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Voltammetric Application of Polypyrrole-Modified Microelectrode Array for the Characterization of DNA Methylation in Glutathione S-Transferase Pi 1

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ABSTRACT

Direct and efficient label-free voltammetric detection of glutathione S-transferase Pi 1 (GSTP1) hypermethylation is reported using a custom-developed 16-channel microelectrode array chip. The microelectrode array chip is used in a dipstick configuration allowing detection of DNA hybridization in a solution volume of only 0.35 mL. Platinum microelectrode disks ($n = 16$) 30 μm in diameter have been modified with a polypyrrole bilayer before any contact with the oligonucleotides. The attachment of 15-mer Probe DNA to the bilayer is random but controlled by the presence of aliphatic tether groups allowing it to form a bidentate complex with the probe DNA. The voltammetric detection procedure of methylated GSTP1-specific target DNA is combined with bisulfite treatment of target DNA. Changes at the interface of the modified microelectrodes in an array configuration are used to record simultaneously cyclic voltammetry on all of the devices. The detection of hybridization is evaluated statistically by a yes or no event by comparing the changes in recorded cyclic voltammograms before and after exposure to the target DNA. All cyclic voltammograms of the methylated target show a greater percentage change than those with the nonmethylated target exposure and show a greater change in cyclic voltammogram area after methylated target exposure. We observe an average percentage difference of $25.6 \pm 4.9\%$ with a variation of 19.1%. These results demonstrate that the fast sensing strategy possesses sensitivity and good specificity. Furthermore, this technology can potentially support rapid, accurate diagnosis and risk assessment of patients with prostate cancer.

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Introduction

DNA methylation is an important epigenetic modification that regulates gene expression (Martienssen 1995). The rate of gene expression and the occurrence of gene silencing are dependent on levels of methylation (Antequera and Bird 1999). DNA methylation in the human genome occurs mostly at the cytosine residues in a 5′—C—phosphate—G—3′ (CpG) dinucleotide at the carbon-5 position, resulting in 5-methylcytosine (Jain, Wojdacz, and Su 2013). Alterations to methylation patterns can also lead to abnormalities

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including the formation of tumors (Antequera and Bird 1999). The efficacy of methods targeting methylation changes for the early detection of prostate cancer and colorectal cancer has been reported previously (Paziewska et al. 2014; Nian et al. 2017).

Early detection for prostate cancer includes prostate-specific antigen (PSA) and digital rectal exam (DRE). The use of PSA and DRE in standard screening procedures (Lippi et al. 2009) has now been cautioned against by the U.S. Preventative Services Task Force due to low sensitivity and specificity and the high number of false positives, necessitating invasive biopsies (PSA: sens. 21–68% based on threshold values/spec. 85–90%; DRE: sens. 59%/spec. 94%, all values approx.) (Bohnen, Groeneveld, and Bosch 2007; Moyer and Services 2012; WebMD, n.d.). Most notably, studies have indicated that 70% of men have a nonaggressive form of prostate cancer that does not increase mortality, but may fail PSA testing (Siegel, Naishadham, and Jemal 2012). Such screening methods have been shown to result in little or no reduction of PCa-specific mortality and has been associated with harms related to over-diagnosis and overtreatment (Chou et al. 2011; Wallner et al. 2013).

The epigenetic biomarker, glutathione *S*-transferase Pi 1 (GSTP1), is a highly promising biomarker for PCa. GSTP1 is a member of the glutathione *S*-transferase (GST) family of enzymes that are involved in the detoxification of xenobiotic and oxygen radicals. Hypermethylation of GSTP1's promoter region is the most described epigenetic alteration in prostate cancer and is only apparent in prostate cancer cells demonstrating greater correlation to prostate cancer (sensitivity: 75%; specificity: 98%) and prostatic intraepithelial neoplasia (Jerónimo et al. 2001; Nelson et al. 2001; Harden et al. 2003; Hoque et al. 2005; Woodson et al. 2008; Hessels and Schalken 2013). Additionally, this biomarker can be detected in biological matrices such as serum/plasma, tissue, or cell lysate as well as through noninvasively obtained biological fluids such as urine (Jerónimo et al. 2002; Wu et al. 2011). Methods of genomic methylation profiling through the use of bisulfite have been developed (Frommer et al. 1992; Zhang et al. 2009) and the application of these methods to nucleic acid detectors has been speculated (Paleček and Bartošik 2012).

