A Universal Electrochemical Biosensor for the Detection of Nucleic Acids based on a Four-Way Junction Structure

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A UNIVERSAL ELECTROCHEMICAL BIOSENSOR FOR THE DETECTION OF NUCLEIC ACIDS BASED ON A FOUR-WAY JUNCTION STRUCTURE

by

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B.S. Northern Arizona University, 2009
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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

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2017
Major Professor: Karin Y. Chumbimuni-Torres
ABSTRACT

Electrochemical hybridization sensors have been explored extensively for analysis of specific nucleic acids. However, commercialization of the platform is hindered by the need for attachment of separate oligonucleotide probes complementary to a RNA or DNA target to an electrode’s surface. This dissertation demonstrates that a single probe can be used to analyze several nucleic acid targets of different lengths with high selectivity, low cost and can be regenerated in 30 seconds by a simple water rinse. The universal electrochemical four-way junction (4J)-forming (UE4J) sensor consists of a universal DNA stem-loop (USL) probe attached to the electrode’s surface and two adaptor strands (m and f) which hybridize to the USL probe and the analyte to form a 4J structure. The UE4J sensor enables a high selectivity by recognition of a single base substitution, even at room temperature. The sensor was monitored with voltammetry and electrochemical impedance spectroscopy using different redox labeling strategies and optimized using synthetic nucleic acid sequences. After the sensor was optimized and fully characterized, it was modified for the detection of the Zika virus. The UE4J sensor presented here opens a venue for a re-useable universal platform that can be adopted at low cost for the analysis of potentially any DNA or RNA targets.
“It’s a trap.”

-Admiral Ackbar

To my mother and father, for supporting me on this journey.
ACKNOWLEDGMENTS

I would like to start by thanking my mentor, Dr. Karin Chumbimuni-Torres for her support and guidance throughout my graduate studies. I appreciate the mutual respect and communication we have shared over the years which has allowed me to take charge of my research projects and invest my own intellectual ideas to advance the research. I appreciate the flexibility she has allowed in the pursuit of my own career goals, even when it has required excessive travel across the country to finish my degree.

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LIST OF ACRONYMS/ABBREVIATIONS

α  Electron transfer coefficient

$\Gamma_{\text{DNA SL probe}}$  DNA SL probe coverage density

%SS  Percent signal suppression

2J  Two-way junction

4J  Four-way junction

A  Area of the electrode

AC  Alternating current

Au  Gold

C  Constant phase element

CE  Counter electrode

CV  Cyclic voltammetry

DI  Deionized water

DNA  Deoxyribonucleic acid

DPV  Differential pulse voltammetry

dsDNA  Double stranded DNA

E  Potential
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>Ep</td>
<td>Peak potential</td>
</tr>
<tr>
<td>E-DNA</td>
<td>Electrochemical DNA sensors</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIS</td>
<td>Electrochemical impedance spectroscopy</td>
</tr>
<tr>
<td>f</td>
<td>Critical frequency</td>
</tr>
<tr>
<td>F</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>FP1</td>
<td>Forward primer 1</td>
</tr>
<tr>
<td>GDE</td>
<td>Gold disc electrode</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>HB</td>
<td>Hybridization buffer</td>
</tr>
<tr>
<td>i</td>
<td>Current</td>
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<td>Forward current</td>
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<td>Reverse current</td>
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<td>Immobilization buffer</td>
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<tr>
<td>K₃[Fe(CN)₆]</td>
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<td>Symbol</td>
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<td>K$_4$[Fe(CN)$_6$]</td>
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<td>Potassium chloride</td>
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<td>LDR</td>
<td>Linear dynamic range</td>
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<td>LOD</td>
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<td>Slope from the calibration curve</td>
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<td>Molecular beacon</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequence based amplification</td>
</tr>
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<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
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<td>Point-of-care</td>
</tr>
<tr>
<td>Q</td>
<td>Integrated peak charge</td>
</tr>
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<td>$R_{CT}$</td>
<td>Charge transfer resistance</td>
</tr>
<tr>
<td>$R_s$</td>
<td>Resistance of the electrolyte solution</td>
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<td>Reference electrode</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Reverse primer for target 84Zik</td>
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<td>RP141</td>
<td>Reverse primer for target 141Zik</td>
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<td>RT-PCR</td>
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<td>Self-assembled monolayers</td>
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<td>SL</td>
<td>Stem-loop</td>
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<td>SNS</td>
<td>Single nucleotide substitutions</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>SPE</td>
<td>Screen printed electrode</td>
</tr>
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<td>ssDNA</td>
<td>Single stranded DNA</td>
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<td>SWV</td>
<td>Square wave voltammetry</td>
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<td>TCEP</td>
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</tr>
<tr>
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<td>Target DNA sequence</td>
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<td>Titanium oxide</td>
</tr>
<tr>
<td>Tris-HCl</td>
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<tr>
<td>UE4J</td>
<td>Universal electrochemical four-way junction</td>
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<td>Universal stem-loop</td>
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<tr>
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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Motivation
The development and commercialization of inexpensive, portable and sensitive sensors for point of care applications (e.g. clinical diagnostics, forensics, environmental monitoring) has long been a focus across many disciplines, undoubtedly due to the success of the blood-glucose sensor.¹⁻³ Sensors using sequence specific hybridization of nucleic acids has transformed disease diagnostics, although commercialized devices have yet to be realized, likely due to difficulty in differentiation nucleic acids that have single nucleotide substitutions (SNS). In recent years significant progress has been made in the design of hybridization sensors to improve selectivity, which is crucial for discriminating nucleic acids that contain a SNS.⁴⁻⁶ However, further improvements are necessary before commercial devices will be available. This dissertation describes the development and in depth characterization of a universal electrochemical biosensing platform based on a four-way junction (4J) structure for selective detection of nucleic acids. The remainder of this chapter provides the theory and background needed to understand following material. Chapter 2 focuses on a comparison in the performance of a conventional sensor with the 4J sensor using electrochemical techniques. In addition, the signaling mechanism of both sensors is investigated to provide a greater understanding of how to manipulate different detection method parameters for an optimal response. Chapter 3 provides a characterization of the universal 4J sensor for the detection of different short nucleic acid sequences. Optimization of the sensor’s components and experimental parameters are explored and this work provides the foundation for future chapters. Chapter 4 describes the ability of the sensor to detect nucleic
acids of different lengths which is applied for detection of real samples in the subsequent chapter. Chapter 5 focuses on the application of the 4J sensor for detection of the Zika virus.

1.2 Biosensors
The blood-glucose sensor, particularly important for management of diabetes, is perhaps the most well-known commercialized biosensor. A biosensor consists of a biorecognition (biological) element connected to a transducer. The biorecognition element interacts with a target of interest to generate a biochemical response that is translated into a measurable signal that can be interpreted by a user via the transducer as shown in Figure 1. Common biorecognition elements include proteins, antibodies, enzymes, nucleic acids or cells among others. The scope of this dissertation focuses on nucleic acid biorecognition elements which provide sequence specific detection of nucleic acids and offers advantages such as low cost, stability and are easy to obtain (i.e. easily synthesized). The most common transducers are optical, electrochemical, magnetic or thermometric. This work focuses on electrochemical transduction because it offers many benefits over other transducing elements such as miniaturization, incorporation in micro-analyses, low cost, portable equipment, and high sensitivity in a simple to use device. The principles for electrochemical transduction are further discussed in section 1.5.
Electrochemical biosensors that use nucleic acids as biorecognition elements exploit the interaction between a nucleic acid sequence of interest and a complementary sequence (probe), to form a target-probe complex that results in a measurable electrochemical signal change. Natural or synthetic deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) recognition probes have been reported for implementation into electrochemical biosensors. DNA probes are less expensive and more stable than RNA probes (which form secondary structures) and thus have been widely adopted for sensing applications.

DNA detection probes are single stranded DNA sequences that are complementary to target sequence of interest. The earliest DNA detection probes were in a linear conformation. Although the linear probes were able to hybridize to a specific target sequence, they lacked the ability to definitely discriminate between nucleic acid sequences containing a SNS. In 2003, Fan et al. reported a DNA stem-loop (SL) probe that offered better selectivity than the linear probe due to the conformational constraints of its design. The DNA SL probe is the equivalent to the
molecular beacon (MB), a fluorophore and quencher labeled hairpin DNA probe (Figure 2A), used in optical assays.\textsuperscript{17} In its hairpin form, the optical signal is quenched as the fluorophore is near the quencher. However, upon hybridization with a complementary target, the quencher and fluorophore are separated, resulting in a fluorescent signal. This design was implemented into an electrochemical format by Fan \textit{et al.}\textsuperscript{16} where the DNA SL probe is attached to an electrode at one end and conjugated with an electrochemically active (redox) label on the other end (Figure 2B). The change in electrochemical signal upon hybridization of the DNA SL probe with the target is measured and this change is dependent on redox label placement. Additional redox labeling strategies are discussed in section 1.5.4.

Figure 2: Schematic for a (A) molecular beacon (MB) fluorescent probe and (B) DNA stem-loop (SL) probe for nucleic acid detection.
An alternate version of these probes was developed for improvements in discriminating SNS using a multicomponent approach, first reported using fluorescence. The multicomponent fluorescent sensor includes a MB probe, labeled with a fluorophore and quencher, along with two adaptor strands. The adaptor strands have a complementary segment to the MB probe and another to the target. In the presence of the target, the adaptor strands hybridize to the MB probe and to the target to form a four-way junction (4J) structure which generates a fluorescent signal as the quencher and fluorophore are separated (Figure 3A). The electrochemical multicomponent approach uses a universal DNA SL (USL) probe that is attached to an electrode along with two adaptor strands (Figure 3B). One adaptor strand is conjugated with a methylene blue (MeB) redox label. In the presence of the target, the 4J structure is formed and an electrochemical is observed upon the redox of MeB.

Figure 3: Schematic illustration of the (A) MB fluorescence sensor and (B) universal four-way junction sensor used for nucleic acid detection.
1.4 Nucleic acid immobilization
The choice of an immobilization substrate is crucial as it can affect hybridization efficiency and selectivity which plays a major role in the performance of the sensor. The ability to control the surface chemistry is important to ensure high hybridization efficiency which is dependent on orientation, accessibility and stability of the DNA probe. The following sections will explore differences in immobilization design which includes: i) noncovalent binding, ii) affinity binding, (iii) covalent binding, and (iv) chemisorption.

1.4.1 Noncovalent binding
Noncovalent binding is a weak interaction between molecules that involves various electromagnetic interactions such as Van der Waals forces or electrostatic interactions among others. Noncovalent immobilization is generally achieved by immersing an electrode into a solution of nucleic acids in order to allow the nucleic acids to bind to the substrate. The binding strength is dependent on the nucleic acid structure, surface charge of the electrode and electrostatic interactions of the nucleic acid bases/phosphate backbone.\(^{22}\) In addition, cationic or conducting polymeric films have been investigated as an entrapment technique for immobilization.\(^{23-24}\) Although these approaches are simplistic and can be performed under mild conditions, the immobilized DNA directly adsorbed onto the electrode tends to have poor hybridization efficiency due to its limited accessibility by a target analyte.\(^{25}\)

1.4.2 Affinity binding
Affinity binding is based on the strength of the binding interaction between a biomolecule to its binding partner or ligand and is reported using an equilibrium dissociation constant (\(K_d\)). A smaller \(K_d\) value reflects a greater binding affinity which can be influenced by non-covalent interactions such as hydrophobic or Van der Waals forces between the two molecules. Affinity
binding for nucleic acid immobilization typically involves the very strong avidin-biotin interaction ($K_d = 10^{-15}$ M). Generally, the nucleic acid detection probe is biotinylated while the avidin is attached to a carbon substrate via adsorption, cross linkage or via another biotin molecule.\textsuperscript{26-27} The strength of this interaction can benefit biosensing schemes as the bond formation is rapid and unaffected by pH or temperature. However, the avidin layer can act as a barrier for redox labels so more complicated signaling techniques are needed.\textsuperscript{28}

1.4.3 Covalent binding
Covalent binding involves the linkage of one end of a nucleic acid without compromising or damaging the original DNA conformation and specificity (i.e. hybridization efficiency). This method of immobilization can be controlled and the nonspecific adsorption of the nucleic acids to the substrate can be reduced. Commonly, a carbodiimide method is utilized where an amino-linked oligonucleotide is fixed onto an activated substrate (contains oxidized groups, i.e. carboxylate).\textsuperscript{29} Other covalent binding immobilization strategies include click chemistry or covalently linked nucleic acids to synthetic polymer films.\textsuperscript{30}

1.4.4 Chemisorption
Chemisorption is a type of adsorption that involves a chemical reaction between a substrate and the adsorbate. Chemisorption approaches for immobilization commonly involve self-assembled monolayers (SAMS), which are spontaneously formed molecular assemblies that occur on surfaces via adsorption. Most commonly, SAMs are created using a thiol conjugated oligonucleotide on gold, although other substrates have been explored (silver, palladium, mercury etc.).\textsuperscript{31-32} Typically, a short chain alkane thiol is incorporated to form a mixed monolayer to minimize nonspecific adsorption by blocking the surface of the substrate from extraneous molecules.\textsuperscript{33} The effect on the alkane length is discussed below. This approach is
elegant as the self-assembly of a thiol labeled DNA probe allows control of orientation and surface coverage of the DNA probe, allowing a higher hybridization efficiency and preserved conformational mobility.

1.4.4.1 Alkane thiol length
The thickness of the SAM which is controlled, largely, by the length of the alkane thiols, can affect sensor stability (storage) and electron transfer efficiency; increased length will cause a decrease in electron transfer efficiency because the redox label is further from the surface of the substrate.\(^{34-39}\) Biosensors have been reported using a mixed monolayer (DNA-conjugated thiols backfilled with thiolated alcohols) that contain 2 to 11 carbons.\(^{16,34,40-41}\) Longer chain alkanes create a stable, well packed monolayer due to enhanced Van der Waals interactions while electron transfer efficiency decreases with alkane chain length bond.\(^{35-36}\) The increased stability of longer chain SAMs has been proposed to be due to the tight packing which reduces the oxidation of the gold-sulfur bond.\(^{42-44}\) Lai et al.\(^{34}\) investigated storage stability of C6- and C-11 based sensors and found that the C11- SAMs have longer shelf lives in solution and under ambient conditions because the improved packing of longer chain alkanes, although the electron transfer rate is reduced as compared to the C6- based sensor. There is a trade-off between sensor stability and electron transfer efficiency as alkane length is increased which can affect sensor performance and signaling. Therefore, depending on the application and architecture of each sensor, this parameter can be chosen accordingly. The sensor developed in this dissertation uses a C6 SAM because it has an adequate stability over time and allows an efficient electron transfer.
1.5 Electrochemical detection

Electrochemical detection requires an oxidation-reduction (redox) reaction, or electron transfer process, between the sensor substrate (electrode) and electrolyte. The flow of electrons results in a current that can be measured using instrumentation described below.

