Chapter 4

Label-Free Electrochemical Detection of Human Methyltransferase from Tumors

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A. L. Furst prepared all samples and collected and analyzed all data. N. B. Muren developed the electrochemical assay, tritium labeling assay, and cell lysis protocol.

Introduction

Epigenetic modifications, including DNA methylation, govern gene expression. Aberrant methylation by DNA methyltransferases can lead to tumorigenesis, so that efficient detection of methyltransferase activity provides an early cancer diagnostic. Current methods, requiring fluorescence or radioactivity, are cumbersome; electrochemical platforms, in contrast, offer high portability, sensitivity, and ease of use. We have developed a label-free electrochemical platform to detect the activity of the most abundant human methyltransferase, DNMT1, and have applied this method in detecting DNMT1 in crude lysates from both cultured human colorectal cancer cells (HCT116) and colorectal tissue samples.

DNA methylation powerfully influences gene expression in cells.[1, 2] DNA methyltransferases are responsible for maintaining a genomic pattern of methyl groups, which are covalently added to cytosine at predominantly 5′-CG-3′ sites. Although essential for many cellular processes, aberrant methylation is associated with cancer. In particular, abnormal activity of DNA methyltransferases can lead to hypermethylation, which can silence tumor suppressor genes and promote cancerous transformations.[3-6] The most abundant mammalian methyltransferase and an important diagnostic target is DNMT1, which preferentially methylates hemimethylated DNA using the cofactor *S*-adenosyl-L-methionine (SAM).[7-10] Current measurements of DNMT1 activity require [methyl-^{3}H]-SAM to observe radioactive labeling of DNA,[8, 11] or expensive fluorescence or colorimetric reagents with antibodies that require large instrumentation,[12, 13] both of which are significant obstacles that impede more widespread assessment of DNMT1 activity.

Traditionally, electrochemistry has been used to overcome such limitations for biomolecule detection, as electrochemical methods are low cost, portable, and require only modest instrumentation.[14, 15] However, electrochemical detection schemes have typically been restricted to measurements of highly purified samples because of the increased congestion and decreased accessibility of surface (vs. solution) platforms. Electrochemistry has been used to detect nucleic acids with high sensitivity and without the need for PCR amplification in bacterial lysate and serum,[16-20] but protein detection remains a challenge.[2, 21-23] In fact, although protein detection from simple serum has been accomplished,[24, 25] to date, no reported electrochemical systems have effectively detected active protein of any kind from crude cell lysate.

We have recently developed a unique electrochemical detection architecture aimed at overcoming the challenges associated with protein detection from complex biological samples. This multiplexed detection system involves a substrate plate consisting of a 15-electrode array and a complementary patterning and detection plate also containing a 15-electrode array, which combines low-density DNA monolayer patterning with the electrocatalytically amplified measurement of DNA charge transport (DNA CT) chemistry at a secondary electrode.[26] The low-density DNA monolayer enables protein access to the DNA even in highly congested lysate samples, while electrocatalytic signal amplification markedly increases sensitivity. We utilize measurements of DNA CT through the DNA helices in the monolayer because of the high sensitivity of this chemistry to perturbations in base stacking caused by mismatches, lesions, and protein binding.[27, 28] Methylene blue, a freely diffusing redox-active probe that is activated by DNA CT, interacts with the DNA stack and thereby reports on the

integrity of DNA CT through the monolayer. We use direct detection from the secondary electrode of the turnover of the electrocatalytic partner to methylene blue, ferricyanide, as a measurement of the amount of DNA charge transport occurring on the substrate electrode.

Here, for the first time, we demonstrate the effectiveness of this platform for the detection of human DNMT1 activity from crude lysates of colorectal tumor biopsies, utilizing a methylation-sensitive restriction enzyme to convert the methylation state of the DNA into an electrochemical signal. This strategy enables the detection of a methyl group, even though methylation itself does not significantly affect DNA CT (Figure 4.1).[29, 30] Electrodes patterned with DNA containing the preferred DNMT1 methylation site (a hemimethylated 5′-CG-3′ site) are first treated with the lysate sample. Electrodes are then treated with a restriction enzyme that is sensitive to methylation at this site. If the DNA is fully methylated by active DNMT1 in the lysate sample, the restriction enzyme does not cut the DNA, and there is an electrochemical signal owing to amplified DNA CT. If, in contrast, the DNA is not methylated by active DNMT1 in the lysate sample, the DNA remains hemimethylated (or unmethylated if this non-preferred substrate is used); the restriction enzyme can then cleave the DNA, significantly decreasing the amount of DNA on the surface, and thus diminishing the electrochemical signal generated from DNA CT. As our electrochemical platform uses electrocatalytic signal amplification involving a freely diffusing electrocatalyst (methylene blue), in contrast to earlier work,[29] the need for redox-labeled DNA is eliminated.

