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EFFECT OF MISMATCH POSITIONS ON ELECTROCHEMICAL DETECTION OF SINGLE-NUCLEOTIDE MISMATCHES

Md. Nazmul Alam

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EFFECT OF MISMATCH POSITIONS ON ELECTROCHEMICAL 
DETECTION OF SINGLE-NUCLEOTIDE MISMATCHES

(Spine Title: Positional Variations in Mismatches)

(Thesis Format: Monograph)

By

Md. Nazmul Alam

Graduate Program in Chemistry
A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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The thesis by
Md Nazmul Alam

entitled:

EFFECT OF MISMATCH POSITIONS ON ELECTROCHEMICAL
DETECTION OF SINGLE-NUCLEOTIDE MISMATCHES

is accepted in partial fulfillment of the
requirements for the degree of
Master of Science

Date

Chair of the Thesis Examination Board
ABSTRACT

In this thesis, I systematically explore the electrochemical behavior of surface-bound 25-mer DNA duplexes that have single-base mismatches at 25 different positions. Each mismatch is located in the target strand. Target and capture strands are hybridized before attachment of the ds-DNA on gold surfaces through Au-S bonding. Electrochemical impedance spectroscopy (EIS) and scanning electrochemical microscopy (SECM) were used to evaluate if it is possible to distinguish mismatches in all 25 positions of the target strand by monitoring the electrochemical response in the presence of anionic redox markers. Our studies clearly show that it is possible to distinguish the electrochemical responses from all 25 mismatch positions using a combined EIS/SECM approach. To our knowledge this is the first systematic study exploring the electrochemical behavior of ds-DNA carrying single-nucleotide mismatches in which the position of the mismatch was varied through all possible positions on the target strand.

Keywords: DNA mismatches detection, electrochemical DNA sensor
DEDICATION

To my parents
ACKNOWLEDGMENTS

I would like to thank my supervisor Prof. Heinz-Bernhard Kraatz for his encouragement, guidance and support during this study. I am very thankful for his patience in leading me in a right direction to finish the research project.

I would like to thank Dr. Piotr Michal Diakowski and Mohtashim H. Shamsi for helping me in learning the basic electrochemical bio-sensing techniques. I would like to take the opportunity to thank the members Dr. Kraatz group.

I would like to thank the faculty and staff of the Department of Chemistry, University of Western Ontario, who provided a great facility to learn throughout my study period.

Finally, I would like to thank my parents, wife and son for their patience and constant support.
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<table>
<thead>
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<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>μA</td>
<td>micro-Ampere</td>
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<tr>
<td>25-mer DNA</td>
<td>25-nucleotide long DNA</td>
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<tr>
<td>A</td>
<td>Adenine</td>
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<td>anthraquinone-2,6-disulfonic acid</td>
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<td>C_{dl}</td>
<td>double layer capacitance</td>
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<tr>
<td>R_{f}</td>
<td>resistance of films</td>
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<td>Scanning Electrochemical Microscopy</td>
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<td>Thymine</td>
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Chapter 1

INTRODUCTION

A nucleic acid is a polymer found in the cells of all living organisms and in viruses, which carries genetic information of life. The chemical structure of nucleic acids is based on nucleotide sub-units. A nucleotide is formed by combination of three important building blocks: the bases, ribose or deoxyribose sugar, and phosphate groups. The bases include two purines (adenine and guanine) and two pyrimidines (cytosine and thymine). These are commonly referred to as A, G, C and T respectively.

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the nucleic acids that carry out the construction of thousands of protein molecules required by living organisms. A single-stranded DNA (ss-DNA) is constructed from chains of the nucleotides joined together through the sugar and phosphate units. Therefore, the backbone of DNA is based on a repeated pattern of the sugar and phosphate group. The order of the bases in a DNA molecule defines the exact information needed to perform their specific functions. The bases have the ability to create specific links between them at the Watson-Crick faces via hydrogen bonding as shown in Scheme 1.1.¹ This allows a ss-DNA to form a double-
helix arrangement through interaction with a complementary strand DNA molecule. The paired double-helical structure is also known as double-stranded DNA (ds-DNA).

Scheme 1.1. Detailed chemical structure of ds-DNA showing hydrogen bonds between specific base pairings along with the presence of sugar units and phosphate groups. Four DNA bases adenine (A), guanine (G), thymine (T) and cytosine (C) are linked with specific hydrogen bonded pairings (black dots). G-C has 3 H-bonds and A-T has 2 H-bonds.

In ds-DNA, association of adenines on one strand with the thymines on the other strand occurs through the specific recognition of the two bases with the formation of two hydrogen bonds. Similarly, guanines bind selectively with cytosines by means of three hydrogen bonds. This association of base pairs is known as base-pairing. Among these pairings, the G:C base-pairing, with its three hydrogen bonds is more stable than the A:T base-pairing with only two hydrogen bonds. Any variation of this base-pairing in DNA sequences is called a DNA mismatch which can arise from insertion, deletion or translocation of a single or of multiple nucleotides. The most common form of variation in the human DNA sequences is a single-nucleotide mismatch in which the two
nucleobases of the complementary strands do not match. Under normal circumstances some DNA repair machinery and polymerase proof-reading mechanisms correct the gene alteration problems, but sometimes these backup systems fail and the mismatch occurs. The presence of a single-base-pair mismatch within the ds-DNA can cause various disorders in a living system. Therefore, identification of DNA mismatches among individuals and populations may allow the diagnosis of genetic diseases and the tailoring of treatment options. The rapid detection of single-base-pair mismatches would enable the monitoring of a wide variety of genetic diseases, viral infections and even estimate probability of certain cancers.

1.1. Electrochemical DNA Sensor: A Device for DNA Detection

A biosensor is an analytical device that consists of a biological recognition element, such as enzymes, antibodies, DNA, whole intact cells or tissue, connected to a physical component that converts a biological recognition event into a readable signal. This signal can be electrochemical, optical or thermal. The main purpose of the biorecognition element is to provide a high degree of selectivity for the sensor by binding with the target analyte from the sample mixture. DNA biosensors are commonly constructed on the basis of the specific affinity of a capture DNA, which is the recognition element, immobilized on the transducer surface for the target DNA. Generally, the capture DNA is a tethered short piece of single-stranded nucleic acid with a known sequence and the target is the free nucleic acid analyte present in the sample. Significant numbers of DNA bio-sensing strategies have been developed based on the specific affinity between the single-stranded DNA, which is known as DNA hybridization. An electrochemical DNA sensor is based on electrochemical transduction
of the recognition signal generated from hybridization of the capture DNA with the target DNA. Generally, the electrochemical measurements are carried out in an electrochemical cell containing three electrodes, namely, the working electrode, reference electrode and counter electrode. The working electrode acts as a transducer, where capture DNA immobilization and target-recognition events take place. The reference electrode, for example Ag/AgCl, has a known and constant potential that serves to control the applied electrical potential on the working electrode. A suitable substrate is chosen to make a transducer surface by immobilizing the capture strands on it under suitable conditions. The recognition element, here the captured DNA strand on the working electrode, is then dipped in solution containing target DNA, which allows capture strands to hybridize with their complementary strands to form double-stranded DNA. The electrochemical signal extracted from the transducer surface provides various information of the target DNA sequence. This general process of electrochemical detection of target DNA by electrochemical technique is described in Scheme 1.2.

Scheme 1.2. General design of an electrochemical DNA sensor. The recognition layer is prepared by immobilizing capture DNA sequences on a transducer surface. Target DNA is captured at the recognition layer, and the resulting hybridization event is transduced into an electrical signal. A) Gold electrode, B) Capture DNA strand immobilized on gold electrode, C) ds-DNA formed upon hybridization with the target DNA strand.
A number of research groups have devised slightly different approaches to determine either the presence or the identity of target DNA by following the detection of hybridization of target DNA to surface supported capture DNA.

1.2. Overview of Electrochemical DNA Sensing Approaches

Electrochemical detection of DNA sequences has become an intense area of research and there are numerous approaches, of which some representative examples will be discussed in the following sections.

1.2.1. Employing Redox Indicators

A large number of electrochemical techniques use redox active compounds to detect target DNA. The hybridization of the capture DNA with the target DNA can be detected by monitoring changes in current signal of the redox indicator. Such indicators are electroactive molecules that preferentially bind to ds-DNA. This approach of electrochemical DNA detection was pioneered by Mikkelsen and co-workers who used [Co(phen)$_3$]$^{3+}$ as a redox indicator.$^{10,11}$ The Co(III) indicator is reversibly reducible to its Cobalt(II) form with the formal potentials within the window over which immobilized DNA is electro-inactive. Moreover, this molecule binds preferentially to ds-DNA resulting in increased redox signal as shown in Figure 1.1.
Figure 1.1. Cyclic voltammograms of [Co(phen)₃]³⁺ obtained at capture DNA-modified carbon paste electrode A) before hybridization and B) after hybridization with a complementary target DNA sequence. Exposure of the sensor to the complementary sequence resulted in a signal increase of 2 μA. Reprinted with permission from {K. M. Millan, S. R. Mikkelsen, Anal. Chem., 1993, 65, 2317-2323}. Copyright {1993} American Chemical Society.

Hybridization to mismatched ss-DNA is studied by carrying out the hybridization at an elevated temperature of 43 °C, which is above the melting temperature of mismatched ds-DNA. This simple but highly efficient strategy has been improved by others to address selectivity and sensitivity of the DNA biosensor. Particular attention was given to the choice of redox indicators and the variation of detection schemes. A number of electroactive indicators have been developed for attaining higher sensitivity and better distinction between ss-DNA and ds-DNA. Takenake et al. reported the use of a ferrocenyl naphthalene diimide compound that binds to the ds-DNA more tightly than usual redox markers. The use of this redox indicator resulted in a detection limit of 10 zmol related to differential pulse voltammetric (DPV) measurement of the hybridization event. In contrast to the normal pulse voltammetry, DPV offers better sensitivity due to minimization of the charging current. Recently, Lorenzo and co-workers synthesized a ruthenium complex, pentaamine ruthenium [3-(2-phenanthren-9-yl-vinyl)-pyridine] as shown in Figure 1.2 A, and studied the interaction of the complex with DNA on gold surfaces (Figure 1.2 B).
Figure 1.2. A) The pentaamine ruthenium complex used by Lorenzo et al. B) Differential pulse voltammograms of the ruthenium complex accumulated on a capture-DNA modified gold electrode before (2) and after hybridization with complementary (1) or non-complementary (3) target. Reprinted with permission from {T. Garcia, M. Revenga-Parra, H. D. Abruna, F. Pariente, E. Lorenzo, Anal. Chem., 2008, 80, 77–84}. Copyright ©2008 American Chemical Society.

As shown in Figure 1.2 B, the current responses due to the only capture DNA and after incubation of the capture DNA in non-complementary DNA are negligible compared with the current due to ds-DNA on the gold electrode. The dramatic increase in peak current for the fully complementary target indicates that the ruthenium complex is accumulated at the ds-DNA layer confined to the electrode surface. This complex binds to DNA in two ways, one is the electrostatic interaction between the negatively charged DNA and the positively charged metal center of the complex, and the other is the intercalation of the ligand via the conjugated phenyl ring moiety to the ds-DNA. Intercalation is a process when ligands of an appropriate size and chemical nature fit within the space between the base-pairs of ds-DNA. Due to high intercalative character of the ligand and both electrostatic as well as redox properties of metal center, using the complex allowed easy discrimination between ss-DNA and fully matched ds-DNA. Moreover, they have reported that this approach was also able to discriminate single-nucleotide mismatches at three different positions in a 25-mer ds-DNA. 

