Exploring the Mechanism of the Electrostatic Denaturation of Double-Stranded DNA

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Exploring the Mechanism of the Electrostatic Denaturation of Double-Stranded DNA

A thesis presented to the faculty of the Department of Chemistry at the University of San Francisco in partial fulfillment of the requirements for a degree of

Master of Science in Chemistry

Written by

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January 2021
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Master of Science in Chemistry

From the

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Acknowledgements

First and foremost, I would like to thank Professor West for his patience and guidance in the duration of my time and research at the University of San Francisco. I learned a lot under his tutelage, and I will be forever grateful for the knowledge he gifted me.

I would also like to thank Professor Margerum. Without his excellent classes on various analytical methods and the importance of standard deviations and averages, my thesis would be sorely lacking in numerical value.

Much gratitude to the Professors that I TA’ed for, who made me a better teacher and impressed upon me the importance of imparting knowledge to the next generation to better the world. On that note, I’d like to give a shout-out to my students. You are all brighter than you give yourself credit for. I fully believe you’ll all go far. And of course, where would we TAs be without the absolutely amazing Angela! The number of times she’s gone above and beyond: providing data sets and grading sheets, keeping us stocked despite last-minute requests, and so on. She’s truly a lifesaver.

My sincere appreciation to Deidre Shymanski for all she has done. I’m about 99% convinced that our department would collapse without her hard work and dedication.

Huge thanks to Jeff Oda, who found us new water baths when the old ones finally gave out, who found us equipment and supplies whenever we ran out, and was all around the chillest dude to ever walk the halls of USF!

I would also like to thank my friends and fellow Chemistry graduate students for their moral support and camaraderie. I’m going to miss all we’ve experienced together, whether it’s grabbing drinks at the Pig’n’Whistle after class, or heading down to Emporium to test our skills at Killer Queen and Pacman, or borrowing a classroom to watch movies together. Andy, Chance, Eddie, Golbon, Hulda, Jeff, Marisa, Matt D., Matt F., Nathaniel, Rory, Ryan, Sara, Umy, I hope you’re all doing great out there and wish you the best in future endeavors.

Of course, I would be remiss to forget the undergraduates of West Lab! To Amanda, Farhad, Idea, Lucas, Namratha, Phaedra, Ziming, my profoundest thanks for your support. Couldn’t have done it without you!

In addition, I would like to thank the faculty and staff at the University of San Francisco for all their help and support.

Finally, I would like to thank the University of San Francisco for giving me the opportunity to explore this fascinating facet of electrochemistry, as well as for the excellent education they imparted to me. Thank you all so much for everything.
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Abstract

Electrostatic melting is an electrochemical tool that can be used to analyze the stability of the DNA double helix, allowing for the detection of various DNA mutations in double-stranded DNA (dsDNA) [1]. Here we explore the influence of the formation of the electrostatic double layer on the electrostatic unzipping of the DNA double helix to better comprehend the mechanism of this type of melting. Previous studies by our lab show that the electrostatic melting curve produced can distinguish between fully complementary 34-bp strands and a version of the same sequence in which one base pair has been replaced with a mismatch pair [2] or detect and characterize the crosslinking of the dsDNA by anticancer drug cisplatin [3]. Additionally, other papers by other research groups show that when the potential on the electrode is alternated between an attractive and repulsive potential over a range of frequencies, the ions in solution are unable to react quickly enough to form the double layer past frequencies higher than 10 kHz [4]. From this, we concluded that replacing the standard melting step with a fast potential pulse routine, which alternates between potentials above and below the threshold potential for melting, could give better insight into the mechanism of electrostatic DNA melting and its dependence on the formation of the double layer. The results of this research show that the time taken for melting to occur (τ) remained constant regardless of frequency. This implies that the mechanism is not wholly dependent on the generation of the electric field caused by the formation of the electrical double layer and that the melting mechanism is not purely electrostatic. Further testing proved that neither thermal melting nor probe desorption is responsible for the loss of signal used to indicate the quantity of DNA adhering to the electrode surface.
Chapter 1: Introduction to the Theoretical Basis for These Experiments

The purpose of this study was to determine the extent of the impact of double-layer formation on electrostatic DNA denaturation, also known as electrostatic melting or e-melting. Currently, e-melting can be used to ascertain the stability of a DNA double helix based on the rate and extent of melting caused by a sufficiently negative potential applied to a DNA modified electrode at temperatures where the dsDNA is otherwise stable. The electrostatic hypothesis assumes that the destabilization and melting of the dsDNA is caused by the extremely high electric fields generated at the negatively charged electrode immersed in electrolyte solution or buffer. By examining the mechanism of e-melting we hope to better understand and optimize this method of determining mismatches or other mutations in double-stranded DNA (dsDNA). Such a method could help to expedite the detection of genetic diseases and certain cancers caused by DNA mismatches, such as sickle-cell anemia, β-thalassemia, cystic fibrosis, and so on. Additionally, electrochemical methods have the potential to be cheaper than other methods, including spectroscopic methods of monitoring DNA melts, and can also be easily miniaturized and multiplexed, leading to smaller – and potentially less complicated – instruments for laboratory research, clinical studies, and possibly even point-of-care diagnostics [5].

This chapter will introduce the basic conceptual aspects relevant to e-melting, starting with basic DNA Biochemistry, with a focus on the structure and function of DNA. Following this, a summary of methods for detecting DNA, especially PCR and DNA biosensors.

DNA Biochemistry

Deoxyribonucleic acid (DNA) is the foundation of all life. The slightest changes in the makeup of DNA can be the difference between one species and another. As such, it is no surprise that DNA has held the scientific realm in fascination since its discovery in the late 1860s by Swiss chemist Friedrich Miescher [6].

As described in Modern Genetic Analysis by Griffiths et. al., DNA has three main purposes in the body. Firstly, every cell in the body has the exact same makeup, which means that the DNA needs to have the ability to be copied exactly with every cell division. Secondly, DNA must be able to carry precise instructions for every function of the body, not unlike a program’s code. Finally, DNA needs to be stable enough that it survives the duration of an organism’s life while also allowing for mutations to occur [7]. It is this last point that this thesis is focused on, or more specifically, that the possibility of DNA mutations
can lead to mismatches in double-stranded DNA (dsDNA), which can lead to a multitude of outcomes: beneficial mutations, harmless mutations, and detrimental mutations, the latter giving rise to various cancers and genetic diseases. As such, identifying DNA mismatches could potentially lead to a faster cancer detection method.

Figure 1 shows a DNA chain made from two polynucleotides. Each polynucleotide consists of a backbone of alternating phosphates and deoxyribose. Attached to each sugar is one of four nitrogenous bases—adenine (A), cytosine (C), guanine (G), and thymine (T). These nucleotides form the links between the 2 polynucleotide chains via hydrogen bonds between matching pairs of nucleotides. Guanine will only bond to cytosine and adenine will only bond to thymine. This is because the areas of potential hydrogen bonding will only align with this configuration. If thymine were to bond to guanine, the additional area of high electron density would destabilize whatever hydrogen bonds were able to form, forming a weak point in the overall structure of the DNA. This thesis hopes to discover a new method to detect the presence of this phenomenon, also known as a DNA mismatch.
Figure 1: Diagram illustrating a portion of DNA made by the author of this thesis using Inkscape. The phosphate backbone and various nucleotides are each highlighted and labeled. Dashed lines denote hydrogen bonds between each matched pair of nucleotides.

Additionally, since the phosphate backbone is hydrophilic and the nucleotide bases are relatively hydrophobic, when a single polynucleotide chain (ssDNA) hybridizes in aqueous solutions to form the paired set of 2 polynucleotide chains (dsDNA), the resulting “ribbon” tends to curl or twist such that the bases are semi-shielded from water by the hydrophilic backbone (see Figure 2). This shape is known as the DNA helix and exists in 3 main forms. Additionally, the phosphate backbone is negatively charged at neutral pH, which supports the current theory of electrostatic melting which assumes that the negatively charged backbone is repulsed by the charge on the electrode surface [8, 9]. This theory is the focus of this work.

Figure 2: Diagram illustrating three representations of the DNA double helix. [7]

There are 3 main forms of dsDNA: B-form, A-form, and Z-form. The first is the most frequent form, the B-form, which occurs at neutral pH and physiological salt concentrations and consists of a right-hand helix where there is a 3.4 Å rise per turn, 10 base pairs (bp) per turn, and a diameter of approximately 19 Å. The B-form also tends to curl in a manner akin to a ribbon coiling around a central shaft (see Figure 3). Similarly, A-form also has a diameter of 19 Å and forms a right-handed helix. However, A-form has a
2.56 Å rise per turn, with 11 bp per turn. It is also most prevalent in RNA-DNA and RNA-RNA structures. Additionally, A-form has the base pairs displaced from the central axis such that they are closer to the major groove. The resulting form resembles a ribbon-like helix with a more open cylindrical core as can be seen in Figure 4 [10].

**Figure 3:** Antiparallel (a), plectonemically coiled (b, c, d) B-form DNA strands. The arrows in (a) are pointed 3’ to 5’, but they illustrate the antiparallel nature of the duplex. The nucleotides arrayed in a 5’ to 3’ orientation on one strand align with complementary nucleotides in the 3’ to 5’ orientation of the opposite strand. [10].
Figure 4: B-form (left), A-form (middle) and Z-DNA (right). The top row shows the side view, the bottom row shows the view from the top [10].

Another difference between the A-form and the B-form is that the deoxyribose is in a different configuration for each form. For the B-form, the deoxyribose is in the C2' endo conformation, whereas it is in the C3' endo conformation in A-form (see Figure 5).
Figure 5: Syn- and anti-conformations of the base relative to the sugar in nucleotides. Left shows the configuration of the deoxyribose in B-DNA while right shows the configuration of the deoxyribose in A-DNA [10].

The final of the three main forms, Z-form DNA, has a radically different structure from the first two. For one, Z-form has a left-handed helical structure, a vertical rise of 19 Å per turn, has 12 bp per turn, and has a pronounced zigzag pattern in the phosphate backbone. The Z-form is predominantly present in DNA sequences with the bases alternate between a purine and a pyrimidine, such as when the sequence contains a repeating set of G and C [10]. To ensure that only one form of DNA is present, these experiments were run at a pH that is close to neutral.

Replication and Mutations

DNA is the blueprint for the vital proteins required for cellular and bodily function. As stated in *Molecular biology of the cell*, these proteins are necessary for a wide variety of functions, from chemical reaction catalysts to muscle generation to transportation within the cell [11]. Thus, if a protein is improperly made, it may lead to cellular or organism death. The most frequent reason for an erroneously made protein is a DNA mutation, usually caused by a DNA replication error [12], an overview of which will follow.

DNA replication is the process by which dsDNA is copied into 2 identical copies. Firstly, the dsDNA needs to already be unzipped for replication to occur. To ensure this, the enzyme helicase unwinds the dsDNA in front of the so-called replication fork, over a short region of approximately 15-20 bp [8, 11]. Helicase helps in opening the double helix and thus provides the appropriate single-stranded DNA template for the DNA polymerase to copy (see Figure 6).
The two strands of double-helical DNA run in opposite directions – one in the 5’ to 3’ direction (leading strand) and the other in the opposite direction (lagging strand). As such, continuous synthesis of two new strands at the replication fork would require that one strand be synthesized in one direction while the other is synthesized in the opposite direction [7, 11]. However, as Cooper [13] states, DNA polymerase catalyzes the polymerization of dNTPs (deoxyribonucleotide triphosphate, the term for the molecule shown in Figure 7, which is the building block for DNA) only in the 5’ to 3’ direction. As a result, the use of another enzyme – specifically single-strand DNA-binding (SSB) proteins, also called helix destabilizing proteins – is required. The SSB stabilizes and straightens the unwound ssDNA, which also prevents the formation of unwanted hairpin helices, the presence of which can impede DNA synthesis (see Figure 8).
Figure 7: Diagram showing the chemistry of the DNA polymerase reaction [14].