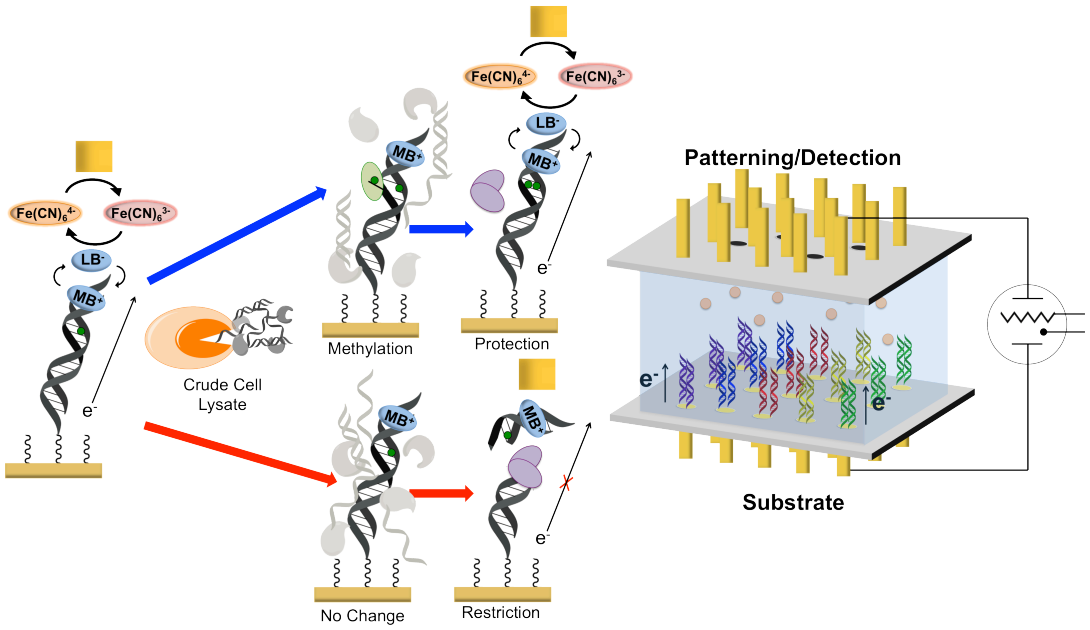

Figure 4.1 Electrochemical platform and scheme for the detection of human methyltransferase activity from crude cell lysates. *Left*: Overview of electrochemical detection scheme. Methyltransferase (green) is added to a surface either in its purified form or as a component of crude lysate, as shown on the left; a multitude of undesired biomolecules are added in the crude lysate mixture along with the methyltransferase of interest. The hemimethylated DNA on the electrode is either methylated (green dot) by the protein to a fully methylated duplex (blue arrows) or is not methylated (red arrows). A methylation-specific restriction enzyme, *BssH*II (purple), is then added. If the DNA is fully methylated, the electrochemical signal remains protected. However, if the DNA remains hemimethylated, it is cut by the restriction enzyme, and the signal is diminished significantly. *Right:* Electrochemical detection platform containing 15 electrodes (1 mm diameter each) in a 5x3 array. DNA is added to the substrate electrode by an electrochemically-activated click reaction initiated by the patterning electrode. Electrocatalytic detection is performed from the secondary (patterning) electrode.

Using this electrochemical platform and assay, we demonstrate the efficient detection of DNMT1 activity in crude lysates from both cultured human colorectal cancer cells (HCT116) and colorectal tissue samples. Femtomoles of DNMT1 in cellular samples are rapidly detected without the use of antibodies, fluorescence, or radioactive labels. Moreover, we distinguish colorectal tumor tissue from healthy adjacent tissue through differences in DNMT1 activity, illustrating the effectiveness of this two-electrode platform for clinical applications.

Materials and Methods

DNA Synthesis and Purification

Oligonucleotides were either synthesized on an Applied Biosystems 3400 DNA synthesizer or purchased from IDT. The terminal C6 alkyne moiety that was incorporated into the 5′ end of one of the strands was purchased from Glen Research. Complementary unmodified strands were purchased from IDT. DNA was deprotected and cleaved from solid support with ammonium hydroxide (60° C for 12 h). Following a preliminary round of HPLC, oligonucleotides were treated with 80% acetic acid in water for 20 minutes. Each oligonucleotide was purified by high-performance liquid chromatography (HPLC) using a gradient of acetonitrile and 50 mM ammonium acetate. Following purification, oligonucleotides were desalted by ethanol precipitation and quantified by ultraviolet-visible spectrophotometry based on their extinction coefficients at 260 nm (IDT Oligo Analyzer). Oligonucleotide masses were verified by matrix-assisted laser desorption (MALDI) mass spectrometry. DNA duplexes were formed by thermally annealing equimolar amounts of single-stranded oligonucleotides in deoxygenated phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) at 90 °C for 5 minutes followed by slowly cooling to 25 °C.

The following sequences were prepared:

Alkyne: 5′-C_2-$(CH_2)_6$-GA CTG AGT ACT GCG CGC ACT GAT AGC-3′

Complement: 5′-GCT ATC AGT GCG CGC AGT ACT CAG TC-3′

Methylated Complement: 5′-GCT ATC AGT GCG C^mGC AGT ACT CAG TC-3′

The *BssHII* restriction site is shown in red.

Western Blot Analysis of Lysate for DNMT1

DNMT1 expression was confirmed by Western blot. Samples were mixed with Laemmli reagent and betamercaptoethanol and probe sonicated for 10 seconds, followed by heat inactivation at 90 °C for 5 minutes. Samples were loaded onto 4-12% polyacrylamide gels in MOPS buffer and run at 175 mV for 1.5 hours. Gels were subsequently transferred to membranes with a dry transfer procedure for 1.5 h. Membranes were then blocked with 5% milk in TBST at room temperature for 1 hour, followed by overnight incubation with a 1^{o} antibody in milk and 3% BSA (w/v) (1:2000 for DNMT1 (New England Biolabs) and 1:1000 for Lamin A (Santa Cruz Biotechnology)) for either DNMT1 or Lamin A detection. The membranes were then rinsed with TBST buffer. Membranes were incubated with goat anti-rabbit 2^{o} antibody (Abcam Incorporated)(1:7500 in 5% milk in TBST) for 1 hour and then rinsed with TBST, followed by scanning on an Odyssey infrared gel scanner. Resulting Western blots are shown in Figure 4.2.

