Ultrasensitive Detection of Nucleic Acids using an Electronic Chip

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Electrical and Computer Engineering
University of Toronto

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Abstract

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The detection of particular genetic sequences aids in the early detection and diagnosis of disease; permits monitoring of the health and state of the natural environment; and informs forensic investigations. To date, gene detection has relied on enzymatic amplification followed by optical readout. Though these technologies have advanced dramatically, the instruments and assays are costly and lack portability. The work presented herein addresses an urgent challenge: molecular diagnostics at the point-of-need.

This work reports the first electronic chip capable of analyzing - directly, without amplification, and with clinically-relevant sensitivity - multiple genes of interest present in a clinical sample. It reports a dramatic acceleration in sample-to-answer times, with clinically actionable findings in minutes where legacy techniques take hours or days.

The key to the sensitivity and speed of the biosensors reported herein lies in their architecture and morphology on multiple lengthscales. It is proven that hybridization-based assays employing a nucleic probe attached to a solid surface can only achieve efficient performance when displayed on a nanotextured surface. It is also discovered that these same sensing elements must reach tens of micrometers into solution to achieve rapid, sensitive detection of nucleic acids in clinical samples.

As a result, the materials integrated onto the sensing chip reported herein are engineered on multiple lengthscales - from the nanometers to the tens of micrometers. Engineering is done
through a combination of low-cost, convenient top-down photolithographic patterning; combined with hierarchically-designed bottom-up growth of electrodeposited sensing elements.

The capstone of this work is a chip that distinguishes among different types of bacteria in an unpurified sample. The chip gives accurate answers in under half an hour when detecting bacteria at a level of 1.5 colony-forming-unit (cfu) per microliter. These speeds and sensitivities enable the application of this technology in point-of-need assays for infectious disease detection.

Ultimately, the work showcases the power of bringing together techniques and principles from materials chemistry, biochemistry, applied physics, and electrical engineering to the solution of an important problem relevant to human health.
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<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
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<tr>
<td>DNA</td>
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<tr>
<td>EIS</td>
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<tr>
<td>HNME</td>
<td>Hierarchical Nanotextured Microelectrodes</td>
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<tr>
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<td>Nanoelectrode Ensembles</td>
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<tr>
<td>NME</td>
<td>Nanotextured Microelectrode</td>
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<tr>
<td>PCR</td>
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<tr>
<td>qrt-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
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<tr>
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<td>Ribonucleic Acid</td>
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<tr>
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1 Introduction

Genes, units of heredity in living organisms, are long stretches of double helical Deoxyribonucleic Acid (DNA) contained within chromosomes. DNA provides the cells with a code for generating proteins through an intermediate process involving another class of nucleic acids, Ribonucleic Acid (RNA). RNA provides the sequence by which amino acids – protein building blocks – are combined in a single polypeptide chain (protein). The information stored in DNA for developing the essential cellular components is transferred to the next generation; however, no two individuals are genetically identical as genetic variations are introduced by single nucleotide polymorphisms [1], copy number variations [2] and epigenetic factors [3]. DNA’s uniqueness and its role in programming cellular function make the knowledge of DNA sequence applicable to areas ranging from disease diagnosis to forensics.

In the present work, we demonstrate a novel approach to nucleic acids detection based on a multiplexed electronic chip. This detection platform enables portable, multiplexed, high speed DNA analysis at a low cost.

This chapter will discuss the underlying principles in decoding the sequence of DNA, and will talk about its most important applications. Furthermore, it will review the existing technologies for DNA sequencing and detection and will explain how they fall short in addressing some of the application-specific requirements. Lastly, this chapter will conclude with the objectives and the organization of this thesis.

1.1 Nucleic Acids Detection

DNA has a double stranded structure, where each strand is a chain of deoxyribonucleotide units composed of three parts – a nitrogenous base, a deoxyribose sugar and a phosphoric acid residue – covalently bonded together. (Figure 1.1) DNA sequence – the order of nitrogenous bases of DNA – contains the necessary information for assembling amino acids into functional proteins. The bases of DNA are pyrimidines, heterocyclic aromatic compounds, or purines, pyrimidines linked to an imidazole ring. Commonly occurring bases are pyrimidine bases – cytosine (C) and thymine (T) – and purine bases – guanine (G) and adenine (A). (Figure 1.2) RNA is another type of nucleic acid that is transcribed from DNA with a few structural differences: deoxyribose is
replaced with ribose, thymine is often replaced with uracil (U), and RNA is often single stranded.
Among nucleic acid bases, uracil and thymine selectively hydrogen bond with adenine, and cytosine selectively binds to guanine. The selectivity by which nucleic acid bases bond together can be used in developing technologies applicable to many industries.

![Figure 1.1: Structure of a single stranded DNA chain.](image)

**Figure 1.1:** Structure of a single stranded DNA chain.

![Figure 1.2: DNA bases A) Adenine (A) and thymine (T) hydrogen bond together, B) Guanine (G) and Cytosine (C) hydrogen bond together.](image)

**Figure 1.2:** DNA bases A) Adenine (A) and thymine (T) hydrogen bond together, B) Guanine (G) and Cytosine (C) hydrogen bond together.

### 1.1.1 Applications of DNA detection and sequencing

The uniqueness of each individual’s genetic code, in addition to the role of nucleic acids in monitoring cellular function, make DNA/RNA sequencing a reliable tool for disease diagnostics,
environmental monitoring, pharmaceutical drug development, forensics, food quality control, and detection of biological warfare agents.

Since DNA plays a significant role in the development of functional proteins and in regulation of cellular functions, presence, absence, variation or expression level of certain genes is closely related to development of certain diseases. As a result, knowledge of individuals’ genetic composition is used in discovery of disease-relevant genes, and developing advanced diagnostic and therapeutic technologies for efficient disease detection and treatment. Diseases that benefit greatly from nucleic acid-based diagnostic technologies are hereditary and multi-factorial diseases involving a genetic factor – cancers [4] and auto-immune diseases [5] – and infectious diseases.

Illnesses caused by genetic disorders are developed due to abnormalities in individual’s genes or chromosomes. Genetic screening is used for detecting chromosomal rearrangements such as microdeletions and interstitial duplications, which are the underlying causes of such disorders.[6] Discovery of tumor-derived DNA or RNA circulating in blood has suggested applications of nucleic acid detection in cancer diagnosis and treatment monitoring.[7] Identifying the DNA or RNA sequences embedded in pathogens such as bacteria and viruses can be used in detection, identification, and drug susceptibility testing of microbial pathogens. [8] Furthermore, the existence of fetal derived DNA in maternal plasma extends the use of DNA detection technology to prenatal diagnostics.

In addition to being an essential part of human and veterinary diagnostics [9], genetic testing plays a significant role in plant pathology through detection and identification of phytopathogens.[10] DNA sequencing and quantitation technologies have a niche in studying the microbial communities in environmental samples such as soil, sediment and water.[11] Furthermore, the efficiency in the design and development of pharmaceutical drugs has been improved through utilization of DNA technologies for correlating drug response with specific genes.[12]

Although genome of different individuals is more than 99% identical, specific regions within the chromosome demonstrate a unique number of short repeated sequences. As the number of these repeated sequences is unique in unrelated individuals, forensic scientists use it to match an
unknown DNA sample to a suspect [13]. As a result, DNA detection technologies are routinely used in identification of criminals in forensic laboratories and in solving paternity disputes.

Effective monitoring of biological contaminants in food products is possible through DNA detection to reveal the trace of cells of different organisms [14].

Biological warfare agents are disease causing pathogens such as bacteria, viruses, or toxins generated by them, which are misused for military or criminal purposes. Bacteria responsible for tularemia, anthrax, brucellosis, plague and botulism [15] have been used as biological warfare agents. Detection of such agents in food and environmental samples has been achieved by bacterial detection and identification using DNA technologies. Different applications of DNA detection is summarized in Figure 1.3.

<table>
<thead>
<tr>
<th>Application</th>
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<tbody>
<tr>
<td>Diagnosis of diseases such as cancer, infectious diseases and hereditary diseases</td>
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<tr>
<td>Plant pathology</td>
</tr>
<tr>
<td>Identification of criminals in forensics laboratories and settling paternity disputes</td>
</tr>
<tr>
<td>Drug development</td>
</tr>
<tr>
<td>Monitoring the quality of food and water</td>
</tr>
<tr>
<td>Detection of biological warfare agents</td>
</tr>
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</table>

Figure 1.3: Summary of applications of DNA detection.

1.1.2 Requirements

In order for a DNA detection technology to be widely used in the previously discussed applications, it must satisfy as many of the following requirements as possible. First, it must be sensitive, capable of detection and identification of small amounts of DNA target present in a sample. Second, it must be selective for identifying nucleic acids of a certain sequence in a sample containing a complex mix of different sequences. Third, it must be multiplexed enabling high throughput analysis to reduce the time and cost of each analysis. Multiplexing can also provide means of comparing relative abundance of different genes in a sample. Fourth, the analysis must be high speed to achieve times scales that are meaningful for each application.
Fifth, miniaturization of the overall device including the sensing and readout components is necessary for point-of-care or in-field applications. Sixth, if the biosensing technology is to be widely used, it needs to be fully automated and integrated within a lab-on-a-chip platform. Seventh, price of the technology – initial investment and operating cost – determines whether the economical benefit provided through DNA detection outweighs its lack of use or use of other technologies.

### 1.1.3 Present-day technologies

#### DNA fingerprinting

DNA fingerprinting is a technique by which a person is identified based on information provided through his/her genome. This technique functions by analyzing particular locations on the chromosomes showing Variable Number Tandem Repeats (VNTRs). VNRTs are a specific location on the genome, where a short DNA sequence is repeated variable number of times. [16] As the number of such repeats is unique between unrelated individuals, their quantity can be used in matching an unknown DNA sample to a suspect or in confirming the relationship of two individuals. This technique has been used in a variety of forensics, and paternity testing applications but it does not provide information about the exact sequence of genes.

#### DNA sequencing

Sequencing technologies allow the determination of exact sequences of nucleotides in a nucleic sample. Most of today’s sequencing techniques are based on a chain termination method developed by Sanger and Coulson. [17] In this method, a single stranded DNA fragment that is to be sequenced is used as a template for synthesis of its complement. During this process, two types of DNA building blocks are available – units that terminate the extension of DNA chain and units that allow extension of the chain. The chain terminating units are similar to DNA building monomers – deoxyribonucleotide triphosphates – with their hydroxyl group replaced with hydrogen. Modified monomers inhibit the addition of new DNA building blocks to the chain, and put a stop to the extension of the DNA chain.

Chain termination units are tagged with fluorescent labels unique to each DNA base. As a result, the original DNA molecule of n bases long is replicated as fragments of 1, 2, 3 …n bases-long
that carry a fluorescent tag. These fragments are then separated by size using gel electrophoresis, creating a pattern reflecting the DNA base sequence. The resulting pattern generated on the gel platform is read out through excitation of fluorescent dyes by lasers and detecting their fluorescence.

Currently available commercial sequencing technologies are based on dye terminator sequencing coupled with capillary-based electrophoresis. [18] These systems allow the sequencing of 20 gigabases of DNA in a single run of 3-4 days with accuracy of 99.9%. In spite of tremendous efforts in making sequencing technologies more affordable and more compact, sequencing machines currently have a footprint of 10 square feet and cost about $400,000. These instruments are still used as mainframe systems in large hospitals and research centers. [19]

Quantitative Real Time Polymerase Chain Reaction (qrt-PCR)

Polymerase Chain Reaction (PCR) is a laboratory technique by which a given DNA segment is selectively amplified. The amount of a particular DNA segment in the sample doubles in subsequent reaction cycles, resulting in millions to hundreds of millions of copies of the desired DNA in one hour, while keeping the amount of undesired DNA unchanged. [20]

PCR can serve as an initial amplification step for DNA finger printing, DNA sequencing or other DNA detection techniques; or it can be directly coupled to optical labeling and readout for DNA detection and quantitation. A technique called quantitative real time PCR (qrt-PCR) has been developed enabling amplification and quantitation of target DNA in a single run.

In contrary to sequencing techniques, qrt-PCR requires a prior knowledge of the sequence of target DNA for selective amplification and detection making it a DNA detection technology rather than a sequencing technology. Using this information, a set of PCR primers – DNA segments capable of initiating the amplification process at a specific location on the DNA – are designed. PCR primers are added to denatured DNA to bind specifically to the desired regions. This marks the sites for amplification. Additionally, DNA building blocks and DNA polymerase – an enzyme enabling DNA replication – are added to the PCR mixture enabling selective target amplification.

As the reactions involved in PCR are temperature-sensitive, a major requirement of a PCR system is the ability to quickly vary the sample’s temperature. In quantitative PCR, fluorescently
labeled DNA segments are incorporated in the reaction mix and are detected using optical detectors to report on the quantity of DNA under amplification.

Currently available PCR systems are fully automatic and provide integrated sample preparation, optical readout and signal analysis modules on a single platform. In a state-of-the-art system, detection and quantitation of up to 16 different DNA targets is possible using a desktop system in one hour.[21] PCR is extremely sensitive allowing detection of DNA from a single cell.[22] As a result of such high sensitivity, PCR is highly susceptible to contaminations present in the experimental environment, making its use problematic in analyzing samples containing large amounts of closely related sequences.[23]

**Hybridization-based assays for the development of the biochip**

DNA biochips are designed to explore a biological sample for particular genetic information. Research on the development of biochips is focused on creation of miniaturized platforms capable of analyzing DNA in a massively parallel approach to increase the analysis speed and throughput while minimizing system’s footprint and cost. Such devices are composed of a fabricated micro-/nano-array of immobilized nucleic acid strands of known sequences called probes. For the purpose of detection and identification of nucleic acid targets of a certain sequence, probe molecules are designed to selectively capture single stranded DNA or RNA targets from solution by forming double stranded DNA. (Figure 1.4)

![Figure 1.4: Schematic representation of a multiplexed DNA biochip used for DNA detection. Single stranded nucleic acid probes are immobilized on the chip. Upon introduction of the complementary target, a measurable signal is triggered.](image)

In addition to a probe recognition layer, DNA chips rely on a transduction mechanism for translating a DNA binding event to a measurable signal. The nature of this signal indicates the type of readout technology employed:
a) Optical detection

Optical microarrays are the most mature biochip technology available to date allowing multiplexed detection of thousands of DNA sequences in a single experiment. In a typical microarray, probe molecules capable of selective DNA capture are immobilized on a glass surface using epoxy-silane or amino-silane or other chemistry. [24] DNA targets conjugated with optical labels are introduced to the microarray. In case of a perfect match, double stranded DNA molecules containing optical labels are formed on specific locations of the microarray surface. In case of a complete or even partial sequence mismatch, DNA-optical label conjugates are washed off the surface so that only double stranded DNA contribute to generation of an optical signal. (Figure 1.5)

![Figure 1.5: Schematic representation of optical detection of DNA. Nucleic acid probes are immobilized on solid surfaces. DNA-optical label conjugates are added to the device creating an optically detectible spot.]

Optical labels can be nanoparticles [25, 26] or chemiluminescence tags [27], but they are mostly the molecules capable of generating a fluorescent signal upon excitation with a light of a certain wavelength [28]. Commercial optical microarrays are capable of analyzing a sample for identification and quantitation of more than 900,000 different sequences. These arrays are capable of distinguishing between genes differing in sequence by a single nucleotide and can quantitate the amount of a particular sequence. [29] In spite of these advantages, microarrays often lack the sensitivity to operate without a PCR amplification step. The complexity added by the PCR step and the high price of optical readout instrumentation, have inhibited the widespread use of microarrays outside the research laboratory.

To overcome the disadvantages of labeled detection – slower diffusion of labeled analyte to the sensor surface, increase in the assay complexity, time, and the reagents used –, to improve the
assay’s sensitivity, and to provide faster analysis at a lower cost, label-free optical detection methods have been explored.

Methods reporting on changes in refractive indices of surfaces as a result of hybridization with complementary targets allow label-free optical detection of DNA.\[30\] The current sensitivity of these devices requires them to be coupled with a PCR system \[31\], but their inexpensive fabrication methods with the possibility of integration in a lab-on-a-chip device make them promising in the field of DNA detection.

Optical readout based on Surface Plasmon Resonance (SPR) is another promising label-free DNA detection approach. In this method, generation of surface plasmons – electromagnetic waves propagating on the boundary of metal-air interface – are manipulated by variations on the sensor’s surface such as DNA hybridization to surface-bound probes. Using these principles, optical DNA detection has been achieved by SPR monitoring \[32\], paving the route for label-free optical detection.

b) Electrical detection

DNA sequencing and detection technologies based on PCR and optical readout are mostly used in large hospital and research labs, but have not been widely spread to doctor’s offices, patient homes or airports. This is mostly due to the high footprint of these instruments along with their high instrumental price and operating cost. Many scientists have envisioned solving these problems by developing biosensors capable of generating an electrical signal directly, without any intermediate steps. As a result, a number of labeled and label-free approaches have been developed for generation of a hybridization-dependant electrical signal. These approaches are explained in great detail in the following chapter.

c) Piezoelectric readout

Another method enabling label-free detection of DNA hybridization involves the use of crystals demonstrating piezoelectric effect as ultrasensitive mass sensors. In a typical assay, a piezoelectric element, often a quartz crystal, is coated with gold and modified with single stranded DNA.\[33\] Capture of target single stranded DNA by the probe recognition layer, results in a measurable change in crystal’s oscillation frequency \[34\]. Developing a fully integrated and multiplexed DNA detection platform employing piezoelectric readout requires additional
progress in microfabrication and microfluidic technologies. This would enable incorporation of pumps, micro-fluidic channels and readout circuitry on a single platform.

1.1.4 Limitations of existing nucleic acids detectors

Among the commercially available technologies, sequencing provides exceptional accuracy, PCR provides exceptional sensitivity and optical microarrays provide a combination of very good selectivity and exceptional multiplexing. Sequencing platforms functioning as mainframe instruments are ideal for major hospitals and research institutes performing pure and applied biological, pharmaceutical and medical research. Sequencing instruments with footprints of tens of square feet and costing hundreds of thousands of dollars are not relevant to low-cost and portable applications.

PCR-based instruments are less expensive and smaller than sequencing equipments but are still irrelevant to applications requiring portability and miniaturization. In addition, analysis of environmental samples containing organisms with closely related but different genes, and analysis of single nucleotide polymorphism are very complex with PCR. Furthermore, high level of multiplexing, enabling simultaneous analysis of hundreds of thousands of genes is not currently possible using PCR.

Optical Microarrays have enabled highly multiplexed DNA detection, which is much more affordable and portable than sequencing and PCR-based platforms. However, DNA microarrays still rely on expensive optical readout and the use of complicated and time consuming algorithms for conversion of optical signals to manageable electrical signals.

In sum, none of the available technologies collectively addresses the requirements described in section 1.1.2: sensitivity, selectivity, multiplexing, speed, miniaturization and automation and cost.

1.2 Thesis Objectives

The objective of this thesis is to overcome the shortcomings of the current technologies to develop a portable, affordable, high-speed selective and multiplexed DNA detection platform.
capable of parallel analysis of thousands of genes, while being sensitive enough for PCR-free operation.

The sensitivity of electronic readout is in principle sufficient to allow direct detection of small numbers of analyte molecules with simple instrumentation. Furthermore, transducers that can directly generate an electrical signal can be easily fabricated in a multiplexed array and coupled to signal analysis and acquisition modules through microfabrication. Electronic instruments for sensitive readout are more compact and less expensive than their optical counterparts, making the development of portable readout equipments less far-fetched.

