

ELECTROCHEMICAL DETECTION OF DNA METHYLATION AND IMPLICATIONS FOR
DETECTION OF CANCER ONSET RESULTING FROM HYPERMETHYLATION

by

Samantha Gonzalez

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by

Samantha Gonzalez

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Abstract:

Epigenetics refers to the process by which genetic material is modified without changing the base coding sequence.¹ One such epigenetic process is DNA methylation, employed by cells to regulate gene expression, often by silencing transcription, which subsequently impedes the expression of targeted genes.² Regulation via DNA methylation is a vital process in facilitating normal development. Abnormally high levels of DNA methylation within cells (ie. hypermethylation) can lead to disease, such as the development of various forms of cancer.³ For instance, hypermethylation in promoter regions coding for tumor suppressor genes results in deactivation of these regulatory genes, affecting downstream cellular processes, and allowing diseased cells to proliferate.²

One strategy for the early detection of cancer is to assess the amount of abnormal methylation at certain genomic loci.¹⁻³ This is typically performed via the use of various techniques, such as quantitative polymerase chain reaction (PCR) and reporters of genomic methylation (RGM).⁴⁻⁵ While these processes provide a significant amount of information, they demand substantial amounts of time, labor, and materials. Electrochemical sensor-based methods to detect DNA and DNA-related processes have the ability to remedy these drawbacks.⁶ Here, we developed an electrochemical sensor to monitor DNA methylation in DNA oligomers of known lengths and sequences with varying levels of methylation.

Gold electrodes were modified with 21-mer single stranded DNA (ssDNA) and then hybridized with complementary strands, creating surface bound double

stranded DNA (dsDNA). The complementary sequences were modified containing up to six 5-methyl cytosine (5-mC) locations. Electrochemical detection of DNA was accomplished utilizing an electrochemically active di-viologen compound, known as C12Viologen, that features differential binding to DNA based on conformation, which is altered by methylation.⁸

Cytosine methylation alters the structure of DNA in solution, which alters the binding of the C12Viologen compound in the oligomers.⁹ These changes in binding were detected using square wave voltammetry (SWV) by monitoring the differences in peak potentials upon exposure to high ionic strength conditions, which forces changes in DNA structure based on 5-mC content.⁹ We show that the electrochemical data demonstrates significant differences among unmethylated and methylated DNA samples.

Introduction and Purpose:

Within the large body of current cancer research, epigenetic processes are of particular interest, as such processes have the potential to modify genetic material and contribute to the onset of various diseases, including cancer.¹ This research focuses primarily on the epigenetic process known as DNA methylation. DNA methylation, at normal levels, operates as a regulatory mechanism necessary for proper human growth and development by silencing transcription and subsequently inhibiting the expression of targeted genes when necessary.¹ At abnormally high levels, DNA hypermethylation occurs, which causes the standard regulatory properties of methylation to be altered in such a way that its silencing

effects are amplified.² This amplification of regulatory processes via hypermethylation ultimately produces negative effects on human development. In the event that hypermethylation results in the silenced transcription for a tumor-suppressing target gene, the tumor-suppressing properties of such a gene would be inhibited, which would eventually lead to the development of cancer.²

As knowledge of the mechanisms of DNA methylation has developed, as well as knowledge of the importance that such a process plays in the development of various forms of cancer, subsequent effort has been directed towards methods of detection of DNA methylation.³⁻⁵ Such methods or techniques include quantitative polymerase chain reaction (PCR) and reporters of genomic methylation (RGM).⁴⁻⁵ These methods, while effective, have drawbacks in that they are time and labor intensive. This project proposes a method of detection of DNA methylation via the use of electrochemical sensors, which is generally less time and labor intensive.⁶ We hypothesize that gold surface electrodes will be effective in detecting DNA methylation by displaying notable differences within electrochemical data based on the various levels of methylation of prepared DNA samples.