Over the past two decades, there has been considerable interest in the development of nucleic acid-based technologies for molecular diagnostics (Drummond, Hill, and Barton 2003) as they may provide high specificity due to complementary base pairing and also exhibit greater resistance to denaturation than proteins (Siddiquee et al. 2010). Hybridization events between the probe and target DNA can be transduced into analytical signals of various types including optical, gravimetric, and electrochemical. Methods involving optical transduction tend to require costly equipment and lack portability. Additionally, these techniques are limited by the particle position resolution and the concentration of the particle in the solution (Cagnin et al. 2009). Electrochemical DNA biosensors couple a biological recognition element to an electrode transducer transforming DNA hybridization events into electrical signals. Electrochemical methods provide superior properties over other methods as they enable fast, simple, low-cost detection and require only a limited volume of the specimen of interest (Kerman, Masaaki, and Eiichi 2004). This electrochemical technology is also desirable as it is amenable to miniaturization, can be accurate and sensitive with simple self-contained instrumentation, and has easily controlled reaction conditions (Wang 2006). In developing such a device, there are two main approaches to electrochemical transduction of DNA hybridization: labeled methods and label-free methods (Odenthal and Gooding 2007). Labeled methods of transduction rely on redox active molecules that bind to DNA and produce an electrical

signal based on whether the DNA was single stranded or double stranded. Label-free methods rely on changes to the electrical characteristics of the DNA-modified interface upon hybridization or on the natural electroactivity of DNA (Odenthal and Gooding 2007).

Previously, we have demonstrated the use of electrostatic modulation of Cl^- ion-exchange kinetics of polypyrrole bilayer film as a simple and direct method of electrochemical detection of hybridization (Thompson et al. 2003). The grafted layer of 2,5-dithienyl-(*N*-3-phosphorylpropyl) pyrrole (pTPTC3PO₃H₂) allows Mg^{2+} cations to form a bidentate complex between the phosphonate group of pTPTC3PO₃H₂ and the phosphate group of probe DNA (Aiyejorun et al. 2005). Unlike covalent immobilization methods, the Mg^{2+} -pTPTC3PO₃H₂-tether offsets the oligonucleotide probe from the surface polymer, providing greater freedom of motion. This reduces the effects of steric hindrance on hybridization events. It also orients the immobilized probe DNA toward the solution, allowing for probe attachment by dipping (Thompson et al. 2003). Through this method, we can detect hybridization events through measuring changes in the chloride ion flux as Cl^- ions transverse the modified platinum electrode upon oxidation and reduction of the polypyrrole bilayer.

We have reported the use of this label-free voltammetric assay in the detection of complementary target DNA strands (Thompson et al. 2003) and the detection of hypermethylation of the prostate cancer biomarker GSTP1 (Saheb, Leon, and Josowicz 2012; Saheb et al. 2014) using commercially available platinum disk electrode with a diameter of 25 μm . In this study, we report on the performance of our 16-channel microelectrode array chip in the detection of methylated GSTP1-specific target DNA. We also statistically examine this voltammetric signal for a *yes* or *no* response to the proposed diagnostic DNA methylation test.

Experimental

Materials and reagents

The monomer of 2,5-dithienyl-(*N*-3-phosphorylpropyl) pyrrole, pTPTC3PO₃H₂, where C3- in this tether group defines the number of methylated groups shortly abbreviated as pTPT was synthesized according to Hartung et al. (2005). Pyrrole (98%), magnesium chloride hexahydrate, potassium chloride, tetrabutylammonium perchlorate (Bu_4NClO_4), acetonitrile, and Tris-HCl buffer (0.1 M, pH 7.2) were all of reagent grade and were used as received. The Epigenetics Bisulflash DNA Modification Kit was purchased from Epigentek (USA) (Epigentek 2015).

All DNA was custom made by Integrated DNA Technologies, Inc. (Coralville, Iowa). The sequences of the probe oligonucleotides (15-mer), nonmethylated target DNA (27-mer), and methylated target DNA (27-mer) strands were received in 1 μmol pellet form. Stock solutions of DNA sequences were prepared using 0.1 M Tris-HCl buffer. The concentrations of probe DNA, nonmethylated target DNA, and methylated target DNA stock solutions were 2.9×10^{-5} , 3.0×10^{-5} , and 1.7×10^{-5} M, respectively. Stock solutions were then diluted with 0.1 M Tris-HCl buffer to a final concentration of 0.1 μM . The DNA sequences (shown in Table 1) reflect the promoter region of the GSTP-1 gene.