Figure 8: The effect of single-strand DNA-binding proteins (SSB proteins) on the structure of single-stranded DNA [11].
The second step is the process by which DNA polymerase synthesizes the complementary strand to the new template strands. An accessory protein acts as a regulated clamp, which keeps the DNA polymerase attached to the template ssDNA (see Figure 9). [11]

Figure 9: The regulated sliding clamp protein that holds DNA polymerase on the DNA [11].
These two steps happen simultaneously, thus these enzymes work as one single replication machine. Essentially, two DNA polymerase molecules work at the fork, one on the leading strand and one on the lagging strand. The dsDNA is opened by a DNA polymerase molecule clamped on the leading strand, acting jointly with one or more DNA helicase molecules running along the DNA in front of it, aided by cooperatively bound molecules of SSB. Whereas the DNA polymerase molecule on the leading strand can operate continuously, the DNA polymerase molecule on the lagging strand must restart at short intervals, using a short RNA primer made by a DNA primase molecule. On the lagging strand, the DNA replication machine leaves behind a series of unsealed Okazaki fragments, which still contain the RNA that primed their synthesis at their 5’ ends (see Figure 10). This RNA is removed, and the resulting gap is filled in by DNA repair enzymes that operate behind the replication fork [11].

**Polymerase Chain Reactions**
Polymerase Chain Reaction (PCR) is a method developed by Kary Mullis in 1983, based on the ability of DNA polymerase to synthesize new strands of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer. This requirement makes it possible to demarcate a specific region of the template sequence that the researcher wants to duplicate. PCR results in billions of copies of the sequence and is currently widely used by clinicians and researchers to diagnose diseases, clone and sequence genes, and carry out quantitative and genomic studies rapidly and with a high degree of sensitivity [15].

First, the sample DNA is subjected to an elevated temperature in order to separate the strands from each other (denaturation). Then, the temperature is lowered, and the DNA polymerase synthesizes the complementary strands for each strand of the original dsDNA in the form of primers (annealing). In the third step, the temperature raises once more so that polymerase can extend the primers (extension), and the cycle is brought to completion. To continue this cycle, the temperature returns to the denaturation
temperature and begins again at the first step. With each step, the dsDNA doubles (see Figure 11) [15], [16].

Qualitative PCR (qPCR) describes a method to detect the presence or absence of a specific DNA product using PCR. In the same vein, quantitative real-time PCR (qRT-PCR) provides information on how much of a specific DNA or gene is present in the sample. qRT-PCR often uses two types of chemical signals to highlight the desired molecule: fluorescent dyes that non-specifically intercalate with double-stranded DNA; and fluorescently labeled sequence-specific DNA. The latter permits detection only after hybridization of the probe with its complementary DNA target. West lab’s research utilizes fluorescently labeled sequence-specific DNA in the form of 18 base pair (18 bp) probe ssDNA hybridized with methylene blue tagged target ssDNA. An example of a colorimetric qPCR-based biosensor comes from Wang et. al, which was based upon a triplex DNA intercalator [17]. Wang et. al. used Ru(phen)₂dppx²⁺ (where phen = 1,10-phenoline, dppx = 7,8-dimethyldipyrido[3,2-a:2',3'-c]phenazine) to convert DNA modified gold nanoparticles to triplex stranded DNA, then used the presence of these triplexes to detect and quantify the previously difficult-to-detect dsDNA [17].

DNA sensing and biosensors

PCR is widely considered the most reliable method for DNA sequence detection, yet it has a few downsides. PCR is more sensitive compared to cultures and staining, can test for anti-microbial resistance, and has an increased ability to detect less common organisms such as viruses. However, PCR has some detriments: PCR has a lower specificity compared to cultures and staining, which implies an increased risk for false positives; is relatively slow as compared to enzyme immunoassays; requires specialized equipment and training; and is prone to airborne contamination [18]. Understandably, some situations require a faster, cheaper, more compact method of DNA sensing without sacrificing the reliability and accuracy of the results of such methods. In hopes of fulfilling such a requirement, our lab turned to a subset of electrochemical sensors: DNA biosensors.

What is an Electrochemical Sensor?

According to Janata [19] in Principles of Chemical Sensors, chemical sensing is a process in which an amplified electrical signal results from the interaction between a target chemical species and the sensor and consists of two main steps: recognition and amplification. In most cases, recognition is provided by chemical interactions, while amplification must be provided by some physical transducer. [19] Electrochemical sensors specifically refer to a subcategory of chemical sensors. Electrochemistry involves
the transfer of charge from the electrode to another phase, either solid or liquid. During this process, chemical changes take place at the electrode, and charge is conducted throughout the bulk of the sample. Both the electrode reactions and the charge transport can be chemically modulated and thus serve as the basis of the sensing process. [19]

What is a Biosensor?

A biosensor is a chemical sensor that measures biological or chemical reactions by generating signals proportional to the concentration of a biological analyte in the reaction [20]. Biosensors are a somewhat recent discovery of the scientific world, given that the first ‘true’ biosensor was created in 1959 by Leland C. Clark – one of the ‘founding fathers of biosensors’ – with his invention of the oxygen electrode [21]. This was later utilized to create the first commercial biosensor, an enzyme-based biosensor, which could measure the glucose levels in the blood, in 1975 [22]. This biosensor consisted of glucose oxidase immobilized in polyacrylamide gel and the Clark oxygen sensor. The enzyme reaction is shown below:

\[
\text{Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}
\]

(1)

The decrease in oxygen reduction current measured with the Clark sensor was proportional to the concentration of glucose. [23]

The field has expanded to the point where it covers multiple disciplines from basic sciences to nanotechnology, electronics, and applicatory medicine and has only continued to grow since its inception. Nowadays, biosensors are used in water quality management, environmental monitoring, prosthetic devices, disease detection, and many other fields [20]. One example is the utilization of the pH-sensitive response of oxide-semiconductor interfaces to detect and monitor kinase activity published by Bhalla et. al. [24]. It is our hope that the work described in this thesis contributes to this rich history.

Biosensors are made up of several key components, which are shown below in Figure 12.
Figure 12: Diagram illustrating the typical components of an electrochemical biosensor [25].

As can be seen in Figure 12, the most common components of an archetypal biosensor include the following:

- **Analyte**: This is the target substance for which quantitative data is desired.
- **Bioreceptor**: This is the molecule that displays a response to the presence of the analyte. This response is usually in the form of a chemical reaction between the analyte and the bioreceptor.
- **Transducer**: A transducer will convert the response of the biosensor into a measurable form that can then be analyzed quantifiably. For example, a change in H\(^+\) ions would be converted into a pH change or a change in energy might be translated into a change in temperature.
- **Electronics & Display**: These last two can be considered a single entity, as their entire purpose is to display the collected data in a way that is user-friendly and matches user requirements, whether that is to produce a table, graph, numerical values, or other such outputs.

**Types of biosensors**

A literature review [25] reveals that there are many biosensor types to consider: optical, acoustic, thermometric, magnetic, immunosensors, and – most importantly for this thesis – electrochemical [20, 25]. Optical sensors are the most common type of biosensor and measure the interaction of an optical field with a biorecognition sensing element, with either measuring changes in light adsorption or measuring light intensity being the ubiquitous methods used. Acoustic biosensors measure the change in the physical
properties of an acoustic wave. Thermometric biosensors measure the temperature change of the solution containing the analyte, typically caused by enzymatic reactions. Magnetic biosensors measure changes in magnetic properties, or magnetically induced effects. Immunosensors are biosensors in which an immunochemical reaction is coupled to a transducer [25, 26]. Finally, electrochemical sensors react with the desired analyte in a way that results in the production of an electrical signal that is proportional to the analyte concentration. They can either be potentiometric – where the sensor measures variations in open circuit potential – or amperometric/voltammetric – where the sensor measures currents generated by the redox reactions of an electroactive species [26].

The biosensors used in this thesis can be classified as both affinity sensors and electrochemical sensors. Affinity biosensors are composed of a biological recognition element that responds to a specific analyte thus producing a measurable electronic signal proportionate to the concentration of said analyte. Some examples of such recognition elements include an antibody, receptor protein, biomimetic material, or DNA interfaced to a signal transducer [27]. In the case of this thesis, the recognition element (bioreceptor) is single-stranded tagged DNA (probe DNA) bound to the electrode surface via thiol bonds. Upon hybridization with complementary DNA tagged with Methylene Blue (target DNA), a change in electrical current is detected by voltammetry. This thesis will be utilizing this type of sensor to detect the change in the concentration of DNA adhered to the electrode surface during e-melts.
Chapter 2: Electrochemical Concepts

A sizable portion of the study of electrochemistry involves the examination of chemical changes induced by an electrical current through the medium being analyzed as well as the electrical energy produced by chemical reactions. Such a field can be utilized for a variety of purposes, including the determination of thermodynamic data of a reaction, the analysis of a solution for trace amounts of metal ions or organic substances, and even the design of new energy sources for public and commercial use. The focus of this thesis will be electrochemical cells, a subset of electrochemistry that focuses on redox-reaction-driven electron transfer.

What is an electrochemical cell?

An electrochemical cell is defined as two electrodes separated by at least one electrolyte phase. An electrode is a point at which current enters or leaves an electrolyte (a substance that gives ions when dissolved in a solution). Additionally, the measurement of electric potential in an electrochemical cell is truthfully the measurement of the difference between two electrodes, rather than the absolute value [28]. In an electrochemical process, electrons flow from one chemical substance to another, driven by the difference in half-cells containing reduction-oxidation reactions (redox reactions), where electrons are transferred from one substance to another. The molecule that loses electrons is oxidized and is known as the reducing agent while the species that gains electrons is known as the oxidizing agent and is reduced in the process. While most chemical reactions require direct contact between all reactants, in redox reactions, as long as each species is part of a continuous circuit, the redox reaction can occur [29].

There are two main types of electrochemical cells: galvanic cells and electrolytic cells (see Figure 13). Galvanic cells describe electrochemical cells where a redox reaction is used to produce an electric current, one example being lithium-ion batteries. Electrolytic cells, on the other hand, are electrochemical cells that employ an external power source to induce a non-spontaneous redox reaction. Electrolytic cells are used to produce hydrogen gas from water, electroplating, and many similar purposes [28, 29]. Our research employs electrolytic cells to induce a signal via methylene-blue tagged dsDNA, where methylene blue is redox capable within the voltage range of these experiments.
One common concept between both the galvanic cell and the electrolytic cell is that of electrochemical equilibrium. In a typical electrochemical cell, the half-reaction that occurs at one electrode produces electrons that are consumed in the half-reaction of the other electrode. Take for example the cell shown in Figure 14.
Figure 14: (a) Diagram of the reaction of a Zinc electrode with aqueous Copper (II) ions in a galvanic cell. (b) The result of (a), showing that the Zn anode loses mass over time while the Copper cathode concurrently gains mass [29].

This galvanic cell consists of a copper strip in a beaker containing an aqueous 1 M solution of Cu\textsuperscript{2+} ions and a zinc strip in a different beaker containing an aqueous 1 M solution of Zn\textsuperscript{2+} ions. The two metal strips are connected by a wire that allows electricity to flow, or, as in this case, allows potential to be measured across a voltmeter. The beakers are connected by a NaCl salt bridge to complete the circuit. When the circuit is completed, the zinc electrode (anode) oxidizes to Zn\textsuperscript{2+} in the left beaker, while in the right beaker Cu\textsuperscript{2+} ions are simultaneously reduced to copper metal at the copper electrode (cathode). As the reaction progresses, the anode loses mass as it dissolves to give Zn\textsuperscript{2+}(aq) ions, while the Cu cathode gains mass as Cu\textsuperscript{2+}(aq) ions are reduced and deposited on the cathode [29, 30]. The half-reactions governing this reaction are shown below:

\[ \text{Zn}(s) \rightarrow \text{Zn}^{2+}(aq) + 2e^- \quad (2) \]

\[ \text{Cu}^{2+}(aq) + 2e^- \rightarrow \text{Cu}(s) \quad (3) \]

Equation 2 is the half-reaction that occurs in the left beaker, while equation 3 occurs in the right beaker. As can be seen in equations 2 and 3, the anode produces 2 electrons per atom of zinc, while the
cathode consumes 2 electrons per atom of copper that is reduced. The half-reactions can be re-written in the following form:

$$Zn_{(s)} | Zn^{2+}_{(aq, 1M)} || Cu^{2+}_{(aq, 1M)} | Cu_{(s)}$$  \hspace{1cm} (4)

In this consolidated form, \(Zn_{(s)}\) represents the anode, the \(Zn^{2+}_{(aq, 1M)}\) represents the solution of the anode (1M concentration), \(Cu^{2+}_{(aq, 1M)}\) represents the solution of the cathode (1M concentration) and \(Cu_{(s)}\) represents the cathode. The single dash line represents a phase change and the double dash lines represent a salt bridge connection.