Background:

While methylation commonly operates as a regulatory mechanism that ensures effective, functional optimization of gene expression, hypermethylation can contribute to the onset of harmful diseases or disorders caused by epigenetic irregularities.¹ Various types of cancer can result from hypermethylation which

silences transcription of tumor-suppressing genes, ultimately depriving those genes of their tumor-suppressing abilities.²

DNA methylation occurs frequently via methylation of cytosine units at the 5-C site (Figure 1).¹⁰ Various methods can be employed to effectively detect 5-methyl cytosine (5-mC) units on DNA. Such methods include polymerase chain reaction (PCR) and tagging approaches.⁴⁻⁵ While polymerase chain reactions and tagging methods are both effective in detecting DNA methylation based on the presence of 5-mC units, electrochemical detection methods do not exhibit the same drawbacks in terms of labor, supplies, and time.⁶

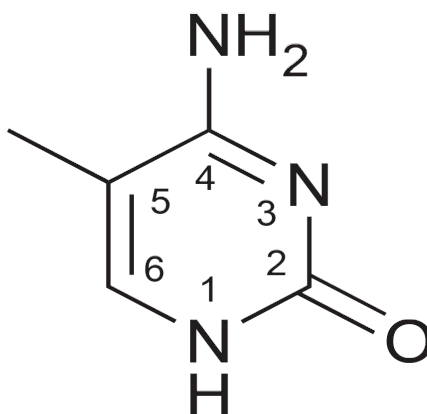


Figure 1: 5-methyl cytosine

One characteristic of electrochemical detection methods is that they involve very small quantities of chemical materials. This characteristic is desirable in terms of supply usage, but also requires the use of electrochemical testing to ensure that the desired molecules adhere to the surface of the electrode when necessary, as surface coverage cannot be determined visually. A ruthenium hexamine compound is used to accomplish the detection of appropriate electrode surface coverage

(Figure 2). After single stranded DNA (ssDNA) is applied, ruthenium hexamine is introduced to the electrode. Ruthenium hexamine will bind to surface bound DNA in certain regions, producing electrochemical signals different than those of DNA containing no bound ruthenium hexamine.¹¹ These signals indicate the amount of electrode surface bound ssDNA based on the quantity of ruthenium hexamine that interacts with surface bound DNA, indicating whether surface coverage is sufficient.¹¹

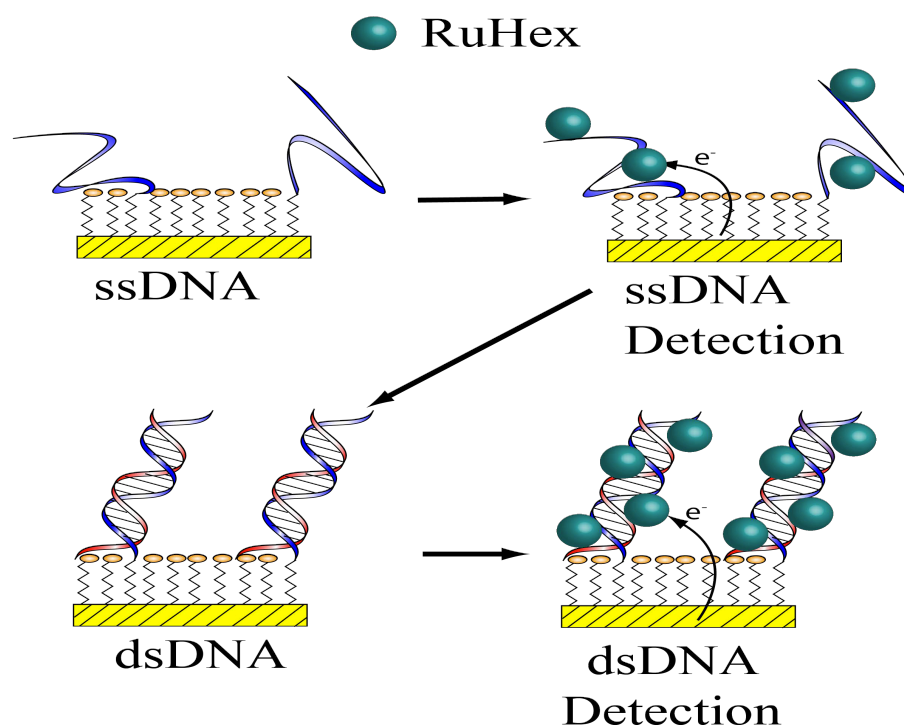


Figure 2: DNA detection on modified gold electrode using ruthenium hexamine.

