Square Wave Voltammetry Detection and CD Spectroscopy Structural Characterization of 5-Methyl Cytosine-Containing Oligomeric DNA Sequences

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December 2022

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DNA methylation, primarily at the 5-carbon in cytosine (5-methyl cytosine) plays a significant role in key processes and functions of the body, such as development of the brain and in neuronal cell differentiation. Lack of DNA methylation control has been linked to cancer, cardiovascular diseases, autoimmune diseases, and imprinting disorders. Due to its role in neuronal development, DNA methylation has also been implicated in the development of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and multiple sclerosis (MS). Because aberrant methylation can arise at such an early stage in disease progression, detection of hypermethylated DNA sequences, or those sequences exhibiting elevated levels of cytosine methylation, may allow for earlier detection of these diseases, resulting in more optimal treatment options.

Here, we describe an electrochemical assay designed to detect cytosine methylation levels in DNA sequences from CD8+ T cells. Methylation in this gene has been linked to multiple sclerosis. Short, defined oligomeric sequences from this gene were immobilized on gold electrodes via thiol linkages and exposed to target, complementary sequences featuring varying numbers (0-4) 5-methyl cytosine modifications forming double stranded DNA hybrids on the electrode. The DNA was then exposed to high ionic strength (0.1 M $MgCl_2$) and a redox active diviologen molecule of the form $C_{12}H_{25}V^{2+}C_6H_{12}V^{2+}C_{12}H_{25}$ (where V^{2+} = viologen, 4-4'-bipyridyl, C12Viologen). Detection of the DNA took place using square wave voltammetry via reduction of C12Viologen, which has been previously shown to bind to DNA in a structure

specific manner. $MgCl_2$ induced structural changes in the DNA, which were related to the amount of cytosine methylation featured in the oligomers. This could be detected via the emergence of a significant reduction current appearing at ~-0.37 V vs. SCE in non-methylated oligomers that decreased in magnitude with DNA featuring increasing methylation content. Statistically significant differences in this current were detected between non-methylated oligomers and all oligomers featuring any amount of cytosine methylation.

The DNA oligomers were characterized using circular dichroism (CD) spectroscopy and UV-Vis thermal melting studies. CD spectra showed that the oligomers adopted typical conformations based on their high GC content (likely favoring the A-form of DNA), and upon exposure to $MgCl_2$, the strong positive bands decreased slightly, with the most change occurring in non-methylated oligomers. This data showed that the structural changes to either non-methylated or methylated DNA were subtle, and likely resulted in a A or BII form where the bases were slightly altered from the initial structure upon exposure to $MgCl_2$. Overall, these changes were consistent with the electrochemical findings, showing that very small structural changes related to DNA methylation could be detected using electrochemical methods, leading to the possibility that this approach could be used as a diagnostic tool to detect cytosine methylation in similar short gene segments.

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A Thesis

Presented to the Faculty of the Department of Chemistry

East Carolina University

In Partial Fulfillment of the Requirements

For the Degree of Master of Science in Chemistry

Victoria G. Preston

December 2022

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ACKNOWLEDGEMENTS

I would like to thank my family for their support and making this thesis possible. I would also like to thank my primary investigator, Dr. Eli Hvastkovs, for his continuous support, patience, guidance, and knowledge, all of which contributed greatly to this project. Finally, I would like to thank the members of my committee: Dr. Robert Hughes for his time and knowledge, Dr. Eric Anderson whose valuable insights and suggestions helped guide this project, and Dr. William Allen who has been a great source of support and advice my during my time at East Carolina.

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LIST OF SYMBOLS AND ABBREVIATIONS

CC	Chronocoulometry
CD	Circular-Dichroism
CpG	Cytosine-phosphate-guanine
CV	Cyclic voltammetry
dsDNA	Double-Stranded DNA
DI H_2O	Deionized water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E-Buffer	Electrochemical buffer: 10 mM Trizma Base/10 mM NaCl @ pH 7.4
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography ultraviolet
LbL	Layer by layer
me	Methyl group
mC	Methylated Cytosine
MCH	Mercaptohexanol
µM	Micromolar
µL	Microliter
mL	Milliliter

mM	Millimolar
mol	Mole
MS	Multiple Sclerosis
nm	Nanometer
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
$Ru(NH_3)_6^{2+}$	Ruthenium hexamine (Ruhex)
SCE	Saturated Calomel Electrode
ssDNA	Single-stranded DNA
SWV	Square-wave Voltammetry
TEA	Triethylamine
THF	Tetrahydrofuran
Tris	Trizma buffer
UV-Vis	Ultraviolet-visible

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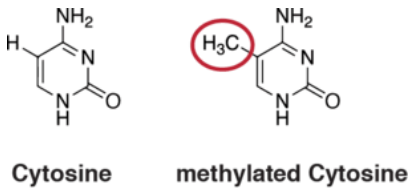
Chapter 1: Introduction

1.1 DNA Methylation

Methylation refers to the transfer of a methyl group ($-CH_3$) to an organic compound. Methylated DNA strands refer to the addition of a methyl group to a DNA strand, often occurring at the fifth carbon atom of a cytosine ring replacing a hydrogen as seen in scheme 1. This results in incorporation of 5-methylcytosine sites in DNA. Methylation in DNA almost exclusively occurs at sites where a cytosine is 5' to guanine at cytosine-phosphate-guanine (CpG) sites. The reaction is catalyzed by a family of enzymes termed DNA methyltransferases.[1] These enzymes transfer a methyl group from S-adenyl methionine to the fifth carbon atom of the cytosine ring.[1]



Scheme 1.1. Cytosine vs methylated cytosine

In biological systems, methylation can regulate protein function, embryonic development, gene expression, and RNA processing.[2] CpG islands are areas in DNA that contain around 1000 base pairs with a particularly high CpG density versus the rest of the genome.[1] These islands have been found to play an important role in gene expression as they regulate transcription factor binding and the chromatin structure.[1] DNA methylation of these islands controls gene expression by inhibiting the binding of transcription factors or by recruiting proteins which are involved in gene repression.[1, 3]

DNA methylation is an epigenetic alteration, or alterations that do not result in changes to the genetic sequence.[4] Epigenetic changes do not alter DNA sequence but rather influence how the DNA sequence is read.[5] DNA methylation does so through gene silencing. It can suppress transcription using either indirect methods, such as recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activities, or by direct methods via inhibiting

the binding of specific transcription factors.[2] DNA methylation plays a critical role in development and in neuronal cell differentiation.[6] It has also been found to play a role in the function of the adult brain, particularly when it comes to the brain and memory and learning related processes.[6,7]

1.2 DNA Methylation & Disease

Due to the significance of DNA methylation in key processes and functions of the body, mental impairment and disease can arise when DNA methylation occurs in areas where it is not supposed to.[7] This can occur as a consequence of environmental risk factors or developmental mutations, such as diet, drugs, hormones, and exposure to environmental chemicals.[7,8] Several studies have been conducted in order to better understand the effect that DNA methylation has on human health, specifically as it relates to neurodegenerative diseases.[3,9] Lack of DNA methylation control has been linked to a plethora of cancers, cardiovascular diseases, autoimmune diseases, and imprinting disorders.[10] Due to its role in neuronal development, DNA methylation has also been implicated in a number or neurological disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and multiple sclerosis (MS).[9,11]

Hypermethylated DNA sequences, or those sequences exhibiting elevated levels of cytosine methylation, may allow for early detection of these diseases. If able to be detected early on, this would allow for earlier treatment of these diseases, possibly giving a better quality of life to those afflicted. For MS, hypermethylated DNA in CD8+ T cells may trigger the development of the disesase.[12,13] This process occurs before classic manifestations in pathology (plaque formation, demyelination) and phenotype (neurological aberrations); therefore, detection of this

process could lead to MS prognosis at very early stages of the disease and would offer vast benefits.

1.3 Common DNA methylation detection techniques

Due to the connection hypermethylation has to many different illnesses, being able to detect such abnormalities in DNA sequences would allow for early detection or confirmation of many diseases. Recognizing the importance of this has led to the development of hypermethylation detection techniques over the past few decades. Such methodologies range from quantitative real-time PCR to HPLC analysis.[14]

Detection of cytosine methylation typically involves an assay comprising bisulfate conversion of altered cytosines.[14] Here, cytosine is converted to uracil after being deaminated whereas methylcytosine remains unchanged due to its low reactivity. PCR is then used to amplify both the originally methylated and unmethylated sequences. The conversion to uracil and subsequent base pairing with thymine vs. guanine for cytosine allows back calculation and determination of methylated sites.[14] A similar technique that does not involve bisulfate conversion is based on the selective digestion of DNA using specific endonucleases.[15] Using two endonucleases, HpaI and MspI, researchers are able to distinguish between methylated and unmethylated sites. HpaI digests CCGG sequences only when they are unmethylated, whereas MspI will digest CCGG sites whether or not a methyl group is present.[15] After digestion of the DNA sequences, electrophoretic analysis of the size of the digestion products can show where DNA methylation is present in the genome.[15] The downfall of both the bisulfate conversion and PCR technique, as well as the digestion-based assay, however, is the necessary knowledge of the

entire composition of the genes being analyzed beforehand. This makes it difficult to analyze the methylation content of unknown genes.

Other techniques include HPLC (high performance liquid chromatography) and the use of antibodies, binding proteins, and metal-complexes, which have a high affinity for methylcytosine and can be read out at as a methylcytosine-positive assay.[14] Using HPLC analysis with an anion-exchange column, the hydrophobic and electrostatic properties of nucleotide bases can be taken advantage of to distinguish between methylcytosine and cytosine.[16] In this technique, after bisulfate conversion, thymine and cytosine correlate with unmethylated and methylated cytosine, respectively. The bases are captured using electrostatic interactions, then separated based on their hydrophobicity.[16] HPLC-UV (high performance liquid chromatography-ultraviolet) is another version of HPLC commonly implemented to detect DNA methylation. First, the DNA sample is hydrolyzed into its individual bases. The dC and 5 mC bases are then separated chromatographically and the fractions are measured. The dC/5 mC ratio is calculated and compared to the original sample, allowing for the amount of methylated cytosine present to be identified.[16] Although these types of assays are reliable and allow the identification of 5 mC in unknown gene sequences, they are typically time-consuming, complicated, and require multiple steps to detect methylcytosine. They also require specialized laboratory equipment and large amounts of DNA (3-10 μg) in order for the sample to be analyzed, neither of which are always readily available.[16]

The techniques listed above are examples of indirect detection methods, which struggle to analyze specific gene sequences. Because of the difficulties each method presents, such as cost and difficulty of technique, researchers have looked to other means of 5 mC identification. In

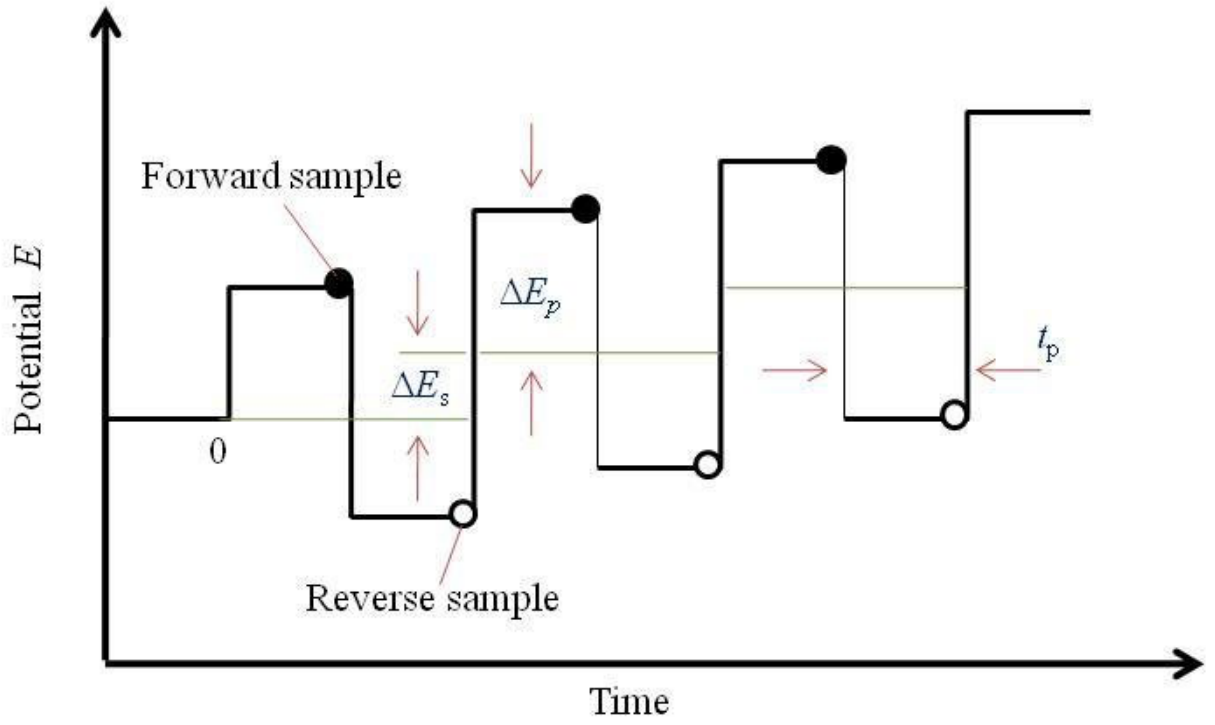
particular, electrochemical methods have been developed, which have shown promise in both cost-effectiveness and reliability, among other benefits.