To create DNA detection devices applicable to point-of-care and in-field applications, a chip-based platform coupled to electronic readout will be developed. More specifically, the following fabrication and detection issues will be addressed.

**Fabrication of multiplexed electronic chips**

In order to take advantage of unique properties of nanomaterials in detection of biomolecules of the same lengthscale, and to enable device miniaturization, we explore the possibility of creating an array of nanotextured electrodes using inexpensive and robust technologies. Furthermore, we answer the question: can this multiplexed electronic chip be used in parallel analysis of disease-related genes?

**Enhancement of detection sensitivity**

A number of current DNA chips relay on PCR for amplification of the analyte prior to its detection. We explore different approaches for enhancing the sensitivity of our system to enable PCR-free detection of clinically relevant amounts of target. Specifically, we ask the following questions: what is the correlation between the sensor’s architecture and its detection sensitivity? Is there a correlation between the properties of the immobilized probe monolayer and the sensitivity of the biosensor?

**Enhancement of system’s dynamic range**

PCR technology demonstrates a dynamic range of 7 orders of magnitude. This enables the development of quantitative or semi-quantitative assays required for a number of medical
applications. We explore different approaches for extending the dynamic range of our DNA detection platform.

Enhancement of system’s speed of operation

For a number of applications including detection of bacterial infections and discovery of biological warfare agents, speed of DNA analysis is extremely important. Most of the existing technologies require long overall assay time in the order of hours. We explore different approaches for achieving total analysis times of less than 30 minutes.

1.3 Thesis Overview

The remainder of this thesis is organized as follows.

Chapter 2 discusses different mechanisms for achieving electrical transduction for DNA biosensing. Chapter 3 discusses the principles of electrocatalytic DNA detection and demonstrates the rationale behind choosing a particular readout method used in the remainder of this work.

Chapter 4 illustrates the design and fabrication of a multiplexed DNA detection platform and tests its performance in analysis of clinically relevant prostate cancer samples. It demonstrates the development of the first multiplexed electrochemical platform used in analysis of patient RNA samples.

Chapter 5 demonstrates the discovery of factors having significant roles in enhancing sensor’s sensitivity. Through manipulation of such factors, we demonstrate the first multiplexed electrochemical platform capable of selectively detecting fewer than 100 DNA target molecules. Chapter 6 discusses ways by which tunability of sensor’s sensitivity can be used to create a device with a dynamic range of 6 orders of magnitude.

Chapter 7 discusses how mathematical modeling is used to design sensors that achieve high-speed DNA detection by decreasing the analyte transport times. Chapter 8 demonstrates the development of hierarchical structures with 100 μm footprints and nanoscale surface features. Furthermore, we showcase the roles these structures played in: 1. decreasing the diffusion-limited transport times of target RNA and 2. improving the sensor’s hybridization efficiency. We
show that through this kind of materials engineering we are able to detect less than 30 bacteria in less than 30 minutes. The thesis concludes with a review of contributions made throughout this work and a discussion of future work.
2 Electrical Nucleic Acids Detection: Literature Review

Previous chapter put forward the challenges involved in addressing the requirements of DNA biosensing. We discussed that electrical detection of nucleic acids has the potential to address such requirements and can be used as the transduction mechanism for biosensing applications. This chapter will review the prior work on electrical DNA detection to adopt a transduction mechanism compatible with a chip-based multiplexed platform.

2.1 Principles of electrical biosensing

All biosensors rely on translating a specific molecular binding event to a usable signal. The essential role of the biosensor is to provide a platform on which capture of specific target molecules results in a measurable signal. In addition to having a variety of applications in different fields, DNA biosensors are particularly interesting since the base pairing between complementary strands is highly selective and robust. In a typical DNA biosensing configuration, single stranded DNA molecules capable of recognizing their complements are immobilized on a surface. The repetitive and uniform structure of DNA molecule allows the organization of such molecules on surfaces to be well defined. The recognition layer, DNA modified surface of the biosensors, affects to a great degree the dynamics of target capture.[35] For this purpose, a number of techniques have been developed for immobilizing DNA on different surfaces - via physical absorption, self-assembly or covalent conjugation- while keeping their affinity for their complementary pair.[36]

Figure 2.1 demonstrates the operation of a hybridization-based sensor: 1. DNA recognition layer is prepared by immobilizing DNA probe molecules on the biosensing platform, 2. Recognition layer is exposed to a solution containing the analyte molecule, 3. After stringent washing to ensure the removal of non-specific analyte, the recognition event is reported using different signal transduction strategies.
Figure 2.1: Schematic representation of electrical DNA hybridization detection.

Different signal transduction strategies based on electrical, optical, or piezoelectric effects play the role of translating molecular recognition events into signals that can be converted to digital information. In cases where the DNA binding is translated directly to an electrical signal, through either an electrochemical reaction or a change in electrical properties of the sensor, electrical signal transduction is achieved. Next section summarizes different classes of electric DNA biosensors.

2.2 Amperometric and voltammetric DNA biosensors

Both amperometric and voltammetric detection involves monitoring DNA hybridization by measuring the resultant changes in the electrochemical current. In both cases, capture of target DNA results in accumulation of electroactive species near the sensing platform generating a measurable Faradaic current. Amperometric techniques monitor the redox current generated by electrochemical reactions at the electrode as the potential is kept constant (Figure 2.2C). Voltammetric techniques use a potential ramp followed by subsequent current measurements to monitor the reaction of electroactive species at the electrode (Figure 2.2A, B). Traditionally, smooth potential ramps similar to that in Figure 2.2A were used for monitoring the redox activity of electrochemical species. More recently, pulsed Voltammetry with a waveform similar to Figure 2.2B is widely used to reduce unwanted charging effects, resulting in more sensitive and reproducible readout.
Sensors rely on different strategies for generating an electrochemical current in case of target capture. A group of sensors rely on hybridization with labeled targets where preprocessing is used for modifying DNA targets with electrochemical labels. As labeling of unknown targets is difficult and adds an additional 2-3 hours to the assay time, several label-free detection strategies are developed. The following sections will introduce some of the leading labeled and label-free detection strategies.

It should be noted that in this thesis, we use the term label-free detection in cases where target molecules are not conjugated with any label prior to hybridization.

**Enzymatic labeling**

A group of amperometric sensors rely on using enzymes as labeling molecules to generate a reductive or oxidative current in the event of DNA capture. Enzymes are protein molecules capable of catalyzing specific chemical reactions. In enzymatic reactions, initial molecules called substrates are converted to new molecules called products (Figure 2.3).
Figure 2.3: Schematic demonstrating electrochemical DNA hybridization detection using enzymatic labeling

Presence of enzymes at the electrode vicinity generates an electrochemical current in different ways:

1. The product of the enzymatic reaction is a redox molecule demonstrating an oxidative/reductive current at a particular potential, which was not present before DNA hybridization.[37]

2. The product of the enzymatic reaction is non-redox but acts as a reducing agent. This allows enzymatic control of metal precipitation by reducing metal ions into metallic films on the surface of the electrode. Using voltammetric techniques, the presence of this metallic coating on the electrode is detected and associated with DNA hybridization.[38]

3. Enzymatic reactions can generate insoluble and insulating products prohibiting the access of solution-borne redox molecules to the electrode. This reduces the electrochemical current following successful DNA hybridization.[39]

Redox labeling

Optical methods often rely on detection of DNA molecules modified with fluorescent labels. Analogous to optical biosensing, redox labels are used in electrochemical biosensing to prepare redox target conjugates. These compounds are capable of generating a characteristic electrochemical response that is present only in case of successful target hybridization.[40] Ferrocene is an electroactive molecule with well-studied electrochemical response and is used for DNA labeling. The detection limits of systems employing ferrocene labeling is determined by
the minimum number of ferrocene molecules that would generate a detectible electrochemical signal.

**Sandwich Assays**

Sandwich assays enable the final DNA duplex to be labeled without having to label the target molecules. In this case, enzymatic labels, redox labels or nanoparticles are added to the DNA duplex by a processing step following the DNA hybridization. Although sandwich assays rely on generation of Faradaic current through enzymatic, redox, or nanoparticles labels, they do not require labeling of target molecules.

In this approach, following the modification of sensor surface with a probe monolayer, target molecules are introduced and hybridized. Probe molecules are designed to be shorter than the target molecules, leaving the top bases of the DNA molecules unhybridized. In order to incorporate an electrochemical reporter on the DNA duplex, DNA molecules complementary to the unhybridized segment of the target molecules are linked to a label. These labeled reporter molecules are reacted with DNA duplexes on the surface of the biosensor. This makes it possible to generate a hybridization-specific electrochemical current.[41] (Figure 2.4)

![Figure 2.4: Schematic representation of a DNA hybridization sensor based on a sandwich assay.](image)

Recent research on nanomaterials has fueled the development of different nanoparticle labels. In particular, colloidal gold nanoparticles are used in creating labeled DNA. As gold nanoparticles have a unique electrochemical signature with well defined oxidation and reduction potentials, their presence can be detected using voltammetry. [42]
Sandwich assays are advantageous over methods involving target labeling and they have achieved detection of as little as 80 molecules. [43] Specificity is a major issue as the labeled reporter DNA gets non-specifically adsorbed on surfaces. In addition, their operation involves several steps, making them labor-intensive and time consuming. This has motivated the development of other label-free methods.

**Electrochemistry of DNA bases**

The purine bases of DNA, guanine and adenine, can be electrochemically oxidized and used for direct detection of target DNA.[44] In a typical assay that relies on monitoring the Faradaic current resulting from guanine oxidation, a large enough potential to oxidize guanine is applied to the probe modified electrode. In order to minimize the background signal from the single stranded probes, guanine bases of the probe DNA are replaced by inosine [45], which is also capable of specific base pairing with cytosine but has a well separated oxidation potential. As a result, guanine-free probe molecules show no electrochemical response, whereas a large electrochemical signal is generated after target hybridization with guanine carrying target strands.

Direct oxidation of DNA bases requires application of large potentials; this places restrictions on the type of samples one can work with. In addition application of large voltages can result in large capacitance currents compromising the Faradaic currents. For this purpose, methods to oxidize target DNA through electrochemical mediators have been developed. A specially interesting approach uses Ru(II) complexes to mediate the oxidation of guanine. [46] The main shortcoming of these assays comes from their sequence dependence, prohibiting them to be used in quantitative biosensing.

**Redox hybridization reporters**

In order to eliminate the need for a redox label, a class of DNA sensors employing solution-borne reporter molecules has been developed. Two classes of reporter molecules are widely used:

1. **Redox intercalators:** Redox molecules having an appropriate size and chemical nature can fit between base pairs of DNA. Intercalators preferably associate with double stranded rather than single stranded DNA, and their presence can be linked to a specific
hybridization event. [47] Voltammetry can be used to measure the Faradaic current associated with intercalator’s presence. Furthermore, the redox reaction of the intercalators can be made catalytic to further improve the device sensitivity.

2. Electrostatic redox reporters

In electrostatic reporter systems, positively-charged electroactive species are electrostatically attracted to the polyanionic DNA. The presence of such compounds in the electrode vicinity is used to generate a Faradaic current. [48] As the magnitude of the resultant current is related to the amount of DNA on the electrode, scanning before and after hybridization reports on occurrence of hybridization.

In order to minimize the accumulation of electroactive species on probe modified electrodes, Peptide Nucleic Acid (PNA) probes are generated for molecular capture. The use of PNA - uncharged analogue of DNA capable of specific base pairing with target DNA- can significantly improve the detection limit of electrostatic biosensors. [49] In addition, the redox reaction of certain electrostatic reporters can be made catalytic to improve the biosensor’s sensitivity.

Electronic beacon

Assays based on electronic beacons use probes with a stem loop DNA structure capable of simultaneous target capture and reporting. (Figure 2.5) Molecular beacons were first used in optical DNA hybridization detection, where hairpin DNA probes with a quenched fluorophore were immobilized on surfaces. In case of DNA hybridization, the probe molecule would undergo a conformational change revealing its fluorescence.[50] This increase in the fluorescence signal reported on the molecular capture event.

Analogous to molecular beacons, electronic beacons are DNA stem-loop capture probes featuring a redox label. In case of successful DNA capture, this stem-loop structure changes conformation altering the electron transfer tunneling distance between the redox label and the electrode. Voltammetry can be used to monitor the changes in the electron transfer efficiency as a result of DNA hybridization. [51]
2.3 *Conductance DNA biosensors*

Direct measurement of hybridization-induced conductance change can be used as a transduction method in electrical DNA sensors. An important class of such devices employs field-effect devices to translate a molecular capture to a change in conductance. Field-effect devices are three terminal electronic devices, where an applied potential at the gate terminal controls the conductance between source and drain terminals. The body of field effect devices is made of semiconducting material, source and drain terminals are coated with metallic contacts and the gate terminal is a metallic electrode separated from the semiconductor body by an insulating film.

The electric field applied at the gate terminal modulates the conductance of the source-drain channel. This electric field is traditionally generated by applying a potential, but it can indeed be provided through accumulation of electric charges. [52, 53] In a field-effect biosensor, surface of the gate insulator is modified with capture probes to enable recognition of complementary DNA strands. In case of successful capture and addition of negatively charged targets on the gate, a measurable conductance change is triggered between source and drain.

Conductivity behavior of nanomaterials is strongly influenced by their surface charge. As a result, a number of highly sensitive field effect devices have been developed using semiconducting nanomaterials. [54, 55] In addition to being ultrasensitive, field-effect devices have the advantage of being reagent free (except for electrolyte) and suitable for integration within lab-on-a-chip platforms. In spite of such advantages, field-effect devices are strongly
affected by the presence of large amounts of non-specific analyte present in biological samples making them unsuitable for analysis of unpurified real-life samples.

2.4 **Impedance DNA biosensors**

In Electrochemical Impedance Spectroscopy (EIS), an AC signal of small magnitude is applied to an electrochemical cell to measure the resistance the cell exerts on current flow, i.e. the impedance. This electrochemical impedance is highly affected by the variations at electrode surface, making EIS a powerful tool in investigation of surface reactions. Such impedance changes are used to translate hybridization events to electrical signals. In a typical assay, EIS is used to monitor the impedance of a probe modified biosensing surface in an electrochemical cell before and after hybridization with a target. Successful hybridization events alter the capacitance of the electrodes resulting in a detectible electronic signal. [56]

Although this method is highly sensitive, it is greatly affected by the high amounts of non-specific analyte in biological samples. This affects the sensor’s specificity and potential use in analysis of biological samples.

2.5 **Conclusions**

In this chapter, we summarized the existing technologies used for translation of DNA hybridization to electrical signal. Since electronic readout requires simple and inexpensive instrumentations, which can be readily integrated on chip devices, it is particularly attractive for development of point-of-care diagnostics and in-field analysis.

Voltammetric and amperometric techniques are particularly interesting since their readout is amongst the simplest. Among these methods, ones that do not require incorporation of covalently bound labels, and can be made catalytic are more rapid, sensitive, and practical. For this purpose, we will use non-covalently bound redox reporters to generate a Faradaic current for voltammetric detection of DNA hybridization. Next chapter will focus on describing different electrocatalytic transduction approaches for achieving label-free electronic DNA detection. Furthermore, it will provide the background for a particular transduction method used in this work.
3 Electrocatalytic DNA hybridization detection: Prior Work

Previous chapter reviewed electrical transduction methods for use in DNA detection. Voltammetric methods coupled with electrostatic redox reporters would enable label-free, rapid and inexpensive DNA detection; However, the sensitivities of many of such methods are limited by the amount of reporter molecules present at the electrode vicinity. To overcome these limitations, we choose a previously developed electrocatatlytic reporter system enabling amplification of Faradaic currents for more sensitive and accurate detection. The remaining sections of this chapter will provide a literature review on electrocatalytic detection of nucleic acids.

3.1 Electrocatalytic readout methods

DNA detection using electrical readout is particularly attractive for development of point-of-need disease diagnostics. Among these methods, label-free electrochemical detection platforms relying on portable, simple and inexpensive instrumentation are excellent candidates for creating practical devices. Majority of such techniques generate a hybridization-related signal by incorporation of a non-covalently bound reporter group, which increases as a result of DNA hybridization. The sensitivity of such techniques is often limited by finite concentration of redox species present at the electrode. Electrocatalysis provides electronic amplification, or gain, which overcomes the sensitivity challenge: hundreds of electrons can readily result from each biomolecular complexation event.

One of such approaches is based on electrocatalytic oxidation of guanine bases of DNA. In this case, guanines are not directly oxidized by an electric potential; instead, they serve as a substrate for electrocatalytic oxidation by Ru(bpy)$_3$$^{3+}$.[57] Ru(bpy)$_3$$^{3+}$ oxidizes guanine by undergoing reduction resulting in the reduced form of the complex, Ru(bpy)$_3$$^{2+}$, which is detectible by Cyclic Voltammetry (CV). In spite of excellent speed, good sensitivity and simplicity, this technique is highly sequence-dependant. This makes it unsuitable for detection of closely related DNA targets present in clinical and environmental samples.
Another approach employs an intercalating redox molecule that reports on the quality of stacking of DNA base pairs. In this assay, the base pair stacks of double stranded DNA mediate the transport of charges from the electrode to the redox intercalator. In case of perturbations in base pair stacks caused by single base mismatches, electrochemical signals are greatly diminished. In a typical assay, methylene blue serves as a redox intercalator that undergoes electrochemical reduction. Methylene blue can be coupled to an oxidizing agent, ferricyanide, to achieve electrocatalysis. This method is very interesting for sensing DNA damage, mutations or mismatches. This system solely reports changes in double stranded DNA and does not allow detection and identification of DNA analyte in a sample solution. [58]

A particularly interesting electrocatalytic system that overcomes the limitations of the previous systems employs Ru(NH$_3$)$_6$$_{3+}$ and Fe(CN)$_6$$_{3-}$ to report DNA hybridization. [59] This approach relies on the primary electron acceptor Ru(NH$_3$)$_6$$_{3+}$, which is electrostatically attracted to the electrode surfaces at levels correlated with the amount of bound nucleic acid. The inclusion of Fe(CN)$_6$$_{3-}$ during electrochemical readout serves to regenerate the Ru(III) substrate, as the Fe(III) species is even easier to reduce, but it is electrostatically repelled from the electrode and thus only undergoes chemical reduction by Ru(II). (Figure 3.1)

![Figure 3.1: Schematic representation of the electrocatalytic system used herein. Ru(NH$_3$)$_6$$_{3+}$ is electrostatically attracted to immobilized single stranded DNA in amounts related to negative charges present on the electrode. Fe(CN)$_6$$_{3-}$ catalyzes the reduction of Ru(NH$_3$)$_6$$_{3+}$ providing signal amplification. [59]](image)

The method described here is a sensitive electrocatalytic process for monitoring DNA/RNA hybridization. Particularly, the versatility and specificity of the system have been demonstrated in detection and identification of synthetic oligonucleotides, PCR products and RNA
transcripts.[59] The label-free nature of this assay, its simplicity and excellent reporting speed make it suitable for use in a practical device. As a result, this system will be used throughout this work as the readout method of choice.

3.2 Electrocatalytic hybridization detection using macroelectrodes

The employment of the Ru(III)/Fe(III) electrocatalytic reporter system for DNA detection was first demonstrated on DNA-modified macroelectrodes. This section will summarize the steps involved in DNA detection using this platform.

3.2.1 Three-electrode electrochemistry

In order to use voltammetric techniques for monitoring the redox currents associated with DNA hybridization, an electrochemical cell is employed. In a three electrode setup (Figure 3.2), electric potential is applied between the working and reference electrodes to generate the desired reactions at the working electrode.

