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Microfabrication of Electrochemical Analytical Devices for Detection of Pathogen Species DNA and Toxic Metal Ions

Spencer Williams

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MICROFABRICATION OF ELECTROCHEMICAL ANALYTICAL DEVICES FOR DETECTION OF
PATHOGEN SPECIES DNA AND TOXIC METAL IONS

By

Spencer Williams

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

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2015
ABSTRACT

Microfabrication of Electrochemical Analytical Devices for Detection of Pathogen Species DNA and Toxic Metal Ions

By

Spencer Williams, Master of Science
Utah State University, 2015

Major Professor: Dr. Anhong Zhou
Department: Biological Engineering

Water quality is a crucial factor in determining the health of an environment, and in turn the health of all those living in the given environment. Early detection of potential hazards in water sources would help to avoid outbreaks of sickness and even death. Current methods for detection of these contaminants are extremely expensive and time consuming. This thesis provides an in-depth investigation into electrochemical techniques that can be used to develop cost effective, portable, and easy to use devices.

The specific contaminants used in the testing process for varying electrochemical techniques include lead ions, cadmium ions, and DNA sequences from a common parasite Cryptosporidium parvum. Lead and cadmium ions were selected because of their convenient and well-known electrochemical properties. C. parvum was chosen because many of the different genotypes genetic sequences are available in public
databases. A novel platform for detection was introduced for quickly distinguishing between different species of *C. parvum*. The platform allowed for improved selectivity and response time of current electrochemical techniques. A microelectrode array was designed and tested for the detection of metal ions, but also has the potential to incorporate detection of pathogenic microorganisms. This array provides a quick and simple test that is capable of detecting such contaminants at low concentrations. Six electrodes were incorporated into one microfluidic device, allowing for multiple tests to be performed simply by switching the active electrode.

(84 pages)
Microfabrication of Electrochemical Analytical Devices for Detection of Pathogen Species DNA and Toxic Metal Ions

Spencer Williams

Water quality is a crucial factor in determining the health of an environment, and in turn the health of all those living in the given environment. Early detection of potential hazards in source water used for drinking would help to avoid outbreaks of sickness and even death. Contaminants, found in contact and drinking water, having negative effects on human health may include pathogenic microorganisms, and metal ion pollutants such as lead or cadmium. Lead and cadmium were selected due to well-known electrochemical properties providing easy references for developing new detection devices. Current methods for detection of these contaminants are extremely expensive and time consuming. The focus of this project was to develop a single device that selectively detects multiple contaminants while maintaining sensitivity. This thesis provides an investigation into electrochemical techniques that can be used to develop cost effective, portable, and easy to use devices. A fabricated microelectrode array is proposed and tested that can be used for the detection of lead and cadmium ions, and also has the potential to incorporate detection of DNA sequences for specific species of pathogenic microorganisms.
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-Spencer Eugene Williams
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1.1 Motivation

The ability to monitor water quality is a vital attribute for communities throughout the world. Water quality, among a variety of factors, helps determine the health of individuals as well as the overall health of surrounding environments. A large focus has been placed solely on drinking water due to the direct consequences of ingesting contaminated sources, but even exposure to contaminated water has shown adverse health effects [1]. Providing more accessible, and efficient methods for detecting pollutants in water would benefit both developed, and developing countries.

Currently, the average person is not equipped to detect invisible contaminants in water because it requires high levels of expertise, and expensive instrumentation. The need for easy to operate, low cost, and portable detection devices is fueling research in a wide variety of disciplines [2]. Electrochemistry is a field of study that provides many promising techniques for obtaining efficient detection of harmful substances in water [3-5]. Incorporating these electrochemical methods into portable, inexpensive devices will provide ordinary people with the ability to monitor water quality, and increase opportunity for a healthy life.

An increasing number of substances carried in water are being recognized as harmful to human health. Greater understanding of health risks associated with contaminants in water increases the need for knowing exactly what the water sources
contain. The focus of this project will be two well-known impurities in water, pathogenic organisms and metal ions that have known negative health effects [6-7]. Solutions for improving the efficiency of detection methods using similar principles and techniques from electrochemistry are discussed for both contaminants.

1.2 Project Overview

1.2.1 Pathogen Species DNA Detection

Biological contaminants in water continue to cause illness and death all over the world in the midst of improving water treatment processes. Waterborne pathogens are one of the main reasons for 4 billion cases of diarrhea that cause 1.6 million deaths annually, and 62.5 million Disability Adjusted Life Years (DALYs) worldwide [8]. Many of these pathogens are known, and they have a variety of resistive attributes to current treatment methods allowing for continued infections. There is a need to detect which pathogens are present, and identify potential hazards and even target hosts.

*Cryptosporidium parvum* is a protozoan parasite that has been recognized as a human pathogen since 1976, and there have been numerous outbreaks reported in more than 40 countries. Symptoms of infection typically include debilitating diarrhea, cramps, weight loss, anorexia, and low-grade fever [9]. Different genotypes or species of *C. parvum* have been identified, and each of these are known to infect a different species of animal based on a short genetic sequence of DNA located in the heat shock protein gene. Each species of *C. parvum* differs by only a few nucleotides in this
sequence, and it is important to distinguish among varying species so that it can be determined who potentially may be infected [10]. Current available technologies usually include flow cytometry [11], polymerase chain reactions [12], or other form of DNA amplification such as rolling circle amplification [13].

Electrochemistry offers techniques that allow for highly specific sequence detection of small strands of DNA, potentially being able to differentiate between single nucleotide polymorphisms (SNPs). Electrochemical biosensors have been developed that utilize the hybridization of single stranded DNA with its complementary strand of DNA to detect a parasite if the complement is present. These sensors provide great recognition, but still have some drawbacks in that they are complex and time consuming [14-15]. A new method introduced in this project uses a novel alignment of single stranded DNA on an electrode surface that does not require additional markers or labels for detection of specific DNA sequences. This new label free platform improves upon current electrochemical biosensors, and will be discussed later in detail.

1.2.2 Toxic Metal Ion Detection

Industrialization is expanding in many countries around the world, increasing the amount of hazardous pollutants being introduced into the environment [16]. Common pollutants are typically in the form of metal ions, and the Environmental Protection Agency (EPA) sets limits for the presence of these ions in drinking water. The EPA limits for a variety of pollutants can be found on their website, epa.gov, and a few of the contaminant regulations are listed in Table 1.1 including the enforceable maximum
contamination level (MCL) allowed, and the environmental MCL goal (MCLG). These metal ions enter organisms and interfere with metabolic processes, producing many physiological problems with the nervous, immune, and gastrointestinal systems [17]. The ability to monitor for hazardous metal ion contamination simply and quickly will help to locate polluted bodies of water so the remediation process may begin.

Table 1.1 - EPA regulations for common contaminants in drinking water.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Maximum Contaminant Level (MCL)</th>
<th>Maximum Contaminant Level Goal (MCLG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic (As)</td>
<td>0.01 mg/L</td>
<td>0</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>0.005 mg/L</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>0.005 mg/L</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>1.3 mg/L</td>
<td>1.3 mg/L</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>0.002 mg/L</td>
<td>0.002 mg/L</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>0.015 mg/L</td>
<td>0</td>
</tr>
</tbody>
</table>

Current standard methods for selective detection of ions include atomic absorption spectroscopy (AAS), and inductively coupled plasma mass spectroscopy (ICP-MS) [18-19]. Both of these methods are expensive and are not convenient for field application because they involve complex operation and detailed sample preparation. AAS is the less expensive option and is easier to operate, but requires a graphite furnace to achieve desired sensitivity. AAS provides great selectivity, but this is also a challenge since a unique light source is required for every individual element to be detected. Therefore, the cost and time required increases for every additional ion to be analyzed.
ICP-MS on the other hand is not only expensive to purchase, but is also expensive to maintain and operate. Some of the advantages to using ICP-MS include multiple element analysis, quick analysis, large linear detection range, and low detection limits [20]. Hence, electrochemical methods are explored as an alternative for detection of hazardous ions due to their sensitivity, selectivity, portability, and lower cost [21].

