\infty} \frac{Z'(x) - Z'(\omega)}{x^2 - \omega^2} dx
\]  \hspace{1cm} (2)

\[
Z'(\omega) = Z'(\infty) + \frac{2}{\pi} \int_{0}^{\infty} \frac{xZ''(x) - \omega Z''(\omega)}{x^2 - \omega^2} dx
\]  \hspace{1cm} (3)

Hence, within a suitable system, the initial data for the imaginary component of EIS can be employed to derive the real part, and conversely, the real component data can be used to determine the imaginary part. When the data is trustworthy, these integrations should align closely with the original data, demonstrating adherence to the prescribed relations. Figure 2-16 illustrates an example of Kramers-Kronig compliance, where the lines represent the real and imaginary parts obtained through the equations in (2) and (3).
The integral was computed numerically using Simpson's rule, relying solely on the raw data. Although the Kramers-Kronig relation generally tracks well with the raw data, notable deviations are observed at the low frequency region. This can be primarily attributed to the lack of raw data at low frequencies. The well-documented challenge of incomplete frequencies in Kramers-Kronig (KK) relation assessments [84] is evident here, yet the data still aligns fairly well with these relations despite the gaps. Additionally, the logarithmic scale tends to amplify variations at these lower frequencies.
making even minor discrepancies more apparent, which might be less discernible on a linear scale. However, as shown in the inset of Figure 2-16, when plotted linearly, the raw data adheres closely to the KK relation across the entire frequency spectrum.

2.5.2.2 Equivalent Circuit Model

Following confirmation of Kramers-Kronig compliance in the impedance spectra, we proceeded to analyze the spectra employing an equivalent circuit model, illustrated in Figure 2-17.

![Figure 2-18: A circuit model to fit the impedance spectra to estimate the relevant parameters.](image)

A simple Randel’s circuit was used as a model where, $R_s$ represents the solution resistance of the electrolyte solution (1X PBS buffer, pH 7.4), $C_{dl}$ denotes the capacitance of the electric double layer formed at the interface between the electrolyte solution and the working electrode. Likewise, $R_{ct}$ represents the charge-transfer resistance, and $C_a$ is the pseudo capacitance associated with the faradaic electron transfer [85]. To calculate the electron transfer rate constant ($k_{et}$), we applied equation (4). The math associated with the derivation of electron transfer rate is explained somewhere else [86].
\[ k_{et} = \frac{1}{2R_{ct}C_{a}} \] \quad \ldots \; (4)

2.5.2.3 Bode Plots and the Fitting Parameters

Figure 2-18 presents the frequency response, including total impedance and phase versus frequency, for three distinct systems: the anchor alone, the anchor hybridized with a competitor with MB at the distal end, and the anchor hybridized with a competitor with MB at the proximal end. In the figure, open symbols denote the experimental data, while solid lines represent the fitted plots obtained from the model. Table 1 provides an overview of the fitting parameters and the calculated electron transfer rate for these systems.
Table 1. Values for parameters obtained by fitting the impedance spectra as shown in Figure 2-12.

<table>
<thead>
<tr>
<th></th>
<th>$R_s$(Ω)</th>
<th>$C_{dl}$(µF)</th>
<th>$R_{ct}$(KΩ)</th>
<th>$C_a$(µF)</th>
<th>$K_{et}$(s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anchor</td>
<td>35.6 ± 0.2</td>
<td>2.99 ± 0.01</td>
<td>832 ± 13</td>
<td>11.0 ± 1.05</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Anchor+Competitor-MB-5'</td>
<td>37.0 ± 0.5</td>
<td>2.50 ± 0.01</td>
<td>242 ± 5</td>
<td>6.48 ± 0.2</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Anchor+Competitor-MB-3'</td>
<td>36.5 ± 0.1</td>
<td>2.84 ± 0.06</td>
<td>5.9 ± 0.3</td>
<td>8.51 ± 0.3</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td>Anchor + Target</td>
<td>38.0 ± 0.3</td>
<td>2.85 ± 0.01</td>
<td>493 ± 7</td>
<td>13 ± 1</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

2.5.2.4 Electron Transfer Rate

For all the systems, the values of $R_s$ and $C_{dl}$ remained nearly identical as they represent background properties of the solution, while the components $R_{ct}$ and $C_a$ characterize the redox process in the electrochemical system [58]. When only the anchor was present, $R_{ct}$ measured at 832 kΩ. However, upon the formation of the anchor-competitor duplex with the redox label MB located at the 5’ end of the competitor, $R_{ct}$ decreased to 242 kΩ. Additionally, when MB was positioned at the 3’ end of the competitor, $R_{ct}$ further decreased to 5.9 kΩ. Furthermore, the rate constant ($k_{et}$) for electron transfer between the redox label MB and the gold electrode increased to $(0.32 ± 0.01)$ s$^{-1}$ when the anchor hybridized with the competitor having MB at the 5’ end. Conversely, when MB was located at the 3’ end of the competitor, $k_{et}$ increased to $(10 ± 0.6)$ s$^{-1}$. These findings suggest that electron transfer efficiency is enhanced when MB is attached to the 3’ end of the competitor ssDNA, bringing the redox label in closer proximity to the working electrode.
2.5.3 OIS Analysis

2.5.3.1 Impedance Analysis from Reflectance Measurement

An electrical potential was applied to the metallic surface plasmon resonance (SPR) interface, while monitoring the light reflectivity at a predetermined incident angle and specific wavelength. By adjusting a DC bias potential to match the formal potential of redox species adsorbed onto the plasmonic interface and applying an AC-modulated potential to induce oscillation between their oxidized and reduced states, a modulated optical signal in reflection was generated, similar to measurements conducted with the Electrochemical Impedance Spectroscopy (EIS) technique. The in-phase (X) and out-of-phase (Y) amplitudes of the reflected optical intensity were tracked using a lock-in amplifier. Subsequently, the total amplitude and phase of the reflected optical intensity across a spectrum of modulation frequencies were determined using equation 5 and equation 6.

\[
\text{Amplitude} = \sqrt{X^2 + Y^2} \quad \ldots (5)
\]

\[
\text{Phase} = \tan^{-1}\left(\frac{Y}{X}\right) \quad \ldots (6)
\]

Figure 2-19 and Figure 2-20 depict the amplitude and phase of the reflected optical signal plotted against the modulation frequency, resembling the Bode plot commonly used in Electrochemical Impedance Spectroscopy (EIS) analysis. Figure 2-19 illustrates the analysis for the anchor and anchor hybridized with competitor, with methylene blue positioned further away from the electrode surface, while Figure 2-20
corresponds to the analysis for the anchor and anchor hybridized with competitor, where
the redox marker is positioned closer to the electrode surface.

Figure 2-20: Amplitude and phase of reflected optical signal for anchor shown in open symbols connected with dashed lines and for anchor hybridized with competitor having methylene blue at the distal end shown in closed symbols connected with solid lines.

Figure 2-21: Amplitude and phase of reflected optical signal for anchor shown in open symbols connected with dashed lines and for anchor hybridized with competitor having methylene blue at the proximal end shown in closed symbols connected with solid lines.
Unlike the magnitude of impedance in EIS spectrum, the amplitude of the reflected optical intensity has a peak at approximately 1 Hz for anchor hybridized with competitor having methylene blue at the distal end of the primer, whereas for the anchor hybridized with the competitor with methylene blue at the proximal end, the amplitude peaks at approximately 2 Hz. This feature is totally different than as seen in EIS spectrum.

However, a similar kind of behavior is seen in the phase vs frequency characteristics in both EIS and Optical analysis. As depicted in figure 2-19 and figure 2-18 (B), the phase shows a similar feature with a slight dip close to 0.1 Hz for anchor hybridized with competitor where the methylene blue is positioned farther away from the electrode surface. Similarly, when the MB is located closer to the electrode surface, the phase has a dip close to 10 Hz as shown in figure 2-20 and figure 2-18 (C).