When electrode surface coverage of ssDNA is too high, the ssDNA molecules are tightly packed on the electrode surface. As a result, hybridization can be

hindered, as complementary strands will have no available space among the tightly packed ssDNA. Conversely, when ssDNA surface coverage is too low, double stranded DNA (dsDNA) surface coverage is low as well, as each complimentary strand can only hybridize to produce one dsDNA oligomer for each surface bound ssDNA oligomer.

Once appropriate electrode surface coverage of ssDNA is verified and hybridization has been attempted via the application of a complementary strand to surface bound ssDNA, hybridization must be verified. Hybridization, like surface coverage of ssDNA, is verified via the use of ruthenium hexamine. The electrochemical signal collected from dsDNA is expected to be much larger than that of ssDNA. Because dsDNA has approximately twice as many suitable binding sites for ruthenium hexamine to bind to as compared to ssDNA, it is expected to produce a signal about twice as large (Figure 2).¹¹

The electrochemical detection method employed in this research involves methylation detection via the use of a bipyridine molecule known as C12Viologen (Figure 3). The C12Viologen molecule exhibits differential binding to DNA based upon DNA configuration.⁸ As a result of the alterations in DNA configuration caused by methylation, C12Viologen is expected to bind differently to methylated DNA as compared to unmethylated DNA.⁸⁻⁹

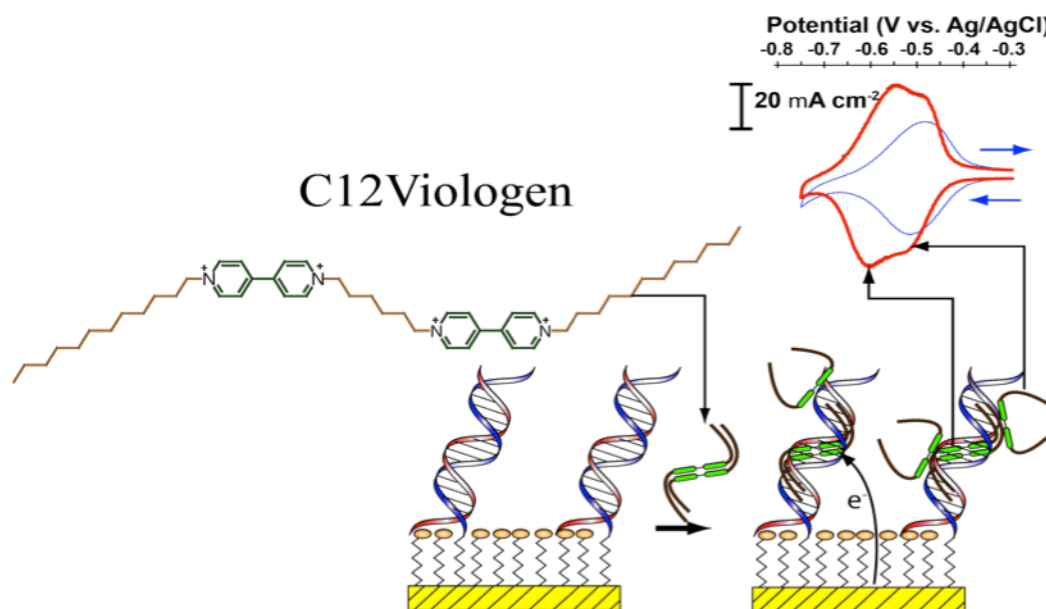


Figure 3: DNA detection using C12Viologen. On double stranded DNA, C12Viologen can show differential voltammetry based on how it binds in the helix.

Methylated DNA can exist in either B-form or Z-form.¹² Methylated DNA bound with C12Viologen hypothetically exists as methylated B-form DNA, while methylated DNA that contains no C12Viologen hypothetically exists as methylated Z-form DNA (Figure 4). Methylated B-form DNA can undergo a B to Z transition, transforming into methylated Z-form DNA via the addition of a high ionic strength salt, such as magnesium chloride (MgCl_2).¹² This B to Z transition of methylated DNA can be detected electrochemically, and will theoretically yield distinct signals for B-form versus Z-form methylated DNA (Figure 4). Unmethylated DNA is expected to display distinct electrochemical signals that do not correspond to the hypothetical expectations of the signals produced by methylated DNA.