The nonmethylated and methylated target DNA sequences were treated using Bisulflash DNA Modification Kit before experimental use. Five microliter aliquots of 0.1 μM target DNA sequences were separately treated according to the protocol outlined in the bisulfite

Table 1. DNA probe and target sequences used in experiments. ^mC identifies the positions of the methylated cytosine.

DNA	Sequence
15-mer probe	5'-TCG CCG CGC AAC TAA-3'
27-mer target (methylated)	5'-TTT ^m CGG TTA GTT G ^m CG ^m CGG ^m CGA TTT ^m CGG-3'
27-mer target (non-methylated)	5'-TTT CGG TTA GTT GCG CGG CGA TTT CGG-3'

Table 2. Bisulfite conversion of DNA sequences.

Methylated 27-mer Target	
5'	TTT ^m CGG TTA GTT G ^m CG ^m CGG ^m CGA TTT ^m CGG 3'
	TTT ^m CGG TTA GTT G ^m CG ^m CGG ^m CGA TTT ^m CGG
Non Methylated 27-mer Target	
5'	TTT C GG TTA GTT G C G C GG C GA TTT C GG 3'
	TTT U GG TTA GTT G U G U GG U GA TTT U GG

kit manual (Epigentek 2015). This produced 20 μL of DNA for every conversion. This process converts all nonmethylated cytosine residues into uracil, while leaving methylated cytosine residues unchanged as shown in Table 2. The resulting DNA is diluted with 330 μL of Tris-HCl buffer and stored in a refrigerator until time of use.

Assembly of platinum microelectrode array chip

The label-free voltammetric 16-channel electrode array used in this study is comprised of a single chip with 16 platinum microelectrodes integrated into a dipstick configuration platform (Figure 1). The 16-channel 30 μm Pt microelectrode array chips were fabricated as published in Kalantari et al. (2010). The Pt microelectrodes were etched into Pyrex wafers deposited with layers of SiO₂ and SiN. These wafers were then attached to a printed circuit board as shown in Figure 1. Each of the 16 electrode channels can be individually functionalized. Once produced, chips undergo a 5-min soak with Piranha solution of a 1:3 ratio of H₂O₂ and concentrated H₂SO₄ to clean residual organic matter. (Hazard note: Piranha solution is very energetic and exothermic; consult appropriate SDS for proper handling.) After rinsing with deionized water, the Pt microelectrodes were electrochemically cleaned in 0.1 M H₂SO₄ (Kalantari et al. 2010).

Electrochemical measurements

All experiments were conducted using a custom-designed multichannel potentiostat integrated with signal processing circuitry and custom LabView-based application software that supports testing, measurement, data transfer, and evaluation of voltammetric sensor data. The instrument is controlled and read by a National Instruments data acquisition system, which provides analog circuitry and digitally controlled gain and multiplexing

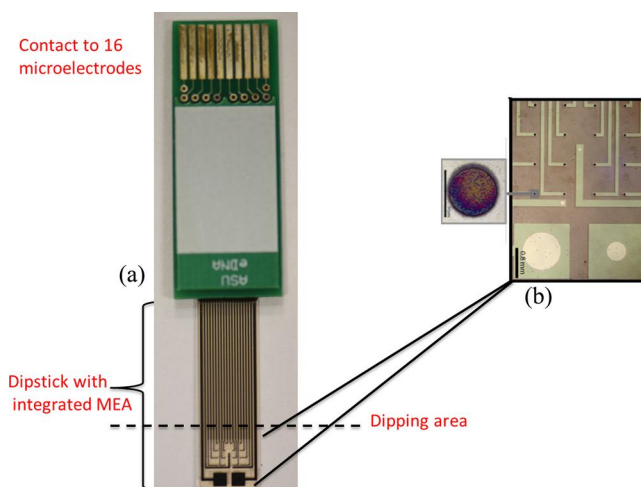


Figure 1. (a) Photograph of the dipstick platform with integrated microelectrode array chip at its bottom. The contact connects the chip to the 16-channel potentiostat. The microelectrode array dipping area (6.5 mm × 5.0 mm) contains the active site, which is comprised of 16 30-μm Pt array electrodes. That configuration allows carrying hybridization test in 0.4 mL solution using a vial as voltammetric cell. (b) Micrographs of 16-channel microelectrode array electrode contacts. The electrode contacts shown are coated with a polypyrrole-pTPTC3PO₃H₂ bilayer that was deposited electrochemically.

functions. Through this software, a potential between +5 and −5 V can be applied and mirrored for each of the 16 electrodes while reading their currents individually. Thus the array chip and 16-channel potentiostat can be used to conduct experiments simultaneously, thereby reducing experimental time and experimental error.