1.5.1 Instrumentation

A potentiostat is electronic hardware used to control an electrochemical cell. The electrochemical cell consists of a three electrode system immersed in a supporting electrolyte (Figure 4) which includes a working electrode (WE), counter electrode (CE), and reference electrode (RE). The potentiostat adjusts the current at the CE to maintain the potential of the WE with respect to the RE, using an electrical circuit by sensing changes in resistance of the cell. Here, the WE is a gold electrode, modified for immobilization and is where the redox reaction of the MeB label incorporated into the 4J sensor is monitored. The CE is an inert conductor, platinum, that completes the cell circuit and facilitates electron transfer. The potential of the CE is not measured but is adjusted to balance the reaction occurring at the WE. The RE has an established electrode potential and is used to maintain a constant potential against the WE (commonly Ag/AgCl). Nitrogen is bubbled into the electrochemical cell to remove oxygen (which interferes in the potential range for redox of MeB) and provide a nitrogen saturated environment while the supporting electrolyte provides sufficient conductivity of the solution.
1.5.2 Voltammetry

Voltammetry involves measuring a current at an electrode as a function of an applied potential. The voltammetry techniques used throughout the course of this work are further discussed below to clarify differences in data acquisition.

1.5.2.1 Cyclic voltammetry

Cyclic voltammetry (CV) is usually the first tool used to identify redox mechanisms, determine formal potentials and electron transfer kinetics among other characterization aspects of a particular system. The electrode potential (E) is ramped linearly versus time in forward and reverse sweeps (Figure 5A). The time it takes for a forward and reverse scan to complete is called the scan rate (V/s). The E is measured between the WE and RE while the current is measured between the WE and CE. This data is plotted as the E versus current (I) to depict a cyclic voltammogram (Figure 5B). In this example voltammogram, the potential is scanned from 0.0 to -0.5 V and the cathodic current increases as the reduction potential of the analyte is
reached. As the concentration of analyte is depleted, the cathodic current decreases. The scan is then reversed (-0.5 V to 0.0 V) to oxidize the reduced analyte, generating a current of reverse polarity.

![Cyclic voltammetry (CV) waveform](image)

**Figure 5:** (A) Cyclic voltammetry (CV) waveform (B) CV response for the redox of methylene blue.

### 1.5.2.2 Differential pulse voltammetry and square wave voltammetry

Differential pulse voltammetry (DPV) and square wave voltammetry (SWV) are techniques which rely on changing the applied potential using pulses (as opposed to sweeping the potential as used in CV) to monitor oxidation or reduction of an analyte. The waveforms for DPV and SWV are shown in Figures 6 and 7. The pulse period (time to complete a pulse) for DPV can range from 0.5 to 5 s whereas the sample period is around 50 ms. The current is sampled twice per cycle (as seen in Figure 6) and the two current values are subtracted to calculate a current per pulse. In contrast, the waveform for SWV is a square wave (symmetrical) imposed on a staircase. The current is sampled twice per cycle, at the end of each pulse (Figure 7), and in this case the pulse period is equivalent to the sample period. The forward current ($i_f$) is sampled during the
first pulse while the reverse current ($i_r$) is sampled from the second pulse and these values are subtracted to generate a current per pulse.

Figure 6: Differential pulse voltammetry waveform

Figure 7: Square wave voltammetry waveform

These techniques allow lower limits of detection and increased sensitivity (as compared to CV) which is necessary for biosensing applications.

1.5.3 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is based on the measure of the resistance to the flow of an alternating current at an interface (electrode/electrolyte). Generally, a low amplitude sinusoidal potential is applied to an electrode over a range of frequencies and the current is
measured. An impedance spectrum, referred to as a Nyquist plot, is generated using the current-voltage ratio at each frequency. The Nyquist plot (imaginary impedance, \(-Z''\), versus real impedance, \(Z'\)) will reveal the redox reaction occurring at the surface of the electrode. An example Nyquist plot is shown in Figure 8.

Figure 8: Nyquist plot

A Nyquist plot may include a large semicircle, which represents the resistance while a straight line indicates diffusion. Diffusion is represented by an element, \(Z_w\), referred to as Warburg impedance. The high frequency region of the semicircle may have an offset on the \(Z'\) axis which is equivalent to the resistance of the electrolyte solution (\(R_s\)) and is dominated by the double layer capacitance (C). The charge transfer resistance (\(R_{CT}\)) can be determined by measuring the diameter of the semicircle on the \(Z'\) axis as the intercept in the low frequency range is equivalent to the sum of \(R_s\) and \(R_{CT}\). Extrapolation of the intercept on the \(Z'\) axis can be difficult due to noise observed in the low frequency region, therefore, in most instances a circuit model is used
to fit the data which may include $R_{CT}$, $R_s$, $Z_w$, or $C$ in various arrangements which is dependent on the reaction at the electrode/electrolyte interface.

EIS measurements are much more time consuming than voltammetry measurements because a large frequency range is used (MHz to mHz) and can take several minutes or hours as compared to seconds. Furthermore, due to the complexity of the technique, most applications of EIS for biosensing only focus on determining the $R_{CT}$ rather than investigating other capabilities of the technique (e.g. capacitive properties derived from the phase angle) because it is all that is required for quantitative measurements. Therefore, a more in-depth analysis of EIS capabilities is not explored here.

In this work EIS is used as a technique for the detection of nucleic acids. Hybridization of a complementary target sequence with the sensor’s components (e.g. DNA SL probe, adaptor strands) can cause changes in interfacial charge, capacitance, resistance and thickness of the electrode’s surface, thus affecting the electron transfer process.

Many electrochemical techniques have been utilized for biosensing applications but the scope of this dissertation will focus on voltammetric techniques and electrochemical impedance spectroscopy (EIS). In order to use these techniques, a redox label (or couple) needs to be incorporated into the sensing scheme which is able to participate in an electron transfer process; the options for redox labels are presented below.

1.5.4 Redox label strategy
The scope of this dissertation will focus on the use of noncovalent or covalent redox labels. Non-covalent redox labels have been reported to indicate events such as hybridization or damage to the DNA at the surface of an electrode.\textsuperscript{22,45-46} Non-covalent labels can interact with DNA
electrostatically, as intercalators or through groove binding among others.\textsuperscript{45-48} This dissertation focuses on noncovalent redox markers that interact with the DNA electrostatically. Commonly, electrostatic redox indicator complexes such as $[\text{Fe(CN)}_6]^{3-/4-}$ (anionic, repelled from negatively charged DNA phosphate backbone) or $\text{Ru(NH}_3)_6^{3+/2+}$ (cationic, attracted to negatively charged DNA phosphate backbone) have been incorporated into sensors and monitored with techniques such as voltammetry or EIS.\textsuperscript{46,49-50} The use of non-covalent labels decreases the cost of the sensor by eliminating expensive chemical modification of detection probes or targets.

Covalently bound redox labels have been incorporated into sensors by attachment to a detection probe or target where the change in redox potential is measured upon hybridization.\textsuperscript{22,45-46} Many research groups have tethered electroactive labels, such as ferrocene or MeB, to an oligonucleotide detection probe or signaling strand.\textsuperscript{16,51} Other complex electroactive labels such as chelates of ruthenium or osmium have been explored but will not be the focus of this project.\textsuperscript{45,52-53} Covalent labels can improve the specificity of the sensor and allows the sensor to be reusable, as the redox marker is bound to the detection probe and not rinsed away upon reuse.\textsuperscript{54}

1.5.4.1 Signal OFF vs. signal ON
Signal OFF detection involves a decrease in current upon target binding (Figure 9A), while signal ON detection results in an increase in current upon target binding (Figure 9B). The design of the redox label attachment and the change in sensor architecture upon interaction with the target will dictate either a signal OFF or signal ON detection. Signal OFF sensing platforms can easily be affected by false positive responses (decrease in current due to matrix rather than hybridization) caused by interactions other than the target binding, so careful design of a signal
ON sensor is important. Figure 10B displays an example of a signal ON electrochemical DNA SL probe. The DNA SL probe, which is attached to a gold substrate via self-assembled thiol chemistry, incorporates a MeB tethered redox label. In the absence of the target, the far proximity of the MeB inhibits efficient electron transfer so only a small peak current is observed using SWV detection. Once the target is introduced, the DNA SL probe undergoes a conformational change as it selectively binds to the target. In this instance, the MeB is now in close proximity to the surface of the electrode, allowing an efficient electron transfer which generates a much higher peak current. Therefore, careful design of the sensing components will control the signaling mechanism (signal OFF or signal ON) for each biosensing platform.

![Figure 9: Schematic of a (A) signal OFF two-way junction sensor and (B) signal ON four-way junction sensor](image)

1.6 References
CHAPTER TWO: COMPARISON OF FOUR-WAY AND TWO-WAY JUNCTION DNA STEM-LOOP PROBE BASED NUCLEIC ACID SENSORS USING ELECTROCHEMICAL TECHNIQUES

2.1 Introduction
There is a high demand for nucleic acid POC sensors that are highly selective, easily operated, cost efficient and sensitive enough to be used for disease diagnostics.\textsuperscript{1-3} Electrochemical analysis offers a simple, portable and inexpensive platform for the development of a POC sensor. The electrochemical DNA sensor developed by Fan \textit{et al.}\textsuperscript{4} has gained significant attention due to its design. This sensor platform utilizes a surface bound DNA SL probe with a covalently bound redox marker that exhibits efficient electron transfer because of its close proximity to the electrode’s surface. As the DNA SL hybridizes with the target, a two-way junction (2J) is formed and the conformational change of the DNA SL probe results in a reduction of current, as the redox marker is positioned farther from the surface. This design causes the 2J sensor to operate as a signal OFF sensing platform with a nonzero baseline. Signal OFF sensing platforms are not preferred as the signal suppression is limited up to 100\% and are susceptible to false positives.\textsuperscript{4-7} To overcome these limitations, the 2J sensor has been “switched” to a signal ON sensing platform by tuning the instrumental parameters of electrochemical techniques.\textsuperscript{5,8} However, despite these improvements, the poor selectivity of the 2J sensor still hinders its implementation into a POC sensor. In order to improve the selectivity, an alternative version of this sensor was developed in an electrochemical format\textsuperscript{9-11} using a multicomponent approach, which was adapted from an optical design using a fluorophore and quencher conjugated to a molecular beacon.\textsuperscript{12-15}
The electrochemical multicomponent sensor includes a USL probe which is immobilized on a gold substrate. Two adaptor strands (m and f) are introduced during hybridization which possess a complementary segment to the USL probe and another complementary segment to the target analyte. In the presence of the target, the adaptor strands hybridize to the USL probe and the target, forming a 4J structure. In the absence of target, the formation of the 4J is not thermodynamically favored and the DNA SL probe remains in its hairpin form. The short target binding arm of the m adaptor strand maintains high selectivity while the f adaptor strand provides high affinity to the target.

We aim to study the signal capability of the 4J sensor in comparison to the 2J sensor using a DNA SL probe with the same sequence in both formats. In the 2J format the DNA SL probe contains a covalently bound MeB redox marker for a signal OFF sensing platform. As seen in Scheme 10A the proximity of the MeB before hybridization results in a large redox current whereas upon the hybridization induced conformational change in the presence of the target, the current decreases due to low electron transfer. The 4J sensor is designed as a signal ON sensing platform with a MeB conjugated m adaptor strand (Scheme 10B). This novel sensor exhibits a zero background signal in the absence of the target. In the presence of the target, the DNA SL probe hybridizes to the adaptor strands which in turn hybridize to the target, positioning the MeB in close proximity to the electrode’s surface generating a large current. The performance of the sensors was monitored using CV, differential pulse voltammetry (DPV) and SWV. The ultimate goal is to generate a specific set of conditions that best suit the architecture of the 4J sensor in comparison with the 2J sensor. Although SWV and CV have been previously used for the
interrogation of the 4J electrochemical sensor, an in-depth study of its performance and comparison among three different voltammetry techniques has not been explored.

![Design of the (A) signal OFF 2J and (B) signal ON 4J sensors.](image)

Figure 10: Design of the (A) signal OFF 2J and (B) signal ON 4J sensors.

### 2.2 Material and methods

#### 2.2.1 Reagents and materials

The DNA SL probe for the 4J sensor (5’-S-S-(CH\textsubscript{2})\textsubscript{6}-T-CGC-GTT-AAC-ATA-CAA-TAG-ATC-GCG-3’), f adaptor strand (5’-GAT-CTA-TTG-TGT-CAC-ACT-CCA-3’) and targets (T\textsubscript{1}: 5’-UGG-AGU-GUG-ACA-AUG-GUG-UUU-G-3’ and T\textsubscript{2}: 5’-CGA-TCT-ATT-GTA-TGT-TAA-CG-3’) were obtained from Integrated DNA Technologies (Coralville, USA) and used as received. The m adaptor strand conjugated with a MeB redox marker (5’-CAA-ACA-CCA-T-
TAT-GTT-AAC-TTT-MeB-3’ and DNA SL-MeB probe for the 2J sensor (5’-S-S-(CH2)6-T-CGC-GTT-AAC-ATA-CAA-TAG-ATC-GCG-MeB-3’) were obtained from Biosearch Technologies, Inc. (Petaluma, USA) and used as received. All aqueous solutions were prepared with deionized water (18 MΩ cm resistivity) using a Millipore Milli-Q system (Lowell, USA).