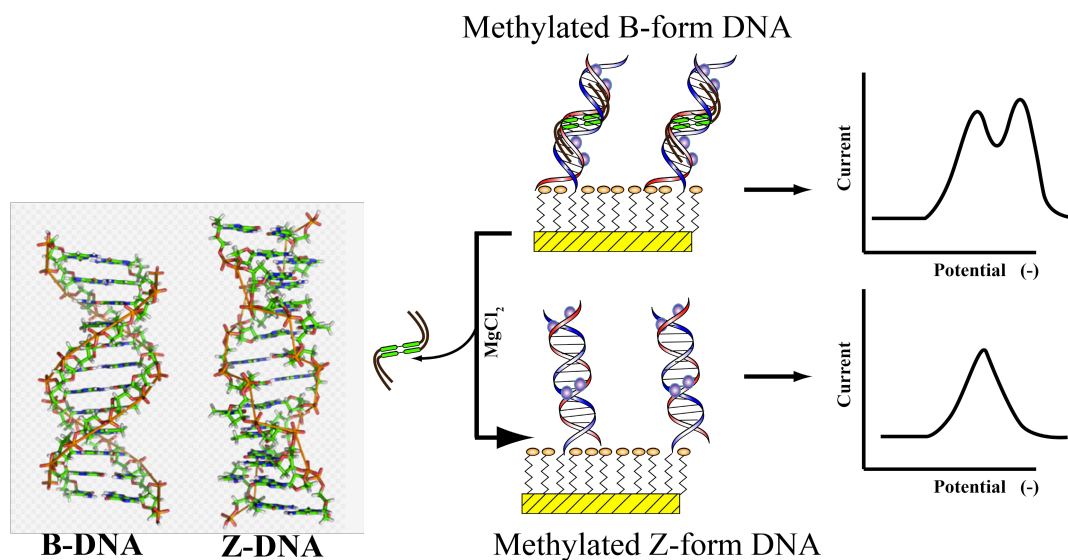


Figure 4: Hypothetical electrochemical detection of methylated DNA using B to Z transition. Methylated cytosine is represented by purple circles.

Materials and Methods:

Gold electrodes were cleaned with piranha solution, which was applied for a total of ten minutes to allow for sufficient removal of residual surface debris. Electrodes were then polished in figure eight motions in a series of three micro polishes to smooth the electrode surface.

Following cleaning and polishing, electrodes were modified with thiolated 16-mer ssDNA of a CG sequence (HS-(CG)), which was applied for 15-20 seconds.

Mercaptohexanol was used as a diluent layer. A 5 μ M solution was prepared by mixing 5 μ L of mercaptohexanol with 5mL of water. Following ssDNA

application, the electrode was rinsed before being suspended in the prepared mercaptohexanol solution for 30-40 minutes.

A standard electrochemical cell was constructed. The DNA modified electrode was used as the working electrode, a saturated calomel electrode (SCE) was the reference electrode, and a platinum wire served as a counter electrode. The electrodes were placed in 10 mL of 10 μ M NaCl buffer, which was purged with argon gas.

ssDNA surface coverage was assessed using ruthenium hexamine introduced to the electrochemical cell. Cyclic voltammetry (CV) and chronocoulometry (CC) were used to monitor electrochemical responses to the binding of ruthenium hexamine to surface bound ssDNA at various, increasing ruthenium hexamine concentrations (0, 1, 2, 5, 10 μ M).

After determining the ssDNA surface coverage, the electrodes were rinsed with 2M NaCl, and then exposed to complementary strands containing 0-4 5-methyl cytosine groups, creating surface bound double stranded DNA (dsDNA) with known levels of methylation. The DNA was in 1M phosphate buffer, pH 7.4. Hybridization took place for 2-3 hours at 37°C. dsDNA electrodes were rinsed with the Tris buffer and placed in fresh buffer in the electrochemical cell.