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As can be seen from Figure 1.3, the peak currents for all three mismatches, middle (2), bottom (3) and top (4), are lower than that of the fully complementary ds-DNA (shown in Figure 1.2 B) and higher than the current obtained for the non-complementary sequence (1). Furthermore, the middle mismatch (2) can easily be distinguished from the other two. The authors reported that the intercalative ability of the complex facilitates the detection of single-base mismatches at three different (top, middle and bottom) locations along the DNA target. The discrimination was possible due to the different duplex formation with the different mismatch positions which leads to different binding to the redox indicator. The use of redox active reporters has found to have excellent specificity with low detection limits. However, a major disadvantage of this approach is the large background noise because of non-specific binding of the redox reporter to the electrode surface or the unintended DNA.
Another method of electrochemical DNA sensors has been developed based on structural changes that occur upon binding of the capture DNA with target DNA. In this approach, an electro-active marker is tethered to one end of an immobilized DNA strand whose other end is attached to the surface. In the absence of the target the capture strand adopts a hairpin loop which brings the redox reporter into close proximity with the surface. This allows for efficient and rapid electron transfer between the redox probe and the surface. Upon binding with the target strand the distance between the redox probe and the surface increases, causing significant decrease in the electron transfer characteristics. This reduction of current signal is proportional to the extent of the hybridization. Plaxco, Heeger and co-workers have first reported a DNA biosensor employing ferrocene or methylene blue labeled capture strands as shown in Scheme 1.3.\(^{15}\)

**Scheme 1.3.** A) A capture DNA possessing a terminal ferrocene group is immobilized on a gold electrode through a gold-thiol bond. In the absence of target DNA, the capture strand forms a structure like a stem-loop which brings the ferrocene redox indicator into close proximity with the electrode surface. Efficient redox reaction of the ferrocene label and consequently higher current is observed in this situation. On hybridization with the target sequence, a large decrease in current is found due to the movement of the ferrocene label away from the surface. B) Voltammograms showing decrease in current (peak height) after addition of target DNA strands. Reprinted with permission from {C. H. Fan, K. W. Plaxco, A. J. Heeger, *Proc. Natl. Acad. Sci. USA*, 2003, 100, 9134}. Copyright {2003} National Academy of Sciences, U.S.A.
In the absence of target DNA, the structural flexibility of single-stranded capture-DNA enables the redox tag to come within close proximity of the electrode surface which results in unhindered electron transfer between the redox species and the electrode. Upon hybridization of the capture strand with the target, the flexible ss-DNA is converted to rigid duplex DNA and pushes the redox probe further away from the electrode surface, resulting in a decrease of signal intensity. Therefore, after hybridization the current signal is off. Because of this “signal-off” approach, this method can give false positive signals by providing reduced current due to the denaturation of the self-hybridized duplex.

The Heeger group continued to improve their “signal-off” device and changed it to a “signal on” device by using a second complementary section on a long immobilized capture probe which forms two stem-loop structures and methylene blue as a redox reporter. The single-stranded capture DNA keeps the methylene blue redox label away from the electrode suppressing the electrochemical signal. When a target DNA hybridizes with the capture strand, the methylene blue is freed from the rigid structure and able to access the electrode resulting in electrical signal. This technique allowed for detection of complementary DNA and the discrimination of mismatched DNA in blood serum vs. normal buffer. Very recently, Plaxcos’ group reported further development of this approach by using a methylene blue labeled ss-DNA capture strand containing a triple-stem structure. This method exploits the large thermodynamic changes in enthalpy and entropy resulting from conformational changes upon target binding. The detail of the approach is shown graphically in Scheme 1.4.
Scheme 1.4. Left: In the absence of target DNA, the probe DNA forms a rigid triple-stem form and keeps the methylene blue (MB) away from the surface inhibiting current flow. Hybridization at three locations is shown in three different colors. Right: in presence of fully matched target DNA, the triple-stem conformation is disrupted and the redox marker MB efficiently interacts with the gold surface. Redrawn with permission from {Y. Xiao, X. Lou, T. Uzawa, K. J. I. Plakos, K. W. Plaxco, H. T. Soh, J. Am. Chem. Soc., 2009, 131, 15311}. Copyright {2009} American Chemical Society. (inset) the chemical structure of methylene blue.

A single-stranded DNA containing three stems serves as a capture strand. This strand was tagged with a methylene blue redox reporter at the 3'-terminus and is immobilized on a gold electrode through a thiol group. In the absence of target DNA, the capture strand is self-hybridized into a distinct double helical form. In this situation, the redox reporter is distant from the electrode. However, hybridization with a fully matched target DNA allows the redox marker to be flexible and come in contact with the electrode surface, which leads to an increase in current response. AC voltammetry was used in this approach because of its ability to get quantitative evaluation more pricisely than the DPV.

As can be seen from Figure 1.4, this approach allows the successful discrimination of full matched DNA from a target DNA that contains a single mismatch.
Figure 1. 4. AC voltammograms of the three-stem DNA sensor for detecting fully matched target (purple line), single mismatched target (green line), and a target containing two mismatches (yellow line). The sharp increase in current for fully matched target against the mismatched targets demonstrates the successful discrimination of the sensor. Reprinted with permission from {Y. Xiao, X. Lou, T. Uzawa, K. J. I. Plakos, K. W. Plaxco, H. T. Soh, J. Am. Chem. Soc., 2009, 131, 15311}. Copyright {2009} American Chemical Society.

The three-stem strategy provides a reagentless, reversible and single-step electrochemical DNA sensor for mismatch detection.

1.2.2. Sandwich Methods

A highly sensitive strategy known as the “sandwich” approach for electrochemical DNA biosensors has been developed using an additional DNA sequence labeled with a redox reporter as shown in Scheme 1.5.
Scheme 1.5. Schematic representation of the “sandwich” approach of DNA sensing. In the first step, a capture DNA modified electrode is hybridized with the target DNA sequence. Signal DNA is allowed to hybridize with the overhang portion of the target DNA. The current resulting from the redox reporter of signal DNA present at the electrode surface signifies the binding of the target DNA.

Here, a portion of the target DNA is allowed to hybridize with the surface immobilized capture DNA. After that, an additional DNA sequence labeled with a redox reporter is added, and which hybridizes with the other part of the target sequence. The third DNA is known as signal DNA that can be modified with various reporter molecules such as enzymes or nanoparticles. If the target DNA is complementary to the capture DNA strand, signal DNA comes closer to the surface giving rise to electrical current. There are many studies on the use of enzymes to label the signal DNA for DNA biosensor. Recently, Lucarelli et al. reported the use of biotin-modified signal DNA to detect DNA mismatches related to mutations in the TP53 gene that cause Li-Fraumeni syndrome. However, the instability of enzymes which leads to false positive responses restricted their use in DNA sensing.19 Ihara et al. described a sandwich assay based on a signal DNA labeled with a ferrocene redox reporter.20 As can be seen from Figure 1.5, the anodic current is significantly higher when the sample contains fully complementary DNA sequence compared to the sample with non-complementary DNA.
This sandwich approach is advantageous over the other labeled technique previously discussed (see section 1.2.1) because it does not require modifying the target DNA strand. Furthermore, the capture and signal strands do not come into contact in the absence of the target, which reduces the background signal and leads to very low detection limit. Very recently the sandwich assay was improved to achieve higher sensitivity by Xia et al.\textsuperscript{21} In this approach, the traditional sandwich method was modified by designing a signal DNA strand that can hybridize to two regions of a target DNA sequence.

As shown in Scheme 1.6, the subsequent hybridization of target DNA with the signal DNA produces a long ds-DNA bearing multiple redox reporters.
Scheme 1.6. Schematic representation of the supersandwich assay on a gold electrode. This approach uses a signal DNA that hybridizes to two regions of target DNA leading to the formation of a long structure containing multiple target molecules and signal probes. Electrochemical measurements (inset) showed large increase in current signal due to the presence of multiple redox reporters on the surface after the formation of a long superstructure. Reprinted with permission from {F. Xia, R. J. White, X. Zuo, A. Patterson, Y. Xiao, D. Kang, X. Gong, K. W. Plaxco, A. J. Heeger, J. Am. Chem. Soc., 2010, 132, 14346}. Copyright {2010} American Chemical Society.

This approach was termed as “supersandwich” assay due to the formation of a large supramolecular structure compared to the traditional sandwich technique which contains only one redox marker in the structure. Scheme 1.6 (inset) shows the electrochemical signal obtained from the methylene blue-tagged signal probe both before and after adding the target DNA sequence. A larger increase in the Faradaic current was obtained after addition of target DNA due to the formation of the supersandwich structure. The authors were able to probe the detection limit to 100 fM of target strand. The traditional sandwich assay has a detection limit of 100 pM. They have also showed the ability of this method to discriminate mismatched DNA from their fully matched counterparts.
1.2.3. Oxidation of DNA

The intrinsic electroactivity of DNA on electrodes was first demonstrated by Palecek more than 50 years ago\textsuperscript{22} who used the redox property of DNA to determine DNA sequences using a dropping mercury electrode. Of the four nucleic acid bases, guanine is the most easily oxidized and can be conveniently used for DNA hybridization detection on the mercury electrode.\textsuperscript{23} Although this technique is highly sensitive, its application is problematic due to high affinity of DNA for mercury leading to nonspecific adsorption and the need for a relatively high potential required to oxidize DNA. Moreover, the presence of guanine bases in the capture strand can result in a big background current. To overcome the limitation, guanines present in the capture strand can be replaced by inosine residues which pair with cytosine similar to the base-pairing with guanine.\textsuperscript{24} The sensor was fabricated by immobilizing the inosine-substituted capture strand on to the carbon paste electrode. When the sensor was allowed to hybridize with target DNA, a sharp guanine oxidation peak was the indication of the presence of the target complementary sequence. The area or height of the guanine peak can then serve as a quantifiable hybridization signal. This approach to DNA detection not only eliminates the need for external indicators, but offers a more reliable detection of DNA, as it relies on the appearance of a new guanine oxidation peak. An approach of indirect guanine oxidation through the use of electrochemical mediators, such as polypyridyl complexes of Ru(II) or Os(II), has been explored.\textsuperscript{25} A greatly enhanced guanine signal was obtained due to the electrocatalytic action of the redox mediator which is shown in Scheme 1.7.
Scheme 1. 7. Schematic representation of guanine oxidation mediated by a ruthenium complex in solution. A fixed potential is applied to the electrode that oxidizes the reduced metal complexes, which then interact with DNA. Guanine residues in DNA can regenerate the reduced mediator by giving one electron. This process continues until all guanine residues have been oxidized. Thus the amplified signal reflects the amount of guanine available for oxidation. Redrawn with permission from {D. H. Johnston, K. C. Glasgow, H. H. Thorp, J. Am. Chem. Soc., 1995, 117, 8933}. Copyright {1995} American Chemical Society.

The authors also reported the ability of this approach to discriminate small structural perturbation due to the presence of mismatches in DNA. Because the guanine-metal electron-transfer rate constant is sensitive to the presence of either fully matched or mismatched sequences in DNA.25

1.2.4. Long-Range Charge Transfer through ds-DNA

A powerful strategy to detect DNA mismatches is by exploiting differences between ss-DNA and ds-DNA to mediate electron transfer. It has been shown that efficient electron transfer occurs through ds-DNA over a distance of more than 40 Å, whereas a ss-DNA does not allow efficient electron transfer over such a long range due to the absence of π-stacking.26 The ability of duplex DNA to carry electron through the π-stack has been used to fabricate a biosensor for DNA mismatch detection by Barton, Hill and co-workers.27,28,29 Thiol-modified single-stranded probe DNA was first hybridized...
with its complementary target DNA sequence to form a duplex. This ds-DNA was then self-assembled on a gold surface forming a tightly packed ds-DNA film. A redox active intercalator, for instance methylene blue or daunomycin, was chosen to add which intercalates into the ds-DNA π-stack. The oxidation and reduction properties of the intercalated redox species via long-range electron transfer was used as an indication of ds-DNA formation. After denaturing the duplex, the current measured at the electrode was significantly reduced due to the presence of only single-stranded DNA. Since the presence of a single-base-pair mismatch influences the electronic coupling between the bases, it was possible to distinguish mismatched ds-DNA from fully matched ds-DNA.

Figure 1.6. Cyclic voltammetry of fully matched and a single-mismatched ds-DNA on a gold electrode in the presence of daunomycin as an intercalator. The decreased current for mismatched ds-DNA is due to the disturbance of electron transfer from the intercalator to the electrode. Reprinted with permission from {S. O. Kelley, E. M. Boon, J. K. Barton, N. M. Jackson, M. G. Hill, Nucleic Acids Res., 1999, 27, 4830}. Copyright {1999} Oxford University Press.

