Electrochemical Assay of GSTP1-related DNA Sequence for Prostate Cancer Screening

by Amir H. Saheb, Michelle Leon, and Mira Josowicz

There is a need in many areas of biochemical research to be able to assay for the presence of nucleic acids in a sample, and further, to assay for the existence of a particular sequence within the nucleic acid. One approach to this problem is the exploitation of hybridization taking place between single-stranded DNA or RNA strands that have a complementary sequence. Herein we demonstrate such a possibility for the detection of prostate cancer.

Prostate cancer (PCa) is the most commonly diagnosed cancer, and the second leading cause of cancer-caused death, in men over the age of 40 years in the United States.[1] The predominant tools for early detection of PCa are prostate specific antigen (PSA) testing and the digital rectal exam (DRE). PSA, a biomarker for PCa, was initially used to monitor treatment response. However, it is uncertain whether extensive use of PSA for PCa screening has prolonged survival of PCa patients. The low specificity of PSA leads to a high number of unnecessary biopsies causing avoidable patient discomfort, uncertainty and an undue burden on the healthcare system. Raising the threshold PSA level at which prostate biopsy is recommended (historically 4.0 ng/ml in the United States) is not advisable due to the significant number of PCa patients with PSA levels below 4.0 ng/ml, 15% of whom will be diagnosed with aggressive disease.[2] As a tool for monitoring treatment response, PSA performs proficiently; however, the development of a more specific tool to aid in early detection of PCa and to guide biopsy acquisition decision is needed. A biomarker with improved specificity as compared to PSA is a public health priority especially for early detection and screening of PCa patients. Attention has turned to the investigation of PCa biomarkers that are independent of PSA to provide additional information and aid clinical decisions.

Epigenetic alterations, including aberrant DNA methylation, are among the most common molecular alterations in human cancer.[3] The most commonly described epigenetic alteration in prostate cancer is hypermethylation of the glutathione-S-transferase π gene (GSTP1).[4,5] Millar and co-workers performed a detailed methylation analysis of the GSTP1 gene in prostate cancer by direct sequencing of the core promoter region followed by Genescan analysis to quantify the methylation status as shown in Fig. 1.[6] In Fig. 1, CpG sites are numbered relative to the start of transcription. In DNA isolated from LNCaP cells, which do not express GSTP1, all 38 CpG sites analyzed (from CpG -28 to CpG +10, relative to the transcription start site) were fully methylated including the transcription factor binding sites.

The basic element of a DNA sensor is the single strand oligonucleotide (ssDNA) probe, immobilized on a transducer surface. The surface property variations, after hybridization with complementary ssDNA (target strand) are subsequently measured by various analytical techniques including optical methods via fluorescence-labeled oligonucleotides,[7-9] quartz crystal microbalance,[10-14] surface plasmon resonance,[15-18] and electrochemical analysis. The development of electrochemical DNA biosensors combining base pair recognition of DNA probes with a miniaturized sensing platform has received increasing attention[19] due to their high sensitivity, quick response time for DNA hybridization detection, cost effectiveness, and portability.

In a typical electrochemical DNA hybridization detector, the ssDNA is immobilized on a conjugated polymer film, such as polypyrrole. Polypyrrole (PPy) is formed by simultaneous electrooxidation of pyrrole and polymerization on a platinum electrode immersed in a solution of an electrolyte containing anion X^-, for example, perchlorate or chloride (Fig. 2). During the electrochemical oxidation of PPy the removal of electrons from the polypyrrole π electron orbitals takes place. The "liberated" electron travels to the positively-charged