Under standard conditions, the cell potential can be represented by the following equation:

$$E^0 = E_{reduction}^0 - E_{oxidation}^0$$  \hspace{1cm} (5)

In this equation, \(E_{reduction}^0\) represents the electrode potential at the cathode and \(E_{oxidation}^0\) represents the electrode potential at the anode.

Thermodynamically, \(E^0\) is related to \(\Delta G^0\) by Equation 6, where \(\Delta G^0\) is the Gibbs free energy at standard conditions, \(n\) is the number of electrons transferred in the balanced redox equation, \(F\) is Faraday’s constant, and \(E^0\) is the potential difference at standard conditions described in Equation 6, as shown below. If \(E^0 > 0\), the reaction is spontaneous, whereas the reverse indicates that the reaction is non-spontaneous.

$$\Delta G^0 = -nFE_{cell}^0$$  \hspace{1cm} (6)

Additionally, \(\Delta G^0\) can be related to the equilibrium constant \(K\) by the following equation:

$$\Delta G^0 = -RT \ln (K)$$  \hspace{1cm} (7)

By substituting Equation 6 into Equation 7, Equation 8 can be derived:

$$E_{cell}^0 = \left(\frac{RT}{nF}\right) \ln(K)$$  \hspace{1cm} (8)

where \(R\) is a constant of 8.314 J/mol K [30]. If the cell is under non-standard conditions, then \(\Delta G\) can be derived from the following equation:
\[ \Delta G = \Delta G^0 + RT \ln (Q) \]  

(9)

where Q is the reaction quotient. Under the same non-standard conditions, equation 6 can be rewritten as:

\[ \Delta G = -nF E_{cell} \]  

(10)

Equations 6, 9, and 10 can be combined to give the following:

\[ -nF E_{cell} = -nF E_{cell}^0 + RT \ln (Q) \]  

(11)

Equation 11 is known as the Nernst equation, after the German physicist and chemist who first derived it, Walter Nernst. When a redox reaction reaches equilibrium, \( \Delta G = 0 \) and \( Q=K \), then \( E_{cell}=0 \). Knowing this, Equation 11 can be rewritten as Equation 12:

\[ E_{cell}^0 = \left( \frac{RT}{nF} \right) \ln (K) \]  

(12)

If \( E^0 \) is positive, then \( K \) is greater than 1 and the reaction favors the products. If \( E^0 \) is negative, then the opposite is true [31].

Nowadays, it is more common to use a 3-electrode cell (see Figure 15) for electrochemical research. In a typical example of such a cell, there are 3 components: the working electrode, the counter electrode, and the reference electrode. The overall chemical reaction taking place in a cell consists of two independent half-reactions, as in the previous example, which describes the chemical changes occurring at each electrode. Customarily, only one of these reactions is of interest and the electrode at which this reaction transpires is referred to as the working electrode. Our lab uses gold electrodes as the working electrodes in our research. The other half of the cell, at which the other reaction ensues, is often standardized using a reference electrode, which, in our research, is a silver-silver chloride electrode (Ag/AgCl) with a potential of approximately 0.197V versus a standard hydrogen electrode (SHE) [28]. Any potentials mentioned in this thesis from this point onwards should be assumed to be versus Ag/AgCl unless stated otherwise. The half-reaction for Ag/AgCl electrodes is shown below:

\[ AgCl(s) + e^- \rightarrow Ag(s) + Cl(aq) \]  

(13)
Finally, the counter electrode (also known as the auxiliary electrode) is an electrode chosen specifically since its electrochemical properties do not affect the behavior of the working electrode. This allows for the potential of the working electrode to be measured against the reference electrode without compromising the stability of the reference electrode [28]. In this thesis, the counter electrode is a Platinum (Pt) electrode.

![Circuit diagram of three-electrode cell assembly.](image)

**Figure 15:** Circuit diagram of three-electrode cell assembly. Our research uses an Ag/AgCl reference electrode instead of the calomel reference electrode shown in the diagram. [32]

**Faradaic and Nonfaradaic Processes**

There are two types of processes that can occur at electrodes. Thus far we have been discussing the first, in which electrons are transferred across the metal-solution interface and cause oxidation or reduction. Such reactions are governed by Faraday’s law, specifically that the amount of chemical reaction caused by the flow of current is proportional to the amount of electricity passed, and are thus known as faradaic processes. Contrarily, nonfaradaic processes refer to processes such as adsorption and desorption, which can occur at the electrode surface without the transfer of electrons, thus changing the structure of the electrode-surface interface and causing changes in potential or solution composition. Despite no charge transfer across the interface, there can still be current flow occurring. Both faradaic and nonfaradaic processes occur when electrode reactions take place. As such, both must be considered when utilizing electrochemical methods to analyze electrostatic DNA denaturation.
Helmholtz Planes and Shielding

The electrode-solution interface has been shown to behave much like a capacitor. At a given potential, there will be a charge on the metal \( q^M \) and a charge in the solution \( q^S \). At all times, the following is true [28]:

\[
q^M = -q^S
\]  

(14)

The charges \( q^M \) and \( q^S \) are often expressed in the form shown below:

\[
\sigma^M = \frac{q^M}{A}
\]  

(15)

In equation 7, \( \sigma^M \) is the electrode charge density and \( A \) is the surface area of the electrode [28]. The interface between the electrode and the solution can be imagined as shown in Figure 16.

**Figure 16:** Proposed model of the Schematic Stern–Gouy–Chapman representation of a negatively charged metal–electrolyte solution interface [33].
The inner Helmholtz plane (IHP), also referred to as compact or Stern layer, corresponds to the layer of specifically adsorbed ions, whereas the outer Helmholtz plane corresponds to the plane of the closest of the solvated ions. The interaction of the solvated ions with the charged metal only involves long-range electrostatic forces, so this interaction is essentially independent of the chemical properties of the ions. Beyond the OHP is what is called the diffuse layer, the charge density of which is $\sigma^d$. Similarly, the charge density for the IHP is $\sigma^i$. The combination of the metal layer and solution layer is collectively called the double layer. The total excess charge density on the solution is given by the following [28, 34]:

$$-\sigma^M = \sigma^i + \sigma^d = \sigma^S$$  \hspace{1cm} (16)

The thickness of the diffuse layer depends on the ionic concentration of the solution [28].

**Electrochemical Techniques**

Our experiments using electrolytic cells are controlled by an external power source or potentiostat. A potentiostat is an instrument that controls the voltage between the working electrode and the reference electrode. The resulting current flowing between the working and counter electrodes is recorded and analyzed [28]. There are several common techniques that a potentiostat can employ, including chronoamperometry, cyclic voltammetry, square wave voltammetry, and fast potential pulse voltammetry [34].

**Chronoamperometry**

Chronoamperometry describes an experiment in which current is measured as a function of time. This thesis uses a form of chronoamperometry called Double Potential Step Chronoamperometry [35]. A graphical representation of this process is shown in Figure 17.
To illustrate this technique, imagine a solid electrode immersed in a redox solution at some known initial concentration. Initially, the electrode is set at a potential (E<sub>i</sub> on Figure 17) that is more positive than the formal potential (E<sup>0'</sup> on Figure 17) for the redox couple, such that only the oxidized form of the redox couple is present in the solution. The solution also contains an excess of inert electrolyte. At time t₀, the potential is stepped to a value significantly more negative than the E<sup>0'</sup> for the redox couple (E<sub>s</sub> on Figure 17), where the oxidized form of the redox couple closest to the surface immediately converts to the reduced form. After a set amount of time has passed, the potential is brought back to E<sub>i</sub> (E<sub>f</sub> on Figure 17) [35].

Chronoamperometry can be used to measure the area of an electrode, the number of electrons involved in the reaction, concentration of the electroactive species, and diffusion constant for the electroactive species [34, 35].
Cyclic Voltammetry

Figure 18: (Top) Graph of the potential step for cyclic voltammetry. (Bottom) Graph of resulting cyclic voltammogram that the top graph would produce for a redox couple in solution [36].

Cyclic Voltammetry (CV) involves the potential being incrementally increased from $E_i$ to $E_s$ and brought smoothly back to $E_i$ (see Figure 18 top image) [28]. Cyclic voltammetry can be used to investigate the redox processes of molecular species. The resulting graph (see Figure 18 bottom image) resembles a duck and can also be used to study electron-transfer initiated chemical reactions [37].
Figure 19: Diagram showing that the scanning waveform of square wave voltammetry comprises of the sum of a simple symmetrical square wave and a staircase wave of the same phase and frequency [38].

Square Wave Voltammetry is performed by superimposing a potential pulse wave form composed of a symmetrical square wave onto a base staircase potential wave form [39, 40] (see Figure 19). The resulting wave form is applied to the working electrode and results in the bell-shaped curve known as a square wave voltammogram (SWV) shown in Figure 20 below.
Figure 20: Square Wave Voltammetry measurements of bare Au electrodes (solid), Lpa-GGH grafted electrode (dash), and Lpa-GGH layer exposure to 10 μM Cu$^{2+}$ solution (dot) [41].

SWV is especially beneficial since this technique has minimal nonfaradaic currents and offers background suppression, slightly greater sensitivity than differential pulse voltammetry, fast scan times and is applicable to a wide variety of electrode materials and systems. SWV is also better for evaluating quantitative parameters for systems that are understood mechanistically and for analyzing systems involving lower concentrations than CV [28].

Fast Potential Pulse Voltammetry

Finally, Fast Potential Pulse Voltammetry (FPP) involves the rapid alternation between two potentials, where the system stays at each potential for a given amount of time (see Figure 21). This is what that we plan on replacing the traditional melt step with and will be discussed further in the experimental chapter. Fast pulses allow for the monitoring of homogeneous reactions as the oxidation potential of both reactants is reached almost at the same time [42].
Figure 21: Example Fast Potential Pulse routine spanning a range of -0.1V to -0.5V with a time period of 10 seconds (Made by author).
Chapter 3: Electrostatic DNA Melting Until Now

Traditional DNA Melts

As stated Levicky et. al. [43], thermal denaturation, or melting, measurements are a classic technique for analysis of thermodynamics of nucleic base driven associations in solution, as well as of interactions between nucleic acids and small molecule ligands such as drugs or carcinogens. As the temperature at which DNA melting occurs is dependent on several factors: the length of DNA; the nucleotide sequence composition; salt concentration of the buffer in which the melting is taking place; and so on, the rate at which DNA melts reveals a lot of information on the stability of the structure as well as its makeup [44]. The most common DNA melting technique is dependent on temperature-induced DNA instability. Krishnan et. al. [45] found that the melting profile can be monitored by optical techniques such as absorption and fluorescence microscopy. Interactions among stacked bases cause a decrease in UV absorption, thus melting of dsDNA at elevated temperatures, which involves breaking the hydrogen bonds of the base pairs and decreasing base stacking, results in an increase in UV absorption, which can then be measured with a spectrophotometer [45]. DNA denaturation (another term for DNA melting) is often induced by an increase in temperature and this process is commonly monitored via UV-Vis spectroscopy, fluorescence spectroscopy, or Raman spectroscopy, as exemplified by the work of Duguid and his colleagues, who utilized Raman Spectroscopy and calorimetric methods to monitor the thermal denaturation of fragments of 160 bp double-stranded calf thymus DNA in its B form [46]. However, Raman spectroscopy requires specialized instrumentation and tends to be relatively expensive, as are other spectroscopy methods. Additionally, since the focus of these studies was still on thermal melting – which utilizes an increase in temperature to break the bonds keeping the helix together - the electrostatic (isothermal) mechanism of DNA denaturation remained relatively unknown.

Bartlett and Fully Electrostatic DNA Melts

Electrostatically induced DNA melts were first reported in 1974 on mercury drop electrodes and studied extensively by the laboratories of Paleček and Nurnberg [9]. Following studies of electrochemically induced denaturation observed on graphite, gold (Au), and platinum (Pt) electrodes, It was discovered that e-melting is dependent on the stability of the DNA duplex and could be used to discriminate mismatched DNA from fully complementary sequences [9]. Then, in 2008, Bartlett et. al. reported a new method of detecting genetic variations in DNA through the use of Raman Spectroscopy and electrostatic melting. This
was achieved by attaching small dye molecules, such as methylene blue, acridine orange, or DAPI, which bind to DNA by electrostatic interactions, intercalation, or a minor-groove interaction. Bartlett et. al. focused on molecules that bind exclusively via electrostatic interactions with DNA to ensure that the binding agent (the dye) was selective for dsDNA [47].