### 2.5.3.2 Electron Transfer Rate from OIS Analysis

A sinusoidal potential modulation with an amplitude of 10 mV was applied to the EC-SPR sensor at a dc bias potential of -0.08 V. The monitoring of in-phase and out-of-phase amplitudes of reflected optical intensity was carried out at frequencies ranging from 0.1 Hz to 200 Hz, akin to the Electrochemical Impedance Spectroscopy (EIS) technique. To determine the electron transfer rate in electrically driven redox reactions, a specific methodology outlined in the literature [86] was employed. This approach involves the integration of impedance data obtained from optical and electrical measurements, which were concurrently collected during a Spectro electrochemical experiment. The underlying equations (7), (8), and (9) succinctly summarize the methodology.
\[ x(\omega) = 2[Z'_t(\omega)A'_{ac}(\omega) - Z''_t(\omega)A''_{ac}(\omega)] \quad \ldots \ (7) \]

\[ y(\omega) = \omega[Z'_t(\omega)A'_{ac}(\omega) + Z''_t(\omega)A''_{ac}(\omega)] \quad \ldots \ (8) \]

\[ y(\omega) = kx(\omega) \quad \ldots \ (9) \]

The analysis shows that it is sufficient to collect the electrical impedance, \( Z_t(\omega) \), of the electrochemical cell and the modulated absorbance signal, \( A_{ac}(\omega) \). \( Z'_t(\omega) \) and \( Z''_t(\omega) \) are the real and imaginary part of the electrical impedance, whereas \( A'_{ac}(\omega) \) and \( A''_{ac}(\omega) \) are the in-phase and out-of-phase components of the modulated absorbance signal measured with a PMT connected to a lock-in amplifier that has been synchronized to the driving potential of the function generator. Using equation (9), the slope of the linear fit will give the electron transfer rate (\( k \)) of the faradaic process.

When MB is positioned in close proximity to the electrode surface, the data for \( y(\omega) \) vs \( x(\omega) \) can be effectively fitted with two distinct straight lines in two separate frequency ranges: one in the higher frequency region and another in the lower frequency region as shown in Figure 2-21 (A) In the higher frequency region, the electron transfer rate was determined to be \( 12.8 \pm 0.6 \text{ s}^{-1} \), slightly exceeding the value obtained through EIS spectrum analysis. Conversely, in the lower frequency region, the electron transfer rate was measured at \( 2.3 \pm 0.3 \text{ s}^{-1} \). Unlike the EIS analysis, the Optical Impedance Spectroscopy (OIS) analysis reveals the presence of two distinct electron transfer rates, suggesting the potential existence of two different electron transfer pathways. However, it's important to acknowledge the possibility that the chosen equivalent circuit for modeling the systems may not be sufficient to fully comprehend the system's behavior.
On the other hand, when the MB is positioned far from the electrode surface, the behavior in the higher frequency region deviated from the parametric relationship outlined in equation (9). Figure 2-21 (B) illustrates that in the higher frequency region, an unexpected negative electron transfer rate was observed, while the lower frequency region conformed to the anticipated pattern, with an electron transfer rate of 0.4 $s^{-1}$, consistent with values obtained from the EIS analysis.

Notably, previous research by Anne and Demaille also observed electron transfer within a system of redox-tagged DNA duplexes when subjected to slowly varying potential perturbations, particularly when the redox-active molecule is positioned at the distal end of the duplex [71]. They attributed the presence of electron transfer at lower cyclic voltammetry (CV) scan rates and its absence at higher scan rates to the bending elasticity of double-stranded DNA (dsDNA), which governs the dynamics of electron transport within the redox-labeled dsDNA layer. In our study, we employed a sinusoidal perturbation and analyzed the changes in optical impedance within the redox-labeled
dsDNA layer, distinguishing our approach from that used by Anne and Demaille. Utilizing the optical impedance of dsDNA layers, further investigations following this approach may provide enhanced insights into the electron transfer pathways within redox-labeled DNA duplexes.

2.5.4 AC Voltammetry and its Optical Analogy

In conducting the study on voltammetric modulation with alternating current, the modulation frequency was empirically established based on the time constant linked to the faradaic process. This involved determining the resonance frequency pertinent to the electron transfer kinetics in the redox process, derived from the electron transfer rate ($K_{et}$), which was found to be 0.32 Hz for DNA duplexes with methylene blue (MB) at the 5’ end, and 10 Hz for those with MB at the 3’ end. Consequently, a sinusoidal potential modulation with a 10 mV amplitude at a frequency of 1 Hz was applied across various direct current bias potentials, ranging from -0.25 V to 0.15 V. During this process, the amplitude of the modulated electrical current was recorded via a potentiostat, in conjunction with the amplitude of the reflected optical intensity, which was monitored and logged by a lock-in amplifier at each bias potential. The results, as depicted in Figure 2-22 (A) and Figure 2-22 (B), distinctly demonstrate a differentiation in both electrical and optical signals between anchor hybridization with a competitor featuring MB at the 5’ and 3’ ends. Notably, trace (c) exhibited a stronger signal than trace (b) at the formal potential of the redox label, which is attributed to the more efficient electron transfer between the redox probe and the electrode when the redox probe is positioned nearer to the electrode surface following hybridization.
Conclusions

The location of the methylene blue (MB) redox label on single-stranded DNA (ssDNA) plays a crucial role in determining both the amount of redox-active species involved in electrochemical reactions post-hybridization and the rate of electron transfer between the redox marker and the electrode. Our studies, incorporating both electrical and optical analyses, revealed that placing the MB redox marker nearer to the electrode surface significantly enhances performance. This positioning leads to an increased density of electrochemically active redox markers and a marked improvement in the rate of electron transfer. Various factors, such as the DNA molecule's conformation post-hybridization, the DNA duplex's elasticity, the surface density of the hybridized probes, and the presence of counterions in the buffer solution, may influence these outcomes. The enhancement in the electro-active probe density and negligible hysteresis on the $\Delta E_p$ in CV when having the competitor with MB closer to the electrode surface, suggests that the

![Figure 2-23: (A) Electric ac voltammetry and (B) reflected optical intensity during ac voltammetry for anchor (a), anchor-hybridized with the competitor with MB at 5’ end (b), and anchor-hybridized with the competitor with MB at 3’ end (c). Scattered symbols represent raw data and solid lines represent Gaussian fit for the data. The amplitude of ac modulation is 10 mV and the frequency is 1 Hz.](image-url)
proximity of the redox marker to the electroactive surface facilitates the charge transfer processes between the methylene blue and the electrode surface. Conversely, when methylene blue is attached at the distal end of the primer, the rigidity of the DNA duplex may impede the access of the probe to the electroactive surface contributing to the enhanced hysteresis on the $\Delta E_p$ and decrease in the peak current. The electron transfer efficiency depends on the accessibility of electrode surface by the redox molecules so, the potential mode of electron transfer for both systems is likely to be contact-mediated or collisional rather than DNA-mediated electron transfer. Further exploration, such as studying electron transfer rates with base pair mismatches in the DNA duplex, could provide deeper understanding of the electron transfer mechanisms. Our findings highlight that increasing the proximity of the redox marker to the electrode significantly boosts signal strength, a critical aspect in designing DNA hybridization sensors.
CHAPTER 3 ELECTRON TRANSFER PATHWAY IN DNA HYBRIDIZATION SENSORS WITH MB COVALENTLY BOUND TO SUGAR PHOSPHATE BACKBONE OF DNA

3.1 Introduction

The capability of DNA duplexes to conduct electrical currents is anticipated to have widespread applications in molecular bioelectronics and nucleic acid biosensors. This is largely due to DNA's ability to self-assemble with a configurable freedom, which is fundamental to creating controllable nanoelectronic devices and biosensing elements [87]. Although there have been successful demonstrations of long-range electron transfer (ET) mediated by double-stranded DNA (dsDNA) between two dye molecules, the efficiency of such electron transfer continues to be debated. Various studies have characterized the DNA duplex bound between two metal electrodes as a conductor [88-90], superconductor [91], semiconductor [92, 93], or even insulator [94, 95]. These findings on the conductivity of DNA were derived under vacuum and dry conditions, yet DNA characteristics are likely to substantially vary in a solution. Consequently, conducting direct electrochemical evaluations of electron transfer in double-stranded DNA under ambient conditions could provide a more comprehensive insight into the electrical properties of DNA.
One prevalent method to investigate electron transfer mechanisms involving DNA duplexes is to use thiol-modified DNA duplexes self-assembled onto gold electrodes. These are further modified with redox-active reporters, which are either covalently attached to the DNA or intercalated within its base pairs. The redox-active molecules interact with the DNA surface through various modes including electrostatic interaction, groove binding, intercalation binding, and specific high-affinity interactions with certain DNA sequences. The diversity of these binding modes leads to a range of electron transfer pathways between the redox marker and the electrode surface, each significantly affecting the observed electron transfer properties [96-98]. Despite over two decades of research, definitive conclusions about the underlying mechanisms of electron transfer through or from electrode-bound dsDNA remain elusive.