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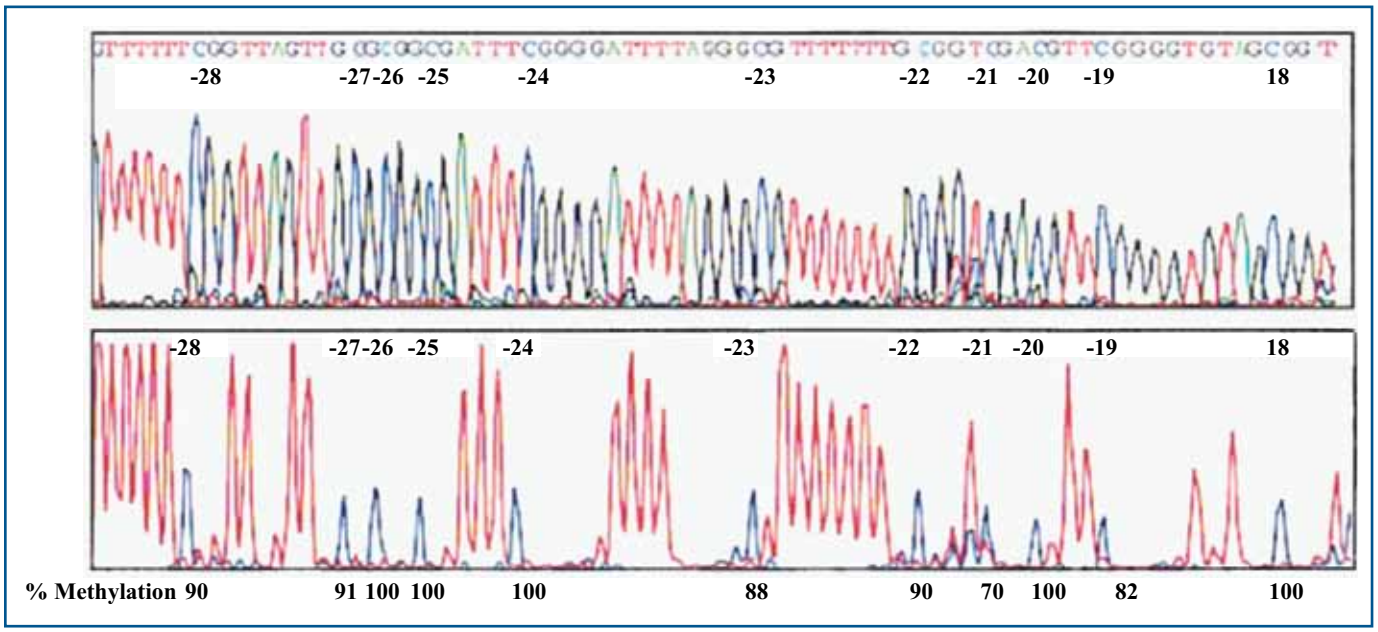

Fig. 1. *Sequence and Genescan analysis of core promoter region of GSTP1 gene. Direct PCR sequence analysis of core promoter region of DNA amplified from bisulphite-treated LNCaP DNA. The sequence is shown at the top of the panel and CpG site numbers are indicated with per cent methylation below each site. (Adapted from Ref. 6.)*

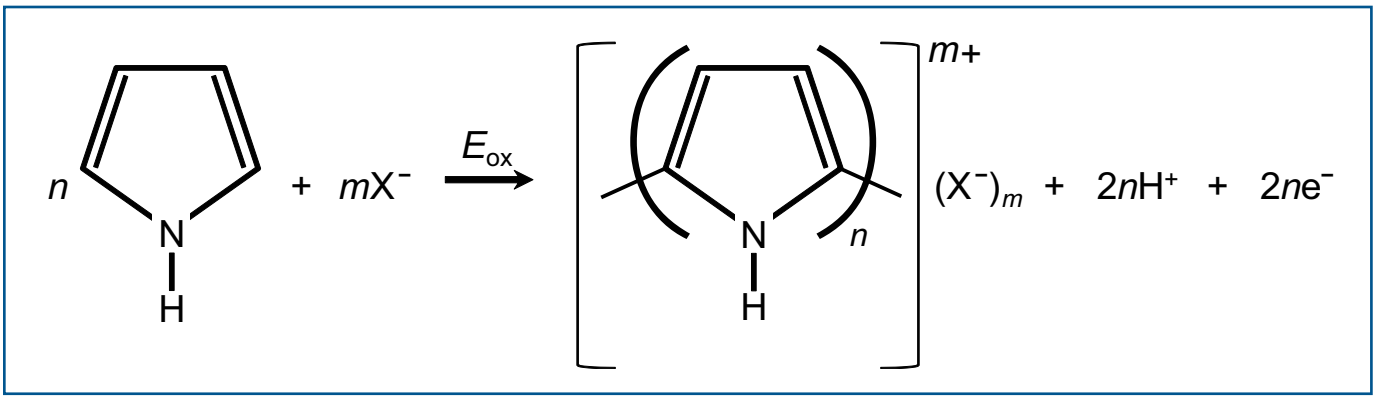

FIG. 2. *Electropolymerization of pyrrole. X^- is the dopant anion that is inserted from electrolyte and m is number of dopant anions.*

Pt electrode on which the PPy is deposited. In chemical terms, the electron-deficient species in PPy is called a cation-radical, while materials scientists prefer to call it a polaron.