Reference electrodes exhibit large input impedance inhibiting them from drawing large currents. As a result, potential of the reference electrode remains constant and close to its open circuit value. This electrode serves as a point of reference in the circuit.

The current is passed between the working and auxiliary electrodes. Auxiliary electrodes are made of non-reactive materials to avoid the generation of substances that could migrate to the working electrode and disturb its operation. In a typical voltammetric experiment, conventional gold working electrodes (macroelectrodes), platinum wire auxiliary electrodes and Ag/AgCl reference electrodes are used as the building blocks of the electrochemical cell. (Figure 3.2)
Figure 3.2: Schematic representation of an electrochemical cell A) electrical connections involved in a three-electrode cell, B) schematic representation of a practical three electrode setup containing (from left) working, reference and auxiliary electrodes.

The bio-recognition events resulting in electrocatalytic signals occur at the working electrodes. In order to translate a molecular recognition event such as DNA hybridization, the working electrode is modified with nucleic acid probes capable of selective analyte capture. This process will be discussed in detail in section 3.2.3.

3.2.2 Voltammetric readout

Electrocatalytic currents generated on DNA-modified electrodes are often measured using voltammetric techniques. Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) are standard techniques used for this purpose. This subsection provides an introductory background on these methods.

Cyclic Voltammetry (CV)

Cyclic Voltammetry is an electrochemical technique where the potential waveform in Figure 2.2A is applied between the working and reference electrodes and the resultant current is measured between working and auxiliary electrodes to generate a current versus potential curve. Usually the potential is varied linearly in time at scan rates of 10 mV/s-1000 V/s. After reaching a certain time, the direction of the scan is reversed.
Figure 3.3 shows a typical CV curve of a reducible redox complex measured using gold macroelectrodes. In this case, if we start scanning from a positive enough potential, where no electrochemical activity is present, only non-faradaic currents are observed. Once the potential is increased passed the standard potential of the redox analyte, appreciable currents start to flow. As the potential is further scanned in the negative direction, the surface concentration of reducible analyte decreases. This increases the analyte flux to the sensor’s surface. As the surface analyte concentration approaches zero, analyte flux reaches a maximum. Following this, the sensor’s surface gets depleted of analyte to undergo reduction, resulting in a decrease in reduction current. The diffusion profile of the analyte in the electrode vicinity results in the peak reduction current observed in Figure 3.3.

![Figure 3.3](image)

**Figure 3.3: Schematic representation of a typical Cyclic Voltammetry (CV) scan of a reducible electrochemical complex. Characteristic reduction and oxidation curves are observed in the CV scan.**

Once the scan direction is reversed towards the positive direction, the already reduced analyte undergoes oxidation generating a Faradaic current of the reverse polarity. As the analyte flux to the electrode increases, the oxidation current increases until the electrode gets depleted of oxidizable analyte. This results in a peak similar to the reduction case with a reversed polarity.

The peak current, $I_p$, of a reversible redox couple analyzed on standard macroelectrodes is given by:

$$ I_p = (2.69 \times 10^5)n^{3/2}AD_0^{1/2}C_0^{1/2}v^{1/2} $$
Where \( n \) is the number of electrons involved in the electrochemical processes, \( A \) is the electrode area, \( D_0 \) is the diffusion coefficient of the analyte, \( C_0^* \) is the initial analyte concentration in the reduced or oxidized form, and \( v \) is the scan rate.[60]

It should be noted that when ultramicroelectrodes, electrodes with dimensions smaller than 25 \( \mu \text{m} \), are used in obtaining CV curves, a different behavior is achieved. These electrodes take advantage of radial diffusion, which results in sigmoidal CV responses. These CV responses demonstrate limiting currents rather than peak currents.

**Differential Pulse Voltammetry (DPV)**

Pulse techniques are used to reduce the electrochemical background currents generated by capacitive charging. Since charging currents decay at a much faster rate than Faradaic currents, if the current is measured at the end of the potential pulse, the effects of charging currents are eliminated.

To further reduce the electrochemical background, DPV samples the current in the beginning and at the end of each pulse and calculates the current difference. This results in increased analysis sensitivity.

**Figure 3.4:** Schematic representation of a typical Differential Pulse Voltammetry (DPV) scan of a reducible electrochemical complex. A characteristic reduction peak is observed.

In a typical DPV curve demonstrating a reductive process, at potentials more positive than the reduction potential no Faradaic processes are present resulting in a current value close to zero. As potential is increased, analytes start to undergo reduction but there is still no appreciable change in the reduction rate throughout the pulse duration. This results in a small differential
current. Beyond a certain point in potential, where appreciable Faradaic currents are generated, currents increase during pulse duration as a result of increased analyte flux. This causes an increasing differential current until the electrode surface experiences depletion of reducible analytes. This results in a distinct reduction peak.

3.2.3 **Probe deposition**

Research groups interested in studying the electrochemical response of DNA-modified surfaces have developed methods for modifying gold surfaces using short oligonucleotides.[61, 62] Particularly, in order to immobilize DNA probes on the gold working electrodes in a robust and reproducible fashion, they are modified to have a –SH (thiol) group on their 5’-terminus. [63]

Upon deposition on gold electrodes, thiolated terminal of the molecules are chemisorped on the substrate, while the DNA portion of the molecules are standing parallel to each other and away from the substrate forming a self-assembled monolayer (SAM). DNA probe monolayer immobilized on working electrodes through thiol-gold chemistry is stable under electrochemical potentials needed for electrocatalytic analysis and can be used for identification of its complementary target sequences.

The density and quality of DNA SAMs are often controlled by coadsorption of short thiolated molecules of mercaptohexanol. In addition, coverage of the electrode by the DNA probe is highly dependent on the amount of divalent cation used in the deposition solution. As a result, probe deposition is typically performed in a solution containing the DNA probe, mercaptohexanol and the divalent cation, Mg^{2+}. In order to achieve efficient DNA hybridization, these elements are suspended in phosphate buffer of PH 7. [59]

3.2.4 **DNA detection**

Detection of DNA hybridization is performed by measuring the electrocatalytic current of a probe modified working electrode generated in the presence of Ru(III)/Fe(III). As such current is related to the amount of DNA-associated negative charge immobilized on the electrode, CV measurements are performed before and after incubation with a sample target. As target incubation is followed by stringent washing, only hybridization with complementary targets results in addition of negative charges and enhancement of the electrocatalytic signal.
Figure 3.5: Hybridization detection by electrocatalysis. CV scans are obtained before (dotted) and after (solid) hybridization of a DNA probe-modified macroelectrode with complementary DNA targets in a solution containing Ru (III) and Fe(III). Inset demonstrates CV curves before (dotted) and after (solid) incubation with complementary targets in the presence of Ru(III) only. Adapted with permission from reference [59] Copyright [2003] American Chemical Society.

Figure 3.5 demonstrates the CV curves of a DNA modified electrode before and after incubation a complementary target. These CV curves show an irreversible electrochemical signal with a distinct reduction peak typical of electrocatalytic signals. Furthermore, it is evident from this graph that there is an increase in the peak current and an increase in the amount of integrated charge as a result of formation of double stranded DNA on the electrode surface.

In order to showcase that the generated signals are indeed catalytic, CV measurements are obtained in a Ru(III)-only solution in the absence of the amplifying Fe(III) complex. (Figure 3.5-inset) These CV curves demonstrate a reversible behavior resulting in distinct reduction and oxidation peaks. In addition, current magnitudes and current changes are significantly smaller than the case where electrocatalysis was present. These differences confirm that Fe(III) indeed catalyzes the electrochemical reaction and its presence is necessary for sensitive electrochemical DNA detection.

The described electrocatalytic scheme coupled with gold macroelectrodes allowed detection of 10 nM target concentrations. [59]
3.2.5 *Electrocatalytic DNA detection using a nanowire platform*

In order to take advantage of sensitivity enhancements provided by nanoscale sensors, the Ru(III)/Fe(III) reporter system was used in an electrochemical setup where the gold macroelectrodes were replaced by Nanoelectrode Ensembles (NEEs). The performance of 2D and 3D gold NEEs in electrocatalytic DNA detection was compared with the performance of gold macroelectrodes. (Figure 3.6)

![Figure 3.6](image)

Figure 3.6 Demonstration of hybridization detection using different electrode platforms. Electrodes are modified with single stranded DNA probes (-) and are exposed to complementary DNA strands (+). CV curves in the presence of Ru(III)/Fe(III) reporter groups are obtained before and after hybridization with complementary targets. Experiments are performed on A) 3D NEEs, B) 2D NEEs, C) planar macroelectrodes. Reproduced with permission from reference[64]. Copyright [2004] American Chemical Society.

It is evident from CV curves of Figure 3.6 that 3D NEEs achieved the highest hybridization-related signal changes among the three electrodes when challenged with identical target
solutions. The detection limit of the 3D NEE platform was measured to be 1 pM, 4 orders of magnitude lower than that achieved using macroelectrodes. [64]

3.3 Conclusions

This chapter reviewed the prior work in the field of electrocatalytic DNA hybridization detection. It discussed in detail an electrochemical reporter system employing Ru(NH₃)₆³⁺, an electrostatic redox reporter, in combination with Fe(CN)₆³⁻, an oxidizing agent providing electrochemical amplification. This reporter group translated DNA hybridization to electrochemical signals through reporting the amount of negative charges accumulated on the electrode surface.

We reviewed the use of the Ru(III)/Fe(III) reporter system in detecting DNA hybridization using probe modified gold macroelectrodes with reported detection limit of 10 nM. Another interesting platform used Nanoelectrode Ensembles (NEEs) modified with capture probes and coupled with the same electrocatalytic readout strategy to report DNA hybridization. This platform enabled detection of 1 pM DNA targets, demonstrating 4 orders of magnitude enhancement in sensitivity.

In spite of the sensitivity enhancements, the NEE-based system does not function in a multiplexed fashion. As a result, it is unfit for clinical applications, where a sample needs to be quickly and simultaneously analyzed for a number of genetic biomarkers. Furthermore, these platforms are fabricated using cumbersome bottom up methods prohibiting their large-scale and low-cost fabrication.

Chapter 4 of this thesis will provide a number of solutions for addressing the multiplexing challenges of this platform. Furthermore, to achieve the sub-femtomolar sensitivity required for medical diagnostics, ways of enhancing the device sensitivity through reengineering the sensor structure will be discussed in chapter 5.
4 Multiplexed Biosensing Using an Electronic Chip

The previous chapters provided a literature review of the field of DNA biosensing and presented a detection assay involving label-free electrical DNA detection. The label-free and catalytic nature of this assay eliminated the need for complex sample preparation and signal acquisition instruments. This made the electrocatalytic readout method ideal for sensitive, high-speed, miniaturized and low-cost sensing.

The system discussed and validated in chapter 3, originally used macroelectrodes as the sensing electrodes and later turned to Nano-electrode Ensembles (NEEs) to enhance the sensitivity. None of these sensing electrodes was made as a multiplexed platform capable of parallel analysis of multiple analytes. Furthermore, the integration of such electrodes within lab-on-a-chip platforms would be difficult and expensive. This chapter will discuss ways of overcoming the challenges involved in multiplexing, miniaturization, and integration of DNA detection platforms.

4.1 The need for multiplexing

Nucleic acids are biomarkers of central importance as their presence, absence or expression level provides important diagnosis and prognosis information for cancer management. Recent studies have identified genetic aberrations leading to over expression of a group of biomarkers in cancer-related cell lines or tumors representing a unique cancer signature.[65] Parallel analysis of such biomarkers can greatly aid physicians in taking efficient and patient-specific therapeutic paths.

Analysis of nucleic acids based on electronic readout has been cited as a promising approach that would enable a new family of chip-based devices with appropriate cost and sensitivity for medical testing.[35] In spite of remarkable advances in this area as well as related fields working towards new biosensing technologies, [43, 55, 66-70] no multiplexed chip has yet shown direct electronic detection of biomarkers in clinical samples. The challenges that have limited the implementation of such devices primarily stem from the difficulty of generating cost-effective multiplexed systems, which are highly sensitive and specific, and allow rapid point of care sample analysis.
In the present chapter, the development of biosensors based on a multiplexed array of microelectrodes will be discussed in detail. The chapter will conclude with the demonstration of a multiplexed system used in parallel analysis of prostate cancer-related biomarkers in cell lines and patient tumor tissues.

4.2 Design and fabrication of multiplexed chips

Conventional photolithography is a well-developed process as it is responsible for the tremendous advances in the microelectronic and optoelectronic industries. In order to make a robust and cost-effective biosensing platform with the possibility of mass production, photolithography is used to create a multiplexed biosensing chip on a Si/SiO$_2$ substrate. This passive silicon chip has the potential to be integrated within lab-on-a-chip (LOC) devices with built-in sample preparation and active signal processing.

Figure 4.1 shows the schematic representation of the passive electronic chip. This chip is developed through photolithography using a three layer photo-mask created using CAD tools. First mask layer is used for patterning the gold electrodes creating an 8-plex array, second layer is used for opening 500 nm circular dielectric vias through a SiO$_2$ passivation layer, third layer is used for removing the insulating layer on the bond pad area.

Figure 4.1: Schematic representation of the microfabricated chip serving as a template for placing microelectrodes in an array format. Apertures are created in the passivation layer at the tip of the gold electrodes. These apertures are electrically addressed via large square gold pads at the chip edge. (Reprinted with permission from reference [71] Copyright [2009] American Institute of Physics)
Following the mask fabrication, a 350 nm thick layer of gold is evaporated using an electron beam evaporation system. This gold layer is photolithographically patterned through a lift-off process using a negative photoresist to create an array of electrically independent gold wires on the Si/SiO$_2$ substrate. The narrow, micrometer-wide end of the wires houses metallic microelectrodes, while the millimeter-wide end provides the means for connection to off-chip readout instrumentations.

The entire chip is covered with a 500 nm-thick, pinhole-free layer of insulating SiO$_2$ using Chemical Vapor Deposition. Finally, 500 nm-5 μm circular apertures are exposed using a stepper mask. Following exposure and development, the apertures are dry etched to expose the underlying gold layer. This step in the chip fabrication defines the template for deposition of metallic electrodes using bottom-up fabrication methods. The fabrication of the designed chips was performed at the Canadian Photonics Fabrication Center (CPFC).

The fabricated chips (Figure 4.2) show sufficient gold to substrate and SiO$_2$ to gold adhesion resulting in a robust platform for solution electrochemistry. Defects in the CVD SiO$_2$ film were minimal, occurring on less than 4 locations per 3” wafer.

![Fabricated multiplexed chip](image)

**Figure 4.2: Fabricated multiplexed chip** A) Photograph of the 3” silicon wafer patterned to create the multiplexed chip device B) SEM image of the patterned silicon chip C) circular aperture imprinted on an on-chip gold wire.

It is extremely important for the dielectric vias to reach all the way to the underlying gold layer. This is validated by measuring the electrochemical response of the pores using Cyclic Voltammetry in a solution containing 3 mM Ru(NH$_3$)$_6^{3+}$, 25 mM sodium chloride and 25 mM...
phosphate buffer. This technique reveals electrochemical reduction at around -0.2 V versus Ag/AgCl as a reference electrode. This is the signature response of Ru(NH₃)₆³⁺ in a pore microelectrode. This confirms the presence of a conductive surface at the bottom of the pore. (Figure 4.3).

![Cyclic Voltammetry](image)

**Figure 4.3: Ru(NH₃)₆³⁺ Cyclic Voltammetry of the pore. CV is generated in a three electrode system with Ag/AgCl as the reference electrode.**

### 4.3 Electrode fabrication through electroless deposition

Electroless gold deposition is used to create three-dimensional metallic electrodes within the pores of the chip-based array. This method is primarily chosen due to its simple operation requiring no external equipment.

For health and safety purposes, plating is performed in a non-cyanide gold bath containing an aqueous mixture of 12 mM hydroxylamine (NH₂OH) and 7 mM hydrogen tetrachloroaurate (III) (HAuCl₄). [72] The bottom section of silicon chips with exposed gold disks are held in the plating solution while being mildly agitated for 5 minutes. As a result, three-dimensional gold electrodes are selectively grown out of the on-chip dielectric pores using an autocatalytic process, creating electrically accessible microelectrodes (Figure 4.4).
Figure 4.4: SEM images of gold Microelectrodes deposited using electroless deposition. Top-view zoomed out image (left), side-view zoomed out image (middle) and zoomed-in image (right) of the same microelectrode are presented.

4.4 Electrode fabrication through electrodeposition

Results of the previous section showed how electroless deposition of gold was used to place microelectrodes at predefined locations on the multiplexed chip. In spite of this success, electroless deposition does not provide an easy way of individual manipulation of electrodes on the same chip. As one of my major research goals is studying the correlation between electrode morphology and biosensing capability, fabrication methods enabling manipulation of individual electrodes on the same chip should be investigated. For this purpose and to improve the electrode to electrode reproducibility of the system, electrodeposition will be explored in this section.

Electrodeposition is a technique used for selective coating of conductive surfaces with a desired material. It uses an electrical current to reduce metallic ions present in a conductive electrolyte solution to create solid metallic structures on a particular conductive surface. Electrodeposition at the cathode is given by:

\[ \text{M}^{+n} + n\text{e}^- \rightarrow \text{M} \]  \hspace{1cm} (2.1)

Where \( \text{M}^{+n} \) represents a metallic ion having oxidation number \( +n \), \( \text{e}^- \) is an electron accepted by the metallic ion, and \( \text{M} \) is the resultant solid metal. Electrodeposition allows systematic control of electrode size, morphology, and composition through manipulation of deposition time and potential, and variation of type and concentration of the metallic ion and supporting electrolyte.
4.5 **Noble metal electrodeposition**

Noble metals are known to create strong chemical bonds with compounds terminating with a thiol (-SH) group.[73] In order to achieve immobilization of nucleic acids on solid substrates, researchers have developed techniques by which DNA molecules can be linked to a thiol group [63]. In this case, the thiolated terminal of the molecules are chemisorbed on the substrate, while the DNA portion of the molecules are standing parallel to each other and away from the substrate forming a self-assembled monolayer (SAM). Since such SAMs form quickly, result in a well-defined and reproducible surface, and are stable under normal laboratory conditions, Platinum, Palladium and Gold electrodes are fabricated using electrodeposition.

4.5.1 **Methods**

During my studies, electrodeposition is performed in a three-electrode electrochemical cell consisting of a series of on-chip gold working electrodes, an off-chip Palladium wire counter electrode and an off-chip Ag/AgCl reference electrode. (Figure 4.5) All experiments are performed using a BAS potentiostat.

All metallic structures are fabricated in an aqueous solution of 5 mM metallic salt mixed with 0.5 M HCl as the supporting electrolyte. These structures are fabricated at a potential of -100 mV versus Ag/AgCl for a duration of 300 s using DC Potential Amperometry (DCPA) technique available through the BAS software. Most experiments are performed by suspending the lower part of the chip in an electrodeposition bath. The bath contains the plating solution, reference and auxiliary electrodes. Potential is applied to the on-chip electrodes by connecting the working electrode terminal of the BAS system to the on-chip gold pads using micrograbber clips.
4.5.2 Results

SEM images of Figure 4.6 demonstrate the microelectrodes created using electrodeposition methods of subsection 4.6.15. Although electrodeposition of all three metals was performed under similar conditions, they differ significantly in size and morphology. Platinum electrodeposition results in smooth hemispherical electrodes (Figure 4.6A), rough hemispherical electrodes are obtained in Palladium electrodeposition (Figure 4.6B) and rough fractal structures are obtained in gold electrodeposition (Figure 4.6C).