In some research, metal complexes like Ru(NH$_3$)$_6^{3+}$ have been employed due to their ability to interact with the negatively charged phosphate backbone of hybridized DNA via electrostatic interaction [99, 100]. In contrast, anionic metal complexes such as Fe(CN)$_6^{3/-4-}$ [101, 102] have been used as redox indicators based on the principle of electrostatic repulsion. Here, the high electrostatic repulsion between the negatively charged phosphate backbone of the DNA strands and the redox marker is exploited for signal generation. The electron transfer mechanism for these electrostatically bound redox-active molecules to the DNA surface and to the electrode surface is believed to be contact-mediated [98, 101, 102]. Similarly, groove binders like positively charged Ruthenium complexes (Ru(NH$_3$)$_5$Cl$^{2+}$, Ru(NH$_3$)$_6^{3+}$) have been suggested to undergo facilitated diffusion and are directly reduced at the electrode surface [103]. Other redox indicators such as daunomycin [104], Co(phen)$_3^{3+}$ [105], doxorubicin [106], meldola’s
blue [107], ethidium bromide [108], Os(bpy)$_2$Cl$_2$ [109], and methylene blue [65, 110] demonstrate a high affinity to the dsDNA structure through intercalation between G-C base pairs.

Daunomycin (DM), known for its ability to covalently intercalate to guanine residues [38], has been used as a redox reporter in studies by Barton and Liu to explore the role of the sugar-phosphate backbone in DNA charge transport. They compared charge transport in DNA with intact sugar-phosphate backbones to those with an intervening DNA mismatch. Their findings highlight remarkably efficient reduction of DM in well-matched DNA duplexes, whether the backbone is intact or contains one or two nicks. Additionally, they observed a significant decrease in DM reduction efficiency when a single base CA mismatch was introduced into the duplex. These results indicate that the pathway for DNA charge transport is likely through the base pair stack, rather than along the sugar-phosphate backbone. Unlike the intercalated redox reporter, for electrostatically bound redox indicators, electron transport is reported to occur either along electronic wires formed by redox species on the DNA surface [63] or via lateral electron transfer pathways due to DNA bending towards the electrode surface [64, 71]. In recent research, Barton et al. utilized the electrostatically binding Ru(NH$_3$)$_6^{3+}$ (RuHex) complex to DNA, arguing that the redox chemistry involved is not mediated by DNA and that the electrochemical readout from RuHex does not change in the presence of a mismatch site within the DNA duplex [65].

The discussion also extends to methylene blue (MB), which is recognized as a versatile redox marker capable of distinctly different interactions with adenine-thymine
(dAdT) and guanine-cytosine (dGdC) rich dsDNA [111]. It's been noted that MB intercalates into dGdC duplexes, a process that is insensitive to ionic strength, whereas groove-binding is the dominant interaction mode, sensitive to ionic strength, for MB with dAdT-rich polynucleotides [111, 112]. Ferapontova et al. have demonstrated that DNA-mediated electron transfer occurs both to MB intercalated into dGdC duplexes and to MB groove bound to dAdT duplexes and they found that the electron transfer rate from the gold electrode to intercalated MB into dGdC-rich duplex is less efficient compared to when MB is groove bound to pure dAdT DNA [87].

Additionally, researchers have identified two simultaneous reduction mechanisms of methylene blue (MB) on DNA-modified electrodes: one involving reduction facilitated by the DNA's base pair stacking and another involving direct reduction of MB on the electrode's surface. Methylene blue, when covalently linked to modified uracil via a flexible C_{12} alkyl chain, gains the flexibility needed to interact with the base stack through intercalation, while also retaining the ability to undergo direct reduction at the electrode surface [110]. The predominant mechanism of MB's reduction is heavily influenced by how the molecular assembly is set up. The analysis suggests that in the context of DNA-mediated electrochemistry, the electron's journey via tunneling through the C_6-alkane link to the electrode surface is a limiting factor for efficient electron transfer. Consequently, electron transfer is slower for reductions involving the base pair stack for intercalated MB compared to direct surface reductions.

Numerous studies have argued that DNA-mediated charge transfer involves charges moving via hopping through an intact \( \pi \)-stack from one guanine base to the next,
with guanine being critical due to its low reduction potential relative to other bases [113, 114]. Evidence supporting the necessity of an intact $\pi$-stack for efficient charge transfer includes reports of significantly slower DNA-mediated charge transfer upon the introduction of base mismatches, believed to disrupt the stacking [102, 115]. Dauphin-Ducharme et al. used a combination of chronoamperometry and chronocoulometry to quantify electron transfer rates from guanine-free, guanine-rich, and mismatch-containing DNA constructs. They employed surface-bound DNA duplexes with covalently attached MB at the phosphate backbone terminus, a common strategy in DNA-based hybridization sensors. Their findings suggest that electron transfer appears to occur via contact-mediated exchange between the reporter and the electrode surface rather than through DNA-mediated transport [116]. They observed that the presence or absence of guanine, thought to be necessary for hopping-based through-DNA charge transfer, does not significantly alter the transfer rate. Similarly, the presence of a mismatch, argued to disrupt the $\pi$-stacking necessary for DNA-mediated transfer, also does not significantly change the transfer rate. Studies on the length dependence of transfer rate from guanine-free duplexes imply that electron transfer might be influenced by the redox reporter's position relative to the electrode surface, suggesting an alternate mechanism where electrons tunnel to the redox reporter when DNA's structural dynamics bring it close to the electrode.

In Chapter 2, analysis using Optical Impedance Spectroscopy, we propose the possibility of alternative pathways for electron transport beyond the contact-mediated electron transfer, particularly for covalently attached MB to the phosphate backbone of DNA duplexes. Additionally, it's clear from the literature that electron transfer pathways
are influenced by various factors including DNA assembly and buffer conditions, the mode of redox marker attachment to DNA, nucleotide length, and the types of nitrogen bases used in the nucleotide chain. Hence, it is critical to explore the electron transfer pathways in the distinct types of DNA constructs we are employing, where the redox marker is located at the distal and proximal ends of the DNA duplex via covalent attachment to the negatively charged phosphate backbone. It is also worth noting that all experiments were conducted under ambient temperature and pressure conditions, and the biosensing construct used is one of the simplest and most widely employed in this kind of biosensing strategy.

In accordance with the information presented in section 2.4 of chapter 2, which discusses the potential mechanisms of electron transfer between the redox molecule and the electrode surface when the redox molecule is covalently linked to the DNA strand, this chapter is exclusively focused on exploring more on the electron transfer pathways through voltammetric analysis. Reduction potential in CV and the change in peak currents at different CV scan rates were explored for two distinct systems: one where the MB is positioned closer to the electrode surface and another where the MB is positioned farther away from the electrode surface.

3.2 Electrode Adsorbed and Freely Diffusing Redox Species

For electrochemically reversible electron transfer processes with freely diffusing redox species, the Randles-Sevcik equation offers an explanation for how the peak current in cyclic voltammetry (CV) depends on the scan rate which suggests that the peak current $i_p$ (A) increases linearly with the square root of the scan rate $v$ ($Vs^{-1}$) as
described in equation 10.

\[ i_p = 0.446nFAc^0 \left( \frac{nFvD_0}{RT} \right)^{1/2} \]  

... (10)

Where \( n \) is the number of electrons transferred in the redox transition, \( F \ (C \text{ mole}^{-1}) \) is the Faradays constant, \( A \ (cm^2) \) is the electrode surface area, \( C^0 \ (mol \ cm^{-3}) \) is the bulk concentration of the species \( D_0 \ (cm^2 \ s^{-1}) \) is the diffusion coefficient of the oxidized species, \( R \ (J \ mol^{-1} \ K^{-1}) \) is the molar gas constant, and \( T \ (K) \) is the absolute temperature.

On the other hand, for an electrode adsorbed species, the peak current is expected to vary linearly with the scan rate as described in equation 2.

\[ i_p = \frac{n^2F^2}{4RT} vA\Gamma \]  

... (11)

Where \( \Gamma \ (mol \ cm^{-2}) \) is the surface coverage of the adsorbed species.

When considering equations 10 and 11 together, they can be combined and simplified as

\[ i_p = Ki^\alpha \]  

... (12)

Where all the parameters except \( v \) is represented by \( K \) and \( \alpha \) is the index of \( v \) such that \( \alpha = 1 \) indicates electrode adsorbed species and \( \alpha = 0.5 \) indicates the diffusion-controlled electron transfer.

Taking natural logarithm on both sides of equation 12 yields

\[ \ln(i_p) = \ln K + \alpha \ln v \]  

... (13)

Therefore, a plot of \( \ln(i_p) \) vs \( \ln v \) can be used to fit a straight line whose slope would give the value of \( \alpha \) and whose y-intercept can be used to find parameters like diffusion coefficient \( (D_0) \) using equation 10, and number of electron transferred in the
redox event \((n)\) from equation 11.