1.4 Electrochemical Analysis Overview

The field of electrochemistry studies the transfer of electrons and its relation to chemical changes.[17] It is a branch of chemistry concerned with the relationship between electrical and chemical changes that are caused by the passage of current.[18, 19] A typical setup for an electrochemical analysis includes electrodes for electrons to move between, an electrolytic solution to separate the electrodes, and a potentiostat. Three electrode setups are preferred as they allow for the potential changes of the working electrode to be measured independently of the changes at the counter electrode, leading to more accurate readings.[20] One of these three electrodes is termed the working electrode, on which the reaction of interest takes place. Another is the reference electrode, which maintains the potential of the working electrode at a constant level and provides reference potential for the potentiostat to apply to the counter electrode and working electrode.[20] Finally, there is the counter or auxiliary electrode, which closes the circuit between the working electrode and the counter electrode and provides the initial potential to the potentiostat.[20] The potentiostat is the source of the voltage applied to the electrodes and works to maintain the potential of the working electrode at a constant level with respect to the reference electrode.[17] It does this by adjusting the current of the auxiliary/counter electrode and interfaces with a computer to provide data output.

Such an electrochemical output can be detected through volumetric techniques.[14] These techniques include cyclic voltammetry (CV), chronocoulometry (CC), and square-wave voltammetry (SWV). CV is a sweeping analysis, which applies a differential potential at a

constant rate and a current is output.[17] It is directly quantifiable, but not as sensitive as SWV.[17] SWV is used most often where there is an increased need for sensitivity versus the standard cyclic voltammetry as it can reduce the contribution from the charging current.[21] This is done by using a staircase potential-time function, as can be seen in Scheme 1.2.[21] The current at the end of each potential step is measured and the current at the reverse step is then subtracted from that measured at the forward step, which considerably diminishes the charging current contribution – i.e. that from movement of ionic species in the solution.[21] This improves both the quality and sensitivity of the data. The remaining current output is largely attributed to the electron transfer process that is often the focus for the experiment.



Scheme 1.2. Square wave voltammetry measurement and waveform scheme. A forward current sample (indicated by the black solid dot) is taken, and a reverse current sample (indicated by the white dot) is taken.

1.5 Electrochemical DNA Detection

In recent years, electrochemical detection methods have become especially popular as a sensitive, rapid, and inexpensive method for assaying DNA sequence recognition or damage.[18,19] Electrochemical detection methods are simple, using relatively inexpensive equipment to detect a current response of hybridized DNA on an electrode.[19] They also offer a way to analyze DNA using small sample volumes, thus adding to the low cost of using such methods.[19] Other techniques require large amounts of input DNA, expensive biological molecules, or hazardous radiolabeling.[14] Response rates are also very quick, unlike other methods, such as HPLC.

DNA can be immobilized on a transducing surface in a number of ways, which is dependent on the type of information that is desired. Such strategies include layer by layer (LbL) electrostatic immobilization or thiol attachment to gold electrodes.[22] In a typical DNA electrochemical sensor where the sequence information is important, a DNA probe of known base sequence is immobilized on an electrode surface often involving thiol attachment to gold forming a self-assembled monolayer.[22] The probe strand is then exposed to the complementary strand, and hybridization can be detected using a number of strategies.[22]

Direct oxidation methods are widely used for hybridization detection due to the simplicity of the method. DNA nucleobases are detected directly using no additions or external modifications.[19] One common direct oxidation technique is guanine oxidation. Guanine is particularly electroactive. The free base is easily oxidized and absorbed on the electrode.[22] Once hybridized, the guanine is much less electroactive. Because less free guanine is available to be oxidized, there is a lower signal and hybridization is assumed.[22] The disadvantage of direct detection techniques is the irreversible damage done to the DNA due to the oxidation of the nucleobases.

Indirect detection methods avoid the direct oxidation of DNA to generate answers to the amount of DNA methylation present in a particular gene sequence. While the electrochemical output does not assay the nucleobase content directly, indirect methods offer the added benefit of not irreversibly damaging the DNA sample, as oxidation of DNA bases is irreversible. An example of this includes sensing DNA hybridization. Indirect detection methods offer a means to detect DNA hybridization without irreversibly damaging the DNA.[22] For instance, electrostatic compounds such as ruthenium hexamine ($Ru(NH_3)_6^{3+}$, RuHex) have been used to assay the doubling in anionic phosphate groups at the electrode surface upon the application of a sufficient reduction potential.[23] Another method of indirect detection includes the use of methylene blue (MB), an aromatic heterocycle that binds to dsDNA via intercalation.[24] DsDNA can be distinguished from ssDNA by comparing the reduction current of the intercalator, in this case MB. The reduction current of the intercalator is proportional to the concentration of DNA present on the electrode, thus hybridized DNA versus ssDNA can be realized.[24]

1.6 Electrochemical Analysis of DNA Methylation

Electrochemical methods have previously been developed to detect DNA methylation. The majority of these methods can be categorized into two overarching categories: direct and indirect detection methods. An example of direct detection has utilized affinity interaction of DNA nucleobases to gold. Here, the DNA in question undergoes bisulfate treatment, followed by asymmetric PCR amplification.[25] The outcome of this process is that unmethylated DNA becomes adenine enriched as the bisulfate treatment does not convert cytosines, which are a, while methylated DNA becomes guanine enriched. The affinity for the bases to gold follows the trend A > C > G > T, which causes the adenine enriched unmethylated DNA to adsorb in higher

amounts to the electrode compared to the guanine enriched methylated DNA.[25] The decreased adsorption from guanine enriched methylated DNA results in lower coulombic repulsion between the negatively charged DNA and redox mediator $Fe(CN)_6^{3-}$. This results in higher currents for the methylated samples and allows a distinction to be made between methylated and unmethylated DNA.[25]

Another example of direct detection of DNA methylation is via direct oxidation of 5 mC. While 5mC and cytosine are normally difficult to distinguish electrochemically via direct oxidation as they both have similar oxidation potentials, specialized nanocarbon electrodes have been used to perform this process. Unlike regular carbon-based electrodes, nanocarbon electrodes are able to maintain a high electrode activity needed to oxidize both 5mC and cytosine due based on widening the potential window.[26] These electrodes allow oxidation of DNA before water oxidation, to distinguish between 5mC and cytosine.[26] Using these electrodes, an endonuclease was employed to shorten the length of the DNA sequences being studied as large sequences are difficult to oxidize directly as they exhibit low electrochemical sensitivity.[26] The overall result was a more sensitive and quantitative detection of DNA methylation without the need for a separation technique such as HPLC.[26]

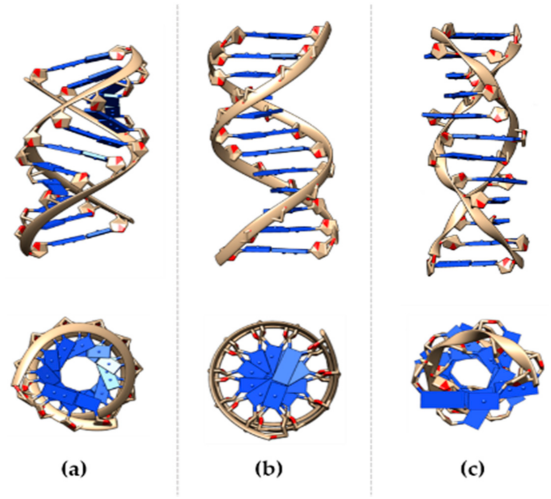
Indirect detection methods have also previously been used to detect DNA methylation. One example is a restriction enzyme digestion-based assay where DNA oligomers were immobilized on a gold electrode. The oligomers contained a distally located redox active molecule that could be detected when the oligomers were fully hybridized. Cytosine methylation impairs the action of restriction enzymes to cleave DNA at selected base pair sites. Following exposure to restriction enzymes, DNA containing methylation still exhibited high currents based

on its complete helical structure, but oligomers exhibiting no cytosine methylation sites exhibited muted currents based on the cutting of the helix and the removal of the redox marker.[14]

Scheme 1.3. Structure of diviologen used in this study. n = 11, giving the compound 12 carbons total at the terminal alkyl region.

The experimental focus here is an indirect electrochemical detection method for DNA methylation analysis. Here, DNA hybridization and subsequent structural changes in the DNA helix were monitored using a redox active diviologen ($C_{12}H_{25}V^{2+}C_6H_{12}V^{2+}C_{12}H_{25}$, C12-Viologen) compound, shown in Scheme 1.3. The main reason for employing this compound is that its electrochemical behavior upon voltametric analysis has been shown to be dependent on DNA structure. This is in contrast to compounds like RuHex that merely assay the doubling of anionic charge on the electrode, but otherwise provide very limited structural information.

C12Viologen has been shown to interact with DNA on the electrode and exhibit electrochemical redox behavior that can provide information about the DNA structure.[27] For instance, the structural changes from covalent DNA damage and hybridization can be studied using this molecule.[28] C12Viologen provides unique voltammetry upon reduction in the presence of DNA, which is based on its ability to form dimers upon one-electron reduction.[27] Current changes at specific potentials denote the C12Viologen DNA binding environment on the electrode surface. These current changes allow for determining the changes of DNA based on covalent molecular damage, de-hybridization, or other structural changes such as changes in the conformation of the DNA structure.