To determine DNA hybridization, ruthenium hexamine was introduced to the cell following the same protocol, and cyclic voltammetry and chronocoulometry were used to produce electrochemical data based on the binding of ruthenium hexamine to surface bound dsDNA. Signals collected from dsDNA were compared to those collected from ssDNA to verify DNA hybridization.

Upon determination of a sufficient hybridization efficiency, the electrochemical cell buffer was replaced and a bipyridine di-viologen compound, C12Viologen, was then introduced to the electrode at concentrations ranging from 1-5 mM, causing it to bind to surface bound dsDNA. Square Wave Voltammetry (SWV) was used to monitor the dsDNA surfaces in these steps. An initial SWV scan was obtained, and after it remained stable, MgCl_2 at desired concentrations (50-2000 mM) was added to the cell. SWV scans were acquired for each addition of MgCl_2 .

Results

Electrochemical data collected via cyclic voltammetry and chronocoulometry from ssDNA and dsDNA upon exposure to ruthenium hexamine is shown below (Figure 5). The data show an increase in the electrochemical signals produced by ssDNA versus dsDNA. The signals produced by dsDNA are much larger than those produced by ssDNA, verifying the increase in the number of bound ruthenium hexamine molecules by hybridized dsDNA. The data confirms successful DNA hybridization.

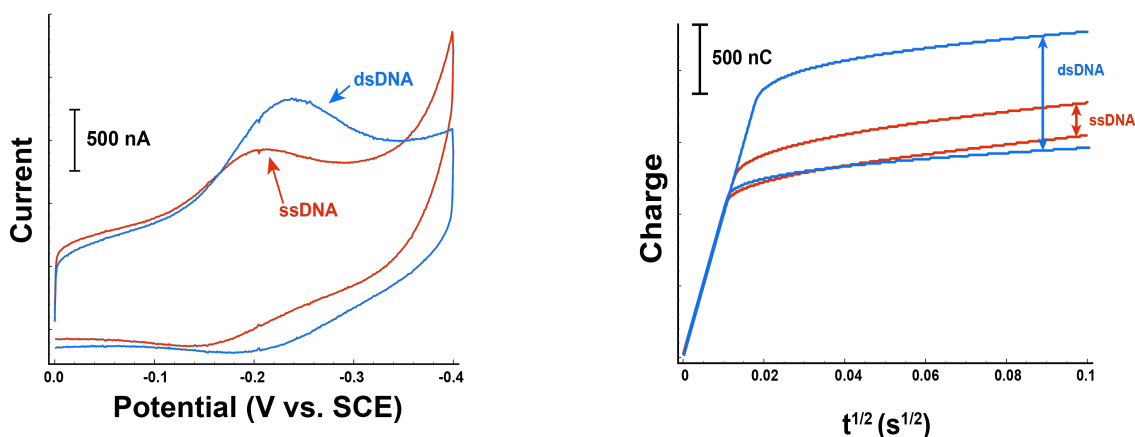


Figure 5: Electrochemical data collected via cyclic voltammetry (left) and chronocoulometry (right) was collected to verify DNA hybridization.

Square wave voltammetry (SWV) was used to electrochemically detect DNA methylation based on the response of bound C12Viologen to the addition of magnesium chloride. Raw data was collected following the introduction of C12Viologen to DNA at various, increasing concentrations of magnesium chloride (0, 50, 100, 500, 1000, 2000 μM MgCl_2) (Figure 6). The data collected from unmethylated (no 5-mC) DNA show an increase in the current at a potential of 0.52 V as the concentration of magnesium chloride increased (A). The data collected from methylated (four 5-mC) DNA show a decrease in the current at a potential of 0.52 V as the concentration of magnesium chloride increased (B).