3.3 Results and Discussions

3.3.1 CV Analysis at Different Scan Rates

Two distinct systems were considered in this study. In one system, the electrode surface was functionalized with an anchor-competitor duplex, positioning the redox tag MB near the surface. In the other system, thiol-modified single-stranded DNA (ssDNA) with MB attached at the other end of the strand was immobilized on the electrode surface, resulting in the redox tag MB being located farther away from the electrode surface. It's worth noting that all the protocols for immobilizing the anchor, hybridizing the anchor and competitor, as well as the buffer conditions, were consistent with those described in Chapter 2.

Figure 3-1 (A) illustrates the cyclic voltammograms obtained at various scan rates for the anchor-competitor duplex system with MB positioned closer to the electrode surface, while Figure 3-1 (B) depicts the cyclic voltammogram for the anchor with MB positioned farther away from the electrode surface.
As seen in Figure 3-1 (A), the shift in the peak-to-peak potential with the increase in scan rate is very small. A typical characteristic of a surface adsorbed species is no shift in peak-to-peak separation with increasing scan rate [80]. A nominal shift in the peak-to-peak separation in this case could be attributed to the delay in the collision of MB molecules with the electrode surface because the MB molecules do not remain completely adsorbed on the electrode surface. In other words, the MB molecules, after hybridization of the competitor with anchor, may form a monolayer very close to the electrode surface but are not fully in contact with the electrode surface. It should be noted that the MB molecule is covalently bound to the phosphate backbone of competitor primer with a chain of six carbon atoms which makes the MB molecules possible to lie very close to the electrode and collide elastically with the electrode surface for electron transfer.

On the other hand, the shift in the peak-to-peak separation with scan rate in Figure 3-1 (B) indicates that the redox species is floating in the bulk solution though bound to the electrode through oligonucleotide. During the time course of a cyclic voltammogram, the MB molecules travel to and away from the electrode surface because of ssDNA elastic bending. The peak-to-peak potential separation with scan rate is significant compared to that in Figure 3-1 (A) which indicates that it is difficult for MB molecules in this particular system to keep up with the fast-changing potential.
Further, as explained in previous section a plot of peak current \( (i_p) \) against both scan rate \( (v) \) and the square root of scan rate \( (\sqrt{v}) \) can provide insights into whether the active species is bound to the surface or freely diffusing. Figure 3-2 and Figure 3-3 depicts the plot of \( i_p \) against \( v \) and \( \sqrt{v} \) for anchor-competitor duplex system with MB at proximal end, and anchor ssDNA with MB at distal end respectively.

The plot of \( i_p \) against \( v \) fitting well with a straight line in Figure 3-2 is a feature of surface adsorbed redox species.

![Figure 3-2: (A) peak current in CV against various scan rate (B) peak current against square root of scan rate for anchor hybridized with competitor with MB located near the electrode surface.](image-url)
Similarly, in Figure 3-3 the plot of $i_p$ against $\sqrt{v}$ being linear is an indication of freely diffusing redox species.

As per equation 13, a plot of $\ln(i_p)$ vs $\ln v$ as shown in Figure 3-4 can be used to find the coefficient $\alpha$ from the slope of linear fit.

The slope ($\alpha$) of the linear fit in Figure 3-4 (A) exhibits a value of 0.99 (or approximately 1), a characteristic often associated with surface-adsorbed species. This observation suggests that the electron transfer mechanism at play is primarily collisional electron transfer rather than DNA-mediated electron transfer. This distinction is important as Barton et al. previously reported instances of DNA-mediated electron transfer, particularly when the redox molecule intercalates within the base pairs of a DNA duplex. However, in our specific scenario, the redox tag is covalently bonded to the DNA duplex.
strand, and the configuration of the system significantly increases the likelihood of the redox species colliding with the electrode surface following the hybridization event between the competitor primer and the anchor primer.

On the contrary, the $\alpha$ value in Figure 3-4 (B) is measured at 0.68. In the context of diffusion-controlled electron transfer, an $\alpha$ value of 0.5 is expected. This deviation from the ideal value suggests that the complex system under investigation involves heterogeneous electron transfer processes, indicating the presence of a certain degree of diffusion-controlled electron transfer within the system.

3.3.2 Effect of Mercapto-6-Hexanol (MCH) in Electrochemical Potential

MCH plays a crucial role in passivating the exposed regions of gold surface that are not occupied by DNAs. Consequently, if the electron transfer mechanism relies on contact mediation, it is anticipated that the redox event would exhibit greater favorability or lower energy demand in the absence of MCH passivation. Figure 3-5 illustrates the cyclic voltammetry (CV) scans for the anchor hybridized with competitor having methylene blue located close to the electrode surface under two distinct conditions: one with the electrode surface passivated by MCH and the other without MCH passivation.
Figure 3-5 shows the cv scans for anchor-competitor duplex with MB molecule positioned closer to the electrode surface when the surface is passivated by MCH (represented by dashed lines, curve (a)) and when the surface is not passivated by MCH (represented by solid lines, curve (b)). The shift in the peak-to-peak potential in CV plots suggests that the activation energy required for the redox event is lower when the surface is passivated by MCH compared to that when the surface is not passivated with MCH. A negative shift in the redox potential refers to a decrease in the electrochemical potential or energy required for a redox (reduction-oxidation) reaction to occur [73, 117]. It can indicate that the redox reaction becomes more favorable or easier to occur under the given conditions.

This indicates that the electron transfer between the redox species and the electrode surface is collisional electron transfer. Had it been DNA mediated electron transfer then passivation of electrode surface would have no effect in the redox potential
of the redox species and hence there would have been no shift in the peak-to-peak potential.

3.4 Conclusions

In conclusion, our investigation indicates that the electron transfer mechanism occurring between methylene blue (MB) and the electrode surface is consistently collisional, regardless of the location of the MB within the DNA duplex. However, in the system where MB is positioned farther from the electrode surface, there is an indication of some level of diffusion-controlled electron transfer.

Furthermore, the observed shift in redox potential in CV plots underlines that the activation energy for redox events is lower when the surface is passivated with MCH, suggesting a collisional electron transfer process. In contrast, DNA-mediated electron transfer would not be affected by surface passivation, which would not lead to shifts in redox potential. Therefore, our study not only contributes to the understanding of electron transfer mechanisms in DNA-based sensors but also highlights the influence of molecular positioning and surface modifications on these processes.

Further investigation, including varying the concentration of the redox marker, adjusting the temperature, and altering the pH of the buffer solution, which all affect the rate of diffusion, could yield deeper insights into the mechanism of diffusion-controlled electron transfer. Additionally, introducing base pair mismatches in the DNA duplex as part of these studies could enhance our understanding of the electron transfer processes.
CHAPTER 4 COMPARATIVE ANALYSIS OF METHYLENE BLUE LABELLED DNA HYBRIDIZATION SENSOR DESIGN FOR SARS-CoV2 DETECTION: AN APPROACH USING ELECTROCHEMICAL AND OPTICAL DETECTION TECHNIQUES

4.1 Detection Schemes

Three different detection schemes were explored; two of them utilized the 30-base thiolated anchor whereas the third scheme utilized the same 30-base thiolated anchor with covalently bound MB at the other end of the strand. These anchors had complementary base sequence to the first 30 bases of a 60-base target with the following sequence: 5’-CAC TAC CTG GCG TGG TTT GTA TGA AAT CAC CGA AAT CAT ACC AGT TAC CAT TGA GAT CTT-3’, which mimicked a specific region (i.e., RNA-dependent RNA polymerase) of the Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) genome. The three schemes are described with the figures below.

Scheme I

Once the anchor is immobilized on the sensor surface, the target analyte is introduced into the flow cell. The protocol for making the target primer ready to inject into the flow cell is described in the following section. Among the 60 bases comprising the target analyte, 30 bases complement the anchor oligo, resulting in half of the target hybridizing with the anchor while the other half remains unbound, as depicted in step 2 of
Figure 4-1. Subsequently, a probe oligo, modified with methylene blue (MB) and complementary to the unbound portion of the target oligo, is introduced into the flow cell as shown in step 3. It is anticipated that if the target primer is hybridized with the anchor, the probe primer will also bind to the target, leading to the observation of a redox signal from MB. However, the cyclic voltammogram presented in Figure 4-2 unequivocally indicates the absence of a redox signal from MB. Numerous experiments were conducted using this approach, yet the redox signal from MB was never detected. This suggests either that the target did not hybridize with the anchor, leaving the probe with nothing to bind to, or that the positioning of MB on the probe was too distant from the sensor surface, hindering its access for electron transfer.