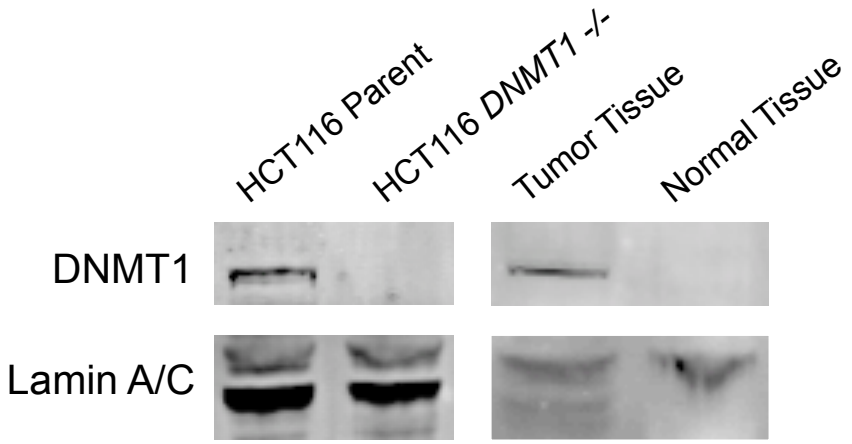

Figure 4.2 Western blot for DNMT1. Shown are gel lanes for HCT116 parent cell lysate, the HCT116 *DNMT1-/-* lysate, the tumor tissue lysate and the adjacent normal tissue lysate. The nuclear protein used as a loading control, Lamin A, is also shown for all samples. DNMT1 is only detectable from the HCT116 parent lysate and the tumor tissue lysate; no DNMT1 is observable in the HCT116 *DNMT1-/-* lysate or the normal tissue lysate.

^{3}H-SAM Methyltransferase Activity Assay

Methyltransferase activity was additionally tested using the conventional method of a ^{3}H-SAM incorporation activity assay. The activity assay followed a published protocol.[8,11] Briefly, 20 μL total reaction volumes were used for the ^{3}H-SAM activity assay. DNA (20 μM), identical to that used as a substrate for the electrochemical assay including the hexynyl terminus, was used. 0.5 μCi ^{3}H-SAM was added, and the reactions were run in DNMT1 activity buffer (50 mM Tris-HCl, 1 mM EDTA, 5% glycerol, pH 7.8). BSA (100 μg/mL) was included for the purified DNMT1 reaction, which was used as a positive standard, along with a negative standard that contained no protein. For the lysate samples, ~2 μL of lysate was included in the reaction mixture, bringing the total protein content for the reaction mixture to 3500 μg/mL. Reactions were incubated at 37° C for 2 h, followed by stopping the reaction with 30 μL of 10% TCA in water. The resulting solutions were spotted onto DE81 filter paper (Whatman) and air-dried for 15 minutes. Filter papers were then individually soaked in 10 mL of 50 mM Na_2HPO_4 for 15 minutes and rinsed with both 50 mM Na_2HPO_4 and 95% ethanol. Filter papers were then heated to 37° C to dry for 15 minutes before liquid scintillation counting. The DNMT1 assay, measured using radioactivity, is shown in Figure 4.3.

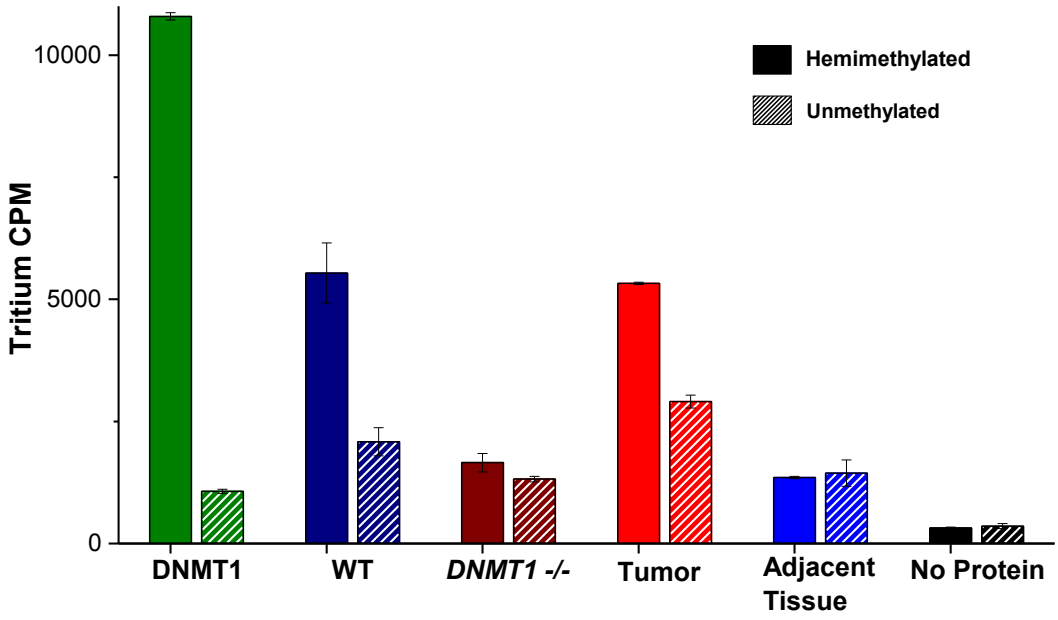

Figure 4.3 ^{3}H-SAM DNMT1 activity assay. The results of a ^{3}H-SAM DNMT1 activity assay are shown, with the counts per minute per sample given. In all cases, the solid bar is data from hemimethylated substrate, and the hashed bar is data from unmethylated substrate. In green is 65 nM DNMT1; in dark blue is parent HCT116 lysate, and in dark red is DNMT1-/- lysate. In red is the tumor lysate, and in blue is lysate from adjacent healthy tissue. In black is the negative control that contains no protein. Error bars represent triplicate scintillation measurements for two replicates of each experimental condition.