Herein, all voltammetric experiments were performed in a single-compartment cell (5 cm³) using a three-electrode cell arrangement at room temperature. The platinum wire was used as a counter electrode and two external reference electrodes: Ag/AgCl in 0.1 M KCl/0.1 M Tris-HCl buffer for aqueous experiments and Ag/Ag⁺ in 0.1 M AgNO₃ in acetonitrile/0.1 M Bu₄NClO₄ in acetonitrile. The abovementioned reference electrodes reflect all electrode potentials mentioned.

Outline of operational steps for modification of microelectrode array

In Figure 2, the final product of modified microelectrode array is illustrated that results from three follow-up steps that are outlined in detail hereafter.

Polypyrrole—pTPTC3PO₃H₂ bilayer deposition

The assembly of the polypyrrole bilayer onto each individual electrode is commenced through the polymerization of pyrrole from a solution of 0.1 M pyrrole in 0.1 M Bu₄NClO₄/ACN. The polymerization of polypyrrole was performed at a constant potential of 700 mV versus Ag/Ag⁺/0.1 M AgNO₃ in ACN/0.1 M Bu₄NClO₄ in ACN and was terminated at 1.0×10^{-6} Coulombs (C). The array chip was then washed with acetonitrile and was soaked in acetonitrile for 10 min to remove excess and unbound pyrrole monomer. The same procedure was then applied to polymerization of TPTC3PO₃H₂ from a solution of 4 mM TPTC3PO₃H₂ in acetonitrile and terminated at 5.5×10^{-7} C.

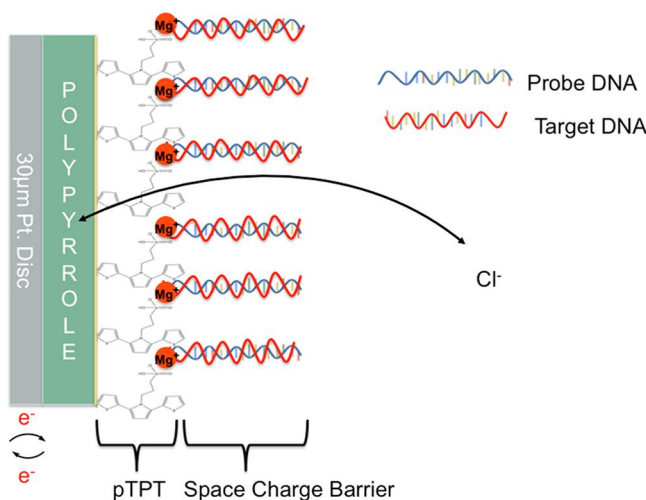


Figure 2. Microelectrode array operational scheme.

Probe DNA attachment

The microelectrode array dipstick was then immersed in 5×10^{-3} M MgCl_2 solution for 15 min. The solution was stirred during incubation. After washing with Tris-HCl buffer and soaking for 10 min in Tris-HCl buffer, the dipstick was placed in 350 μL of 0.1 μM of probe DNA in a microcentrifuge tube. The sample was then heated on a thermostatic bath at 45°C for 30 min. After washing again with Tris-HCl buffer and soaking for 10 min in Tris-HCl buffer, the microelectrode array was ready for the first voltammetric test. Three consecutive cyclic voltammograms were taken of the array chip in Tris-HCl buffer using a potential range of +0.3 to -0.3 V (versus Ag/AgCl) at a scan rate of 50 mV s^{-1} .

Target DNA hybridization and methylated DNA detection

Following the procedure outlined above, the microelectrode array dipstick was placed in a 350 μL of nonmethylated target DNA that has been pretreated with the bisulfite conversion kit and incubated at the optimum parameters of 45°C for 30 min. Optimization of this step was done in our previous paper to ensure maximum yield in probe attachment and target–probe DNA hybridization (Saheb, Patterson, and Josowicz 2014). The chip was then washed and soaked in Tris-HCl buffer. Three consecutive cyclic voltammograms were taken of the array chip in Tris-HCl buffer using a potential range of +0.3 to -0.3 V at a scan rate of 50 mV s^{-1} . This procedure is also repeated for the pretreated methylated target DNA.

Results and discussion

The viability of this array system in detection of GSTP1 methylation relies on the (a) selection and treatment of probe and target sequences and (b) the adaptation of techniques used in the electrochemical detection of DNA hybridization by individual microelectrode to an array platform.