Prior to Bartlett’s work, methods for identifying mutations tended to use either solution-based or surface methods. Solution-based methods included single-strand conformational polymorphism identification, the use of denaturing gradient gel electrophoresis, or quantitative polymerase chain reaction (qPCR)-based approaches. The latter did not require a chromatographic separation step and could be used to detect several sequences simultaneously. The most widely used methods for monitoring DNA melting are based on fluorescent detection schemes such as those used in Molecular Beacons, Taqman, Scorpions, or Hybridization Probes [1]. Other sensing approaches included the measurement of mass changes using a quartz crystal microbalance [48], nano-mechanical detection using microcantilevers [49], local refractive index changes using surface plasmon resonance [50], electrochemical changes using either impedance measurements or redox labels [51, 52], and fluorescence [53].

In a typical mutation detection experiment, the preferred method is to utilize fluorescence spectroscopy to monitor the melt. This is mainly done by immobilizing probe-ssDNA to the surface, then hybridizing the immobilized probe with target-ssDNA. The melt is then observed via fluorescence, though alternate methods have included optical wave guides [54], optical scan arrays [55], and temperature gradient assay platforms [1, 56]. Bartlett’s 2008 paper, conversely, employs surface-enhanced Raman scattering (SERS) combined with either thermal or electrochemical cycling to determine the presence of mutations in the human CFTR gene, and specifically differentiate between the presence of a single nucleotide mutation, a more common triplet deletion, and the wild type (non-mutated version). Additionally, to prove the veracity of this technique, Bartlett assessed it against samples of CFTR DNA that had been produced via asymmetric PCR and thus contained an unknown amount of triplet deletion CFTR [1].

In 2012, Bartlett further improved the SERS-based approach by introducing a method for label-free DNA mutation detection. At this point, label-free detection was of great interest to the scientific community, with a particular focus on the employment of impedance spectroscopy to achieve this. However, impedance spectroscopy has difficulty producing reliable and reproducible results when considering minor changes in environmental conditions. Other label-free biosensors were developed at this time, but few were capable of discriminating between mutation types [47]. For example, Nasef et al.’s work in Labelless electrochemical melting curve analysis for rapid mutation detection [57] established that differential pulse voltammetry combined with thermal denaturation of DNA discriminated between a perfectly complementary DNA sequence and a sequence containing a mismatch, especially when used with DNA that had been altered such that the target strand was non-covalently bound to methylene blue, a
compound that can undergo redox reactions to produce a signal [57]. However, while a single specific mutation could be discriminated from a fully complementary dsDNA, these experiments failed to show flexibility for situations beyond this explicit set of circumstances [47]. To counter this disadvantage, Bartlett suggested that since electrochemical melting discriminates between sequences based on the stability of the duplex, the label-free method that he was introducing could be applied to a wide range of targets of interest, thus suggesting that such a method had potential for integration into a point-of-care device. Bartlett’s method used DNA binding agents to selectively detect dsDNA at a SERS substrate without requiring prior synthetic modification of the detection target [47].

Bartlett’s melts also operated at a much more negative voltage than our lab (-200mV to -1600mV). Bartlett avoided unwanted desorption by using a disulfide linker which allowed for 6 thiol-gold bonds to form, thus binding the DNA to the electrode surface with six times the strength compared to my experiments [47]. Our lab was unable to employ a similar technique due to financial restraints and as such needed to determine the appropriate voltage required to ensure melting would occur while minimizing thiol desorption to the greatest extent. Denny Ho, a previous graduate student of West lab, explored the effect of the melting potential of a traditional melt on thiol desorption [2], and we continued similar experiments to ascertain the effect of FPP routine on probe desorption.

Another way that our experiments differ from Bartlett’s is the method of data acquisition. Bartlett’s work was focused on measuring optical signals via SERS against a scanning range of potentials to extract thermodynamic data to analyze the melts [47], [1]. However, our lab compares the peak heights of the obtained SWVs at regular intervals and constant potential in order to extract both kinetic and thermodynamic data in the form of $\Delta$, the extent of melting, and $\tau$, the time taken to melt. As such, the focus of this thesis diverges from that of Bartlett’s work.

**Previous Experiments from West Lab**

As previously mentioned, our lab explored the capabilities of purely electrostatic methods of monitoring the stability of dsDNA and detecting the presence of mismatches in dsDNA. For example, Denny Ho’s work was focused on establishing the ability of electrochemical analysis to monitor e-melting of dsDNA [2]. His work presented a method for the simultaneous melting and monitoring of surface-bound dsDNA using a purely electrochemical approach via a sequence of potential pulses to propagate the melt and SWVs taken at set intervals between pulses to monitor the signal strength provided by methylene blue moieties attached to the target strand of the dsDNA being examined. This allowed both e-melting and detection to be conducted without the need for parallel spectroscopic monitoring. Ho used the same low ionic strength buffer as my experiments to minimize counterion screening and maximize the Debye length.
in the electrical double-layer (approximately 3 nm in low ionic strength buffers). However, his electrodes were prepared using the “passive backfill method”, and his pulse routines varied from mine (see Figure 22) [2].

Figure 22: Schematic of the electrochemical melting routines used in this work. Each potential pulse is held for a fixed pulse time, tp. After each pulse, the potential returns to an equilibration potential of −0.1 V for 10 s before a square wave voltammogram is acquired. During an e-melting experiment, the pulse potentials either (a-b) decrease throughout the routine or (c-d) remain constant for the duration of the melt. Zoomed-in snapshots of the melting routines for (a) and (c) are shown in (b) and (d), respectively [2].
Ho’s experiments can be classified into three sections. First, he acquired scanning potential melts to investigate the effects of pulse potential on the electrochemical melting of DNA duplexes. He obtained this information by varying the pulse potential from $-0.1$ V to $-1.0$ V in 10 mV steps per pulse for the total duration of 480 seconds before returning to an equilibrium voltage of $-0.1$ V and performing a SWV, as illustrated in Figure 22(a). The peak heights of each of the baseline-subtracted SWVs were normalized to the initial peak current, plotted versus the pulse potential, and fitted to equation 17, resulting in the melting curve displayed in Figure 23(b), which portrays scanning potential melts at different step times. From 0 to $-400$ mV, no loss in signal is observed [2].

\[ i_{\text{peak}} = \Delta e^{-\frac{t}{\tau}} + (1 - \Delta) \]  

(17)

Figure 23: Square wave voltammograms and e-melting curves using a scanning potential: (a) Overlay of baseline-subtracted square wave voltammograms acquired during scanning potential pulse routine on electrode modified with 34-bp fc dsDNA hybridized at 55°C. The arrow indicates the direction of progressively more negative pulse potentials. (b) Normalized peak currents of the SWVs in plotted versus the pulse potential for pulse times of 480 s (circle, purple), 60 s (squares, blue), and 5 s (diamond, green). Each data point is proportional to the amount of MB-tagged target still present at the electrode surface after a pulse of the given potential. Solid lines are sigmoidal fits and the inset shows 1st derivative of sigmoidal fits vs potential [2].

The melting potentials were $-570 \pm 30$ mV for a pulse time of 480 s (Figure 23(b), purple circles), $-680 \pm 50$ mV for a pulse time of 60 s (Figure 23(b), blue squares), and $-750 \pm 50$ mV for a pulse time of 5 s (Figure 23(b), green diamonds). For pulse times greater than 480 s, no additional positive shifts in the
melting curves were observed [2]. These results set the standard for future research with melt steps to last 480 s to ensure that the DNA had enough time to fully melt and diffuse away.

Ho’s next set of experiments determined the feasibility of e-melting to identify mismatches in dsDNA. Scanning potential melting curves were obtained for 34-bp duplexes with a single mismatch at the 13th base pair from the electrode surface (referred to as mis13 dsDNA or mismatch duplex) and an 18-bp fc duplex. Ho hypothesized that if melting potential directly correlates to thermodynamic stability, then the single mismatch duplex and the 18-bp duplex should melt at less negative potentials than the 34-bp fc duplex. Using a pulse time of 480 seconds, the scanning potential melting curve for each duplex was obtained and are compared in Figure 24. The melting potential of the mismatched 34-bp duplex was −550 mV, compared to a melting potential of −570 mV for the fully complementary 34-bp duplex (see Figure 24). Ho determined that e-melting amplified the difference in melt curve comparable to a small difference in melting temperature, thus proving that e-melting is more capable of determining the presence of a mismatch compared to a thermal melt [2]. This thesis will go into more detail about this discovery in the next chapter.

Figure 24: Melting potentials for the three duplexes used in this work. The error bars show ±1 standard deviation from triplicate measurements. [2].
Finally, Ho’s work utilized a constant pulse routine to determine the ideal parameters to maximize the quality of melt data obtained. First, the probe is adhered to the electrode surface along with the mercaptohexanol pacification layer. Second, the probe hybridizes with target strands to form dsDNA. A negative potential is then applied causing repulsion between the negatively charged phosphate backbone of the DNA and the negative potential on the electrode, which causes the DNA helix to unzip. Ideally, the probe strand stays secured to the gold surface via the thiol bond and only the target sequence and attached MB group will drift away into the surrounding solution, resulting in a decrease in the signal as the MB would no longer be close enough to the electrode surface to undergo redox reactions. This process of unzipping and removal of the target via the application of a destabilizing negative potential is called electrostatic denaturation. SWVs are taken every eight minutes and used to monitor the MB signal decay. The peak height of each SWV is calculated and plotted versus time to form the resulting curve of signal decay (see Figure 25).

![Figure 25](image_url)

**Figure 25:** (Left) Overlaid SWV taken in the process of a melt. The highest peak is from the first SWV, with each following SWV showing a reduced peak from the previous as some target is lost to solution which results in some signal loss. (Right) Peak heights of SWV plotted against time, showing the resulting exponential decay curve of the signal caused by the electrostatic melt, which could be fit to equation 17 (Own data).

Constant potential melts of the 34-bp fc dsDNA were obtained using a pulse time of 480 s and various pulse potentials from −400 mV to −750 mV and it was concluded that the duplex melted faster and to a greater extent at more negative potentials (see Figure 26). Ho also explored the pulse time, via 34-bp fc dsDNA melt curves run at −600 mV and various pulse times from 10 s to 720 s. The time constant was
found to increase with increasing pulse time, reinforcing the theory that faster pulsing enhances mixing at the electrode, thus removing melted target from the electrode surface more efficiently.

**Figure 26:** (a) Constant potential melting curves of 34-bp fc duplex at different potentials with a pulse time of 480 s. The pulse potentials were −750 mV (red) or −600 mV (green), −500 (orange), −400 (blue), or a control at open cell potential (black). (b) Constant potential melting curves of three different DNA duplexes: 34-bp complementary duplex (circle, blue), 34-bp duplex with a single mismatch (diamond, red), and 18-bp complementary duplex (square, green). The pulse potential was −500 mV (open symbols) or −750 mV (closed symbols) with a pulse time of 480 s. Solid lines are exponential fits [2].

Ho determined that a more negative melting potential resulted in a faster melt and that the speed of the melt hindered the ability to fit the data to equation 17, thus reducing the quality of data obtained from these experiments. Additionally, at potentials higher than -500 mV, a significant amount of probe desorption was observed (see Figure 27) [2].
Figure 27: Comparison of melting and desorption during constant potential melting routine obtained for the 18-bp complementary duplexes with either MB-tagged target (circles, green) or MB-tagged probe (diamonds, purple). The pulse potential was −500 mV (open symbols) or −750 mV (closed symbols) with a pulse time of 480 s [2].

Ho found that, at −500 mV, desorption accounts for ~7% of the total signal loss after 8 min. On the other hand, at −750 mV, desorption accounts for roughly 65% of the signal loss after 8 min [2]. Based on Ho’s work, it was determined that a standard melting potential of −0.5 mV and a pulse time of 480s were the ideal conditions to maximize the quality of my research.
Chapter 4: DNA Electrodes and Optimization

As previously stated, it is the hybridization of the probe DNA with the target DNA that produces a detectable signal as the Methylene Blue tag on the target DNA undergoes redox reactions when a current is introduced. This produces a signal peak during a square wave voltammogram on the electrode containing the tagged DNA.