An immobilization buffer (IB) was prepared with 50 mM sodium phosphate dibasic/ sodium phosphate monobasic dihydrate (Na2HPO4/NaH2PO4•2H2O), 250 mM sodium chloride (NaCl) and adjusted to pH 7.4 using 1 M sodium hydroxide (NaOH). The hybridization buffer (HB) contained 50 mM Trizma hydrochloride (Tris-HCl), 25 mM NaCl, 50 mM magnesium chloride (MgCl2) and was adjusted to pH 7.4 using 1 M NaOH. Gold disc electrodes (GDEs) were cleaned in a piranha solution (1:3 ratio of H2O2:H2SO4, CAUTION: extremely exothermic reaction) and then polished with alumina slurry (1.0 μm, 0.3 μm and 0.05 μm) on a microcloth. The GDEs were then sonicated in water and ethanol to remove any residual alumina particles trapped at the surface of the electrode. Finally, the areas of the GDEs were calculated upon analysis in 0.5 M sulfuric acid (H2SO4) via CV from 1.6 to -0.1 V at a scan rate of 100 mV/s.16

2.2.2 DNA SL probe immobilization and hybridization
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM) was used for the reduction of the disulfide bonds of the DNA SL probes by mixing the solution at room temperature for 1 hour. The respective DNA SL probe solution was diluted to 0.1 μM with IB and 15 μL of the solution was incubated on the electrode for 30 minutes at room temperature. Both DNA SL probes were immobilized at a concentration of 0.1 μM to ensure optimal 4J hybridization and to serve as an experimental constant to compare both sensors.11 Following immobilization, the GDEs were rinsed with IB to remove any unbound probes and then dried with nitrogen. Next, 15 μL of MCH
(2 mM prepared in IB) was incubated for 30 minutes to reduce nonspecific adsorption. For hybridization, a solution (15 µL) containing HB along with 50 nM of T₁, 0.25 µM of the m adaptor strand and 0.50 µM of the f adaptor strand for the 4J or 50 nM T₂ for the 2J were incubated on the electrode for 1.5 hours at room temperature.

2.2.3 Electrochemical analysis
Electrochemical measurements were performed with a CHI660D Electrochemical Workstation (CH Instruments, Austin, USA) at room temperature. A three electrode setup was used where the modified GDE was used as the working electrode, a platinum wire as the counter electrode, and Ag/AgCl (3 M KCl) was used as a reference electrode (CH Instruments, Austin, USA). Three electrochemical techniques were used: CV, DPV and SWV. CV measurements were recorded with scan rates ranging from 0.1 to 600 V/s. DPV measurements were taken at pulse widths ranging from 1 to 250 ms and an amplitude of 0.05V following Lai et al.⁸ SWV measurements were recorded at frequencies ranging from 1 to 5000 Hz and an amplitude of 0.07 V. The electrochemical cell was saturated with nitrogen prior to analysis and at least three electrodes were used to provide statistically significant results.

The DNA SL probe surface coverage, \( \Gamma_{\text{DNA SL probe}} \), was calculated using the DNA SL probe conjugated with a MeB redox marker by integrating the charge under the MeB reduction peak using CV (Equation 1)¹⁷

\[
\Gamma_{\text{DNA SL probe}} = \frac{Q}{nFA}
\]  

where \( Q \) is the integrated charge of the CV reduction peaks, \( n \) is the number of electrons transferred (\( n = 2 \) for MeB), \( F \) is the faraday constant, and \( A \) is the area of the electrode.
\( \Gamma_{DNA \, SL \, probe} \) is reported as the average value obtained at various scan rates (\( \nu = 10, 50, \) and 100 mV/s).

The signal suppression (% SS) was calculated using equation (2) for the 2J sensor:

\[
\% SS = \left( \frac{I - I_0}{I_0} \right) \times 100
\]  

(2)

where \( I \) is the current obtained upon hybridization and \( I_0 \) is the current obtained in the absence of the target.

In addition, the Laviron equation (3) was used to calculate the electron transfer rate constant \( (k_s) \) of MeB before and after hybridization

\[
\log k_s = \alpha \log(1 - \alpha) + (1 - \alpha) \log\alpha - \log\left( \frac{RT}{nFR} \right) - \alpha(1 - \alpha) \frac{nF\Delta E_p}{2.3RT}
\]  

(3)

where \( k_s \) (s\(^{-1}\)) is the electron transfer rate constant, \( \alpha \) is the electron transfer coefficient, \( \nu \) (V/s) is the scan rate using CV and \( \Delta E_p \) (V) is the difference between cathodic and anodic peak potentials.\(^{18} \) A plot of \( E_p \) vs \( \log \nu \) generates a linear response when \( \Delta E_p > 200/n \) mV for the 2 electron \( (n) \) transfer for MeB; the slope can be used to determine \( \alpha \) and the intercept can be used to calculate \( k_s \).\(^{18} \)

### 2.3 Results and discussion

#### 2.3.1 Sensors performance for cyclic voltammetry

CV has been recently used to evaluate the performance of 2J DNA SL probe based sensors\(^{7,8} \) as well as to monitor the electrocatalysis of a 4J electrochemical sensor.\(^{10} \) However, a direct comparison between the electrochemical 2J and 4J sensors has not been investigated. Here, CV was used to evaluate the performance of the 2J and 4J sensors at scan rates ranging from 0.1 to 600 V/s, since the signal can be affected by the scan rate which is dictated the electron transfer
rate of the redox marker. In addition, the DNA SL probe density was calculated by integrating the charge under the MeB reduction peak of the CV using equation 1. Since the same DNA SL probe at the same concentration was used for the 2J and 4J sensors, the DNA SL probe coverage is equivalent among these sensors and was calculated to be 1.21(±0.2) x 10^{12} molecules/cm^{2}.

At this probe coverage, the 2J sensor was interrogated using CV at a scan rate of 1.0 V/s, resulting in a slight signal suppression (8%) after hybridization (b) with 50 nM T_{2} (Figure 11A) which was calculated using equation 2. The 4J sensor exhibited a 41% SG at 1.0 V/s upon hybridization with 50 nM T_{1} (Figure 12A: b). At 500 V/s the separation between anodic and cathodic peak potentials increased significantly for the 2J sensor as the MeB reduction potential was shifted to -0.422 V before hybridization and -0.395 V (vs. Ag/AgCl) after hybridization (Figure 11B), likely allowing the faradaic current to dictate the electron transfer rates.\textsuperscript{18,19} This peak to peak separation is also evident in the 4J sensor as the reduction potential of MeB shifted from -0.255V to -0.513 V (vs. Ag/AgCl) upon hybridization when the scan rate was increased from 1 V/s to 500 V/s (Figure 12A-B). The cathodic peak current increased linearly for the 2J sensor with increased scan rate until around 500 V/s before and after hybridization (Figure 11C). The cathodic peak current of MeB from the 4J sensor increased upon hybridization until reaching saturation after 500 V/s, with a linear range from 0.1 to 200 V/s (Figure 12C). For the 4J there is a background signal of zero before hybridization since no redox marker is present (Figure 12C: a). The increase in peak current upon increasing scan rate is expected because the scan rate can control the behavior of the sensor by altering the electron transfer rate.\textsuperscript{19} The corresponding %SS of the 2J sensor upon hybridization of 50 nM T_{2} increased with respect to the scan rate and plateaued around 59% SS at 100 V/s (Figure 11 D). The signal gain of the 4J sensor increased
upon hybridization with 50 nM T₁ as the scan rate was increased, achieving the highest signal gain (1100%) at 600 V/s (Figure 12D).

At slower scan rates the electron transfer rate of the 2J sensor is due to the MeB reduction from a portion of hybridized and unhybridized probes. The 2J sensor achieved maximum %SS at higher scan rates (>20 V/s) likely due to the remaining signal from the unhybridized DNA SL probes, as the electron transfer from hybridized probes were not sustained at high scan rates (due to increased distance of MeB redox marker). This is evidenced by the estimated electron transfer rate constants for the reduction of MeB before (kₛ) and after (kₛ’) hybridization. The kₛ for the 2J sensor was 203 s⁻¹ while kₛ’ was 82 s⁻¹. Upon hybridization, the 2J sensor exhibits a decrease in electron transfer kinetics because the location of the MeB redox marker on hybridized probes is significantly farther from the surface of the electrode. Therefore, at higher scan rates the electron transfer kinetics are dictated by the portion of unhybridized probes which have a MeB redox marker close to the electrode. The procedure for determining electron transfer rate coefficients is discussed in section 2.4 (Figures 16-17).

For the 4J sensor kₛ’ was 199 s⁻¹ which indicates the placement of the MeB redox marker on the m adaptor strand is positioned in close proximity to the surface of the electrode upon hybridization and formation of the 4J structure. The hybridized 4J sensor reached a maximum signal around 600 V/s as the proximity of the MeB redox marker on the m adaptor strand supported efficient electron transfer at significantly high scan rates. It is important to note also that due to the placement of the redox marker on the 4J sensor, no unhybridized probes contributed to this signal as observed for the 2J sensor.
Figure 11: Cyclic voltammetry data from the 2J sensor recorded before (a) and after (b) hybridization with 50 nM Target 2 at scan rates of (A) 1.0 V/s and (B) 500 V/s. The scan rate dependence for (C) the peak current upon reduction methylene blue and (D) the resultant signal suppression upon hybridization.
Figure 12: Cyclic voltammetry data from the four-way junction sensor recorded before (a) and after (b) hybridization with 50 nM Target 1 at scan rates of (A) 1.0 V/s and (B) 500 V/s. The scan rate dependence for (C) the peak current upon reduction methylene blue and (D) the resultant signal gain upon hybridization.

Both sensors include the same SL probe; the 2J sensor has the MeB redox marker attached to the SL probe whereas the 4J sensor has the MeB redox marker attached the intermediate m adaptor strand. The redox marker placement has resulted in differences in signaling mechanisms between the two designs. For both sensors, the hybridization is a competition between the closed hairpin conformation of the SL probe and the target-induced hybridized structure (2J or 4J). Even when the sensor has achieved a saturated signal, a small percentage of the probes remain unhybridized.
while the majority have been hybridized to form a 2J or 4J structure. The %SS and %SG of both sensors are dependent on this concept of conformational change of the SL probe in the presence of the target. The signal is suppressed upon hybridization of the 2J sensor because of the placement of the redox marker, which is moved farther from the surface. At slower scan rates the electron transfer kinetics are dictated by hybridized and unhybridized probes whereas at high scan rates the unhybridized probes are responsible for the signal because of the fast electron transfer kinetics. For the 4J sensor the placement of the MeB redox marker is near the surface upon hybridization, resulting in a signal gain, so the hybridized structures are solely responsible for the signaling mechanism. Here, CV was a crucial technique used to estimate the rate transfer constants for each sensor and also provided probe coverage information which was shown to be dependent on the sensor design and scan rate.

2.3.2 Sensors performance for differential pulse voltammetry and square wave voltammetry

DPV has been used for analysis of many DNA SL probe based 2J sensors but has not previously been used to analyze the 4J sensor or elucidate differences in behavior among the two sensors. Here, the performance of the 2J and 4J sensors were monitored using DPV at pulse widths ranging from 1 to 100 ms. The highest peak current for the 2J sensor before and after hybridization (Figure 13A: a-b) was observed at short pulse widths (1 ms) followed by a rapid decrease in current, approaching negligible values at longer pulse widths (>250 ms) (Figure 13A). The 4J sensor exhibited similar behavior upon hybridization (Figure 13C: b). This is expected as the pulse width dependence of the capacitive and faradaic current is highly dependent on the electron transfer rates due to how the current is sampled. Recall, the background current of the 4J sensor is zero due to the absence of the redox marker (Figure 13C: b).
a). The 2J sensor achieved 80% SS at a pulse width of 1 ms (Figure 13B) while the 4J sensor obtained 89% SG (Figure 13D). The corresponding DP voltammograms for pulse widths of 1 ms for both sensors demonstrate the % SS or %SG of the 2J sensor upon hybridization with 50 nM T₂ and for the 4J sensor upon hybridization with 50 nM T₁ respectively (Figure 13 insets).

Figure 13: Differential pulse voltammetry peak current of methylene blue recorded before (a) and after (b) hybridization with 50 nM Target 2 for the two-way junction (2J) sensor (A-B) and Target 1 for the four-way junction (4J) sensor (C-D). The pulse width dependence of peak current upon reduction of methylene blue (A, C) and signal change (B) and gain (D) upon hybridization of the sensors are shown. The insets display the response of the (A) 2J and (C) 4J sensors at a pulse width of 1 ms.

SWV has been previously used to interrogate 2J and 4J electrochemical sensors, although a direct comparison between the two has not been explored. The relationship between current and
frequency in SWV is dependent on the electron transfer rate. SWV has been previously used to identify a “critical frequency” \((f)\) that represents the maxima in the peak current described by a \((i_p)/f\) vs \(f\) relationship.\(^{21}\) The critical frequency is dependent on the architecture of the sensor and its target induced conformational change. Consequently, the current dependence of each sensor with respect to the SW frequency (1-5000 Hz) was investigated. The 2J sensor exhibited an increase in current for the entire range of frequencies in its unhybridized state (Figure 14A: a), whereas the current in the hybridized state increased until around 500 Hz and remained relatively constant until 5000 Hz (Figure 14A: b). The current generated from the unhybridized probes (even at signal saturation) continued to increase as the electron transfer rate is fast and can keep up with the increased frequency. However, upon hybridization, a portion of the unhybridized probes \((k_s = 203 \text{ s}^{-1})\) support fast electron transfer while the electron transfer rate of the hybridized probes \((k_s' = 82 \text{ s}^{-1})\) is not sufficient to keep up with an increased frequency.

Figure 14C displays the signal of the 4J sensor before hybridization is zero as the redox marker is not present, allowing a unique signal ON sensing platform (Figure 14C: a). The 4J sensor was able to operate adequately at frequencies ranging from 1 to 5000 Hz upon hybridization (b), although an increase in signal was observed from 1 to 1500 Hz, where the signal stabilized and achieved the most efficient electron transfer (Figure 14C). The critical frequency was observed at 4000 Hz where the current then began to decrease (Figure 14C) as the electron transfer \((k_s' = 199 \text{ s}^{-1})\) was not sufficiently high enough to operate at upper frequencies. The signal suppression of the 2J sensor reached maximum around 86% at 500 Hz (Figure 14B) and plateaued at higher frequencies. In contrast, the signal gain of the 4J sensor achieved at 1500 Hz was 268% (Figure 14D). The corresponding SW voltammograms for frequencies of 1500 Hz for the 4J and 2J
sensors demonstrate the signal suppression and gain upon hybridization with 50 nM T$_1$ and 50 nM T$_2$ respectively (Figure 14 insets).

Figure 14: Square wave voltammetry (SWV) response upon reduction of methylene blue (MeB) recorded before (a) and after (b) hybridization with 50 nM Target 2 for the two-way junction (2J) sensor (A-B) and Target 1 for the four-way junction (4J) sensor (C-D). The frequency dependence of the peak current upon reduction of MeB for the 2J (A) and 4J (C) sensors. The signal suppression of the 2J sensor (B) and the signal gain of the 4J sensor (D) upon hybridization of the sensors are shown. The insets display the SWV response of the 2J sensor at frequencies of (A) 1 Hz and (B) 1500 Hz. The SWV response of the 4J sensor at frequencies of (A) 1 Hz and (B) 1500 Hz.