Obviously, the existing π system of the PPy that is interrupted by this "defect," still maintains its properties as a vehicle for electrical charge transport. The positive "defect" is redistributed within the π system much like a Gaussian distribution, and is not stationary. In summary, the PPy becomes positively-charged and needs to be compensated by a negatively-charged entity in order for the system to maintain charge neutrality.[20-22]

An important property of this material is that it can be reversibly reduced and reoxidized by controlling the potential applied to the polymer-modified platinum electrode. A convenient method for repetitive sweeping the electrode potential, *E*, from the positive to negative direction while monitoring current, *i*, is cyclic voltammetry. When a positive potential is applied to the PPy-modified electrode, the electrons leave the electrode. Simultaneously, the chloride anions from the electrolyte penetrate the bulk of the polymer to compensate for the electrochemically-generated charge imbalance at the electrode.[23,24] During the reduction of PPy, the polymer gains electrons that are being used by the polymer "to fill" the positive "defects" (polarons) and the dopant anions, X^-, are expelled from the PPy back to the electrolyte. Again, that process helps to preserve the charge neutrality in the polymer. Furthermore, the cyclic voltammetry traces the quantity of electrons that are transferred to the electrode or from the electrode when the potential *E* is applied to the modified electrode (working electrode). Therefore, it is possible to determine the quantity of charge that is accepted and removed from the polymer during the potential cycling from the area under the recorded *i–E* curve.

Consequently, repetitive cycling of the PPy film should result in identical cyclic voltammograms (CVs). This electrochemically-controlled anion-exchange process forms the basis for understanding the operation of the anticipated development of the DNA hybridization detector. Therefore we need to conduct one more thought experiment. Suppose, we create a barrier at the PPy–solution interface that carries negative charge. Will the electrostatic barrier affect the quantity of the anions penetrating the bulk of the PPy film? The answer is no, because the electrostatic barrier will affect the kinetics of the exchange of the anions but not the quantity. Therefore, the slower kinetics of negative ions penetrating bulk of the PPy layer affects the shape of the recorded CV. DNA hybridization can be diagnosed from the changes of the CV shape.

A proper layout of the modified electrode is needed to create an electrostatic barrier for chloride ions on top of the PPy layer. In this context, it is useful to think of a DNA molecule as a polyanion. In DNA, the building blocks consisting of nucleic base and deoxyribose are linked to each other as esters via a phosphoryl moiety. This arrangement leaves the remaining acid hydroxy group unbound and negatively charged under appropriate pH conditions. Such a site can be bound to any other negatively charged entity via multivalent ions, such as Mg^{2+}. The single strand, ssDNA, is capable of forming a double helix with a complementary strand by establishing hydrogen bonds between complementary nucleic bases. The shape of the macromolecule becomes cylinder-like; the inner core consists of the electrically neutral nucleic bases, while the external coat contains ionizable phosphate groups. The hybridization process places the negative charges on the outer part of the double helix. Consequently, the presence of the negative charges on the outer part of the double helix defines the type of the electrostatic barrier for the PPy layer. To link that electrostatic barrier to the PPy-modified electrode we need to introduce another polymer on top of the PPy layer. It is required that this polymer be of limited thickness and contain a functional group that is able to link the phosphate groups of the DNA probe using a complex bond formation.

We chose a grafted thin layer of 2,5-bis(2-thienyl)-*N*-(3-phosphorylpropyl) pyrrole, $pTPTC3PO_3H_2$. The presence of the phosphonic acid groups on the $pTPTC3PO_3H_2$ allows the link with the phosphate groups of the ssDNA probe through the divalent magnesium ions. The assembled electrochemical label-free DNA hybridization detector is then exposed to a solution of a target DNA (non-complementary or complementary ssDNA) and the CV response is tested. The analytical signal of the detector, $i = f(E)$, is recorded before and after exposure to the target DNA. If the target DNA strand is complementary, the barrier height increases, hence, the chloride ion exchange between the PPy and the solution becomes kinetically hindered resulting in a shape change of the CV. Thus our label-free electrochemical detection of DNA hybridization is based on this measured CV shape change.