DNA thiol monolayers

At one point in history, a theory formed that attaching DNA to the surface of a sensor would help to remove the one-tube-per-experiment limitation that had constrained efficacy at the time. This enabled scientists to run experiments in parallel by optical readers [58]. One example, by Fodor et. al., involved attaching DNA to a chip [59]. However, the length of DNA presented by this method was severely limited by rates of error in photochemistry and DNA synthesis [59]. Other methods involved the attachment of DNA to gel layers or glass, which allowed for the attachment of longer chains. Unfortunately, these methods resulted in random orientation, which complicated comparative analyses such as single-base mismatch determination. Furthermore, as Bamdad theorizes in *A DNA Self-Assembled Monolayer for the Specific Attachment of Unmodified Double- or Single-Stranded DNA* [58], DNA immobilized on glass may be prone to nonspecific binding. Gold readily forms bonds to thiol groups, making attaching thiol groups to DNA a viable solution to avoid these issues. Attaching a thiol group to the end of the DNA sequence in question would ensure the orientation of the DNA attached to the gold surface. Alkyl thiolates self-assemble, by chemisorption, onto gold to form layers of single-molecule thickness (see Figure 28) and can be selective. For an example of this selectivity, thiols terminated with nitrilo triacetic acid (NTA) have been shown to self-assemble with ethylene glycol-terminated thiolates and yield a composite surface that specifically binds histidine-tagged proteins while resisting nonspecific adsorption. [58]
Figure 28: Formation of the thiol-based monolayer on a gold surface for DNA immobilization and hybridization. ssDNA = single-stranded DNA, cDNA = complementary DNA, dsDNA = double-stranded DNA [60].

DNA sensing and the purpose for tagging the DNA

DNA bound to the gold electrode surface via gold-thiol bonds is not capable of undergoing redox reactions at the range available on these electrodes [2]. To be able to monitor the melt, a redox tag was attached to the target DNA so that the modified dsDNA strands produce a signal during the SWV part of the melt program.
Figure 29: Comparison of melting and desorption during constant potential melting routine obtained for the 18-bp complementary duplexes with either MB-tagged target (circles, green) or MB-tagged probe (diamonds, purple). The pulse potential was $-500$ mV (open symbols) or $-750$ mV (closed symbols) with a pulse time of 480 s. [2]

Figure 29 compares melting curves run at pulse potentials of $-500$ mV (purple open symbols) and $-750$ mV (purple closed symbols) alongside desorption curves (green symbols, closed for $-750$ mV, open for $-500$ mV) at the same potentials. As can be seen, the dsDNA is more prone to thiol desorption when the pulse potential is set at $-750$ mV, with desorption reaching a maximum of approximately 10% at $-500$ mV versus desorption being responsible for roughly 65% of signal loss when pulsing at $-750$ mV [2]. As such, $-500$ mV is the better melting potential to use to allow for e-melting to occur while minimizing probe desorption.

Proof of ability to identify DNA mismatches
The West lab showed that electrostatic DNA melts help detect the presence of a DNA mismatch in dsDNA. One set of electrodes with 34-bp dsDNA (sequence shown in Table 1) adhered to the surface alongside a passivation layer of mercapto-1-hexanol (MCH) were pulsed with a constant potential of -500 mV against an Ag/AgCl reference electrode. A second set of electrodes with the same DNA layer conditions were pulsed with a constant potential of -750 mV. This was repeated with a set of 34-bp dsDNA (sequence shown in Table 1), which included a single mismatch, and with a shorter strand of dsDNA amounting to 18-bp (sequence shown in Table 1). The results of this experiment are shown in Figure 30 which shows that there is a visible shift in the melting curve upon including a single DNA mismatch to the dsDNA, shown in the general change in curve shape from open symbols (fully complementary dsDNA) to closed symbols (single mismatch dsDNA). \( \tau \) and \( \Delta \) derived from these curves using equation 17 gives further insight, as the general trend West lab observed was that \( \tau \) decreases and \( \Delta \) increases upon the introduction of a mismatch to the dsDNA. This proves that electrostatic melts can be used to determine the presence of a DNA mismatch. Additionally, there does not seem to be any major difference between the fully complementary 34 base pair dsDNA (fc 34-bp dsDNA) and the fully complementary 18 base pair dsDNA (fc dsDNA), thus our use of 18-bp dsDNA in place of 34-bp dsDNA will not influence our results in any significant way and should still provide ample insight into the mechanism of electrostatic DNA melting regardless of its shorter length.

### Table 1: Table of DNA Sequences used in Ho et. al.’s Paper [2]

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>34-bp Fully Complementary Duplex</td>
<td><strong>Probe</strong> 5’HS-C6-TGG ATC GGC GTT TTA TTC TTG TTC AGA TAT TCA A 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Target</strong> 3’ (MB) - ACC TAG CCG CAA AAT AAG AAC AAG TCT ATA AGT T 5’</td>
</tr>
<tr>
<td>34-bp Single Mismatch duplex</td>
<td><strong>Probe</strong> 5’ HS-C6-TGG ATC GGC GTT CTA TTC TTG TTC AGA TAT TCA A 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Target</strong> 3’ (MB) - ACC TAG CCG CAA AAT AAG AAC AAG TCT ATA AGT T 5’</td>
</tr>
<tr>
<td>18-bp Duplex (MB on target)</td>
<td><strong>Probe</strong> 5’ HS-C6-TTG ATC GGC GTT TTA TTC 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Target</strong> 3’ (MB) - AAC TAG CCG CAA AAT AAG 5’</td>
</tr>
<tr>
<td>18-bp Duplex (MB on probe)</td>
<td><strong>Probe</strong> 5’ HS-C6-T (T-MB) G ATC GGC GTT TTA TTC 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Target</strong> 3’ A A C TAG CCG CAA AAT AAG 5’</td>
</tr>
</tbody>
</table>
Figure 30: Constant potential melting curves of three different DNA duplexes: 34-bp complementary duplex (circle, blue), 34-bp duplex with a single mismatch (diamond, red), and 18-bp complementary duplex (square, green). The pulse potential was $-500$ mV (open symbols) or $-750$ mV (solid symbols) with a pulse time of 480 s. Solid lines are exponential fits [2].

The next question that arose was that of the best tag to use for this experiment. Our lab eventually decided on tagging the target strand with a methylene blue group, which has a redox potential ranging between $-0.4V$ and $-0.1V$. Levicky discovered that, by including a passivation layer of MCH, the performance of such electrodes is much improved [61]. Once the probe is adhered to the electrode surface and hybridized with the target strands to form dsDNA, a negative potential is applied to the electrode. This negative potential repels the negatively charged phosphate backbone of the DNA, causing an unzipping of the DNA helix. The probe strand remains secured to the gold surface via the thiol bond and only the target sequence will diffuse into the surrounding solution, resulting in a decrease in the signal as the MB drifts away from the electrode surface. This process of unzipping and removal of the target is called electrostatic denaturation. A SWV is taken every eight minutes in order to monitor the MB signal decay. The measured peak heights of each SWV plotted versus time results in the curves of signal decay (see Figure 31 and equation 17).
Figure 31: (Left) Overlaid SWV taken in the process of a melt. The highest peak is from the first SWV, with each following SWV showing a reduced peak from the previous as some target is lost to solution resulting in signal loss. (Right) Peak heights of SWV plotted against time, showing the resulting exponential decay curve of the signal caused by the electrostatic melt, which could be fit to equation 17 (Own data).

Further experiments in our lab were done to explore the efficacy of electrostatic DNA melts in determining various DNA characteristics. For example, one experiment involved the determination of the presence of hairpins in the DNA. Following some rigorous research and experimentation on the matter, it was determined that, while hairpins can be identified on some occasions, the results proved to be too inconclusive, with a tendency for the standard deviation to be too high [2].

Other factors that can affect electrostatic denaturation include the effect of surface density, ionic strength, and buffer concentration. For example, the surface density can affect the ratio of hybridization. Peterson et. al. found that for high probe density films, the efficiency of hybridization is low. They also found that for probe densities below $3 \times 10^{12}$ molecules/cm$^2$, the initial hybridization rate was faster, reaching a maximum within 15 min. Peterson theorized that the shape of the hybridization isotherms at densities above $5 \times 10^{12}$ molecules/cm$^2$ is most likely due to repulsive electrostatic and steric interactions that increase with increasing probe density (see Figure 32) [62].
Figure 32: Target hybridization kinetics as a function of probe density. The probe density, determined by SPR, varies from $2 \times 10^{12}$ to $12 \times 10^{12}$ molecules/cm$^2$. Heating the probe film prior to hybridization increases the hybridization efficiency. All runs are 1 µM target in 1 M NaCl with TE buffer [62].

Bartlett [9] studied the effect of ionic strength on electrostatic denaturation and determined that as ionic strength increases, the strength of the electric field produced by the shielding of the double layer decreases, as shown in Figure 33. Our research concurs with this conclusion. As ionic strength decreases, electrostatic repulsion between the negative phosphate backbones of the two DNA strands increases. Conversely, an increase in ions will increase the shielding of the negative charge on the DNA backbone, thus reducing the electrostatic repulsion and increasing the stability of the duplex [63].
Our lab explored the effect of cisplatin on melting curves in the past, with a focus on the effect of cisplatin on dsDNA stability. Cisplatin is an alkylating antineoplastic drug that is used in combination with other antineoplastic agents for the treatment of metastatic testicular cancer and ovarian cancer. It is also used in the treatment of advanced bladder cancer and is effective against a variety of other tumors including cervical and bone cancer [64]. In an aqueous solution, the chloride ligands of cisplatin are replaced with H$_2$O, as shown below:

$$Pt(NH_3)_2Cl_2 + 2H_2O \rightarrow [Pt(NH_3)_2(H_2O)_2]^+ + 2Cl^-$$ (18)
This aquated form of cisplatin can bind to DNA mainly through the N7 atoms of purine bases with a preference for binding to guanine over binding to adenine. Cisplatin has a variety of effects on the stability of dsDNA, ranging from stabilizing dsDNA if bound via intrastrand cross-linking between guanines separated by one base, to destabilizing DNA if the cross-linking results in a perturbation of the base stacking [3].

**Figure 34**: Kinetic melting curves and exponential fits of dsDNA with cisplatin (data: orange squares; fit: dashed line) and without cisplatin (data: blue circles; fit: dotted line) for the three preparation methods: (a) backfill method with 0.5 μM probe DNA, (b) insertion method, and (c) co-deposition method. All denaturation experiments were performed in 10 mM Tris (pH 7.2) at 30 °C [3].

In all three graphs of Figure 34, the orange squares showing dsDNA treated with cisplatin plot a curve showing less melting than the blue circles (untreated dsDNA). This proves that the introduction of cisplatin to the DNA tended to improve helix stability, thus reducing the extent of melting and increasing the time taken to melt. In addition, West Lab found the stabilizing effect of cisplatin to be heavily reliant on the method by which the DNA monolayer was prepared. The methods of co-deposition and insertion – assumed to produce a more homogenous surface coverage – resulted in a decrease of the extent of melting and the time constant. Conversely, for the backfill method, the addition of cisplatin resulted in an increase in both the time constant and extent of melting. These results suggest that the process of cross-linking by cisplatin is dependent on the DNA surface density, heterogeneity, and interstrand distances [3].

**Commonly assumed mechanism of electrochemical DNA melts**
Thus far, it was assumed that the electrostatic mechanism involves the negatively charged backbone being repulsed by the high electric field produced by the formation of the electrostatic double-layer, causing the dsDNA to unzip [65]. However, this electrostatic mechanism is the most commonly assumed theory for electrostatic melting, though there is some evidence that this mechanism is not correct [4, 9].