The adjustment of DPV and SWV parameters has been previously used to convert signal OFF sensors into a signal ON sensing platform since the signal OFF sensors have limited signal suppression and are susceptible to false positive responses. This phenomenon was
investigated in order to directly compare the signal gain of both the 2J and 4J sensors in a signal ON format. The pulse width dependence of DPV on the 2J sensor performance showed a “switch” in sensing format from OFF to ON at 50 ms and remained ON at higher pulse widths (250 ms), allowing the sensor to achieve a signal gain upon hybridization (Figure 15A: a-b).

Finally, the SW frequency dependence on the “switching” ability of the 2J sensor was evaluated and compared to the signal gain of the 4J sensor in the signal ON format. The 2J sensor “switched” from signal OFF to ON around 7 Hz and was only operational in the signal ON format until around 12 Hz (Figure 15C). In this range of frequencies, the current generated from the hybridized probes is dominant while the current from the unhybridized probes is hindered because of its fast electron transfer, allowing the 2J sensor to operate as a signal ON sensor. The 2J sensor reached its maximum signal gain (~8%) upon hybridization (a-b) at 10 Hz (Figure 15D). The 4J achieved a significantly higher signal gain (~250%) with SWV and allowed a larger range of operating frequencies in the signal ON format. Furthermore, the 4J sensor exhibits a zero baseline in the absence of the target whereas the 2J sensor displays a nonzero baseline that will limit its possible signal gain in the signal ON format. Although the 2J sensor can be “switched” from a signal OFF to a signal ON sensing platform, it is apparent that tuning the pulse width using DPV offers a greater advantage than with a tuned SWV frequency. DPV has been shown to be a well suited electrochemical technique for either sensor and allows a conversion between signal OFF and ON platforms for the 2J sensor. In contrast, tuning the SW frequency has been shown to be more advantageous for analysis with the unique architecture of the 4J sensor versus that of the 2J sensor as it operates in a signal ON format over a wider range of frequencies while offering an impressive signal gain.
Figure 15: The conversion of the two-way junction (2J) sensor from a signal OFF to ON platform was dependent on the pulse width (A) and frequency (C) when analyzed with differential pulse voltammetry and square wave voltammetry respectively before (a) and after (b) hybridization with 50 nM Target 2. The signal (%) changes between the signal OFF and ON states dependence on pulse width (B) and frequency (B) are displayed.

2.4 Effect of scan rate on sensor performance
It is important to understand differences in the signaling between the 2J and 4J based sensors in order to realize variance in design architecture and elucidate future improvements or modifications. Therefore, the electron transfer kinetics of MeB for the 2J and 4J sensing platforms was investigated using the cyclic voltammetry data presented in Section 2.3.1. The logarithm dependence of scan rate (v) versus $E_p$ was studied for the 2J sensor before (Figure
16A) and after (Figure 16B) hybridization. As the scan rate increases, $E_{pa}$ increases while $E_{pc}$ decreases (Figure 16). When $v$ is greater than 20 V/s, there is a linear relationship between $E_p$ ($E_{pa}$ and $E_{pc}$) and log $v$. For the 4J sensor there is no redox marker in place until after hybridization, due to its placement on the m adaptor strand. Thus, Figure 17 depicts the dependence of $E_p$ to log $v$ after hybridization. When $v > 40$ V/s, a linear relationship between log $v$ and $E_p$ is observed. The slopes of the linear regions for oxidation and reduction peaks from Figures 16-17 are represented by $2.303RT/(1-\infty)nF$ and $-2.303RT/\infty nF$ respectively, according to the Laviron theory.\textsuperscript{18}

![Graphs](image)

**Figure 16**: Plot of peak potential ($E_p$) vs. log scan rate ($v$) for the 2J sensor (A) before and (B) after hybridization with 50 nM T\textsubscript{1} acquired from the redox of methylene blue (MeB) using cyclic voltammetry. The slope and intercepts from each plot are used to calculate the electron transfer kinetics of MeB.
Figure 17: Plot of peak potential ($E_p$) vs. log scan rate ($v$) for the 4J sensor after hybridization with 50 nM T$_2$ acquired from the redox of methylene blue (MeB) using cyclic voltammetry. The slope and intercepts from each plot are used to calculate the electron transfer kinetics of MeB.

The charge transfer coefficient ($\alpha$) was calculated from Equation 4

$$\frac{k_a}{k_c} = \frac{\alpha}{1-\alpha}$$

where $k_a$ and $k_c$ are the slopes from Figures 17-18 for $E_{pa}$ and $E_{pc}$ respectively. A value of 0.423, 0.498 and 0.460 was estimated for $\alpha$ of the 2J sensor before hybridization, 2J sensor after hybridization and for the 4J sensor after hybridization, respectively. These values were used in the Laviron equation (equation 3) to calculate the electron transfer rate constants for each platform before ($k_s$) and after ($k_s'$) hybridization (Table 1).
Table 1: Electron transfer rate constants for the 2J and 4J sensors before ($k_s$) and after hybridization ($k_s'$)

<table>
<thead>
<tr>
<th>Sensor Platform</th>
<th>$k_s$ (s$^{-1}$)</th>
<th>$k_s'$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2J</td>
<td>203 ± 4</td>
<td>82 ± 1</td>
</tr>
<tr>
<td>4J</td>
<td>n/a</td>
<td>199 ± 3</td>
</tr>
</tbody>
</table>

For the 2J sensors $k_s$ value of 203 s$^{-1}$ and $k_s'$ value of 82 s$^{-1}$ was obtained (Table 1). The electron transfer kinetics are dependent on the conformation of the probe and the proximity of the MeB redox marker to the surface of the electrode. For the 2J sensor, before hybridization the SL probe remains in a hairpin configuration with the MeB redox marker positioned close to the electrode surface, resulting in a fast electron transfer (203 s$^{-1}$). A decrease in electron transfer kinetics (82 s$^{-1}$) is observed upon hybridization because as the SL probe opens to form a rigid duplex structure with the target, the distance of the MeB redox marker to the electrode surface is increased. The $k_s'$ value of the 4J sensor after hybridization was 199 s$^{-1}$. This value is similar to that of the 2J sensor before hybridization, indicating the design of the 4J positions the MeB redox marker on the m adaptor strand the same distance from the electrode as when the MeB redox marker is attached to the SL probe. This is an important observation as future studies can focus on the attachment of the redox marker on the m adaptor strand to investigate the effect on the electron transfer kinetics for the unique signal ON universal sensing platform.

2.5 Conclusions
Here, a comparison of a 4J and 2J DNA SL based sensor was conducted using multiple electrochemical techniques: CV, DPV and SWV for the first time. The characterization provides
important insight for parameters chosen for each electrochemical technique which are unique to the architecture of each sensor. In general, all techniques explored here provided valuable data to understand the benefit of tuning specific electrochemical parameters and allowed a vigorous comparison between the two sensing formats. The signal OFF 2J sensor was converted to a signal ON sensor by modifying the pulse width and frequency during DPV and SWV analysis respectively. However, the signal gain in the signal ON state was limited because of a nonzero baseline and was only achieved at a narrow operational window of “switching” potentials necessary for converting between signal OFF and signal ON formats. The 2J sensor achieved a higher signal gain in the signal ON state under DPV whereas the 4J sensor exhibited a superior signal gain for SWV over a wider range of operational parameters with a zero baseline. The characterization presented here provides insight into the signaling of the 4J sensor and a greater understanding of its performance compared to state of the art 2J sensors which will allow future performance modifications for implementation into real world biosensing.

2.6 References
CHAPTER THREE: A SINGLE ELECTROCHEMICAL PROBE USED
FOR ANALYSIS OF MULTIPLE NUCLEIC ACID SEQUENCES


3.1 Introduction
Nucleic acid detection using hybridization techniques has received significant attention due to its valuable applications in clinical diagnostics, national defense and forensics.1-3 Inspired by the undoubted success of the blood glucose meter, electrochemical methods have been explored as a valuable approach for nucleic acid detection due to the potential for *on-site* testing while offering a fast, simple and inexpensive analysis.4-12 Fan and colleagues initiated the field of electrochemical nucleic acid analysis by introducing a DNA SL probe conjugated with a redox marker.13 The DNA SL probe remains in a hairpin configuration until the stability is disrupted upon hybridization with a fully matched sequence, forming a thermodynamically favored duplex structure. Limits of detection down to aM concentrations were reported.14-15 Despite this success, to the best of our knowledge no commercial electrochemical sensor for nucleic acid analysis is available up to date. One significant challenge is the absence of a universal probe which could be used for the analysis of many nucleic acid sequences while maintaining high selectivity with minimum modifications of the assay conditions.16 Optimizing the performance of this universal platform could lead to low cost bulk manufacturing and use of the same electrode in a variety of applications. Universal platforms for recognition of nucleic acids have received ever growing
attention for fluorescent formats.\textsuperscript{17-21} Previously, we demonstrated how a DNA 4J-forming multicomponent probe can be used for analysis of multiple nucleic acid analytes.\textsuperscript{18} The approach is based on a MB probe, a fluorophore and a quencher labeled hairpin DNA strand (Figure 3A).\textsuperscript{22,23} Two adaptor strands hybridized to both a universal MB probe and the target DNA or RNA sequences to form a 4J structure.\textsuperscript{11,12} In this structure, the MB probe acquires an elongated conformation with the fluorophore separated from the quencher, resulting in high fluorescence. The MB probe does not hybridize directly to the nucleic acid analyte and therefore, can be used for analysis of potentially any sequence if the adaptor strands are tailored for each new analyte. Importantly, this approach enabled high selectivity of nucleic acid recognition, even at ambient temperatures.\textsuperscript{17,18} The high selectivity can be attributed to the short hybrid of one of the adaptor strands to the target which is extremely sensitive to a single mismatched base pairing, allowing the detection of SNS in folded target analytes, which is not possible by the conventional DNA SL probe, e.g. MB probe.\textsuperscript{18,24,25} The fluorescent platform was then adopted for electrochemical nucleic acid analysis.\textsuperscript{26,27} The first sensor used a redox reaction of electrochemically active markers which was inhibited (signal OFF) upon 4J complex formation on the surface of the electrode.\textsuperscript{26} The major drawback of this approach is the signal OFF sensing format, which can easily be affected by false positive responses caused by interactions other than the target binding.\textsuperscript{8,13,28,29} A second sensor used a signal ON format via electrocatalysis of glucose oxidase with covalently bound MeB redox markers.\textsuperscript{27} This sensor was shown to recognize target analytes with impressively low detection limits. Here we demonstrate how the multicomponent design enables detection of multiple analytes by utilizing the same electrode-bound probe. This study in
combination with low limits of detection (LOD) demonstrated previously,\textsuperscript{26,27} will eventually enable widespread use of electrochemical techniques in nucleic acid analysis.

For this study, we have chosen a signal ON format (Figure 3B). The sensor utilized a thiol bond between a USL probe and a gold substrate. Two adaptor strands, m and f, were introduced along with the target during hybridization to form a 4J structure. The m adaptor strand was conjugated with MeB for electrochemical detection using SWV. The following parameters were optimized using electrochemical techniques in conjunction with spectroscopic ellipsometry: optimal USL probe and adaptor strand concentrations, immobilization and hybridization time, selectivity and conditions for sensor regeneration. The focus of this work is to demonstrate for the first time the ability of the universal electrochemical four-way junction (UE4J) sensor to be re-used for cost effective analyses of multiple analytes using a USL probe.

### 3.2 Experimental

#### 3.2.1 Reagents and solutions

The USL probe, f adaptor strands (f1and f2), miRNA-122, target DNA (T-DNA) and mismatch sequences were purchased from Integrated DNA Technologies (Coralville, USA) and used as received (Table 2). The m adaptor strand modified with a MeB redox marker (seven carbon (C7) linker) was purchased from Biosearch Technologies, Inc. (Petaluma, USA) and used as received (Table 2). Gold SPE and GDE were purchased from DROPSENS (Spain) and CH Instruments (Austin, USA), respectively. All aqueous solutions were prepared with deionized water (18 MΩ cm resistivity) using a Siemens PURELAB Ultra system (Lowell, USA). A PBS solution was used as the IB and was prepared with 50 mM Na\textsubscript{2}HPO\textsubscript{4}, 50 mM NaH\textsubscript{2}PO\textsubscript{4}•2H\textsubscript{2}O, 250 mM NaCl and adjusted to a pH of 7.4 using 1 M NaOH. The HB was prepared with 50 mM Tris-HCl, 25 mM NaCl, 50 mM MgCl\textsubscript{2} and adjusted to pH 7.4 using 1 M NaOH.
Table 2: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name [a]</th>
<th>Sequences [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>USL probe</td>
<td>5’-S-S (CH\textsubscript{2})\textsubscript{6}TTTTTTTTTTGC\textsubscript{GTTAACATA}CAATAGATCGG-3’</td>
</tr>
<tr>
<td>f1</td>
<td>5’-GATCTATTGTGTCACACTCCA-3’</td>
</tr>
<tr>
<td>f2</td>
<td>5’-GATCTATTGATCCGTATCCAG-3’</td>
</tr>
<tr>
<td>m-MeB</td>
<td>5’-CAAACACCATTATGTTAACTTTTTTTTT TT-MeB-3’</td>
</tr>
<tr>
<td>T-DNA</td>
<td>5’-CTGGATACGGATATGGTGGTTGG-3’</td>
</tr>
<tr>
<td>miRNA-122</td>
<td>5’-UGGAGUGUGACAAUGGUGUUG-3’</td>
</tr>
<tr>
<td>Mismatch</td>
<td>5’-UGGAGUGUGACAAUGGUGUUUG-3’</td>
</tr>
</tbody>
</table>

[a] MeB, methylene blue, redox label. [b] SNS sites are underlined; self-complementary regions of USL probe are in italic; complementary fragments are in the same color.

3.2.2 Preparation of electrodes
Gold SPE and GDE were used as substrates for USL probe immobilization. GDEs were chemically cleaned by immersion in a piranha solution (1:3 ratio of H\textsubscript{2}O\textsubscript{2}:H\textsubscript{2}SO\textsubscript{4}) for ten minutes. Then, the electrodes were manually polished on a microcloth with alumina slurry (1.0 μm, 0.3 μm and 0.05 μm), rinsed with water and sonicated in ethanol to remove any residual alumina particles trapped at the surface of the electrode. The GDEs and SPEs were finally activated in 0.5 M H\textsubscript{2}SO\textsubscript{4} via CV from 1.6 to -0.1 V at a scan rate of 100 mV/s and their corresponding areas were calculated.\textsuperscript{31}

3.2.3 Immobilization and hybridization
The USL probe was immobilized on the electrode’s surface via a gold-thiol bond. The disulfide bonds of the USL probe were reduced with 1 mM TCEP by shaking the solution at room temperature for 1 hour. The solution was then diluted with IB to yield 0.1 μM of the USL probe and 15 μL of this solution was drop casted on the electrode and incubated at room temperature.
for 1.5 hours. The electrodes were rinsed with IB and dried with nitrogen. Then, 15 μL of 2 mM MCH in IB was drop casted on the electrode and incubated for 30 minutes to minimize nonspecific adsorption on the electrode surface. Hybridization was performed using the desired concentration of target miRNA-122 or T-DNA by preparing with an equimolar of m and f adaptor strands (1 μM) in HB. Next, 15 μL of this solution was drop casted to the electrode and incubated for 2 hours at room temperature.