Electrodeposition of each metal occurs at a different rate, in which gold is the fastest and platinum is the slowest reducing metal among the three. This is consistent with previous reports of gold having the most positive reduction potential making it more easily reducible.[60]

Results of Figure 4.6 point towards a correlation between electrodeposition rate and morphology. This serves as a foundation for building highly tunable microelectrodes using each metal.

Figure 4.5: Schematic representation demonstrating on-chip electrodeposition. Potential is applied to apertures via large gold pads. External reference and auxiliary electrodes are added to a solution covering the electrodes to create a three-electrode electrochemical cell.

Figure 4.6: SEM images demonstrating electrodeposition of microelectrodes of different composition. A. Platinum electrode deposited from H$_2$PtCl$_6$, scale bar: 3 μm, B. Palladium electrode deposited from H$_2$PdCl$_4$, scale bar: 5 μm C. Gold electrode deposited from
HAuCl₄, scale bar: 2 μm. All of the electrodeposition trials were performed at -100 mV with respect to an Ag/AgCl electrode in a 0.5 M HCl electrolyte solution.

4.6 Palladium electrodeposition

An extensive analysis reveals that Pd provides the best performance; the electrodes made from this material are highly reproducible, stable, and well-behaved when modified with thiolated probe molecules for capture of target sequences. This is consistent with prior work demonstrating that this metal can participate in stronger interactions with thiolated species than Au or Pt.[74]. As a result, we choose Palladium to study how electrode nanostructure size and topography is manipulated using the axes of time, electroplating potential, reagent concentration, and supporting electrolytes.

4.6.1 Methods

During my studies, electrodeposition is performed using methods described in subsection 4.5.1. Briefly, electrodeposition is performed in a three-electrode electrochemical cell consisting of a series of on-chip gold working electrodes, in addition to an off-chip Palladium wire counter electrode and an off-chip Ag/AgCl reference electrode.

The structures of Figure 4.7A are fabricated (from left) at 0 mV, -100 mV and -250 mV in 5 mM H₂PdCl₄ and 0.5 M HCl for 150 s. Structures of Figure 4.7B are fabricated by (from left) 20, 125 and 500 seconds of plating at -100 mV in 5 mM H₂PdCl₄ and 0.5 M HCl. The structures in Figure 4.7C are plated in a solution of 5 mM H₂PdCl₄ and 0.5 M HClO₄ at -100 mV for (from left) 5 s, 10 s and 40 s. Structures of Figure 4.7D are fabricated (from left) in 5mM H₂PdCl₄ + 0.060M HCl, 5mM H₂PdCl₄ + 0.030M HCl, 5mM H₂PdCl₄ + 0.015M HCl at -100 mV for 150 s.

4.6.2 Results

Figure 4.7 shows how electroplating time, electroplating potential, reagent concentration, and supporting electrolytes are manipulated to program electrode size and morphology.

When low potentials are used, very smooth microelectrodes are generated (Figure 4.7A). When higher potentials are used, microelectrodes with extensive nanostructuring are produced. Increasing the plating potential accelerates the electrodeposition kinetics. This promotes
generation of rougher structures, where growth proceeds outwards faster than internal voids can be filled in.

The top-view diameter and height of the electrodes are proportional to the duration of electrodeposition. (Figure 4.7B) The longer the deposition time, larger amounts of ions are reduced to form a larger structure.

The supporting electrolyte used for electrodeposition also exer tes a significant effect on microelectrode nanostructuring (Figure 4.7B and C). Deposition in the presence of HCl produces structures that are dense and nanorough on the size scale of 100-300 nm, while the use of HClO₄, or the absence of electrolyte, yields finer structures nanorough on the size scale of 20-50 nm.

Figure 4.7D indicates that HCl is an inhibitory electrolyte that slows the growth of the nanotextured microelectrodes (NMEs) by suppressing the ionization of the palladium (II) salt. When HClO₄ is used as an electrolyte, growth proceeds without inhibition and therefore the fast electrodeposition observed in the presence of this electrolyte yields more fractal structures as the growth of tree-like structures occurs efficiently. (Figure 4.7C)[75]

Figure 4.7: Programmable nanostructured microelectrodes (NMEs). (A) SEM images illustrating the effect of deposition potential on structure morphology/degree of
nanostructuring. Structures are fabricated (from left) at 0 mV, -100 mV and -250 mV in HCl for 150 s. (B) SEM images illustrating the effect of deposition time on the structure size. The same aperture is imaged after 20, 125 and 500 seconds of plating at -100 mV in HCl. (C) SEM images illustrating the effect of deposition time and supporting electrolyte type on plating rate. The structures are plated in HClO₄ as supporting electrolyte at -100 mV for (from left) 5 s, 10 s and 40 s. (D) SEM images illustrating the effect of palladium ion/supporting electrolyte ratio on NME morphology. Structures are fabricated (from left) in 5mM H₂PdCl₄ + 0.060M, 5mM H₂PdCl₄ + 0.030M HCl, 5mM H₂PdCl₄ + 0.015M HCl at -100 mV for 150 s. All scale bars correspond to 2 microns.[75]

Furthermore, our experimental data demonstrate that electrodeposition current is proportional to the square root of the electrodeposition time. This kind of behavior indicates that electrodeposition processes in our system involve instantaneous nucleation followed by 3D growth. Nucleation is mainly dependant on the properties of the electrode substrate, whereas growth kinetics is highly dependent on the electrodeposition parameters[31]. Furthermore, our experiments indicate that high growth rates result in sensors with open fractal morphologies; however, lower growth rates result in closely packed hemispherical structures. Particularly, we observe that increasing the palladium to HCl concentration ratio and increasing the electrodeposition overpotential increase the electrodeposition rate and move the structures towards fractal morphology. Such morphological variations are caused as a result of counterbalancing effects related to electrodeposition rates[39] and the rate and direction of diffusion of adatoms on the growth surface during deposition.[36]

4.7 Electrochemical response of on-chip microelectrodes

In order to test the possibility of using these electrodes as robust and reproducible electrochemical biosensors, their electrochemical response is measured. Two classes of Palladium electrodes are chosen and analyzed using two different electrochemical compounds. Nanorough hemispherical electrodes (Figure 4.8 top) are scanned in a 10 mM solution of Fe(CN)₆⁴⁻, and nanostructured fractal electrodes (Figure 4.8 bottom) are scanned in a 3 mM solution of Ru(NH₃)₆³⁺ using Cyclic Voltammetry in a three electrode setup described in section 3.2.14.5.2.
Figure 4.8: SEM images of eight structures are shown to illustrate the high degree of reproducibility from electrode to electrode. In addition, cyclic voltammograms (CVs) for 10 mM Fe(CN)$_6^{4-}$ are shown for the structures at top, and cyclic voltammograms (CVs) for 3 mM Ru(NH$_3$)$_6^{3+}$ are shown for the structures in the bottom row.

It is evident from the results of Figure 4.8 that the fabricated electrodes exhibit ideal microelectrode behavior and maximized current density and signal magnitude while keeping
importantly, the fabrication of these electrodes is highly reproducible, with microelectrodes consistently exhibiting peak limiting currents differed by less than 5%.

4.8 Electrode functionalization using thiolated DNA probes

Using methods similar to those described in section 3.2.2 for immobilization of thiolated probes on gold macroelectrodes, we modify the electrodes of Figure 4.7 with DNA probes. Precisely, a 10 μL solution containing 5 μM single stranded thiolated DNA, 20 mM MgCl2, 25 mM sodium chloride and 25 mM phosphate buffer (25/25) is deposited on the on-chip electrodes for one hour in a dark humidity chamber at room temperature.

In order to verify the adsorption of DNA probes on the NME surface, we monitor the oxidation of a negatively charged electroactive compound, Fe(CN)6^{4-}. This is demonstrated in Figure 4.9.

![Figure 4.9: Verification of thiolated DNA immobilization using electrochemical techniques. CV scans of NMEs in 10 mM Fe(CN)_6^{4-} before (red) and after (black) functionalization with thiolated DNA.](image)

From CV curves of Figure 4.9, it is evident that deposition of negatively charged DNA on the electrode prohibits the access of Fe(CN)_6^{4-} and reduces the Faradaic current. This is explained by electrostatic repulsion of Fe(CN)_6^{4-} from the negatively charged DNA film. This behavior is line with results described in literature in immobilization of DNA on gold macroelectrodes. [76] This confirms that interaction of thiolated DNA with Palladium electrodes is similar to its interaction with gold.
4.9 Electrocatalytic readout using on-chip microelectrodes

In order to validate that the currents generated from DNA-modified microelectrodes are indeed catalytic, we look at the CV scans of such electrodes in the presence and absence of the catalytic complex Fe(CN)$_6^{3-}$. (Figure 4.10)

![CV scan](image)

**Figure 4.10: Demonstration of Electrocatalysis of on-chip microelectrodes. CV scans are generated on a double stranded DNA-modified palladium electrode using a solution of 3 μM Ru(NH$_3)_6^{3+}$ (dotted) and a solution of 3 μM Ru(NH$_3)_6^{3+}$ and 2 mM Fe(CN)$_6^{3-}$ (solid).**

Figure 4.10 demonstrates the CV curves of a double stranded DNA modified palladium electrode in the presence of Ru(NH$_3)_6^{3+}$ only and in the presence of Ru(NH$_3)_6^{3+}$ and Fe(CN)$_6^{3-}$. This figure clearly demonstrates the signal amplification provided through Electrocatalysis. Where Ru(NH$_3)_6^{3+}$ is the only reporter molecule, there is no distinct Faradaic current, and the generated current is due to the electrochemical background. Once Fe(CN)$_6^{3-}$ is added, there is a clear generation of reductive current rising above the electrochemical background. Since these currents are generated on ultramicroelectrodes, reduction peaks demonstrated in Figure 3.5 are replaced with limiting currents.

4.10 Detection of prostate cancer-related nucleic acids

The ultimate goal of this chapter is detecting gene fusions that occur in early stages of prostate cancer development. Recent studies indicate that a chromosomal translocation causes ERG and TMPRSS2 genes to fuse and create a hybrid gene ERG:TMPRSS2[77-81]. This hybrid gene
results in expression of about 20 different sequence types, which are present in at least 50% of prostate tumors.[65, 82, 83]

As a result, distinguishing the fusion sequences from their half-complementary wild type counterparts is extremely important in prostate cancer diagnosis. For this purpose, we use our chip-based platform in analyzing samples containing synthetic DNA targets with sequences representing 1. ERG: TMPRSS2 fusion gene, 2. ERG wild type gene, and 3. control target. The sequences of these targets are presented in subsection 4.10.1.

4.10.1 Methods

Electrodes are fabricated using protocols described in subsection 4.6.1 to create the structures demonstrated in Figure 4.7 C3.

Following electrode fabrication, identical solutions containing nucleic acid probes are deposited on three different chips. These solutions typically contain: thiolated single stranded fusion probe of sequence SH-ATA AGG CTT CCT GCC GCG CT, magnesium chloride, 25 mM sodium phosphate (pH 7), and 25 mM sodium chloride (25/25 buffer). These solutions are deposited on the area of the chips housing the electrodes using a manual pipette. Chips are incubated with these solutions in a dark humidity chamber for 30-60 minutes at room temperature.

Chips are washed after probe deposition for 10 minutes using 25/25 buffer. Probe-modified electrodes are scanned using DPV in a catalytic solution containing 10 µM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻. Following the first electrochemical readout and washing the chips with an aqueous solution of 25 mM sodium chloride and 25 mM phosphate buffer (25/25), the three chips are incubated with three different samples at 37°C for one hour. These samples typically contain 100 fM of DNA targets in 25/25 buffer. The sequences of DNA targets included in each sample are listed below:

ERG:

5’ TCA TAT CAA GGA AGC CTT AT 3’

TMPRSS2:ERG type III fusion:

5’ AGC GCG GCA GGA AGC CTT AT 3’
Non complementary control:

5' TTT TTT TTT TTT TTT TTT TT 3'

To determine whether any of the on-chip probe sequences are successfully hybridized, the reductive catalytic current of each NME is measured after hybridization. The change in peak reductive current is represented as ΔI, and is documented for different probe/target combinations. Calculation of ΔI is demonstrated in Figure 4.13.

Figure 4.11: Calculation of ΔI. $I_{ds}$ represents the background subtracted current after hybridization. $I_{ss}$ represents the background-subtracted current before hybridization. ΔI is a quantitative measure of the difference between $I_{ds}$ and $I_{ss}$.

4.10.2 Results

The data presented in Figure 4.12 is gathered in collaboration with Zhicha Fang and it demonstrates the selectivity of the chip-based platform in detecting closely related sequences. As mentioned in the previous subsection, only the fusion sequence TMPRSS2:ERG is fully complementary to the immobilized probe, while the wild type sequence, ERG, is half complementary to the immobilized probe.

The bars of Figure 4.12 represent the average ΔI as a result of incubation with the three different oligonucleotide sequences. It is evident that the fusion target generates a much larger response than the wild type target. Furthermore, the response of the fully non-complementary target is
negligible compared to the response of the fusion target. There is a higher signal change seen from hybridization with the wild type gene than the fully non-complementary target. This is most likely due to the cross-hybridization of the complementary portion of some of the wild type target molecules with the immobilized probe.

![Graph](image)

**Figure 4.12:** Differentiation of related nucleic acid sequences using the multiplexed chip. TMPRSS2:ERG represents a fusion sequence fully complementary to the immobilized probe. ERG represents a wild type sequence only half complementary to the immobilized probe, and non-comp represents a sequence of no complementarity to the immobilized probe. All target sequences have a concentration of 100 fM. [84]

In order to use this platform for detecting and distinguishing sequences that differ only by a single nucleotide, more stringent hybridization and washing protocols should be employed. Using this approach, the same platform was shown to discriminate between miRNA sequences differing by a single nucleotide. This indicates that the platform is of high selectivity and has the potential to be used in detecting single nucleotide polymorphism. [5]

### 4.11 Multiplexed detection of prostate cancer gene fusions

Previous sections demonstrated how microfabrication and nanofabrication techniques were combined to integrate NMEs on a silicon chip capable of electrochemical analysis. This section presents results of using this chip for multiplexed analysis of prostate cancer biomarkers.
The biomarkers selected herein are a group of gene fusions resulting from a chromosomal translocation that joins two separate genes. These occur frequently in early stages of prostate cancer development, making them excellent diagnostic markers. [65, 82] Furthermore, there are at least 20 variations of such fusions resulting in the expression of related but different messenger RNA (mRNA) sequences. As there is a correlation between fusion type and aggressiveness and metastatic potential of the tumor, these biomarkers have prognostic value and can be used in choosing effective therapeutic directions.

Prostate cancer diagnosis and treatment can benefit greatly from a multiplexed sensing platform capable of detecting and identifying the type of prostate cancer-related gene fusions. As a result, we will use our multiplexed chip to analyze biological samples containing different types of gene fusions.

4.11.1 Methods

A group of five mRNA samples are analyzed for three of the most common types of prostate cancer gene fusions: type I, type III and type VI. Among these five samples, three cancer cell lines are tested using the NME chip: VCaP, NCI-H660, and DU145. Previous reports indicated that VCaP [65] was type III positive, NCI-H660 [85] was type III and type VI positive and DU145 [65] was fusion negative. The sequences of the prostate cancer gene fusion probes are summarized in Error! Reference source not found.

<table>
<thead>
<tr>
<th>Fusion type</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>SH-CTG GAA TAA CCT GCC GCG CT</td>
</tr>
<tr>
<td>III</td>
<td>SH-ATA AGG CTT CCT GCC GCG CT</td>
</tr>
<tr>
<td>VI</td>
<td>SH-ATA AGG CTT AGT TCA AA</td>
</tr>
</tbody>
</table>

In addition, we analyze two patient samples that contain mRNA extracted from tumor tissues collected by radical prostatectomies. The mRNAs are extracted from cell lines and patient tissue samples with the Invitrogen Dynabeads mRNA Direct Kit. Screening of tumor samples using
conventional PCR followed by RNA sequencing indicated that one of the samples was positive for type I fusion, while the other contained the sequence of type III fusion.

After selecting an appropriate set of samples, nucleic acid probes complementary to each fusion segment and terminated in an –SH group are synthesized and deposited on NMEs. For multiplexed experiments, 0.5 μL of solutions containing thiolated single stranded probe, magnesium chloride, 25 mM sodium phosphate (pH 7), and 25 mM sodium chloride (25/25 buffer) are deposited on each NME using a manual pipette. Deposition is performed in a dark humidity chamber for 30-60 minutes at room temperature.

For experiments presented in this section, five chips are fabricated to have electrodes demonstrated in Figure 4.7-C3. Each chip features 8 individually addressable NMEs: two NMEs are modified using type I probe, two NMEs are modified using type III probe, two NMEs are modified using type VI probe, and two are left blank as negative control. Each chip is challenged with one of the five cancer cell lines or patient samples. In order to obtain a set of statistically reliable data, each experiment is repeated at least three times on 15 different chips.

Following probe deposition, Differential Pulse Voltammetry (DPV) is used to record the catalytic reductive current of the probe modified electrodes. DPV is typically performed in a three-electrode setup using a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and pulse period of 100 ms, translating to scan rate of 50 mV/s. In order to obtain a potential window where catalysis is observed, scanning is started at 0 Volts and is terminated at -0.45 Volts.

Probe modified electrodes are first scanned using DPV in a catalytic solution containing Ru(NH3)6^{3+} and Fe(CN)6^{3-} (Figure 4.13-dotted line). Following the first electrochemical readout and washing the chips with an aqueous solution of 25 mM sodium chloride and 25 mM phosphate buffer (25/25), mRNA samples suspended in 25/25 buffer are deposited on the chip and incubated at 37°C for one hour.

To determine whether any of on-chip probe sequences are successfully hybridized, the reductive catalytic current of each NME is measured again (Figure 4.13-solid line). The change in peak reductive current is represented as ΔI, and is documented for different probe/target combinations. Calculation of ΔI is demonstrated in Figure 4.13.
Figure 4.13: Calculation of $\Delta I$. $I_{ds}$ represents the background subtracted current after hybridization. $I_{ss}$ represents the background-subtracted current before hybridization. $\Delta I$ is a quantitative measure of the difference between $I_{ds}$ and $I_{ss}$.

4.11.2 Results

Figure 4.14 compares the change in DPV responses of two probe-modified NME chips when challenged with different RNA targets. Both electrodes are modified with type III probe complementary to the mRNA sequence present in VcaP cell lines. However, only one electrode is challenged with VcaP RNA, while the other is challenged with a sample with a mismatched mRNA sequence.

These results demonstrate the specificity by which the NME chip device operates: the matched sample results in a peak current increase of 80%, while the mismatched sample results in nearly no increase. This confirms that there is minimal cross talk between the sensors of the same chip, and that our device is suitable for multiplexed analysis.

It should be noted that the DPV scans of devices prepared using exact same procedures are not identical. They differ slightly in peak current, peak potential and magnitude of the background current. These changes result from 1: minor structure variations form electrode to electrode and 2: differences in the density and organization of probe monolayer of two devices.
Figure 4.14: Selective detection of VcaP RNA. DPV signals in an electrocatalytic solution of 10 \( \mu \text{M} \) Ru(NH\(_3\))\(_6\)\(^{3+}\) and 4 mM Fe(CN)\(_6\)\(^{3-}\) are monitored before (dotted) and after (solid) incubation with 10 ng of complementary (left panel) and non-complementary (right panel) RNA samples. Reprinted with permission from reference [84]. Copyright 2009 American Chemical Society.