The testing of samples is rarely limited to only one ion, and there is currently no efficient method for analysis of multiple ions simultaneously. The focus of this project was in developing a single device that selectively detects multiple ions while maintaining adequate sensitivity. In addition to satisfying the necessary performance factors, other factors were considered such as cost of the device, ease of use, and cost of required instrumentation. The project combined electrochemical instruments and methods with a newly fabricated device that can achieve detection of multiple ions simultaneously. This combined design provides an opportunity to maintain acceptable detection limits and sensitivity, while limiting costs and difficulty of use. The ability to efficiently detect trace amounts of hazardous pollutants improves the monitoring of water quality, and in turn benefits human health.

The availability of such electrochemical methods to the general public, will allow for frequent testing of more water systems in a variety of locations. Since metal ions tend to accumulate in organs over time leading to toxicity, consistent monitoring is important to determine the health of water systems, and to avoid potentially harmful
situations. There is a need for more effective and efficient methods, and research is ongoing to develop sensitive electrodes for the detection of multiple ions [22].

1.2.3 Electrochemical Techniques

A wide variety of electrochemical techniques are available with electrochemical analyzing instruments. All electrochemical experiments done with regard to this thesis were performed using two instruments. One is a VMP2 Multi Potentiostat from BioLogic, and the other a CH Instruments Electrochemical Analyzer. The first being a large benchtop unit, and the second is a smaller, more portable unit that could potentially be used for field experiments. The CHI Electrochemical Analyzer is also more affordable when compared to instruments such as the AAS and ICP-MS as will be shown later in the cost analysis. The specific techniques used for analysis of pathogen species, and toxic metal ion detection will be introduced in this section.

A three-electrode setup was used for both instruments, and the basic layout of all electrochemical experiments can be seen in Figure 1.1. The three electrode system consists of a working electrode (WE), counter electrode (CE), and a reference electrode (RE). For the purposes of this work, a gold electrode was used for the WE that acted as the anode, a platinum wire electrode was used for the CE that acted as the cathode, and a silver/silver-chloride electrode was used as the RE to help measure the voltage potential between the WE and CE.
Figure 1.1 – A diagram of the basic 3 electrode system for general electrochemical techniques. The silver/silver-chloride reference electrode (RE), gold working electrode (WE), and platinum wire counter electrode (CE) are shown.

Electrochemical impedance spectroscopy (EIS) is a powerful technique that allows for converting small changes at an electrode/electrolyte interface into a measurable electrical signal [23]. The changes being monitored and analyzed will be the binding of single stranded DNA to a gold WE, and whether or not hybridization occurs when potential complimentary strands of DNA are introduced.

The electron transfer through a DNA monolayer on the electrode surface can be described as an equivalent circuit model known as the Randles model [24]. This model has four components that can be obtained using EIS; the electrolyte resistance ($R_\Omega$), charge-transfer resistance ($R_{ct}$), double-layer capacitance ($C_d$), and Warburg impedance ($Z_W$) due to mass transport at the electrode surface. A specific kinetic parameter, the
standard charge-transfer rate constant \( (K_{a0}^0) \), relating to the electron transfer can be obtained by using the \( R_{ct} \) value inserted into Equation 1.1 \([25]\). Where \( R \) is the universal gas constant, \( T \) is 298 K, \( A = 0.0314 \) cm\(^2\), \( n = 1 \), and \( F \) is Faraday’s constant. The \( \alpha \) is assumed to be 0.5, and \( C_R^\alpha = C_0 \) where \( C_0 \) is the concentration of the bulk electrolyte solution. The \( K_{a0}^0 \) was used to compare varying electrode surfaces, and analyze whether or not hybridization was occurring.

\[
R_{ct} = \frac{RT}{n^2 F^2 K_{a0}^0 C_0^{(1-\alpha)} C_R^\alpha} \quad \text{(Equation 1.1)}
\]

The \( R_{ct} \) values are obtained from the semicircular portion of the Nyquist plot that shows the imaginary component versus the real component of impedance over a range of frequencies. Figure 1.2 shows a sample Nyquist plot along with the corresponding equivalent circuit. EIS is a promising method for DNA detection compared to other methods since impedance is inversely related to current, and this permits detection in low target concentration ranges when changes in current are too small to detect. EIS also provides advantages by allowing for label free DNA detection that makes for a less expensive, more simple detection method \([26-28]\).
Figure 1.2 – Diagram of a typical Nyquist plot with monolayer DNA on the electrode surface. The inset shows the corresponding Randles equivalent circuit. $R_\Omega$ is the electrolyte resistance, $R_{ct}$ is the charge-transfer resistance, $(C_d)$ is the double-layer capacitance, and $(Z_w)$ is the Warburg impedance.

Differential Pulse Anodic Stripping Voltammetry (DPASV) is a technique that is currently being used in research for detecting trace amounts of heavy metal ions in solution [29-32]. This technique is a specific application of differential pulse voltammetry (DPV) where the fundamental method measures current over a certain voltage potential range. The voltage changes in a series of steps, and the measured
current is plotted versus the potential. There is a pre-concentration time period where positively charged ions in solutions are accumulated onto the negatively charged WE surface. Metal ions have specific oxidation potentials that can be utilized once they have been concentrated onto the electrode surface. As the potential changes, a peak in current will occur at the oxidation potential of the corresponding ion. The intensity of the peak current will be directly related to the concentration of ions present in the solution. A diagram depicting the overall process of DPASV can be seen in Figure 1.3. This technique allows for highly specific detection and quantification of ions in solution.

![Graph showing differential pulse anodic stripping voltammetry](image)

**Figure 1.3** – A diagram of differential pulse anodic stripping voltammetry. The inset shows measured current vs. potential during the stripping phase to observe oxidation.
Cyclic Voltammetry (CV) is a technique similar in function to the previously mentioned DPV. However, in CV there is a complete scan in both directions so both oxidation and reduction of an ion can be observed. The change in voltage potential is also a direct linear sweep instead of a series of potential steps, and the curve produced can be seen in Figure 1.4.

Figure 1.4 – A diagram showing one cycle of Cyclic Voltammetry. Measuring the current versus the change in potential. The inset shows voltage potential over time for the same cycle.
CV was used to verify the functionality of fabricated electrodes by using potassium ferricyanide, which is easily oxidized and reduced at known potentials. According to the Randles-Sevick equation [25] seen in Equation 1.2 there is a linear relationship between the oxidation or reduction peak current $i_p$ and the scan rate $v^{1/2}$. In the equation, $n$ is the number of electrons transferred, $A$ is the area of the electrode, and $C$ is the concentration of the potassium ferricyanide in solution. The slope of the line for peak current $i_p$ versus the scan rate $v^{1/2}$ yields the diffusion coefficient ($D_0$) of the electroactive species to or from the electrode surface. The $D_0$ was used to confirm that the electrolyte mass transfer for the electrodes in the fabricated device was similar to commercial electrodes in a bulk solution.

$$i_p = (2.69 \times 10^5)n^{3/2}AD_0^{1/2}Cv^{1/2}$$ (Equation 1.2)

1.2.4 Device Fabrication

In order to bring novelty to the field of electrochemical detection, a new device needs to be fabricated. The techniques and principles involved are becoming well established, but the application of these methods has plenty of room for advancement. Current methods and devices typically only use or provide one WE for experiments, and commercially available gold electrodes are near 100 dollars per electrode [33-35]. Utilizing knowledge of the microfabrication process in concert with the principles of electrochemistry, a new device is fabricated and tested that incorporates six WEs onto a glass slide. The six electrodes will have individual access to each through microfluidic
channels, but also will be connected to a bulk solution in order to perform electrochemical tests. This way each electrode can be prepared individually for a unique purpose, or they can be prepared uniformly all at once. The structure and design of this device will be discussed in detail in Chapter 3.