**3.2.4 Characterization and optimization of USL probe, adaptor strand concentrations and experimental timing**

The concentrations of USL probe were varied from 0.05 μM to 1 μM and incubated with 1 μM m-MeB, 1 μM f1, and 50 nM miRNA-122. Next, the concentration of the adaptor strands (m-MeB and f1) was varied in equimolar concentrations (0.1 to 1.0 μM) and incubated with 50 nM miRNA-122 upon immobilization of 0.1 μM USL probe. Finally, the concentration of the m-MeB adaptor strand was held constant at 0.25 μM and the concentration of the f1 strand was changed from 0.1 μM to 1 μM in the presence of 50 nM miRNA-122. Immobilization time was varied from 15 to 75 min whereas hybridization time was varied from 15 to 120 min.

**3.2.5 Electrochemical measurements**

SWV was performed with a CHI660D Electrochemical Workstation (CH Instruments, Austin, USA). A typical 3-electrode system was used where the modified gold (Au) SPE or GDE served as the working electrode, a platinum wire was used as the counter electrode, and Ag/AgCl (3 M KCl) was used as a reference electrode (CH Instruments, Austin, USA). SWV measurements were recorded in a buffer solution at a potential range from 0.0 to −0.5 V, frequency of 100 Hz and amplitude of 0.07 V. Nitrogen was bubbled in the electrochemical cell to remove oxygen.
before measurements were performed at room temperature. At least three electrodes were used for each experiment to provide statistically significant results.

### 3.2.6 Ellipsometric measurements

Spectroscopic ellipsometry is an optical technique that can be used to characterize thin films (e.g. thickness, roughness, composition, dielectric properties). Ellipsometry operates by measuring a change of polarization of light as an incident beam (light source) interacts with a sample. The change in polarization is dependent on the properties of a sample (thickness, refractive index, etc.) and can be used on films on the nanometer scale because the incident radiation can be focused. Figure 18 displays the basic setup of an ellipsometry experiment. Briefly, a light source emits electromagnetic radiation that passes through a polarizer, which allows light of a specific electric field orientation to pass, before interacting with the sample. The linearly polarized light is reflected from the sample’s surface and passes through a modulator (to elliptically polarize) and then analyzer before reaching the detector. The detector determines the change in polarization by converting light to an electronic signal.

![Schematic of Spectroscopic Ellipsometry](image)

**Figure 18:** Schematic of Spectroscopic Ellipsometry
Once the measurement is taken on a sample, a mathematical model is created using software provided by the instrument manufacturer based on the materials used to calculate the thickness of the film and corresponding optical constants. Ellipsometry has been utilized for many applications such as semiconductors, electronics and biosensors because it provides a non-destructive analysis of samples. Spectroscopic ellipsometry was used in this work to elucidate changes in DNA film thickness upon sensor modification (e.g. hybridization) to provide a greater understanding of the properties of the sensor at a nanometer scale.

Ellipsometric measurements were performed with a V-Vase ellipsometer from J.A. Woollam Co. (Lincoln, USA). Spectroscopic ellipsometry has previously been used to calculate the thickness of DNA layers before and after hybridization. The experimental data obtained for the thickness of the USL probe was modeled using the WVASE software package (J.A. Woollam Co.) and assessed using the mean square error (MSE). Generally, a MSE < 10 is acceptable and smaller MSE values indicate good agreement of the model with the experimental data. A spectroscopic scan from 300 to 800 nm was first performed on clean glass slides (1.254 mm) coated with 5 nm titanium oxide (TiO) and 100 nm Au (Infolab Inc., Herndon, USA) at incident angles of 65°, 70°, and 75°. Then, measurements were recorded after USL probe immobilization, MCH backfilling, hybridization with adaptor strands (m and f1) and miRNA-122 on the clean Au slides. A Cauchy layer was used to model the DNA layer thickness on the gold slides and measurements were performed in triplicate.

3.3 Results and Discussion
3.3.1 Thickness of the USL
Ellipsometry was used to monitor the thickness of the DNA layer on Au coated glass slides. The thickness of immobilized USL probe on the electrode’s surface was 11.16 ± 0.32 Å. This value is
about half of the value reported for a linear single strand DNA probe of similar length, which correlates with our assumption of the SL folded conformation of USL probe shown in Scheme 1 B.\textsuperscript{32,33} Upon addition of MCH, the thickness was 10.78 ±1.12 Å, indicating an insignificant change of the DNA orientation. Hybridization with the adaptor strands (m and f1) and the miRNA-122 yielded a measured thickness of 37.85 ± 1.31 Å, demonstrating a significantly increased thickness caused by 4J structure formation. This thickness is comparable to previously studied conventional double-stranded complexes of a similar length.\textsuperscript{34} In the presence of the target containing a SNS the thickness was 9.77 ± 0.26 Å, which is comparable to the thickness of the original USL probe. This reflects negligible interaction of the USL probe with the adaptor strands in the presence of a mismatched target. In contrast, a previous ellipsometric study with a conventional hybridization probe yielded a slight increase of thickness upon exposure to a mismatched target when compared to the thickness of the probe.\textsuperscript{33} It is worth mentioning that the ellipsometric measurements provide only about 20% of the theoretical film thickness. For example, if the USL probe was extended to a linear conformation the theoretical thickness would yield about 235 Å.\textsuperscript{35} This could indicate that both the USL probe alone and in complex with a target do not form closely packed monolayers.\textsuperscript{33}

3.3.2 Optimization of the concentrations of the USL probe and adaptor strands
The blank response of the sensor in the absence of the USL probe, SWV frequency dependence of the current density and buffer optimizations are presented in Appendix A (Figures 33-35). The concentrations of the USL probe during the immobilization step was optimized to achieve highest signal in the presence of analyte. As illustrated in Figure 19A, the current density (j) as the concentration of the USL probe during the immobilization step increased from 0.05 μM to its
maxima at 0.1 μM. A decrease in current density was observed at higher concentrations than 0.1 μM, likely due to a steric crowding effect and electrostatic repulsion of the neighboring DNA structures. Thus, using 0.1 μM USL probe for immobilization produced near optimal density of the USL probe on the surface of the electrode for formation of the 4J structure. Next, concentrations of m-MeB and f1 adaptor strands were optimized during the detection step using 50 nM miRNA-122 as a target analyte. The concentration of m-MeB and f1 adaptor strands were varied simultaneously in equimolar concentrations (0.10 μM, 0.25 μM, 0.50 μM, 0.75 μM and 1.0 μM) followed by the detection of current from the MeB redox marker (Figure 19B). The current density increased as the equimolar concentration of adaptor strands m-MeB and f1 increased from 0.1 μM to 0.25 μM reaching a plateau at high concentrations. Consequently, the m adaptor strand concentration was held constant at 0.25 μM and the concentration of the f strand was changed from 0.1 μM to 1 μM in the presence of 50 nM miRNA-122. The highest signal observed was with 0.25 μM m and 0.5 μM f1 (Figure 19C), which is comparable to the signal observed in Figure 1B and was subsequently used for further studies.
Figure 19: (A) Current density (j) obtained from SWV in the presence of 1 mM of adaptor strands (m-MeB and f1) and 50 nM miRNA-122 obtained with different concentrations of the USL probe during immobilization. (B) Optimization of the adaptor strand concentration, which are simultaneously analyzed at equimolar concentrations, at fixed concentration of USL probe (0.1 mM). (C) 0.25 mM of m-MeB strand fixed and variable f strand concentrations along with 50 nM miRNA-122.
3.3.3 Experimental timing

Next, the immobilization time of the USL probe was varied in intervals from 15 - 75 min. The purpose of this experiment was to minimize electrode preparation time without significant loss of the signal intensity. Following the immobilization, MCH was added for 30 min and hybridization with adaptor strands and target was carried out for 120 min. As seen in Figure 20A, the current increased from immobilization times of 15 to 30 min where the signal remains relatively constant up to 75 min. The purpose of this experiment was to minimize analysis time without compromising the signal. Therefore, an immobilization time of 30 min was adequate for further analyses. Similarly, the hybridization time was investigated from 15 to 120 min. The current increased from hybridization times of 15 to 90 min until it reached saturation to 120 min (Figure 20B). Consequently, 90 min was a sufficient incubation time to produce a high signal and was used for subsequent experiments.
Figure 20: Optimal time for (A) immobilization of 0.1 µM USL probe and (B) hybridization with 0.25 µM m strand, 0.50 µM f strand and 50 nM miRNA-122 target.

3.3.4 Sensor regeneration

It is known that DNA probes can be regenerated from their complexes with analytes by heating or changing the pH. In contrast, Lubin et al. reported the regeneration of a DNA SL probe sensor with high reproducibility by rinsing with water for 30 sec. This is most likely due to the DNA SL probe conformation which shifts thermodynamic equilibrium from the duplex to dissociated state. Similarly, we demonstrate here that the 4J structure dissociates at room
temperature after rinsing with water, likely due to the incorporation of the DNA SL probe. To demonstrate the distinctive DNA SL probe regeneration characteristic, the sensor was re-used up to seven times after the original hybridization with over 97% recovery (Figure 21) by rinsing the sensor with deionized water for 30 sec following hybridization. The electrodes showed stability over one week when stored in IB at 4°C. A representative voltammogram is presented in Appendix A before and after regeneration (Figure 36).

![Figure 21](image.png)

Figure 21: Regeneration of the UE4J sensor using Au screen printed electrodes by a simple 30 second rinse with deionized water following hybridization.

### 3.3.5 Sensor selectivity
The selectivity of the sensor was studied with a target containing a SNS (Table 2). The current density was close to the background (Figure 22, curve c) when compared to the fully matched target at the same concentration (50 nM; Figure 22, curve b). Furthermore, even when the mismatched target was used in four-fold excess (200 nM) the current density still remained low
(Figure 22d), thus reflecting the capability of the sensor to detect a fully matched target even in four times excess amount of a single base mismatched analyte, a property important in practice.\textsuperscript{36} The high selectivity is attributed to the sensor design: the m strand with a shorter target binding arm does not hybridize to the mismatched target and has the capability to discriminate a SNS.\textsuperscript{18,24,28}

![Graph showing sensor response](image)

Figure 22: Selectivity of UE4J sensor. Sensor response after (a) the immobilization of USL probe and backfill with MCH (b) incubation with m and f1 along with miRNA-122 (5'-UGG-AGU-GUG-ACA-AUG-GUG-UUU-G-3'; 50 nM) (c) addition of a single base mismatch (50 nM) and (d) an excess of single base mismatch (200 nM)

These results are in agreement with our previous findings using the 4J sensor in a fluorescent format, which offers a higher selectivity at ambient temperatures than the DNA SL probe alone.\textsuperscript{17,18,30} This performance is hard to achieve by conventional hybridization sensors when analyzing folded target analytes such as miRNAs used in this study.\textsuperscript{25,38} The hybridization between a DNA SL probe and folded target analyte is inefficient because the hairpin structure is
energetically favored over the formation of a duplex, which can be circumvented by using a multicomponent sensing approach such as the 4J structure. Therefore, the UE4J sensor would be ideal for a highly selective analysis of folded structures.

3.3.6 LOD and the universality of UE4J

Although we did not focus on achieving a low LOD, we determined the detection limit of the UE4J sensor as follows. The current density \( J \) increased with increasing concentrations of miRNA-122 from 5 to 75 nM as shown in Figure 23A. The linear dynamic range (LDR) was from 5 nM to 50 nM and the response became nonlinear beyond the upper level, indicating electrode saturation. The LOD was calculated to be 3.2 nM as three times the standard deviation of the blank divided by the slope from the calibration curve \( (S_b/m) \).

Finally, the target-binding arms of strands m and f can be changed to tailor the sensor to each new target sequence using one optimized USL probe. Previously, the 4J sensor was shown to successfully detect different miRNAs but its affinity for DNA detection was not explored using the same probe. To demonstrate this ability, the sensor was incubated with a new target DNA (T-DNA: 5’-CTGGATACGGATATGGTGTTTG-3’ ) sequence using the same USL probe with the same m adaptor strand and the new adaptor strand, f2, equipped with analyte binding arms complementary to T-DNA (Table 2). As shown in Figure 23B, the current density increased with increasing concentrations of T-DNA from 5 to 75 nM. The LDR was from 5 to 50 nM and the response became nonlinear beyond the upper level, indicating electrode saturation.

The LOD was calculated to be 0.65 nM. In addition, a longer DNA target (60 nt) and target containing a SNS were analyzed and are discussed further in Appendix A to demonstrate the applicability of long analyte detection (Figure 37). These results illustrate that the same USL
probe can be used for the detection of various nucleic acids by only modifying the target binding arm of the adaptor strands. The universal character of the 4J design in conjunction with the ability to regenerate the DNA SL probe, provides a user friendly format for analyzing a variety of nucleic acids. This multicomponent format eliminates the labor intensive immobilization of a new probe for each new target and in conjunction with the low cost of synthetic oligonucleotides, the costs for detection of many analytes with a single UE4J will be reduced compared to conventional formats.