Scheme 1.4.a. A-DNA showing the right-handed double helix and top-view **b.** B-DNA showing the right-handed helix and top-view **c.** Z-DNA showing left-handed helix and top-view.[29]

Methylated DNA sequences adopt slightly altered structures compared to non-methylated sequences. DNA methylation imparts higher hydrophobicity to the DNA helix, which will force the helix to alter its structure slightly to minimize any solvent/methyl group exposure. As the methylation does protrude into the major groove, this initially means that the groove dimensions are altered slightly upon solvation of the DNA. Under higher ionic strength conditions, methylated DNA has been shown to undergo significant structural modifications.[29] For instance, methylated DNA may adopt a left-handed Z-form helix to minimize the unfavorable solvent interactions.[29] Z-form has also been shown to occur with alternating dinucleotide repeating purine-pyrimidine or pyrimidine-purine sequences.[29] The major groove in Z-form is almost non-existent, while the minor groove is narrow.[29] This is in contrast to the canonical right-handed B-form (shown in Figure 1.4). Other DNA transitions have also been observed when ionic strength of solution is increased, such as promotion to the A-form transition or even very limited changes at all.[30] These changes have been shown to be due to the amount of methylation and DNA sequence. A-form has been shown to occur in low (<75%) humidity conditions, such as during crystal formation, and is also associated with RND-DNA heteroduplexes.[29] A-form is more rigid and compact than B-form.[29] Because methylation has been shown to cause an increase helical rigidity as the helix fights unfavorable solvent

interactions, A-form could also be expected as the ionic strength of solution is increased for methylated DNA.[31]

1.7 Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectroscopy is an optical spectroscopic method that uses differences in absorption of left- and right- circularly polarized light, allowing for chemical structure to be studied. Optically active chiral molecules absorb preferentially one direction of circularly polarized light over the other.[32] This difference in absorption between the left and right circularly polarized light is measured and quantified in order for structure of the molecule being studied to be elucidated.[32] In a typical CD instrument, a light source passes through a monochromator and is linearly polarized.[32] The polarized light then travels through a modulating device, such as a photoelastic modulator, transforming the linear light into circular polarized light.[32] The incident light on the sample will then switch from left-handed and right-handed circularly polarized light. The absorption changes and the differentiation molar absorptivity are calculated as the light switches directions.[32]

This instrument allows for the study of the structure and conformational changes of DNA that result from environmental changes, such as temperature and pH.[33] For our purposes, the environmental changes of study will be ionic strength. Wavelengths between 210-350 nm will be used to detect structural changes in the DNA, as the bases of DNA absorb light at these wavelengths.[33] This method has a number of advantages over other techniques, such as NMR spectroscopy or crystallography, in that only relatively small sample amounts are needed and conditions similar to those in the cell are easily replicable.[33] Replicating conditions in from the

electrochemical setup as well as in the cell, CD spectroscopy will allow changes in structure of the DNA due to high ionic strength to be observed and confirmed.

1.8 Conclusion:

The importance of DNA methylation in disease has been heavily indicated through numerous studies. Being able to detect DNA methylation before classic manifestations of disease are seen could allow for an early diagnosis and treatment of many different diseases. For the research here, short, defined DNA oligomers spanning a sequence from the CD8+ T cell promoter region were utilized. Methylation in this sequence has been implicated in MS.[12] DNA was immobilized on gold electrodes using a capture binding to complementary target strands exhibiting varying amounts of cytosine methylation. C12Viologen was used as an indirect electrochemical probe to detect changes in DNA structure based on changes in its surface morphology. C12Viologen voltammetry was monitored before and after addition of magnesium chloride to the electrochemical solution. The addition of $MgCl_2$ impacts the ionic strength and promotes changes to the helical structure. Because cytosine methylation, especially under high ionic strength conditions, has been shown to alter the DNA structure, it was envisioned that C12Viologen voltammetry would be dependent on the methylation content present in the DNA. This voltammetry would be dependent on any morphological changes in DNA structure, such as a possible change from B to Z form. It will be shown that these drastic structural changes did not occur, but unique C12Viologen voltammetry based on the presence of methylated DNA oligomers was seen. Circular-dichroism (CD) spectroscopy was used to elucidate the methylation-related DNA structural transitions taking place based on exposure to $MgCl_2$

We show that this electrochemical detection approach is based on extremely subtle DNA structural changes, which shows how sensitive the C12Viologen detection approach can be. This approach was able to be used to detect and quantify the level of cytosine methylation in short DNA oligomers of a known sequence rapidly and reversibly. Spectroscopic information was used to validate the structural transitions and provided evidence to help understand the binding of C12Viologen to DNA based on methylation content and $MgCl_2$ exposure. Overall, this project presents a step forward toward a rapid DNA methylation assay to monitor biologically relevant DNA sequence that may facilitate the diagnosis of a devastating disease at an extremely early stage in its development.

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CHAPTER 2: EXPERIMENTAL

2.1 Electrochemical DNA methylation analysis

DNA Sequences and other materials:

Short, defined DNA oligomers with a sequence from the CD T 8+ promoter region were purchased from IDT DNA Technologies (Ames, IA). The sequences for the DNA are as follows:

Thiolated Probe: 5' – HS – GCG AGG ATG CGT GGG ACC CGG AGC GG– 3'

Non-thiolated probe: 5' – GCG AGG ATG CGT GGG ACC CGG AGC GG – 3'

Complimentary strands:

0-5mC: 5' – CCG CTC CGG GTC CCA CGC ATC CTC GC – 3'

1-5mC: 5' – CC^{5me}G CTC CGG GTC CCA CGC ATC CTC GC – 3'

2-5mC: 5' – CC^{5me}G CTC CGG GTC CCA CGC ATC CTC^{5me} GC – 3'

2b-5mC: 5' – CCG CTC C^{5me}GG GTC CCA C^{5me}GC ATC CTC GC – 3'

4-5mC: 5' – CC^{5me}G CTC C^{5me}GG GTC CCA C^{5me}GC ATC CTC^{5me} GC – 3'

Precursor compounds needed for C12Viologen synthesis (see below) were from Sigma. Mercaptohexanol, ruthenium hexammine ($Ru(NH_3)_6^{2+}$) and Trizma (Tris) buffer were from Sigma. All other compounds were reagent grade and used as received.

Buffers/Solutions

Immobilization buffer was phosphate buffer (1.0 M) prepared with equal molar amounts of potassium phosphate monobasic (Sigma-Aldrich) and potassium phosphate dibasic (Sigma-

Aldrich) with 1 mM EDTA in DI H_2O. Hybridization buffer was 10 mM Tris + NaCl (1.0 M) and 1 mM EDTA in DI H_2O adjusted to pH 7.4.

Electrochemical buffer (E-Buffer) was comprised of 10 mM Tris Base (Sigma-Aldrich) and 10 mM NaCl (Sigma-Aldrich) in DI H_2O. This solution was adjusted to pH 7.4.

Synthesis of C12Viologen:

4,4'-bipyridine in an 11:1 molar excess with 1,6 dibromohexane was refluxed in tetrahydrofuran (THF) for 8 hr. The product ($V^+C_6H_{12}V^+$, where V = the 4,4'-bipyridine or viologen) was removed via filtration. This di-viologen base molecule was then refluxed in excess 1-bromododecane in 1.5 ml DMF:MeOH in a 2:1 ratio under argon for 24 hr. The precipitate was filtered and rinsed with cold DI water.[1] The final product had the general formula $C_{12}H_{25}V^{2+}C_6H_{12}V^{2+}C_{12}H_{25}$ where V^{2+} is 4,4'-bypridyl and is termed C12Viologen.

DNA Preparation:

Thiolated oligomers were reconstituted in 100 μL of 50 mM dithiothreitol (DTT) with 2 μL of 2% triethylamine (TEA). This solution sat at room temp. for 10 min. After 10 min, 500 μL of acetone with 2% $LiClO_4$ was added. The solution was put on ice for 20 min. The solution was then centrifuged at 5000 rpm for 5 min. The supernatant was removed. The precipitate was the reduced thiol modified DNA. It was dried under Ar gas to remove any residual acetone and water.

The DNA was reconstituted in 10 mM Tris-1mM EDTA with a pH of 8.0 (TE Buffer). From these concentrated oligomer stocks, several 50 μL aliquots were diluted to 2 μM in

immobilization buffer. The non-thiolated complimentary oligomers were reconstituted to 1 mM in TE Buffer before diluting several 50 μL aliquots to 2 μM in hybridization buffer.

Electrode Preparation:

Gold electrodes were purchased from CH Instruments (Austin, TX). A drop of piranha solution (1:2 concentrated hydrogen peroxide to conc. sulfuric acid) was pipetted onto the electrode surfaces (10 μL droplet, 5 min), then rinsed with DI H_2O. Each electrode was polished on Buehler polishing pad paper using alumina polishing powder with three different sizes for approx.. 2 min each starting with 1 μm, then 0.30 μm, and finally 0.05 μm. After, the electrode was rinsed with DI water and briefly sonicated to remove residual polishing compound. Electrodes were placed in 0.5 M H_2SO_4 and electrochemically cleaned using cyclic voltammetry (0 to 1.6 V at 250 mV s^{-1}). After the cleaning cycle, the electrodes were cycled to 0 V to 1.4 V for 10 additional cycles to quantify the gold surface area according to a published procedure.[2] The electrodes were rinsed with absolute ethanol, and dried under Ar.

The capture probe thiolated DNA was immediately exposed to a clean electrode. This was done by pipetting a drop of the DNA on the electrode surface for 30 s. The electrode was then rinsed with DI water and then exposed to a mercaptohexanol (MCH, 5 μL in 5 mL H_2O) solution for 30 min. The mercaptohexanol solution acts as an anti-fouling agent to prevent the unwanted accumulation of DNA on the electrode. After the addition of MCH, the electrode was assayed for ssDNA content (see below).

ssDNA electrodes were rinsed in E-buffer. Hybridization of the ssDNA was performed by placing the complimentary strand DNA aliquot tube (0.5 mL centrifuge tube) onto the

electrode tightly and inverting. The electrode was placed in an incubator at 37 °C for 1 hr. dsDNA electrodes can be stored at 4 °C overnight before analysis.

DNA analysis:

Electrochemical analyses were performed using a CH Instruments (Austin, TX) Model 660A Potentiostat with a saturated calomel reference electrode (SCE in saturated KCl) and a Pt counter electrode. The modified gold electrodes were the working electrode.

Hybridization analysis: A ruthenium hexamine (RuHex) solution was prepared by adding ~1 mg of RuHex to 1 mL DI H_2O and vortexing the solution. The RuHex acts as a redox mediator. The exact concentration is known. Electrodes were placed in a glass cell containing 10 mL of E-buffer. Chronocoulometry (CC, -0.3 to -0.7 V scan, 60 Hz frequency, 1 mV step, 0.1 pulse width (sec), $1x10^{-5}$ A sensitivity, 0.00025 s sample interval, and 2s quiet time) and cyclic voltammetry (CV, 0 to -0.50 V scan, scan rate 0.1 V s^{-1}, $1x10^{-6}$ A sensitivity) were performed in the presence of 0-5.0 μM RuHex. The solution was purged with Ar for 1 min between each addition of RuHex. This analysis was performed after ssDNA immobilization and dsDNA hybridization, and DNA was quantified following an established protocol where the RuHex binds to DNA phosphates in a 1:3 ratio.[3]

DNA Methylation Analysis: Square-wave voltammetry (SWV, -0.3 to -0.7 V scan, 2 Hz frequency, 25 mV amplitude, 4 mV step, 2 s quiet time, and $1x10^{-6}$ A/V sensitivity) analysis was performed on the dsDNA electrodes as it is a more sensitive method due to the differential removal of background (non-Faradaic) currents.[2] A fresh C12Viologen stock solution was made by weighing a few mg of the compound and dissolving in approximately 5 mL of DI H_2O and heating to 35 °C. The exact concentration was known. Electrodes were exposed to 0 - 7 μM of

C12Viologen. Ar was used to purge the cell for 3 min between each SWV run. After a stable SWV was obtained, $MgCl_2$ (volume added to make cell concentration 0.1 M) from a concentrated solution was added to the electrochemical cell and voltammograms were obtained at specific time points from 30s to 600s with Ar purging between.

2.2 CD Spectroscopy Analysis

DNA Sample Preparation

DNA was hybridized by adding 50 mM of NaCl and equal molar amounts, 10 each μL, of non-thiolated probe strand and the respective complimentary strand to a PCR tube. The tubes were placed in a PCR thermocycler (Thermo) and hybridized by heating to 95^{o}C for 5 min before cooling slowly to room temperature. The hybridized DNA stock solutions were all 500 μM.