Using the simple addition of an intercalative redox probe Barton’s group was able to detect different types of mismatches except a GA mismatch. They speculated that this is caused by the thermal stability of the GA mismatch and fully matched DNA is too close to discriminate.30,31 Later, they have developed an electro-catalytic approach that allowed
the differentiation of a fully matched DNA from the GA mismatch based on the electro-
catalytic reduction of ferricyanide by MB that intercalated into ds-DNA. Scheme 1.8
illustrates the detection by a methylene blue / ferricyanide electro-catalytic cycle on the
DNA monolayer.

Scheme 1.8. A) Schematic representation of the electrocatalytic DNA hybridization
sensor based on long-range charge transfer developed by Barton and co-workers. The
intercalator methylene blue (MB) is electrochemically reduced to leucomethylene blue
(LB\textsuperscript{+}) via the charge transfer. [Fe(CN)\textsubscript{6}]\textsuperscript{3-} in solution oxidizes the LB\textsuperscript{+} back to MB to
complete the electrocatalytic cycle. B) Catalytically enhanced current (red line) is shown
compared with current obtained only due to the presence of methylene blue (blue line)
and current in absence of the redox marker from cyclic voltammetric measurement.
Redrawn with permission from {E. M. Boon, D. M. Ceres, T. G. Drummond, M. G. Hill,

While most of the other approaches require stringent washing steps to discriminate
between fully matched and mismatched target, this strategy simplifies the operation of
electrochemical DNA sensor due to the absence of washing steps. This is because the
LB\textsuperscript{+} produced by reduction of MB prefers to stay in solution. However, the DNA films in
this process must be densely packed on the electrode to avoid any non specific redox
reaction between the redox probe and electrode surface. In order to prevent the direct
electron transfer of the redox probe with the electrode surface and favor charge transfer
through ds-DNA, Gooding and co-worker developed another strategy by using an anionic intercalator, anthraquinone-2,6-disulfonic acid (AQDS).\textsuperscript{33} They demonstrated that the negatively charged intercalator is repelled by the same-charge of DNA film and intercalates near the top of the ds-DNA. The use of this intercalator allows the discrimination of fully matched and mismatched DNA. Recently, Mehrgardi \textit{et al.} reported the use of a four negatively charged complex molecule, Copper-phthalocyanine tetrakisulfonic acid-tetra sodium salt, for discriminating DNA mismatches based on the long-range charge transfer strategy.\textsuperscript{34} They were able to differentiate even thermodynamically stable GA and GT mismatches through diminution of the electrochemical signal due to intercalation of the negatively charged redox probe at the top end of the DNA film. In the same paper, they reported that this approach is, however, unable to detect the position of mismatches in ds-DNA as shown in Figure 1.7 A.

\begin{figure}[ht]
\centering
\includegraphics[width=\textwidth]{Figure1_7.png}
\caption{A) Differential pulse voltammograms for the hybridization of capture strand with the complementary (bold), G-T mismatched target DNA at two different positions, bottom mismatch (gray) and top mismatch (dashed). Reprinted with permission from {M. A. Mehrgardi, R. Daneshtalab, \textit{J. Electroanal. Chem.}, 2011, 650, 214} Elsevier Publications. B) The chemical structure of copper-phthalocyanine tetrakisulfonic acid-tetra sodium salt.}
\end{figure}
1.2.5. Electrochemical Impedance Spectroscopy Based DNA Sensors

Electrochemical impedance spectroscopy (EIS) is a very useful technique for studying biomolecular interactions on a transducer surface.\textsuperscript{35} EIS is based on the direct or indirect alteration of the electrical properties of the transducer surface upon formation of a recognition complex between the capture and target DNA. This electrochemical technique has been used as a signal readout device for various approaches of DNA detection. The impedance measurements usually rely on changes in the barrier to the redox conversion due to the recognition of analyte DNA by the capture strand. The ferrocyanide/ferricyanide solution is often chosen as a redox couple for this purpose. This method circumvents the need to modify capture or target DNA with any redox reporter such as electro-active hybridization indicators, enzymes or nanoparticles. Furthermore, EIS is well suited to provide information about surface phenomena such as electron transfer processes, capacitance, and bulk properties such as diffusion-limited current in a single experiment. Kafka \textit{et al.} have reported the use of EIS as a tool to detect hybridization events between immobilized capture DNA and non-modified target DNA.\textsuperscript{36} They have used a thiol-modified 18-mer capture DNA immobilized on a gold electrode as a sensor surface and allowed the sensor to hybridize with the complementary target DNA strand. Hybridization caused an increase in the charge transfer resistance for electron transfer between ferri/ferrocyanide and the electrode surface (Figure 1.8).
Figure 1. 8. A) Single-stranded capture DNA (grey) was immobilized on gold electrode to detect target DNA (black) in presence of ferri/ferrocyanide ([Fe(CN)₆]³⁻/⁴⁻) as a redox probe. B) Impedance spectroscopy results from a capture-strand modified electrode (○) and the electrode after target hybridization (●) is shown in the form of Nyquist plots. Higher diameter of the semicircle resulted from the higher charge transfer resistance for conversion of solution-based redox probe, ferri/ferrocyanide, due to hybridization on the surface. Reprinted with permission from {J. Kafka, O. Panke, B. Abendroth, F. Lisdat, Electrochim. Acta, 2008, 53, 7467}. Copyright {2008} Elsevier Publications.

The increase in from the increase in diameter of the semicircles in the Nyquist diagrams charge transfer resistance after hybridization is clearly visible. The diameter after hybridization of target DNA was about three times higher than that of the unhybridized surface. The authors have also shown that this technique is able to discriminate the presence of mismatched or non-complementary sequences from a fully matched target strand. However, this method of DNA detection using EIS is inadequate to provide information on the position of single-nucleotide mismatches of the target DNA. Kraatz et al. were able to detect single mismatches at different positions by using EIS.³⁷ For this purpose, a thiol-modified capture DNA strand is hybridized with the target DNA strand prior to immobilization onto a gold electrode (1.6 mm diameter). The pre-hybridized ds-DNA forms a film on the gold surface which is then incubated in a metal ion solution, preferably Zn²⁺ solution in Tris-buffer at pH 8.6. Reduction of the impedance for the
conversion of redox species in solution was shown after the addition of Zn$^{2+}$ both for fully matched or mismatched ds-DNA. It was observed that the presence of a mismatch in the ds-DNA provides a reproducibly lower charge transfer resistance ($R_{ct}$) compared to the perfectly matched ds-DNA. The decrease in the $R_{ct}$ has been rationalized by the improvement of electron transfer kinetics for Zn$^{2+}$ modified DNA film or enhanced penetration of the negative redox species to the film by reducing the phosphate negative charges on ds-DNA in the presence of Zn$^{2+}$. The difference in the charge transfer resistance, $\Delta R_{ct}$ caused by the addition of Zn$^{2+}$ ions in the ds-DNA film characterizes the presence of different target DNA sequence in the film. They have used three 20-mer target DNA strands containing mismatches at three different positions, top, middle and bottom. By comparing values of the $\Delta R_{ct}$, the three different mismatch positions were distinguished. Figure 1.9 demonstrates the decrease in charge transfer resistance after addition of Zn$^{2+}$ ion in the ds-DNA film consisting of a middle mismatch. Additionally, the $\Delta R_{ct}$ of the mismatched ds-DNA was shown to be smaller than that of the fully matched DNA. Thus, $\Delta R_{ct}$ was found to be diagnostic for mismatch detection. This paper reported that the $\Delta R_{ct}$ of the ds-DNA strands decreases from 190 (22) $\Omega \cdot \text{cm}^2$ for a fully matched to 95 (20), 30 (20), and 85 (20) $\Omega \cdot \text{cm}^2$ for a mismatch at the top, middle and bottom positions with respect to the gold surface. It can be noted that the $\Delta R_{ct}$ for the middle mismatched ds-DNA film was the least out of the three different positions.
Figure 1.9. A). Schematic representation of a ds-DNA film before and after addition of Zn$^{2+}$. B). Nyquist plots obtained from a solution based redox reporter, ferri/ferrocyanide, in the case of two different ds-DNA films before (○, □) and after (●, ■) the addition of Zn$^{2+}$ ions in the films. The change of the diameters of the semicircles for a fully complementary (○, ●) sequence was higher than that for the middle mismatched (□, ■) sequence. This variation of charge transfer resistance ($R_{ct}$) indicates the presence of mismatch in the target DNA strands. Redrawn with permission from {Y. T. Long, C. Z Li, T. C. Sutherland, H.-B. Kraatz, J. S. Lee, Anal. Chem., 2004, 76, 4059}. Copyright {2004} American Chemical Society.

Therefore, the evaluation of the difference in charge transfer resistance, $\Delta R_{ct}$, obtained from different ds-DNA films gives excellent discrimination between a fully matched and one containing a mismatch at three different positions. Using this approach, the H-B. Kraatz group was also able to develop a reliable technique for mismatch detection by developing 10-μm-diameter electrode arrays on gold-covered Si chip.$^{38,39}$ The schematic view of the microchip is shown in Scheme 1.9.
Scheme 1. Schematic representation of the design of a gold microelectrode array. Yellow spots in the middle of black squares are the gold electrodes of 10 μm diameter. Electrodes are separated by 50 μm from each other.

The application of the microelectrode array for the detection of mismatched DNA by EIS was useful in discriminating mismatches at very low concentration (10 fM) of calf thymus DNA. They reported that the array provided more reliable detection of mismatches compared with the previous study on disk gold electrode. The microarray provided well-distinguishable $\Delta R_{ct}$ with lower standard deviation for a fully matched and a middle-mismatched ds-DNA, which are 63.5 (5.5) $\Omega \cdot \text{cm}^2$ and 8.2 (1.3) $\Omega \cdot \text{cm}^2$ respectively. In addition, better film formation with fewer defects was observed with the use of this microelectrode array. Recently, Shamsi et al. reported the use of this approach...
with gold microelectrodes (50 μm diameter) to detect the effect of positions and nature of the mismatch pair in a 25-mer ds-DNA film. The three mismatch positions were reported to be clearly distinguishable from the fully complementary duplex as shown in Figure 1.10.

![Figure 1.10](image.png)

**Figure 1.10.** Comparison of the difference in charge transfer resistance, $\Delta R_{ct}$, as a function of mismatch position. Three different types of mismatches in ds-DNA films A-C (white), A-A (light grey), and A-G (dark grey) at position 5 (bottom), 13 (middle) and 18 (top) with respect to the electrode surfaces are shown in this figure. [M. H. Shamsi, H-B. Kraatz, *Analyst*, 2010, 135, 2280] – Reproduced by permission of The Royal Society of Chemistry.