In a paper by Rant et. al., the orientation of DNA (both ssDNA and dsDNA) was manipulated via the potential on the electrode surface. DNA was bound to the gold electrode surface using a thiol group on the 5’ end while a fluorescent tag was bound to the 3’ end. This fluorescent tag was quenched when close to the electrode surface, and free to fully fluoresce when away from the electrode surface (see Figure 29). By monitoring the fluorescence, they were able to show that alternating between an attractive and a repulsive potential caused the orientation of the electrode-bound DNA to lie flat against the electrode surface, then stand perpendicular to the electrode surface. DNA motion was found to follow the double-layer response, since as pulse time grew shorter and shorter, the DNA responded more and more sluggishly. When the pulse time fell below the response time of the double-layer, which resulted in the cessation of the formation of double-layer, the DNA stopped responding. This occurred at a frequency of approximately 10 kHz (~0.1 ms) (see Figures 35 and 36).

Rant’s work gives rise to the hypothesis that if e-melting is purely electrostatic, melting should cease or drastically decrease when using pulses faster than the response time of the double-layer. Accordingly, we devised experiments to test this hypothesis. The experimental approach and the results of these experiments will be discussed next.
Figure 35: Electrically induced, persistent switching of a DNA layer (double-stranded, 24-mer) on a gold surface monitored by optical measurements. (Left) The fluorescence intensity observed from the dye-labeled DNA layer alternates upon periodically reversing the electrode charge. (Right) Negatively biased electrodes repel the likewise charged DNA strands, bright fluorescence is emitted from the dye attached to the DNA's top end. The positive surface charge attracts the strands and due to the close proximity to the metal efficient energy transfer from the excited dye to the Au results in a substantial quenching of fluorescence. Note that the layers maintain their functionality over millions of cycles (>13.8 h) showing outstanding persistency. No indications for desorption of molecules have been found. Salt in solution: [Tris] = 10 mM. Molecule surface density: $5 \times 10^{15}$ m$^{-2}$ [4].

Figure 36: Response of the fluorescence modulation amplitude as a function of the frequency of the driving electrical AC potentials. Upon hybridization, the double-stranded 24-mer DNA layer (ds24) shows substantially enhanced switching compared to the single-stranded conformation (ss24), accompanied by a shift of its cutoff frequency to higher values. Salt in solution: [Tris] = 10 mM, [NaCl] = 50 mM. The lines are drawn by best fit. (Inset) Correlation between the cutoff frequencies of the DNA switching and the electrochemical charging current. Data points are for solution salt concentrations of 3, 5, 7, 10, 15, 25, 40, 60, 100 mM, from left to right, respectively. Molecule surface density: $3 \times 10^{15}$ m$^{-2}$ [4].
Chapter 5: Experimental

Materials

All chemicals were purchased from Sigma-Aldrich. All oligonucleotides used were purified by dual high-performance liquid chromatography (LGC Biosearch Technologies, Petaluma, CA).

Five buffers were used in this work: 9M Mercaptohexanol (MCH) solution and probe solution were both made with PBS Buffer (10 mM phosphate buffer, 2.7 mM KCl, and 1.14 M NaCl, pH 7.4); experiments were run in E-Buffer (10 mM Tris Base, pH 7.2); electrodes were rinsed in R-Buffer (5 mM Tris and 10 mM NaCl, pH 7.2) between each step of electrode preparation; hybridization occurred in H-Buffer (10 mM Tris Base, 1 M NaCl and 1 mM EDTA, pH 7.2); and DNA was stored in DNA Buffer (10 mM Tris, 1 mM EDTA at pH 8). Buffers were set to their respective pH using 2M HCl and 2M NaOH when required. All experiments were run against an Ag/AgCl (1M KCl) reference and a platinum counter electrode.

Methods

Gold electrode cleaning

First, the working electrodes (referred to hereafter as electrodes) were cleaned on a 73 mm diameter Microcloth polishing pad using a 0.05-micron alumina slurry. Electrodes were cleaned for 3 min in a Figure-eight pattern, swapping direction every 30 s. The electrodes were then sonicated in deionized water (DI water) for one min, then in ethanol for 1 min. This overall sonication process was repeated a total of 3 times, followed by a final sonication in DI water.

The next step in the cleaning process was chemical cleaning, where the electrode was submerged in 0.5 M H$_2$SO$_4$, 10 mM KCl solution and a cyclic voltammogram was performed using the AfterMath software under the following conditions: Initial potential 0.24 V scanned to 1.54 V at 0.1 V per second for 120 cycles. The electrodes were washed in deionized water and then placed in 0.5 M H$_2$SO$_4$ solution without any KCl and run through a cyclic voltammogram program with the same conditions as listed above. Finally, the electrodes were rinsed with ethanol, allowed to air dry, and then stored dry in a 96 well plate.

Modification of the Gold Electrode Surface
These electrochemical measurements were performed using a VersaStat 4 potentiostat and VersaStudio version 2.42.3 software (Ametek Scientific Instruments, Berwyn, PA). All electrochemical measurements were conducted at a temperature of 30 °C.

The three main methods of probe deposition utilized here were the traditional backfill method, the co-deposition method, and the insertion method. With all three methods, the disulfide bond on the probe was first reduced by mixing the probe with tris-2-carboxyethyl (TCEP) and leaving the mixture to rest for one hour.

The traditional backfill method, also known as the backfill, pulse-assisted method, was the most common method employed in our lab. The reduced probe solution was diluted to 0.5 μM, and each electrode was pulsed in this solution at 30°C from 0.5 to -0.2 V at 10 ms intervals for 15 min. This process was carried out at 30 °C. Electrodes were then rinsed in R-Buffer for one min and placed in 9 mM MCH solution overnight at 4°C. After, the electrodes were placed in a 10 μM MB target solution for 2 hours to enable hybridization.

The co-deposition method involved diluting the reduced probe solution to a final concentration of 0.5 μM probe and mixing this solution with 9 μM 6-mercapto-1-hexanol (MCH) in a solution of PBS Buffer. This mixture was pulsed onto the freshly cleaned electrodes via a fast potential pulse (FPP) program from an initial voltage of +500mV to -200mV at 10 ms intervals for 15 mins at 30 °C.

Finally, the insertion method utilized passive probe deposition. Gold electrodes were incubated in 9mM MCH for 2 hrs. Subsequently, these electrodes were rinsed in R-buffer for 1 min, then placed in 10 μM probe solution (diluted with PBS buffer) for 22-24 hrs. The next day, the electrodes were rinsed in R-buffer for 1 min, and then incubated in 9 mM MCH for 15-17 hrs. The electrodes were again rinsed in R-buffer for 1 min then placed in 2.5 mM target for 2 hrs in the dark.

For all three methods, the final step was to rinse the electrodes in R-buffer for 1 min and wipe the sides for dry storage.

**Melting of the Gold Electrodes**

The electrochemical melting routines consisted of a programmed sequence of potential pulses, between which square wave voltammetry (SWV) was used to monitor the amount of methylene blue-tagged DNA on the electrode surface. In the traditional method, the pulse potentials were kept constant. After each pulse period, the electrode was equilibrated at −0.1 V for 10 s to provide better quality SWVs, the parameters of which were as follows: initial potential = −0.1 V, final potential = −0.45 V, amplitude = 25 mV, frequency = 250 Hz, and increment = 6 mV. All data in this work was performed in triplicate. The
baseline subtracted SWV peak currents were normalized to the initial (pre-melt) peak current and plotted versus time.

In the traditional electrochemical melt method, as visualized in Figure 37(a), the electrode is kept at a voltage of -0.5 V for 8 min, then the potential is stepped up to -0.1 V and the electrode is allowed to equilibrate. Following this, a SWV is produced by scanning from -0.1 V to -0.45 V and monitoring the resulting current. Then the voltage is brought back to -0.5 V and the process repeats from step 1 until enough SWVs to map the melt have been collected.

![Figure 37](image)

**Figure 37:** (a) Schematic of the electrochemical melting routines used in this work. Each potential pulse is held for a fixed pulse time, tp. After each pulse, the potential returns to an equilibration potential of −0.1 V for 10 s before a square wave voltammogram is acquired [2]. (b) Schematic of Fast Pulse Potential step that will replace the circled parts in (a).

Our goal was to replace the pulse step of the traditional melt (see Figure 37(a), pulse step encircled in dark blue) with a fast pulse potential step (see Figure 37(b)), where the potential was pulsed between -100 mV and -500 mV. The theory was that, if the potential is pulsed rapidly enough between a potential at which melting occurs (-500 mV) and a potential at which melting does not occur (-100 mV), the extent of melting (Δ) should drop to zero and the time taken to melt (τ) should increase exponentially.

The peak heights of the resulting SWVs (see Figure 38(a)) were plotted to form an exponential decay curve (see Figure 38(b)). This decay curve was fitted to match the following equation:

\[ i_{peak} = \Delta e^{-\frac{t}{\tau}} + (1 - \Delta) \]  \hspace{1cm} (19)

In equation 19, Δ is the extent of melting, and τ is the time taken to melt.
(a) Current (µA) vs. E (V vs. Ag/AgCl in 1 M KCl)

(b) Normalized Peak Current vs. Time (s)

rss=0.043
**Figure 38:** (a) SWVs from an e-melt. The current is produced by the oxidation of the methylene blue tag attached to the target strand of the dsDNA (b) Peak heights from the top graph of SWVs, plotted versus time. Grey line is best fit, plotted to fit equation 18.

The DNA sequence used in these experiments is an 18 bp sequence, as listed in Table 2. Mis5 refers to the mismatch sequence, while the tagged probe sequence was used in probe desorption experiments used to determine how much of the melt was due to probe desorption rather than DNA denaturation.

<table>
<thead>
<tr>
<th>Table 2: DNA Sequences Used in this Research:</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-bp Regular Duplex (MB on target)</td>
</tr>
<tr>
<td>Probe: 5’HS-C6-TTG ATC GGC GTT TTA TTC 3’</td>
</tr>
<tr>
<td>Mis5 Probe: 5’HS-C6-TAG ATC GGC GTT TTA TTC 3’</td>
</tr>
<tr>
<td>Target: 3’ (MB) - AAC TAG CCG CAA AAT AAG 5’</td>
</tr>
<tr>
<td>18-bp Tagged Probe (MB on probe)</td>
</tr>
<tr>
<td>Probe: 5’HS(MB)-C6-TTG ATC GGC GTT TTA TTC 3’</td>
</tr>
</tbody>
</table>

In the course of this research, our lab used two concentrations of probe to get an idea of how the double-layer responded across various surface densities of dsDNA: 0.5 µM and 0.0625 µM. Electrodes were also prepared via the insertion method, as a second method of probe deposition. A range of pulse time periods were explored, from a pulse time period of 1000 ms to a pulse time period of 0.002 ms, which translates to a frequency range of 1 Hz to 25,000 Hz. Additionally, in order to verify that we were operating at a speed faster than the response speed of the double layer, it was necessary to determine the average response time of the double layer. This was done using chronoamperometry at -100 mV and 10,000 time points per second.

Again, the end goal of these experiments was to gain further insight into the exact mechanism of electrostatic DNA melting by determining the extent of the dependence of e-melting on the formation of the double-layer.
Chapter 6: Impact of Double-Layer Formation on Electrostatic Denaturation

As previously stated, the main hypothesis of this thesis is that if the mechanism of e-melting is purely electrostatic, then melting should cease, or decrease, when the pulse period of the FPP melts is shorter than the double layer response time. The first set of tests were to establish a trend for how e-melting changes as the frequency of the fast pulse potential routine increases. This was achieved by repeating the FPP melt with a wide range of pulse periods, from 0.002 ms to 1000 ms, which corresponds to a frequency range of 0.5 Hz to 250,000 Hz at a temperature of 30°C. The potential was pulsed between -0.1 and -0.5 V and with two pulses per cycle for the 1000 ms pulse time, one full cycle is 2000 ms. At each frequency, melts were performed in triplicate, and the average calculated Δ and τ for each frequency is plotted below against frequency. As can be seen in Figure 39, as the frequency increased beyond 1 kHz, the extent of melting did decrease as expected. However, rather than dropping significantly, as was hypothesized to be the case if the mechanism was purely electrostatic, the extent of melting only dropped by ~28%, from an average of 0.60±0.09 (14% RSD) to an average of 0.4±0.1 (23% RSD). For comparison, the expectation was that the extent would drop to zero if the mechanism had been purely electrostatic. Instead, Δ dropped from approximately 60% to approximately 40%.

Figure 39: Average extent of melting versus frequency of pulses. The blue dash line indicates the average extent of melting for frequencies less than 1 kHz and the orange line indicates the average extent of melting.
for frequencies of 1 kHz or greater. The standard deviation for each set of triplicate data is shown in the form of error bars.