CD Spectroscopy Instrumental Analysis

CD spectroscopy was performed using a Jasco J815 spectrophotometer. The instrument was operated from 200 to 350 nm, which spans the wavelength range where DNA absorbs UV light. The data pitch was 0.2 nm, the scanning speed was 50 nm min^{-1}, and the bandwidth was 1.00 nm. The CD used had a pathlength of 1 mm and total volume of 400 μL. Hybridized DNA (3 μM) in E-buffer was added to the cell and a baseline spectrum was obtained. After the baseline run, $MgCl_2$ was added in 200 mM steps up to 800 mM obtaining a spectrum after each addition. Spectra were also obtained over extended periods of time to monitor the extent of the DNA transitions.

2.3 UV-Vis Thermal Analysis

Thermal UV-Vis DNA Prep

All melting analyses were performed using a Varian Cary 300 UV-Vis Spectrophotometer equipped with a temperature control device. Matching quartz cuvettes (Starna Cells) had total volumes of 75 μL. To each cuvette, 3 μM hybridized DNA in E-buffer was added (0, 1, 2, 2b, and 4 methyl hybrids + a blank). The absorbance was measured at 260 nm with the following parameters: data interval of 0.50 ^{o}C, and spectral band width of 1 nm, and the ramp temperature from 25 °C to 95 °C at a rate of 2 °C min^{-1}.

2.4 Data Processing

CH Instruments data, Jasco data, and Varian data were saved as .txt or .csv files and imported and collated in Microsoft Excel. Graphical analysis was performed using both Excel and Origin Pro software and data plots and figures were modified using Adobe Illustrator.

2.5 References

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CHAPTER 3: ELECTROCHMEICAL DNA METHYLATION ANALYSIS RESULTS

3.1 Electrochemical DNA Hybridization Analysis Results

Upon DNA immobilization on the gold electrodes, hybridization was assayed utilizing ruthenium (III) hexamine (Ruhex) that has been shown to bind to the immobilized DNA based primarily on electrostatic attraction. The cationic compound binds three phosphates for every one molecule, and it is reduced at ~-0.2 - -0.3 V vs. SCE, so it acts as a convenient indirect DNA hybridization marker. Comparing the signal obtained for ssDNA to dsDNA immobilization allows the assertion of hybridization as the doubling of anionic phosphate groups at the electrode surface draws more Ruhex to the Au surface.[1] Figure 3.1 shows a CV comparison plot of the electrode response for ssDNA and dsDNA modified gold electrodes in the presence of Ruhex.

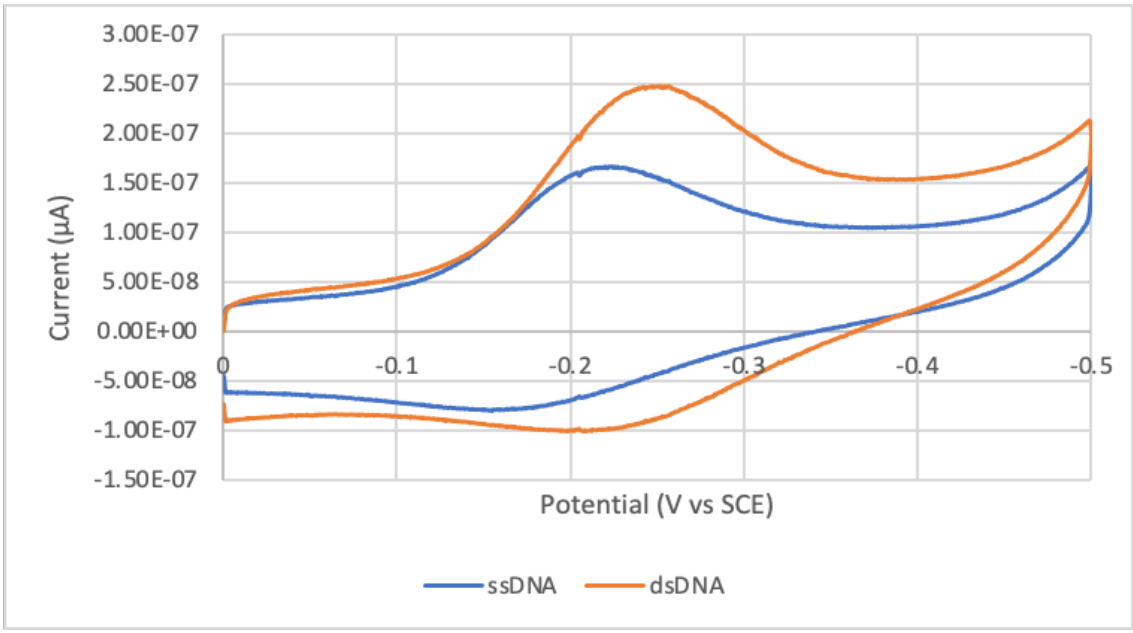


Figure 3.1. CV plot showing reduction of Ruhex with ssDNA (blue) versus with dsDNA (orange).

The figure shows the reduction of Ruhex from +3 to +2 on the sweep from 0 to -0.5 V and the reoxidation back to 0 V (vs. SCE). The plot comparison shows that the reduction peak is

significantly larger when dsDNA was assayed compared to ssDNA, which denotes more Ruhex reduction in the presence of dsDNA. This confirms the hybridization forming dsDNA on the electrode by doubling of anionic phosphate groups at the electrode surface, which forces more Ruhex to the surface and larger reduction waves. Integration of the reduction wave allows quantification of the Ruhex via the modified Cottrell equation:[2]

$$Q = nFA\Gamma$$

Where Q is the integrated charge of the reduction wave, n = 1 (the number of electrons transferred), F is Faraday's constant (9.65 x 10^4 C/mol), A is the electrode area in cm^2, and Γ is the surface coverage of Ruhex. Solving for Γ allows the calculation of the amount of Ruhex, which can be converted to DNA coverage per cm^2 via knowing that the number of DNA bases involved. Integrated Ruhex charge can also be obtained more easily via the use of chronocoulometry where the charge is assayed over time via a direct stepping of potential from 0 to -0.45 V. Analysis of this information typically results in a surface coverage of ~1.5 x 10^{12} DNA hybrids cm^{-2}. This hybrid density has been shown to be optimal for the use of C12Viologen (see below).

Another aspect of the CV plot that show that dsDNA was properly immobilized is that the peak potential difference between reduction and oxidation is less than 59 mV. Here, the figure shows that it is approximately 20 mV. 59 mV is the expected difference if the molecule would diffuse to the surface in accordance with the Nernst equation, while 0V represents a molecule that is tethered to the electrode surface and cannot diffuse. Here, Ruhex is not perfectly tethered to the electrode, so the difference suggests a difference in the interaction between the +3 and the +2 forms. Indeed, this is also manifested in the magnitude difference of the reduction and oxidation peaks. The smaller oxidation suggests that the +2 form does not

interact with the DNA as strongly as the +3 form, which is expected based on the primarily electrostatic interaction. Overall, the importance of this data is merely to show that dsDNA hybrids are formed on the electrode surface for subsequent DNA analysis.

3.2 Electrochemical DNA Methylation Analysis Results

As discussed in Chapter 1, SWV is a potential step method where current is measured after both a forward and reverse potential pulse and the plotted output is the difference between the two.[3] The technique is more sensitive than CV, as the difference technique removes the contribution from the charging current.[3] This allows one to monitor the electron transfer process

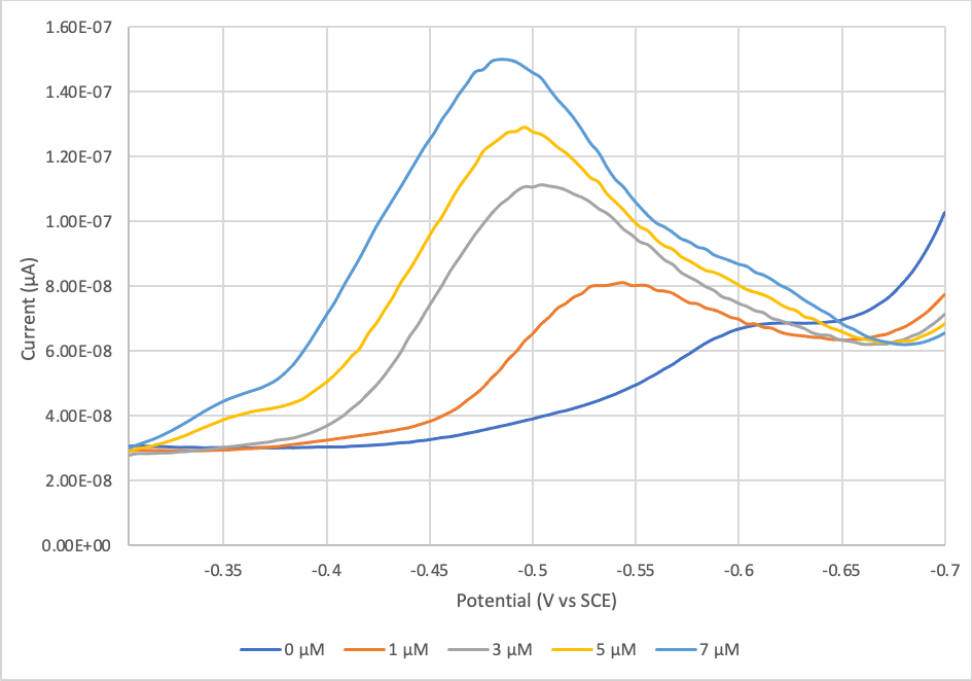


Figure 3.2. Raw SWV overlay showing the response of 0-me DNA after the addition of 0 µM (blue), 1 µM (orange), 3 µM (gray), 5 µM (yellow), and 7 µM C12 Viologen.

of interest vs. other contributors. After confirming the presence of dsDNA, C12Viologen was used to detect methylation-based dsDNA changes. Our strategy to accomplish this was to utilize C12Viologen and its ability to bind to DNA in different ways as a DNA structural probe as

compared to Ruhex, which is attracted to DNA primarily based on electrostatic attraction. Figure 3.2 shows a raw SWV plot of the tested DNA sequence containing no cytosine methylation (0-me dsDNA) exposed to C12Viologen from 0-7 μM. The figure shows how C12Viologen seems to bind in two different populations: one around -0.6 V emerging early and saturating and one around -0.48 V. This dual wave voltammetry for C12Viologen suggests that it is bound in two environments. We have previously shown that these environments are an electrostatically bound species that is reduced at -0.48 V, which is close to the formal potential of the viologen compound without DNA present, and a minor groove bound species that is reduced at more negative potentials. The shift to more negative potentials suggests that the oxidized form of the viologen is engaging in stabilizing interactions offered by groove binding.[4]

After obtaining the 7 μM C12Viologen SWV scan, 100 mM $MgCl_2$ was added to the solution. This was done to expose the DNA to high ionic strength and to promote any morphology changes that accompany this ionic strength exposure. SWV were acquired after addition of the $MgCl_2$ at specific time intervals from 0 – 600 s. These SWV plots were overlayed and an example of this for the same 0-me dsDNA as outlined in Figure 3.2 is shown in Figure 3.3a.