1.3 Project Goals and Aims

The goal of this thesis was to address the need for inexpensive, portable, and easy to use methods of detecting harmful substances present in water. To meet this goal, different approaches were explored that potentially will be combined together for a highly efficient, multiple use device that can be easily distributed. The first approach is to improve the technique currently used for electrochemical detection of DNA hybridization. This is to be accomplished by designing and testing a new structure for the immobilized DNA on the surface of the electrode. The second approach is to design and fabricate a device that not only detects trace amounts of toxic metal ions, but also has the potential to incorporate the detection of DNA hybridization developed in the first approach. The completion of these two approaches would provide an efficient alternative to costly, bulky, and difficult to use methods.
CHAPTER 2
LABEL-FREE MISMATCH DETECTION OF PATHOGEN DNA ON SOLID SUBSTRATE

2.1 Introduction

A new label-free platform for specific DNA sequence detection that has the potential to overcome drawbacks of current conventional electrochemical methods is introduced in this chapter. This label-free platform brings a novel approach to DNA sequence detection by immobilizing DNA horizontally on the electrode surface offering low steric interference, excellent mismatch discrimination, and quick response time. Electron transfer characteristics of the horizontal immobilized DNA sequences were investigated and compared to the conventional method of vertically aligned DNA. The potential for this new platform to be incorporated into a microelectrode array would provide an inexpensive and simple method for distinguishing between various genotypes for a variety of pathogens.

The specific sequences of single stranded DNA (ss-DNA) immobilized to the electrode surface will further be referred to as probes, and the provided unknown DNA strands to be detected in solution are the targets. The conventional platform for a DNA hybridization assay, includes target DNA molecules in solution colliding with vertically aligned probes that are tethered to the gold electrode. As the reaction proceeds, the hybridization of the probes and targets form a dense canopy that acts as a resistive layer that increases the activation energy of further reactions. The increase in required activation energy leads to a decrease in the diffusion rate of the targets to the electrode
surface. The reaction rate of hybridization can be increased by making the probe DNA more accessible to the targets, and thus addressing the rate limiting step that governs most hybridization reactions.

Proper detection for conventional methods lead to slow reaction times, depending heavily on the length of the probe and location of any mismatched nucleotides. Considering the drawbacks of existing techniques, the newly proposed horizontal platform offers a rapid, sensitive, label-free, and controllable method for DNA mismatch detection. This method consists of a mixed monolayer of thiolated poly-T single stranded DNA posts, and 6-mercapto-1-hexanol (MCH) that allow for probe DNA to be exposed horizontally over the DNA posts when two poly-A tails are attached to both sides of the probe DNA strand. For proof of principle, DNA sequences of the heat shocked protein \( (hsp70) \) from the human genotype (Genebank Access No. AF221535) of \( C. parvum \) were used, and sequences from the bovine genotype were used to demonstrate differentiation capabilities. Electrochemical characterization of the DNA assembly on the electrode surface, and the mismatch detection capabilities were investigated.
2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Chemicals were of analytical reagent grade and were supplied by Sigma-Aldrich unless otherwise mentioned. Distilled water was used in all solutions. All DNA sequences (Table 2.1) used was synthesized and purified from University of Utah Core Facility (Salt Lake City, USA). A three-base mismatched DNA target between base positions 1511 to 1550 of the bovine isolate of *C. parvum* (U71181) was also used. DNA samples were diluted to 1 µM concentration stock solutions. 1 mM 6-mercapto-1 hexanol (MCH) was prepared in distilled water. 1 M KH$_2$PO$_4$ (pH 4.5) was used as the immobilization buffer and hybridization buffer containing 50 mM Tris-HCl, 250 mM NaCl, 10 mM MgCl$_2$, pH 7.4 at 25°C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe-DNA</td>
<td>5'-(POLY-A$<em>{28}$)-TACCAGTGCTTTTATCAACACGAGATATTCAAGATACC-(POLY-A$</em>{28}$) - 3'</td>
</tr>
<tr>
<td>Probe-DNA 2</td>
<td>5'-SH- TACCAGTGCTTTTATCAACACGAGATATTCAAGATACC-3'</td>
</tr>
<tr>
<td>T-DNA</td>
<td>5'-GGT ATC TTG AAT GTA TCT GCT GTT GAT AAA AGC ACT GGT A-3'</td>
</tr>
<tr>
<td>(Human Genotype)</td>
<td></td>
</tr>
<tr>
<td>NT-DNA</td>
<td>5'-GGT ATC TTG AAT GTG TCT GCT GTT GAT AAG AGT ACT GGT A-3'</td>
</tr>
<tr>
<td>(Bovine Genotype)</td>
<td></td>
</tr>
<tr>
<td>Poly-T DNA Post 1</td>
<td>DP 1 : 5'-SH-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'</td>
</tr>
<tr>
<td>Poly-T DNA Post 2</td>
<td>DP 2 : 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT- SH-3'</td>
</tr>
<tr>
<td>SNP(5' - end) - DNA</td>
<td>5'-'GGT CTC TTG AAT GTA TCT GCT GTT GAT AAA AGC ACT GGT A-3'</td>
</tr>
<tr>
<td>SNP(Middle) - DNA</td>
<td>5'-'GGT ATC TTG AAT GTA TCT ACT GTT GAT AAA AGC ACT GGT A-3'</td>
</tr>
<tr>
<td>SNP(3' - end) - DNA</td>
<td>5'-'GGT ATC TTG AAT GTA TCT GCT GTT GAT AAA AGC ACT TGT A-3'</td>
</tr>
</tbody>
</table>
2.2.2 Preparation of Gold Electrode Surface

Gold electrodes were cleaned with 25% H$_2$O$_2$ and 75% H$_2$SO$_4$ by volume (piranha solution) for 10 minutes to remove organic impurities and then rinsed with distilled water. The gold surface was then polished by hand to restore a smooth surface, using a three step polishing kit (CH Instruments, Austin, USA).

2.2.3 Immobilization of DNA Posts on Gold Surface

The freshly cleaned gold electrodes were immersed in a solution mixture (1:1) 0.5 µM DP-1: DP-2 (Poly-T DNA posts) in immobilization buffer for 90 minutes at room temperature. The modified electrode was rinsed with distilled water to remove any DNA that was non-specifically adsorbed on the surface. The electrodes was then dried with compressed nitrogen gas, and immersed in 1 mM MCH solution for 2 hours. After immersion in MCH, the electrode was rinsed with distilled water and immersed in immobilization buffer overnight, at 4°C. The poly-T DNA strands immobilized on the gold surface were termed DNA posts. The electrodes were taken directly from the 4°C refrigerator and used for electrochemical measurements.