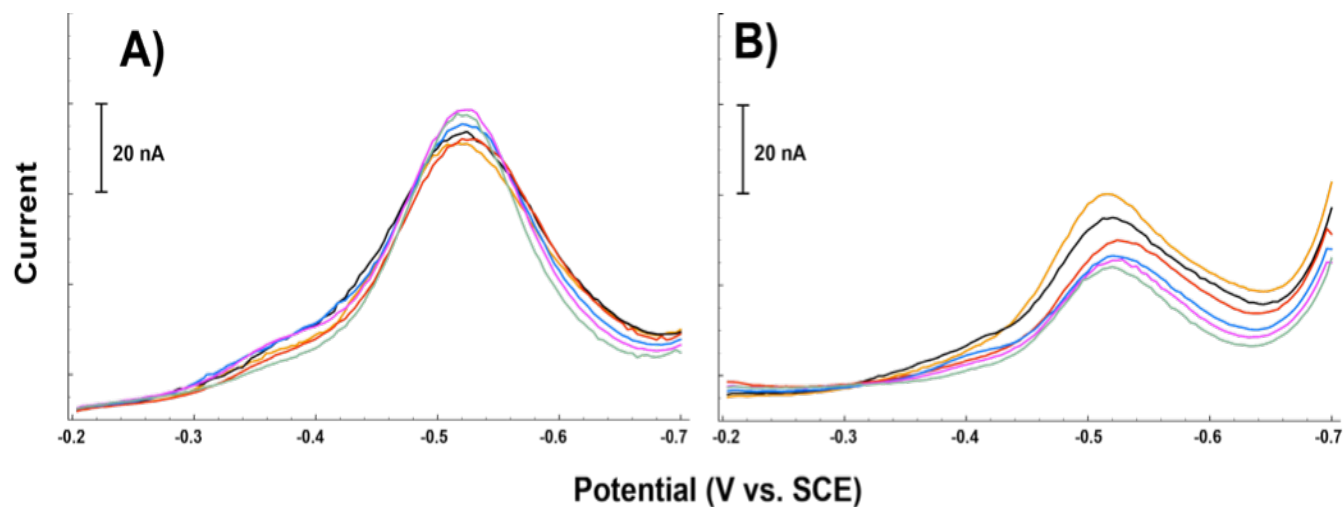


Figure 6: Raw square wave voltammetric (SWV) C12Viologen responses on DNA containing A) no 5-mC and on DNA containing B) four 5-mC on the target strand upon exposure to 50-2000 μM MgCl_2 .

Background subtracted electrochemical data more clearly depicts the responses of unmethylated (no 5-mC) and methylated (four 5-mC) DNA (Figure 7). Figure 7 (C) shows the increase in current corresponding to the addition of magnesium chloride to unmethylated (0 5-mC) DNA while Figure 7 (D) shows the decrease in current corresponding to the addition of magnesium chloride to methylated (four 5-mC) DNA.