In previous work, Josowicz and co-workers have demonstrated the unambiguous identification of hybridization event based on single-stranded oligonucleotide probes of known sequence immobilized on a single Pt-microelectrode *a priori* to an addition of sample with fully complementary target DNA.[25-29] The DNA sensor relies on the immobilization of single-stranded DNA (ssDNA) probe on a transducer to create a ssDNA recognition detector. The DNA detector recognizes its complementary ssDNA target and forms a hybrid with it resulting in useful analytical data. An electropolymerized-conducting-polymer bilayer consisting of polypyrrole (PPy) and poly[2,5-dithienyl-(N-3-phosphoryl-propyl)-pyrrole] (pTPT), which acts as electrochemically-controlled ion-exchanger during the CV. In a chloride-containing buffer (pH = 7.2) the rate of exchange of chloride ion, and thus the shape of CV, is affected by the electrostatic barrier at the electrode/buffer interface (Fig. 3). Upon hybridization of the ssDNA probe with its complementary ssDNA target, the charge at the sensing layer increases, hindering the exchange of chloride ions, which in turn reduces the current flow and affects the shape of the CV.

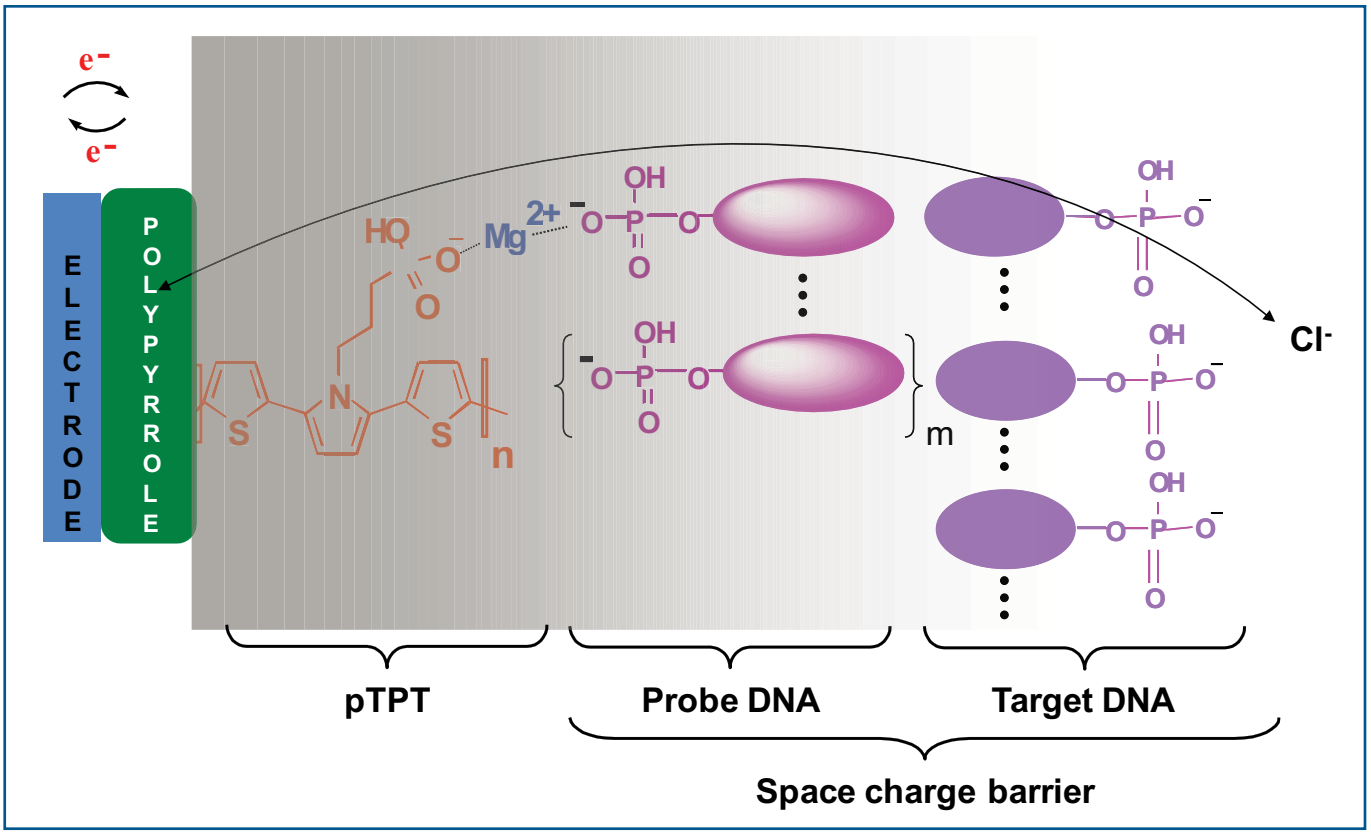

FIG. 3. *Key steps in the preparation of the DNA hybridization sensor.*

Detection of a GSTP1 Sequence

The specificity of the biorecognition of our electrochemical DNA hybridization method was studied with synthetic 27-mer strands of oligonucleotide of the sequence as shown in Table I. The probe consisted of a 15-mer strand of ss-DNA.