Methodology of the applied detection in GSTP1 sequence

As mentioned in the introduction, the specificity of biorecognition of our electrochemical DNA hybridization method was studied with synthetic 27-mer strands of oligonucleotide of the sequence, which relates to a hypermethylated region of the GSTP1 gene (Hartung et al. 2005). The correlation between GSTP1 hypermethylation and the occurrence of prostate cancer enables the practical use of these sequences for rapid detection of hypermethylation and by implication, prostate cancer.

The application of a bisulfite treatment to target DNA oligomers is a crucial step in detecting methylation of DNA. The bisulfite treatment deaminates cytosine residues and converts them into uracil residues (Hayatsu, Wataya, and Kazushige 1970). This treatment when applied to our nonmethylated target alters the sequence making it noncomplementary to the probe oligonucleotide sequence. This reduces the ability of the target DNA to hybridize to the probe, thereby diminishing the effect of probe exposure to nonmethylated sequence on the area of cyclic voltammogram. Bisulfite treatment of methylated target DNA, however, is ineffective as the bisulfite is nonreactive with methylcytosine residues, thereby circumventing alterations to the DNA sequence. This difference in chemical reactivity enables our modified microelectrode array to detect methylation of DNA sequences. Bisulfite treatment of DNA can provide increase sensitivity, which is only surpassed by its more costly and time-intensive genetic sequencing (Herman et al. 1996).

Characterization of platinum microelectrode array chip

The electrochemical quality of the bare Pt microelectrode surface area was evaluated through cyclic voltammetry of 2 mM $K_4Fe(CN)_6$ /2 mM $K_3Fe(CN)_6$ in a 0.1 M KCl (1:1) mixture. A cyclic voltammogram of each channel was taken simultaneously, which is shown in Figure 3. The areas of sigmoidal shape cyclic voltammograms are compared to each other for uniformity. The channels produced an average redox current of 26.1 ± 0.5 with variation of 2.1%. The limit of detection and limit of quantitation were established in our previous work (Saheb, Patterson, and Josowicz 2014).

Suitability of the microelectrode array as a methylation detector in GSTP1

To evaluate the microarray detection capacity, we recorded three cyclic voltammograms after the immobilization of probe and repeated after the probe interactions with the non-methylated and methylated target solutions as outlined above. Three cyclic voltammograms were taken to ensure stabilization; thus only the last cyclic voltammogram cycle of each series was used in the subtraction calculation. The cyclic voltammogram recorded after the probe immobilization was used as a baseline to measure the change in cyclic voltammogram areas for each modified microelectrode array following sequential exposures to the nonmethylated and methylated target DNA solutions.

As a method of qualitative analysis, we compared the cyclic voltammograms of postprobe–target interactions to the initial baseline cyclic voltammogram. An overlay of the final cyclic voltammogram of each modified microelectrode array after each exposure is shown in Figure 4. The cyclic voltammogram overlay depicts a change in the area of each channel after both nonmethylated and methylated target DNA exposure. When comparing the change between each target DNA exposure (baseline—nonmethylated target vs.