DNA Monolayer Formation

The two-electrode array was constructed as previously reported.[26] The multiplexed setup consisted of two complementary arrays containing 15 x 1 mm diameter gold rod electrodes embedded in Teflon. Gold surfaces were polished with 0.03 micron polish before monolayer assembly. Mixed monolayers were formed on one of the plates using an ethanolic solution of 1 M 12-azidododecane-1-thiol (C_{12}thiolazide) and 1 M 11-mercaptoundecylphosphoric acid (Sigma Aldrich). Surfaces were incubated in the thiol solution for 18-24 h, followed by rinsing with ethanol and phosphate buffer (5 mM phosphate, pH 7.0). The water-soluble $[Cu(phendione)_2]^{2+}$ (phendione=1,10-phenanthroline-5,6-dione) was synthesized by mixing two equivalents of phendione with copper sulfate in water. Covalent attachment of DNA to mixed monolayers containing 50% azide head group and 50% phosphate head group through electrochemically-activated click chemistry was accomplished by applying a sufficiently negative potential to the secondary electrode. Specifically, a constant potential of -350 mV was applied to a secondary electrode for 25 minutes, allowing for precise attachment of the appropriate DNA to a primary electrode. 40 μL of 100 μM catalyst and 80 μL of 50 μM DNA in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) were added to the platform for covalent attachment.

Cell Culture and Lysate Preparation

HCT116 cells, either parent or *DNMT1*$^{-/-}$(Vogelstein Lab),[9] were grown in McCoy's 5A media containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL

streptomycin in tissue culture flasks (Corning Costar, Acton, MA) at 37° C under a humidified atmosphere containing 5% CO_2.

Approximately 6 million cells were harvested from adherent cell culture by trypsinization, followed by washing with cold PBS and pelleting by centrifugation at 500*g* for 5 minutes. An NE-PER nuclear extraction kit (Pierce from Thermo Scientific) was used for cell lysis, with buffer then exchanged by size exclusion spin column (10 kDa cutoff; Amicon) into DNMT1 activity buffer (50 mM Tris-HCl, 1 mM EDTA, 5% glycerol, pH 7.8). Cell lysate was immediately aliquoted and stored at -80° C until use. A BCA assay (Pierce) was used to quantify the total amount of protein in the lysate. The total protein concentration at which the lysate was frozen was 3,500-5,000 μg/mL.

Tissue samples were obtained from CureLine. Colorectal carcinoma as well as healthy adjacent tissues were obtained. Approximately 150 mg of tissue were homogenized manually, followed by nuclear extraction, buffer exchange, storage, and quantification, as described above. The total protein concentration at which the lysate was frozen was 35,000-50,000 μg/mL.

Electrochemistry

All electrochemistry was performed on a bipotentiostat (BASinc.) with two working electrodes, a platinum wire counter electrode, and an AgCl/Ag reference electrode. Constant potential amperometry was performed for 90 seconds with an applied potential of 320 mV to the secondary electrode and -400 mV to the primary electrode relative to an AgCl/Ag reference electrode with a platinum counter electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1

mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide. Scans were taken at each of the 15 secondary pin electrodes, and the reported variation in the data represents the standard error across three measurements of three electrodes, all at a given condition.

To incubate electrodes with desired proteins, a 1.5 mm deep Teflon spacer was clipped to the primary electrode surface. Each electrode is isolated in an individual well that holds 4 μL of solution. For methyltransferase activity detection, three electrodes on the device were always incubated with 65 nM DNMT1 with 160 μM SAM and 100 μg/mL BSA as a positive control. For electrodes incubated with lysate, lysate was either directly combined with SAM to a final SAM concentration of 160 μM or the lysate was diluted in DNMT1 activity buffer to the desired total protein concentration and subsequently combined with SAM to a final SAM concentration of 160 μM. For the tissue lysate, 50 μg/mL BSA was also added. Each electrode had the desired solution added to the well and incubated at 37° C for 1.5 h in a humidified container. The primary electrode array was then treated with 1 μM protease solution in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) for 1 h. The surface was then thoroughly rinsed with phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) and scanned. The electrodes were subsequently incubated with the restriction enzyme *BssH*II at a concentration of 1500 units/mL for 1.5 h at 37° C in a humidified container. *BssH*II was exchanged into DNMT1 activity buffer by size exclusion column (10 kDa, Amicon). The electrodes were again rinsed with phosphate buffer and scanned.

Results and Discussion

Electrochemical Platform

Using a 15-pin setup, low-density DNA monolayers were formed on one set of electrode surfaces by DNA patterning from a secondary electrode. DNA substrates were optimized for length to balance on/off signal differential with the ability of proteins to access the binding site. For monolayer formation, thiol monolayers with 50% azide and 50% phosphate head groups first were prepared on the gold pins. We have previously characterized such low-density monolayers, and have found the total DNA coverage to be 20 pmol/cm^2.[31] Subsequently, specific DNA sequences were tethered to individual pins using electrochemically-activated Cu^{1+} click chemistry.[26] The secondary electrode activates the inert copper catalyst precursor only at specific locations on the primary electrode surface. Multiple sequences of DNA with different methylation states in the restriction enzyme binding site (either hemimethylated or unmethylated) were therefore patterned onto particular electrodes (Figure 4.1). The multiplexed array allows five experimental conditions to be run in triplicate, enabling simultaneous detection from healthy tissue and tumor tissue, along with the incorporation of a positive control of pure DNMT1.