Although the $\Delta R_{ct}$ of the middle mismatched ds-DNA differs from that of the top and bottom positions, the top and bottom mismatches cannot be distinguished by using the $\Delta R_{ct}$ values. Moreover, the higher $\Delta R_{ct}$ values for the middle mismatched ds-DNA compared to top or bottom positions are in contrast to the previous report where the $\Delta R_{ct}$ for middle mismatched position was the lowest of any other ds-DNA films. Another important finding of this study was that this approach of EIS based mismatch detection is able to discriminate the nature of mismatches present in ds-DNA.
The three different types of mismatches at the middle position, which are A-C, A-A, A-G, were distinguishable based on the different \( \Delta R_{ct} \) values of 470 (40) \( \Omega \cdot \text{cm}^2 \), 560 (20) \( \Omega \cdot \text{cm}^2 \) and 750 (10) \( \Omega \cdot \text{cm}^2 \) respectively. EIS based detection was further expanded to get information on the presence of a mismatch as well as the type of neighboring base in the target DNA sequence.\(^4^1\) The use of a small molecule naphthyridine-azaquinolone (Npt-Azq) instead of using \( \text{Zn}^{2+} \) enables them to detect the presence of the A-A mismatch closest to a guanine base in a target sequence. The \( \Delta R_{ct} \) for the mismatched sequence was found to be 11.7 (2.9) \( \Omega \cdot \text{cm}^2 \), while the fully matched ds-DNA did not show any change of the charge transfer resistance. A slightly different effort was recently taken by the Kraatz group for DNA mismatch detection using EIS technique. Instead of using negatively charged capture DNA strand, neutral peptide nucleic acid (PNA) oligomers were chosen as capture strands that circumvents the issue of background signal due to the binding of metal ions with the capture strand.\(^4^2\) The Kraatz’s group reported that a single C-T mismatch can be detected after exposing the capture PNA- target DNA duplexes to \( \text{Ni}^{2+} \) using EIS. Employing the same promise, this group detected different types of mismatches even in the presence of human serum and red blood cell lysate with the detection limit down to 10 fM.\(^4^3\)
1.2.6. Scanning Electrochemical Microscopy based DNA Sensors

Scanning electrochemical microscopy (SECM) is a non-contact scanning probe technique that uses an electrochemical tip (microsensor) to image biomolecules on various surfaces.\textsuperscript{44} SECM has shown a high level of performance for investigating immobilized biomolecules and biological processes at solid-liquid interfaces.\textsuperscript{45,46} In SECM, detection of DNA hybridization is accomplished through Coulombic interactions between a negatively charged mediator in solution and the phosphate groups of the immobilized DNA strands. The electrostatic repulsion between the anionic species generates a modulation of the current that originates from the redox conversion of the anionic species, which is used to develop a simple and sensitive assay for DNA detection. The first report by Yamashita and his co-workers discussed the visualization of DNA hybridization events by means of SECM.\textsuperscript{47} Schuhmann and co-workers have studied the use of SECM to visualize the status of surface-bound DNA strands on gold electrodes.\textsuperscript{48,49} In these studies, they immobilized single-stranded capture DNA on a gold wafer at different positions. Some of the spots were allowed to hybridize with their complementary ss-DNA while others remained unhybridized. SECM tip was then scanned over the sample in a solution containing $[\text{Fe(CN)}_6]^{3-}$ as a redox marker, and the current profile was obtained (Scheme 1.10).
Scheme 1.10. SECM technique to detect DNA hybridization. A) A steady-state current is observed due to the diffusion of solution based redox marker, $[\text{Fe(CN)}_6]^{3-}$, and conversion into $[\text{Fe(CN)}_6]^{4+}$ B) The tip-generated $[\text{Fe(CN)}_6]^{4+}$ recycles into the parent form as the tip approaches the Au surface causing an increase in tip current. C) The recycling of the redox mediator is hindered at the ss-DNA modified surface due to the charge interactions between the tip-generated $[\text{Fe(CN)}_6]^{4+}$ and the negative charge on oligonucleotide backbone, which results a drop in tip current. D) Hybridization increases the density of negative phosphate groups and causes enhanced repulsion of the redox mediator leading to further drop in tip current. Redrawn with permission from \{F. Turcu, A. Schulte, G. Hartwich, W. Schuhmann, Angew. Chem. Int. Ed., 2004, 43, 3482\}. Copyright \{2004\} John Wiley and Sons.

The current obtained from different spots corresponds to the types of DNA immobilized on the surface. They successfully employed the label-free SECM technique for electrochemical recognition and visualization of DNA hybridization. F. Zhou and co-workers reported the ability of SECM to discriminate single- and double-stranded DNA with the addition of methylene blue which binds only with duplex form of DNA.\textsuperscript{50} They found that this approach can discriminate a mismatched ds-DNA from the fully complementary strand only at elevated temperature, more than 45 °C. Bard and Kraatz have shown that the addition of metal ions in double stranded DNA facilitates penetration
of a solution based redox mediator towards the surface thus leading to an enhanced current. This happens because of the formation of metal-DNA (M-DNA) monolayers that reduce negative charges on the DNA film. A few years later, the H.-B. Kraatz group reported the ability to discriminate the presence and position of a single-nucleotide mismatch in ds-DNA upon addition of Zn\(^{2+}\) ions in the ds-DNA films using SECM. As shown in Figure 1.11, the current profile data is a clear indication of the presence of mismatches at three different positions of ds-DNA films.

**Figure 1.11.** A) Schematic representation of fully matched and three different mismatch positions in ds-DNA used in the study. B) Typical SECM image obtained over a ds-DNA microarray on a Au substrate in the presence of [Fe(CN)\(_6\)]\(^{3-}\) redox marker. C) Current profiles obtained on four different ds-DNA spots are the characteristics of the position of the DNA mismatches. Reprinted with permission from [P. M. Diakowski, H.-B. Kraatz, *Chem. Commun.*, 2009, 10, 1189] – Reproduced by permission of The Royal Society of Chemistry.
The emerging interest in developing electrochemical DNA biosensors for mismatch detection shows that there is an urgent need to evaluate positional parameters of single-nucleotide mismatches in a target sequence in more detail. Most of studies carried out to date focused on the detection of different types of mismatches at a single position. Although our group has reported the feasibility of detecting single-nucleotide mismatches in different positions in principle, previous studies did not attempt to evaluate mismatched positions in detail. There are no known studies that evaluate mismatched positions within a single DNA target in detail and precisely.

1.3. Objective

My objective is to evaluate the presence of single-base-pair mismatches in a 25-mer ds-DNA at 25 different positions and how it affects the EIS / SECM response in order to obtain a precise “mismatch map”.

1.4. Approach

In order to reach the research objective, I will follow the DNA mismatch detection approach developed by Kraatz and co-workers. In this approach, DNA capture strand is hybridized with its fully complementary or single-mismatched DNA strand and then immobilized onto clean gold surfaces. Electrochemical measurements (EIS and SECM) will be carried out on the ds-DNA films both before and after Zn$^{2+}$ addition. I will use a 25-mer target DNA with a single-nucleotide mismatch whose position will be varied along the ds-DNA as shown in Scheme 1. EIS and SECM for all duplexes will be recorded and compared to the fully matched system in an effort to extent information about the differences of the electrochemical behavior for all samples.
Scheme 1. Schematic views of all the ds-DNA films on gold electrodes that will be investigated in this study. 25 mismatched (M) DNA strands with the variation of mismatch positions from M1 to M25 will be compared with the fully complementary strand (FC). Red symbols represent the position of mismatches along the duplex.
Chapter 2

EXPERIMENTAL

2.1. Chemicals and Reagents

Tris(hydroxymethyl)aminomethane (TRIS) was purchased from Boehringer Mannheim Biochemicals (BMB Indianapolis, IN). K₄[Fe(CN)₆] and K₃[(Fe(CN)₆]₄, and perchloric acid (70%) were obtained from EM Science (Gibbstown, NJ). Hexaamineruthenium(III) chloride (Ru(NH₃)₆Cl₃) and Zn(ClO₄)₂·6H₂O were obtained from Aldrich Chemicals (Milwaukee, WI). All solutions were prepared with deionized water (18 MΩ cm resistivity) purified by a Millipore Milli-Q water purification system. In this study, we have employed a 25-mer DNA sequence called Folmer primer LCO149053 as a capture DNA. Mismatched sequences were designed by changing a nucleotide base of the fully complementary strand of the capture DNA at 25 different positions. The 5'-disulfied-labeled DNA capture sequence, unlabeled fully complementary sequence and single-base-pair mismatched sequences were synthesized using a fully automated DNA synthesizer and purified by HPLC at BioCorp (UWO OligoFactory, the University of Western Ontario, London, ON). The list of DNA strands is shown in Table 2.1.
Table 2.1. 25-mer DNA strands: fully complementary (FC) and single-nucleotide mismatches (M) varying the positions from 1-25. The positions of the mismatches in the complementary target are shown in red.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mismatch</th>
<th>Mismatch type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-(CH₂)₆-S-S-(CH₂)₆-5'</td>
<td>GGT CAA CAA ATC ATA AAG ATA TTG G-3'</td>
<td>Capture strand</td>
</tr>
<tr>
<td>3'-CCA GTT GTT TAG TAT TTC TAT AAC C-5'</td>
<td>Full Complementary (FC)</td>
<td></td>
</tr>
<tr>
<td>3'-CGA GTT GTT TAG TAT TTC TAT AAC C-5'</td>
<td>M-1 (GG)</td>
<td></td>
</tr>
<tr>
<td>3'-CCA GTT GTT TAG TAT TTC TAT AAC C-5'</td>
<td>M-2 (GG)</td>
<td></td>
</tr>
<tr>
<td>3'-CGG GTT GTT TAG TAT TTC TAT AAC C-5'</td>
<td>M-3 (TG)</td>
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<tr>
<td>3'-CCA GTT GTT TAG TAT TTC TAT AAC C-5'</td>
<td>M-25 (GG)</td>
<td></td>
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</table>

2.2. Preparation of Gold Microelectrodes

Gold microelectrodes were constructed in several steps. First, one end of a borosilicate glass capillary was sealed in such a way that a conical shape was obtained inside the sealed part of the glass. A piece of straight gold wire (50 μm diameter) is placed at the bottom of the sealed glass. The capillary glass was then put under vacuum,
and gently sealed onto the wire using a butane flame. Heating was carried out carefully and slowly to avoid bending the glass. The sealed gold wire was then joined to a copper wire with conductive silver adhesive paste. The capillary was checked by a microscope to make sure that the gold wire inside was completely sealed. The sealed end of the capillary was cut to expose the gold wire. The exposed side of the capillary was polished with sand papers of decreasing particle size. During polishing, the sand papers were soaked with water to reduce the strain on the glass. The electrodes were then cautiously polished sequentially with 0.3 and 0.05 μm alumina slurries on a wet polishing cloth. After each polishing, the electrodes were washed extensively with Milli-Q water and sonicated to remove alumina completely from the electrode surface. The electrodes were, furthermore, dipped in ethanol and sonicated to get rid off any organic substance on the surface. The presence of any air bubble or crack on the electrode surface was always inspected with an optical microscope. Immediately prior to DNA immobilization, the electrodes were electrochemically cleaned by repeated cycling in 0.1 M H₂SO₄ or 0.05 M KOH between 0 and 1.4 V versus Ag/AgCl with a scan rate of 500 mV until stable voltammograms were obtained.

2.3. Preparation of ds-DNA Films on Gold Electrodes

Preparation of the ds-DNA films on gold electrodes was carried out in two steps. First, 25 μL of 100 μM solution of the capture strand containing hydroxyalkyl disulfide linker was mixed with 25 μL of the complementary or single mismatched DNA sequences in a sterilized microtube. 50 μL of 50 mM Tris-ClO₄ buffer (pH 8.6) was added to the DNA mixture in order to form duplex DNA. The final ds-DNA concentration is 50 μM. The mixture was heated at 65 °C for 5 minutes and then kept at
room-temperature for 2 hours. A clean and dried gold electrode was then dipped in 10 μL of the pre-hybridized ds-DNA in a microtube which was then sealed with paraffin film to avoid evaporation. This microtube was kept at 4 °C for 3 days. Incubation time was 3 days because well ordered film formation can take several days although dense monolayers assemble in less than 1 h. At least ten gold electrodes were incubated each time according to the same procedure. The electrodes were taken out from the DNA solution and washed with Tris-ClO₄ buffer solution before putting them in an electrochemical cell for impedance measurements. In order to modify the ds-DNA film on gold electrodes with Zn²⁺ ions, the films were incubated in a 0.4 mM solution of Zn(ClO₄)₂ in Tris-ClO₄ buffer for 12 hours at 4 °C.