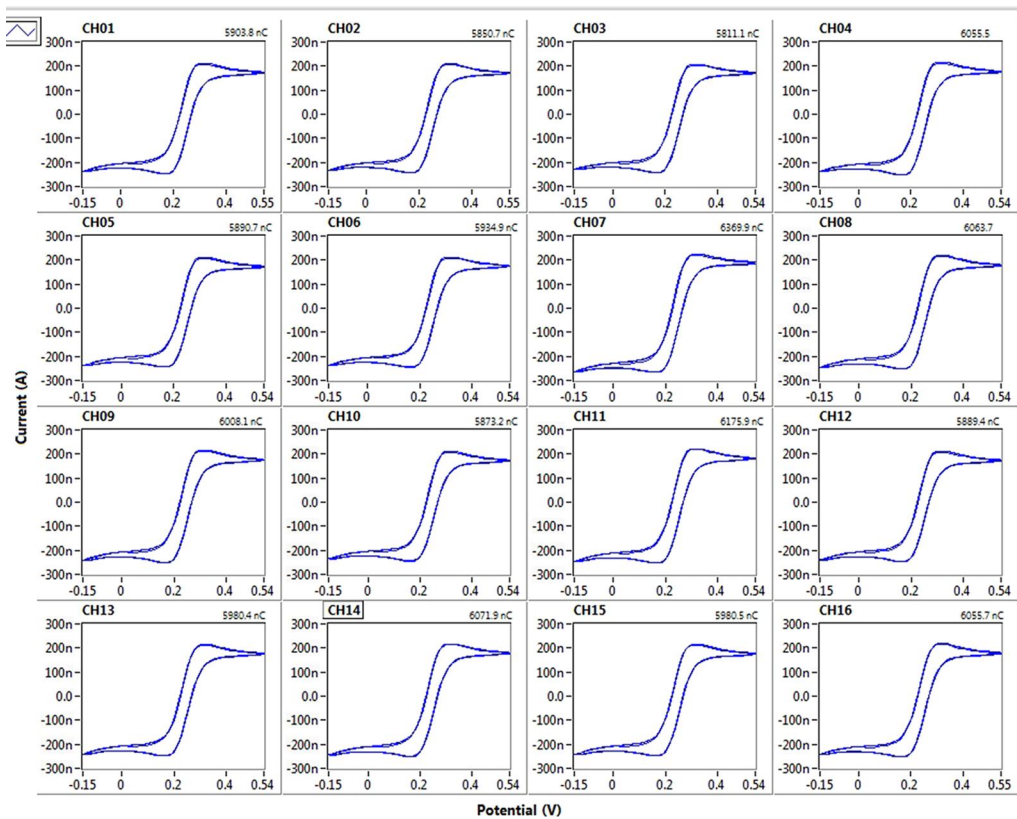


Figure 3. Cyclic voltammogram of 16-channel microelectrode array Pt surface in 2 mM $K_4Fe(CN)_6$ / 2 mM $K_3Fe(CN)_6$ in 0.1 M KCl (1:1) mixture.

baseline—methylated target), the change is shown to be greater after exposure to the methylated DNA.

The detection signal for each channel of the modified microelectrode array chip was quantified from the area of the recorded cyclic voltammograms as the percent difference. The mean and relative standard deviation were also calculated. The area of each cyclic voltammogram was obtained directly from the LabView software. The difference between cyclic voltammogram areas for each exposure trial and their baseline cyclic voltammogram is used to calculate the percentage difference as shown in the following equations:

$$\text{difference for non-methylated target (\%)} = \frac{(A_{\text{probe}}) - (A_{\text{non-methylated target}})}{A_{\text{probe}}} \times 100 \quad (1)$$

$$\text{difference for methylated target (\%)} = \frac{(A_{\text{probe}}) - (A_{\text{methylated target}})}{A_{\text{probe}}} \times 100 \quad (2)$$

In Figure 5, the percent differences of all cyclic voltammograms of the methylated target and nonmethylated target are compared following Eqs. (1) and (2). It is seen that a greater percentage change is always observed for methylated target exposure. The total difference