This particular single-stranded target DNA was chosen because it relates to the GSTP1 gene in a region which shows hypermethylation of its five cytosines residues (Fig.1). A baseline cyclic voltammogram (CV) in TRIS-HCl was acquired of the electrode with the probe DNA attached (Fig. 4, red curve a). The electrochemical probe was immersed in a solution of the non-complementary target DNA (0.1×10^{-6} mol L^{-1}) for 30 min and a cyclic voltammogram (CV) acquired as shown in Fig. 4 (blue curve b). As the non-complementary target DNA will not completely hybridize with the probe DNA, a significant change versus the cyclic voltammogram was not expected to occur when the cyclic voltammogram was run in TRIS-HCl buffer as shown by the blue curve. However, after the probe was immersed in complementary target DNA (0.1×10^{-6} mol L^{-1}) for 30 min (Fig. 4, green curve c), a larger change in the CV was apparent as the hybridization event at the electrode surface due to dsDNA was hindering exchange of chloride ions and thus the flow of current resulting in the CV. The change in the CV can be quantified by subtracting the curves after hybridization with target DNA (complementary and non-complementary) from the curve after attachment of the probe DNA as shown in Fig. 4 (curves d and e). As is observed in the subtraction of the CVs, the change is greater for the complementary target DNA (curve d) versus the non-complementary target DNA (curve e). In summary, our results indicate that the addition of negative charge to the surface of the electrode, in the form of complementary target DNA, further hinders the chloride ion exchange as seen from the decrease of CV current. Thus, non-complementary and complementary interactions can be clearly distinguished. We hypothesis that heating the target DNA to 50°C will cause an even greater decrease in the CV current versus the non-complementary target as this will make the DNA less likely to hybridize to mismatched bases.

Moreover, to support the electrochemical data acquired through the CVs, infrared spectroscopy was used as a means of detecting the grafting of Mg^{2+} and the individual hybridization events for both methylated complementary target DNA and unmethylated complementary target DNA at the cytosine residue. Fourier Transform IR (FTIR) spectra were collected on a BIORAD FTS-6000 spectrometer with a BIORAD UMA 500 IR microscope run in the ATR mode using a germanium crystal. All the FT-IR spectra of the cast films were measured using a 1.5 mm diameter Pt disc electrode as a background. Polypyrrole films were electropolymerized from a solution of 0.1 M pyrrole holding at 0.8 V for 4 mC. Afterward, pTPTC3-PO_3H_2 polymerization was performed from a 4 mM solution of TPTC3-PO_3H_2 holding at 0.8 V for 2 mC. Attachment of Mg^{2+} was made by placing

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Table I. DNA Inventory

DNA	Sequence
Probe	5' TCG CCG CGC AAC TAA 3'
Target (Non-Complementary*)	5' GCC TTT GGG TCC ATT TAA TTC GCC TTT 3'
Target (Complementary*)	5' TTT CGG TTA GTT GCG CGG CGA TTT CGG 3'

*In reference to probe

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the electrode in a 0.5 mM $MgCl_2$ solution for 10 min. Probe and target DNAs were immobilized on the electrode by immersing the electrode in the respective 10^{-8} molar solution of DNA (in Tris-HCl buffer) for 30 min.

FTIR spectra for probe ssDNA (curve a), methylated complementary target DNA (curve b) and unmethylated complementary target DNA (curve c) after hybridization are shown in Fig. 5. Detailed analysis of these data indicated that there were distinguishing factors between the hybridized target DNAs and single-stranded probe DNA that was attached to the electrodes. This is due to the double amount of sugar-phosphate regions present in the duplexed target DNA samples. Other observations included a tendency of greater peak shifts between the probe DNA and unmethylated DNA samples than between the probe and methylated DNA samples in the 800-1200 cm^{-1} spectral region.