Figure 4.15 summarizes the results of multiplexed analysis of 5 samples for three different types of prostate cancer gene fusions. Bars demonstrate the average \( \Delta I \) generated as a result of incubating probe-modified NMEs with a particular sample. For example, the first bar of Figure 4.15 represents the average \( \Delta I \) value of at least three different NMEs modified with type I probe and incubated with a DU145 sample. The error bar is the standard error between the \( \Delta I \)s generated from at least three different trials.

DU145 is used as the negative control since it is known not to contain any of the targets under test. The average \( \Delta I \) value of each sample is compared to the corresponding DU145 value to determine the presence of a particular sequence. If its value is larger than the DU145 value it is considered detected. For example, in the patient sample containing the type I fusion, only \( \Delta I \) corresponding to the NME modified with type I probe is larger than that of DU145. Similarly in patient with type III fusion, only \( \Delta I \) value of NME with probe III modification shows a signal larger than that of DU145.

We compare the electronic analysis of patient samples and cell lines with results of conventional techniques such as PCR and sequencing. From this comparison, we conclude that our assay predicts the presence of the correct sequence every time.
Figure 4.15: Multiplexed profiling of prostate cancer related gene fusions in clinical samples and cell lines. Patient samples and three cell lines are analyzed for three different types of prostate-cancer related gene fusions: type I, type III, and type VI. 10 ng mRNA isolated from two tumor samples are tested, one that is positive for the type I fusion, and one that is positive for the type III fusion. In addition, three cell lines are tested: VCaP (type III positive), NCI-H660 (type III and VI positive), and DU145 (fusion negative). RNA hybridization is performed by incubating probe modified chips with mRNA targets for 60 minutes. The RNA solutions have a concentration of 1 ng/ul. Error shown is the standard error determined from multiple trials. Sequences that are also observed by direct sequencing are highlighted as black bars. Reprinted with permission from reference [84]. Copyright 2009 American Chemical Society.

4.12 Conclusions

Prior to this work, there were only a handful of reports demonstrating multiplexed detection of DNA using electronic readout. [86] However, none of these devices were shown useful in analysis of DNA biomarkers in biological samples containing large amounts of non-specific analyte.

The present chapter demonstrated how top-down microfabrication was combined with bottom-up nanofabrication. The result was a simple, inexpensive and scalable method for fabrication of a multiplexed sensing array.
We then proceeded to demonstrate the use of this multiplexed chip in analysis of prostate cancer biomarkers in cancer cell lines and mRNA derived from cancer patient tissues. We compared the results of multiplexed electronic profiling of biomarkers with results of a conventional DNA sequencing. We concluded that our chip-based platform was able to profile these important and relevant biomarkers in complex biological sample in a parallel fashion, demonstrating an accurate multiplexed biosensing platform.
5 Ultra-sensitive biosensing

The previous chapter demonstrated that through a combination of different fabrication methods we developed a scalable and inexpensive silicon chip capable of parallel detection of DNA biomarkers in biological samples. In spite of overcoming the multiplexing and miniaturization challenge, we realize that a detection platform is only useful if it has sufficient detection limit for analysis of samples relevant to applications described in section 1.1.1.

Specifically, to get around the complexities introduced by PCR amplification, this chapter will focus on developing methods for improving the detection limit of the electronic chip enabling amplification-free detection.

5.1 Role of electrode nanostructuring on detection limit

Materials at the nanoscale are thought to be ideal for detection of biomolecules due to increased surface area to volume ratio and/or efficient interaction with biomolecules of the same length scale. In the route towards building a highly sensitive biosensing platform, we will study how the architecture of biosensing electrodes in nanoscale correlates with their detection capability. For this purpose, two Palladium sensors - one with a highly fractal structure showing fine nanotexturing, and the other with a smoother hemispherical geometry - are chosen and fabricated using methods of section 4.6.1. (Figure 5.1)

Following the fabrication, we compare the nucleic acid detection performance of these Pd microelectrodes, which have similar top-view diameters but feature different levels of nanostructuring. Thiolated DNA probes are immobilized on the structures and their electrocatalytic reduction current is measured before and after hybridization with 100 pM solutions of 20-nucleotide long synthetic targets. Measurements are performed using methods of sections 4.10.
Figure 5.1: Correlation of the biosensor architecture and sensitivity. SEM images of two NMEs with different morphologies are shown. DPV scans were obtained before (dotted) and after (solid) hybridization of DNA modified NMEs with 100 pM complementary targets. DPV scans are performed in a solution containing 10 μM Ru(NH$_3$)$_6^{3+}$ and 4 mM Fe(CN)$_6^{3-}$.

DPV scans of these electrodes are shown in Figure 5.1. We realize that tree-like structures show ΔI of 115%, while the smooth hemispherical structure show ΔI of 16%. We hypothesize that this factor of 7 enhancement in ΔI, could generate a much more pronounced improvement in detection limit since the fractal structure may already be in the saturation regime.

Although results of Figure 5.1 confirmed a correlation between electrode architecture and its biosensing capability, it does not provide enough evidence that nanostructuring is indeed responsible for the enhanced detection capability. To address this issue, we will proceed to investigate systematically the impact of nanostructuring on performance by varying microelectrode surface nanostructure and investigating nucleic acids detection sensitivity.

In order to create electrodes that are identical in every aspect except for the degree of nanostructuring, different plating conditions of section 6 are combined in sequence creating composite structures that demonstrate a high degree of structural control. For example, a smooth sphere is generated using a low deposition potential (Figure 5.2), and then a higher deposition potential is used in combination with a different electrolyte to “decorate” the smooth surface with nanostructures.
Figure 5.2: Control of Pd microelectrode nanostructuring as visualized using scanning electron microscopy (SEM). Palladium structures are fabricated by electrodeposition through reduction of H$_2$PdCl$_4$ A) at 0 mV in HCl for 150 s B) at 0 mV in HCl for 150 s followed by plating in HClO$_4$ for 1 second at -250 mV C) at 0 mV in HCl for 150 s followed by plating in HClO$_4$ for 2 seconds at -250 mV and D) at 0 mV in HCl for 150 s followed by plating in HClO$_4$ for 5 seconds at -250 mV. The scale bar represents 5 μm. [87]

We compare the electrocatalytic nucleic acid detection performance of two different Pd microelectrodes that possess different surface structures: one has a smooth hemispherical geometry (Figure 5.2A), and the other has the same underlying structure, but also possesses a layer of finely nanotextured structures (Figure 5.2B). Following modification with nucleic probes, electrocatalytic reduction current is measured before and after hybridization with dilute attomolar to picomolar solutions of synthetic complementary DNA targets.

Figure 5.3 demonstrates that while both electrodes showed similar microelectrode behavior and limiting current magnitude (Figure 5.3B), the nanostructured microelectrode showed a superior sensitivity in detecting DNA hybridization, with a strong signal change observed in the presence of, for example, 10 fM of a target while none was observed with the smooth microelectrode. [87]
Figure 5.3: Comparison of the sensitivities of a smooth (left) and nanorough (right) Pd microelectrodes. A) Signals obtained before (dotted) and after (solid) incubation of probe-modified microelectrodes with 10 fM of a synthetic target sequence for 60 minutes are compared by monitoring the limiting reductive current in a Ru(III)/Fe(III) electrocatalysis solution. Cyclic Voltammetry scans are performed in a solution containing 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻. Scale bars represent 4 μm B) Representative cyclic voltammograms for each type of bare microelectrode in a solution containing 3 mM Ru(NH₃)₆³⁺. [87]

The importance of nanostructuring in the construction of biosensors with low detection limits is readily apparent in these experiments, which are the first to directly analyze this parameter in the context of biosensing applications.
In order to take advantage of the structure dependence of biosensing sensitivity, we elect the highly nanostructured fractal structure of Figure 5.1 as the sensor of choice for the remaining sensitivity optimizations.

### 5.2 Optimizing sensitivity by using different nucleic acid probes

With a view of creating a DNA sensor having high sensitivity and specificity, we will design and compare different probe structures: one made of conventional DNA with its phosphodiester backbone, the other employing peptide nucleic acid (PNA) having a pseudopeptide backbone. Both of these probes are known to hybridize with complementary DNA strands according to Watson-Crick hydrogen-bonding rules; however, unlike DNA, PNA is a charge-neutral molecule. As a result, its electrocatalytic background signal in case of an unhybridized probe monolayer is expected to be dramatically lower than that of DNA [49].

![Figure 5.4 Formation of a PNA (left)/DNA (right) complex](image)

#### 5.2.1 Methods

To capture specific analytes in solution, single-stranded DNA and PNA strands terminated with a single thiol endgroup (as described elsewhere [63]) are immobilized on the electrodes. Probe deposition is performed from a solution containing single stranded nucleic acid probes dissolved in 25/25 buffer at room temperature.
To evaluate the hybridization of the electrode-bound probe strands with target analyte strands, the electrodes (DNA- or PNA-modified) are incubated with 10 pM complementary targets or 100 pM non-complementary targets (negative control) for one hour at 37˚C. The electrodes are then removed from the analyte solution, rinsed, and immersed in an electrocatalytic solution described in chapter 3 to enable electrical readout of hybridization.

### 5.2.2 Results

Figure 5.5 demonstrates differential pulse voltammetry (DPV) traces for the DNA- vs. PNA-functionalized chips. PNA-modified electrodes showed a 70% lower background current compared to DNA-modified electrodes. As expected, the PNA-only electrode produced little electrocatalytic signal due to its uncharged nature.

As a consequence of elevated signal and lowered background, the use of PNA produces a dramatic 3-fold enhancement in signal-to-background ratio compared to DNA probe in analyzing 10 pM complementary target. The use of the uncharged probe layer thus enhances capture of the negatively-charged analyte molecules, providing a further advantage to the use of PNA.

![Figure 5.5: Comparison of PNA and DNA probe monolayer in DNA hybridization detection. Schematic illustration (left) and differential pulse voltammetry measurements (right) representing hybridization with a DNA probe (top) versus a PNA probe (bottom). DPV scans are performed in a solution containing 10 μM Ru(NH$_3$)$_6^{3+}$ and 4 mM Fe(CN)$_6^{3-}$. Reprinted with permission from reference [71] Copyright [2009] American Institute of Physics](image)
In addition, we perform a suite of control experiments that challenge the specificity of the system and reveal any artifacts arising due to nonspecific adsorption of target molecules from the analyte solution. These results are summarized in Figure 5.6.

![Figure 5.6: Comparison of the sensitivity and specificity of DNA and PNA probes. Matched targets are 10 pM of synthetic oligonucleotides complementary to the deposited nucleic acid probes. Mismatched targets are 100 pM of synthetic oligonucleotides non-complementary to the deposited nucleic acid probes. DPV scans are performed in a solution containing 10 μM Ru(NH$_3$)$_6^{3+}$ and 4 mM Fe(CN)$_6^{3-}$ and quantitated to find ΔI. (Reprinted with permission from reference [71] Copyright [2009] American Institute of Physics).](image)

Figure 5.6 indicates when we introduced mismatched targets at an elevated concentration of 100 pM, the signal change under these nonspecific conditions remained well below 14 percent; proving the PNA system to be selective in analyte capture. Furthermore, the signal changes resulted from incubation of non complementary targets with DNA- and PNA-modified electrodes is in some cases negative. This is often associated with small changes in the monolayer as a result of washing and application of electric potential. This effect is more evident in DNA probe devices since the electrocatalytic system accurately reports on the quantity and conformation of the immobilized DNA probe molecules.
5.3 Optimizing sensitivity by changing the density of probe monolayer

To decrease the extent of non-specific adsorption on the electrode surface and to find a more favorable density of capture probe, we functionalize the system using a mixed-monolayer of probe and spacer/blocker molecules. Thus, we add to our use of PNA probe a mercaptohexanol (MCH) blocker/spacer, a short, uncharged molecule. [88] We believe that an optimal MCH concentration would exist: its concentration high enough to favor specific hybridization of complementary DNA over nonspecific adsorption while allowing a sufficient PNA probe density to capture analyte DNA and produce detectable signal changes.

In order to test the possible sensitivity enhancements provided by introducing a mixed monolayer, we incorporate different concentrations of MCH in the PNA probe solution. After deposition of the mixed PNA/MCH solution, the fractal NMEs of Figure 5.1 are challenged with a 100 fM solution of complementary target. The ΔI of sensors modified with varying density of capture probes is determined as a result of hybridization with a complementary target. These results are summarized in Figure 5.7

Figure 5.7: ΔI comparison of PNA devices having different MCH spacer concentrations. Probe deposition is performed from a solution containing 5 μM single stranded PNA with varying concentrations of MCH dissolved in 25/25 buffer. DPV scans are performed in a solution containing 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻ and quantitated to find ΔI.
As shown in Figure 5.7, 0.1 μM MCH produces a 300% larger signal change than 1 μM MCH and 50% enhancement relative to the case where no MCH is employed. The use of this spacer/blocker layer thus provides a powerful additional implement in the quest for exceptional sensitivity.[71]

5.4 Ultrasensitive DNA hybridization detection using joint structure and monolayer optimizations

The ultimate goal of this sensing platform is the analysis of clinical samples containing a small number of copies of a particular strand within the large background present in a heterogeneous sample. In this section, we will critically evaluate the detection limit of our optimized system of nanostructured fractal electrodes modified with a mixed MCH/PNA monolayer.

For this purpose, we analyze solutions containing between 1 aM and 1 fM complementary target alongside solutions containing 100 fM non-complementary targets. To demonstrate further the advantages presented by the PNA probe, we evaluate the limit of detection of the DNA probe device by looking between 100 fM and 100 pM side by side of 100 pM non-complementary targets. The results of this study are presented in Figure 5.8.
Figure 5.8 Comparison of detection limit of PNA and DNA devices. (a) ΔI of a PNA device (left) and a DNA device (right) at different concentrations of complementary target. The system background associated with hybridization with non-complementary target is shown for reference. The devices contained 0.1 μM MCH acting as a spacer. DPV scans are performed in a solution containing 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻ and quantitated to find ΔI. Errors represent standard deviation of average ΔIs between different trials. (b) Sequences of the nucleic acid targets and probes used herein. Reprinted with permission from reference [71]. Copyright [2009] American Institute of Physics.

As shown in Figure 5.8A, we obtained a 10 aM limit of detection using our optimized chip-based platform as opposed to 10 pM detection limit realized with our DNA device. This 6 orders of magnitude enhancement in sensitivity corresponds to the specific detection of fewer than 100 copies of the target sequence.
5.5 Conclusions

Previous work on electrocatalytic DNA detection using three-dimentional Nanoelectrode Ensembles (NEEs) demonstrated a detection limit of 1 pM. [49] This chapter demonstrated five orders-of-magnitude improvement in detection sensitivity through reengineering the recognition layer of the biosensor: the biosensor architecture and its functionalization.

It was shown that sensors having nanoscale features on the order of 10-20 nm were more sensitive than sensors of similar surface area with a smooth surface structure. Furthermore, when the surface of these sensors was functionalized with Peptide Nucleic Acid (PNA) probes instead of DNA, sensitivity was further enhanced. This was expected as the uncharged nature of PNA resulted in a reduced electrochemical signal in the absence of complementary target.

In addition, we incorporated a spacer molecule within the probe monolayer to improve the selectivity of target capture and to enhance the hybridization efficiency of analyte capture. As a result, the system employing optimal three-dimensional biosensors featuring nanoscale elements modified with a mixed PNA-spacer monolayer demonstrated a detection limit of 10 aM. This presented a record sensitivity in label-free electrical DNA detection using a multiplexed platform.
6 **High dynamic range biosensing using a multiplexed chip**

Previous chapter demonstrated ways of enhancing the biosensing sensitivity by reengineering the sensor-biomolecule interface. In spite of exceptional sensitivity, results of Figure 5.8 demonstrated that the tree-like sensor shown in the left panel of Figure 5.1 experienced saturation after two orders of magnitude. This brings our attention to the next challenge: dynamic range.

In certain applications of nucleic acid detection related to disease diagnosis and biological research, it would be sufficient to determine whether or not a certain biomarker is present in a sample. On the other hand, an important class of applications requires the knowledge regarding relative abundance of different genes in a single sample. For this purpose, the detection platform needs to be multiplexed and quantitative. Development of a multiplexed system was discussed in chapter 4. The present chapter will report on ways of combining multiplexing with tunability of sensitivity in creating a quantitative biosensing platform with a wide dynamic range.

6.1 **Integration of electrodes of different sensitivity on the same chip**

In order to overcome the limitations of the current system in quantitative and semi-quantitative analysis, we look into different strategies for tuning and subsequently expanding the dynamic range. An important strategy involves manipulating the ligand binding affinities of the capture probes to develop a sensing platform with a suitable dynamic range.[19, 89] This approach mostly focuses on tuning the binding efficiencies (K_d) of proteins and ligands through manipulating conformational equilibria within the protein. It is shown that the K_d value of maltose binding protein (MBP) and maltose can be tuned within two orders of magnitudes using this approach. This strategy can be combined with the multiplexed chip platform to combine a group of sensors of varying binding affinities on the same chip. Particularly, one approach is to functionalize each sensing element with a capture probe of certain affinity to tune and subsequently combine the dynamic range of individual sensors to achieve a wide overall dynamic range. Although this approach can be employed in antibody-antigen based sensors, it is
difficult to implement in nucleic acid hybridization-based sensors. Particularly, it is difficult to manage precise manipulation of $K_d$ values in the desirable range while maintaining high degree of probe-target selectivity for on chip biosensors that are operated at the same temperature. As a result, other strategies for integrating sensors of varying sensitivity and selectivity are investigated.

A powerful strategy for tuning the dynamic range of the biosensor involves predictable manipulation of sensitivity of each sensing element. We had previously shown (section 5.1) a correlation between sensitivity and structure of palladium electrochemical sensors. With this in mind, we will develop a simple approach for combining sensors of different sensitivity on the same chip for expanding the system’s dynamic range beyond saturation.

We envision an array of sensing elements with each element being responsive at a different concentration regime. Ultrasensitive electrodes would show a log-linear response when challenged with low amounts of target molecules, while less sensitive sensors would be active when detecting high target concentrations. For this purpose, we sequentially deposit electrodes of different morphology on the same chip using protocols of section 4.6. Particularly, we electroplate the first NME using methods of Figure 4.7A1, we wash the sample thoroughly in deionized water, deposit another NME using methods of Figure 4.7A2 followed by another wash, and finally deposit another NME using methods of Figure 4.7C3. The SEM images of these structures are demonstrated in Figure 6.1.
Figure 6.1 Fabrication of three different NMEs on the same chip A. Zoomed out SEM image demonstrating the presence of NMEs at the end of the gold leads, B. Top view, zoomed out (top), zoomed in (middle) and side view (bottom) SEM images of NMEs.[75]

Figure 6.1 demonstrates creation of three diversely-structured electrodes on a single chip: 1. A finely nanotextured fractal structure, with features ranging from 25-50 nm (right), 2. A hemispherical structure with larger features ranging from 200-300 nm (middle) and 3. A smooth hemispherical structure (left).
6.2 High Dynamic Range biosensing using tunable electrodes in a multiplexed array

6.2.1 Methods

Following the fabrication of chips having the NMEs demonstrated in Figure 6.1, on-chip sensing electrodes are functionalized using thiolated PNA probes. PNA deposition is performed from a solution of single stranded thiolated PNA mixed with mercapto hexanol dissolved in 25/25 buffer at room temperature for one hour. PNA modified electrodes are incubated with synthetic targets having a sequence complementary or non-complementary to the designed PNA probe at 37°C for one hour.