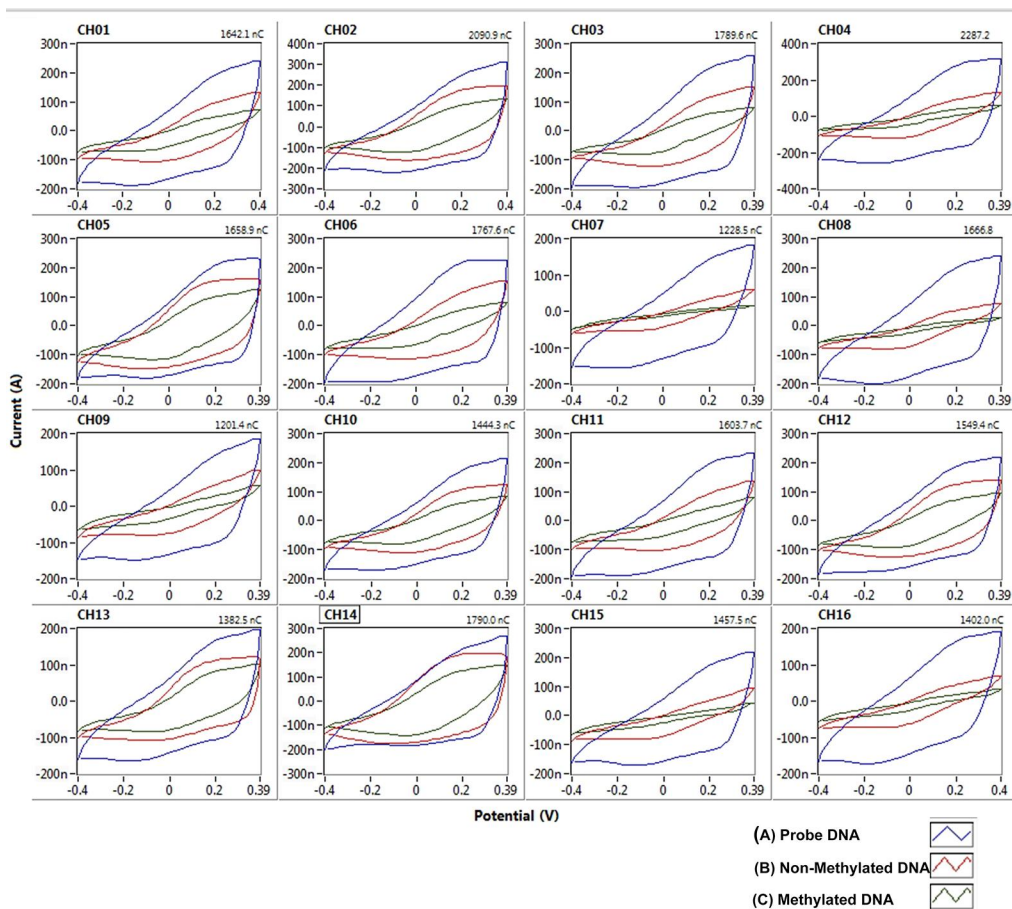


Figure 4. Comparison between cyclic voltammograms of microelectrodes array modified with polypyrrole/pTPTC3PO₃H₂/Mg²⁺ bilayer recorded in 0.1 M Tris-HCl buffer (pH 7.2) at a scan rate of 50 mV/s. Curve (a): baseline cyclic voltammogram—after attachment of the probe DNA and no target DNA exposure. Curve (b): nonmethylated target exposure—after exposure to the pretreated nonmethylated target DNA. Curve (c): methylated target exposure—after exposure of the same microarray chip to pretreated methylated target DNA.

for each individual electrode was calculated and compared by:

$$\text{total percentage difference} = \text{Eq. (2)} - \text{Eq. (1)} \quad (3)$$

Figure 6 shows a greater change in total cyclic voltammogram area after exposure to the methylated target. The average percent difference was $25.6 \pm 4.9\%$ with variation of 19.1%.

The operational structure of our array comprises of a polypyrrole-pTPTC3-PO₃H₂ bilayer that serves as a chloride ion exchanger and also anchors the probe DNA oligonucleotides (through the Mg²⁺ linkage) to the bilayer surface. The immobilized probe DNA provides target DNA sequence recognition, which ultimately increases the space charge barrier upon hybridization. Changes in the space charge barrier results in a reduction in the Cl⁻ ion exchange occurring at the polypyrrole layer which is reflected in the change in the area and shape of the cyclic voltammograms.

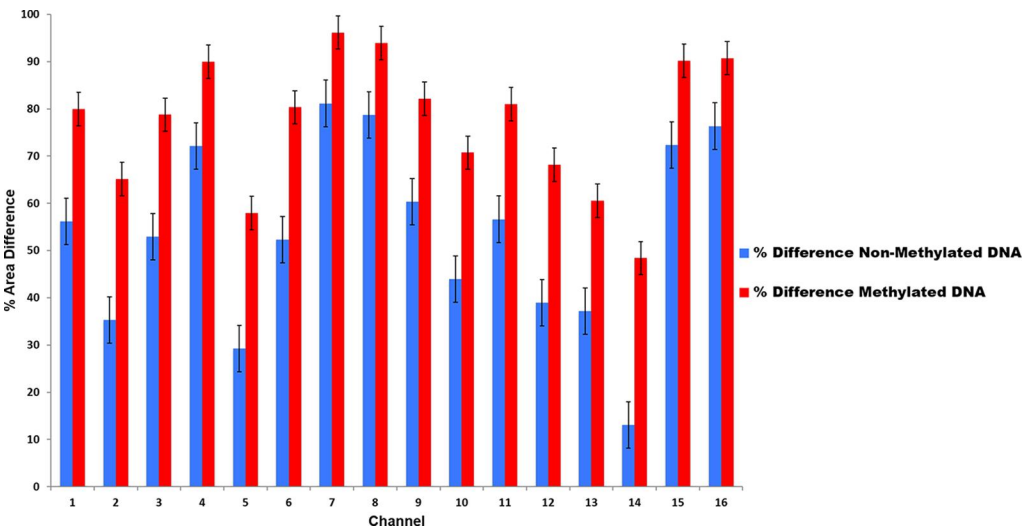


Figure 5. Percent difference of cyclic voltammogram area of nonmethylated target versus percent difference of cyclic voltammogram area of methylated target. The error bars show the standard deviations.