Conclusion

A label-free detection scheme for a short GSTP1 related DNA sequence (27-mer) based on conducting-modified polypyrrole films deposited at a microelectrode surface was presented above. The addition of negative charge to the PPy-pTPTC3-PO_3H_2-Mg^{2+}/probe DNA-modified electrode surface due to phosphonic acid groups of the complementary strand hinders chloride ion exchange and causes a decrease in cyclic voltammetry current. The microelectrode sensor exhibited a reduction in the current upon exposure to the complementary target DNA related to the GSTP1 gene. Hypermethylated sequences of the GSTP1 gene were shown to be prevalent in prostate cancer patients and may thus serve as a specific genomic sensor for early detection of prostrate cancer. Similar experiments are planned with microelectrodes using target DNA which is methylated at the cytosine residues. This will allow us to use a bisulfite conversion kit which consists of treating DNA with bisulfite, converting unmethylated cytosines to uracil but not changing methylated cytosines. The methylated cytosine residues are left as cytosine and will bind to its complementary guanine base on the probe DNA of our sensors. The direct application of this electrochemical DNA hybridization probe on clinical samples offers great promise for its translational use in early PCa diagnosis, prognostic assessment of tumor behavior, as well as monitoring response to therapeutic agents.

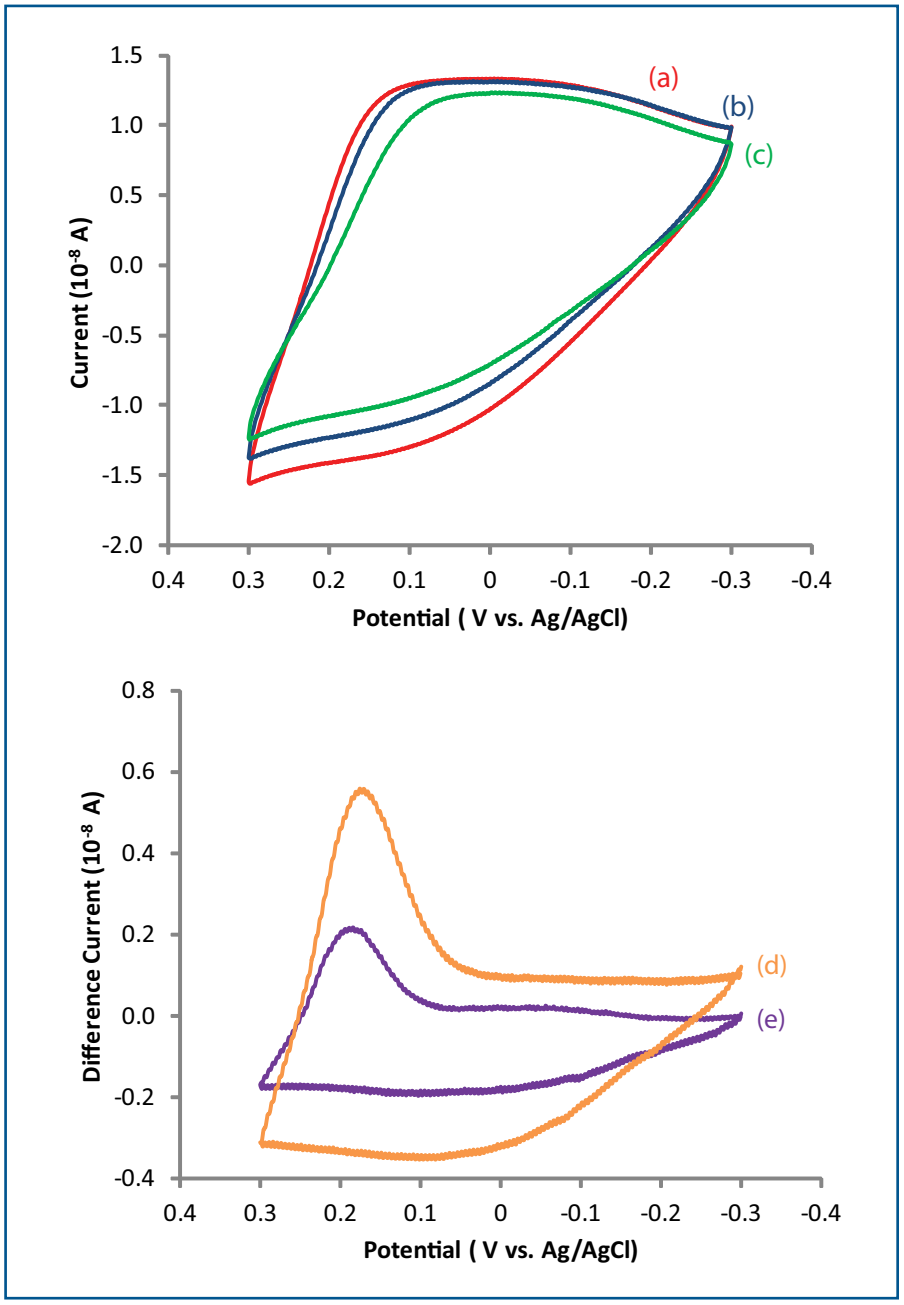

FIG. 4. *Electrochemical responses of the DNA hybridization sensor on a 25 micron Pt disc electrode: (a) after immobilization of DNA probe, (b) after exposure to the non-complementary target DNA, (c) after exposure to the complementary target DNA, (d) subtraction of the responses to complementary DNA and probe DNA (curves a-c), and (e) subtraction of the responses of non-complementary DNA and probe DNA (curves a-b). All voltammograms were recorded in TRIS-HCl pH 7.2 buffer at 50 mV s^{-1} scan rate.*

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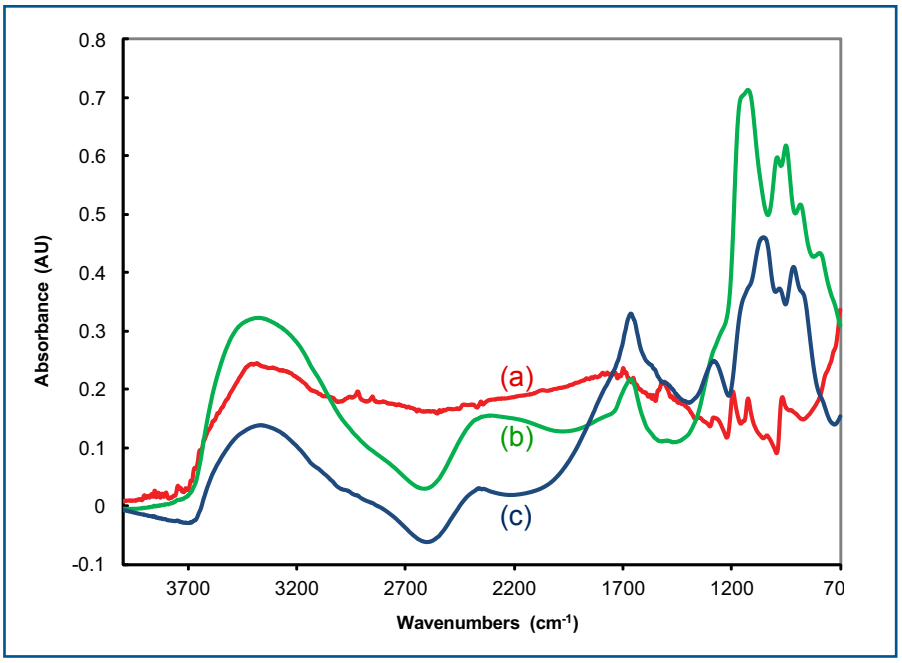

FIG. 5. *IR spectra of (a) Probe DNA, (b) hybridized methylated complementary target DNA, and (c) hybridized unmethylated complementary Target DNA recorded from modified macroelectrode after placing 2μL of DNA samples. All data were collected using a 1.5mm diameter Pt disc macroelectrode after PPy, TPTC3-PO_3H_2 polymerization and attachment of Mg^{2+}.*

Amir H. Saheb received his BS along with his MS and PhD degrees in analytical chemistry from the Georgia Institute of Technology in Atlanta, GA. In 2009, Dr. Saheb joined Albany State University as an Assistant Professor of Chemistry. Dr. Saheb conducts biomedical research into developing medical diagnostic sensors for the early detection of prostate cancer in direct collaboration with researchers at the Georgia Institute of Technology. He may be reached at asaheb@asurams.edu.