2.2.4 Probe DNA Addition to Gold Surface

The electrodes with DNA posts on the surface were further used as a platform for DNA mismatch detection. The prepared electrodes were incubated in 40 µL (0.5 µM probe- DNA) solution for approximately 10 minutes, unless stated otherwise. This created bridges of DNA across the established posts due to the poly-A anchors on the probe-DNA, linking itself with the DNA posts, exposing the capture site. The electrodes
were rinsed in hybridization buffer for 5 minutes to avoid DNA adsorption. The same hybridization procedure was adopted for TDNA or NT-DNA hybridization. The gold electrodes with the probe-DNA were immersed in either 0.5 µM T-DNA or 0.5 µM NT-DNA in ligase buffer for approximately 10 minutes as well, unless stated otherwise.

2.2.5 Electrochemical Measurements

DNA hybridization and platform formation cause an increase in the electrical resistance of a conducting surface that is reflected in electrochemical impedance spectroscopy (EIS) measurements. The specifically designed probe-DNA when linked to Poly-T posts forms a gapped-duplex structure. The effect of mismatch and probe-DNA density on the gapped-duplex structure was studied using EIS.

Experiments were performed using the three-electrode setup; consisting of a platinum wire CE, a silver/silver-chloride RE, and a gold WE all obtained from CH Instruments. EIS measurements were carried out in a solution of 0.5 mM potassium ferricyanide + 0.5 mM potassium ferrocyanide + 0.01 M phosphate buffered saline (PBS) under an AC amplitude of 0.01 V, and a frequency range from 100 KHz to 0.01 Hz.

Kinetic parameters related to the electron transfer at the DNA modified electrode surface can be obtained by EIS analysis that include the heterogeneous standard charge-transfer rate constant ($K_a^0$), and the charge transfer resistance ($R_{ct}$).
2.3 Results and Discussion

2.3.1 Design of Gapped-Duplex Based DNA Biosensor

DNA of 96 nucleotides in length was used as the probe-DNA, consisting of a 40 nucleotide detection zone or capture site, and two 28 nucleotide anchors at both ends completing the single strand DNA seen in Figure 2.1(a). The nucleation sites at the capture site, of the horizontally aligned probe-DNA, are now more accessible to T-DNA. T-DNA, 40 nucleotides in length, is complimentary to the capture site. Figure 2.1 shows the most probable conformational orientation of the gapped duplex.

The proposed hybridization assay is a two-step process. The first step involves probe-DNA linkage (96 nucleotides) to the previously immobilized poly-T posts. Secondly, the probe-DNA linked surfaces were allowed to react with the T-DNA (40 nucleotides). The surface impedance changes associated with the probe-DNA linkages and target DNA hybridization were monitored using EIS techniques. Electron transfer resistance ($R_{ct}$) values, a measure of resistance offered by the surface to the flow of redox agent, were then obtained. An increase in $R_{ct}$ and a change in calculated $K_\alpha^{\theta}$ values were observed during T-DNA hybridization. An increase in the $R_{ct}$ values were observed after probe-DNA linkage due to the formation of gapped-duplex assembly between poly-T posts and probe-DNA anchors observed in Figure 2.1(b). The gapped-duplex assembly was employed to discriminate between human and bovine genotypes of $C$. 
C. parvum. NT-DNA indicates the three-base mismatched target DNA sequence from the bovine isolate of C. parvum, which was used as an alternate target DNA.

Figure 2.1 - Proposed platform for DNA mismatch detection: a) the probe-DNA with capture site (complementary to target DNA) and anchors with Poly-A (complementary to Poly-T post); b) gapped duplex assembly via linkage of anchors from the probe-DNA to the Poly-T posts; c) target DNA (T-DNA) hybridization with capture site of probe-DNA.
2.3.2 EIS Measurements of DNA/Electrode Interface

Figure 2.2 illustrates the $R_{ct}$ changes ($1/K_a^0$ vs hybridization time) during target-probe duplex formation for proposed and control experiments. Upon introduction of the fully complimentary T-DNA, seen in Figure 2.1(c), to the probe-DNA linked assembly, the $K_a^0$ values increased steadily and a 355% increase was observed after 50 minutes. The superior reaction rate is attributed to the easy availability of probe-DNA capture sites, making the reaction diffusion dominated for a prolonged time. A negligible change of 19.4% was observed during the three-base mismatched bovine NT-DNA hybridization. Hence, by displaying a $K_a^0$ change of 355% during T-DNA and negligible changes during NT-DNA hybridization, the gapped duplex assembly has demonstrated high selectivity and sensitivity.

In comparison, the conventional method with vertically immobilized probes displayed lower $1/K_a^0$ changes and slower reaction rates. Reaction rates for the conventional method were also seen to decrease significantly after a short time period. For example, the reaction rate during T-DNA hybridization decreased after the initial 30 minutes. This rate limiting phenomenon typically occurs when the reaction kinetics are no longer diffusion limited. The changes in $K_a^0$ of the human genotype (T-DNA) versus the bovine genotype (NT-DNA) for both the conventional, and the proposed method are depicted in Figure 2.2.
Figure 2.2 - EIS results, represented as a change in $K_a^0$, for complimentary (T-DNA, human genotype) and three-base mismatched DNA (NT-DNA, bovine genotype) detection using the conventional and proposed methods. EIS measurements were carried out in a solution of $0.5 \text{mM } K_3\text{Fe(CN)}_6 + 0.5 \text{mM } K_4\text{Fe(CN)}_6 + 0.01 \text{ M PBS}$ under an AC amplitude of 0.01 V and a frequency range from 100 kHz to 0.01 Hz.

While the conventional method recorded a 260 % change in $K_a^0$ for the T-DNA, a 190% change was also observed for the NT-DNA. During the first 20 minutes of conventional hybridization, the bovine NT-DNA (108%) and human T-DNA (128%) hybridization rates are comparable. This inability of the conventional method to discriminate genotypes with mismatches until a steady state is reached, is due to
densely packed and inaccessible probe-DNA capture sites on the electrode. Distribution of probes per unit area is termed as probe density. In the proposed method, a control for probe–DNA density can be achieved by varying the probe-DNA linkage time. DNA surface density ($\Gamma_{\text{DNA}}$) on the electrode surface was estimated with chronocoulometry, $[\text{Ru(NH}_3\text{)}_6]^{3+}$ being the indicator. The amount of resistance offered to a redox response is reflected in the cathodic peak currents. Based on the $\Gamma_{\text{DNA}}$ values, it was evident that the 20-25 minute probe-DNA linkage created a denser probe distribution than a 0.5 minute long probe-DNA linkage as expected. The $\Gamma_{\text{DNA}}$ values were found to increase from $0.132 \times 10^{-11}$ to $3.993 \times 10^{-11}$ mol/cm$^3$ in 25 minutes.

The diffusion coefficient ($D_0$), calculated from the cathodic peak currents using the Randles-Sevick equation, is another electron transfer related parameter that reflects the density of DNA linked to a surface. The bare DNA-posts on the gold electrode gave rise to a $D_0$ of $7.15 \times 10^{-6}$ cm$^2$/s. The $D_0$ was found to decrease after probe-DNA hybridization at the modified gold surface, as the DNA layer created a barrier to block diffusion of the electroactive ferrocyanide and ferricyanide ions. After 0.5 minutes of hybridization, the $D_0$ value decreased to $6.664 \times 10^{-6}$ cm$^2$/s. The subsequent hybridization of probe-DNA under longer hybridization times (0.5-25 minutes) led to a decrease in $D_0$ values. The EIS measurements on T-DNA hybridized probe-DNA further validated probe density control. Figure 2.3 represents the rate of change of $R_{\text{ct}}$ values at various probe densities. Surfaces with denser probe distribution took longer time to attain a hybridization steady state; reflected as negligible changes in $\Delta R_{\text{ct}}$ values. The
ΔR_{ct}(t)^{10} values reached equilibrium earlier and slower than ΔR_{ct}(t)^{2} and ΔR_{ct}(t)^{0.5}. This clearly shows the competence of the proposed methodology to control probe-DNA density on an electrode surface.