In order to measure the DNA hybridization-dependant change in electrocatalytic current (ΔI), Differential Pulse Voltammetry responses of these electrodes are monitored before and after hybridization in a solution of 10 μM Ru(NH3)_6^{3+} and 4 mM Fe(CN)_6^{3-}. Moreover, to find the detection limit of each structure, we vary the concentration of the complementary target from 1 aM to 1 nM and compare the resultant ΔI with the case of InM non-complementary target (Figure 6.2)

6.2.2 Results

The sigmoidal curves of Figure 6.2 demonstrate ΔI value versus analyte concentration for three different electrode structures. This ΔI value represents the increase in the electrode's reductive current in case of hybridization with a complementary target. In addition, ΔI value generated when a probe modified electrode is incubated with a non-complementary target is measured and displayed (dotted grey line) in Figure 6.2. This value is considered as the worst case background: samples generating a ΔI value 3 times larger than this value are considered detected. In other words, the detection limit of each structure is the concentration at which the corresponding average ΔI is three times larger than the average background value.
Figure 6.2: Comparison of the detection limit and dynamic range of three probe-modified Pd NMEs with different levels of nanostructuring. ΔI values represent current changes collected in voltammetry sweeps performed in 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆⁻³ and represent averages from 6-8 trials. Error bars represent standard error; 100 fM of a non-complementary sequence is used as a control to evaluate background levels (average background shown as gray line). Device responses are normalized to the plateau currents. [75]

Following this kind of analysis we realize that the detection limit observed with these structures are dramatically influenced by the fineness of the nanostructuring: the limit of detection of the finely nanostructured microelectrodes is 10 aM, while that for moderately-nanostructured sensor is 10 fM, and that for the smooth structure is 100 fM.

Since these three types of structures, which are generated on a single chip, respond in distinct concentration regimes, their multiplexing can be used to realize 6-7 orders of magnitude dynamic range. This feature is not available in other array-based platforms that have access to only a single type of sensor nanostructure[55, 90]. Expanded dynamic range is a powerful tool for
monitoring disease-associated nucleic acids such as cancer-related genes that are known to vary over many orders of magnitude in patient samples.\[91\]

### 6.3 Mechanistic study of correlation of device morphology and sensitivity

In order to explore the physical mechanism leading to enhanced sensitivity in the most finely nanostructured devices of Figure 6.2, we will investigate two hypotheses: (1) nanostructured electrodes' increases surface area enhances redox currents relative to smooth structures and increases sensitivity (the area hypothesis); (2) nanostructured electrodes' surface textures enhance accessibility during hybridization, leading to faster and more efficient binding of analyte (the accessibility hypothesis).

To evaluate the area hypothesis, we measure the working area of each differently-nanostructured electrode, using the type of electrochemical data shown in Figure 6.3.

**Figure 6.3 CV scans of three different electrodes in 3 mM solution of Ru(NH$_3$)$_6^{3+}$**

Figure 6.3 demonstrates that the more finely nanostructured NME results in a smaller limiting current. As the limiting current of a microelectrode is proportional to its working area, it is evident that the more finely nanostructured NME has smaller apparent working areas than the less nanostructured electrodes. Furthermore, more accurate surface area measurements of the three structures is performed in our laboratory by Dr. Xiaomin Bin using cyclic voltammetry in acidic solutions. In this method, cyclic voltammetry is used to form a layer of palladium oxide on the electrode in an anodic scan and reduce the resultant palladium oxide during a cathodic scan.
Specifically, the cathodic peak involved in reduction of palladium oxide is integrated to calculate the amount of charge, and compared with experimental values\cite{92} to obtain the surface area of the palladium microelectrodes. Using this analysis, it is found that surface areas of the three electrodes are in the range $6-9 \times 10^{-5}$ cm$^2$ with the fractal structure being closer to the lower surface area range. \cite{93} This confirms that the fractal electrode does not provide increased surface area, and a resultant increase in capture sites and signal magnitude. It should be noted that other non-electrochemical methods such as Brunauer-Emmet-Teller (BET) may be more suitable in characterizing porous materials and should be addressed in future work.

We then examine the accessibility hypothesis: that incorporating nanoscale structural features into sensing elements results in the display of probe molecules in a more favorable conformation for hybridization, promoting more efficient complexation with the target sequence. The previous observation that finely nanotextured NMEs, with features ranging from 20-50 nm, had a limit of detection three orders of magnitude lower than NMEs with moderate (100-300 nm) nanostructuring, could arise from such an effect. To evaluate this hypothesis, we monitor directly the kinetics of hybridization at two differently-nanostructured biosensing electrodes. The results are shown in Figure 6.4.

For this purpose, Peptide nucleic acid modified NMEs were prepared as described in section Figure 6.2. Following probe deposition NMEs were immersed in a solution containing 10 μM Ru(NH$_3$)$_6^{3+}$, 4 mM Fe(CN)$_6^{3-}$, 100 fM DNA target, 25 mM sodium phosphate (pH 7), and 25 mM NaCl. The electrocatalytic signals were obtained at time intervals shown in Figure 6.4 and used to calculate $\Delta I$. All measurements were performed at 37°C.
Figure 6.4 Hybridization kinetics observed for two different types of NMEs. NMEs with moderate (red) and fine (blue) nanostructuring are studied by measuring the time evolution of ΔI while immersing the PNA-modified NMEs in solutions containing 100 fM of synthetic target, 10 μM Ru(NH₃)₆³⁺, and 4 mM Fe(CN)₆³⁻.[75]

Figure 6.4 demonstrates the evolution of ΔI in real time as the NMEs are suspended in mixtures containing DNA targets and the electrochemical reporter compounds. The more nanostructured fractal sensor exhibited a very fast response, rising to 70% of its maximal signal within 2 minutes, whereas the coarser spherical structure took 9 minutes to generate a similar response. The fact that the denser spherical structure took significantly longer to generate the same signal-change, indicates that hybridization is slower when probes are displayed on this structure.

Previous studies have shown that there is a non-linear correlation between the densities of the probe monolayers on solid surfaces and their capture efficiencies.[17] As a result, the differences in the hybridization kinetics of the different sensors may be related to the differences in the densities of probe monolayers. To address this issue and further validate the accessibility hypothesis, detailed analysis was performed by Bin et. al. at our laboratory.[93] Throughout this study, density of single stranded DNA molecules on the differently structured electrodes was measured using a chronocoulometric method. This method is based on using Ru(NH3)₆³⁺ as an electrochemical reporter that associates at a ratio of 3:1 with single negatively charged DNA phosphate groups present on the electrode surface. The charges generated during
chronocoulometry measurements are used to extract the number of electrode-bound DNA. This number is divided by the surface area measured using electrochemical methods monitoring the reduction of palladium oxide in acidic conditions. Furthermore, to access the efficiency of target capture of probe molecules immobilized on different surfaces, the surface coverage of the electrodes after hybridization with a complementary target was accessed using the same method. Hybridization efficiency was determined using this approach to find the fraction of molecules that hybridize to their complementary target. The results of such assessments are summarized in Figure 6.5.

Figure 6.5: Surface coverage of single stranded DNA modified electrodes (black bars) and their hybridization efficiency in capturing target DNA (gray bars). Reproduced with permission from reference[93][64]. Copyright [2010] American Chemical Society.

Figure 6.5 confirms our previous results indicating that the finely nanostructured fractal structure is the most efficient among the three electrodes in capturing complementary DNA targets. In spite of this finding, we realize that the fractal structure also has the highest coverage among the three electrodes. This indicates that differences in surface coverage is also linked to differences in hybridization efficiency.
The role of probe surface coverage is further investigated by monitoring the hybridization efficiency of the fractal structure as a function of single stranded DNA surface coverage. This is demonstrated in Figure 6.6.[93]

![Figure 6.6: Hybridization efficiency as a function of probe coverage on the finely nanostructured fractal electrode. Reproduced with permission from reference[93]. Copyright [2010] American Chemical Society.](Image)

It is seen that as the hybridization efficiency of the fractal electrode does not change noticeably as its surface coverage is decreased towards the surface coverage of the smooth hemispherical electrode. This indicates that the accessibility and optimal display of the probes on the electrode surface are important factors in promoting efficient target capture.

The results of this section lead us to believing that the architecture of the electrodes-its overall morphology and surface structure-and their surface coverage influence the arrangement of probe molecules on the electrode surface. This influences the accessibility of the probe molecules which in turn determines the hybridization efficiency.
6.3.1 Conclusions

Previously, we demonstrated highly sensitive electronic DNA detection using a multiplexed platform. However, this system generated saturated hybridization response after a 100-fold increase in analyte concentration. qrt-PCR systems, the gold standard for quantitative DNA detection, has a dynamic range of 7. This indicated the need for developing ways of expanding the system’s dynamic range.

This chapter focused on addressing the dynamic range challenge by engineering a chip combining biosensors of different sensitivity. We demonstrated that by arraying structures of varying sensitivity on the same chip, we developed a device, which was responsive in the 1 aM-100 fM range without entering into saturation. Prior to this work, such wide dynamic range was not available in array-based platforms that had access to only a single type of sensor nanostructure.

In order to study the structure-dependant sensitivity of our sensors, we explored the mechanism by which electrode architecture affected its sensitivity. For this purpose, we monitored the real-time hybridization of target molecules by probe monolayers deposited on biosensors of different morphologies. Our study concluded that probe molecules immobilized on nanostructured electrodes were more accessible than probes immobilized on flat surfaces resulting in faster hybridization response.
7 High-speed biosensing through electrode lengthscale engineering

In chapter 5, we developed a sensing system with a record sensitivity of 10 aM; however, all of our detection level experiments were performed using short synthetic targets. The questions that we would like to answer are: will our nanotextured microelectrodes be effective in analysis of long RNA targets present in clinical samples? Will the diffusion of larger molecules be too slow for accumulation on sensor surfaces in times relevant to disease diagnostics?

To answer these questions, we will look at mathematical modeling [78, 94, 95] to find out how long it would take for a single bacterial RNA to make it to the sensor surface. We will see how this time is correlated with sensor size and geometry, and we will design a sensor that is capable of accumulating enough molecules on its surface for successful detection in a 30 minute timeline. In order to move towards system miniaturization, we look into designing sensors with optimal geometries and sizes, which can achieve high-speed biosensing with the smallest possible footprints. For this purpose, we turn into mathematical modeling.

7.1 Mathematical Modeling of electrodes for efficient target capture

A particularly interesting model calculated the number of molecules that were irreversibly adsorbed on the sensor surface as a function of time.[94] This assumption would tremendously simplify the analysis and would be applicable to our case of DNA hybridization detection since double stranded DNA molecules are highly stable under the laboratory conditions used in our experiments. In this case, the maximum number of molecules adsorbed on the surface as a function of time, i.e., \(N\) would be given as:

\[
N(t) = \int_{0}^{t} f(\tau) d\tau = \int_{0}^{t} \int j d\sigma d\tau
\]  

(7.1)

Where \(J\) is the total flux (molecules/s), \(j\) is the flux per unit area, \(A\) is the sensor area and \(t\) is the time. For a three-dimensional hemispherical sensor, \(N\) is given by:
\[ N(a, t) = 2\pi N_A c_0 D \left( at + 2a^2 \sqrt{\frac{t}{\pi D}} \right) \] (7.2)

And for a two-dimensional disk sensor, \( N \) is given by:

\[ N(a, t) = 4D N_A c_0 at \] (7.3)

Where \( N(a,t) \) is the number of molecules accumulated on the sensor, \( N_A \) is the Avogadro’s number, \( c_0 \) is the initial analyte concentration, \( D \) is the diffusion coefficient of molecules in the solution, \( a \) is the radius of the hemisphere or the disk and \( t \) is the total accumulation time.

In order to choose the appropriate sensor geometry and size, we first need to find application-specific values for \( c_0 \)- analyte concentration- and \( D \)- diffusion coefficient of a particular target molecule. The discussion of how these values are chosen to model a clinically relevant sensor will follow in the next section.

### 7.1.1 Choosing a clinical model system

We are interested in using our sensors in detecting clinically relevant nucleic acid targets. mRNA contained within infectious bacteria can be used in detection and identification of these harmful pathogens in patients to guide physicians in prescribing specific antibiotics. This is useful in preventing the spread of infections in hospitals and communities, and it can slow down the development of antibiotic resistant bacteria.

Escherichia coli (E. coli) is a gram positive bacterium responsible for causing gastroenteritis, urinary tract infections[96], and neonatal meningitis[97] in humans. Detection and identification of these bacteria using current methods can take up to two days- a timeline unacceptable in preventing the spread of infections in hospitals. To address this issue, we design our device to be capable of detecting clinically relevant amounts – 1 colony forming unit (CFU) per microliter – of E. coli in 30 minutes.

As a specific molecular capture probe, we design a thiol-terminated DNA probe with a sequence complementary to an E. coli specific mRNA. This mRNA target- RNA polymerase β mRNA transcript (\( rpoB \))- is 4000 nucleotide-long with a calculated diffusion coefficient of 1.25
μm²/s[98]. On the other hand, synthetic RNA targets used in our previous detection limits calculations were 20 nucleotides long with a diffusion coefficient of 150 μm²/s. [98]

As the bacterial targets diffuse at a much slower rate—at least two orders of magnitudes less-than the synthetic targets, we believe there would be a further need for optimizing sensors’ geometry and size. To demonstrate this point, we use equation 7.2 to determine the time required to accumulate one synthetic target molecule on sensors of different diameters. Using similar analysis, we determine the required time to accumulate one bacterial target molecule on the sensing surface. The results of this analysis are summarized in Figure 7.1.

![Figure 7.1](image)

Figure 7.1 Accumulation times of synthetic and biological RNA on hemispherical sensors of different diameters.[99]

Figure 7.1 indicates that sensors smaller than 100 μm are inefficient in clinical diagnostics, and require analysis-to-result times of more than 30 minutes.

7.1.2 Comparison of disk and hemispherical electrodes for bacterial detection

Using conclusions of the previous section, we use $D = 1.25 \mu m²/s$ in equations 6.2 and 6.3 to find the required sensor size and geometry for accumulating detectible amounts of bacterial RNA in less than 30 minutes. Figure 7.2 demonstrates how long it would take for hemispherical sensors (solid lines), having diameters of 100 μm, 10 μm, 1 μm and 100 nm to accumulate one target molecule from solutions of varying concentrations. This is then compared with times it would
take for disk electrodes (dotted line) of the same diameter to accumulate one target molecule from the same solutions.

Figure 7.2 Accumulation time versus concentration plotted for 100 μm, 10 μm, 1 μm, 100 nm hemispherical (100um-h, 10um-h, 1um-h, 100nm-h) and disk electrodes (100um-d, 10um-d, 1um-d, 100nm-d). Accumulation time refers to the time required to capture 1 copy of specific bacterial RNA from a 2 fM solution.

The curves of Figure 7.2 demonstrate that regardless of sensor diameter, three-dimensional hemispherical sensors are capable of analyzing solutions that are at least 200 times less concentrated than two-dimensional disk electrodes. This indicates that sensitivity enhancements achieved by hemispherical electrodes are beyond their factor of two increase in surface area. Comparing the behavior of three-dimensional spherical sensors of different sizes reveals that accumulation of at least one molecule from a 2 fM bacterial target in less than 30 minutes is only possible with sensors having a diameter of at least 100 μm. Next section will discuss ways by which three-dimensional hierarchical sensors are fabricated.
7.2 Fabrication of smooth micro-scaffold electrodes

Fabrication of micro-scaffold electrodes is performed on a template similar to that explained in section 4.2 with the exception that 5 μm apertures are imprinted - instead of 500 nm apertures- on the passivated silicon chips. (Figure 7.3)

Figure 7.3 Schematic representation demonstrating fabrication of micro-scaffold electrodes. Electrodeposition is used to reduce gold ions on predefined on-chip apertures.\cite{99}

This modification was made since 500 nm apertures were unable to support structures with a 100 μm footprint.

Among noble metals, gold shows the most stability and robustness in fabrication of sub-millimeter structures. In addition, gold electrodeposition enables creation of electrodes that rise from the template into the solution rather than growing close to the chip surface. We envision this to be a powerful tool for improving the diffusion-driven molecular transport.

Following template fabrication, gold electrodes are deposited from a solution containing 20 mM HAuCl₄ and 0.5 M HCl using DC potential Amperametry at 0 Volts. In order to achieve sensors being 100 μm wide and 50 μm high, we perform gold deposition for duration of 5 minutes. This technique results in structures demonstrated in Figure 7.4. This puts the microscaffold electrodes at an advantageous position when analyzing long bacterial targets relying on diffusive transport.
Figure 7.4 SEM images of micro-scaffold electrodes created for increasing detection speed of the biosensing platform A) zoomed out top-view (left) and side-view (right) SEM images B) zoomed in SEM images [99]

Top- and side-view SEM images of Figure 7.4 demonstrate the footprint and the height of the sensors to be 100 μm and 50 μm respectively. Moreover, zoomed in SEM images demonstrates structures that are substantially smooth on length scales less than 100nm.

A major advantage of the micro-scaffold electrodes is in their ability to reproducibly grow away from the substrate and reaching beyond 50 μm into the solution. This was not achievable using the fractal electrodes demonstrated in Figure 4.7. These electrodes grew close to the chip surface, and failed to rise beyond a few microns above the substrate.

7.3 Micro-scaffold electrodes for bacterial detection

7.3.1 Methods

In this section, we modify micro-scaffold electrodes with thiol-terminated single-stranded probe sequences to achieve specific molecular recognition (Figure 7.5). We introduce unpurified lysed bacterial samples on the chip to test the capability of these electrodes in bacterial detection. Furthermore, we employ an electrocatalytic assay described in section 3.1 for readout of hybridization-induced reductive currents. The detailed procedures are presented below.

Probe Deposition

Probe deposition is performed in solutions containing single stranded thiolated DNA and magnesium chloride dissolved in 25/25 buffer. The probe is made complementary to the RNA
polymerase β mRNA (\textit{rpoB}), a transcript with a high expression level in bacteria[100] that has a sequence exhibiting significant variation from species to species, making it an ideal target for bacterial detection and identification. Furthermore, mRNA is an optimal target molecule if detection of viable organisms is of interest. The mRNA contained within dead cells remains intact for only minutes before being degraded by RNAse. The use of mRNA reduces the occurrence of false positive results due to presence of dead cells. This requires a fast lysis system and minimal time between lysis and detection to accurately analyzes cells, which were alive before lysis.

![Figure 7.5](image)

**Figure 7.5** Schematic representation of immobilization of DNA probes on micro-scaffold electrode and their use in detection of 4000 nucleotide targets.

**Preparation of bacterial RNA**

Following sensor fabrication and modification, they are challenged with solutions of unpurified bacterial lysates generated from cultured \textit{E. coli} using a reagent-free approach that relies on a rapid electrical lysis technique [101] amenable to in-line integration with electronic chips (Figure 7.6).[101]

To achieve bacterial lysis, samples of E.Coli are grown in Lysogeny Broth (LB-Broth) at 37°C for 20 hours. After growth over night, bacterial samples are centrifuged at 3000 rpm for 5min. The supernatant is discarded, and replaced with an equal amount of 1x saline Phosphate Buffered Saline (PBS) (pH 7.4). Bacteria are re-suspended by vortexing.

Overall scheme of lysis procedure is as shown in Figure 7.6. Roughly 1.5mL of bacterial sample suspended in PBS buffer is loaded into a 3mL syringe and placed in a syringe pump and connected to the lysis chamber using HPLC tubing. Full lysis of samples is achieved with a flow rate of 20µL/min and application of 400V.
Figure 7.6 Schematic representation of electrical bacterial lysis using a microfluidic device. Bacteria suspended in phosphate buffer saline (PBS) are loaded into a syringe. This is placed in a syringe pump and connected to the microfluidic lysis chamber using HPLC tubing. Full lysis of samples is achieved with a flow rate of 20μL/min and application of 400V. Immediately following lysis, samples are transferred to the multiplexed sensing chip for analysis.[99]

Hybridization of probe modified devices with lysed samples

Immediately after lysis, samples are deposited on probe modified electrodes. After 30 minutes of incubation of the bacterial lysate with the chip-based sensors at 37°C, they are washed thoroughly using PBS buffer.