Electrochemical measurements were obtained by constant potential amperometry over 90 seconds. Electrodes were measured after treatment with methyltransferase, either in its purified form or as a component of crude lysate, and again after treatment with 1500 units/mL of the restriction enzyme *BssH*II. Lysate was prepared from cultured cells through a simple treatment of cell disruption followed by buffer exchange. Purified DNMT1 was first used to establish the sensitivity and selectivity of this platform (Figure

4.4) and was subsequently included alongside lysate activity measurements as a positive control. The DNA-mediated signal remains fully ‘on’ after restriction when the electrode is preliminarily treated with a minimum of 65 nM DNMT1 protein on a hemimethylated DNA substrate in the presence of the SAM cofactor, although protein is easily detectable at a 15 nM concentration with 48±3% signal protection. For electrodes treated with 65 nM DNMT1 in the absence of additional SAM, only 33±5% signal protection is observed. Similarly, little DNA protection (31±6% signal protection) is observed with the unmethylated substrate. This is explained by the strong preference of DNMT1, as a maintenance methyltransferase, for a hemimethylated substrate.

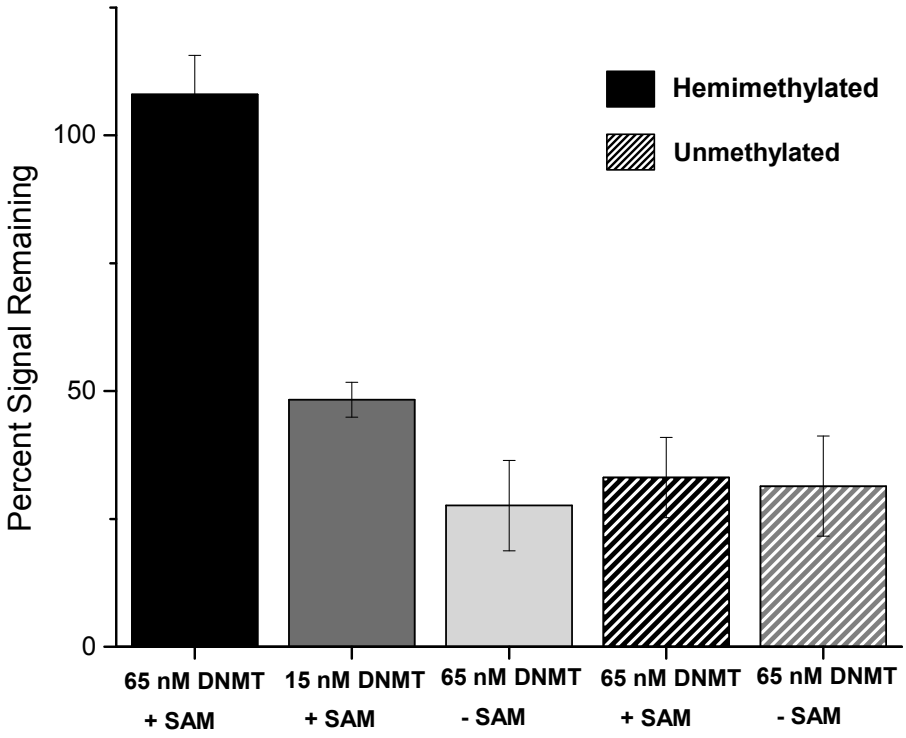

Figure 4.4 Substrate specificity and detection limits for purified DNMT1. The data are reported as the percent signal remaining by comparing the signal after protease treatment and after restriction enzyme treatment. DNMT1 (65 nM) on hemimethylated DNA with 160 μM SAM afforded full protection of the DNA on the surface (black), while electrodes treated with 15 nM DNMT1 on hemimethylated DNA with 160 μM SAM maintained 48±3% signal (dark grey). DNMT1 (65 nM) on hemimethylated DNA without SAM (light grey), on unmethylated substrate with 160 μM SAM (black hashed), or on unmethylated substrate without 160 μM SAM (grey hashed) showed no signal protection. The constant potential amperometry was performed for 90 seconds with an applied potential of 320 mV to the secondary electrode and -400 mV to the primary electrode relative to an AgCl/Ag reference electrode with a platinum auxiliary electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide. Error bars represent the standard deviation over three measurements for three experiments.

Figure 4.5 shows the raw data collected for two individual electrodes treated with crude lysate, one in which the signal is 'on' in the presence of the SAM cofactor and one in which the signal is turned 'off' in the absence of cofactor, due to DNA restriction in the absence of methylation. Additionally, the reproducibility of the platform is shown (Figure 4.5), along with quantification of the 15 individual electrodes of a single assay. Interestingly, high concentrations of lysate were found to diminish the electrochemical signal, likely due to crowding on the DNA-modified electrode, and thus limiting access and binding of the methyltransferase of interest. Multiple concentrations of lysate were tested (Figure 4.6); a concentration corresponding to 4,000 cells per electrode was sufficiently dilute to allow access of DNMT1 to the DNA on the surface while still containing sufficient DNMT1 to produce measurable activity.