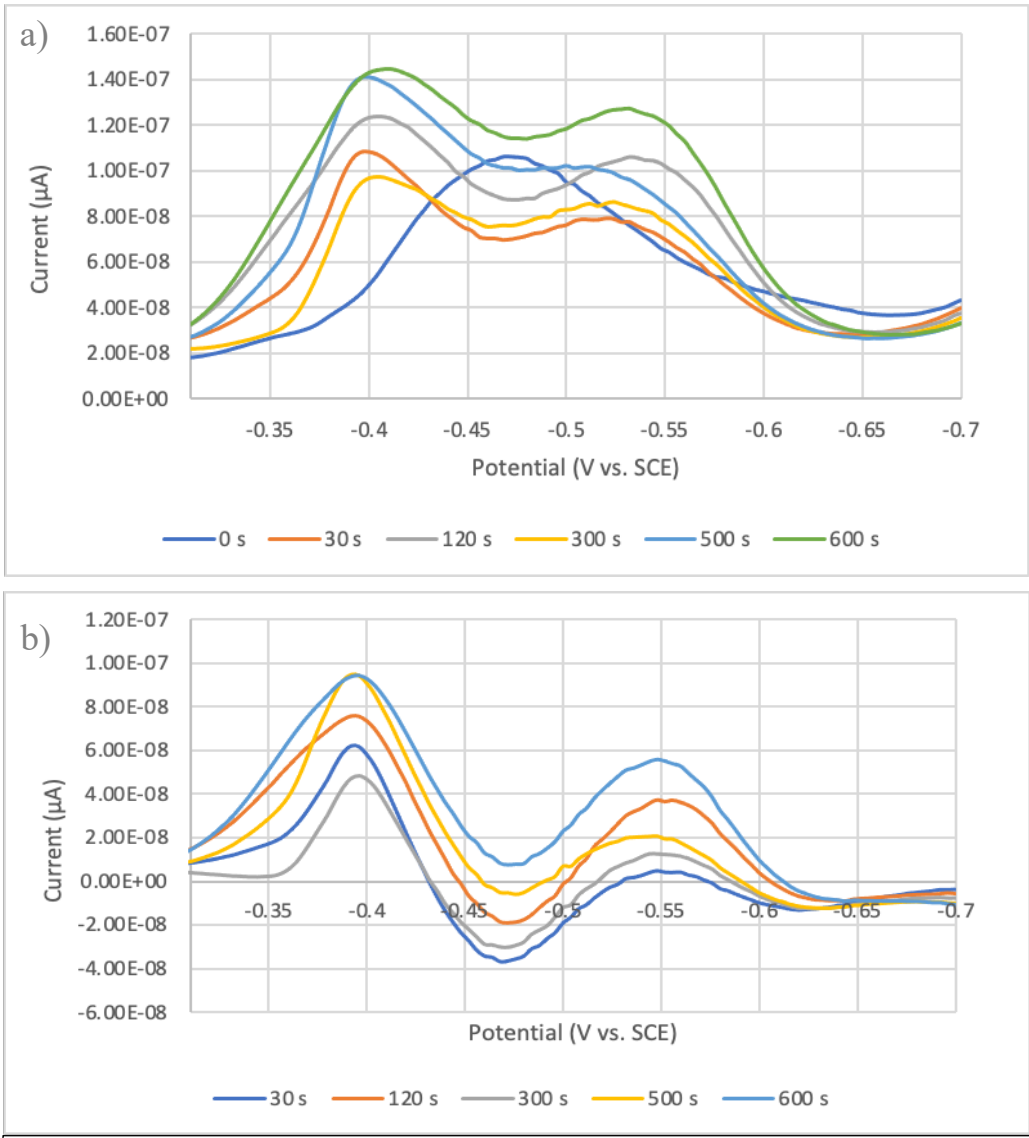


Figure 3.3a. Raw SWV overlay showing the response of C12Viologen at 0-me dsDNA after addition of 0.1 M MgCl2 at 0 s (blue) 30 s (orange), 120 s (gray), 300 s (yellow), 500 s (blue), and 600 s (green). **b.** Background subtracted SWV overlay showing the same timed responses subtracted from the 0s response with 30 s (blue), 120 s (orange), 300 s (gray), 500 s (yellow), and 600 s (blue).

To see the potential changes more clearly, the 0s SWV was treated as baseline and subtracted from the subsequent timed plots. Figure 3.3b shows these difference plots from 30 – 600 s $MgCl_2$ exposure to the DNA. Data plotted in this manner shows the differences in specific potentials, which is indicative of the changing C12Viologen-DNA binding environment. For

instance, decreases at -0.48 V suggest that a significant number of viologen bound in this environment before $MgCl_2$ exposure are not bound there anymore. Growth at the -0.38 V and -0.55 V potentials suggest increases in different C12Viologen bound populations on the DNA. We turn to what these potentials suggest about the DNA structure in the discussion below, but overall, this shows that the $MgCl_2$ influences the DNA oligomers. This same process to immobilize the DNA, "saturate" it with C12Viologen, and then expose it to $MgCl_2$ was repeated with the other tested oligomers containing 1, 2, and 4 methylated cytosines in the sequences. The difference SWV plot results using 4-me dsDNA are shown as a comparison Figure 3.4. The figure shows that the response from MgCl2 exposure for the 4-me containing oligomer is significantly different than when the DNA did not contain any cytosine methylation.

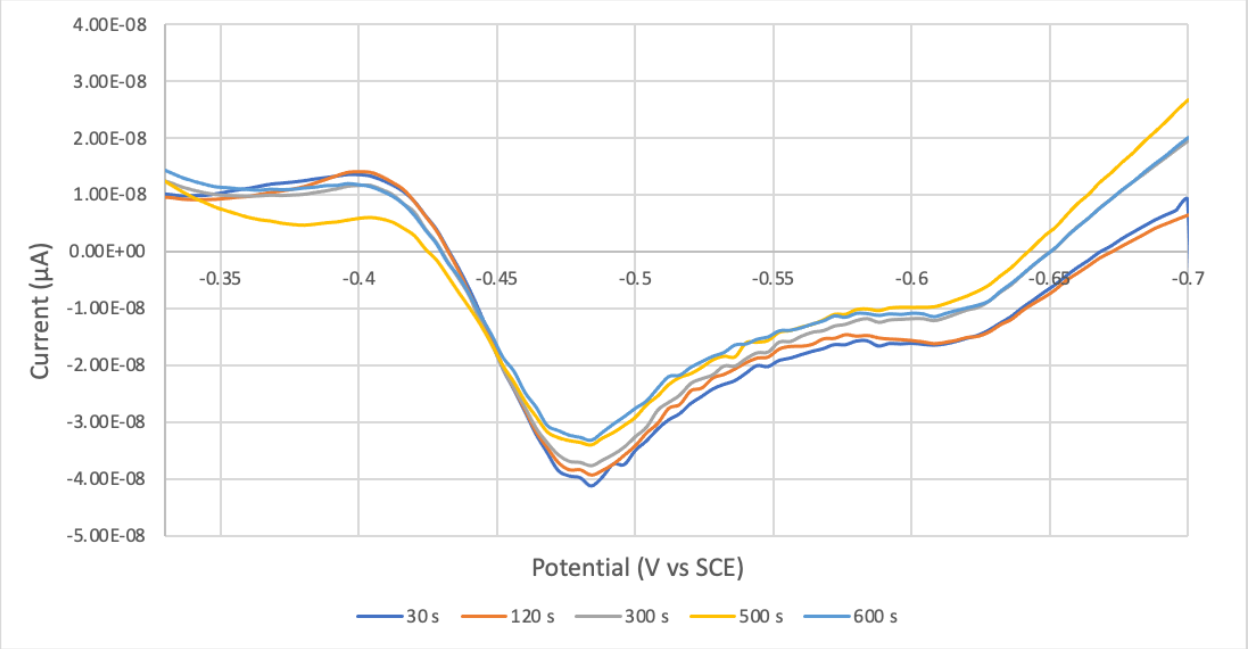


Figure 3.4. Background subtracted SWV overlay showing the response of C12Viologen at 4-me dsDNA after addition of 0.1 M MgCl2 subtracted from the 0s response with 30 s (blue), 120 s (orange), 300 s (gray), 500 s (yellow), and 600 s (blue).

The relatively unchanging plot suggests that the changes induced by $MgCl_2$ exposure occur very quickly.

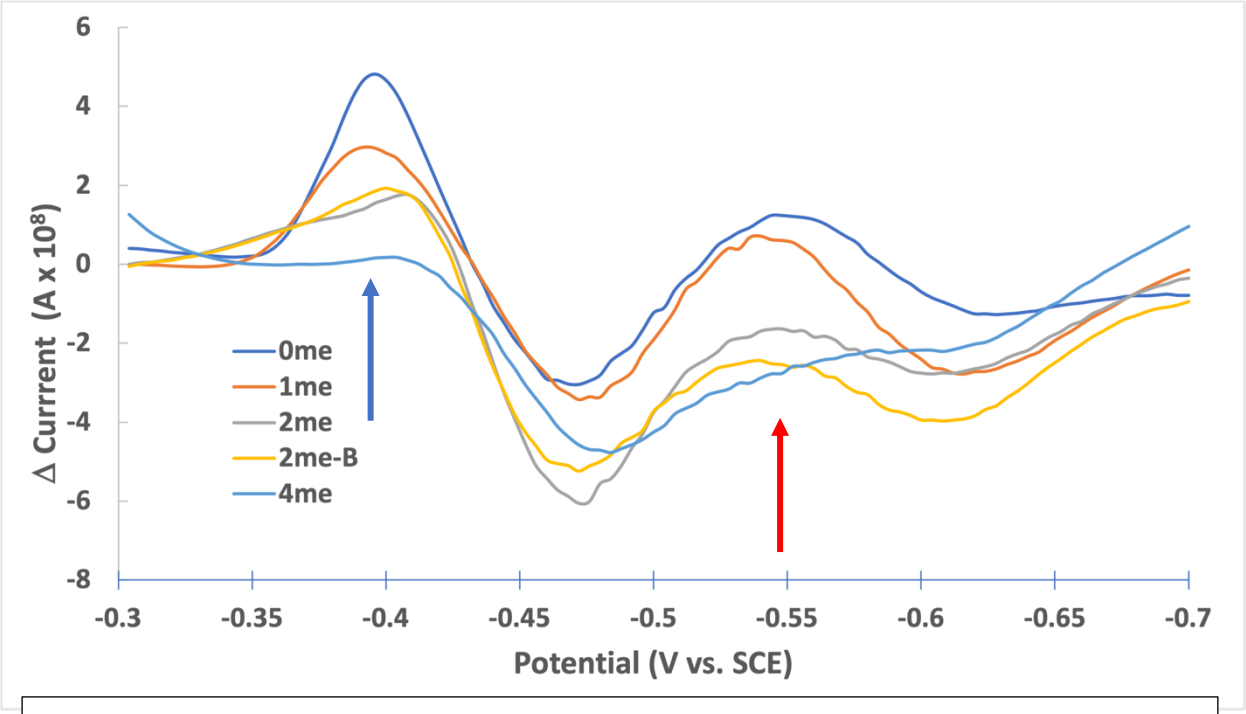


Figure 3.5. Background subtracted SWV overlay showing the responses 0-me (blue), 1-me (orange), 2-me (gray), and 2b -me (yellow), and 4-me (blue) dsDNA with C12Viologen at 300 seconds after 0.1 M $MgCl_2$ has been added.

The difference SWV for all methylated oligomers at 300 s $MgCl_2$ exposure is shown in Figure 3.5. The oligomers tested had 0, 1, 2, or 4 methylated cytosine modifications. The oligomers containing 2 methyl groups were split into two sets. The oligomer labeled 2-me contained one methyl groups near the 5' and 3' ends of the oligomer, while the 2b oligomer contained the methylation near the middle of the sequence. The sequences are outlined in Chapter 2.

The figure above shows how $MgCl_2$ affects the methylated oligomers each in a slightly different manner. The changes in the three main potentials (~ -0.38 V, -0.48 V, and -0.55 V) are evident in the plots. The general trend is a decrease at the -0.48 V for all oligomers, and methylation dependent trends of decreasing current at the -0.55 V (red arrow) and -0.38 V (blue

arrow) potentials. The current changes at either potential show clear methylation dependent responses, but the -0.38 V changes were more reliable and reproducible. Current changes at this potential show large changes at 0-me to no change when 4 methyl groups were tested. These are difference plots, so a flat line plot suggests no current change compared to before $MgCl_2$ addition – i.e., the range on the 4-me plot from -0.3 to -0.42 V. Figure 3.5 also shows that the difference between the 2-me modified oligomers was negligible, shown in the similarity between these difference plots. Because of this, these data were treated as one large 2-me group and not different types in the subsequent data analysis.