Figure 4-1: Illustration of scheme I for the detection of SARS-CoV2

Figure 4-2 is the cyclic voltammogram for anchor, anchor-target, and anchor-target-probe configuration.
After the immobilization of anchor followed by the injection of target into the flow cell, a competitor primer with base sequence complementary to the anchor primer is introduced into the flow cell as shown in figure 4-3. The competitor primer is the same competitor primer described in chapter 1 which has the MB attached at the proximal end such that the MB is positioned closer to the electrode surface upon hybridizing with the complementary anchor. If the 30 bases of the target is hybridized with the anchor then there remains no room the competitor to hybridize with the target and hence no redox signal is expected after the introduction of competitor into the flowcell.
Nevertheless, traces of signal from the MB were observed following the introduction of the competitor into the flow cell, as illustrated in the cyclic voltammogram in Figure 4-4. This observation suggests the presence of either free, unhybridized anchor primers with some competitors hybridized to them, or the binding of a few bases of the competitor to the complementary bases of the upper portion of the target, allowing the MB to access the electrode surface for electron transfer.

Figure 4-3: Illustration of scheme II for the detection of SARS-CoV2.
Scheme III

For the third scheme, the anchor was further modified with MB at the other end. A redox signal from MB is anticipated after the immobilization of anchor on the sensor surface. Because of the flexibility of a ssDNA the MB attached to the distal end of the anchor can access the electrode surface for electron transfer. This anchor being complementary to the first 30 base of the target can hybridize with the target to form a duplex which is more rigid and upright than ssDNA because of which the MB molecule is positioned away from the electrode surface upon hybridization of target with anchor. An illustration of the scheme is shown in figure 4-5.

Figure 4-5: Illustration of scheme III for the detection of SARS-CoV2.

4.1.1 Hybridization of Target Analyte

A 60-base target sequence represents a considerable length for DNA-based sensing platforms. With such an extended sequence, single-stranded DNA (ssDNA) can
undergo various self-binding mechanisms, leading to the formation of hairpin structures, loops, and stem-loop conformations. Therefore, it is necessary to prepare long ssDNA strands in a manner that prevents the formation of these secondary structures.

To achieve this, a 650 nM target solution was subjected to a temperature of 90 °C for 10 minutes, followed by immediate immersion in an ice bath for 2 minutes. Subsequently, the solution was allowed to equilibrate to room temperature over a period of 2 hours in the ice bath. This controlled thermal treatment ensured that the target ssDNA adopted the desired conformation without the formation of hairpin, loop, or stem-loop structures, preparing it for injection into the flow cell.

4.1.2 Sensor Surface Regeneration

Like many other DNA sensors within this category, the sensor was regenerated through a straightforward 30-second rinsing with deionized (DI) water, as mentioned in the literature [118]. 10 ml of DI water was injected into the flow cell for 30 s, followed by a rinse with 1X PBS buffer. Cyclic voltammograms to confirm that the DI rinse indeed breaks the H-bond between the DNA nucleotides and unzips the DNA strands are shown in Figure 4-6. The change in pH and ionic concentration of the system may play a significant role in the disruption of the H-bond when rinsing with DI water.
In Chapter 2, Section 2.4.1, we explored the distinctions in cyclic voltammograms arising from variations in the accessibility of the redox molecule to the electrode surface. The results illustrated that in cases where the redox species was in close proximity to the electrode surface, no discernible peak-to-peak separation between the cathodic and anodic potentials was observed.

Conversely, when the redox tag was positioned farther from the electrode surface, a significant peak-to-peak separation was evident. Consequently, this observation led to the conclusion that the peak-to-peak separation between the cathodic and anodic potentials can serve as a valuable indicator for assessing the accessibility of the redox tag to the electrode surface, thereby establishing its utility as a sensing parameter.
4.2.2 Phase vs Frequency

In Chapter 2, Section 2.4.2 of the dissertation, we conducted electrochemical impedance spectroscopy (EIS) to investigate the kinetics of electron transfer and the changes associated with duplex formation. The Bode plots clearly demonstrated that as the AC frequency approached the time constant of the redox reaction, the phase angle exhibited a decreasing trend, eventually reaching a minimum point.

The frequency at which this phase angle minimum occurred proved to be a valuable parameter for qualitatively distinguishing between systems with varying configurations or electron transfer kinetics. Notably, our observations indicated that in systems where the redox species could access the electrode surface, the phase angle reached its minimum at a frequency falling within the range of 1 to 10 Hz.

Furthermore, our findings suggested a correlation between the frequency at which the phase angle minimum occurred and the rate of electron transfer. Systems exhibiting phase angle minima at higher frequencies demonstrated faster rates of electron transfer. Hence, we concluded that plotting the phase angle against the frequency of applied AC potential perturbation can serve as a useful tool for differentiation when assessing whether redox species can access the electrode surface or not.
4.3 Results and Discussions

4.3.1 CV Analysis

Figure 4-1 (A) displays the cyclic voltammograms obtained for various systems. A clear redox signal was observed in CV for anchor-MB. The flexibility of ssDNA conformation can be attributed to the access of electrode surface by MB to transfer electron. Following the protocol explained in preceding section, the target analyte was subjected to a heating step to reach 90°C for 10 minutes, then rapidly cooled in an ice bath for 2 minutes, and

![Figure 4-7: CV scan for Anchor-MB (solid blue trace), Anchor-MB hybridized with target (red trace), and after regeneration of sensor (dashed blue trace).](image)

finally, the solution is brought to room temperature before being introduced into the flow cell. After the introduction of the target analyte, a complete suppression of the redox signal was achieved after the hybridization of the target with the anchor. Subsequently, the redox signal was restored after the sensor regeneration.
The structural conformation attained by the target after heat and cold treatment may potentially promote more effective hybridization with the anchor, resulting in a more rigid anchor-target duplex that restricts MB molecule access to the electrode surface.

4.3.2 Phase Analysis from EIS Spectrum

The phase-versus-frequency response to the applied AC potential perturbation is graphed in Figure 4-7.

A noticeable distinction emerged in the phase-versus-frequency plot between the anchor and anchor-target duplex systems. Specifically, a phase minimum was evident at around 5 Hz when no target was present in the flow cell. However, this characteristic phase minimum was absent after the hybridization of the target with the anchor. This distinctive feature of the phase minimum holds promise as a potential detection tool for

Figure 4-8: Phase against the frequency of ac potential for Anchor-MB (solid blue trace), Anchor-MB hybridized with target (red trace), and after regeneration of sensor (dashed blue trace).
DNA/RNA hybridization sensors. Furthermore, although not fully restored, the phase minimum was partially regained following the sensor's regeneration.

4.4 Conclusions

The CV and phase analysis from EIS spectrum show a clear distinction between the presence and absence of the hybridization of target analyte with the capture probe regardless of the protocols followed for hybridization. The CV analysis reveals a notable distinction in the redox signal between the presence and absence of the hybridization of the target analyte with the capture probe. Similarly, the phase analysis of the EIS data also provides a notable difference in the phase between the absence and presence of target analyte. In addition to this, the redox signal in CV and the phase minimum in EIS were restored after sensor regeneration. This is a crucial observation since efficient sensor regeneration is essential for the long-term performance and reusability of the biosensing platform.

Both signal suppression and sensor regeneration are pivotal factors in the context of a biosensor, as they directly influence its sensitivity and practicality. The results of this study highlight the need for further refinement in the hybridization protocol to optimize the overall sensor performance. Nonetheless, we present a simple yet robust DNA-based biosensing strategy which can be further optimized to be used in detecting the genome sequence of not only SARS CoV2 but also other genomes of virus and pathogens.
CHAPTER 5 SUMMARY AND FUTURE PERSPECTIVES

5.1 Summary

The study involved the utilization of both electrochemical modulation of surface plasmon wave (ECM-SPW) and electrochemical transduction techniques to investigate various redox assemblies consisting of single and double-stranded DNA molecules. The impact of the positioning of the redox label, Methylene Blue (MB), covalently attached to the sugar-phosphate backbone of DNA, on the electrochemical response of a DNA hybridization sensor was investigated. Both electrochemical and optical analyses were employed to examine how the location of the redox-active molecule on the DNA affected the sensor's performance. The results consistently demonstrated that positioning the redox marker closer to the electrode surface significantly improved the electrochemical response. This proximity facilitated more efficient charge transfer processes between MB and the electrode surface. Conversely, when the redox marker was placed farther from the electrode surface, the rigidity of the DNA duplex upon hybridization hindered its access to the electroactive surface, resulting in a reduced electrochemical response.