2.4. Chronocoulometry Using Hexamine Ruthenium(III) Chloride

The DNA immobilized electrodes were placed into a cell containing a fresh solution of 10 mM Tris-ClO₄ (pH 7.4) and 0.1 M NaCl, which was then purged into N₂ gas. The double layer capacitance of the system (Q₉) was determined by performing chronocoulometric scans on the electrolyte solution with no redox marker. The starting potential (E₀) for all chronocoulometry experiments was 0.15 V and the final potential (Eₙ) was -0.4 V versus Ag/AgCl. After the Q₉ scan, the solution was changed to freshly prepared 100 μM hexamine ruthenium(III) chloride (RuHex) in 0.1 M NaCl, 10 mM Tris-ClO₄ (pH 7.4) buffer and purged for 10 min. The chronocoulometry scans were then repeated in order to determine the amount of ds-DNA attached to the gold surface.
2.5. Electrochemical Impedance Spectroscopy Measurements

After three days of incubation in ds-DNA, the gold electrodes were taken out of the microtubes and rinsed by dipping the electrodes in Tris-ClO$_4$ (pH 8.6) buffer solution for one minute in order to get rid of loosely attached DNA. The electrodes were then mounted into a conventional three electrode cell system. Ag/AgCl electrode as a reference electrode, a platinum wire as a counter electrode and a miniature salt bridge (agar plus 1 M KNO$_3$) were other components of the cell. All impedance measurements were carried out in the presence of the redox couple ferri/ferrocyanide with a concentration of 2 mM each in 50mM Tris-ClO$_4$ buffer (pH 8.6). The cell was enclosed in a grounded Faraday cage and the experiments were carried out at room temperature. All impedimetric measurements were performed with CHI-650c electrochemical system (CHI, Austin, TX). Frequencies from 100 kHz to 0.1 Hz were applied. The ac amplitude was 5 mV. The Open Circuit Potential (OCP) was used as the applied potential that was closed to 250 mV (formal potential of the redox probe). After measuring the impedance of B-DNA on gold, the electrodes were rinsed by dipping in Tris-ClO$_4$ buffer (pH 8.6) for one hour in order to remove any ferri/ferrocyanide attached with the DNA film. The DNA-modified electrodes were immersed in 0.4 mM Zn(ClO$_4$)$_2$ solution in Tris-ClO$_4$ buffer (pH 8.6) for 24 hours. The electrodes were rinsed with the buffer and mounted into the cell for EIS measurement under the same condition mentioned above. All experimental results were fitted to a Randles’ equivalent circuit, using ZSimpWin 3.20 software (Princeton Applied Research)$^{58}$, which allowed us to extract resistive and capacitive values from the experimental data.
2.6. Scanning Electrochemical Microscopy

2.6.1. Preparation of Platinum Tip Electrode

Platinum microelectrodes were prepared by sealing 25 μm diameter Pt wires into glass capillaries. One end of the glass capillary was pulled by a heating coil puller and then sealed by a Bunsen lamp. A cone shape inside the pulled capillary favorably fitted the inserted Pt wire in a straight position. The thinner glass wall at the pulled end facilitates the sharpening of the glass sheath. The glass sheath was sharpened by 3.0 and 0.05 μm diameter diamond pads. Further, a used hard drive disk covered with 0.05 μm diameter alumina was used as a polishing wheel, which was spun at high speed. The quality of the tip was monitored by cyclic voltammetry in 0.1 M H₂SO₄ and the RG (ratio of diameter of glass to the diameter of Pt wire) value was estimated by comparing the SECM approach curve with theoretical curves.

2.6.2. Fabrication of Gold Substrate

Gold-deposited silicon wafers were prepared for using them as a substrate to immobilize ds-DNA for SECM measurements. Highly polished (2 micron thickness) silicon wafers were used as supports for ion beam gold deposition (vapor deposition). At first, a 20 nm Ti film was deposited (9.6 kV; 1.7 A; rate 0.4 Å/s) to improve the adhesion of the 200 nm gold film (9.6 kV; 1.9 A; rate 1 Å/s) on the native SiO₂. Thorough cleaning pretreatment was carried out in steps, starting from cleaning in Piranha solution at 90 °C for 5 min, rinsing with MilliQ water prior to drying under nitrogen. The average grain size was on the order of ~50 nm and the surface appears to be very smooth. The texture of the gold films was described as Au(111).
2.6.3. Scanning Electrochemical Microscopy Measurements

A three-electrode system was used, with the 25 μm diameter Pt tip as the working electrode, a Cu wire as the counter electrode, and a saturated Ag/AgCl electrode as the reference electrode. The fabricated gold wafers were cut into 1 cm x 1 cm small pieces. The gold substrate was cleaned in Piranha solution (70% sulfuric acid + 30% hydrogen peroxide) and washed with copious amounts of deionized water followed by drying with nitrogen. DNA microarrays were prepared using a spotting device (SpotBot, Arraylt, Sunnyvale, CA) employing a 100-μm-diameter pin (SMP3, Arraylt). Typical arrays consisted of 5 spots x 6 spots and a single spot on the sixth row. All spots were separated by 160 μm. The substrate was then placed on top of a wet filter paper inside a Petri dish which was wrapped with parafilm and incubated at 4° C for 48 hours.

SECM measurements were carried out with a CHI-900b (CH Instruments, Austin, TX). Approach curves were measured at a typical approach rate of 10 μm s⁻¹, and a tip scan rate of 25 μm s⁻¹ was employed for SECM imaging. All the measurements were performed in 20 mM Tris-ClO₄ (pH 8.6) containing 1 mM K₄[Fe(CN)₆] and 50 mM NaClO₄. The tip electrode was approached towards an unmodified region of the gold substrate before starting to capture SECM images. After proper positioning and approach, an image was obtained by scanning the tip over the substrate. The ds-DNA modified substrate was then washed with Tris-ClO₄ buffer (pH 8.6). The Zn-modified ds-DNA film was prepared by exposing the ds-DNA film to a solution of 0.4 mM Zn(ClO₄)₂ in 50 mM Tris-ClO₄ buffer (pH 8.6) for 12 hours. The Zn-incubated ds-DNA modified substrate was washed with the Tris-ClO₄ buffer solution and SECM measurements were carried out in presence of 1 mM K₄[Fe(CN)₆] and 50 mM NaClO₄. A current profile was obtained by extracting values from an SECM image with the help of Gwyddion software.
3.1. DNA Film Formation on Gold

A film of ds-DNA was prepared by incubation of the prehybridized ds-DNA on a clean gold surface. The self assembly of disulfide-containing DNA molecules allows them to chemisorb onto the gold surface and results in an -S-(CH$_2$)$_6$-ds-DNA film adjacent to hydroxylalkyl-S groups. It has been reported that thiol-derivatized ss-DNA exhibit some non-specific physisorption through the nitrogen atoms and polar side groups of DNA molecules. However, for ds-DNA, because the single strands of DNA are paired with one another, the probability of non-specific interactions will be very low. The adjacent hydroxylalkyl-S group generated from the disulfide linker serves as a spacer that is necessary for forcing the DNA to stand up and allowing a greater DNA loading of the surface. Long et al. studied XPS of ds-DNA immobilized gold surface and demonstrated that this process of DNA immobilization produces a densely packed array of duplexes. Therefore, the final structure of ds-DNA film is most likely to arrive from specific chemisorption through the covalent Au-S bond formation. The adjacent hydroxylalkyl-S group generated from the disulfide linker serves as a spacer that is necessary for forcing the DNA to stand up and allowing a greater DNA loading of the surface. The schematic diagram of the DNA film on gold electrode surface is depicted in Scheme 3.1.

The density of DNA strands on a gold surface was determined using chronocoulometry (CC) by determining the double-layer charge \( (Q_{dl}) \) on the surface in the absence and presence of a trivalent redox cation, \([\text{Ru(NH}_3)_6]^{3+}\). This cation electrostatically binds to the single- negative charged DNA phosphate groups in the phosphodiester backbone of the oligonucleotide strand in the ratio of 1:3. Electrical voltage is applied across the formal potential to reduce the ruthenium ion at the diffusion limited rate, and the resulting charge flow is measured. The total charge is the sum of the double layer charge \( (Q_{dl}) \), the charge due to reduction of surface confined ruthenium ion and the reduction of ruthenium diffusing from solution. This allows accurate determination of the amount of ruthenium bound to the DNA, and the number of DNA strands bound to the surface. Figure 3.1 is a plot of a typical chronocoulometric experiment for the measurement of the charge associated with the electrode surface.
Figure 3. Chronocoulometric response curves for ds-DNA modified electrode in the absence of (black) and presence of (red) RuHex. The lines are the fits to the data to determine the intercepts at \( t = 0 \). The intercepts of the plot of charge \( (Q) \) versus \( t^{1/2} \) was the measured value of charges on the modified electrodes.

As can be seen by the increase in the intercept, the charge associated with the electrode surface increases upon addition of RuHex. Using the charges determined during the chronocoulometry experiments, the amount of RuHex on the surface of the electrode was determined. By calculating the amount of DNA attached to the electrode surface, we found that we have \( 1.11-1.8 \times 10^{12} \) ds-DNA molecules/cm\(^2\) on the gold surface, which is in accordance with previous literature reports.
3.2. Electrochemical Impedance Spectroscopy for Mismatch Detection

We have employed electrochemical impedance spectroscopy (EIS) to probe single-base mismatches at all twenty-five different positions in the Folmer primer, a 25-base oligonucleotide. In each of these cases, the nucleobase mismatches were introduced in the complementary strand. The disulfide-modified capture ss-DNA was hybridized with the target strand to form ds-DNA prior to immobilization onto gold electrodes, as described in the experimental chapter. This approach was chosen, since it enables the immobilization of ds-DNA directly onto the surface and eliminates problems associated with surface hybridizations, resulting in the formation of ds-DNA films. We prepared films of the fully matched combination (FC) and films containing mismatches in twenty-five different positions in the complementary strand. Mismatch positions in the ds-DNA films were counted numerically from the side of the electrode surface. For example, a ds-DNA film possessing a mismatch closest to the gold surface is named M1. A mismatch in position 2 is named M2, and so on. EIS measurements were carried out on all ds-DNA films in 50 mM Tris-ClO₄ at pH 8.6 in the presence of a 1:1 mixture of [Fe(CN)₆]³⁻/⁴⁻ (4 mM), as the solution-based redox probe. After the EIS measurements, the ds-DNA films were exposed to a 0.4 mM Zn²⁺ solution at pH 8.6 according to the previously reported procedure, and the EIS measurements were repeated. Figure 3.2 shows representative EIS data in the form of a Nyquist plot for a fully complementary ds-DNA film and a ds-DNA film containing a mismatch position 6 (M6), collected over a frequency range from 0.1 to 100 kHz. The impedance data are also represented as Bode plots in which the absolute values of impedance and the phase angle are plotted against the frequency (Figure 3.3).
Figure 3.2. A representative Nyquist plot ($Z_{re}$ vs. $-Z_{im}$) obtained from ds-DNA films of a fully matched (black) and a ds-DNA with a single-mismatch at position-6 (red) in the absence (◊) and presence (●) of Zn$^{2+}$. The measurements were performed in the presence of 2 mM [Fe(CN)$_6$]$_3^{-}$ and 2 mM [Fe(CN)$_6$]$_4^{3-}$ in 50 mM Tris-ClO$_4$ solution (pH 8.6), upon application of a biasing potential of 0.25 V vs. Ag/AgCl and the voltage frequencies from 0.1 Hz to 100 kHz. Solid lines correspond to the theoretical fit of the experiment data by a modified Randles equivalent circuit. Inset: The modified Randles’ equivalent circuit used for data fitting. WE, CE, and RE denote working electrode, counter electrode, and reference electrode, respectively. $R_{ct}$, $R_x$ and $R_s$ are interfacial charge transfer resistance, ds-DNA film resistance and uncompensated solution resistance, respectively, while $C_{dl}$ is capacitance associated with the double layer charge at the film modified electrode and CPE is a constant phase element.

In the Nyquist plots, the observed semicircle portion indicates an electron transfer limited process. The diameter of the semicircle provides an estimate of the charge transfer resistances between the redox reporters in solution and the electrode surface for all of the ds-DNA films. In the Bode plot, it is easy to understand how the impedance depends on the frequency. As shown in the Figure 3.3 A, total impedance of the ds-DNA films before Zn$^{2+}$ addition reaches to the maximum at lower frequency compared to that of the ds-DNA films after Zn$^{2+}$ addition. Thus it takes lower measurement times to get charge
transfer resistance values for Zn\textsuperscript{2+} systems. Similarly, highest phase angle is obtained at relatively lower frequency for the ds-DNA films in presence of Zn\textsuperscript{2+}.

![Graph](image)

**Figure 3.3.** Variation of (A) total impedance (|Z|) and (B) phase shift with frequency in the form of Bode plot from ds-DNA films of a fully matched (black) and a ds-DNA with a single-mismatch at position-6 (red) in the absence (○) and presence (♦) of Zn\textsuperscript{2+}. Solid lines correspond to the theoretical fit of the experiment data by a modified Randles’ equivalent circuit shown in Figure 3.2 (inset).