![Graph A](image)

![Graph B](image)

Figure 23: Calibration curve for miRNA-122. (B) Calibration curve for T-DNA. Insets: Respective SWVs corresponding to each concentration of miRNA-122 and T-DNA at 1, 5, 15, 25, 30, 40, 50, and 75 nM. SWVs were performed in HB. The errors bars represent the standard deviation of the signal generated from three separate electrodes. The standard deviation is represented by the error bars, which accounts for the current density generated from three separate electrodes.
3.4 Conclusion
A systematic characterization of a UE4J sensor has been performed in this study to provide new insights for the future use of a portable universal electrochemical sensor for SNS discrimination at room temperature. The next step is to focus on achieving a lower LOD for the analysis of biological samples as it is a current limitation of this sensor. We also envision that the sensor reported here has advantages over conventional designs for the analysis of folded nucleic acids such as bacterial 16S rRNAs. To achieve unfolding, RNA strand f can be designed with a long analyte-binding arm. This will unwind possible secondary structures and liberate an RNA fragment to bind the SNS specific m strand, as it was reported earlier for fluorescent sensors.24,25 The novelty of this sensor are that (i) enables signal ON detection with zero signal background in the absence of the target,; (ii) it is highly selective toward a SNS at room temperature; (iii) it is able to be regenerated by simply rinsing with water due to the incorporation of the DNA SL probe, and (iv) it provides a universal format allowing a cost effective analysis of multiple analytes. Indeed, changing the target-binding arms of strands m and f is sufficient to tailor the sensor to each new target sequence. The characteristics of the UE4J sensor reported here with SNS discrimination at room temperature and the possibility to detect folded endogenous RNA in a re-usable format are highly significant since it promises to deliver a new efficient tool for a highly specific and cost-effective detection of DNA and RNA analytes in a portable electrochemical format.

3.5 References
CHAPTER FOUR: A UNIVERSAL AND LABEL-FREE IMPEDIMETRIC BIOSENSING PLATFORM FOR DISCRIMINATION OF SINGLE NUCLEOTIDE SUBSTITUTIONS IN LONG NUCLEIC ACID STRANDS

4.1 Introduction
A great number of hybridization-based sensors have been explored for the detection of specific DNA or RNA sequences in clinical molecular diagnostics of human diseases, environmental monitoring and food safety.1-4 Among various strategies, electrochemical transduction is routinely adopted for nucleic acid detection because of the simplicity, high sensitivity, low cost and portability.5-8 First reported by Fan et al.,9 a class of electrochemical DNA sensors (E-DNA) which included an immobilized SL probe with a covalently bound redox marker was developed. This class of E-DNA sensors undergo a conformational change upon target binding and the change in current is measured.9-11 Although E-DNA sensors offer good sensitivity, they fall short when detecting SNS using folded probes.

The selectivity of SL folded probes has been improved by the development of multicomponent probes for optical reporters.12-13 This approach utilizes adaptor strands along with the conventional MB probe, a hairpin-folded DNA strand conjugated with a fluorophore and quencher on opposite ends.14-15 The multicomponent approach exhibits improved selectivity in a broad range of temperatures (5-40 °C) compared to the MB or SL probe due to the design of the short analyte binding arm of one adaptor strand.12-13,16 The short analyte binding arm will not bind at the SNS site and will in turn destabilize the 4J structure, resulting in the SL probe to be thermodynamically favored in its hairpin conformation rather than the 4J.
We have used this multicomponent approach to detect DNA or RNA targets (~22 bases) with the ability to discriminate SNS and have characterized the sensor using voltammetry techniques.\textsuperscript{17} Other electrochemical 4J sensor designs were explored for the analysis of microRNAs (~22 bases), but the previous designs involved the use of multiple covalently bound redox labels or enzyme assisted amplification strategies.\textsuperscript{18-19} The signal output of these sensors is limited for applications which require low limits of detection because the current is restricted to a single redox marker at the surface of the electrode for each hybridized SL probe or require excess complicated steps. Although many amplification techniques have been developed to overcome this challenge, the user-friendly aspect has been eliminated as more steps and costs are incorporated to achieve lower limits of detection.\textsuperscript{20-21} Therefore, electrochemical impedance spectroscopy (EIS) was also investigated for detection of nucleic acids to achieve low limits of detection (fM-aM) without use of additional amplification steps or expensive bound redox markers.\textsuperscript{22} EIS is a powerful technique that monitors interfacial changes upon surface modifications.\textsuperscript{23-25} A few studies have explored EIS detection of varied analyte lengths, e.g. 17, 21, and 27 base DNA analytes reported by Wang \textit{et al.}\textsuperscript{26} or PCR products of 90 bases reported by Minaei \textit{et al.}\textsuperscript{27}

In this work, we utilize EIS to monitor interfacial changes for the detection of nucleic acids of varied lengths (22 bases, 60 bases, 200 bases) with a universal electrochemical 4J platform to explore the performance and the impact of target length on discrimination of SNS, which has not yet been explored. The sensor includes an immobilized USL probe attached to a gold substrate via a thiol bond and two adaptor strands (m and f) as seen in Figure 24. The adaptor strands have a SL binding arm and target-binding arm complementary to the target. In the presence of the
target, the USL probe changes its conformation to form a bulky 4J structure that serves as a barrier to the redox couple in solution, resulting in a large charge transfer resistance.

Figure 24: A schematic of the design of the label-free impedimetric electrochemical 4J sensor.

The change in electron transfer resistance was used to monitor the hybridization for nucleic acid targets of varied lengths. An equivalent circuit model was used to analyze the performance of the sensor upon fabrication and hybridization with the nucleic acid analytes. The developed multicomponent sensor could be used for an inexpensive, selective and label-free detection of potentially any RNA or DNA analyte of varied length for environmental monitoring and clinical diagnostics.

4.2. Materials and Methods

4.2.1 Reagents and materials
Solutions were prepared with deionized water (18MΩ cm resistivity) using a Milli-Q Integral Water Purification System from EMD Millipore (Massachusetts, USA). The oligonucleotides
used in this study were purchased from Integrated DNA Technologies (Coralville, USA) and used as received (Table 3). An IB was prepared with 50 mM Tris-HCl, 250 mM NaCl. A HB was prepared with 50 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl₂. The IB and HB were adjusted to a pH of 7.4 using 1 M NaOH.

Table 3: Nucleic acid sequences

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>USL probe f</td>
<td>5’-S-S-(CH₂)₆-TTTTTTTTTTCGCGTTAACATACAATAGATCGCG-3’</td>
</tr>
<tr>
<td>m</td>
<td>5’-CAAAACACCATATGTTAAC-3’</td>
</tr>
<tr>
<td>T-22</td>
<td>5’-UGGAGUGUGACAAUGGUGUUUG-3’</td>
</tr>
<tr>
<td>T-60</td>
<td>5’-UGGAGUGUGACAAATGGTTTTTGTTCTAAAACTATCAAACGCC-3’</td>
</tr>
<tr>
<td>T-200</td>
<td>5’-UGGAGUGUGACAAATGGTTTTTGTTCTAAAACTATCAAACGCC-3’</td>
</tr>
<tr>
<td>SNS-22</td>
<td>5’-UGGAGUGUGACAAUGGCUUUUG-3’</td>
</tr>
<tr>
<td>SNS-60</td>
<td>5’-UGGAGUGUGACAAATGGTTTTTGTTCTAAAACTATCAAACGCC-3’</td>
</tr>
<tr>
<td>SNS-200</td>
<td>5’-UGGAGUGUGACAAATGGTTTTTGTTCTAAAACTATCAAACGCC-3’</td>
</tr>
</tbody>
</table>

1 The bases that compose the stem of the USL probe are in italics; the SNS site is underlined; the hybridized portion of the targets are in bold

4.2.2 Instrumentation

A CHI660D Electrochemical Workstation (CH Instruments, Austin, USA) was used to perform EIS and CV. Gold screen printed electrodes (DROPSENS, Spain) served as the WE in a three electrode system which included an external Ag/AgCl (3 M KCl) reference electrode and platinum counter electrode (CH Instruments, Austin, USA). EIS and CV measurements were
recorded in HB containing 5 mM \( \text{K}_3[\text{Fe(CN)}_6]/\text{K}_4[\text{Fe(CN)}_6] \). EIS measurements were taken over a frequency range of 10 kHz to 10 Hz at an AC potential of 0.15 V in a faraday cage to reduce electrical noise. The experimental data is presented as Nyquist plots which were fitted by an equivalent circuit using instrumental software. CV measurements were obtained at a scan rate of 100 mV/s. At least three electrodes were used in each experiment to acquire statistically significant data.

4.2.3 Electrode preparation
The WE was activated by using CV from 1.6 to -0.1 V at a scan rate of 100 mV/s in 0.5 M \( \text{H}_2\text{SO}_4 \) and its area was calculated.\textsuperscript{28} The WE was rinsed with DI water and dried with nitrogen prior to use.

4.2.4 Immobilization and hybridization
The immobilization of the USL probe was achieved using a gold-thiol bond. First, 1 mM TCEP was added to the USL probe and was vortexed for 1 hour to reduce the disulfide bonds. This solution was diluted to 0.1 \( \mu \text{M} \) in IB and 15 \( \mu \text{L} \) of the solution was drop casted and incubated on the electrode for 30 minutes at room temperature.\textsuperscript{17} Then, the electrodes were rinsed using IB and dried with nitrogen. Next, 2 mM MCH (15 \( \mu \text{L} \)) was drop casted and incubated on the electrode for 30 minutes to reduce nonspecific adsorption, rinsed with IB and dried with nitrogen. The target solutions (T-22, T-60, and T-200) were diluted in HB to appropriate concentrations and mixed with 0.25 \( \mu \text{M} \) of the m adaptor strand along with 0.5 \( \mu \text{M} \) of the f adaptor strand and 15 \( \mu \text{L} \) was drop casted and incubated on the electrode for 90 minutes.\textsuperscript{17}
4.3 Results and Discussion

4.3.1 Electrochemical behavior of a USL probe modified electrode

EIS was utilized to investigate the changes at the electrode/electrolyte interface upon modification of the WE. Figure 25A displays the Nyquist plots for the electrode (a) bare (b) modified with USL probe (c) backfilled with MCH and (d) upon hybridization with the adaptor strands and T-22 (1 pM) in the presence of 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆].

![Nyquist plots](image)

Figure 25: (A) Nyquist plots of the gold electrode (a) before and (b) after immobilization of the USL probe (c) after backfilling with MCH and (d) upon hybridization with adaptor strands (m and f) and 1 pM target (T-22) in an electrochemical cell containing 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] and hybridization buffer. The frequency ranged from 10 kHz to 10 Hz at an AC potential of 0.15 V. (B) An equivalent circuit model representing the interface of the electrochemical 4J sensor and electrolyte including resistance of the solution (Rₛ), charge transfer resistance of the redox couple in solution (R_CT), a constant phase element (C) and a Warburg impedance element (Z_w).

The impedance is shown as the sum of the real Z (Z’) and imaginary Z (Z”) components, which represent the resistance and capacitance of the electrochemical cell. The Nyquist plot generates a semicircle representative of the charge transfer resistance (R_CT) due to the electron-transfer kinetics of the redox couple to the electrode’s surface. The bare electrode is represented by a straight line in the lower frequency region (Figure 25A, line a). This demonstrates a diffusion
controlled reaction at the WE for the redox couple $K_3[\text{Fe(CN)}_6]/K_4[\text{Fe(CN)}_6]$. A small semicircle was observed in the higher frequency region upon immobilization of the USL probe followed by a straight line in the lower frequency region that represents a mixed electron transfer and diffusion controlled reaction at the interface (Figure 25A, line b). Following immobilization of the USL probe, MCH was used to backfill the electrode to reduce nonspecific adsorption as exhibited in Figure 25A, line c. A larger $R_{\text{CT}}$ was generated due to inhibition of the electron transfer reaction of the redox couple $K_3[\text{Fe(CN)}_6]/K_4[\text{Fe(CN)}_6]$ as the surface of the WE was modified. Next, the sensor was hybridized with the adaptor strands (m and f) and 1 pM of T-22 (Figure 25A, line d). The $R_{\text{CT}}$ further increased as the formation of the 4J decreased the electron transfer efficiency of the redox couple. Therefore, the change in $R_{\text{CT}}$ which is strongly dependent on any modification of the electrode’s surface, such as target binding, was monitored as a signal for the sensors response. The equivalent circuit model that represents the electrochemical process at the 4J sensor/electrolyte interface was used to interpret the impedimetric spectra (Figure 25B). In this modified Randles circuit, the $R_{\text{CT}}$ (dependent on the electron-transfer kinetics of the redox couple to the electrode’s surface) is in parallel with a constant phase element (C), which accounts for the Helmholtz double layer and surface roughness between the film and solution, and to the Warburg impedance ($Z_w$), which represents a diffusion-limited electrochemical process. These circuit elements are in series with the solution resistance between the modified working electrode and reference electrode ($R_s$).

4.3.2 EIS detection of varied target lengths
The conformational change of the USL probe upon target binding affects the charge transfer resistance of the redox couple ($K_3[\text{Fe(CN)}_6]/K_4[\text{Fe(CN)}_6]$) to the surface of the electrode. The
USL probe is in a hairpin conformation in the absence of the target (Figure 24). In the presence of the target and addition of adaptor strands (m and f), the USL probe opens to form a 4J structure. The target induced conformational change results in a bulky 4J structure that blocks the redox couple from accessing the surface of the electrode, resulting in an increased resistance. In addition, the resistance is affected by the repulsion between the negatively charged anions of the redox couple with the negatively charged phosphate backbone of the nucleic acid strands. The conformational switch of the USL probe in the presence of the target and adaptor strands was monitored upon incubation with different concentrations of T-22 (0.5, 1.0, 2.5 and 5.0 pM) as shown in Figure 26A-B. The ΔR_CT increased linearly as the concentration of T-22 increased from 0.5 pM to 5 pM (Figure 26A). To explore the response of the sensor for targets of varied lengths, the sensor was hybridized separately with a target containing 60 bases (T-60) and 200 bases (T-200) (Figure 26C-F). Longer targets resulted in an increase of resistance for the same target concentrations (Figure 26). The ΔR_CT of the hybridized USL probe increased linearly as the concentration of T-60 increased from 0.1 pM to 5 pM (Figure 26C) and as the concentration of T-200 increased from 0.01 pM to 5 pM (Figure 26E).
Figure 26: Analytical performance of the 4J electrochemical sensor. (A) A calibration plot for the $\Delta R / \Omega$ versus log concentration of T-22. (B) Nyquist plot upon (a) USL immobilization and hybridization of T-22 at concentrations of (b) 0.5 pM (c) 1.0 pM (d) 2.5 pM and (e) 5.0 pM. (C) A calibration plot for the $\Delta R / \Omega$ versus log concentration of T-60. (D) Nyquist plot upon (a) USL immobilization and hybridization of T-60 at concentrations of (b) 0.1 pM (c) 0.25 pM (d) 1.0 pM and (e) 5.0 pM. (E) A calibration plot for the $\Delta R / \Omega$ versus log concentration of T-200. (F) Nyquist plot upon (a) USL immobilization and hybridization of T-200 at concentrations of (b) 0.01 pM (c) 0.10 pM (d) 1.0 pM and (e) 5.0 pM.
It is interesting to note that as the length of the target increased from T-22 to T-60, an increase in sensitivity was observed. The slope of the calibration plot for T-22 was 223 Ω/pM and a three-fold increase was observed for detection of T-60 (650 Ω/pM). For T-200 the sensitivity further increased by six-fold with respect to T-22 (1265 Ω/pM). As can be observed, lower limits of detection (LOD) were observed for longer targets which were calculated as three times the standard deviation of the blank divided by the slope from the calibration curve ($S_b/m$). The LOD was calculated as 300 fM, 120 fM and 90 fM for T-22, T-60 and T-200, respectively. The difference in the LOD and sensitivities are likely due to increased steric hindrance and surface coverage for longer targets which prohibit the redox couple from achieving an efficient electron transfer.