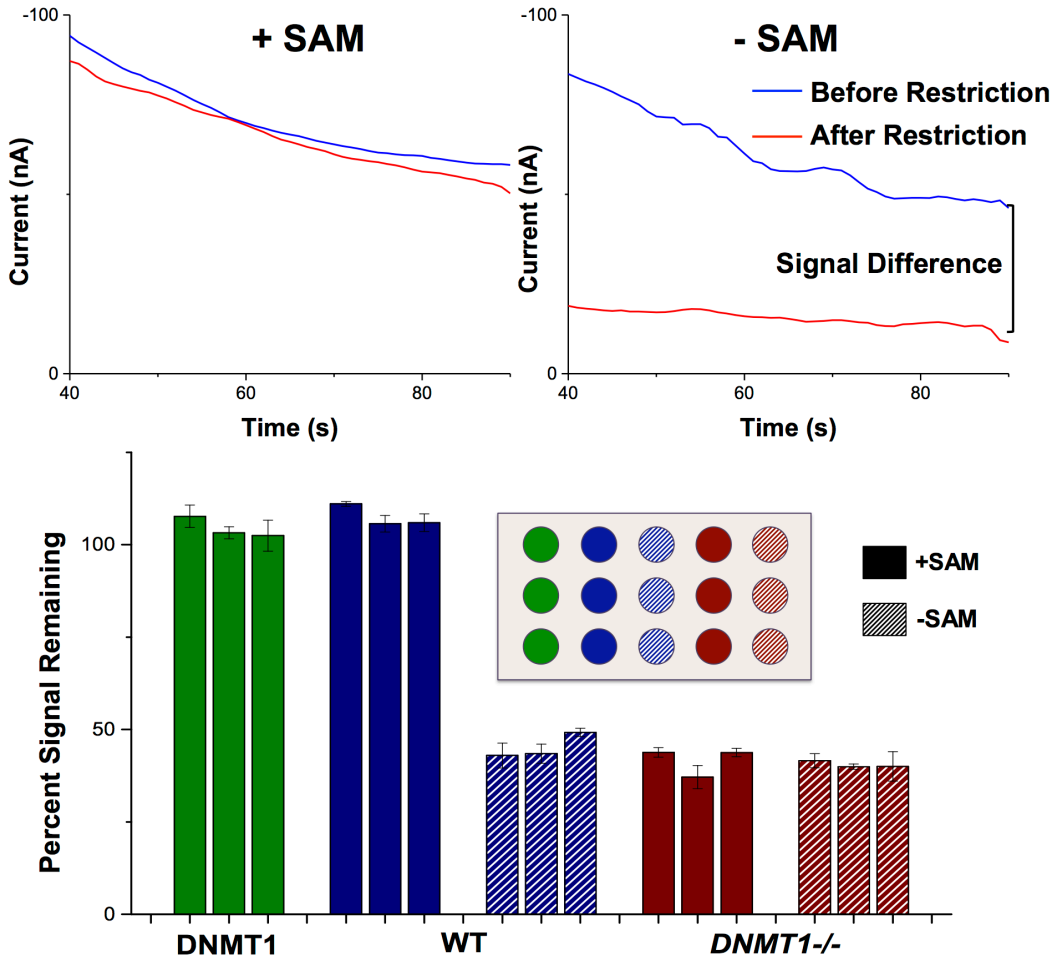

Figure 4.5 Detection and reproducibility of DNMT1 activity in cell lysates using electrochemical platform. *Top:* Raw data from single electrodes in the presence or absence of SAM cofactor. In blue is the preliminary scan after an electrode modified with hemimethylated DNA has been treated with parent lysate, followed by treatment with 1 µM protease in phosphate buffer, both at 37° C. In red is the scan after the electrode was treated with 1500 units/mL *BssH*II for 1.5 h at 37° C. On the left is the final scan from an electrode with 160 µM SAM added to the lysate; the signals essentially overlay, indicating an 'on' signal. On the right is the final scan from an electrode without SAM added to the lysate; this produces an 'off' signal. The constant potential amperometry was run for 90 seconds with an applied potential of 320 mV to the secondary electrode and -400 mV to the primary electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 µM methylene blue and 300 µM potassium ferricyanide. *Bottom:* Reproducibility within the two-electrode multiplexed array. Orientation of the tested conditions on the 5x3 array is shown (inset) with circular electrodes colored to correspond with activity data represented in the bar graph. Both the electrodes treated with 65 nM purified DNMT1 on hemimethylated DNA with 160 µM SAM (green) and those treated with parent (WT) lysate in the presence of SAM (solid blue) show full signal protection. On electrodes

without added SAM (blue striped and red striped) and on *DNMT1* $^{-/-}$ lysate-treated electrodes in the presence of SAM (solid red), no signal protection is observed. Bar graph data are raw without standardization and error bars represent the standard deviation over three measurements.