Electrocatalytic readout

Hybridization of the target mRNA is evaluated for the micro-scaffold sensors using the electrocatalytic reporting strategy described in chapter 3. Briefly, DPV is used to measure the electrocatalytic current of a probe-only modified electrode in a solution of 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆⁴⁻. Following hybridization, the electrodes are scanned again in the same solution. The change in the peak reductive current is evaluated by calculating ΔI as described in Figure 4.13.

Quantitation of bacterial RNA in lysed samples

In order to quantitate the concentration of bacteria in solutions used for detection, bacteria are grown on agar plates for 20 hours. Following culture on agar plates, the grown to visible bacterial colonies are counted and are divided by the volume of bacterial mixture used for
culture. This is used to derive the number of Colony Forming Units (CFU) of bacteria per liter (CFU/L).

To find the concentration of mRNA present in each sample droplet in moles per liter, Quantitative PCR is used. PCR determines that on average 800 copies of RNA polymerase β mRNA (rpoB) are present in 1 CFU. Target concentrations in moles/L are derived as:

\[
\text{Concentration}\left(\frac{\text{moles}}{L}\right) = \frac{x\text{CFU}}{L} \times \frac{800\text{ molecules}}{\text{CFU}} \times \frac{1}{N_A}
\]

(6.4)

Where \(N_A=6.02 \times 10^{23}\) molecules/mole.

### 7.3.2 Results

Figure 7.7 demonstrates the Differential Pulse Voltammetry (DPV) scans of a DNA-modified micro-scaffold electrode having a diameter of 100 μm incubated with different concentrations of lysed E. coli. The peak DPV current rises as more concentrated lysates are introduced on the sensor. It is evident that the 100 micron sensor is able to detect as low as 5 CFU/μL, corresponding to a concentration of 6.5 fM. Appreciable signals are not observed with lower bacterial counts.

Furthermore, Figure 7.7 presents the DPV scans of a DNA modified microelectrode having a diameter of 10 μm. This sensor generates a positive \(\Delta I\) only when challenged with RNA at concentration of 500 CFU/μL or higher. This is in line with our previous hypothesis regarding the need for sub-millimeter structures for bacterial analysis.
Figure 7.7: Comparison of 10 μm and 100 μm sensors in bacterial detection. Differential Pulse Voltammetry scans of a DNA-modified micro-scaffold electrode and a DNA-modified ultramicroelectrode are measured. All measurements are performed in Ru(III)/Fe(III) electrocatalytic solutions before and after addition of lysed bacterial samples. These samples contain 5, 50, and 500 CFU/μL of E. coli bacteria.

Equation 7.2 predicts the accumulation of six molecules on a 100 micron hemispherical sensor from a 6.5 fM solution in 30 minutes. In principle, it is possible to generate a detectible signal upon hybridization of one 4000-nucleotide long RNA polymerase β mRNA strand using the electrocatalytic system presented in section 3. This is justified by the large amount of negative charge of \(-4000\ e\) accumulated on the electrode surface due to a single hybridization event. As a result, we hypothesize that sensor’s detection can be further optimized by incorporation of nanoscale features similar to those developed in section 5.1.

7.4 Conclusions

Previous chapters focused on developing a multiplexed platform for ultrasensitive detection of DNA. We engineered electrodes with unique morphologies that improved DNA hybridization efficiency. In addition, we developed new methods for functionalizing the electrode surface for enhancing the sensor’s detection limit. Using these approaches, we achieved sensitivity of 10 aM, equivalent to detection of fewer than 100 molecules in solution. Previous detection limit measurements dealt with analyzing 20 nucleotide-long synthetic DNA targets.

Practical devices should detect much longer DNA strands of approximately 4000 nucleotides. Such large molecules diffuse at least 100 times slower than the shorter synthetic molecules. As a
result, the time limiting step of the overall assay becomes diffusion-driven analyte transport. This chapter used mathematical modeling to come up with a device architecture for overcoming the challenge of biosensing speed.

We explored mathematical modeling of biosensors for their high-speed operation. We adopted the analytical solution of the diffusion equation to model our sensors and discovered that we needed three-dimensional electrodes with a 100 μm footprint for detection of bacterial RNA. To address this issue, we created sensors having 100 μm footprints that reached 50 μm above the surface of our multiplexed chip platform.

In order to validate the results of our theoretical approximations, we measured the detection sensitivity of two similar sensors differing solely in size. Our results showed that 100 μm sensors required 100 times less bacterial RNA to produce the same ΔI as 10 μm sensors. This confirmed the correlation between sensor's overall size and its ability to capture specific molecules from a sample solution.
8 **High-speed ultrasensitive detection of bacteria**

According to recent statistics, healthcare-associated infections (HIAs) affect approximately 1.7 million people per year in the USA, causing 99,000 deaths.[102] In addition to being a significant cause of mortality and morbidity, HIAs place a significant financial burden on the healthcare system. According to Center for Disease Control (CDC), they cost US hospitals approximately 30 billion dollars every year.[103] One way of reducing the rates of such infections is through coupling patient screening with appropriate infection control programs.

Diagnosis of infectious diseases is currently dominated by methods based on detection and identification of human pathogens via microscopic examination and phenotypic characterization of microorganisms such as bacteria. These methods rely on culture-based enrichment steps for reaching the required purity and concentration for microscopic evaluation. The overall process involving the enrichment steps, bacterial identification, and drug susceptibility testing takes a minimum of 48 hours and can take up to four weeks when testing the drug resistance in clinical samples of Mycobacterium tuberculosis.[104] [21, 29][21, 29][21, 29][21, 29][21, 29] These long wait times often delay treatments and are inefficient in proper infection control.

Furthermore, genetic methods for analysis of pathogens provide accurate means of species identification, virulence and toxicity evaluation, and drug susceptibility testing.[105] Particularly, through identification of conserved regions within the genome, it is possible to identify different species and even different strains of a particular species. As a result, patients can be tested for bacterial strains capable of toxin generation and antibiotic resistance. Achieving similar capabilities with phenotypic characterization is time consuming and at times labor- and resource-intensive and especially difficult to achieve in developing countries. These place genetic testing at an advantageous position in the field of infectious disease diagnosis.

Detecting bacteria in samples that are collected non-invasively is particularly interesting for research on the development of new assays. Bacteria that infect the respiratory tract including streptococcus pyogenes, staphylococcus, mycobacterium tuberculosis can be detected non-invasively through the analysis of nasal and throat swabs. The required limits of detection in case of respiratory pathogens is highly application specific and is in the range: 100-10,000 CFU/mL.[106, 107] Furthermore, urinary tract infecting bacterial pathogens can be monitored
non-invasively through analysis of urine samples. The important bacterial pathogens in this group are different strains of Escherichia coli (E. coli), which are often present at levels of 1000 CFU/mL in urine.[108]

Furthermore, genetic detection and identification of bacteria have applications in microbial analysis of foods. Particularly, detection of four major pathogens, salmonella spp., listeria monocytogenes, E. Coli O157 and campylobacter spp. are important for food safety monitoring. Assays that have limits of detections as low as 1-5 CFU of target bacteria in 25 grams of liquid or solid foods are useful in this field. Genetic testing devices would have advantages over the existing phonotypical testing if they demonstrate sufficient sensitivity alongside low-cost, portability and automated operation.

Previous chapter demonstrated that through a combination of mathematical modeling and material design, we were able to detect bacterial RNA in analysis times relevant to clinical diagnostics. For clinical and food safety monitoring applications, we need to further improve our detection sensitivity. As a result, this chapter will discuss ways of designing novel sensing materials for achieving high-speed detection at a higher sensitivity. Specifically, this chapter will discuss the design and fabrication of hierarchical electrodes that are controllable in nano- and microscale. Furthermore, it will demonstrate the sensitivity and speed of operation of these hierarchical biosensors by presenting proof of principle experiments in analyzing samples containing E. coli.

8.1 Fabrication of sub-millimeter hierarchical electrodes with nanometer features

We showed in section 5.1 that palladium nanostructures deposited on smooth hemispherical electrodes significantly improved their sensitivity. For the purpose of enhancing the sensitivity of our micro-scaffold electrodes, we introduce a thin overcoating of palladium by electrodeposition on structures shown in Figure 7.4.

Palladium electrodeposition is performed in a bath containing 5 mM H₂PdCl₄ and 0.5 M HClO₄ at a potential of -250 mV for 10 seconds. Palladium is chosen for surface modification as this noble metal is particularly versatile in producing different nanostructured morphologies.
depending on deposition conditions [109]. SEM images of these palladium-coated gold electrodes are presented in Figure 8.1.

![SEM images of hierarchical structures demonstrating the effect of palladium coating on micro-scaffold electrodes. Hierarchical electrodes are manipulated in micro- and nano-scale to engineer an ideal sensor for detection of long bacterial RNA. Microscale dimension is optimized to accumulate enough molecules on the sensing surface in 30 minutes by diffusion. Surface coating is manipulated to control the hybridization efficiency of the sensor.][99]

Figure 8.1 demonstrates how our two-level electrodeposition method produces a layer of nanostructures ranging from 10-50 nm, and a hierarchical nanostructured microsensor structure (HNME) with size features spanning the microscale to nanoscale.

### 8.2 Comparison of hybridization efficiency of smooth and hierarchical electrodes

In this section, we use electrochemical methods to compare the hybridization efficiency of two electrode structures: smooth micro-scaffolds and nanorough HNMEs. This allows us to confirm that decoration of large gold structures with palladium nano-grains is indeed effective in enhancing biosensing sensitivity.

Hybridization efficiency reflects the percentage of single stranded probes that can successfully hybridize with their complement to create double stranded DNA. It is possible to quantitate the number of DNA molecules present on the sensor surface by precisely measuring the number of nucleotide phosphate residues.[110] In this method, chronocoulometry is used to determine the amount of cationic redox reporter, Ru(NH$_3$)$_6$$^{3+}$, attracted to the DNA-modified electrode surface.
As the signal generated by this method is directly proportional to the amount of negative charges on the electrode, it can be used to determine the hybridization efficiency of probes in DNA capture.

This section will summarize how this method is used for comparison of hybridization efficiency of smooth gold electrodes of Figure 7.4 with hierarchical electrodes of Figure 8.1. Hybridization efficiency measurements are performed by Dr. Xiaoamin Bin and are described in detail elsewhere.[111]

Figure 8.2A (left) demonstrates SEM images of smooth gold electrodes alongside the rough hierarchical gold electrodes with a palladium surface coating. It is evident that our ~ 100 µm-sized sensors are substantially smooth on lengthscales shorter than 100 nm. Figure 8.2A (right) demonstrates SEM images reflecting the effect of palladium coating on the sensor surface morphology. It is evident that surface modification produces a layer of nanostructures ranging from 10-50 nm. The result is a hierarchical nanostructured structure with size features spanning the microscale to nanoscale.

Surface coverage indicates the number of molecules present on the sensor per surface area. In order to compare the hybridization efficiency of smooth micro-scaffold electrodes and rough hierarchical electrodes, their surface coverage is measured before and after hybridization with target DNA. Surface coverage measurements are performed suing methods described elsewhere.[111] Figure 8.2B summarizes the results of surface coverage studies of the two electrodes.
Figure 8.2: Comparison of hybridization efficiency of smooth and rough 100 μm sensors. A) SEM images of the smooth and rough electrodes. Smooth electrodes are fabricated using gold electrodeposition. Rough electrodes are fabricated using a two-step electrodeposition with palladium deposition following gold deposition. B) Surface coverage of smooth and rough electrode are measured before (ss) and after hybridization with a complementary (ds) target. Hybridization efficiency is the ratio of double stranded surface coverage to single stranded surface coverage.[99]

It is evident from Figure 8.2 that with the increased electrode nanostructuring, probe surface coverage and the hybridization efficiency of a synthetic oligonucleotide improve dramatically. As a result we hypothesize that hierarchical structures would be ideal for high speed and ultrasensitive biosensing. Hierarchical structures with 100 μm footprints and nanorough surface structure would enhance the detection sensitivity and speed by decreasing the diffusion-driven transport times and increasing the hybridization efficiency. The remaining sections of this chapter will investigate the validity of this hypothesis.

### 8.3 Electrochemistry of sub-millimeter hierarchical electrodes with nanometer features

In order to examine the capability of the HNMEs of section 8.1 in electrochemical sensing, we look at their CV responses in the presence of 3mM Ru(NH$_3$)$_6^{3+}$. The behavior of these HNMEs
are very similar to the behavior of conventional macroelectrodes. It is notable that these sensors, which are fractal in nature and exhibit small morphological differences from sensor to sensor, demonstrate very reproducible and controllable properties.

![Figure 8.3: Electrochemical reproducibility of hierarchical electrodes. CV scans are generated by scanning four different electrodes in 3 mM solution of Ru(NH₃)₆³⁺. Electrochemistry is performed in a three-electrode setup featuring Ag/AgCl reference electrode. Scale bar represents 50 μm.[99]](image)

8.4 Hierarchical electrodes for rapid bacterial detection

8.4.1 Methods

In order to investigate the detection limit and the detection speed of multi-lengthscale electrodes developed in section 8.1, we fabricate electrodes with varying footprint sizes. Gold electrodes having a top-view footprint of 10 μm, 30 μm and 100 μm are created and their surfaces is modified using a 50 nm thick film of nanostructured palladium. All electrodes are deposited on chips with 5 μm apertures in a bath containing 20 mM HAuCl₄ and 0.5 M HCl using DC potential Amperometry at 0 Volts. Electrodeposition time is manipulated to achieve the desired electrode size: 15 seconds for 10 μm structures, 65 seconds for 30 μm, and 300 seconds for 100 μm. Following deposition of gold micro-scaffolds, a palladium overcoating is deposited on all three electrodes.

To achieve a similar overcoating on micro-scaffolds of different sizes, palladium electrodeposition is performed at the same potential but for different durations. As a result,
surfaces of different area are coated using grains of the same size and structure. Palladium electrodeposition is performed in a bath containing 5 mM H₂PdCl₄ and 0.5 M HClO₄ at a potential of -250 mV for 10 seconds, 3 seconds, and 1 second for 100 μm, 30 μm and 10 μm structures respectively.

The detection of hybridization is performed using methods of section 7.3.1. Briefly, the electrodes are modified with DNA probes complementary to the RNA polymerase β mRNA (rpoB) specific to E. coli. Non-complementary probes are also deposited on the electrodes in order to measure the system’s background in the presence of mismatched targets. Equation 7.4 is used to find the concentration of mRNA in each sample. Serial dilutions are performed to create samples of varying concentrations used in the following section.

8.4.2 Results

In order to confirm the correlation between the sensor’s overall size and detection speed, we develop two sensors of varying footprints. Figure 8.4 compares the ΔI of 10 μm sensors and 100 μm sensors as a result of 5 and 30 minutes incubations with 100 CFU/μL of complementary targets. In addition, we use a probe that is non-complementary to the target mRNA sequence to find the sensors’ background (Figure 8.4, grey dotted line).

We compare the ΔI generated through incubation with a complementary target with the sensors’ background to determine whether the specific target is detected or not. If sensor’s ΔI is at least as large as 3 times the background value, target is detected with confidence. As a result, we realize that detection of 50 CFU/μL of target RNA (65 fM) is possible in 5 minutes using the larger sensor, whereas it requires 30 minutes in case of the smaller sensors. This is in line with the calculated values demonstrating that no molecule would be accumulated on the smaller sensor in 5 minutes whereas 18 molecules would be accumulated on the larger sensor, making it more suitable for rapid analysis of bacterial mRNA.
Figure 8.4: Comparison of detection speed of HNMEs of different sizes. Average ΔI is calculated using DPV scans before and after hybridization of a probe complementary to E.coli with a 50 CFU/μL E.coli target. System background is the average ΔI value resulting from incubation of a non-complementary DNA probe to an E.coli sample.

To further investigate the role of electrode overall size on detection limit and detection speed, we create 3 electrodes of varying sizes on a single chip. This chip device has HNMEs with overall footprints of 10 μm, 30 μm and 100 μm. (Figure 8.5) Incorporating the three types of sensors on a single chip enables us to measure the response of each sensor type under the exact same conditions.

Figure 8.5: SEM images demonstrating the fabrication of HNMEs of different sizes on the same chip. HNMEs with gold base and palladium coating are fabricated with footprints of approximately 10 μm, 30 μm and 100 μm. Top panel demonstrates zoomed out view of on-
chip electrodes. Middle panel demonstrates side view images of each electrode. Bottom panel demonstrates top view images of each electrode.[99]

For this purpose, we modify two chips with DNA probes complementary to the \( rpoB \) mRNA (P32) and one chip with a probe non-complementary to the E.coli mRNA target (P33). We challenge the P32 modified devices with E. coli lysates of 1.5 CFU/\( \mu \)L or 150 CFU/\( \mu \)L. In addition, we challenge the P33 modified device with E.coli lysates of 150 CFU/\( \mu \)L. Modification of the chip with P33 allows us to measure the system’s background.

Figure 8.6 demonstrates the resultant \( \Delta I \) as these 3 sensors are challenged with bacterial lysates. Bar graphs demonstrate the average \( \Delta I \) value generated by complementary targets. The dotted grey lines demonstrate the average \( \Delta I \) value generated by non-complementary targets. This represents the sensor’s background.

![Graph showing \( \Delta I \) values for different concentrations of E. coli lysates and different chip sizes.](image)

**Figure 8.6: Comparison of sensitivities of HNMEs of different sizes.** Average \( \Delta I \) is calculated using DPV scans before and after hybridization of a probe complementary to E. coli mRNA. System background (dotted line) is the average \( \Delta I \) value resulting from incubation of a non-complementary DNA probe to an E. coli sample.[99]

In order to find out whether a molecule is detected or not, we compare its average \( \Delta I \) value to the sensor’s background. If this value is at least as large as three times the background value we consider it detected.

Figure 8.7 summarizes the results of this analysis and compares them with the calculated number of molecules accumulated on the sensor in 30 minutes. This table indicates that as long as a single \( rpoB \) mRNA is present on the sensor, its detection is possible. This is in line with our
previous hypothesis that our electrocatalytic reporter system is capable of transuding a detectible signal when encountering a charge addition of \(-4000e\). It is noteworthy that the calculated values in Figure 8.7 are rounded down to demonstrate the accumulation of whole molecules. For example, the number of molecules accumulated on a 100 µm sensor in 30 minutes from a 1.5 CFU/µL solution is calculated to be 1.86. This means that in principle it may be possible to detect down to a concentration of 0.8 CFU/µL as this value corresponds to accumulation of exactly one molecule.