The electrochemical investigations revealed that regardless of the location of MB within the DNA duplex, the electron transfer pathway was primarily contact-mediated rather than DNA-mediated. When MB was in close proximity to the electrode surface, it exhibited characteristics typical of a surface-adsorbed redox species.
Conversely, when MB was positioned further away from the electrode surface, a diffusion-controlled electron transfer mechanism became apparent. Optical impedance analysis revealed two distinct electron transfer rates—one in the higher frequency region and another in the lower frequency region, suggesting the existence of two different electron transfer pathways. The utilization of the ECM-SPW device and optical impedance spectroscopy techniques opened up new avenues for exploring electron transfer mechanisms in label-based DNA sensors.

Ultimately, the dissertation concluded with the development of a simple yet robust detection strategy capable of identifying a 60-base genome sequence specific to the SARS-CoV-2 genome. Phase analysis from the electrochemical impedance spectra emerged as a potential parameter for distinguishing the presence and absence of the target analyte. This sensing strategy opens a path for a simple electrochemical transduction platform for the detection of multiplexed target which can be useful in clinical and diagnostic settings.

5.2 Future perspectives

The biosensor market was valued at over USD 28.5 billion in 2022, with glucose sensors being a major contributor, and is anticipated to surpass USD 58 billion by 2032 [119]. The growing understanding of the fundamental science underpinning the electrochemical and surface characteristics of DNA, alongside technological advancements in nucleic acid (NA) detection, will inevitably give rise to an increase in marketable electrochemical NA sensing platforms. These platforms are poised for application in point-of-care testing (POCT), clinical diagnostics, as well as environmental
and agricultural analysis [1].

The unforeseen emergence of the COVID-19 pandemic has highlighted the need for reliable and rapid testing methodologies. Enhancements in integrating electrochemical and optical detection, as discussed in this dissertation, could lead to the development of POCT devices addressing current challenges in detection speed and accuracy.

In designing biosensors, a critical aspect is the affordability of production. Steps toward making biosensors cost-effective include miniaturizing electrochemical biosensors and incorporating inexpensive materials. Recent studies have shown interest in utilizing Aluminum film in Surface Plasmon Resonance (SPR) [120] due to its effective SPR response in the ultraviolet (UV) light spectrum [121-123]. Furthermore, UV-vis spectroscopy reveals that DNA molecules exhibit distinct absorption peaks at 260 nm and 280 nm within the UV range [124]. Consequently, employing Aluminum film combined with DNA/RNA aptamers in Evanescent Coupled Mode-Surface Plasmon Wave (ECM-SPW) technology is promising for biosensing applications.

DNA molecules' capability to undergo chemical modification to attach a variety of functional groups offers the potential for precise positioning of nano cargoes. DNA nanotechnology further provides a route for nanofabrication, leveraging customizable nanoscale architectures through base pairing specificity, thus serving as versatile three-dimensional templates. This has led to the development of novel devices and systems such as carbon nanotube transistors, quantum computing devices, and photonic devices for advanced light manipulation, as well as biophotovoltaics and biobatteries [125]. The assembly process, which occurs in biofriendly conditions such as aqueous solution with
no extreme chemicals, pH values, and temperature, further adds environmental
sustainability.

Moreover, various enzymes, including invertase [126], lipase [127], alkaline
phosphatase [128], glucose oxidase [129], and Horseradish peroxidase (HRP) [130], have
been effectively incorporated with DNA hybridization complexes to significantly amplify
electrochemical signals. These enzymes are capable of producing 10 to 1000 fold higher
redox-active products [131], leading to a substantially increased redox current at the
electrode surface. Strategically positioning these enzymes near the electrode surface can
further boost the access to redox-active products, thereby enhancing the overall redox
current.


APPENDICES

Appendix A: Gold thin film characterization

1. Mathematica script to estimate film thickness and optical constants of thin metal films.

Clear[λ, φ, φ, α, x, y, z]

"Wavelength (nm)"; λ=660;

"Base angle of prism in degrees";

φ=60;

"Refractive outside prism, typically air";

nair=1.000;

"Medium before layers, BK-7";

n0=\(1+1.03961212/(1-0.00600069867/(λ/1000)^2)+0.231792344/(1-0.0200179144/(λ/1000)^2)+1.01046945/(1-103.560653/(λ/1000)^2))^{.5};\)

"Layer 1";

n1=x-y I;
t1=z;

"Layer 2";

n2=x-y I;
t2=0;

"Layer 3";

n3=x-y I;
t3=0;
"Layer 4";
n_4 = x - y I;
t_4 = 0;

"Layer 5";
n_5 = x - y I;
t_5 = 0;

"Medium after layers, e.g. buffer, air";
n_m = 1.329898027;

"Read experimental data";
ang = Import["C:/.../Theta", "csv"];
ref = Import["C:/.../R", "csv"];

θ = ang[[All, 1]];
R = ref[[All, 1]];

"Start calculations";

\[ N_e = n_{air} \sin \left( \frac{\pi}{180} \theta \right) \cos \left( \frac{\pi}{180} \phi \right) + \sqrt{n_0^2 - \left( n_{air} \sin \left( \frac{\pi}{180} \theta \right) \right)^2} \sin \left( \frac{\pi}{180} \phi \right); \]
\( \chi_0 = \text{Abs} \left[ \text{Re} \left[ \sqrt{n_0^2 - N_e^2} \right] \right] - \text{IAbs} \left[ \text{Im} \left[ \sqrt{n_0^2 - N_e^2} \right] \right] ; \)

\( p\eta_0 = n_0^2 / \chi_0 ; \)

\( \chi_1 = \text{Abs} \left[ \text{Re} \left[ \sqrt{n_1^2 - N_e^2} \right] \right] - \text{IAbs} \left[ \text{Im} \left[ \sqrt{n_1^2 - N_e^2} \right] \right] ; \)

\( p\eta_1 = n_1^2 / \chi_1 ; \)

\( \delta_1 = \frac{2\pi}{\lambda} t_1 \chi_1 ; \)

\( \chi_2 = \text{Abs} \left[ \text{Re} \left[ \sqrt{n_2^2 - N_e^2} \right] \right] - \text{IAbs} \left[ \text{Im} \left[ \sqrt{n_2^2 - N_e^2} \right] \right] ; \)

\( p\eta_2 = n_2^2 / \chi_2 ; \)

\( \delta_2 = \frac{2\pi}{\lambda} t_2 \chi_2 ; \)

\( \chi_3 = \text{Abs} \left[ \text{Re} \left[ \sqrt{n_3^2 - N_e^2} \right] \right] - \text{IAbs} \left[ \text{Im} \left[ \sqrt{n_3^2 - N_e^2} \right] \right] ; \)

\( p\eta_3 = n_3^2 / \chi_3 ; \)
\[ \delta_3 = \frac{2\pi}{\lambda} t_3 \chi_3; \]

\[ \chi_4 = \text{Abs} \left[ \text{Re} \left( \sqrt{n_4^2 - N_e^2} \right) \right] - \text{IAbs} \left[ \text{Im} \left( \sqrt{n_4^2 - N_e^2} \right) \right]; \]

\[ p\eta_4 = \frac{n_4^2}{\chi_4}; \]

\[ \delta_4 = \frac{2\pi}{\lambda} t_4 \chi_4; \]

\[ \chi_5 = \text{Abs} \left[ \text{Re} \left( \sqrt{n_5^2 - N_e^2} \right) \right] - \text{IAbs} \left[ \text{Im} \left( \sqrt{n_5^2 - N_e^2} \right) \right]; \]

\[ p\eta_5 = \frac{n_5^2}{\chi_5}; \]

\[ \delta_5 = \frac{2\pi}{\lambda} t_5 \chi_5; \]

\[ \chi_m = \text{Abs} \left[ \text{Re} \left( \sqrt{n_m^2 - N_e^2} \right) \right] - \text{IAbs} \left[ \text{Im} \left( \sqrt{n_m^2 - N_e^2} \right) \right]; \]

\[ p\eta_m = \frac{n_m^2}{\chi_m}; \]

\[ M_{1a} = \cos[\delta_1]; \]
M1b = \( I \sin(\delta_1) / p \eta_1 \);
M1c = \( I p \eta_1 \sin(\delta_1) \);
M1d = \( \cos(\delta_1) \);
M2a = \( \cos(\delta_2) \);
M2b = \( I \sin(\delta_2) / p \eta_2 \);
M2c = \( I p \eta_2 \sin(\delta_2) \);
M2d = \( \cos(\delta_2) \);
M3a = \( \cos(\delta_3) \);
M3b = \( I \sin(\delta_3) / p \eta_3 \);
M3c = \( I p \eta_3 \sin(\delta_3) \);
M3d = \( \cos(\delta_3) \);
M4a = \( \cos(\delta_4) \);
M4b = \( I \sin(\delta_4) / p \eta_4 \);
M4c = \( I p \eta_4 \sin(\delta_4) \);
M4d = \( \cos(\delta_4) \);
M5a = \( \cos(\delta_5) \);
M5b = \( I \sin(\delta_5) / p \eta_5 \);
M5c = \( I p \eta_5 \sin(\delta_5) \);
M5d = \( \cos(\delta_5) \);