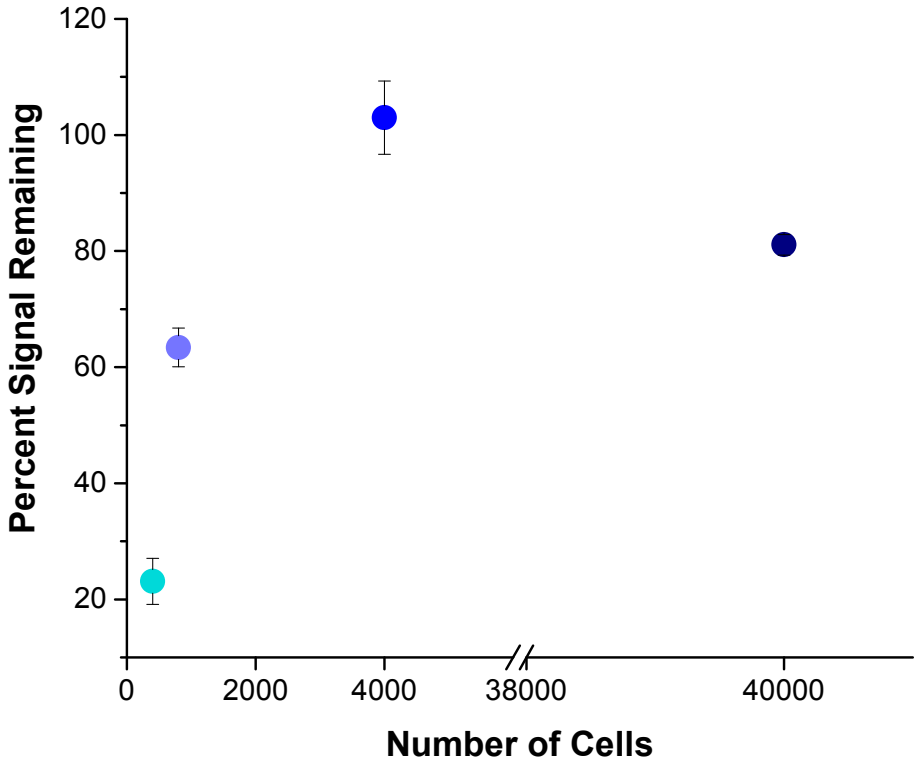

Figure 4.6 Signal protection from differing amounts of lysate. All electrodes on this array were modified with hemimethylated DNA, and values are normalized to 100% protection of the DNMT1 electrodes. All samples include 160 μM SAM. The darkest blue is lysate from approximately 40,000 cells. The subsequent points are from 4,000 cells, 800 cells, and 400 cells. Full protection is afforded from approximately 4,000 cells. The constant potential amperometry was performed for 90 seconds with an applied potential of 320 mV to the secondary electrode and -400 mV to the primary electrode relative to an AgCl/Ag reference electrode with a platinum counter electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide. Error bars represent the standard error for three electrodes over three measurements.

To further combat signal decreases caused by undesired DNA-binding proteins, after electrodes are treated with lysate, a protease treatment step is incorporated to remove remaining bound protein before the electrochemical measurements. This protease step further ensures that there is no remaining protein bound either to the DNA or directly to the surface that could interfere with electrochemical measurements. Methyltransferase activity is then determined by the percent signal remaining after *BssH*II treatment. If the DNA is cut by the restriction enzyme, the signal is low, indicating little methyltransferase activity. It is noteworthy that the percent signal remaining is always non-zero because, even after restriction, a DNA fragment remains that can generate an electrochemical response with the noncovalent methylene blue redox probe; electrochemical amplification is proportional to the amount of bound methylene blue, and therefore to DNA length.

Differential Detection of DNMT1 Activity from Multiple Crude Cultured Cell Lysates

We then tested the ability of the platform to differentiate between lysate from a parent (HCT116 wild type) colorectal carcinoma cell line and a cell line that does not express DNMT1 (HCT116 *DNMT1*$^{-/-}$). As shown in Figure 4.7, specific detection of DNMT1 activity is dependent on both the methylation state of the substrate and the presence of the cofactor SAM. The 'signal on' specificity for the hemimethylated DNA substrate indicates unambiguous DNMT1 activity (maintenance methylation), and not activity by other human methyltransferases, DNMT3a or DNMT3b, which do not show this substrate preference (*de novo* methylation).[7] Signal is dependent on the presence of DNMT1 (purified or from parent lysate) as well as the cofactor SAM (Figure 4.7b) and

the hemimethylated substrate (Figure 4.7a). The remaining electrodes, treated either with parent lysate without SAM, or *DNMT1*$^{-/-}$ lysate independent of the cofactor, had significantly attenuated signals after restriction enzyme treatment.