![Figure 2.3](image)

**Figure 2.3** - The rate of change of ΔR_{ct} over time for various probe densities.  

R_{ct}(t)^x represents the change in ΔR_{ct} values for x minutes of probe-DNA hybridization in 50 mM Tris-HCl + 250 mM NaCl + 10 mM MgCl_2, pH 7.4.

2.3.3 Specificity Evaluation of Gapped-Duplex DNA Biosensor

The effect of mismatch position and type of mismatch with regard to hybridization specificity was investigated. In order to interrogate the effect of mismatch position, three probes were designed that had variations of single nucleotide polymorphisms (SNPs) at the distal end (SNPS’-DNA), middle (SNPM-DNA), and proximal
end (SNP3' -DNA) of the human genotype target-DNA. All the three SNP variants gave negligible changes in $K_a^0$ (2.55, 1.58, 5.76% as compared to the 355% change in T-DNA), as shown in Figure 2.4.

![Figure 2.4 - Percentage change in $K_a^0$ during SNP mismatches at different locations on the target DNA strand. SNP5'-DNA indicates a mismatch at the 5’ end, SNP_M-DNA indicates the middle, and SNP3'-DNA indicates the 3’ end. All were compared with complementary hybridization in 50 mM Tris-HCl + 250 mM NaCl + 10mM MgCl$_2$, pH 7.4.](image-url)
2.4 Conclusions

A novel label-free, DNA mismatch detection platform was introduced. The sensitivity, selectivity, and the response time of the platform was demonstrated using electrochemical impedance spectroscopy. EIS and chronocoulometry were also used to monitor the probe density on the electrode surface. The increments in $R_{ct}$ values for varying hybridization times were considered as an indication of increased surface impedance. This proposed method has the ability to overcome major shortcomings associated with conventional hybridization methods. After evaluation of hybridization efficiency, DNA mismatch discrimination, and probe density control, the platform was determined to be a strong candidate for label free DNA mismatch detection. This method can be employed to improve the specificity of DNA mismatch systems that necessitates long probe DNA, and also has the potential to be incorporated into an electrode array device for detection of multiple genotypes simultaneously.
CHAPTER 3
ELECTROCHEMICAL AND COLORIMETRIC DETECTION OF METAL IONS

3.1 Introduction

To accomplish the goals set forth for creating easy to use, portable, and less expensive devices for trace detection of metal ions, the principles of electrochemistry were applied in a newly micro-fabricated device. The process for development of this device involved investigation into a variety of ideas prior to deciding on the design. These ideas provided opportunity for lowering costs, and simplifying use of the device. A few areas investigated for improvement of the device includes colorimetric methods of detection, screen printed electrodes, and micro-fabricated electrode arrays.

Colorimetric methods are commonly used for higher concentrations of ions, so the colorimetric tests were performed in combination with screen printed electrodes. Combining these two methods provided the possibility of a rapid initial screening for higher concentrations, followed by immediate electrochemical detection for much lower concentrations. Creating a microelectrode array would provide a simple device that can perform multiple tests quickly, and can be used for a variety of applications. Each electrode could be used to test different samples, or they could be used to confirm the exact concentrations of one sample. The benefits and drawbacks of each idea will be discussed along with the corresponding results.
3.2 Colorimetric and Screen Printed Electrode Detection

3.2.1 Materials and Methods

Electrochemical Detection was performed using a CH-Instruments Electrochemical Analyzer in combination with the screen-printed electrodes (DS110) purchased from DropSens. Heavy metal reference standard solutions of lead and cadmium were purchased from Fisher Scientific at a concentration of 1000 ppm. Differential pulse anodic voltammetry (DPASV) was used for all electrochemical measurements. For the colorimetric detection, grade 1 chromatography paper was purchased from Whatman GE Healthcare, along with rhodizonic acid, disodium salt, 97% purchased from Sigma Aldrich for lead tests.

Wax ink was used to print channels into the chromatography paper since the paper is hydrophilic and the hydrophobic wax created barriers to direct the flow. Two circular zones, one for detection and one for sample injection, were connected via the wax channels. This system allowed for easy monitoring of the interaction of the sample with dye. A change in color is observed when lead reacts with sodium rhodizonate, and a smart phone was used to take pictures for the colorimetric analysis using free ImageJ software. The paper containing wax channels was laminated onto the screen printed electrode aligning the detection zone with the WE so that electrochemical tests could be performed along with colorimetric. A diagram depicting the basic design of this device can be seen in Figure 3.1. The combination of electrochemical and colorimetric
detection offers the simplicity of monitoring color change while maintaining the lower detection limits of electrochemistry.

![Screen Printed Electrode Layout](image)

**Figure 3.1** – The layout of the screen printed electrode is shown, along with the process of adding the paper/wax channel via lamination. A hole was punched in the laminate over the injection zone to allow for addition of solution samples to be tested.

3.2.2 Results and Discussion

The first step was to determine what range of ions could be detected using colorimetric techniques that could be analyzed simply with a cell phone camera and free image analysis software. Lead ions were detected using sodium rhodizonate to test the
feasibility of the method. Once sodium rhodizonate was added to the detection zone, the 
filter paper changed from white to a bright orange color. Solutions with varying lead ion 
concentrations were then added to the detection zone, and the interaction was 
monitored using the cell phone camera.

A cell phone image was taken at each step of the process and the results are 
shown in Figure 3.2. The resulting image was imported into ImageJ and converted to 
gray scale. The mean gray value was calculated for each region by taking the sum of the 
gray values for all the pixels, and dividing by the total number of pixels. The mean gray 
values were then plotted versus the concentration of lead ions, and the results are 
shown in Figure 3.3. High lead ion concentrations were required in order to obtain 
noticeable color changes, and there was no linear relationship for the range of ions 
yielding a 0.71 \( R^2 \) value from a linear fit. The concentration range tested resulted in a 
curvilinear relationship, but no replications were performed because the concentration 
range was too large to be considered useful. From these results it was determined that 
this colorimetric technique would only be suitable to detect the presence of lead ions at 
high concentrations (>10 mg/L), and would not be effective for determining specific 
concentrations.
Figure 3.2 – Visual results obtained from a smart phone camera of the colorimetric lead detection tests. Chromatography paper was used with 0.1 M sodium rhodizonate.

<table>
<thead>
<tr>
<th>Blank paper with printed wax channels</th>
<th>80 µL of 0.01 M Sodium Rhodizonate was added to each large well</th>
<th>20 µL of designated lead ion concentration was added to each small well</th>
<th>Approximately 20 minutes of time passed to allow solutions to interact</th>
<th>Excess solution was absorbed by gently using a Kimwipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/L Lead</td>
<td><img src="image" alt="Image of 10 mg/L Lead" /></td>
<td><img src="image" alt="Image of 250 mg/L Lead" /></td>
<td><img src="image" alt="Image of 500 mg/L Lead" /></td>
<td><img src="image" alt="Image of 1000 mg/L Lead" /></td>
</tr>
</tbody>
</table>

Figure 3.3 – The image on the left shows the cell phone picture below and the grayscale analysis directly above. The calibration curve is shown on the right.