The corresponding Nyquist plots for the difference in $\Delta R_{CT}$ for hybridization of the targets (of different lengths) at a concentration of 1 pM are shown in Figure 27A. As the length of the target is increased, the observed $\Delta R_{CT}$ is significantly increased (Figure 27A). The response for varied target lengths is based on the 4J structure formed, where the longer targets have significantly longer unhybridized portions which act as a barrier to the redox couple in solution, thus increasing the charge transfer resistance. In addition, the longest target (T-200) had a larger linear dynamic range (0.01 pM to 5 pM) compared to T-60 (0.1 pM to 5 pM) and T-22 (0.5 pM to 5.0 pM). This extended linear dynamic range can be attributed to the significantly larger excess portion of the target which reduces charge transfer efficiency of the redox couple in solution, even at low concentrations.
4.3.3 CV response for varied target lengths
To further investigate the electrochemical processes occurring at the interface, CV was performed. The cyclic voltammograms are shown in Figure 27B for the redox of 5 mM 
K₃[Fe(CN)₆]/K₄[Fe(CN)₆] after hybridization of the USL probe, adaptor strands and (a) T-22 (b) T-60 or (c) T-200. The peak current decreases and the separation of peak potentials (ΔEₚ) increase upon hybridization of T-22 compared to that of T-60 (Figure 27B, line a vs. line b). Once again, these changes are due to the self-assembled monolayer consisting of MCH and 4J structures formed upon hybridization which have a negatively charged phosphate backbone that repel the negatively charged anions of the redox couple and decrease electron transfer efficiency. Upon hybridization with T-200 (Figure 27B, line c), the bulky 4J structure prohibits easy access of the redox couple to the surface of the electrode, resulting in a slight decrease peak current. However, the change in current using CV is only slight as the length of the target is increased and is not discernable between T-60 and T-200. However, the Nyquist plot (Figure 27A) shows a clear distinction in signal between the different target lengths (T-22, T-60 and T-200) and demonstrates the benefit of EIS detection when analyzing targets of varied lengths.
Figure 27: (A) Nyquist plots and (B) cyclic voltammetry response upon immobilization of 0.1 μM USL probe, backfilling with MCH and hybridization with 0.25 μM m, 0.5 μM f along with 1 pM of (a) T-22 (b) T-60 and (c) T-200.

4.3.4 Selectivity for varied target lengths
The selectivity of the sensor was studied using EIS upon hybridization of the USL probe with target analytes of varied lengths (SNS-22, SNS-60, SNS-200) containing a SNS along with the adaptor strands (m and f). The $R_{CT}$ for all SNS targets (1 pM) was consistent with that of the MCH background (Figure 28, lines a-b). Additionally, even when SNS targets were used at concentrations in a hundred-fold excess (100 pM), the $R_{CT}$ remained low (Figure 28, line c) and consistent with that of the signal from the unhybridized sensor. Upon hybridization of the USL probe and adaptor strands with the fully complementary target (T-22, T-60, T-200), a significant change in $R_{CT}$ was observed (Figure 28d). The improved selectivity of the electrochemical 4J sensor is due to its unique design. The m adaptor strand possesses a short target binding arm that prohibits formation of the 4J structure (destabilizes the 4J) in the presence of an analyte containing a single nucleotide substitution.\textsuperscript{12-13,16} The results shown here are consistent with our
previous studies\textsuperscript{14,17,29-30} using the 4J (in solution and immobilized) to demonstrate the potential for single base mismatch differentiation and this is the first time we evaluate the selectivity of longer targets for the electrochemical 4J sensor.

Figure 28: Sensor response upon (a) immobilization of 0.1 μM USL probe and MCH along with 0.25 μM m and 0.5 μM f for hybridization with (A) SNS-22 at concentrations of (b) 1 pM (c) 100 pM and (d) 1 pM fully complementary target (T-22) (B) SNS-60 at concentrations of (b) 1 pM (c) 100 pM and (d) 1 pM fully complementary target (T-60) (C) SNS-200 at concentrations of (b) 1 pM (c) 100 pM and (d) 1 pM fully complementary target (T-200).
4.4 Conclusions
In this work, we show the development of a novel, label-free electrochemical sensor for the detection of multiple nucleic acid sequences of different lengths with a low limit of detection and capability to be used for SNS differentiation. The principle of this sensing platform relies on the conformational change of a USL probe to form a bulky 4J structure in the presence of the analyte which leads to detectable changes in electron transfer resistance at the electrode/electrolyte interface. This work demonstrates that the electrochemical 4J sensor can achieve low limits of detection (fM) and does not require complex signal amplification techniques as previously reported. In addition, the sensing platform is sensitive to targets of varied length which is an important aspect for a universal sensor to demonstrate applicability to shorter analytes such as microRNAs (~22 bases) as well as longer analytes such as pathogenic bacteria. Furthermore, the unique design of the electrochemical 4J sensor allows a highly selective and universal platform not offered by conventional sensors, eliminating the need to optimize a new SL probe for each new target. The novel sensor presented here combines highly sensitive impedimetric transduction, a USL probe for the detection of varied length targets, the absence of exogenous labels and high selectivity which could revolutionize point-of-care testing.

4.5 References
CHAPTER FIVE: RAPID DETECTION OF THE ZIKA VIRUS (ZKV-359) WITH A UNIVERSAL FOUR-WAY JUNCTION ELECTROCHEMICAL SENSOR

5.1 Introduction
Zika virus (ZIKV) is a flavivirus transmitted by mosquitoes that has quickly spread throughout the Americas and caused many health concerns.\textsuperscript{1-4} ZIKV typically causes mild influenza-like symptoms such as fever, rash and headaches or more serious complications such as Guillain-Barré syndrome and fetal microcephaly.\textsuperscript{5-8} In addition, around 80\% of ZIKV infections are asymptomatic and the other 20\% have non-specific symptoms that mimic those of other flaviviruses, such as dengue and chikungunya, which make its clinical diagnosis problematic.\textsuperscript{9} Due to the clinical difficulty of differentiating flaviviruses, significant interest and research efforts are being explored to develop new diagnostic tests.

Routinely, ZIKV is detected in serum by analyzing viral RNA or antibodies,\textsuperscript{10-11} although cross reactivity among the flavivirus antibodies has hindered their discrimination. Therefore, molecular testing methods (using viral RNA) have shown to be more reliable.\textsuperscript{8,10,12-13} Molecular testing is usually performed using reverse-transcription polymerase chain reaction (RT-PCR) or similar PCR methods which can provide a sensitive analysis in as little as 90 minutes.\textsuperscript{14-16} However, these methods are only useful in a limited time frame because the viremia decreases over time and, in general, PCR techniques require trained personnel, expensive equipment and tedious sample preparation which proves difficult in underdeveloped areas and remote locations. Therefore, further improvements to sensitivity and development of methods capable of
differentiating flaviviruses are still needed for simple and inexpensive ZIKV POC diagnostics. Recently, Pardee et al.\textsuperscript{17} reported an inexpensive molecular detection platform for the ZIKV combining nucleic acid sequence based amplification (NASBA) with a RNA probe based sensor. However, this technology still has limitations as a diagnostic tool such as a long detection time, complex steps and manipulation of the sensor design for colorimetric discrimination of different SNS.

Here, a rapid diagnostic tool for the detection of ZIKV was developed using a universal electrochemical sensing platform combined with NASBA. The sensor is sequence specific and can easily discriminate between SNS due to the inherent design of the sensor which includes a USL probe and two adaptor strands that hybridize to a target sequence to form a 4J structure. One adaptor strand is labeled with MeB for electrochemical detection (Figure 3B). The benefits of improved selectivity and universal character for analysis of different lengths and sequences of nucleic acids was previously demonstrated.\textsuperscript{18} This sensing platform is inexpensive, portable and could be used for rapid POC diagnostics to discriminate ZIKV from other flaviviruses.

5.2 Materials and Methods

5.2.1 Reagents and materials

The IB consisted of 50 mM Tris-HCl, 250 mM NaCl and the HB consisted of 50 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl\textsubscript{2}. The buffers were adjusted to a pH of 7.4 using 1 M NaOH. The nucleic acid sequences used in this study are listed in Table 4. Viral RNA was amplified for a target containing 84 bases (T-84Zik) and 141 bases (T-141Zik) using NASBA.
Table 4: Oligonucleotide sequences

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>USL probe f</td>
<td>5'-S-S-(CH$_2$)$_6$TTTTTTTTTTCGTTAACATAAAATAGATCCG-3'</td>
</tr>
<tr>
<td>m</td>
<td>5'-CTCGACTTTTTATGTTAACTTTTTTTTTTTTTTTTT-MeB-3'</td>
</tr>
<tr>
<td>T-84Zik</td>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGA CGAGAAGAGCGAAGCGAGGUACGCUGUAAUCACCAAGAGC-3'</td>
</tr>
<tr>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGACGAAGC</td>
<td></td>
</tr>
<tr>
<td>SNS-84</td>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGA CGAGAAGAGCGAAGCGAGGUACGCUGUAAUCACCAAGAGC-3'</td>
</tr>
<tr>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGA CGAGAAGAGCGAAGCGAGGUACGCUGUAAUCACCAAGAGC</td>
<td></td>
</tr>
<tr>
<td>SNS-141</td>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGA CGAGAAGAGCGAAGCGAGGUACGCUGUAAUCACCAAGAGC</td>
</tr>
<tr>
<td>5'-GGGAGAAGGGCAUACGGGAUGAUUGAAUGAAAACUGA CGAGAAGAGCGAAGCGAGGUACGCUGUAAUCACCAAGAGC</td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>5'-AATTCTAATACGACTCATAATAGGGAGAAGGGCATACGCGGA TGATTGATA-3'</td>
</tr>
<tr>
<td>RP84</td>
<td>5'-GCTCTTGTTGATTAGCGGA</td>
</tr>
<tr>
<td>RP141</td>
<td>5'-CCTGTCTTGGTACAGTC-3'</td>
</tr>
</tbody>
</table>

1 The bases that compose the stem of the USL probe are in italics; the SNS site is underlined; the hybridized portion of the targets are in bold

The NASBA reaction was prepared by developing a master mix for controls and five different concentrations (1 pg, 0.1 pg, 0.01 pg, 0.001 pg and 0.0001 pg) for each target of 10 kb RNA of Zika virus (MR-766) for antisense amplification of 84 bases target and 141 bases target. All solutions were prepared in DNase, RNase and Protease free water, in an ice bath. 46.9 µL of 3X NASBA reaction buffer, 23.1 µL of 6X nucleotide mix, 7 µL of 5 µM forward primer1 (FP1) and 7 µL of 5 µM reverse primer84 (RP84) to target antisense amplification of 84 bases; reverse primer 141 (RP141) to target antisense amplification of 141 bases, were mixed using mini-centrifuge. The solutions were then partitioned in 7 microtubes (12 µL each) and then 3 µL of DNase, RNase and Protease free water was added to each of the two controls tubes, while 1.6 µL
of the Zika virus RNA (0.0001 pg, 0.001 pg, 0.01 pg, 0.1 pg and 1 pg) was combined with 0.4 µL of DNase, RNase and Protease free water and added to the respective tubes. All of the tubes were incubated for 2 minutes at 65 °C for annealing, then cooled down in a 41°C water bath for 10 minutes. Next, 5 µL of NASBA enzyme cocktail of wet kit for NEC-1-24 (Lifescience; SKU: NEC-1-24) was added to each tube and incubated at 41 °C for 90 minutes. The concentration of the NASBA reacted analyte mixture was measured using a Promega Quantus™ Fluorometer.

Gel electrophoresis was performed after the 90 minute incubation, using 2% agarose gel (agarose in a buffer containing 89mM Tris, 89 mM Boric acid, and 2 mM EDTA) that was casted in an owl gel system and left to cool. Once the gel solidified, samples were prepared for gel loading. The marker was prepared by mixing 2 µL (1 µg) 50-1000 bases ssRNA Ladder (500 µg/ml) 3 µL (DNase, RNase, and Protease free water), and 5 µL 2X RNA ladder loading buffer (BioLabs, Catalog No. N0364S), then heated at 65 °C for 5 minutes. Then it was chilled in ice for 2 minutes and loaded in the gel. Negative controls and NASBA products were prepared for loading by mixing 5 µL of each with 5 µL of 2X RNA ladder loading buffer, heated at 65 °C for 5 minutes, and was then chilled in ice for 2 minutes before it was loaded in the gel. Gel electrophoresis was performed in 1X TBE buffer at 75 volts. The gel was imaged by Bio Rad molecular imager (Model No. Universal Hood II).

5.2.2 Instrumentation

Electrochemical measurements (SWV) were recorded using a CHI660D Electrochemical Workstation (CH Instruments, Austin, USA) from 0 to -0.5 V at a frequency of 100 Hz. A three electrode system was used with a gold disc electrodes that served as the WE, a Ag/AgCl (3 M
KCl) RE and platinum wire as the CE (CH Instruments, Austin, USA). At least three electrodes were used in each experiment to acquire statistically significant data.

5.2.3 Sensor fabrication

The WE was immersed in a piranha solution (1:3 ratio of H₂O₂:H₂SO₄) to remove any organic material on the surface of the gold and then manually polished on a microcloth with a set of alumina slurries (1.0 μm, 0.3 μm and 0.05 μm). Excess polish was removed upon sonicating the WE in water for three minutes followed by ethanol. The area of the WE was determined by using CV from 1.6 to -0.1 V at a scan rate of 100 mV/s. The WE was rinsed with DI water and dried with nitrogen prior to use.

The USL probe was immobilized on the WE via a gold-thiol bond. First, the disulfide bond of the USL probe was reduced using 1 mM TCEP by vortexing for 1 hour. Then, the solution was diluted with IB to a final concentration of 0.1 μM. 15 μL of the solution was drop casted and incubated on the electrode for 30 minutes at room temperature. Next, the electrodes were rinsed using IB and dried with nitrogen. Next, 2 mM MCH (15 μL) was drop casted and incubated on the electrode for 30 minutes to reduce nonspecific adsorption, rinsed with IB and dried with nitrogen. The target solutions (T-84Zik or T-141Zik) were diluted in HB to appropriate concentrations and mixed with 0.25 μM of the m adaptor strand along with 0.5 μM of the f adaptor strand. 15 μL of the hybridization solution was drop casted and incubated on the electrode for 1.5 hours.