<table>
<thead>
<tr>
<th>Sensor footprint</th>
<th>10 µm</th>
<th>30 µm</th>
<th>100 µm</th>
<th>10 µm</th>
<th>30 µm</th>
<th>100 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria/µL</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Calculated # of molecules</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
<td>9</td>
<td>34</td>
<td>186</td>
</tr>
<tr>
<td>Successful detection</td>
<td>x</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Figure 8.7: Correlation of sensor’s footprint with its bacterial detection capability. Calculated number of molecules is derived from equation 7.2. Successful detection is judged by data presented in Figure 8.6[99]

It should be noted that the limit of detection reported herein is based on the number of RNA transcripts detected during each experiment. As a result, it could include mRNA contained within dead cells and could vary based on the number of RNA transcripts contained within each organism. We suspect that the majority of mRNA targets of nonviable bacteria is degraded, and that it does not provide an overestimation of sensitivity. Furthermore, there is a correlation between bacterial growth stage and RNA transcript levels of each cell. In some cases, the RNA copy number increases five times from logarithmic stage to stationary stage.[26] As we perform our experiments with bacteria in the stationary stage, our limit of detection could be in fact few times larger than reported if the bacteria in the sample are in a different growth stage. For accurate determination of limit of detection and proper calibration, electrochemical sensing using our chip-based platform should be performed alongside PCR for detecting bacteria at different growth stages. These experiments are planned as future work for the research group.
8.5 Conclusions

Prior to this work, most sensing platforms relied on electrodes that were controllable in a single lengthscale. Most of such sensors were functional and controllable in micro-, nano- or the millimeter-scale. In the current chapter we demonstrated how new material architecture methods were invented to simultaneously manipulate sensors in micro- and nano-scale.

This was particularly important since sensors needed to have a geometry that would allow them to accumulate enough targets on their surface and would enable them to efficiently capture such target molecules. We validated the role of sensor geometry on hybridization efficiency using a quantitative electrochemical method that measured the fraction of single stranded DNA molecules that successfully hybridized to their complement. We found that nanoscale features on the order of 10-20 nm significantly improved the hybridization efficiency.

Furthermore, we revisited the effect of overall sensor size on the system’s sensitivity by constructing hierarchical structures having 10, 30 and 100 μm diameters on a single platform. It was evident that only the 100 μm sensors were capable of ultrasensitive detection in hybridization times of 30 minutes.

We demonstrated a nucleic acid sensing platform for detection of bacteria at levels of 1500 CFU/ml. This system integrated high-speed approaches in lysis, analyte capture, and signal readout. As a result, the entire analysis was performed in less than one hour: electrical lysis at the rate of 10 μL/min was approximately five minutes long, target capture during hybridization was achieved in 30 minutes, and electrochemical readout was approximately ten minutes long. In addition to bacterial detection, we demonstrated bacterial identification through speciation. This is important in a number of applications including diagnosis and treatment of urinary tract infections, where identifying E. coli versus staphylococcus is clinically relevant in selection of antibiotics. In spite of this success, the selectivity of the system remains to be tested in identification of different strains of bacterial species.

Current experiments demonstrated detection of the DH5α strain of E. coli - non pathogenic strain often used in research laboratories for safety reasons- using a probe complementary to the rpoB mRNA. This probe can also be used to detect the more relevant pathogenic strains such as O157 since the rpoB gene is conserved in E. coli; however, this probe will not distinguish among
different strands of each species. Moreover, differentiating antibiotic resistant strains from non-resistant strains is an important medical problem and needs to be addressed in future work. Other important work involving sub-speciation are: detecting rifampicin-resistant and isoniazid-resistant strains of mycobacterium tuberculosis and detection of antibiotic strains of staphylococcus aureus.

To address these important selectivity concerns, studies distinguishing among strains are planned for the research group in future. Steps would include (1) Identifying differentiating sequences from the published literature and appropriate design of capture probes (2) Synthesizing and purifying those sequences and modifying them with a thiol group (3) Ensuring compatibility of these oligomers with the chip and the assay (4) Obtaining the necessary biosafety certification and growing the different strains of bacteria in our laboratories (we have found that using fresh lysates is important, so it is much desired to do the culturing in-house) (5) Proving the selectivity of the assay in identification of sub-species that may differ from each other by a single nucleotide.

In addition to selectivity, the overall sensitivity of the assay should be further improved for some medical diagnostic and most of food monitoring applications. This can be achieved through integration of separation and concentration techniques within the chip-based platforms. Some of such processing steps include employing bacterophages[71, 87][71, 87][71, 86][71, 87] and paramagnetic beads for separating and concentrating bacteria in clinical or food samples.[15, 24, 85]
9 Conclusions

Diagnostic technologies are essential in improving people’s quality of life since they are the first step in proper prevention and treatment of disease. The newly available genetic information has identified nucleic acids sequences that are able to signify the presence of disease and predict its course. The detection, identification and quantitation of such sequences at the patient bed side, rather than centralized hospital labs, are believed to revolutionize the healthcare industry. In spite of tremendous advances in biosensing technologies, there is still not a single handheld device capable of genetic analysis of clinical samples. This thesis showcases an engineering approach to building a portable device for sensitive analysis of clinical samples in a parallel fashion.

In this concluding chapter, the findings of this work are briefly summarized. Furthermore, we portray how these findings have impacted the field of DNA biosensors. Finally, the prospects of further research that build on the findings of this thesis are discussed.

9.1 Thesis findings

DNA biosensors were fabricated using electrodeposition on a silicon chip. This allowed us to assemble a number of different sensors on a single platform and couple them to electrical readout instrumentation. In order to use this platform for parallel analysis of nucleic acid samples, we attached single-stranded nucleic acid molecules, capable of capturing their complement, on the biosensor surface. We coupled this platform to an electrochemical reporter system capable of translating the addition of negative charges induced by DNA hybridization to a measurable electrical signal. Throughout this work, significant challenges had to be addressed for developing a previously unavailable platform capable of multiplexed, sensitive, high-dynamic range and rapid detection of specific nucleic acids contained in cells.

- Electrodeposition through conductive pores resulted in metallic electrodes with diverse size and morphology if deposition time, applied potential, metal concentration, or electrolyte were varied. The size of microelectrodes was controlled with the highest precision by controlling deposition time. Increasing the ratio of metal salt to electrolyte concentration during the reaction also increased the structure size; the presence of more free metal likely accelerated deposition and increased the device size. The surface
structure and degree of nanostructuring was varied significantly by changing the applied potential and supporting electrolyte. When low potentials were used, very smooth microelectrodes were generated. When high potentials were used, microelectrodes with extensive nanostructuring were produced. Deposition in the presence of HCl as a supporting electrolyte produced structures that were dense while the use of HClO₄ (or the absence of electrolyte) produced sparse fractal structures.

- The sensitivity by which DNA hybridization was detected was highly dependent on the sensor morphology. Fractal electrodes with nanoscale feature of 10-20 nm were proven to have the lowest detection limit when challenged with synthetic DNA targets. Furthermore, if we used PNA probes – uncharged nucleic acid molecules capable of selective DNA/RNA capture – instead of DNA probes, we improved the detection limit of our system by 6 orders of magnitudes. Furthermore, we saw that the surface density of probe molecules on the sensor had an effect on its detection sensitivity.

- We found that our sensors typically displayed a saturated response after 2 orders of magnitude increase in analyte concentration. It was also discovered than sensors of different morphology displayed a log linear response in varying concentration ranges. We combined sensors that were active in different concentration regimes on a single platform to extend the system’s dynamic range to 6 orders of magnitudes.

- By referring to published mathematical models that calculated the number of molecules accumulated on a surface, we found that sensors smaller than 10 μm would take hours to accumulate a single bacterial mRNA. We concluded that we needed 3D sensors that were approximately 100 μm-wide to detect and identify bacteria in times relevant to clinical diagnostics, i.e. 30 minutes.

- It was found that creating hierarchical structures that were 100 μm-wide and decorated with 10-20 nm grains was essential in bacterial analysis. These structures were not only rapid in analyte capture but also resulted in highly sensitive devices. Employment of hierarchical structures in RNA analysis, allowed detection and identification of clinically amounts of bacteria in 30 minutes.
9.2 Thesis contributions

The above findings led to the following contributions:

- The first multiplexed electronic system capable of DNA detection in clinical samples was developed. The sensitivity of this system was 10 aM, equivalent to detection of fewer than 100 molecules in solution. This sensitivity was achieved for the first time using a direct, amplification-free multiplexed electronic chip.[71, 84]

- Spatial multiplexing was used to integrate electrodes of different sensitivity on the same platform realizing a system with a 6 log-linear dynamic range. Prior to this work, no multiplexed electrical DNA sensor reported such wide dynamic range.[75, 108]

- Hierarchical electrodes, having a 100 μm footprint decorated with 10-20 nm grains were used for the first time as DNA sensing electrodes. These electrodes had a dual role of overcoming the long transport times associated with large biomolecules and improving the sensor’s hybridization efficiency. Throughout this unique material engineering, we were able to achieve detection of 1.5 CFU/μL of bacteria in times as short as 30 minutes, making this technique one of the fastest bacterial detection and identification platforms reported to date.

9.3 Future work

This section provides an overview of the remaining challenges in developing practical biosensing devices. Addressing such challenges involves defining research problems that can be addressed by researchers involved in engineering, chemistry, biochemistry and materials research.

Increasing system’s throughput and degree of multiplexing:

Previous chip designs integrated 8 electrodes on a silicon platform. This chip was suspended in an electrochemical cell containing electrolyte solution along with reference and auxiliary electrodes. A new chip design featuring a larger number of electrodes on the same area, along with on-chip reference and auxiliary electrodes, will improve the system’s throughput and make it more compact and portable. As a first step in addressing the throughput issue, we designed a new microchip with an array of 42 electrodes along with on-chip reference and auxiliary
electrodes. (Figure 9.1) The testing and use of this system for detection of relevant nucleic acids will be addressed by members of the research group in the future. Furthermore, we envision that new and improved versions of this chip with active components for on-chip data acquisition, storage and transmission be implemented by the members of this research group along with other researchers in the field.

![Figure 9.1 Electronic chip featuring 42 working electrodes and a reference and a counter electrode.](image)

**Figure 9.1 Electronic chip featuring 42 working electrodes and a reference and a counter electrode.**

**Automating and integrating sample preparation**

It is of urgent importance for point-of-need applications to integrate sample preparation and sensing in a single, convenient, single-use cartridge. Other researchers in our research group have created cartridges insertable into readout instruments. (Figure 9.2A) To create a mass producible and cost-effective platform, it is desirable to create a cartridge that can be microfabricated using a single process. This should be addressed in the future work.

![Figure 9.2 Fully integrated DNA analysis platform. A) Cartridge combining the sensing chip with the lysis module B) Cartridge and readout computer are inserted in the portable readout module.](image)

**Figure 9.2 Fully integrated DNA analysis platform. A) Cartridge combining the sensing chip with the lysis module B) Cartridge and readout computer are inserted in the portable readout module.**

**Active molecular transport**
To achieve faster molecular transport, electric field [112], magnetic field [113], fluid flow [114] and mixing[115] were previously used. It is important to develop different fabrication methods to integrate field generating electrodes and microfluidic networks on the sensing array. These fabrication and integration challenges can be addressed in future work.

Multiplexed Immunoassays based on electronic chips

Immunoassays have been widely used in detection and quantitation of biochemical analyte in complex samples. Protein detection using immunoassays has been used in detection and quantitation of cancer-related biomarkers. For example, high levels of Prostate Specific Antigen (PSA), related to prostate cancer development, have been identified using immunoassays.[116]

In spite of recent advancements, existing technologies depend on expensive optical readout instrumentations for quantitating fluorescence signals[117, 118]. Inexpensive and portable analysis is not possible using these platforms. This presents the opportunity to extend our platform to multiplexed protein detection. This advance will rely on selecting clinically relevant protein biomarkers and antibodies for their capture. Biosensing electrodes will be reengineered to enable efficient antibody-protein interactions. New electrochemical transduction methods can be developed in future to maximize sensitivity while minimizing the background effects from non-specific adsorption.

Multiplexed platforms for parallel protein and genetic analysis

The reliability of molecular diagnostics will be improved if we simultaneously monitor the presence and quantity of disease-related proteins and nucleic acids. For example, prostate cancer development is correlated with gene fusions along with an increase in the concentration of PSA. The use of each biomarker alone can lead to high false positive rates; however, simultaneous monitoring of these biomarkers results in much more reliable diagnostic technologies.

For this reason, developing a multiplexed chip with functionalized electrodes for selective protein and DNA capture is of high importance. Furthermore, such chip can be coupled to a single reporter group for signal transduction of protein binding and DNA hybridization. This will lead to a unique molecular diagnostics tool with exceptional reliability.

Multiplexed platforms for detection of viral infections for halting pandemics
Viral infections spread quickly and are responsible for the recent HIV and flu pandemics. Symptomatic diagnostic methods are often inefficient in preventing the spread of many of such infections. For example, seasonal flu and its deadly variations often present undistinguishable symptoms at their early stages. As a result, genetic analysis is essential in taking the appropriate measures for preventing the spread of dangerous strains of viruses across the human populations.

Development of handheld, sensitive, selective and integrated genetic analyzers for detection and identification of viruses is essential. To address this need, the biosensing platform can be reengineered to analyse viral genomes at the point-of-need. For this purpose, isolation of viral DNA from its surrounding protein protection layer should be achieved and integrated with the sensing array. Furthermore, probes capable of selective capture of viruses should be designed.

### 9.4 Final Remarks

Prior to this work, the following question could have been posed: would it be possible to analyze genetic diagnostic biomarkers using a portable, simple-to-operate instrument at a cost lower than $100 in less than 30 minutes?

Previously, the answer to this question was: it is unknown if and when this would be possible. At the moment, we can say that it is indeed possible to make a device capable of sensitive and selective DNA detection, which is portable and inexpensive enough to be used as a handheld diagnostic device. This has been achieved through this work by combining innovations in electrical engineering, materials engineering, and biochemistry.
10 Publication List and Other Contributions

10.1 Refereed journal publications


10.2 Patents

Nanostructure microelectrodes and biosensing devices incorporating the same.
WO/2010/025547, 2010

10.3 Conference presentations


References


Appendix 1-Detailed Methods

Electrodeposition of Nanotextured Microelectrodes (NMEs)

1. Place chips in a beaker and sonicate them in acetone for 5 minutes. Make sure they are not overlapping.
2. Rinse chips one by one in IPA for 5 seconds, and water for 5 seconds, and blow dry with a stream of N2.
3. Place 2 mL of the desired plating solution in a petri dish.
4. Immerse reference and auxiliary electrodes in the plating solution. Contact the outlet for reference electrode to the white wire coming from the potentiostat and contact the auxiliary electrode to the red wire coming from the potentiostat.
5. Contact an NME lead using a minigrabber and immerse the bottom 2 mm of the chip in solution. Contact the minigrabber wire to the black alligator clip of the potentiostat.
6. Open Epsilon software and choose new experiment. Choose DCPA as the electrodeposition technique and use the desired plating time and potential.
7. After the first plating raise the chip and move the minigrabber to the second lead. Continue steps 5-6 until all leads are plated
8. Once all leads are plated raise the chip and gently remove it. Rinse the chip in water for 5 second and dry it with a duster.

Probe deposition

1. Take the chips gone through electrodeposition and place each chip in a petri dish. Place the dishes in a humidity chamber. Make sure humidity chamber contains some water to avoid drying of the probe solution.
2. Make the probe solution according to Table 1 for a typical experiment or using other methods described in this thesis. It is necessary to make 10 µL of probe solution per each 4 lead chip.
3. Using a pipettor, place 10 µL of the probe solution prepared in step 2 on the bottom portion of each chip covering all NMEs.
4. Cover the humidity chamber and store the humidity chamber according to the information in table 2 in a dark area.

**Table 0-1-Typical probe solution preparation**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Concentration</th>
<th>MCH concentration</th>
<th>MgCl₂ Concentration</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>5 uM</td>
<td>0</td>
<td>100 mM</td>
<td>25 mM NaCl/25 mM NaPhos (25/25)</td>
</tr>
<tr>
<td>PNA</td>
<td>500 nM</td>
<td>100 nM</td>
<td>0</td>
<td>25 mM NaCl/25 mM NaPhos</td>
</tr>
</tbody>
</table>

**Table 0-2-Typical probe deposition**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Deposition Temperature (°C)</th>
<th>Deposition Duration (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>PNA</td>
<td>4</td>
<td>16-24</td>
</tr>
</tbody>
</table>

Hybridization and detection

1. Wash the chip after probe deposition step with 25/25 buffer twice, 5 minutes each.

2. Set up a 3-electrode system. Scan the NMEs with the solution 1 and then solution 2 listed in Table 3 and parameters in Table 4 using Cyclic
Voltammetry (CV). All of the solutions are made with 25/25 buffer (pH 7) and degassed with nitrogen/Argon for 15-20 minutes.

3. Prepare a target solution of desired concentration in 25/25 buffer (pH 7) (When hydridizing DNA/RNA target to DNA probe include 100 mM MgCl₂ in the target solution).

4. Add 20 µl of the target solution to each chip and place the chips in the humidity chamber.

5. Place the humidity chamber (with the chip inside) in the 37°C incubator for 1 hour or as described by experimental methods.

6. Take the humidity chamber out of the incubator and wait 5 minutes for the chips to cool down to room temperature. Afterwards, wash the chips twice with 25/25, 5 minutes each time.

7. Set up a 3-electrode system. Scan the NMEs using CV with the same solutions as in step 2.

8. Save and analyze the data by calculating ΔI.

### Table 3-EC Solution preparation

<table>
<thead>
<tr>
<th>Solution</th>
<th>Solvent</th>
<th>Final solute concentration</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 mM NaCl/ 25 mM NaPhosphate</td>
<td>5 µM Ru(NH₃)₆³⁺</td>
<td>Ru</td>
</tr>
<tr>
<td>2</td>
<td>25 mM NaCl/ 25 mM NaPhosphate</td>
<td>5 µM Ru(NH₃)₆³⁺/4 mM Fe(CN)₆²⁻</td>
<td>Ru/Ferri</td>
</tr>
</tbody>
</table>
### Table 4-EC CV parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Solution 1</th>
<th>Solution 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Potential (mV)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Switching Potential 1 (mV)</td>
<td>-400</td>
<td>-400</td>
</tr>
<tr>
<td>Final Potential (mV)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># of Segments</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Scan Rate (mV/s)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Quiet Time (s)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Full Scale</td>
<td>100 nA</td>
<td>100 nA</td>
</tr>
</tbody>
</table>

**Bacterial Growth and Lysis**

1. Grow E. coli bacteria from a commercial stock solution by adding 10 µL of stock solution to 10 mL of Lysogeny Broth (LB-Broth) at 37°C for 20 hours in a shaking incubator.

2. After growth, bacterial samples are centrifuged at 3000 rpm for 5 minutes. The supernatant is discarded, and replaced with an equal amount of 1x saline Phosphate Buffered Saline (PBS) (pH 7.4). Bacteria are re-suspended by vortexing. This process is repeated once again to completely replace the LB broth with PBS.

3. 1.5mL of bacterial sample suspended in PBS buffer is loaded into a 3mL syringe and placed in a syringe pump and connected to the lysis chamber inlet using HPLC tubing.

4. The sample is inserted into the lysis chamber inlet and leaves the lysis chamber into a large pipette tip without applying any electric potential. This process washes the lysis chamber and all the tubes involved.
5. Full lysis of samples is achieved with a flow rate of 20\(\mu\)L/min using a syringe pump and application of 400V. 400 V is applied using a Keithly Source meter between electrodes present in the inlet and outlet of the lysis chamber.

6. Lysed samples are collected at the outlet of the lysis chamber in a large pipette tip.

7. Following obtaining enough lysed sample, the potential is turned off and the sample is moved from the inlet to the outlet through the lysis chamber.

8. After washing the chamber with unlysed sample, 100 \(\mu\)L of sample is collected and diluted. Different dilutions are placed on agar plates and incubated at 37\(^{\circ}\)C for 20 hours. After bacterial growth to visible colonies, they are counted to obtain the number of colony-forming-unit of bacteria in one mL (CFU/mL).