$R^2 = 0.710$
Next, the screen printed electrode capabilities were tested for detection of lead and cadmium ions. The prepared range of solutions containing both ions were deposited onto the electrode surface as 80 microliter droplets. DPV was used to measure the current as the potential, in reference to the silver/silver-chloride electrode, was gradually changed from -1.1 V to -0.4 V. Peaks in current representing oxidation were observed for the lead ions at about -0.57 V, and for cadmium at about -0.85 V. The intensity of these peaks correlate directly to the concentration of ions in the solution. The concentrations that were tested ranged from 50 µg/L which is the equivalent of parts per billion (ppb) all the way up to 10 mg/L or parts per million (ppm).

The linear response range for both lead and cadmium ions on the screen printed electrode were determined to be in the low ppm range starting just below 1 ppm and increasing up past 10 ppm. The resulting DPV curve showing the response in this low ppm range is shown in Figure 3.4. The height of the corresponding peak currents for lead and cadmium were plotted against concentration, and a regression was fitted to the data to assess the linearity of the data. The linear regression for the lead ions is shown in Figure 3.5, and the coefficient of determination (R²) was 0.96 indicating that the equation generated by the regression accounts for 96% of the variation in the data. A very similar linear regression was observed for cadmium in Figure 3.6, and the R² was 0.92. No detectable peaks were observed at concentrations lower than 0.5 ppm using the screen printed electrodes. The desired linear range for electrochemical detection of
these ions was in the ppb range, therefore it was determined that the screen printed electrodes would not be suitable to meet the aims of this thesis.

Figure 3.4 – DPV results obtained using the screen printed electrodes for varying concentrations of lead (Pb$^{2+}$) and cadmium (Cd$^{2+}$) ions.
Figure 3.5 – Peak current response plotted versus the concentration of lead ions setting up a calibration curve. Results were obtained using screen printed electrodes.

Figure 3.6 – Peak current response plotted versus the concentration of cadmium ions setting up a calibration curve. Results were obtained using screen printed electrodes.
3.3 Fabrication and Testing of Electrode Array Device

3.3.1 Materials and Methods

The microfluidic electrode array was fabricated on 2 by 1 inch glass microscope slides that were 1 mm thick obtained from Fisher Scientific. Glass slides were cleaned by two 20 minute sonication baths, first in acetone, then in ethanol, and they were then dried with compressed nitrogen gas. A mask was designed in AutoCAD and made in a machine shop that allowed for six WEs (2 mm$^2$ area) with connecting wires to six larger contact pads. Thin layers of gold were used as the electrode surface since it has a high stability and good conductivity. Using the mask, a thermal evaporation sputter coating system was used to deposit the electrodes to the glass. In order to maximize adhesion of the gold, an initial layer of about 15 nm chromium was deposited onto the glass slide, followed by a 100 nm layer of gold. The glass slide now contained six gold electrode surfaces to be used for electrochemical detection as seen in Figure 3.7.

Figure 3.7 – A picture of the glass slide after the gold has been deposited using thermal evaporation.
To create individual access to each electrode, a polydimethylsiloxane (PDMS) layer with microfluidic channels was added to the glass slide. A mold for the PDMS was created using photolithography and a mask designed in AutoCAD that was printed onto transparent paper. AZ 400K positive photoresist was spin coated onto a silicon wafer to a depth of 1 mm. The layer of photoresist undergoes a sequence of baking and exposure to light that develops the desired regions exposed by the mask. The undeveloped photoresist can then be removed leaving a 1 mm thick mold of the desired pattern. PDMS with a 10:1 ratio of elastomer to the cross-linking agent is mixed and poured onto the mold, and allowed to cure. The PDMS is easily peeled off the silicon wafer leaving the desired microfluidic channels exposed on the PDMS layer. Holes are then punched in the PDMS allowing for inlet and outlets to the six microfluidic channels. Then the PDMS layer is aligned and bound to the glass slide using oxygen plasma to activate the surfaces allowing for a tight seal.

The last step in the device fabrication included adding one more layer of PDMS that creates a reservoir for a bulk solution to interact with all six of the electrodes. The reservoir is able to hold about 3 mL of solution, which will be enough for the desired electrochemical experiments. A simplified diagram of the fabrication process from the side view can be seen in Figure 3.8. The six contact pads can be connected to the measurement system using conductive copper tape, and the RE and CE will be introduced to the bulk solution in the reservoir for all experiments. Images of the final device can be seen in Figure 3.9.
Figure 3.8 – Simple diagram of the fabrication process for the microfluidic electrode array device.

Figure 3.9 – The left shows the whole glass slide with two PDMS layers, and the right is zoomed in on the microfluidic channels for each electrode.
Careful preparation of the electrode specific to the desired contaminant is critical to ensure proper detection. The fabricated device contained six WEs that could be prepared individually or all together, and those were compared against a commercially available gold WEs from CH Instruments for about 80 dollars apiece. Both the fabricated device and the commercial electrode utilized the same CE and RE for all experiments. For the detection of lead and cadmium ions, a bismuth coating was deposited onto the gold WE surface. Bismuth increases the sensitivity of the gold electrode for ions such as lead and cadmium, and is far less toxic than the previously used mercury. Proper bismuth coatings can improve the sensitivity of the electrode from ppm to ppb.

The addition of bismuth to the electrode surface only adds one step to the process, and takes about 100 seconds. The desired WE is connected to the electrochemical analyzer, and immersed in solution containing 40 mg/L of bismuth ions. A constant negative potential of -1.1 V is then applied for 100 seconds allowing for the positively charged bismuth ions to accumulate on the negative gold surface. The electrode can then be rinsed with double distilled water, and is ready for detection of lead and cadmium ions. As long as the potential remains more negative than the oxidation potential of bismuth, which is about -0.5 V, the ions will remain on the electrode surface for multiple experiments.

A similar approach is used when testing for lead and cadmium with the bismuth coated gold WE. The electrode is immersed in a solution containing low concentrations
of either lead or cadmium ions, and then connected to the electrochemical analyzer unit. A constant negative potential of about -1.1 V is applied to the WE, and positively charged lead and cadmium ions are accumulated on the electrode surface. This potential remains constant for approximately 300 seconds in order to allow enough ions onto the surface. After 300 seconds of preconditioning time, the potential is then gradually increased using a series of discrete potential steps until the lead and cadmium ions have been oxidized. Both ions have an oxidation potential that is more negative than bismuth so there is no risk of oxidizing and removing the bismuth layer. The potential range can be adjusted according to the specific ions to be detected. This DPASV technique was used for all testing of metal ions, and a very similar method was used for the characterization of the electrodes on the fabricated device.

3.3.2 Characterization of Fabricated Electrodes

In order to confirm the functionality of the fabricated electrodes on the glass slide, they were tested using two common electrochemical measurement techniques. The two methods used included cyclic voltammetry, and differential pulse voltammetry. Both techniques are used to observe oxidation and reduction reactions either in solution or on the electrode surface. The results obtained from the fabricated electrodes were compared against standard commercial gold electrodes.

Cyclic voltammetry experiments were performed in a solution of 0.01 M phosphate buffered saline (PBS) containing 2.5 mM potassium ferricyanide. The oxidation and reduction of the potassium ferricyanide is observed using CV, and this
process is a standard electrochemical experiment that verifies the fabricated electrodes are functional. As seen in Figure 3.10, the CV curve, averaging all six fabricated electrodes, demonstrates consistent oxidation and reduction of ferricyanide in solution. The curve from the fabricated electrodes is comparable to Figure 3.11 where the commercial electrode curve is shown. This confirms that the mass transfer in the microfluidic channels is sufficient for the flow electrons, and that fabrication of each electrode is uniform, and can produce consistent results. The CV results at each of the six electrodes on one device were tested in triplicate, and results are seen in Figure A.1.