The results show a reduction of the area of cyclic voltammogram after both nonmethylated and methylated target DNA exposures. This reduction, however, is significantly greater after the methylated target DNA exposure. This suggests that greater specific

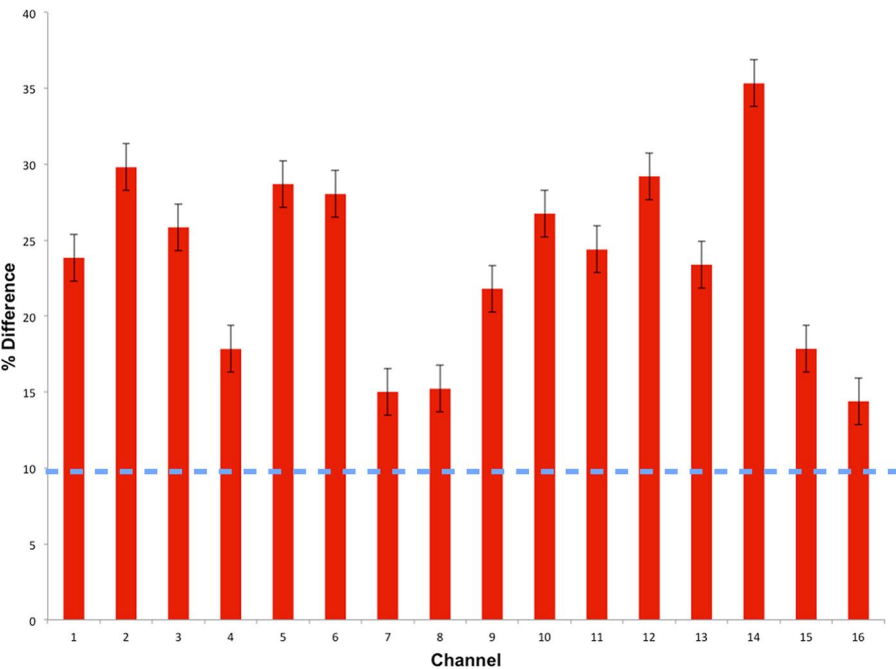


Figure 6. Total percent difference of cyclic voltammogram areas. The error bars show the standard deviations.

binding occurred in the methylated target DNA exposure than the nonmethylated target DNA exposure. These findings mirror previous work that shows noncomplementary target DNA has a less significant effect on the exchange of Cl^- ions within the polypyrrole layer because of its inability to hybridize with the probe molecules. It is believed that cyclic voltammogram area changes observed after probe exposure to nonmethylated target DNA suggest minor nonspecific binding events of noncomplementary DNA sequences and more apparent hybridization of unconverted nonmethylated DNA oligonucleotides. The results demonstrate that the unconverted DNA has a greater effect, as noncomplementary DNA and, it can be seen as a noncompetitive in binding to probe DNA (Aiyejorun et al. 2005). We attribute this change to unsuccessful bisulfite conversion of the nonmethylated target DNA oligos. Future testing of new bisulfite protocols must be done to ensure a higher conversion efficacy. Despite these effects, our sensor has been able to show significantly greater sensitivity to methylated target DNA.

Conclusion

We evaluated the performance of the microelectrode array in its detection and quantification of DNA methylation through hybridization events of methylated complementary single-stranded DNA versus nonmethylated noncomplementary single stranded using a commercially available bisulfite conversion kit. The hybridization events were monitored through changes in the chloride ion flux to the platinum electrode modified with the polypyrrole bilayer and DNA probe before and after the hybridization event took place. The reduction of chloride ion exchange in methylated (complementary) targets was always higher as compared to the signals resulting from interaction of the probe with nonmethylated (non-complementary) targets. Hybridization of methylated target DNA sequences was detected with an average percent difference of $25.6 \pm 4.9\%$ with variation of 19.1%. These results indicate that this assay provides very good sensitivity to binding events and high specificity for the GTSP1 methylated target DNA above the previously determined 10% threshold value of hybridization efficiency (Kalantari et al. 2010). They also confirm that it is possible to distinguish the specific from the nonspecific binding interactions and moreover provide a *yes–no* response on DNA methylation.

The significance of this array lies in its ability to perform a highly specific and sensitive assay several times simultaneously, thereby providing improved statistical precision in DNA methylation profiling. This study highlights the potential use of this technology in developing a noninvasive, small, fast, easy-to-use, and low-cost DNA hybridization detection device for rapid screening of hypermethylated DNA as a prostate cancer biomarker. Another potential useful application of this technology is its use in the detection of multiple gene methylation patterns, thereby offering increased diagnostic possibilities. We look to support these findings with the evaluation of microarray using human specimens.

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