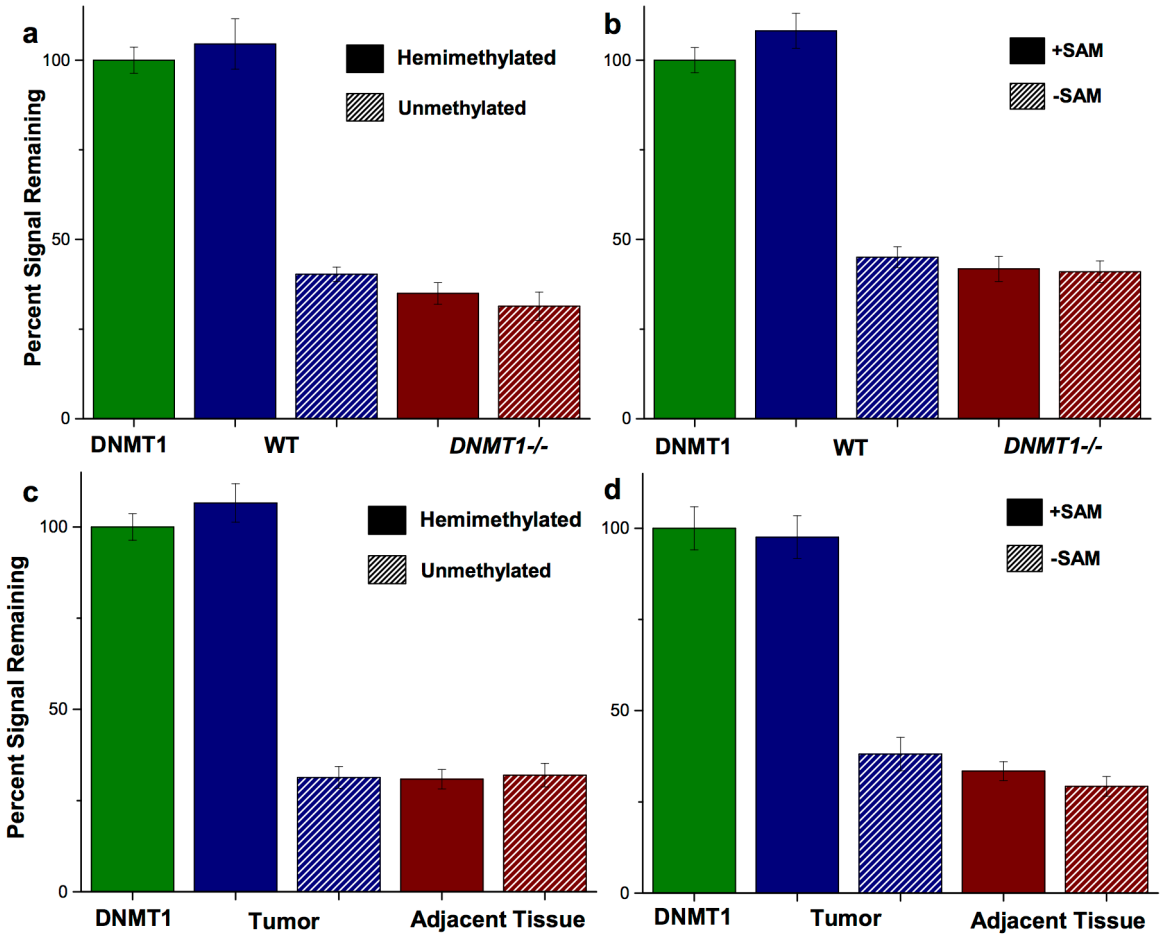

Figure 4.7 Dependence of lysate activity on the DNA substrate and cofactor. The positive control is 65 nM purified DNMT1 on hemimethylated DNA with 160 µM SAM (green). All values are normalized to 100% protection of the purified DNMT1 electrodes. Upper panels show cultured cell lysate substrate (a) and cofactor (b) dependence. Electrodes treated with parent (WT) lysate on the hemimethylated substrate in the presence of 160 µM SAM (blue) showed full signal protection, while on an unmethylated substrate or in the absence of SAM (striped blue) no signal protection is observed. Independent of conditions, electrodes treated with *DNMT1* $^{-/-}$ lysate (red solid and striped) showed no signal protection. Lower panels show biopsy tissue substrate (c) and cofactor (d) dependence. Electrodes treated with tumor lysate on the hemimethylated substrate in the presence of 160 µM SAM (blue) showed full signal protection, while on an unmethylated substrate or in the absence of SAM (striped blue) no signal protection is observed. Independent of conditions, electrodes treated with normal tissue lysate (red solid and striped) showed no signal protection. The data shown is the aggregation of three independent replicate experiments, with three electrodes per condition per experiment.

Detection of DNMT1 Activity from Human Tumor Tissue

Human biopsy tissue samples were similarly evaluated, and tumor tissue was readily distinguished from adjacent normal tissue (Figure 4.7). Tissue biopsy samples were purchased from a commercial source and were thus handled and stored using conventional methods (snap freezing in liquid nitrogen after removal and storage at -80° C for upwards of one month). The optimal amount of tissue for detection from these samples was found to be ~500 μg per electrode; typical colon punch biopsies yield 350 mg of tissue.[32] Samples of colorectal carcinoma tissue as well as the adjacent healthy tissue were prepared just as the cultured cell lysate was, and showed differential activity with our electrochemical platform. The tumor sample, which showed greater signal protection, was sensitive both to substrate and to cofactor, consistent with high DNMT1 methyltransferase activity, and similar to the cultured parent colorectal carcinoma cells. In contrast, the normal tissue sample showed low methyltransferase activity, as seen through the reduced electrochemical signal (Figure 4.7). These data clearly indicate that tumors can be effectively differentiated from healthy tissue through electrochemical DNMT1 measurement with our platform. By western blot, the relative abundance of DNMT1 in the tumor tissue as compared to healthy tissue was quantitatively consistent with the electrochemical results (Figure 4.2).

Lysate activities were also tested by a ^{3}H-SAM assay, and relative activities of the various samples were comparable to those determined electrochemically (Figure 4.3). However, as is typical for such radioactivity assays, activity measurements observed among trials of the ^{3}H-SAM assay were extremely variable, much more so than with the electrochemical platform. Activity differences between the tumor and healthy tissue

were seen only at concentrations of ~1 mg of tissue per sample, significantly higher than what is needed for electrochemical detection. The time required to obtain the data was additionally substantially longer.

Implications

DNMT1 is an important clinical diagnostic target due to its connection to aberrant genomic methylation, which is linked to tumorigenesis. Direct detection of methyltransferase activity from crude tissue lysates provides an early method of cancer screening and can also inform treatment decisions. However, current approaches for the detection of methyltransferase activity rely on radioactive or fluorescent labels, antibodies, and obtrusive instrumentation, which limit their application in laboratories and clinics. Although electrochemical approaches generally overcome these limitations, direct detection of proteins from crude samples remains challenging because of the complexity of crude biological lysates, as well as the sensitivity required to analyze the limited material of small clinical biopsy samples.

Our electrochemical assay for DNMT1 methylation effectively circumvents these problems. Methylation is detected through the presence or absence of DNA surface restriction followed by electrocatalytic amplification. We avoid clogging the platform through the formation of low-density DNA monolayers, enabling target DNA-binding proteins in the lysate ample access to the individual DNA helices on the surface. Our platform is also sensitive and selective without the use of radioactivity, fluorescence, or antibodies through the combination of electrocatalytic signal amplification and the sensitivity of DNA CT chemistry to report changes to the integrity of the DNA. This allows for detection of DNMT1 from both cultured colorectal carcinoma cells and tissue biopsy specimens. No difficult or time-consuming purification steps are necessary, and, for each electrode, only ~4000 cultured cells or ~500 μg tissue sample are required. Importantly, because of the multiplexed nature of this platform, we are able to assay for

substrate specificity while simultaneously measuring normal tissue and tumor tissue lysates. Therefore, with our platform, healthy tissue is easily distinguished from tumor tissue using very small amounts of sample. More generally, this work represents an important step in new electrochemical biosensing technologies.

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