Analysis of the peak current at the most positive potential C12Viologen population (at -0.38 V, blue arrow in Figure 3.5) as a function of cytosine methylation for these oligomers is shown in Figure 3.6. The figure shows a clear current decrease at this potential based on cytosine methylation. Based on the mean i_p values and their respective standard deviations there was a statistically significant difference between 0 methylation and all methylation modifications, as well as between 1-2 and 1-4 methylation modifications ($p < 0.02$). The difference between 2 and 4 methyl modifications was not quite statistically significant ($p < 0.06$). The least squares analysis of the Figure 3.6 plot allowed polynomial fitting, shown as the dashed line in the figure. The best fit equation was:

$$i_p = 4x10^{-9}\,[me]^2 + 3x10^{-8}\,[me] + 5x10^{-8}\ (R^2 = 0.99)$$

Here, i_p is the peak current and [me] is the methylation content in an oligomer. This fit would allow an estimation of the methylation content on similar DNA oligomers.

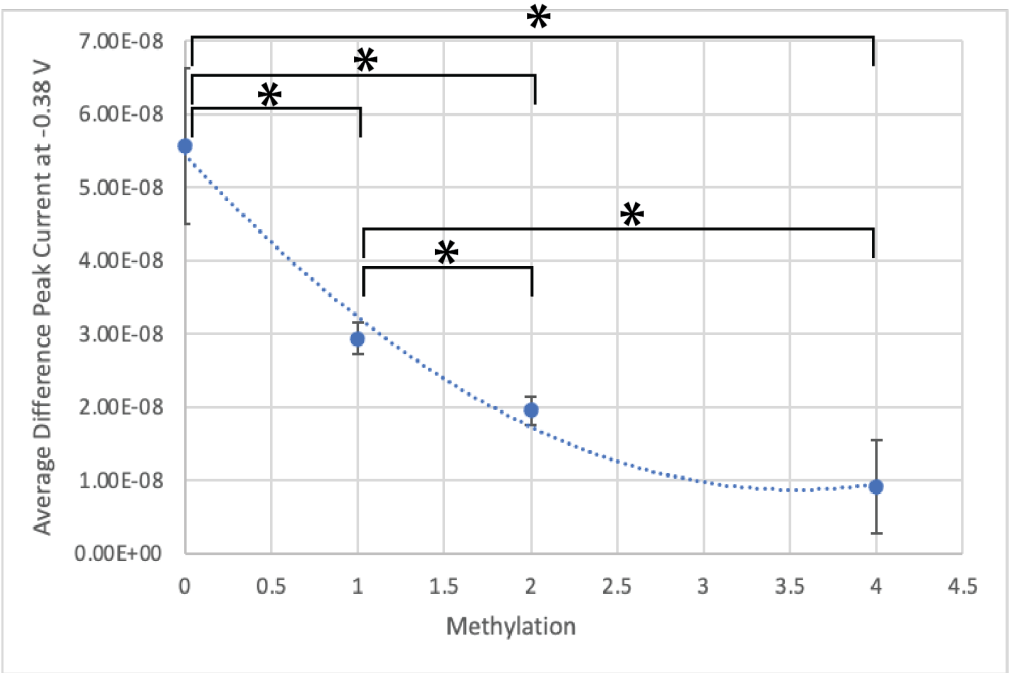


Figure 3.6. Average difference peak current analysis at -0.38 V as a function of cytosine methylation. Error bars represent s.d. for n = 3 trials. * Denote statistically significant differences ($p<0.02$).

3.3 Discussion of Electrochemical DNA Methylation Results

It has been previously shown that changes in currents at different potentials upon C12Viologen reduction in the presence of DNA suggest that the binding environment has changed for the viologen molecule as a result of DNA structural changes. Changes at the -0.38 V potential were shown to arise after DNA damage with benzo[*a*]pyrene (B[*a*]P) metabolites. B[*a*]P is known to bind to DNA in a number of ways, one of which results in a protrusion of the hydrophobic molecule into the minor groove. This was postulated to provide a hydrophobic external DNA binding site where the C12Viologen could aggregate. Reduction of the individual viologen groups to their respective cation radicals ($V^{2+} + e^- \rightarrow V^{\bullet+}$) allowed viologen subunits to

form stabilizing dimers via overlap of now singly occupied π* orbitals.[4] This resulted in a large increase at potentials positive shifted of the formal potential (~ -0.48 V vs. SCE).[4] The positive potential shift compared to the formal potential is due to the stabilization of the reduced radical cation $V^{\bullet+}$ state based on dimer formation over the oxidized +2 state.[4-6] Currents at -0.48 V suggest that C12Viologen is bound electrostatically to the DNA based on phosphate charge compensation, and as mentioned previously, currents at -0.55V are due to more stabilizing interactions, also related to π* dimer formation, but within the minor groove of the DNA. The negative shift is due to the +2 state stabilization via favorable interactions offered by the DNA groove.[4]

These previous findings offer some insight into how C12Viologen behaves at methylated DNA oligomers based on the data acquired here. We see the prominent $MgCl_2$ induced current changes occur at -0.38 V, which suggests that high ionic strength conditions impact some external site on the DNA helix that can facilitate viologen π* dimer formation upon reduction. This site is enhanced in cases where there is less cytosine methylation as compared to more. Additionally, C12Viologen seems to bind in a +2 stabilized population resulting in increases at -0.55 V, once again in a methylation dependent manner enhanced by less methylation on the oligomers (Figure 3.5).

Exposure to high ionic strength conditions has been shown to facilitate the B- to Z-form transition for short highly methylated oligomers but not for the non-methylated analogs.[7] High ionic strength conditions leading to conformational transitions are thought to play a major part in methylated DNA packing within chromatin.[8] Based on these reports it was expected that $MgCl_2$ exposure would lead to a similar transition for the methylated oligomers bound on the gold electrode studied here, and due to the ability of C12Viologen to interact with DNA based on

structural considerations, this would provide the means to detect methylated vs. non-methylated DNA sequences. The data shows, however, that exposure to the high ionic strength solution promoted the most C12Viologen electrochemical change and presumably DNA structural changes in the non-methylated oligomers vs. methylated ones.

To make sense of this seeming anomaly, it is important to note that the impacts of cytosine methylation on DNA structure are not straightforward, and significant differences have been determined. For instance, cytosine methylation has been shown to promote the Z-form transition, but it has also been shown to promote the A-form transition or no transition from the canonical B-form as well.[9] It has been shown that cytosine methylation provides very little structural changes to the DNA outside of impacts in the sugar-phosphate backbone.[9] Additionally, it has also been shown that cytosine methylation can provide stabilizing or destabilizing impacts to DNA as measured by thermal melting or force experiments based on the overall DNA sequence.[10] In short DNA sequences, methylation seems to increase rigidity in the oligomer as the increased hydrophobicity forces the oligomer to adopt a somewhat protective conformation to reduce the aqueous exposure to the alkyl groups in the major groove.[9,10] However, this can lead to enhanced flexibility if the DNA bends at the methylated site, and this has been shown to impact DNA and how it can adopt tight turns in chromatin packing.[8]

It is the enhanced rigidity imparted by the methylation that is important here as it presumably significantly impacts the oligomers immobilized on the electrode, which provides an explanation to the observed C12Viologen electrochemical responses. First, it is highly unlikely that the $MgCl_2$ is causing a major B- to Z-form transition in these immobilized oligomers, which is elucidated further in Chapter 4 describing CD spectroscopy experiments.

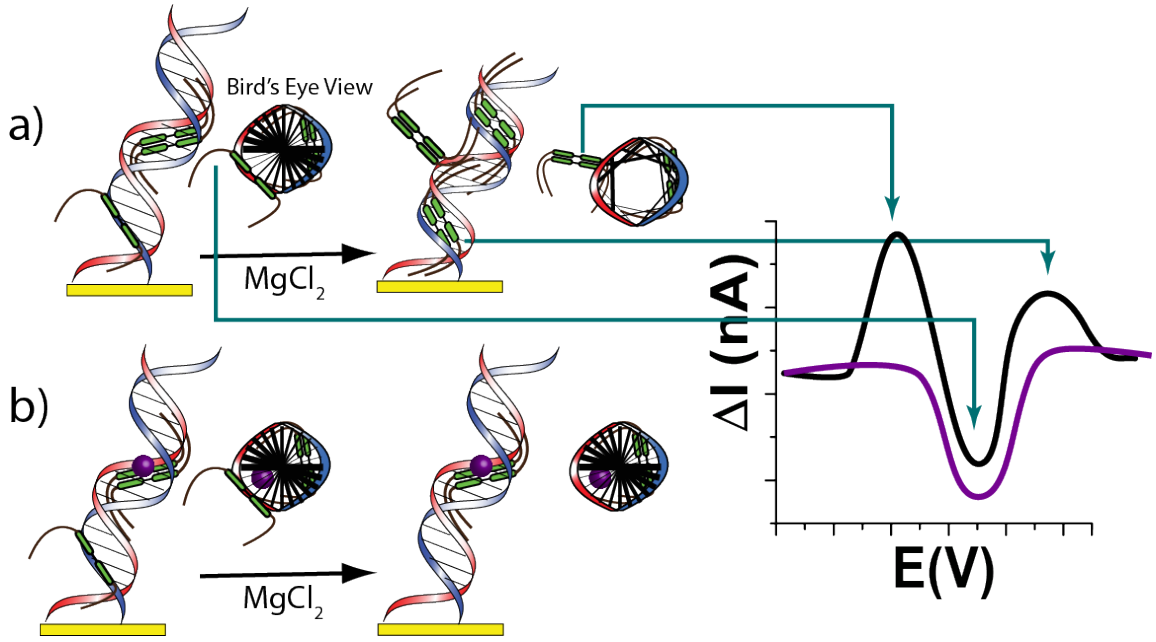
Based on the electrochemical results, however, it is likely that the higher ionic strength imparted by the addition of the Mg^{2+} does cause some transition of the unmodified DNA. Electrochemical results here suggest that the $MgCl_2$ exposure causes less morphological DNA change as cytosine methylation content increases. This can be explained by an oligomer shortening to more of an A-form, which has been shown under high ionic strength based on elimination of water and screening of anionic charge from the binding of the higher charge cations.[8, 11] Increasing the salt molarity from 0.01 M to 0.1 M as used here has shown to decrease DNA length by 20% for monovalent ions, and this is even more pronounced for divalent ions. If the DNA is shortened, this forces the bases within the DNA to shift, and it has been shown that this shortening is accompanied by the bases shifting toward the major groove causing a narrower, but deeper, minor groove and a shallow major groove. Here, the major groove becomes shallower as the bases protrude almost to the edge of the helix, and the minor groove becomes deeper.[12]

This speculation is rather preliminary, but considering these possible morphology changes, the protrusion of the bases to the edge of the major groove could provide the external aggregation site for the C12Viologen that results in the large -0.38 V population upon reduction after Mg^{2+} exposure. The narrowing and deepening of the minor groove would impart the helix with favorable dimensions to promote minor groove binding of the C12Viologen, which would cause an increase of the negatively shifted -0.55 V population at that site upon reduction following Mg^{2+} exposure.

Current increases at the -0.38 V location decrease as methyl groups are added and very little change from 0 s to 300 s Mg^{2+} exposure was seen at that potential when 4 methyl groups were present on the oligomers. The decreases in current as a function of cytosine methylation

suggests that the added ionic strength simply does not affect the DNA morphology as much compared to when no methyl groups were present. Comparing 0 me to 4 me, the 4 me oligomer does not change significantly over the monitored time to acquire SWV plots outside of a decrease at the -0.48 V potential. Decreases at -0.48 V are likely the removal of electrostatically bound C12Viologen replaced by a divalent cation with centrally located charge. This type of charge compensation is similar to Mg^{2+} displacing Na^{+} as predicted by counterion condensation theory and has been seen when using cations like spermine/spermidine that feature multiple positive charges spread over a longer structure.[13, 14]

The methylation dependent decreases in current change up to 300 s on the SWV difference plots suggests that addition of methyl groups in this environment counters any DNA structural changes that would be facilitated in the absence of methylation. Methylation causes



Scheme 3.1. Scheme comparing electrochemical response of a) 0-me dsDNA versus b) 4-me dsDNA after the addition of C12Viologen and $MgCl_2$. Methyl groups are demonstrated by the purple spheres. C12Viologen is demonstrated by the green molecules.

rigidity in the DNA sequence, and it is possible that this imparted rigidity counters the DNA shortening and adopting the base shifts as the methylated DNA adopts structures to keep water out of the hydrophobic major groove. If methylated DNA remains somewhat unchanged as a consequence of the added hydrophobicity, this could explain the lack of current change seen when C12Viologen was reduced in the presence of the modified DNA oligomers vs. the unmethylated ones.