For an accurate quantitative analysis of all the components involved in the electrochemical system, the experimental impedance spectra were modeled using an equivalent circuit consisting of electrical components. A modified form of the Randles’ equivalent circuit\textsuperscript{67} was used for the ds-DNA modified electrodes and is shown in the right hand corner of the Figure 3.2. The circuit was constructed on the basis of the physical components involved in our ds-DNA films. The EIS response at various frequencies is shown as symbols while the fit of the equivalent circuit to the experimental data is given as a solid line. The same model was used to fit all ds-DNA films described in this thesis. The circuit consists of an ohmic resistance of the electrolyte solution (R\textsubscript{s}) between the reference electrode and the working electrode. The R\textsubscript{s} is in series with the...
double layer capacitance (\(C_{dl}\)) on the ds-DNA film modified electrode. \(C_{dl}\) represents a fixed layer of oppositely charged ions close to the gold interface. The charge transfer resistance (\(R_{ct}\)) is resulted from the interfacial electron transfer resistance. The addition of \(R_x\) and a constant phase element (CPE) to the Randles’ circuit accounts for the presence of pinholes in the film structure. \(R_x\) represents the non-Faradaic impedance due to an interfacial resistance in parallel with the double-layer capacitance. This resistance is due to the movement of mobile ions in and out of the ds-DNA film. \(^6\) \(R_x\) is quite separate from the \(R_{ct}\), which describes the flow of charges across the modified interface into the electrode. The CPE acts as a nonlinear capacitor representing the in-homogeneity of the film and the electrode surface. \(^6\) The Warburg impedance of the general Randles circuit is not included in this circuit since no diffusional behavior was observed with the microelectrodes within the frequency range used.

As shown in Figure 3.2, before the addition of Zn\(^{2+}\), the diameter of the semicircle for FC (\(\Phi\)) is higher than that for M6 (\(\Phi\)). The higher charge transfer resistance for the fully mismatched duplex was previously attributed to the film uniformity. \(^3\) Presence of a mismatch results in a disordered and flexible ds-DNA film which leads to lower steric hindrance and better charge transfer to the redox markers. Upon addition of Zn\(^{2+}\), decreases in the charge transfer resistances were observed both for FC and M6. The lower impedance of ds-DNA film after incubation in Zn\(^{2+}\) is expected from previous observations. \(^6\) Influence of the Zn\(^{2+}\) on ds-DNA films in our electrochemical system is depicted in Scheme 3.2. The ds-DNA film immobilized on gold surfaces is resisting the negatively charged [Fe(CN)\(_6\)]\(^{3-}/4^-\) redox species to penetrate through the film and reach the electrode surface.
Scheme 3. Schematic representation of the effect of Zn$^{2+}$ ions on the charge transfer resistance between the redox species and the ds-DNA modified gold electrode. Upon Zn$^{2+}$ addition, electrostatic repulsions between the redox probe $[\text{Fe(CN)}_6]^{3-/4-}$ and the negatively charged DNA films decreases, resulting in better penetration of the redox species through the film and reduced charge transfer resistance.

The resistance offered by the DNA film is attributable to the physical existence of the film itself (steric hindrance) and the electrostatic repulsion between the negatively charged redox reporters and the phosphate backbone. The Zn$^{2+}$ ions may interact non-covalently as counterions or coordinate to the phosphate backbone and/or coordinate to specific ligand sites on the nucleobases. The covalent interaction of Zn$^{2+}$ ions with nucleobases is reported to happen at the N7 of purines or the N3 of pyrimidines or the exocyclic base keto groups as shown in Scheme 3.
Scheme 3. Affinities of Zn$^{2+}$ ions for the various binding sites (designated by arrow) within nucleotide unit of the DNA molecule.

Thus, the reduction of negative charge due to Zn$^{2+}$ binding with ds-DNA film enables the film to be more diffusive for the anionic redox reporters, $[\text{Fe(CN)}_6]^{3-/4-}$, which give rise to the lower charge transfer resistance, $R_{ct}$. The change in the charge transfer resistance, $\Delta R_{ct}$, before and after addition of Zn$^{2+}$ to a specific ds-DNA film is considered to be an indication of the presence of a mismatch. EIS measurements were carried out on all 25 ds-DNA films and the results are plotted as Nyquist plots in Figure 3.4. For the convenience, five mismatch positions were grouped into a single graph and the data for FC is shown in every graph for the comparison.
Figure 3.4. Representative Nyquist plots ($Z_{re}$ vs. $-Z_{im}$) obtained from all of the 25 ds-DNA films consisting of a single-nucleotide mismatch at different positions both before (A) and after $Zn^{2+}$ addition (B). The mismatch positions are shown in legends of each plot. Measurement conditions are described in Figure 3.2. Solid lines correspond to the theoretical fit of the experiment data by the equivalent circuit shown in Figure 3.2.
As shown in Figure 3.4, before addition of Zn$^{2+}$, it is difficult to discriminate the mismatched sequences just by comparing the Rct values between two mismatch positions, because many of the semicircles overlap with each other. After adding Zn$^{2+}$ to the films, the Rct values decrease for all of the films but to different extents, which also do not provide a meaningful way to distinguish one mismatches from the other. That is why, the ΔRct values for all the films were examined in detail from the fitting results of the experimental data as listed in Table 3.1.

### Table 3.1. Values of the electrical components obtained by fitting the EIS data with the equivalent circuit. Data were taken from fully complementary (FC) and 25 mismatch positions (from M1 to M25) in ds-DNA films. Slandered deviations obtained from ten measurements are shown in the parenthesis.

<table>
<thead>
<tr>
<th>Zn present/absent</th>
<th>R, Ω·cm$^{-2}$</th>
<th>C$_d$, μF·cm$^{-2}$</th>
<th>Rct, Ω·cm$^{-2}$</th>
<th>CPE (Y$_0$) x 10$^4$</th>
<th>n</th>
<th>R$_*$, Ω·cm$^{-2}$</th>
<th>ΔRct, Ω·cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC present</td>
<td>0.03</td>
<td>3.5 (3.1)</td>
<td>1089 (130)</td>
<td>2.4 (2.1)</td>
<td>0.91 (0.02)</td>
<td>1.5 (0.7)</td>
<td>905</td>
</tr>
<tr>
<td>absent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>0.04</td>
<td>2.9 (0.5)</td>
<td>184 (40)</td>
<td>3.0 (0.4)</td>
<td>0.92 (0.02)</td>
<td>1.0 (0.1)</td>
<td>(130)</td>
</tr>
<tr>
<td>M1 present</td>
<td>0.10</td>
<td>3.0 (2.9)</td>
<td>916 (150)</td>
<td>2.3 (0.9)</td>
<td>0.91 (0.02)</td>
<td>2.5 (2.1)</td>
<td>749</td>
</tr>
<tr>
<td>absent</td>
<td>0.10</td>
<td>1.8 (0.2)</td>
<td>167 (45)</td>
<td>1.6 (0.5)</td>
<td>0.93 (0.03)</td>
<td>1.9 (0.8)</td>
<td>(175)</td>
</tr>
<tr>
<td>present</td>
<td>0.01</td>
<td>2.4 (0.9)</td>
<td>771 (80)</td>
<td>2.3 (1.2)</td>
<td>0.91 (0.03)</td>
<td>1.9 (0.8)</td>
<td>(175)</td>
</tr>
<tr>
<td>M2 present</td>
<td>0.01</td>
<td>2.2 (0.3)</td>
<td>211 (110)</td>
<td>1.9 (0.9)</td>
<td>0.91 (0.02)</td>
<td>2.4 (0.7)</td>
<td>(110)</td>
</tr>
<tr>
<td>absent</td>
<td>0.02</td>
<td>1.7 (0.1)</td>
<td>428 (60)</td>
<td>2.7 (0.7)</td>
<td>0.90 (0.01)</td>
<td>1.6 (0.1)</td>
<td>263</td>
</tr>
<tr>
<td>present</td>
<td>0.02</td>
<td>1.9 (0.3)</td>
<td>165 (25)</td>
<td>2.1 (0.4)</td>
<td>0.92 (0.02)</td>
<td>1.7 (0.1)</td>
<td>(70)</td>
</tr>
<tr>
<td>M3 present</td>
<td>0.02</td>
<td>1.7 (0.1)</td>
<td>519 (90)</td>
<td>2.7 (0.7)</td>
<td>0.90 (0.01)</td>
<td>1.6 (0.1)</td>
<td>309</td>
</tr>
<tr>
<td>absent</td>
<td>0.02</td>
<td>1.9 (0.3)</td>
<td>210 (50)</td>
<td>2.1 (0.4)</td>
<td>0.92 (0.02)</td>
<td>1.7 (0.1)</td>
<td>(145)</td>
</tr>
<tr>
<td>present</td>
<td>0.07</td>
<td>2.2 (0.6)</td>
<td>334 (45)</td>
<td>2.9 (0.8)</td>
<td>0.90 (0.02)</td>
<td>1.6 (0.4)</td>
<td>218</td>
</tr>
<tr>
<td>M5 present</td>
<td>0.07</td>
<td>2.3 (0.7)</td>
<td>116.4 (45)</td>
<td>2.6 (0.8)</td>
<td>0.90 (0.03)</td>
<td>1.5 (0.1)</td>
<td>(20)</td>
</tr>
<tr>
<td>absent</td>
<td>0.07</td>
<td>2.4 (0.8)</td>
<td>463 (75)</td>
<td>3.1 (0.6)</td>
<td>0.92 (0.01)</td>
<td>1.2 (0.1)</td>
<td>246</td>
</tr>
<tr>
<td>present</td>
<td>0.07</td>
<td>3.2 (1.5)</td>
<td>217 (55)</td>
<td>2.4 (0.4)</td>
<td>0.92 (0.01)</td>
<td>1.2 (0.1)</td>
<td>(35)</td>
</tr>
<tr>
<td>M6 present</td>
<td>0.10</td>
<td>2.6 (0.8)</td>
<td>730 (80)</td>
<td>5.9 (4.5)</td>
<td>0.93 (0.02)</td>
<td>1.6 (0.9)</td>
<td>398</td>
</tr>
<tr>
<td>absent</td>
<td>0.10</td>
<td>2.6 (1.0)</td>
<td>332 (45)</td>
<td>4.8 (2.0)</td>
<td>0.89 (0.06)</td>
<td>1.2 (0.2)</td>
<td>(50)</td>
</tr>
<tr>
<td>present</td>
<td>0.11</td>
<td>3.1 (1.6)</td>
<td>607 (75)</td>
<td>2.8 (1.0)</td>
<td>0.89 (0.05)</td>
<td>1.7 (0.5)</td>
<td>401</td>
</tr>
<tr>
<td>M7 present</td>
<td>0.06</td>
<td>2.3 (1.8)</td>
<td>206 (65)</td>
<td>4.1 (1.2)</td>
<td>0.91 (0.02)</td>
<td>1.3 (0.1)</td>
<td>(40)</td>
</tr>
<tr>
<td>absent</td>
<td>0.08</td>
<td>2.9 (1.4)</td>
<td>613 (75)</td>
<td>2.2 (0.8)</td>
<td>0.92 (0.02)</td>
<td>2.2 (0.8)</td>
<td>452</td>
</tr>
<tr>
<td>present</td>
<td>0.05</td>
<td>5.3 (0.7)</td>
<td>161 (70)</td>
<td>2.3 (0.7)</td>
<td>0.89 (0.01)</td>
<td>1.8 (0.3)</td>
<td>(50)</td>
</tr>
<tr>
<td>M8 present</td>
<td>0.06</td>
<td>2.1 (0.5)</td>
<td>725 (75)</td>
<td>2.8 (0.6)</td>
<td>0.90 (0.05)</td>
<td>1.2 (0.1)</td>
<td>619</td>
</tr>
<tr>
<td>absent</td>
<td>0.05</td>
<td>1.6 (0.5)</td>
<td>106 (45)</td>
<td>3.2 (2.5)</td>
<td>0.93 (0.04)</td>
<td>1.3 (0.1)</td>
<td>(30)</td>
</tr>
</tbody>
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50
<table>
<thead>
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<th>Table 3.1. Continued...</th>
</tr>
</thead>
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<tr>
<td><strong>Zn</strong></td>
</tr>
<tr>
<td>present/absent</td>
</tr>
<tr>
<td><strong>M11</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M12</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M13</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M14</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M15</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M16</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M17</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M18</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M19</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M20</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M21</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M22</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M23</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
</tr>
<tr>
<td><strong>M24</strong></td>
</tr>
<tr>
<td>absent</td>
</tr>
<tr>
<td>present</td>
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</table>
The solution resistance, $R_s$, remains constant at 0.01-0.05 $\Omega \cdot \text{cm}^2$ both before and after $\text{Zn}^{2+}$ addition, as would be expected for measurements under identical conditions of electrolyte concentration and temperature. Moreover, the distance between the Pt counter electrode and the gold electrode was kept approximately constant in order to minimize variations in the solution resistance $R_s$. There are minor variations in the $C_{dl}$ values among the ds-DNA films. This irregular change was in the order of the measuring error. The values of $R_x$ are similar for almost all of the ds-DNA films before addition of $\text{Zn}^{2+}$. This indicates that the number and size of the assumed pinholes are almost the same in all of the films. However, a slight decrease in $R_x$ was observed in most of the films after addition of $\text{Zn}^{2+}$. This can be attributed to the reduction of the negative charge of the phosphate backbone of DNA upon binding with $\text{Zn}^{2+}$, which provides a less restrictive movement of ions in or out of the film. Since the exponential modifier, $n$, is greater than 0.9, the CPE can be considered as a capacitor according to the previous report. Only the charge transfer resistance, $R_{ct}$, was found to decrease significantly once the metal ion is added. The difference in charge-transfer resistance, $\Delta R_{ct}$, between a ds-DNA film before and after $\text{Zn}^{2+}$ addition is listed in the last column of the Table 3.1. $\Delta R_{ct}$ provides an excellent parameter that discriminates different mismatches in the ds-DNA. A comparison between $\Delta R_{ct}$ as a function of all the 25 different mismatch positions is shown in Figure 3.5. Interestingly, over 25 mismatches, $\Delta R_{ct}$ possesses two minima at position M5 and M18, which is in accordance with earlier reports by Shamsi et al., who reported significant drops in $\Delta R_{ct}$ at these positions. It is noticeable that all 25 ds-DNA films containing a single-base-pair mismatch exhibit lower values of $\Delta R_{ct}$ compared to that of the fully matched film. This may be attributed to differences in film packing.
Figure 3.5. A plot of the differences in the charge transfer resistance ($\Delta R_{ct}$) as a function of mismatch positions. $\Delta R_{ct}$ is the difference between the charge transfer resistance observed in ds-DNA film before and after Zn$^{2+}$ addition. Error bars represent the standard deviation as given in the Table 3.1.