To further confirm that the fabricated electrodes on the microfluidic device were comparable to the commercial electrodes, another set of experiments were performed using CV. The parameter analyzed was the diffusion coefficient ($D_o$) that was obtained by plotting the anodic or cathodic peak current responses versus varying scan rates. CV curves were collected at five different scan rates, and they are shown in Figure 3.12. The peak currents were plotted against the square root of the scan rate to obtain the linear relationship as seen in Figure 3.13.
Figure 3.10 – CV curve showing the average of all six electrodes on the fabricated device in a 0.01 M PBS solution containing 2.5 mM ferricyanide. The error bars shown represent the standard deviation of the six electrodes.

Figure 3.11 – CV curves for the commercial electrode in 2.5 mM ferricyanide. The error bars represent standard deviation of triplicate testing.
Figure 3.12 – CV curves at varying scan rates in 0.01 M PBS solution containing 2.5 mM ferricyanide. The average was taken over all six electrodes, with the error bars representing the standard deviation.

Both the anodic and cathodic peaks are shown in Figure 3.12 with well-fitting linear regressions, having R² values above 0.99, indicating that 99% of the variance in the data was accounted for by the model. The model was used to calculate $D_o$ values of $7.1 \times 10^{-7}$ for oxidation, and $6.4 \times 10^{-6}$ for reduction. The same process for the commercial gold electrode produced a slightly higher $D_o$ value of $8.1 \times 10^{-6}$ for oxidation, and a significantly similar value of $5.61 \times 10^{-6}$ for reduction (Figures A.2-3).
Figure 3.13 – Plot showing the peak currents for the anodic and cathodic peaks of the CV curve versus the square root of the scan rate. Averages were taken of all six fabricated electrodes on a device, and the error bars represent standard deviation.

Differential potential voltammetry was also used to confirm the functionality of the fabricated electrodes. Experiments were performed using the same potassium ferricyanide solution from the CV tests done previously. DPV is typically used to observe either oxidation or reduction, not both, and in this case the focus will be oxidation.

Comparison of the average of six fabricated electrodes to commercial electrode yielded similar results, as seen in Figure 3.14 and Figure 3.15, respectively. Consistency between
the six fabricated electrodes, similar to that of CV, was observed for the DPV experiments and the results are shown in Figure A.4.

Figure 3.14 – DPV curve showing the average of all six electrodes on the fabricated device in a 0.01 M PBS solution containing 2.5 mM ferricyanide. The error bars shown represent the standard deviation.
Figure 3.15 – DPV curves for the commercial electrode in 0.01 M PBS solution containing 2.5 mM ferricyanide. The error bars represent standard deviation of triplicate testing.

3.3.3 Results and Discussion

The main objective of the fabricated device was to detect low concentrations of lead and cadmium ions. DPASV was used to examine the capabilities of the fabricated gold electrodes for detection of metal ions. The detection limits using bare gold electrodes are not low enough, so a bismuth modification to the electrode surface was used to enhance the detection of lead and cadmium. Bismuth ions in solution were introduced at a high concentration of 40 mg/L to the electrode surface, and a negative potential was applied for a period of 100 seconds. The positively charged bismuth ions (3+) were adsorbed to the negatively charged electrode surface. A visible change to the
electrode surface is observed after the bismuth coating has been applied as seen in Figure 3.16. With a bismuth coated surface, the electrode is prepared for enhanced detection of lead and cadmium ions.

Figure 3.16 – The fabricated device after bismuth ions have been deposited onto one of the electrodes. The altered electrode is seen in the white circle.

Once the electrode had bismuth ions coated onto the surface, the 1 M potassium chloride solution containing lead or cadmium ions to be tested is added to the device. Another period of deposition with a negative potential was applied to the electrode surface for 300 seconds. This time period allowed for any positive free ions in solution to accumulate onto the electrode surface, and after 300 seconds the potential was increased until the oxidation potential of the desired ion was achieved. The oxidation potential of lead and cadmium on a bismuth coated commercial gold
electrode are approximately -0.64 V and -0.87 V, respectively. The results on the fabricated electrodes demonstrated similar oxidation peaks for both ions.

Calibration curves were generated for both lead and cadmium ions by plotting the peak current values of the oxidation peaks versus the corresponding concentrations of the ion. The calibration curves were obtained separately for lead and cadmium, but both ions can be detected simultaneously in solution due to the individual oxidation potentials being different. Four concentrations were prepared in a 1 M KCL solution starting at 1 µg/L and going up to 100 µg/L.

The DPASV results for lead are shown in Figure 3.17, and Figure 3.18 shows the corresponding calibration curve. A well-fitting linear regression, with an R² value of 0.991, was performed to obtain a model that was representative of the data. Similarly, the DPASV results obtained for the detection of cadmium, seen in Figures 3.19-20, demonstrate a linear relationship with an R² value of 0.967. A major concern is the consistency of results between multiple tests, as can be noted by the lack of replicate testing for the calibration curves. The lack of consistency is most likely due to the need for a better stirred system since microfluidic channels partially restrict the flow of ions from the bulk solution to the electrode surface. However, the results do show that the fabricated electrodes with microfluidic channels are capable of detecting very low concentrations of lead and cadmium ions.
Figure 3.17 – DPASV curves for varying concentrations of lead used to obtain a calibration curve.

Figure 3.18 – Calibration curve for lead ions. Linear fit is shown with an $R^2$ value.
Figure 3.19 – DPASV curves for varying concentrations of cadmium used to obtain a calibration curve.

Figure 3.20 – Calibration curve for cadmium. Linear fit is shown with an $R^2$ value.
3.4 Conclusions

In conclusion, the combination of colorimetric techniques with screen printed electrodes proved incapable of detecting lead and cadmium at concentrations low enough to be useful in typical scenarios where parts per billion are required. The linear range for electrochemical detection on the screen printed electrodes was low ppm, and the colorimetric detection was high ppm. Applications of colorimetric methods could potentially be used as a very inexpensive and simple initial test for cases where higher concentrations of lead may be a concern.

The fabrication of an electrode array device proved to be the more feasible approach for detecting hazardous ions down in the ppb range. The device provides a quick and portable method for detecting trace amounts of lead and cadmium in water samples. The benefit of being able to modify the gold electrode surface individually allows for six unique application on each device. The bismuth coating demonstrated the ability to allow for detection at low concentrations, and may be a solution that could also be applied to the screen printed electrodes to increase their detection capabilities.
CHAPTER 4
SUMMARY, COST ANALYSIS, AND FUTURE WORK

4.1 Summary

Electrochemical methods provide a promising alternative to current methods of detection for harmful contaminants. Using basic principles of electrochemistry a variety of unique applications are explored to lower the time, cost, and expertise required to analyze water samples for contamination. A few potential applications were investigated in this thesis, to determine detection efficiency of specific contaminants including toxic metal ions, and individual genotypes of *C. parvum*. These methods in combination with microfabrication processes allow for novel devices to be created for more available forms of detection.

A novel platform was introduced that improved the speed and sensitivity of electrochemical detection methods for specific DNA sequences. The platform worked by aligning probe DNA horizontally, using uniform DNA posts, on the surface of an electrode to increase the rate at which DNA hybridization occurred. The horizontal platform also allowed for high specificity in detection because nonspecifically bound DNA did not stay on the surface and provide false positive results.