"Matrix multiplications":

MLa = M1a;
MLb = M1b;
MLc = M1c;
MLd = M1d;
MPa = MLa * M2a + MLb * M2c;
\[ MP_b = ML_a * M_{2b} + ML_b * M_{2d}; \]
\[ MP_c = ML_c * M_{2a} + ML_d * M_{2c}; \]
\[ MP_d = ML_c * M_{2b} + ML_d * M_{2d}; \]
\[ ML_a = MP_a; \]
\[ ML_b = MP_b; \]
\[ ML_c = MP_c; \]
\[ ML_d = MP_d; \]
\[ MP_a = ML_a * M_{3a} + ML_b * M_{3c}; \]
\[ MP_b = ML_a * M_{3b} + ML_b * M_{3d}; \]
\[ MP_c = ML_c * M_{3a} + ML_d * M_{3c}; \]
\[ MP_d = ML_c * M_{3b} + ML_d * M_{3d}; \]
\[ ML_a = MP_a; \]
\[ ML_b = MP_b; \]
\[ ML_c = MP_c; \]
\[ ML_d = MP_d; \]
\[ MP_a = ML_a * M_{4a} + ML_b * M_{4c}; \]
\[ MP_b = ML_a * M_{4b} + ML_b * M_{4d}; \]
\[ MP_c = ML_c * M_{4a} + ML_d * M_{4c}; \]
\[ MP_d = ML_c * M_{4b} + ML_d * M_{4d}; \]
\[ ML_a = MP_a; \]
\[ ML_b = MP_b; \]
\[ ML_c = MP_c; \]
\[ ML_d = MP_d; \]
\[ MP_a = ML_a * M_{5a} + ML_b * M_{5c}; \]
\[ MP_b = ML_a * M_{5b} + ML_b * M_{5d}; \]
\[ MP_c = ML_c * M_{5a} + ML_d * M_{5c}; \]
\[ MP_d = ML_c * M_{5b} + ML_d * M_{5d}; \]
\[ B_p = MP_a + \mu M_{\mu} MP_b; \]
\[ C_p = \text{MPc} + \eta_m MPd; \]

\[ R_p = \text{Re} \left[ \frac{\eta_0 B_p - C_p}{\eta_0 B_p + C_p} \right] \text{Conjugate} \left[ \frac{\eta_0 B_p - C_p}{\eta_0 B_p + C_p} \right]; \]

\[ T_p = \text{Re} \left[ \frac{4\eta_0 \text{Re}[\eta_m]}{\eta_0 B_p + C_p} \right] \text{Conjugate} \left[ \frac{1}{\eta_0 B_p + C_p} \right]; \]

\[ A_p = \text{Re} \left[ \frac{4\eta_0 \text{Re}[B_p \text{Conjugate}[C_p] - \eta_m]}{\eta_0 B_p + C_p} \right] \text{Conjugate} \left[ \frac{1}{\eta_0 B_p + C_p} \right]; \]

\[ \eta_{\text{air}} = n_{\text{air}}^2 / \sqrt{n_{\text{air}}^2 - \left( n_{\text{air}} \sin \left[ \frac{\pi}{180} \theta \right] \right)^2}; \]

\[ \eta_{\text{prism}} = n_0^2 / \sqrt{n_0^2 - \left( n_{\text{air}} \sin \left[ \frac{\pi}{180} \theta \right] \right)^2}; \]

\[ T_{\text{air/prism}} = \text{Re} \left[ \frac{4\eta_{\text{air}} \eta_{\text{prism}}}{(\eta_{\text{prism}} + \eta_{\text{air}})^2} \right]; \]

\[ R_p = \text{Re} \left[ \frac{\eta_0 B_p - C_p}{\eta_0 B_p + C_p} \right] \text{Conjugate} \left[ \frac{\eta_0 B_p - C_p}{\eta_0 B_p + C_p} \right] \left( T_{\text{air/prism}} \right)^2; \]

\[ \phi = \text{Re} \left[ \left( \frac{(R - R_p)}{R_p} \right)^2 \right]; \]

\[ \alpha = \text{Norm}[\phi]; \]

\[ \text{NMinimize}[\alpha, ((x, 0, 3), (y, 0, 5), (z, 0, 60))]; \]

\[ x=\ldots; \]

\[ y=\ldots; \]

\[ z=\ldots; \]

\[ \text{Export ["C:/\ldots ", R_p, "csv"];} \]

\[ \text{ListPlot}[[R_p, R], \text{PlotRange} \to \text{All}]; \]

\[ \text{Null} \]
2. Python script for Kramers-Kronig compliance and EIS analysis.

[1] # This program takes raw experimental impedance data and plots the numerical Kramers-Kronig relations.
[2] # It then fits the data to a circuit model to find solution resistance, charge transfer resistance, double layer capacitance, and pseudo-capacitance.
[3] # To use, simply adjust the file location in the first non-commented line after the preamble.
[4] # preamble
[5] import csv
[6] import numpy, scipy
[7] import math
[8] import matplotlib.pyplot as plt
[9] from scipy.optimize import curve_fit
[10]
[11] # read from file and process data
[12] file = open('C:/Users/Thomas Hulse/py_projects/KK_Compliance/AC impedance_anchor.csv', "r") # adjust file name as needed
[13] data = list(csv.reader(file, delimiter=""))
[14] file.close()
[15]
[16] # find line where data starts
[17] # always after three blank lines, the impedance data begins
[18] i = 0
[19] j = 0
[20] while i < 50:
[21]   if(data[i][0]==' '):
[22]     j += 1
[23]   if(j == 3):
[24]     break
[25]   i += 1
[26]
[27] # create arrays for frequencies and real/imaginary impedances. also calculate total impedance and phase
[28] frequencies = numpy.array([float(row[0]) for row in data[i+1:]])
[29] ZReal = numpy.array([float(row[1]) for row in data[i+1:]])
ZImag = numpy.array([float(row[2]) for row in data[i+1:]])
totalImp = numpy.sqrt(ZReal**2 + ZImag**2)
phase = -360/2/math.pi*numpy.arctan(ZImag/ZReal)

# create the imaginary part from real part by KK-relations
KKImag = [0]*len(frequencies)
integrandArray = [0]*len(frequencies)
integrandDenom = [0]*len(frequencies)

# compute imaginary component of Z using Simpson's Rule for KK-relations
i = 0
# for each data point...
while i < len(frequencies):
    # compute the integrand for the KK-relations at every point, avoiding the singularity
    integrandDenom = frequencies**2-frequencies[i]**2
    integrandDenom[i] = 1
    integrandArray = (ZReal - ZReal[i])/(integrandDenom)

    # avoid the singularity
    integrandArray[i] = 0

    # use Simpson's rule to numerically evaluate integral
    KKImag[i] = -2*(frequencies[i])/numpy.pi*scipy.integrate.simps(integrandArray, frequencies, .1)
    i += 1

# create the real part from imaginary part by KK-relations
KKReal = [0]*len(frequencies)
integrandArray = [0]*len(frequencies)
# compute real component of Z using Simpson's Rule for KK-relations
i = 0
# for each data point...
while i < len(frequencies):
    # compute the integrand for the KK-relations at every point, avoiding the singularity
    integrandDenom = frequencies**2 - frequencies[i]**2
    integrandDenom[i] = 1
    integrandArray = (frequencies*ZImag - frequencies[i]*ZImag[i])/(integrandDenom)
    # avoid the singularity
    integrandArray[i] = 0
    # use Simpson's rule to numerically evaluate integral
    KKReal[i] = 2/numpy.pi*scipy.integrate.simpson(integrandArray, frequencies, .1) + ZReal[0]
    i += 1

# plot (being careful to convert to angular frequency)
plt.xlabel("log(\u03C9)")
plt.ylabel("Z'(\u03C9) & Z''(\u03C9)")
# scatter + plot the real parts
plt.scatter(numpy.log10(2*numpy.pi*frequencies), ZReal, label='Z' Raw Data', s = 20)
plt.plot(numpy.log10(2*numpy.pi*frequencies), KKReal, color='red', label = 'Z' Kramers-Kronig', linewidth = 2.5)
# scatter + plot the imaginary parts
plt.scatter(numpy.log10(2*numpy.pi*frequencies), ZImag, label='Z'' Raw Data', s = 20)
plt.plot(numpy.log10(2*numpy.pi*frequencies), KKImag, color='black', label = 'Z\'\' Kramers-Kronig', linewidth = 2.5)