Presumably, mismatched ds-DNA films are more loosely packed compared to fully matched films. Thus, changes in probe diffusion in and out of the film are smaller for a disordered film when evaluated before and after Zn$^{2+}$ addition. The lowest $\Delta R_{ct}$ obtained at two valleys of the trend were 218 (20) $\Omega\cdot$cm$^2$ for M5 and 301 (70) $\Omega\cdot$cm$^2$ for M19. These two mismatched films have on average one third of the $\Delta R_{ct}$ value compared to that of FC, which is 905 (130) $\Omega\cdot$cm$^2$. When the mismatch is at the middle of the target DNA, the $\Delta R_{ct}$, 713 (60) $\Omega\cdot$cm$^2$, is more than double that of the M5 and M19 films. Similar quantitative differences of $\Delta R_{ct}$ among these three mismatch positions were also observed in the previous study.$^{40}$ The $\Delta R_{ct}$ for the M1 mismatch (proximal end) was
found to be quite similar to that of the fully matched film. This result was consistent with a recent report on DNA mismatch detection with a surface plasmon resonance sensor.\textsuperscript{75} It was reported that the mismatch at the proximal position showed no significant difference with the fully complementary strand. The Figure 3.5 also showed that the $\Delta R_{ct}$ for M2, M3 and M4 mismatches gradually decreased until the lowest point of the valley, which is M5. Therefore, the mismatch positions (M1-M4) in the bottom region are well distinguishable which can be rationalized as a result of the different extent of disruption in film structure because of the shift of mismatch positions from the electrode surface. It is noted that a detailed comparative study for these mismatch positions as performed here has not been reported in the literature. The less likely distinguishable mismatches at the four top (distal) positions, such as M22, M23, M24 and M25 have slightly lower $\Delta R_{ct}$ than that of fully matched film. This can be anticipated having least role of these mismatches in film deformation because of the long distance from the electrode surface. In contrast to this result, Ito \textit{et al.} reported increasing charge transfer resistance for an extreme top mismatch position.\textsuperscript{76} Ito \textit{et al.} studied three 15-mer target DNA strands containing a single-mismatch at the proximal (1\textsuperscript{st}), middle (7\textsuperscript{th}) and distal positions (15\textsuperscript{th}) by EIS. The charge transfer resistances obtained from the proximal and middle mismatch positions were quite similar to the fully complementary strand. This finding was in line with our results. However, they found abnormally high charge transfer resistance for the distal mismatch position than fully matched DNA, which was assumed to be due to the structural distortion of the duplex and the hydration around the distal mismatched bases. In our study, we did not observe such an increase in resistance for the distal mismatch positions.
Apart from these observations, the mismatch positions in proximity of the top, centre and bottom mismatch positions, have similar responses, therefore, these positions are less likely to be distinguishable from each other. This pattern of $\Delta R_{ct}$ versus mismatch positions provides very important information about the validity of the approach for mismatch detection at different positions. It is worth mentioning that the little fluctuations in the overall trend of the $\Delta R_{ct}$ values might be due to the variations of mismatch types, which were not controlled in this study. In order to obtain more information about the effect of positional variations of mismatches, SECM measurements were carried on ds-DNA films a gold microarray.
3.3. Scanning Electrochemical Microscopy

In an effort to discriminate all 25 different mismatch positions in films of ds-DNA by SECM, the pre-hybridized DNA strands were immobilized in an array format with the help of a spotbot on a flat gold substrate (Au on a Si wafer) as described in the experimental section and SECM experiments were carried out. A typical image obtained from the SECM measurement on the ds-DNA array in the presence of the solution-based redox marker \( K_4\text{Fe}([\text{CN}]_6 \) is shown in Figure 3.6. Individual spots represent different ds-DNA films deposited by the automated spotbot and correspond to the fully matched ds-DNA (FC) and 25 different mismatches sequentially from mismatch position-1 (M1) to position-25 (M25). The spots for FC were repeated three times in the first and last row in order to compare the current signals obtained from identical DNA strands and to evaluate potential problems associated with tilting of the measurement stage. As can be seen from the image, it was possible to visualize a large number of ds-DNA spots electrochemically in a single microarray format. The color values shown in the bar of the images help to visualize the DNA spots on gold substrate by giving lower current signals on the spots compared to the higher current for gold-background, which is due to a higher resistance at the ds-DNA spots. After the addition of Zn\(^{2+}\) to the ds-DNA films, an overall enhancement of the tip current was observed. However, the current signals obtained from the different spots do not provide rational differences between them. This is presumably due to the tilting of the substrate.
Figure 3.6. Representative SECM images measured over a ds-DNA microarray on a gold substrate in the absence (A) and in the presence of Zn$^{2+}$(B). Fully complementary (FC) and the 25 mismatched ds-DNA were immobilized on a single gold wafer. Experiments were carried out in 1 mM K$_4$[Fe(CN)$_6$], 50 mM NaClO$_4$, 20 mMTris–ClO$_4$ (pH 8.6), with 25 mM Pt tip vs. Ag/AgCl, $E_T = 0.5$ V. Spots in the measured SECM plots correspond to the labels and mismatch positions as indicated on the right.
Another way to evaluate the ds-DNA microarray is to carry out experiments in which the tip is approached to the surface and the current response is monitored. The approach curve is a normalized current-distance profile, which can be generated by withdrawing the tip electrode from the surface and then approaching the center of the spots. This curve provides information about the nature of the substrate and the spots. If the surface is conductive, the current will rise as a function of distance, while for an insulating substrate the current will decrease to zero since the redox probe cannot be recycled efficiently because there is no reactivity on the insulating substrate (see Chapter 1). Approach curves obtained at the unmodified conductive gold surface and as expected, the normalized tip current is enhanced when the tip comes closer to the substrate surface. Figure 3.7 shows some of the approach curves measured on mismatches located near the electrode surface (bottom mismatch positions M2, M5 and M8) of the DNA microarray.

Figure 3.7. Typical normalized approach curves observed above the individual ds-DNA spots for FC (black), M2 (blue), M5 (pink), M8 (red) measured in absence (A) and presence (B) of Zn$^{2+}$. The tip approach current was normalized by the tip steady-state current at an infinite distance from the substrate. The normalized current is plotted against d/a, where d is the tip/substrate separation and a represents the tip radius. Solid lines represent simulated approach curves for dimensionless rate constants in the absence of Zn$^{2+}$, $\lambda = 0.048, 0.048, 0.065, 0.065$, and presence of Zn$^{2+}$, $\lambda = 0.045, 0.048, 0.075, 0.08$ respectively.
A negative feedback was observed for all of the ds-DNA spots, although the fully matched (FC) ds-DNA and the mismatched DNA at the extreme bottom (e.g. M2) showed higher negative feedback compared to the others. Furthermore, after addition of Zn$^{2+}$, the approach curves were more distinguishable from each other. The approach curves for M5 and M8 showed slightly positive feedback current that are distinguishable from the curve for FC. In the same way, the approach curves obtained in the absence of Zn$^{2+}$ (Figure 3.8) for a middle mismatched (M13) and a top mismatched (M18) ds-DNA are indistinguishable from that of the fully matched ds-DNA.

Figure 3.8. Typical normalized approach curves observed above the individual ds-DNA spots for FC (black), M13 (blue), M18 (pink) measured in absence (A) and presence (B) of Zn$^{2+}$. The tip approach current was normalized by the tip steady-state current at an infinite distance from the substrate. The normalized current is plotted against d/a, where d is the tip/substrate separation and a represents the tip radius. Solid lines represent simulated approach curves for dimensionless rate constant in the absence of Zn$^{2+}$/A = 0.048 for all three positions, and presence of Zn$^{2+}$/A = 0.048 for FC and M-13, 0.065 for M-18 respectively.

After Zn$^{2+}$ addition, the feedback current for M18 is slightly less negative. This means that the added Zn$^{2+}$ facilitated discrimination of slightly different films formed from different mismatch positions in ds-DNA. Since the current-distance profiles tend to
follow the diffusion-limited heterogeneous kinetics, fitting of an experimental current-distance curves to theory allows estimating the kinetic parameters for the process of the mediator regeneration at the substrate. A steady-state diffusion problem for SECM geometry was solved using the finite element method (COMSOL Multiphysics software) according to the know procedure described elsewhere. After that, numerical solutions were carried out to obtain theoretical approach curves for different values of the dimensionless rate constant, \( A \). Experimental approach curves were fitted for different values of \( A \). This non-linear fitting gave an apparent rate constant (\( k^0 \)) for electron transfer across the ds-DNA film according to the equation \( A = k^0 a/D \), where \( k^0 \) is the standard rate constant, \( a \) is the tip radius and \( D \) is the diffusion coefficient. The \( k^0 \) values for all mismatch positions and fully matched ds-DNA obtained by fitting the experimental approach curves in the presence of Zn\(^{2+}\) are shown in the Figure 3.9. Significant differences between the rate constant values for the test samples were observed in presence of Zn\(^{2+}\). For the convenience of explanation, we can divide the whole figure into two different patterns of \( k^0 \) values. The first group containing fully matched, middle mismatch and extreme top or extreme bottom mismatches showed similar apparent rate constant values. In the second group, the mismatches at around position 5 and 18 provided similar electron transfer kinetics, which is significantly lower than that of the first group.
Mismatch positions

Figure 3. Variation of apparent rate constant ($k^0$) with respect to the position of mismatches in ds-DNA film. The error bar shows standard deviation of three individual experiments.