A scheme attempting to tie together the electrochemical data with these DNA biophysical explanations is seen in Scheme 3.1. The scheme provides a comparison of DNA morphological changes (both side and top view) resulting in the electrochemical responses for 0-me (a) versus 4-me dsDNA (b) before and after the addition of $MgCl_2$. The scheme outlines how unmodified DNA becomes shorter with altered bases resulting in changes in C12Viologen binding. For unmethylated DNA, the base shifts provide both the hydrophobic external site that causes the -0.38V and -0.55 V current increases denoted with arrows on the SWV plot. For methylated DNA oligomers, the methylation causes the DNA to be rigid and unchanging, which results in minor SWV changes upon $MgCl_2$ exposure. The primary difference is loss of electrostatically bound viologen, denoted with the arrow to -0.48V. This loss is seen in the 0-me case as well, as it is simply a result of screening anionic phosphate groups and not a methylation related process.

3.4 Conclusions

It was expected that the current changes would be dependent on methylation as compared to the lack of it. It was expected that the DNA morphological changes would be more significant due to addition of methylated cytosines. That simply was not the case here. However, C12Viologen was useful as an electrochemical probe to assess oligomer methylation content,

and it was seen that a statistically significant difference in current at -0.38V was noted when comparing unmethylated oligomers to those that have at least one methyl modification. Based on presumably subtle DNA morphological changes based on methylation content in the presence of higher ionic strength, this electrochemical assay is a very convenient and sensitive method with which to discern the content of cytosine methylation within DNA oligomers. Subsequent chapters provide additional evidence showing that any morphological changes in the oligomers upon exposure to Mg^{2+} are not dramatic, which shows the utility of this electrochemical assay and lends promise that it could be used as a hypermethylation detection strategy.

3.5 References

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CHAPTER 4: SPECTROSCOPIC STUDIES

4.1 CD Spectroscopy

The electrochemical sensor responses outlined in Chapter 3 suggest that cytosine methylation leads to a detectable response difference that can be monitored. The electrochemical response differences were facilitated via exposing the DNA to higher ionic strength conditions. The reason why $MgCl_2$ was chosen to facilitate any methylation-dependent changes in the DNA is because methylated DNA has been shown to adopt different conformations such as the left-handed Z-form where non-methylated DNA has been shown to stay in the right-handed B-form upon exposure to high ionic strength. Ionic interactions are much stronger using divalent cations vs. monovalent ones. Therefore, it was envisioned that the addition of $MgCl_2$ would impact the ionic strength and promote a helical transition that could be detected using the C12Viologen. To determine the structural changes that are induced in the oligomers via the exposure to Mg^{2+}, circular dichroism (CD) spectroscopic studies were performed. As mentioned in Chapter 1, CD is an extensively used tool to determine the structure of DNA by monitoring its absorbance of rotating, plane polarized light. These measurements have been used to study DNA structure and conformational changes that result from environmental changes, such as temperature, pH, and ionic strength.[1] To perform validation, we attempted to follow previously published CD studies while exposing our DNA oligomers to similar conditions as utilized in the electrochemical studies. DNA oligomers containing between 0-4 cytosine methyl groups were exposed to increasing concentrations of $MgCl_2$ ranging from 0 mM to 800 mM and monitored from 210-350 nm. In Figure 4.1, the CD spectra of 0-me dsDNA before and after the addition of increasing concentrations of $MgCl_2$ is shown.

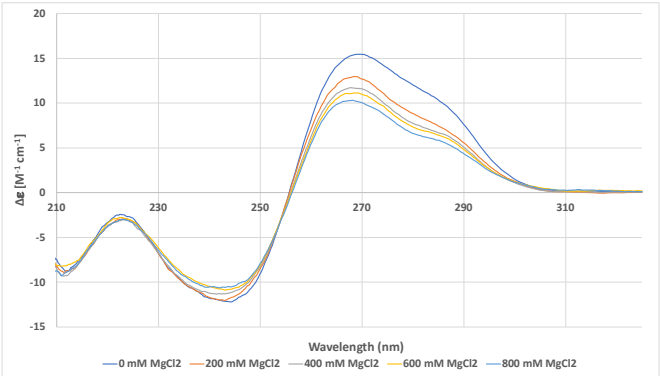

Figure 4.1. CD spectra showing the responses of 0-me dsDNA after the addition of 0 mM $MgCl_2$ (blue), 200 mM $MgCl_2$ (orange), 400 mM $MgCl_2$ (gray), 600 mM $MgCl_2$ (yellow), and 800 mM $MgCl_2$ (blue).

CD spectra are wholly dependent on DNA structure and sequence.[1] Genomic DNA from different species generate slightly different spectral features based on the base pair composition.[1] Figure 4.1 shows a standard response for DNA that is relatively rich in GC base pairs. The oligomers used here are 76% GC content, and the response mirrors that from genomic DNA reflecting the same content.[1] The important aspects of the spectra are the negative band ca. 245 nm and the longer wavelength positive band ~268 nm with the prominent hump at ~285 nm. Upon exposure to increasing concentrations of Mg^{2+}, the negative band increases slightly, while the positive bands decrease. In addition to the magnitude changes in these bands, there is a slight blue shift in the positive bands by a few nm. For instance, the strong positive band absorbance at 268 nm shifts to 265 nm at the highest salt concentrations here.

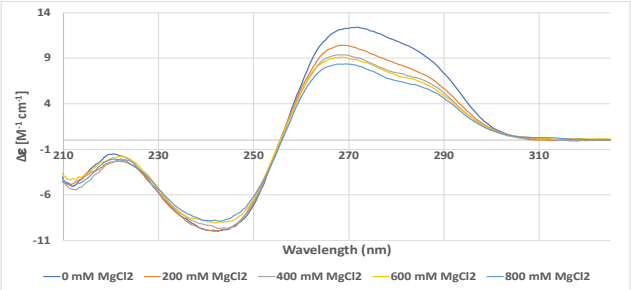

Figure 4.2. CD spectra showing the responses of 4-me dsDNA after the addition of 0 mM $MgCl_2$ (blue), 200 mM $MgCl_2$ (orange), 400 mM $MgCl_2$ (gray), 600 mM $MgCl_2$ (yellow), and 800 mM $MgCl_2$ (blue).

The response for the 4-me dsDNA oligomers under the same conditions is shown

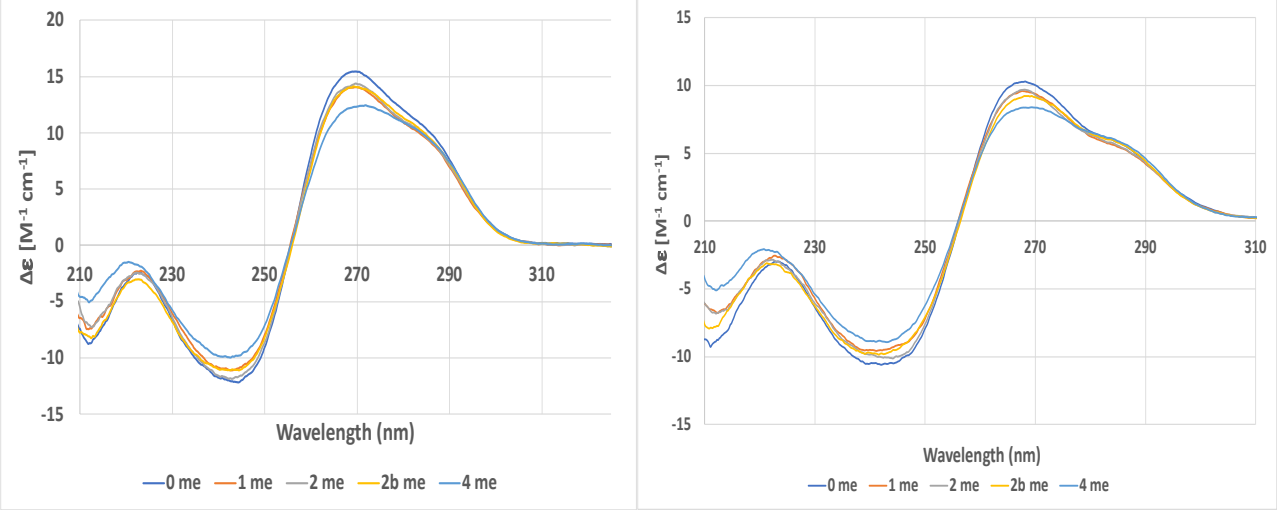

Figure 4.3. a) CD spectra showing the responses of 0-me (navy), 1-me (orange), 2-me (gray), and 2b -me (yellow), and 4-me (light blue) dsDNA with 0 mM $MgCl_2$. **b).** CD spectra showing the responses of 0-me (navy), 1-me (orange), 2-me (gray), and 2b -me (yellow), and 4-me (light blue) dsDNA after the addition of 800 mM $MgCl_2$.

in Figure 4.2. The figure shows the same general response with a few minor differences. For instance, the intensity of the positive band at 268 nm is red shifted and less intense. This suggests some minor structural differences due to the presence of cytosine methylation in the oligomers even before $MgCl_2$ exposure. Overlays for all the oligomers before and after addition of 800 mM $MgCl_2$ are shown in Figure 4.3 a-b.

Table 4.1. Relationship between percent ellipicity difference and methyl cytosine content in studied oligomers.[a]

Oligomer Methylation Content	**% Difference in ellipticity[b,c]**
0 me	35.1
1 me	33.0
2 me	34.4
2b me	35.4
4 me	31.9

a – % difference before and after addition of 800 mM $MgCl_2$
b - at major positive band λ_{max}
c - n = 3, s.d. = +/- 0.02%

Figure 4.3 shows that the CD monitored changes to the oligomers before and after $MgCl_2$ exposure are slight. The most obvious change is the positive band ellipticity intensity decrease upon exposure to the salt. These percent decreases comparing before and after $MgCl_2$ exposure are summarized in Table 4.1. The decrease in the intensity in the λ_{max} ~268 nm peak is roughly similar for 0-me – 2-me oligomers but exhibits the least amount of change in 4-me oligomers.

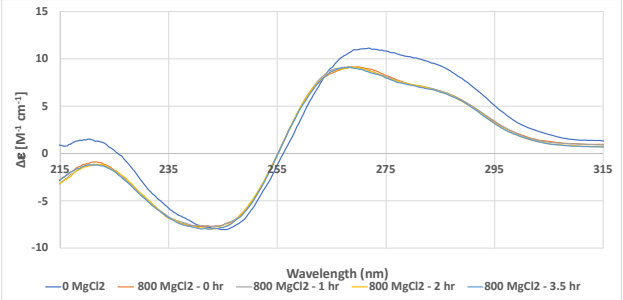

Figure 4.4. CD Spectra of 4-me dsDNA with no $MgCl_2$ (blue) and after the addition of 800 mM $MgCl_2$ at t = 0s (orange) or after t = 2.5 hours (green).

The impact of $MgCl_2$ exposure time on the DNA was investigated as well. The CD spectra results showing these data are plotted in Figure 4.4. The figure shows that the CD spectral changes from any DNA structural changes due to $MgCl_2$ exposure are immediate and essentially unchanging over time.