References

1. A. Jemal, T. Murray, A. Samuels, A. Ghafoor, E. Ward, and M. Thun, *CA Cancer J. Clin.*, **53**, 5 (2003).
2. I. M. Thompson, D. K. Pauler, P. J. Goodman, C. M. Tangen, M. S. Lucia, and H. L. Parnes, *N. Engl. J. Med.*, **350**, 2239 (2004).
3. P. A. Jones and S. B. Baylin, *Nat. Rev. Genet.*, **3**, 415 (2002).
4. W. H. Lee, R. A. Morton, J. I. Epstein, J. D. Brooks, P. A. Campbell, and G. S. Bova, *Proc. Natl. Acad. Sci. USA*, **91**, 11733 (1994).
5. M. Nakayama, M. Gonzalgo, S. Yegnasubramanian, X. Lin, A. De Marzo, and W. Nelson, *J. of Cell. Biochem.*, **91**, 540 (2004).
6. D. Millar, K. Ow, C. Paul, P. Russell, P. Molloy, and S. Clark, *Oncogene*, **18**, 1313 (1999).
7. J. R. Epstein, I. Biran, and D. R. Walt, *Anal.Chim. Acta*, **469**, 3 (2002).
8. T. A. Taton, G. Lu, and C. A. Mirkin, *J. Am. Chem. Soc.*, **123**, 5164 (2001).
9. R. K. Rogers, A. Apostol, J. S. Madsen, and W. C. Spencer, *Anal. Chim. Acta*, **444**, 51 (2001).
10. F. Patolsky, A. Lichtenstein, and I. Willner, *J. Am. Chem. Soc.*, **123**, 5194 (2001).
11. K. Feng, J. Li, J. H. Jiang, G. L. Shen, and R. Q. Yu, *Biosens. Bioelectron.*, **22**, 1651 (2007).
12. D. D. Atti, S. Tombelli, M. Minunni, and M. Mascini, *Biosens. Bioelectron.*, **21**, 1876 (2006).
13. S. H. Chen, V. C. H. Wu, Y. C. Chuang, and C. S. Lin, *J. Microbiol. Methods*, **73**, 7 (2008).
14. G. Hui-Shi, K. Jong-Min, K. Seung-Jin, C. Sang-Mok, and K. Woo-Sik, *Langmuir*, **25**, 648 (2009).
15. B. P. Nelson, T. E. Grimsrud, M. R. Liles, R. M. Goodman, and R. M. Corn, *Anal. Chem.*, **73**, 1 (2001).
16. P. Gong, C. Y. Lee, L. J. Gamble, D. G. Castner, and D. W. Grainger, *Anal. Chem.*, **78**, 3326 (2006).
17. P. Guedon, T. Livache, F. Martin, F. Lesbre, A. Roget, G. Bidan, and Y. Levy, *Anal. Chem.*, **72**, 6003 (2000).
18. M. Manesse, V. Stambouli, R. Boukherroub, and S. Szunerits, *Analyst*, **133**, 1097 (2008).
19. K. Kerman, M. Kobayashi, and E. Tamiya, *Meas. Sci. Technol.*, **15**, R1 (2004).
20. M. Levi, C. Lopez, E. Vieil, and M. Vorotyntsev, *Electrochim. Acta,* **42**, 757 (1997).
21. A. Diaz and J. Bargon, *Handbook of Conducting Polymers,* T. A. Skotheim, Editor, Dekker, New York (1986).
22. S. Sadki, P. Schottland, N. Brodie, and G. Sabouraud, *Chem. Soc. Rev.,* **29**, 283 (2000).
23. A. Hillman, M. Swann, and S. Bruckenstein, *J. Electroanal. Chem.,* **291**, 147 (1990).
24. A. Bard and L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications,* Wiley & Sons, New York (2001).
25. L. A. Thompson, J. Kowalik, J. Janata, and M. Josowicz, *J. Am. Chem. Soc.*, **125**, 324 (2003).
26. T. Aiyejorun, J. Kowalik, J. Janata, and M. Josowicz, *J. Chem. Educ.*, **83**, 1208 (2006).
27. J. Hartung, J. Kowalik, C. Kranz, J. Janata, M. Josowicz, A. Sinha, and K. McCoy, *J. Electrochem. Soc.*, **152**, E345 (2005).
28. C. Riccardi, H. Yamanaka, M. Josowicz, J. Kowalik, B. Mizaikoff, and C. Kranz, *Anal. Chem.*, **78**,1139 (2006).
29. T. Aiyejorun, L. Thompson, J. Kowalik, J. Janata, and M. Josowicz, *Electrochemistry of Nucleic Acids and Proteins*, Elsevier Publishers, 331 (2005).