This pattern of rate constants can be rationalized by a structural variation of ds-DNA films due to the presence of mismatches at different positions. Mismatches of the second group yield the most distorted films, which enable better diffusion of the redox marker into the ds-DNA film, as is also evident from the EIS measurements described earlier. Since films containing mismatches at the extreme ends showed electron transfer kinetics similar to the fully matched ds-DNA, they might form similar film structure. This assumption is also supported by the EIS results discussed in previous section. Therefore, SECM provides a clear map of surface reactivity from the chemical imaging of the ds-DNA microarray to the assessment of surface reaction kinetics.
CONCLUSIONS AND FUTURE WORK

In summary, a systematic study of single-nucleotide mismatch positions in 25-mer ds-DNA films was carried out with EIS and SECM. Twenty five pre-hybridized ds-DNA films each of which has a single-mismatch sequentially from bottom to top with respect to the electrode surface were investigated. The approach takes advantage of the distinct differences of an anionic redox probe to diffuse in and out of a ds-DNA film, which translates into differences in the observed charge transfer resistance $R_{ct}$, which is influenced by the presence and position of nucleotide base mismatch and the presence of Zn$^{2+}$ ions. Using $\Delta R_{ct}$ as a sensing parameter, results presented here clearly show that a single-nucleotide mismatch, irrespective of its position, is easily distinguishable from fully matched ds-DNA films. All films containing mismatched DNA exhibit lower values of $\Delta R_{ct}$. The $\Delta R_{ct}$ values gradually decrease as the mismatch positions shift from the proximal end of ds-DNA film to about one forth of the DNA sequence, where the lowest $\Delta R_{ct}$ was obtained. After that, the $\Delta R_{ct}$ values start to increase again towards the middle mismatch position making a valley like trend. Another valley like change of $\Delta R_{ct}$ was observed for the mismatches at the second half of the ds-DNA films. Although most of the mismatch positions can be discriminated, the differences in $\Delta R_{ct}$ between mismatches were not distinguishable if the mismatch positions are extreme proximal or distal or in the middle. This pattern of changes was attributed to the different film structures and flexibility due to the presence of a mismatch at different positions. Results from the
SECM experiments give similar results and show clear differences in the amperometric feedback current as a function of mismatch positions due to changes in the electron transfer kinetics. To our knowledge this is the only systematic study where a comparison is performed between mismatches at all possible positions of a ds-DNA film. The work presented here indicates that in order to discriminate positions exactly, additional work will be necessary. For example, it was shown earlier that small molecule binding to mismatched DNA can be detected and enhances the electrochemical signature of the mismatch. Potentially, this is a strategy that would enable us to evaluate mismatches more accurately and this should be explored in more detail in the future.
REFERENCES


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APPENDIX

4.1. Preparation of the gold electrodes

The prepared gold electrodes are polished by following the procedure mentioned in experimental section and electrochemically cleaned before using them as a transducr surface of a DNA biosensor. The electrochemical cleaning was carried out by cyclic voltammetry (CV) in a 0.1 M solution of sulfuric acid and 0.05 M of potassium hydroxide, respectively. The cleanness of an electrode is confirmed by the characteristic anodic peak current near +1.2 V (vs. Ag/AgCl) and a single cathodic peak near +0.9 V in 0.1 M sulphuric acid solution. Figure 4.1 is a representative CV of a clean gold electrode in sulfuric acid.

![Figure 4.1. A representative cyclic voltammogram of a gold electrode in 0.1 M H2SO4 (0.5 V/s scan rate; Ag/AgCl reference electrode). The electrodes were cycled until a stable CV was obtained. The area of reduction peak is integrated to calculate the charge, the integrated area of reduction peak is used to calculate the surface roughness of the electrodes. CV was scanned from 0.0 V to 1.4 V.](image)
In sulfuric acid, a monolayer of chemisorbed oxygen is formed and reduced during each cycle. This oxygen formation in turn is corresponding (one-to-one correspondence) to the quantity of surface gold atoms. A value of $390 \pm 10 \ \mu C \ cm^{-2}$ has been experimentally determined for the reduction charge per microscopic unit area for polycrystalline gold.\textsuperscript{1} From a cyclic voltammogram recorded in 0.1 M $H_2SO_4$, the reduction charge can be calculated by integrating the reduction current peak. The reduction charge was then divided by $390 \ \mu C \ cm^{-2}$ to get surface areas of the microelectrodes.\textsuperscript{2} This area was divided by the theoretical surface area of the microelectrode, $1.98 \times 10^{-5} \ cm^2$ to obtain the roughness factor ($R_f$). The electrodes with $R_f$ values between 1.5-2 were chosen to be worked with. After electrochemical pre-treatment, the electrodes were immediately rinsed with a direct stream of ultrapure water, dried with nitrogen before incubating in ds-DNA.

4.2. Immobilization of DNA probe on gold surface

The formation of a recognition layer on a transducer surface with capture DNA strands is one of the most critical parts of DNA biosensor fabrication. The rate of hybridization at the transducer surface is directly related to the accessibility of the capture strands by the target DNA. Therefore, it is desirable to attach one end of the capture sequences covalently to the surface in order to give enough flexibility for binding with target sequences and help coping with the changes in their conformations.\textsuperscript{7} Gold is usually chosen as a transducer surface for several reasons. Being a noble metal, Gold can be handled in air without the formation of an oxide layer on the surface and can withstand
harsh chemical cleaning procedures such as those used to remove organic contaminants. Gold also provides well defined surfaces such as bulk gold, either polycrystalline or single crystal, or thin films deposited on various substrates. One of the most elegant approaches for making DNA recognition layers on gold surfaces is through the formation of thin films of DNA strands modified with sulfur containing groups, for example, thiols (R-SH), disulfides (R-S-S-R) and sulfides (R-S-R). A thin film is formed by spontaneous adsorption or chemical binding of sulfur containing groups of DNA molecules by aligning themselves by Van der Waals forces onto a substrate. The self-assembly of the sulfur containing organic compounds on variety of metals was first characterized by Nuzzo et al. For instances, the chemisorptions of the thiol containing compounds can react with gold according the following reaction:

\[ RSH + Au \leftrightarrow RS-Au + e^- + H^+ \]

As a result, a covalent bond is formed from the interaction of thiol with gold surface, which gives a robust and stable thin film of capture strand on gold substrate. It was reported that the strength of the metal-sulfur bond is on the order of 40-50 kcal mol\(^{-1}\). Furthermore, films prepared by this method are stable over the potential range between -0.8 and 1.4 (vs. Ag/AgCl), whereas outside this range capture strands are desorbed. To improve DNA hybridization, a mixed monolayer of thiol-DNA with a hexanethiol is usually used. The capture DNA sequences (usually a short chain oligonucleotide) can be modified with the asymmetric disulphide group at the 5' terminal of the sequence during the synthesis. When the synthesized hexanethiol containing disulphide linked DNA (eg, \(\text{OH-(CH}_2\text{k}_6\text{-S-S-(CH}_2\text{k}_6\text{-DNA)}\)) is exposed to clean gold surface, the S-S bond is broken and a mixed film is obtained. Herne, Tarlov and coworkers studied extensively the film
formation of thiol labeled single-stranded DNA on gold surfaces.\textsuperscript{8,9} They have described
that maximum hybridization efficiency can be obtained by creating mixed films which
actually controls the surface coverage of DNA on the surface.

4.3. Chronocoulometric determination of ds-DNA on gold surface

In chronocoulometric experiments, the integrated charge $Q$, is plotted as a
function of time $t$ in order to get quantitative information of surface bound redox probe
according to the integrated Cottrell expression,

$$Q = \frac{2nFAD_0^{1/2}C_0^*}{\pi^{1/2}} t^{1/2} + Q_{dl} + nFA\Gamma_0$$

where $n$ is the number of electrons per molecule for reduction, $F$ the Faraday constant
(C/equiv), $A$ the electrode area (cm$^2$), $D_0$ the diffusion coefficient (cm$^2$/s), $C_0^*$ the bulk
concentration (mol/cm$^2$), $Q_{dl}$ the capacitive charge (C), $nFA\Gamma_0$ the charge from the
reduction of $\Gamma_0$ (mol/cm$^2$) of adsorbed redox marker and $\Gamma_0$ is the amount of redox
marker confined near the electrode surface. The quantity of redox marker is determined
from the difference in chronocoulometric intercepts for the identical experiments in the
presence and absence of redox marker. ds-DNA surface density on the electrodes was
then calculated using the equation (2)\textsuperscript{10}

$$\Gamma_{\text{DNA}} = \Gamma_0 \left( \frac{z}{m} \right) (N_A)$$

Where $\Gamma_{\text{DNA}}$ is the surface density of the immobilized DNA on the surface of the
electrode (molecules/cm$^2$), $m$ the number of bases in the DNA strands, $z$ the charge of the
redox marker and $N_A$ is Avogadro's number.
4.4. Basics of EIS

In an ideally resistive electrical circuit, the relationship between voltage (E), current (I) and resistance (R) is governed by Ohm’s law:

\[ R = \frac{E}{I} \]

However, if an electrical circuit consists of capacitative (C) components along with the resistive component, in this case an alternating (AC) voltage is applied. In case of AC applied potential:

Excitation potential signal, \( E_t = E_0 \sin(\omega t) \)

Response current signal, \( I_t = I_0 \sin(\omega t + \phi) \)

Therefore, impedance, \( Z = \frac{E_t}{I_t} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \phi)} \)

Where, \( \omega \) is the excitation frequency and \( \phi \) is the phase shift. Electrochemical impedance is measured by applying an alternating (AC) voltage at a fixed potential to an electrochemical cell and measuring the current though the cell. EIS experiment involves the application of a sinusoidal electrochemical potential to the sample that covers a wide range of frequencies. This multi-frequency excitation allows the measurement of several electrochemical reactions that take place at different rates as well as the measurement of the capacitance of the electrode. The complex impedance is usually presented as a Nyquist plot obtained by plotting real impedance \( (Z_{re}) \) on x-axis and imaginary impedance \( (Z_{im}) \) on y-axis. Faradaic impedance measurements are commonly carried out in presence of a redox probe such as a mixture of Ferricyanide/ferrocyanide. To model the electrochemical properties of the redox probe, the experimental data is fitted with a Randle’s equivalent circuit. Randle’s equivalent circuit consists of an element
corresponding to the bulk solution resistance \( (R_s) \), double layer capacitance \( (C_{dl}) \), interfacial charge transfer resistance \( (R_{ct}) \) and a Warburg's impedance \( (Z_w) \) which represents the diffusion of the redox probes in solution. For EIS measurement, a conventional three electrode cell is used with a potentiostat having a frequency analyzer.

4.5 Basics of SECM

Scanning electrochemical microscope (SECM) is a type of instrument which belongs to the family of scanning probe microscopes. This technique is widely being used to collect information about the electrochemical activity of conducting, insulating and semiconducting surfaces dipped in solution. Scheme 4.1 shows a graphical diagram of an SECM which consists of mainly four parts: a small electrochemical cell, a potentiostat (an electrochemical measuring instrument), a high resolution positioning system, and a computer.

**Scheme 4.1.** Schematic representation of an SECM set-up. Probe electrode is moved on a microarray with the help of a positioner that can be controlled by software. The cell consists of three electrodes, working electrode (Pt), reference electrode (Ag/AgCl) and a counter electrode (Pt).

The sample of interest is usually mounted at the bottom of the cell. The working electrode of the cell is moved by the mechanically driven positioning elements with a
resolution as high as tenth of nanometers. The computer allows controlling the positioning and electrochemical data-acquisition system, and displays the SECM data. In SECM technique, the working electrode, commonly an ultramicroelectrode, is scanned in x, y, or z direction in close proximity to the surface to be investigated. Here, the steady state faradaic current at the working electrode near a surface immersed in an electrolytic solution is used to monitor the electrochemical nature and process at the surface. Since SECM can provide information on the local electrochemical activity of the substrate, this tool is very useful in addressing problems in the area of electrochemical biosensors.
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Description of Material: Figure 1
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