As shown in Figure 40, the time constant of the melts also did not follow any obvious anticipated trend. If the mechanism was purely electrostatic and dependent on the formation of the double layer, then $\tau$ would have increased exponentially. Our results do not indicate any pattern in the changes in $\tau$.

![Figure 40: Graph of the average time taken to melt ($\tau$) versus the frequency of the fast pulse potential routine. There is no consistent change in $\tau$ across various frequencies.](image)

The average $\tau$ was found to be $1600 \pm 452$ with a larger 28% RSD. These results imply that the mechanism of e-melting is not purely electrostatic since purely electrostatic e-melting would give $\tau$ increasing exponentially and $\Delta$ decreasing to zero, once the pulse time dropped below the reaction time of the ions in solution. Since $\Delta$ only decreases by an average of 28% and $\tau$ shows no significant change, while electrostatics is only a small factor to be considered. Furthermore, as these experiments were run at 30°C, one must consider a thermal melting component to factor into the melt. In other words, both electrostatic and thermal melting account for the signal loss observed, and thermal melting does not depend on pulse frequency. Thermal melting is reliant on heat energy to break the bonds between the two DNA strands, whereas e-melts are reliant on the presence of a negative voltage to unzip the dsDNA. As such, this data has only been used to determine the best standard pulse times for the experiments beyond this point.
Negating the thermal component of melting

As a result of the initial experiments, three pulse periods, 1000 ms, 0.01 ms, and 0.002 ms, were chosen as the standard pulse times for the remaining experiments. The first time period (1000 ms) is a time period in which the double layer would have time to respond to the potential on the electrode surface and be able to generate the electric field thought to be the main driving forces of e-melting. The smallest pulse period (0.002 ms) was assumed to be far below the response threshold of the ions in solution, thus the double-layer would not form and was the fastest frequency available on VersaStat 4. Finally, 0.01 ms was chosen as a midpoint between the two extremes to generate more data about the behavior of e-melts at a frequency close to the response time of the double layer. From Rant’s work, we determined that the approximate response time of the double layer was around 1000 Hz, or 1 ms, based on Figure 41, which shows the correlation between the cutoff frequencies of the DNA switching and the electrochemical charging current [4]. To ensure the approximation made was accurate for our setup, DL response time experiments were also carried out.

Figure 41: Correlation between the cutoff frequencies of the DNA switching and the electrochemical charging current. Data points are for solution salt concentrations of 3, 5, 7, 10, 15, 25, 40, 60, 100 mM, from left to right, respectively. Molecule surface density: $3 \times 10^{15}$ m$^{-2}$ [4].

Double layer (DL) response time determination
To verify that the lowest time period chosen was below the threshold of the DL response time, chronoamperometry experiments were carried out in order to determine the exact response time of the ions in solution. 4 types of electrodes were run against a chronoamperometry routine: clean and unmodified gold electrodes at -20 mV; electrodes with a passivation layer of MCH at -100 mV; electrodes with probe DNA and a passivation layer of MCH at -100 mV; and electrodes with dsDNA and a passivation layer of MCH at -100 mV. All chronoamperometry experiments took a data point every 0.0001 s and were performed at 25°C. The resulting chronoamperograms are shown below in Figure 42.
Figure 42: Chronoamperomograms from DL response time determination experiments on: (a) bare gold (clean, unmodified) electrodes; (b) gold electrodes with an MCH passivation layer; (c) gold electrodes with 2 concentrations of probe DNA and an MCH passivation layer; and (d) gold electrodes with 2 concentrations of fully complementary dsDNA and an MCH passivation layer. (e) shows one chronoamperomogram of each type of electrode plotted on the same graph to show the similarities of the general shape for each type.

Once the chronoamperomograms of each type of electrode were obtained, the response time of the double layer was determined by fitting the resulting decay curve to the same equation used for plotting the signal decay of the melting curves (equation 19). The calculated $\tau$ was equal to the time constant of the double layer and the resulting values of this determination have been displayed in Table 3 below.
Table 3: Average Calculated Time Constants for the Response Time of the Double Layer Using Various DNA-Modified Electrodes:

<table>
<thead>
<tr>
<th></th>
<th>τ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bare Gold Electrode</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0022</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.0002</td>
</tr>
<tr>
<td>RSD</td>
<td>11.2%</td>
</tr>
<tr>
<td><strong>MCH Only</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.000814</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.000097</td>
</tr>
<tr>
<td>RSD</td>
<td>11.9%</td>
</tr>
<tr>
<td><strong>MCH + 0.5 µM probe</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.00073</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.00008</td>
</tr>
<tr>
<td>RSD</td>
<td>10.3%</td>
</tr>
<tr>
<td><strong>MCH + 0.0625 µM probe</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0008</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.0002</td>
</tr>
<tr>
<td>RSD</td>
<td>29.2%</td>
</tr>
<tr>
<td><strong>MCH + 0.5 µM dsDNA</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.00073</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.00008</td>
</tr>
<tr>
<td>RSD</td>
<td>10.3%</td>
</tr>
<tr>
<td><strong>MCH + 0.0625 µM dsDNA</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0008</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.0002</td>
</tr>
<tr>
<td>RSD</td>
<td>29.2%</td>
</tr>
<tr>
<td><strong>Collective Results for Fully Modified Electrodes</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0008</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.0002</td>
</tr>
<tr>
<td>RSD</td>
<td>23.4%</td>
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</tbody>
</table>

These results for the fully modified electrodes (electrodes with either concentration of dsDNA and an MCH passivation layer) show that the DL response time is approximately 0.8 ms. This response time is much greater than the shortest pulse time utilized in the melt experiments (0.002 ms). It is also greater than the intermediate pulse time period of 0.01 ms, which may explain some of the results gathered in the last portion of this thesis. As such, it is clear that the mechanism of e-melting is not reliant on the formation of the double layer.

Thus far, the results above strongly suggest that e-melting is not as reliant on the formation of the double layer as these results show that the double layer didn’t have time to form in the 0.01 ms and 0.002 ms FPP melts. However, these initial investigations still leave many questions unanswered. As such, a second battery of tests were designed to further expand on our lab’s knowledge of the potential factors that affect e-melting.
Single Mismatch Experiments

Some initial experiments were to compare the behavior of mismatch dsDNA to that of fc dsDNA when both are subjected to FPP melts. Using fully complementary dsDNA (fc DNA) and single-mismatch DNA (mis5 DNA), which utilized the same target sequence as the fc DNA. These measurements were performed for low density and high density DNA monolayers by adjusting the probe DNA concentration from 0.0625 uM to 0.5 uM, respectively, and were prepared using the backfill method. The results of these tests are depicted in Figure 43. We hoped to see that mismatch DNA could be detected using an FPP melt and how that compares to fc dsDNA undergoing an FPP melt.

| 18-bp Regular Duplex (MB on target) | Probe: 5’HS-C6-TTG ATC GGC GTT TTA TTC 3’ |
| Mis5 Probe: 5’HS-C6-TAG ATC GGC GTT TTA TTC 3’ |
| Target: 3’ (MB) – AAC TAG CCG CAA AAT AAG 5’ |

Table 4: DNA Sequence Comparison of fc DNA and mis5 DNA:

![Graph showing the results of the experiments](image-url)
Figure 43: Comparison of fc dsDNA FPP melt data to that of mis5 dsDNA FPP Melt data. (Top) A graph comparing the extent of melting for fc dsDNA and mis5 dsDNA. (Middle) A graph comparing the time constant for fc dsDNA and mis5 dsDNA. (Bottom) A graph showing fc dsDNA FPP melts overlaid with mis5 dsDNA melts, at pulse periods of 0.002 ms and 0.01 ms.
There are two key conclusions from the results in Figure 43: firstly, the introduction of a mismatch in the dsDNA results in the decrease of $\tau$ regardless of the pulse time; and second, the change in $\Delta$ upon the insertion of a mismatch was not consistent with regards to pulse time. There was an average decrease of $\tau$ by $22\% \pm 6\%$ (26% RSD) for the higher concentration and an average decrease of $\Delta$ by $56\% \pm 16\%$ (29% RSD) for the lower concentration. More specifically, for a pulse period of 1000 ms, $\tau$ decreased by $16\% \pm 1$ (7% RSE, this is average $\pm$ calculated standard error and relative standard error) and $\Delta$ decreased by $35\% \pm 5\%$ (15% RSE) with the addition of a mismatch for 0.5$\mu$M electrodes whereas $\tau$ decreased by $70\% \pm 6\%$ (8% RSE) and $\Delta$ decreased by $50\% \pm 5\%$ (10% RSE) with the addition of a mismatch for 0.0625$\mu$M electrodes. For a pulse period of 0.01 ms, $\tau$ decreased by $24\% \pm 3\%$ (11% RSE) and $\Delta$ decreased by $10.4\% \pm 0.5\%$ (5% RSE) with the addition of a mismatch for 0.5$\mu$M electrodes whereas $\tau$ decreased by $59\% \pm 2\%$ (4% RSE) and $\Delta$ increased by $6.4\% \pm 0.3\%$ (4% RSE) with the addition of a mismatch for 0.0625$\mu$M electrodes. Finally, for a pulse period of 0.002 ms, $\tau$ decreased by $27\% \pm 3\%$ (11% RSE) and $\Delta$ increased by $39\% \pm 6\%$ (16% RSE) with the addition of a mismatch for 0.5$\mu$M electrodes whereas $\tau$ decreased by $38\% \pm 6$ (15% RSE) and $\Delta$ decreased by $64\% \pm 16\%$ (26% RSE) with the addition of a mismatch for 0.0625$\mu$M electrodes.

Overall, introducing a mismatch caused the most variation in $\tau$ when utilizing a pulse period of 1000 ms and preparing the electrodes with the lower concentration of DNA while the biggest difference in $\Delta$ was seen with the lower concentration of DNA and a pulse time of 0.002 ms. In all cases across the two concentrations of DNA and the various pulse periods, $\tau$ always decreased with the introduction of a mismatch. This supports the theory that introducing a mismatch would reduce the stability of the dsDNA, thus causing it to be more easily denatured. These results aligned well with previous results from our lab.

As previously mentioned, unlike the change in $\tau$, the change in $\Delta$ upon the insertion of a mismatch was not consistent with regards to pulse time. At 0.002 ms, the extent of melting increases by $39\% \pm 6\%$ (16% RSE) at higher concentrations of DNA and increases by $38\% \pm 6$ (15% RSE) at lower concentrations of DNA upon the inclusion of a mismatch, which abides by the rule that the presence of a mismatch in dsDNA causes structural instability, thus more of the dsDNA would melt as a result. Contrarily, at 1000 ms, $\Delta$ decreases (35% $\pm 5\%$ (15% RSE) for 0.5$\mu$M electrodes, 50% $\pm 5\%$ (10% RSE) for 0.5$\mu$M electrodes), which conflicts with the logical conclusion, since, as previously stated, introducing a mismatch would result in the destabilization of the dsDNA, which should cause the dsDNA to more readily unzip and result in an increase for the extent of melting. The results at 0.01 ms seems to exemplify both extremes, as the inclusion of a mismatch causes the decrease of $\Delta$ at high concentrations and the increase of $\Delta$ at lower concentrations. At this point of our research, no conclusions can be drawn as to why these results are so mixed. One possibility is that the thermodynamics are influenced primarily by the thermal melting component, whereas the pulsed potential has a large impact on the rate of melting, by lowering the activation energy for melting.
The effect of the surface density of the dsDNA on the behavior of the double layer and the melts was also compared in these 30°C experiments (see Figure 44). This was achieved by comparing the FPP melts of 0.5 µM dsDNA and 0.0625 µM concentration dsDNA. Previous experiments in our lab had shown that a concentration of 0.5 µM produces a suitably strong signal and the resulting melting curve is easier to fit to equation 17 [2]. The second concentration, 0.0625 µM, was selected because this concentration produces a similar DNA monolayer density as the insertion method, which involves introducing the MCH first, then “inserting” the thiol-DNA second. General trend is that when the concentration decreases, the extent of melting decreases, while τ increases.