All experiments were performed using synthetic DNA to help simplify sample preparation. Converting this new platform to detecting actual organism DNA requires a more intensive sample preparation including lysing cells to extract the DNA, using enzymes to cut up the DNA, and then using PCR to amplify the desired sequence. The
new method allows the sample preparation to stop there however and does not require further modifications such as adding a label to the DNA, thus creating an inexpensive and simple alternative to current methods. The uniform posts in the new method also allow for preparation of a generic electrode surface that can be used to detect a variety of sequences. This allows for an easy platform to be created where multiple sequences can be detected simultaneously using individual electrode surfaces.

A fabricated six-microelectrode array allowing for individual modification of each electrode while maintaining the ability to test all electrodes was introduced. Characterization of the device electrodes was done in order to confirm that they had comparable function and ability to commercially obtained electrodes. The fabricated device was then tested for detection of lead and cadmium ions, and potentially could be applied to DNA detection of specific genotypes using the newly introduced platform. Lead and cadmium were successfully detected at low concentrations in the ppb range, but further testing is required to confirm the sensitivity and selectivity of the device.

Colorimetric methods in combination with screen printed electrodes were unsuccessfully investigated for the detection of lead and cadmium at concentrations in the ppb range. Detection of higher concentrations were achieved, but the colorimetric method required very high concentrations and the screen printed electrodes were only able to detect low ppm. However, the application of colorimetric methods could still be valuable as an initial screening for dangerously high concentrations. The screen printed
electrodes could also potentially be improved with the addition of a bismuth coating as was done for the fabricated electrode device.

Electrochemical techniques are continuing to open new doors for unique sensors that improve current methods while lowering costs, increasing accessibility, and making sensors easier to use. With the introduction of a novel platform for DNA detection, and a newly designed electrode array, this thesis provides potential routes for fulfilling a specific need. The need addressed is that of inexpensive and portable devices for detection of contaminants in water that pose threats to human health, and the surrounding environment.

4.2 Cost Analysis

An overall cost analysis is shown in Table 4.1 to give an idea as to the approximate costs of different instruments and methods for both DNA sequence detection as well as heavy metal ion detection. Instruments that are typically found in research laboratories are shown such as the ICP-MS, AAS, and genetic sequence analyzer all costing upwards of $10,000. In comparison, an electrochemical analyzer, or potentiostat can generally be purchased for less than $10,000 and the one used for the experiments in this thesis cost about $2,000. This electrochemical analyzer considerably less expensive than the alternative, but there are still less expensive options. A similar instrument can be built for about $200, which would lessen the cost of electrochemical experiments even further.
In addition to lowering the cost of instrumentation required, the methods proposed also allow for reducing the costs of required materials. The largest price difference occurs by eliminating the need for any kind of label to be added to the DNA strands for sequence detection. As can be seen in Table 4.1, the cost of fabricating DNA with a label attached is over 20x more expensive than the same DNA strand without a label. A commercial electrode can be purchased for about $80 per electrode, and the fabricated device with six electrodes can be made for less than $10 per device.

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Materials</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP-MS</td>
<td>DNA w/Label</td>
<td>~ $580</td>
</tr>
<tr>
<td>AAS</td>
<td>DNA w/o Label</td>
<td>~ $25</td>
</tr>
<tr>
<td>Genetic Sequencer</td>
<td>Thiolated DNA</td>
<td>~ $320</td>
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<tr>
<td>Electrochemical Analyzer</td>
<td>Commercial Au Electrode</td>
<td>~ $80</td>
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<td>Homemade EC Analyzer</td>
<td>Pt Wire Electrode</td>
<td>~ $75</td>
</tr>
<tr>
<td></td>
<td>Ag/AgCl Electrode</td>
<td>~ $35</td>
</tr>
<tr>
<td></td>
<td>Fabricated Device(6 Au WEs)</td>
<td>&lt; $10</td>
</tr>
</tbody>
</table>

4.3 Future Work

The most immediate future work needs to include further testing of the capabilities of the microelectrode array device for the detection of lead and cadmium ions. Potential interferences need to be addressed, and actual samples need to be collected and tested. Consistency needs to be developed for the detection these ions at low concentrations, and device capabilities need to be confirmed using commonly accepted methods such as ICP-MS. When comparing ICP-MS to electrochemical methods it is important to note that ICP-MS breaks everything down and measures
individual atoms present, while electrochemical methods only detect available ions in solution.

Ideas that could be explored to improve the consistency of responses include controlling the acidity of the sample being tested, or implementing a pump system into the device that would provide a constant flow of ions from the solution over the electrode surface. Controlling the acidity of the samples is important because metal ions tend to form complexes with other ions in neutral or basic environments. These environments render the metal ions unavailable for electrochemical detection, and is a possible reason for inconsistent results. Using a constant pH that was acidic (between 2 and 4) would help dissolve complexes leaving the metal ions free in solution to be detected. Control of the pH would also allow for determination of bioavailable ions compared to total ions present in a solution. Knowing the state of given ions in solution helps determine if there are immediate or potential hazards.

There are a variety of directions that can be explored with the colorimetric detection method in combination with screen printed electrodes. Incorporation of the bismuth coating to the screen printed electrode could be done to see if the detection limits could be improved. Further research needs to be conducted in order to determine if there are other possible ways to use colorimetric reactions to detect ions at low concentrations.

The novel DNA detection platform can also be tested on the fabricated device to see if DNA from six individual genotypes could be detected. Probe DNA from six
different species could be immobilized on to the six different working electrodes, and then an unknown sample could be introduced to each electrode to determine where the match was located. This would be done by immobilizing the standard posts on each of the six electrodes, and then adding the unique sequences to the posts one electrode at a time. Adding an unknown sequence to each of the electrodes will only yield a response on the corresponding match, and the genotype can then be determined.

Another application and benefit of aligning DNA horizontally on the electrode surface using standard DNA posts, is the potential to re-use the posts again. Since the posts are bound to the gold surface via strong thiol bonds, and the probe DNA sequences are held in place by hydrogen bonds formed during hybridization, the probe DNA sequence can be removed without removing the posts. The removal of the probe DNA can be done using either sodium hydroxide solutions or heating the solution to de-hybridize the DNA from the posts. Experiments need to be performed to determine which method is more effective, and how the efficiency of the electrode changes after each use. The potential for re-usable posts would make the device even more cost effective, and simplify the process of testing a large number of DNA sequences.
References


[31] Velia Sosa, Nuria Serrano, Cristina Arino, Jose Manuel Diaz-Cruz, Miquel Esteban, Voltammetric determination of Pb(II) and Cd(II) ions in well water using a sputtered bismuth screen-printed electrode, Electroanalysis 26 (2014) 2168-2172.


Appendix A
Figure A.1 – Cyclic Voltammetry results of all six electrodes on the fabricated device using a 0.01 M PBS solution containing 2.5 mM ferricyanide. Error bars represent standard deviation from triplicate testing for each electrode.
Figure A.2 - Plot showing the peak currents for the anodic and cathodic peaks of the CV curve versus the square root of the scan rate. Error bars represent standard deviation of triplicate testing.
Figure A.3 – Cyclic Voltammetry curves for the commercial gold electrode at varying scan rates in a 0.01 M PBS solution containing 2.5 mM ferricyanide. The error bars represent the standard deviation of triplicate testing.
Figure A.4 – Differential Pulse Voltammetry results of all six electrodes on the fabricated device using a 0.01 M PBS solution containing 2.5 mM ferricyanide. Error bars represent standard deviation from triplicate testing for each electrode.
Appendix B
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