# legend + show
plt.legend(loc="center right")
plt.show()

# define various circuit models for real impedance, imaginary impedance, total impedance, and phase
# note: R1 is solution resistance, R2 is charge transfer resistance, C1 is double layer capacitance, C2 is pseudo-capacitance

# shorthand for frequencies
x = frequencies

# model for real part
def modReal(data, R1, R2, C1, C2):
    R1 = ZReal[0]
    # analytical solution of circuit
    result = R1 + (-R2/(C1*C2*((2*math.pi*x)**2)) + R2*(1/((2*math.pi*x)*C1) + 1/((2*math.pi*x)*C2))/((2*math.pi*x)*C1)**2) + 1/((2*math.pi*x)*C2))
    # force all parameters to be positive and reasonable (no massive capacitances)
    if(R1 > 0 and R2 > 0 and 1 > C1 > 0 and 1 > C2 > 0):
        return result
    else:
        return 1e10
# model for imaginary part

def modImag(data, R1, R2, C1, C2):
    R1 = ZReal[0]
    # analytical solution of circuit
    result = -(R2**2/((2*math.pi*x)*C1) + 
               1/((2*math.pi*x)*C1) + 1/((2*math.pi*x)*C2))/((2*math.pi*x)**2)/(C1*C2*(2*math.pi*x)**2)/(R2**2 + 
               (1/((2*math.pi*x)*C1) + 1/((2*math.pi*x)*C2))**2)
    # force all parameters to be positive and reasonable (no massive capacitances)
    if(R1 > 0 and R2 > 0 and 1 > C1 > 0 and 1 > C2 > 0):
        return result
    else:
        return 1e10

# model for total impedance

def modTotImp(data, R1, R2, C1, C2):
    R1 = ZReal[0]
    # real and imaginary parts added in quadrature
    result = numpy.sqrt((R1 + (-
                          R2/(C1*C2*((2*math.pi*x)**2)) + R2*(1/((2*math.pi*x)*x)*C1) + 1/((2*math.pi*x)*x)*C2))/((2*math.pi*x)*x)*C1)/(R2**2 + 
                          (1/((2*math.pi*x)*x)*C1) + 1/((2*math.pi*x)*x)*C2))**2 + 
                          (-R2**2/((2*math.pi*x)*x)*C1) + (1/((2*math.pi*x)*x)*C1) + 
                          1/((2*math.pi*x)*x)*C2))/(C1*C2*(2*math.pi*x)**2)/(R2**2 + 
                          (1/((2*math.pi*x)*x)*C1) + 1/((2*math.pi*x)*x)*C2))**2)
    # force all parameters to be positive and reasonable (no massive capacitances)
    if(R1 > 0 and R2 > 0 and 1 > C1 > 0 and 1 > C2 > 0):
        return result
    else:
        return 1e10
# model for phase in degrees

def modPhase(data, R1, R2, C1, C2):
    R1 = ZReal[0]
    result = -360/2/math.pi*numpy.arctan(-
        R2**2/((2*math.pi*x)*C1) + 1/((2*math.pi*x)*C1) +
        1/((2*math.pi*x)*C2))/((C1*C2*(2*math.pi*x)**2))/(R2**2 +
        1/((2*math.pi*x)*C1) + 1/((2*math.pi*x)*C2))/((2*math.pi*x)**2)) +
    R2*(1/((2*math.pi*x)*C1) +
        1/((2*math.pi*x)*C2))/((2*math.pi*x)*C1))/((2*math.pi*x)*C2))/((2*math.pi*x)**2))
    if(R1 > 0 and R2 > 0 and 1 > C1 > 0 and 1 > C2 > 0):
        return result
    else:
        return 1e10

# fitting phase model
# initial guess for parameter values
initialParameters = numpy.array([ZReal[0], 1e5, 2e-6, 1e-6])

# find fitted parameters with curve_fit
fittedParameters, pcov = curve_fit(modPhase, frequencies, phase, initialParameters, maxfev=5000)
R1, R2, C1, C2 = fittedParameters
print(fittedParameters)

# third data set, third equation
y_fit_totImp = modTotImp(frequencies, R1, R2, C1, C2)
# fourth data set, fourth equation
y_fit_phase = modPhase(frequencies, R1, R2, C1, C2)

# fitted plots
# plotting total impedance vs frequency
plt.scatter(numpy.log10(frequencies),
            numpy.log10(y_fit_totImp), label = 'Raw Data', s = 20)
plt.plot(numpy.log10(frequencies),
         numpy.log10(y_fit_totImp), color = 'red', label = 'Fit',
         linewidth = 2.5)
plt.xlabel(r"log($\Omega$)"")
plt.ylabel("Z")
plt.legend(loc="center right")
plt.show()

# plot phase vs frequency
plt.scatter(numpy.log(2*numpy.pi*frequencies), phase,
            label = 'Raw Data', s = 20)
plt.plot(numpy.log(2*numpy.pi*frequencies),
         y_fit_phase, color='red', label = 'Fit', linewidth = 2.5)
plt.xlabel("ln($\Omega$)"")
plt.ylabel("\phi")
plt.show()
EDUCATION

**PhD in Physics**  
Expected: April 2024  
University of Louisville  
Louisville, KY

**MS in Physics**  
August 2020  
University of Louisville

**MS in Physics**  
July 2017  
Tribhuvan University  
Kathmandu, Nepal

**BS in Physics**  
May 2012  
Tribhuvan University

WORK EXPERIENCE

**Part-time Lecturer**  
March 2024 – Present  
Department of Physics and Astronomy  
University of Louisville  
Louisville, KY

**Adjunct Faculty-Physics Lab Instructor**  
Jan 2024 – Present  
Bellarmine University  
Louisville, KY

**Graduate Teaching Assistant**  
2018 to 2023  
University of Louisville

PROJECT AND RESEARCH WORK

**Graduate Research**

- Investigation of signal intensity variations in a DNA hybridization sensor based on the positional attachment of redox molecules to the DNA duplex.
- Fabrication and characterization of gold film for Surface Plasmon Resonance Platform.
- Fabrication and optical characterization of magnetic superlattice film.
PUBLICATION AND WORKSHOP

Submitted Manuscript:

**Title:** Optical Impedance Spectroscopy using Electrochemically Modulated Surface Plasmon Wave as an Alternative to Electrochemical Interrogation of DNA-based Sensors.

**Authors:** Anil Sharma, Thomas Hulse, Sergio B. Mendes, Aymen H. Qatamin, Monica Moreno, Klester S. Souza, Marcelo B. Pereira, Paulo M. Roehe, Fabricio S. Campos, Leandro B. Carneiro, A.M.H. de Andrade

Conference Presentations:

- Virtual-Oral presentation at American Physical Society (APS) March Meeting, March 21, 2023  
  **Title:** Electron Transfer Rate in Nucleotides and Applications in Detection of Genetic Material of SARS-COV2 Virus.  
  https://meetings.aps.org/Meeting/MAR23/Session/LL03.7

- Poster presentation at Graduate Student Council (GSC) Regional Conference, University of Louisville, KY, USA, March 22, 2023  
  **Title:** Electron Transfer Rate in Nucleotides and Applications in Detection of Genetic Material of SARS-COV2 Virus.

- Three Minute Thesis (3MT) presentation, University of Louisville, KY, USA, Nov 18, 2022  
  **Title:** There is more we can do with DNA.

Workshop:

- Lesker University Vacuum Technology Workshop by Kurt J. Lesker Company coordinated by KY Multiscale and the Micro/Nano Technology Center, University of Louisville, KY, USA, Nov 14, 2019. Course topics included Physical Vapor Deposition and Thin Film Growth

LEADERSHIP ROLES

**Graduate Student Council (GSC) Representative**  
Department of Physics, University of Louisville  
Louisville, KY  
Sep 2022 – Aug 2023

- Managed communications between the Graduate Student Council (GSC) and students in the Department of Physics and Astronomy, disseminating information related to events, conferences, research, and travel grants.

**Vice-President**  
Nepalese Student Association (NSA), University of Louisville  
Louisville, KY  
Aug 2019 – Aug 2021

- Conducted General Assembly meetings.
- Coordinated the planning and execution of events including the ‘Nepalese Dashain and Tihar Program’ and the ‘Nepalese New Year Program’.

PROFESSIONAL ASSOCIATIONS

Member of American Physical Society (APS)  
2022