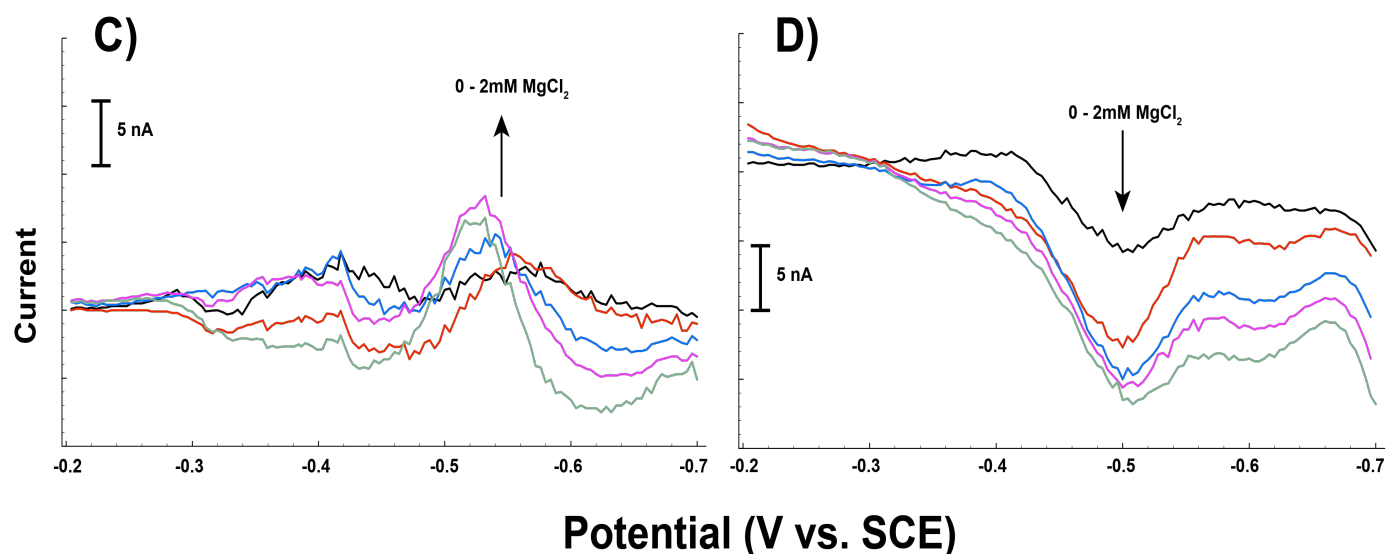


Figure 7: Background subtracted data corresponding to C) DNA with no 5-mC and D) DNA with four 5-mC on the target strand. Background refers to 0 μM MgCl₂. The arrows denote the potentials at which the current change occurred in response to the increasing concentration of MgCl₂ from 50-2000 μM .

Discussion

Data collected via cyclic voltammetry and chronocoulometry to measure surface coverage of ssDNA followed by hybridization of surface bound DNA indicated both successful surface coverage and successful hybridization (Figure 5). The cyclic voltammetric signal given by dsDNA was significantly larger than that of ssDNA, indicating that there were, in fact, more suitable binding sites available for the binding of ruthenium hexamine after the application of the complimentary strand, signifying successful hybridization. Similarly, the chronocoulometric signal

collected from ssDNA was much smaller than that of dsDNA, further verifying successful hybridization of surface bound DNA.

Data collected via square wave voltammetry displays the response of C12Viologen to the addition of magnesium chloride at increasing concentrations from 0-2000 μM (Figures 6 & 7). The data corresponding to the unmethylated (no 5-mC) DNA displays an increase in current upon the addition of magnesium chloride at a certain potential (-0.52 V), while the data corresponding to the methylated (four 5-mC) DNA displays a decrease in current upon the addition of magnesium chloride at the same potential (-0.52 V).

This square wave voltammetric data corresponds to hypothetical expectations based on the characteristics of the B to Z transition typical of methylated DNA (Figure 4). As the concentration of magnesium chloride increased, the response of C12Viologen on unmethylated DNA produced an increase in the electrochemical signal. The response of C12Viologen on methylated DNA produced a decrease in the electrochemical signal. This decrease in the electrochemical signal of methylated DNA complies with expectations, as the signal decrease reflects the loss of C12Viologen that accompanies a B to Z form transition for methylated DNA. The same transition does not occur with unmethylated DNA, which is reflected in the increasing signal produced by unmethylated DNA within the experimental data.

The differences in the electrochemical signals produced by methylated DNA versus unmethylated DNA suggest that the method involving gold surface electrodes used in combination with C12Viologen binding was successful in detecting DNA methylation. Though these results are preliminary, they are a positive indicator that

the method at the center of this study may prove to be a plausible and effective application for the detection of DNA methylation levels.

Conclusions:

Gold electrochemical sensors were successful in detecting DNA methylation via the production of differential electrochemical signals based on differences in levels of methylation of DNA. The unmethylated, or zero 5-mC, target DNA sequences exhibit an increase in peak current at -0.52 V whereas the methylated, or four methyl, target DNA sequences exhibit a peak current decrease at -0.52 V. The electrochemical responses are consistent with DNA structural changes, which lead to changes in C12Viologen binding behavior on dsDNA oligomers with various levels of methylation.

DNA oligomers in this study were of a short repeating CG sequence. CG rich sequences adopt unique conformations, which may have an additional impact on C12Viologen binding to the oligomers used¹³. Future studies may be conducted using DNA oligomers of different sequences, lengths, levels of methylation, etc.

The results of this study have significant implications regarding methods of detection of DNA methylation, providing an avenue for further research and development of a method of detection that is more efficient in terms of cost, labor, and time, as compared to existing methods. This particular method of detection may prove useful in the early detection of certain cancers, particularly those that result from hypermethylation that consequently silences the expression of tumor-suppressing genes.

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