5.3 Results and Discussion

5.3.1 Control samples

Multiple control samples were tested to investigate the response of the sensor using SWV. The first control sample was used to test the blank response (no target present, adaptor strands only)
of the 4J sensor. The response was monitored after (a) immobilization of the USL probe, backfilling with MCH and upon (b) hybridization with a sample containing only the adaptor strands (m and f) without the target sequence (Figure 29A). No peak current was observed for the blank sample, indicating there is no interaction between the adaptor strands and USL probe in the absence of the target, thus preventing false positive responses. Next, a dummy electrode (no USL probe present) was used as a control to investigate if any nonspecific adsorption was occurring at the surface of the electrode. The WE was (a) backfilled with MCH and then incubated with a hybridization solution containing adaptor strands (m and f) along with 50 nM (b) NASBA T-84Zik and (c) NASBA T-141Zik (Figure 29B). A peak current was not observed upon incubation of the sensor with either NASBA target, indicating that nonspecific adsorption is not affecting the response of the sensor, even in a complex matrix (e.g. primers, nucleotide mixture, enzymes). Finally, a negative control (hybridization with no target present) was tested to ensure matrix components were not contributing to any observed signal upon hybridization. A SWV was recorded after (a) immobilization of the USL probe, backfilling with MCH and upon hybridization with a solution containing the adaptor strands and the NASBA matrix (excluding RNA) for b) T-84Zik and (c) T-141Zik (Figure 29C). Once again, no peak current was observed, indicating the components of the NASBA matrix (e.g. primers, nucleotide mixture, and enzymes) do not generate a signal.
Figure 29: Control samples for the 4J sensor. (A) SWV response for blank after (a) immobilization of DNA SL probe and backfilling with MCH and (b) hybridization with adaptor strands (m and f). (B) Dummy electrode upon (a) backfilling with MCH and hybridization with m, f and (b) 50 nM NASBA T-84Zik and (c) 50 nM NASBA T-141Zik. (C) Negative control upon (a) immobilization of DNA SL probe and backfilling with MCH and hybridization with m, f and NASBA matrix (excluding viral RNA) for (b) NASBA T-84Zik and (d) NASBA T-141Zik.

5.3.2 Sensor response
Since no peak current was observed for the control samples, the response of the sensor was investigated upon hybridization with (A) synthetic T-84Zik (B) NASBA T-84Zik (C) synthetic T-141Zik (D) NASBA T-141Zik (Figure 30). Hybridization of synthetic samples was compared with that of the NASBA samples to compare sensor response and investigate matrix effects. The LOD of all targets (synthetic and NASBA) was around 1 nM. The response of the synthetic T-84Zik was linear at concentrations ranging from 1-25 nM while the response of the NASBA T-84Zik was linear at concentrations ranging from 25-75 nM (Figure 30A-B). This shift in the LDR is likely due to the matrix interferences from the NASBA samples as further evidenced by the decrease in sensitivity between the targets (10.149 µA/cm²·nM for synthetic versus 6.741 µA/cm²·nM for NASBA). The matrix effects are also elucidated by the signal saturation for the NASBA target which reaches a maximum peak current density of around 7 µA/cm² whereas the
synthetic target saturates around a peak current density of 15 µA/cm². A similar trend for the sensor’s response is observed for the longer target (T-141Zik). The synthetic T-141Zik responded linearly at concentrations ranging from 5-75 nM while the response of the NASBA T-141Zik was linear at concentrations ranging from 10-75 nM (Figure 30C-D). The LDR also slightly shifts between the synthetic and NASBA targets and the sensitivity is significantly decreased for the NASBA target (9.880 µA/cm²•nM for synthetic versus 3.841 µA/cm²•nM for NASBA). The matrix effects are more pronounced for T-141Zik (comparing synthetic to NASBA response), likely due to the increased length of the target which also generates steric hindrance effects between neighboring hybridized 4J structures. These effects caused by the NASBA sample matrix can further prohibit or delay hybridization for T-141Zik, requiring higher concentrations to achieve an adequate signal for detection.

Figure 30: Response of the sensor to (A) synthetic T-84Zik (B) NASBA T-84Zik (C) synthetic T-141Zik and (D) NASBA T-141Zik.
5.3.3 Selectivity

The selectivity of the 4J sensor was investigated using a synthetic target containing a SNS with a length of 84 bases (SNS-84) and 141 (SNS-141) bases. The response was recorded using SWV after (a) immobilization of the USL probe and backfilling with MCH and upon hybridization with the adaptor strands along with (b) 50 nM of the SNS target (c) 200 nM of the SNS target and (d) 50 nM of the fully complementary target for T-84Zik (Figure 3A) T-141Zik (Figure 3B). No peak current was observed upon hybridization of the target containing a SNS, even in excess, for both target lengths. However, the fully matched target sequences produced a significant peak current indicating a 4J structure was formed upon hybridization of the USL probe with the adaptor strands and target. The ability to discriminate a SNS has been shown in our previous work\textsuperscript{18} and holds true for this sensor, even as the target length is increased.

Figure 31: Response of the sensor to a synthetic target containing a single base substitution (SNS) for (A) T-84Zik and (B) T-141Zik upon immobilization of the DNA SL probe and passivation with MCH (a) and hybridization with 0.25 µM m-MeB and 0.50 µM f and the respective SNS at (b) 50 nM (c) 200 nM and (d) 50 nM of the fully complementary target.
5.3.4 Hybridization timing

For POC applications, a quick response time is desired so that the patient can be diagnosed and treated as soon as possible. Therefore, the response of the sensor for hybridization with the Zika targets was investigated for synthetic and NASBA targets. The hybridization time was varied from 1 to 90 minutes with (a) synthetic and (b) NASBA targets for (A) T-84Zik and (B) T-141Zik (Figure 32). A discernable signal was observed in as little as one minute for both synthetic and NASBA targets and the current density kept increasing as hybridization time was extended to 90 minutes. The signal was equivalent for synthetic and NASBA targets up until 15 minutes of hybridization, where the signal started to increase for the synthetic sample and remained relatively constant for the NASBA targets. This signal variance is likely due to the complex matrix of the NASBA samples which could decrease the hybridization efficiency of the sensor. Although 90 minutes of hybridization generated the highest signal, a response was observed in as little as 1 minute which is the minimum time needed for a positive result for the presence of Zika.

Figure 32: Varied hybridization timing upon hybridization of 50 nM (A) T-84Zik and (B) T-141Zik along with 0.25 µM m-MeB and 0.50 µM f for (a) synthetic targets and (b) NASBA targets using the USL probe.
5.4 Conclusion
Here, we developed a universal 4J electrochemical biosensing platform for the detection of the Zika virus. Proper control experiments confirmed the design of the sensor was adequate and able to operate in complex matrices without generating false positive responses. The viral RNA was amplified using NASBA and the sensor was able to detect sequences of 84 and 141 bases while discriminating targets containing a SNS. This is important in practice as the Zika virus needs to be discriminated from other flaviviruses (e.g. dengue). Even though matrix effects were observed, a positive signal was obtained for both target lengths and did not hinder the sensor from detecting the Zika virus. In addition, the sensor was shown to provide a response in as little as one minute which is important for clinical diagnostics and POC applications for Zika detection. Finally, the ability of detecting secondary structures using this sensing platform has been shown for real world samples. This is an extremely important attribute as conventional probes do not have this ability due to thermodynamical constraints (e.g. stability of the secondary structure versus that of the probe). In the 4J structure, the length of the target binding arm on the f adaptor strand can be extended to help unwind secondary structures of longer targets to facilitate hybridization in a matter of seconds.

This dissertation has provided experimental evidence of the key advantages of using the 4J structure in an electrochemical format for biosensing applications: (i) universal character achieved by tailoring the sequence of the target binding arms on the adaptor strands (ii) detection of varied length targets and secondary structures (iii) high selectivity which allows SNS discrimination in targets of different lengths (iv) signal ON sensing scheme with zero background signal (v) easy regeneration of the USL probe using a 30 sec rinse with DI water for
a reusable sensor and an (vi) adjustable LOD or LDR which is achieved by altering detection techniques and parameters. In a broader scope, this platform would save time and expenses for optimizing a new probe for every new target of interest and instead can be easily modified for potentially any RNA or DNA sequence (of various lengths) for a wide variety of applications such as detection of viruses, bacteria, microRNAs and gender determination of skeletal remains.

5.5 References
APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER THREE
Blank Response in the Absence of Immobilized USL Probe

The response of the UE4J sensor was monitored in the absence of the USL probe. As seen in Figure 33, no peak current was observed upon (a) backfilling the electrode with MCH and (b) after hybridization with the adaptor strands m-MeB, f and 50 nM miRNA-122. The lack of signal indicates that the adaptor strands and target do not absorbed to the electrode surface in the absence of the USL probe.

![Figure 33: SWV current density (j) upon (a) backfilling the electrode with MCH and (b) hybridization with the adaptor strands m-MeB, f, and 50 nM miRNA-122.](image)

Current Density Dependence on Frequency

The frequency used for square wave voltammetry was optimized for electrochemical analysis. Upon hybridization, the peak current density was monitored at frequencies ranging from 1 to 150 Hz. As the frequency increased from 1 to 75 Hz, the current density increased as seen in Figure
34. A constant current density was observed from 75 to 150 Hz. A frequency of 100 Hz was chosen for analysis, since the peak shape was better defined at that frequency.

![SWV current density (j) dependence on frequency from 1 to 150 Hz](image)

**Figure 34:** SWV current density (j) dependence on frequency from 1 to 150 Hz

**Buffer Optimization**

A set of buffers were used for immobilization and hybridization to monitor the efficiency of the four-way junction formation (Table 5). All buffers were adjusted to pH 7.4 using 1 M sodium hydroxide (NaOH). Figure 35 demonstrates the current density monitored after (a) immobilization of the USL probe and MCH and (b) upon hybridization with the adaptor strands m-MeB, f, and target using the following buffer combinations IB#1, HB#1 (c) IB#1, HB#2 (d) IB#2, HB#3 and (e) IB#1, HB#3 (see Table 5 for buffer composition). The highest current density was observed with IB#1 and HB#3 (Figure 35e). The presence of NaH₂PO₄•2H₂O and Na₂HPO₄ during immobilization resulted in a higher peak current, likely due to greater stability.
of the DNA backbone in comparison to that observed with Tris-HCl based buffers. During hybridization, it is important to have a high concentration of MgCl₂ in order to stabilize the negative charges on the DNA phosphate backbone. Here, Tris-HCl was used as hybridization buffer instead of phosphate buffer to avoid precipitation of phosphate with magnesium. Therefore, IB#1 and HB#3 were used for all analyses to maximize hybridization efficiency.

Table 5: Composition of buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components [a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilization #1</td>
<td>50 mM Na₂HPO₄, 50 mM NaH₂PO₄·2H₂O and 50 mM NaCl</td>
</tr>
<tr>
<td>Immobilization #2</td>
<td>50 mM Tris-HCl and 250 mM NaCl</td>
</tr>
<tr>
<td>Hybridization #1</td>
<td>50 mM Tris-HCl, 0.5 mM EDTA, 20 mM KCl, 100 mM NaCl and 50 mM MgCl₂</td>
</tr>
<tr>
<td>Hybridization #2</td>
<td>50 mM NaH₂PO₄·2H₂O, 50 mM Na₂HPO₄, 25 mM NaCl and 20 mM MgCl₂</td>
</tr>
<tr>
<td>Hybridization #3</td>
<td>50 mM Tris-HCl, 25 mM NaCl and 50 mM MgCl₂</td>
</tr>
</tbody>
</table>

[a] All buffers were adjusted to pH 7.4 using 1 M NaOH

Figure 35: Current density (j) upon (a) USL probe and backfilling with MCH and (b) after sensor hybridization with adaptor strands m-MeB, f, and target using IB#1, HB#1 (c) IB#1, HB#2 (d) IB#2, HB#3 and (e) IB#1, HB#3 at pH 7.4 using SWV. For buffer compositions see Table S1 above.
Sensor Regeneration

The sensor was regenerated by rinsing with deionized water for 30 sec after hybridization. Figure 36 shows the SWV data upon (a) immobilization of the USL probe and backfilling with MCH, (b) the first hybridization with adaptor strands m-MeB, f, and target miRNA-122, (c) regeneration of the sensor by rinsing with deionized water for 30 sec and (d) the second hybridization with adaptor strands m-MeB, f, and target miRNA-122.

Figure 36: SWV current density upon (a) immobilization of the USL probe and backfilling with MCH (b) hybridization with the adaptor strands m-MeB, f, and target (c) regeneration of the UE4J sensor by rinsing with deionized water for 30 sec and (d) second hybridization.

Detection of Long Analytes

A long DNA analyte (T-DNA-2) and corresponding sequence containing a SNS (Mismatch 2) were purchased from Integrated DNA Technologies (Coralville, USA) and were used as received
(Table 6). The selectivity of the sensor in differentiation of Mismatch 2 against T-DNA-2 was investigated.

Table 6: Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-DNA-2</td>
<td>5'-GTTTCCTTAGCAGAGCTGGAGTGGACTGACAATGGTGTTTGTGTCTAAACTATCAAACGCC-3'</td>
</tr>
<tr>
<td>Mismatch 2</td>
<td>5'-GTTTCCTTAGCAGAGCTGGAGTGGACTGACAATGGTGTTTGTGTCTAAACTATCAAACGCC-3'</td>
</tr>
</tbody>
</table>

[a] SNS sites are underlined

The sensor was incubated with the same USL probe and MCH, then hybridized with the adaptor strands m-MeB, f1, and 75 nM T-DNA-2. As shown in Figure 37, the current density increased upon hybridization (b). The current density was close to the background (Figure 37, curve c) with Mismatch 2. Furthermore, even when the long target containing a SNS was used in four-fold excess (300 nM) no current signal was observed (Figure 37d). This reflects the capability of the sensor to detect longer oligonucleotides, even in four times excess amount of a single base mismatched analyte.
Figure 37: SWV current density upon (a) USL probe alone (b) hybridization with strands m-MeB, f1 and 75 nM T-DNA-2 (c) addition of a single base mismatch (75 nM) and (d) an excess of single base mismatch (300 nM).
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