**Figure 44:** (Top) Graph illustrating the trend observed of the extent of melting at 30°C when concentration of dsDNA is reduced. (Bottom) Graph portraying the observed trend of the time constant at 30°C for the same change of conditions.
Experiments at 25°C

As previous experiments in our lab have shown that some thermal melting occurs at 30°C, the initial experiments described previously were only used as a reference to determine the parameters of the experiments run at 25°C. In order to ensure that the only cause of signal loss was electrostatic melting, we lowered the temperature for these experiments to 25°C, a temperature at which the thermal component of melting is negligible, and ran a set of FPP melts with pulse times of 0.002 ms, 0.01 ms, and 1000 ms. These melts were performed for both a DNA concentration of 0.5 µM and a DNA concentration of 0.0625 µM. The results are shown in Figure 45 and give a general trend: as the pulse time increases, so does both the extent of melting and the time constant. These graphs lead to the conclusion that when the surface density is reduced, the extent of DNA denaturation is less and takes longer than the melt of a denser DNA monolayer. There must be some contribution of steric interactions to the overall amount of denaturation.
Figure 45: (Top) Graph illustrating the trend observed of the extent of melting across three different pulse time periods for DNA concentrations of 0.5 µM and 0.0625 µM at 25°C. (Bottom) Graph portraying the time constant for the same conditions as the top graph.

Figure 46 shows the comparison of each data set across the two temperatures, 25 °C and 30 °C.
Figure 46: (Top) Graph illustrating the differences between extents of melting for FPP melts run at 25°C and 30°C across three different pulse time periods for DNA concentrations of 0.5 µM and 0.0625 µM. (Bottom) Graph portraying the differences in time constants for FPP melts for the same as the top graph.

Generally, the extent of melting is less at 25°C than at 30°C, whereas there is no consistent change in $\tau$ with the temperature decrease. Table 5 shows a summary of the changes in $\Delta$ and $\tau$ with respect to the decrease in temperature.
Table 5: Summary of Changes in Δ and τ with respect to the decrease in temperature:

<table>
<thead>
<tr>
<th>Pulse Time (ms)</th>
<th>Change in Δ</th>
<th>Change in τ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>Average 17</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>RSE 15</td>
<td>7</td>
</tr>
<tr>
<td>0.01</td>
<td>Average 34</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>RSE 7</td>
<td>8</td>
</tr>
<tr>
<td>0.002</td>
<td>Average 49</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 11</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>RSE 22</td>
<td>14</td>
</tr>
</tbody>
</table>

For 0.0625 µM concentration

<table>
<thead>
<tr>
<th>Pulse Time (ms)</th>
<th>Change in Δ (%)</th>
<th>Change in τ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>Average 8</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>RSE 10</td>
<td>8</td>
</tr>
<tr>
<td>0.01</td>
<td>Average 40</td>
<td>-37</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RSE 4</td>
<td>4</td>
</tr>
<tr>
<td>0.002</td>
<td>Average 44</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>St. Dev. 9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>RSE 21</td>
<td>9</td>
</tr>
</tbody>
</table>

On average, the thermal component of melting accounts for an average of 33% of the extent of melting for the 0.5 µM concentration and an average of 31% of the extent of melting for the 0.0625 µM concentration. In terms of time constants, the thermal component of melting accounts for an average of 20% at the higher concentration and 48% at the lower concentration. There also seems to be more uncertainty in our results at higher temperatures. Additionally, the extent of melting is more impacted by the higher temperature for the higher concentration. One reason for this could be that the steric interference at the higher concentration causes more of the DNA to melt, hence accounting for the higher extent of melting at 30°C for the 0.5 µM concentration, where steric interference has a greater influence. In contrast, the time constant is more impacted by the higher temperature at the lower concentration. One potential reason for this result could be attributed to the higher temperature contributing to the speed of ions in the buffer solution, thus causing the double layer to form more quickly and thus reducing the time constant.
Experiments at extended range of voltage

One theory to explain the results of the FPP melts was that the process of rapidly pulsing between the two set voltages was resulting in the double layer reacting to the voltage as if the voltage was averaged between the 2 set voltages. To test this theory, another variation on the initial experiments was carried out. This variation involved modifying the FPP routine such that the voltage was pulsed between +0.3 V and -0.5 V extremely fast, leaving the double layer unable to fully respond, regulating in an “effective” potential of -0.3 V, a potential where electrostatic melting still occurs, albeit more slowly. On the other hand, rapid pulsing between -0.5 V and +0.3 V would average to an effective potential of -0.1 V where no electrostatic melting is expected to occur (data not shown). All data was collected at 25 ℃ and is portrayed below in Figure 47.

![Graph showing extent of melting vs concentration for different time intervals and normal vs extended ranges.](image-url)
From Figure 47, it is clear that changing the voltage range of the FPP melts does not slow the melt, as indicated by the trend showing that extending the range results in a decrease in $\tau$, implying that the rate of melting increased. In terms of extent of melting, however, the only consistent trend is in the lower concentrations, where the extent decreases at lower pulse periods. This could be attributed to the fact that our research has already established that the extent decreases when the time per pulse is shorter than the DL response time. As the previous chapter shows, this trend is more evident at lower concentrations than higher ones, most likely due to the increased steric interactions at higher concentrations. In any case, the results do not conclusively confirm the role of the electrochemical double-layer in electrostatic melting.

Some electrodes were prepared using the insertion method, in order to compare the FPP melt curves produced by the backfill method versus those of the insertion method, the latter method having shown to produce very homogenous DNA coverages. Furthermore, the method produces low DNA densities. These conditions should reduce electrostatic and steric effects commonly observed for the higher concentration electrodes prepared with the backfill method. The insertion method gave a 3% increase in $\Delta$ and a 53% increase in $\tau$ over the backfill method carried out using the 0.0625 $\mu$M concentration for 0.002 ms FPP.
melts. For 0.01 ms FPP melts, there was a 28% decrease in $\Delta$ and a 9% increase in $\tau$ for electrodes prepared via the insertion method over the 0.0625 $\mu$M concentration backfill method for 0.01 ms FPP melts. Finally, for 1000 ms FPP melts, there was a 46% decrease in $\Delta$ and a 44% decrease in $\tau$ over the 0.0625 $\mu$M concentration backfill method. A graphical comparison is shown in Figure 48. As the 0.0625 $\mu$M backfill method is meant to give a comparable DNA surface density as the insertion method, the differences seen are due to the larger spacing between DNA in the homogeneous monolayers from the insertion method compared to the more heterogenous layer formed during the backfill method.

![Figure 48](image-url)

**Figure 48**: (Top) Graph comparing the extent of melting for various methods of electrode preparation. (Bottom) Graph comparing the time constants for various methods of electrode preparation. 0.5 $\mu$M and 0.0625 $\mu$M refer to the concentrations of probe DNA used for the probe deposition step in the backfill method.
method, while Insertion refers to electrodes that were prepared using the insertion method. The DNA surface density from the pulse probe deposition of 0.0625 μM DNA is thought to be relatively comparable to the DNA surface density produced by the insertion method.

**Probe Desorption Measurements**

In addition to the extended range measurements, previous experiments from our lab showed that, under mild conditions (25°C and -500 mV potential), desorption of the thiol monolayer was minimal. However, these results were for melts carried out with a constant applied potential of -500 mV, not FPP, hence it was necessary to determine the extent of desorption during an FPP melt. This was done by adhering methylene blue tagged probe to the electrode surface, then running an FPP melt to see if the signal decayed in the process. The presence of signal decay would indicate the occurrence of probe desorption.

**Table 6: DNA Sequence Used for Probe Desorption Tests:**

| 18-bp Tagged Probe (MB on probe) | Probe: \(5'\) HS-C6-T(T-MB)G ATC GGC GTT TTA TTC 3' |

**Figure 49:** Graph of 0.5 μM tagged probe FPP melt curves with a pulse period of 0.002 ms and 1000 ms.
As can be seen in Figure 49, there is, at best, negligible signal decay, which indicates that, for a concentration of 0.5 μM, probe desorption is not significant and thus can be ignored. This same experiment was also repeated for concentrations of 0.0625 μM and for tagged probe electrodes prepared via the Insertion method. These results are shown in Figure 50.

Figure 50: (Top) Graph of 0.0625 μM tagged probe FPP melt curves with a pulse period of 0.002 ms and 1000 ms. (Bottom) Graph of tagged probe FPP melt curves on electrodes prepared by the Insertion method run with a pulse period of 0.002 ms and 1000 ms.
For both 0.0625 μM electrodes and for electrodes prepared via the insertion method, there is evidence that probe desorption has occurred during the melting process. For the 0.0625 μM electrodes, probe desorption accounts for an average of 23 ± 4% (16% RSD) of DNA lost, while, for the insertion electrodes, probe desorption accounts for 28 ±7% (27% RSD) of DNA lost. However, despite these values indicating that probe desorption accounts for a small portion of the DNA lost, this loss is small compared to the extent of melting found in typical electrostatic melting experiments as presented in this work, further cementing the theory that e-melting is not purely electrostatic.
Chapter 8: Summary and Future Work

The purpose of this thesis was to explore electrostatic DNA denaturation on gold electrodes and provide insight into the mechanism of this process. The method was to apply a purely electrochemical routine and monitor the signal produced by the methylene blue modified dsDNA attached to the electrode surface via square wave voltammograms. The square wave voltammograms were taken every 480 seconds and the original constant voltage melt step was replaced with a system set to alternate between -100 mV (a non-melting voltage) and -500 mV (a melting voltage) at various frequencies, so as to determine the extent of the effect of the double layer on the melting process. The voltammetric peak currents were plotted versus time to construct melting curves, which were then fit to an exponential function with two parameters, the time constant (τ) and the extent of melting (Δ). Two methods, commonly used in this field, were used to prepare DNA monolayers were compared: the backfill method, consisting of pulse-assisted adsorption of probe DNA followed by incubation in MCH overnight; and the insertion method, consisting of passive adsorption of MCH followed by DNA (overnight) and once again, MCH (overnight).

On the whole, it is clear that the commonly assumed electrostatic mechanism is not the sole reason for destabilization of the dsDNA. The results here show that it only accounts for a portion of the overall e-melting process. Furthermore, the results of our research show that the formation of the double layer accounts for approximately 20-30% of the total melt. Even when reducing the temperature to 25°C, a temperature at which the thermal component of melting is negligible, the average extent of melting is 28% ± 2% (8% RSD) for an FPP melt with a pulse time period of 0.002 ms (averaging results for both 0.5 μM and 0.0625 μM). This means that some of the melt must be attributed to an as-of-yet unknown factor that is neither double-layer driven, not thermally driven. As shown in other papers in this field, the mechanism of e-melting is still an area of debate [2, 4, 9]. The impact of the electric field on the phosphate backbone and pH gradients near the electrode surface have been explored, and while these processes contribute to the melting process, they do not fully explain the melting phenomenon [2], so the factors studied in this thesis may just be another theory that partially explains electrostatic DNA melting, but does not give a complete account. Perhaps the mechanism of e-melting is a conglomerate of the impact of the electric field on the phosphate backbone, the impact of pH gradients near the electrode surface on the stability of dsDNA, and charge injection into the base stack or similar factors, some previously explored and some waiting to be explored. At the very least, the experiments outlined in this thesis provide further insight into the exact extent of the impact of the DL formation on e-melting.

Other inferences that can be drawn from this body of work are that the presence of a mismatch can be detected via FPP melts, which is especially evident in 0.002 ms FPP melts. Furthermore, the double
layer does not respond as if the voltage pulsed is an average of the two extremes of the fluctuating voltage in an FPP melt, as proven by the melts carried out under an extended range of voltage. Likewise, probe desorption measurements proved that, even when accounting for probe desorption, melting still occurs in large enough percentages to show that the mechanism of e-melting is not purely electrostatic.

**Future Work**

In future, some potential variations of our experiments that could potentially expand the current knowledge of the electrostatic melting mechanism include variations of FPP melts involving uncharged DNA mimics such as Morpholino or PNA which may further verify whether the phosphate backbone acts as a conductor for the negative charge on the electrode, causing the DNA to unzip due to the carried charge. Other potential variations include relocating the mismatch in the mismatch DNA to identify whether the position of the mismatch is identifiable via FPP melts.
References


[35] Department of Education Open Textbook Pilot Project, the UC Davis Office of the Provost, the UC Davis Library, the California State University Affordable Learning Solutions Program, and Merlot,