4.2 CD Discussion

The CD spectrum characteristics for these DNA oligomers are consistent with those seen from GC-prevalent DNA and similar synthetic oligomers.[1,2] GC rich DNA is known to adopt a slightly altered A-form configurations compared to more balanced base composition DNA in aqueous conditions.[1,2] A-form DNA exhibits specific CD spectral characteristics such as a significant positive band shifted toward 260 nm, a shallower negative 245 nm band, and a negative band at 210 nm.[1] Canonical B-form DNA features equally intense positive and negative bands at 275 nm and 245 nm, respectively, crossing over at 260 nm.[1,2] Based on this, it is clear that these oligomers do not adopt a canonical B-form nor canonical A-form under the conditions utilized here. Z-form DNA is characterized by a sharp negative band at 285-290 nm with a

positive band around 260 nm.[1] Similarly, based on these characteristics, it is clear that the DNA oligomers do not adopt the Z-form helix under the conditions utilized here. Previous studies have shown that cytosine methylation can promote Z-form transition, as well as A-form or induce no transition at all.[3]

The CD spectral characteristics recorded here are consistent with some A-form characteristics for each oligomer.[2] Additionally, the changes induced via exposure of the oligomers to the concentrations of $MgCl_2$ used here were gradual. It has been shown that any B- to A-form transition is cooperative and abrupt, and that gradual CD changes like those seen here are consistent with structural changes within the DNA form and not a transition to another form.[1,2] Therefore, these data are consistent with the oligomers likely adopting some partial A-form, or at least a non-canonical B-form initially before undergoing slight structural changes upon exposure to $MgCl_2$. These changes are less prevalent with increasing amounts of cytosine methylation.

Increasing mono- or divalent salt concentrations have been shown to force subtle helical changes based primarily on charge compensation with the anionic phosphates and more intricate water displacement considerations upon cation binding.[4] It was shown that increasing salt concentrations like what was used here caused a decrease in the positive CD bands with minimal changes elsewhere in the spectra.[5] The decreases in the ~268 nm band were thought to be due to changes in base pair winding and pitch angles moving along the helix. An older viewpoint of this change was the formation of a C-form DNA, which is now thought to be a subset of the B-form helix.[4,5] This form is BII, as opposed to the BI canonical form, and features fewer base pairs per turn (9 vs. 10), a shorter base pair rise and shorter DNA length, resulting in a double

helix with a deep and narrow minor groove and shallower major groove where bases are adjusted toward the outside of the helix.[4]

Overall, the CD spectra allow some elucidation for the electrochemical results of Chapter 3. The data here are consistent with $MgCl_2$ inducing subtle changes similar to what was described when transitioning from a BI to BII helical form. The important aspects of this transition are that the DNA length decreases slightly, which is accompanied by a narrow and deep minor groove with base displacement toward the major groove. Cytosine methylation likely prevents some of these changes in a complicated exchange preventing DNA length decrease (i.e. DNA rigidity) by fighting the displacement of the bases toward the major groove exterior.[6] This would force the methyl groups residing in the major groove to be more exposed to the aqueous exterior of the helix, which is thermodynamically unfavorable.[6] Electrochemically, it was noted that the most significant changes were seen for 0-me when exposed to the $MgCl_2$. The large positive shifted C12Viologen population seen for 0-me oligomers was postulated to be due to its aggregation on the exterior of the helix. If this DNA undergoes some transition where bases are displaced near the major groove exterior of the helix, this could explain the increased current seen upon monitoring 0-me oligomers. These changes were less prevalent as cytosine methylation increased, similar to what was seen here in the CD studies monitoring decreases at ~268 nm. Finally, a narrow, deep minor groove may facilitate binding of C12Viologen in that location and result in current increases at -0.55 V (see Ch. 3). Overall, while not initially obvious, the CD data here suggest subtle helical structural changes upon $MgCl_2$ exposure dependent on cytosine methylation and are consistent with the electrochemical results in Ch. 3.

4.3. UV-Vis Thermal Melting Studies

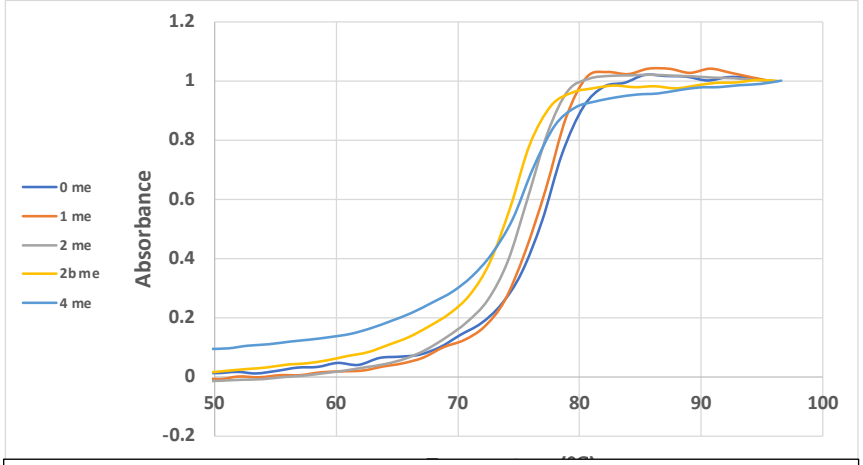

Figure 4.5 Normalized melting curve of 0-me dsDNA (blue), 1-me dsDNA (red), 2-me dsDNA (green), 2b-me dsDNA (purple), and 4-me dsDNA (blue).

UV-Vis Thermal melting experiments were also conducted on the dsDNA oligomers in order to study the stability of the DNA. Of interest was whether the stability would be impacted by the addition of methyl groups to the DNA. The absorbance was monitored at 260 nm as a function of temperature. Melting plots can be seen in Figure 4.5 for the 0-4-me dsDNA.

The data shows how the melting of the helices is impacted by the inclusion of cytosine methylation. These types of melting curves allow comparisons of the relative DNA helical stability under identical conditions. The melting point (T_m) for a helix is taken at the point where the dsDNA is 50% unwound, roughly the temperature at 0.5 along the y-axis for the melting curve. The curves show that the melting process is cooperative resulting in a two-state process. The steepness of the plots denotes the ease in which the helices transition from duplex to ssDNA. The plot shows that the melting of the DNA exhibits some dependence on the presence of cytosine methylation, with 4-me DNA melting at 74.5 °C, followed by 2b-me at 74.3 °C, 2-me at 75.7 °C ,1-me at 76 °C, and finally 0-me, the most stable, melting at 76.9 °C under these conditions. The odd response from the 2b-me oligomer with split methyl groups along its

structure may indicate a more intricate melting process for this oligomer. Regardless, the stability of the dsDNA was subtly affected by the presence of methyl groups.

4.4 UV-Vis Thermal Melting Discussion

A difference in response dependent upon the number of methyl groups present on an oligomer can be seen using the electrochemical technique described in Chapter 3. These differences in response were able to be identified by utilizing the hydrophobicity of methylated DNA compared to non-methylated sequences under higher ionic strength conditions, used to promotes helical transitions. UV-Vis thermal was used to determine if the stability of the oligomers was affected by the number of methyl groups present. The data suggest that as more methyl groups are added, the stability of the oligomers decreases. Because this experiment was not run under similar conditions as the electrochemistry and CD spectroscopy previously mentioned, it cannot be determined whether the addition of $MgCl_2$ affects the stability of the oligomers differently.

Though there was a slight difference in melting temperatures, there was not a dramatic difference between the melting temperatures of 4-me dsDNA, at 74.5 °C, and 0-me dsDNA, at 76.9 °C. It would be difficult to use thermal melting as the means to detect cytosine methylation in similar short DNA sequences. In fact, T_m experiments like this have shown that cytosine methylation may impart stability or destabilize the duplexes, and this depends on base sequence.[3] Coupled with the slight changes that were detected comparing 0-4-me dsDNA using CD spectroscopy, this shows how the electrochemical assay ultimately provides a uniquely sensitive and convenient method with which to discern the methylation status of DNA oligomers.

4.5 References

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CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

5.1 Future Studies

The electrochemical detection method described in this paper outlines the means to detect differences in DNA methylation by utilizing the morphological changes of DNA caused by increased ionic strength of solution. DNA methylation plays a key role in gene expression, as well as the development and functioning of the adult brain.[1,3] Because of the role it plays in such processes, DNA methylation has been implicated in the development of diseases, such as cancers, autoimmune disorders, and neurodegenerative diseases.[2,4] Hypermethylation has been connected to many such diseases, so being able to detect such methylation patterns early on could allow for an earlier diagnoses of said diseases. This, in turn, would allow for better treatment and management of the disease.

Electrochemistry has shown promise as a relatively low cost, rapid, and simple method to detect DNA morphologies.[5,6] As such, new techniques are continually being developed, such as the one discussed here, in the hopes that electrochemical sensors could potentially be used as a point-of-care clinical tool. More work is needed, however, to bring such sensors to that point. For this technique to be used as a diagnostic tool, testing of this technique using human versus manufactured DNA is needed. Real-time and advanced sampling strategies will also be needed in order to prevent false negatives. A sensor which is able to fully run on its own without human intervention would also be warranted to complete routine methylation analysis on a much larger scale and consistently in clinics.

5.2 Conclusions

A new electrochemical technique was devised to detect different methylation statuses of DNA. The importance of DNA methylation in disease has been heavily indicated through numerous studies. Being able to detect DNA methylation before classic manifestations of disease are seen could allow for an early diagnosis and treatment of many different diseases, including MS. Hypermethylated DNA in CD8+ T cells have been implicated in those who suffer from MS and are speculated to play a role in the development of the disease. Here, an electrochemical technique to distinguish the number of methyl groups on CD8+ T cell DNA sequences was developed. The detection method developed here allowed a differentiation of methyl groups on DNA using C12Viologen, a redox active di-viologen molecule. This molecule has been shown to bind to DNA in a structure specific manner, allowing the study of DNA structural changes.

Methylated DNA sequence have been shown to undergo Z-form transitions from normal B-form helices after high ionic strength condition exposure.[7] We originally envisioned that this transition could be exploited by using $MgCl_2$ to detect electrochemically methylated oligomers using C12Viologen as it had been shown to be susceptible to DNA structure. The results showed that we were able to distinguish between oligomers with different methylation status electrochemically, with 0-me dsDNA undergoing the most dramatic transition and 4-me dsDNA undergoing the least.

CD spectroscopy was used to further study the DNA structure when exposed to MgCl2, and somewhat validate the sensor responses. These studies showed the that the transition from B-form to Z-form did not occur. Instead, the data were consistent with some partial A-form or an altered B-form, possibly BII, transitions in the DNA were likely taking place upon $MgCl_2$ exposure. CD spectroscopy also showed that the 0-me dsDNA underwent the most structural

change versus the methylated DNA. This supported the electrochemical data, which showed that the greatest structural change was seen with the non-methylated oligomer versus the methylated oligomers. Comparing 0-me to 4-me oligomers, the C12Viologen response at the 4 me oligomer did not change significantly upon SWV analysis over the monitored time outside of a decrease at the -0.48 V potential. Taken all together, these data are consistent with enhanced rigidity in the methylated oligomers versus the non-methylated oligomers. Methylation is known to cause rigidity in the DNA sequence, which is a result of the oligomer attempting to keep water out of the more hydrophobic major groove countering any morphology changes that DNA seeks to adopt due to the higher ionic strength of the environment.

Even though the expected Z-form transition did not take place, the electrochemical technique described here was able to detect differences in the methylation status of oligomers. Overall, it was shown that C12Viologen exhibits voltammetry differences based on the DNA target tested. $MgCl_2$ is shown to change the DNA structure on the electrode, as confirmed by CD spectroscopy. C12Viologen binding was affected by target strand methylation content, and the differentiation of the number of methyl groups on the DNA was possible using C12Viologen and SWV electrochemical analysis methods. This method has important ramifications for diagnostic tools, and further development may allow it to be used as a point-of-care clinical tool.

5.3 References

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