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Electrochemical DNA biosensor for detecting cancer biomarker related to glutathione *S*-transferase P1 (*GSTP1*) hypermethylation in real samples

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ABSTRACT

An electrochemical genosensor for the detection of hypermethylation of the glutathione *S*-transferase P1 (*GSTP1*) gene, a specific marker of prostate cancer, was reported. This new sensor was used in combination with a single-use carbon graphite working electrode and differential pulse voltammetry, with the results of sample analysis based on the guanine oxidation signals obtained at +1.0 V before and after hybridization between probe and synthetic target or denatured PCR samples. The detected DNA hybridization was also characterized by electrochemical impedance spectroscopy with potassium ferri/ferrocyanide as a redox probe. The protocol consisted of 2 different modes: (i) capture probes selective for methylation-specific and unmethylated *GSTP1* sequences were immobilized onto the sensor directly, and hybridization was formed on the electrode surface; (ii) probe/target or probe/noncomplementary target couples were mixed in solution phase, and the transducer was modified through simple adsorption. The limit of detection (S/N = 3) was calculated as 2.92 pmol of target sequence in a 100-μl reaction volume. The optimum analytical detection parameters for the biosensor, as well as its future prospects, were also presented.

1. Introduction

Prostate cancer is the most frequently diagnosed type of cancer and the second leading cause of cancer-related mortality in men in recent years (Jemal et al., 2010). The etiology of prostate cancer involves several genetic and environmental factors. One of the many genetic factors contributing to the progress of prostate cancer is a member of the glutathione *S*-transferase (GST) superfamily (Sundberg et al., 1998).

Glutathione *S*-transferase P1 (*GSTP1*) is mainly related to the metabolism, detoxification, and elimination of potentially toxic compounds in the cell. These functions of *GSTP1* help protect mammalian cells from the electrophilic metabolites of carcinogens and reactive oxygen species, and thus against cancer risk. Suppression of *GSTP1* activity or any alteration of this gene could result in DNA damage and increased cancer incidence. Methylation of CpG islands in the promoter region of genes is a frequently acquired epigenetic event in the pathogenesis of many human cancers. This modification inhibits the expression of the affected genes and leads to gene deletions or mutations that can cause loss of gene function (Esteller et al., 2001). The genes that are exposed to methylation during the early phases of tumorigenesis could potentially be used as prognostic markers (Roupret et al., 2007, 2008). Many studies have reported the use of *GSTP1* methylation as a biomarker to detect prostate cancer (Bastian et al., 2005; Herman et al., 2008). Hypermethylation of the *GSTP1* gene promoter region is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or benign hyperplasia of prostate (Lee et al., 1994; Millar et al., 1999). Thus, promoter hypermethylation of *GSTP1* is the best DNA-based biomarker for the disease.

To date, many different approaches have been developed to analyze DNA methylation, such as methylation-specific PCR (MSP), methylation-sensitive restriction digestion, bisulfite sequencing, and microarray analysis (Gebhard et al., 2006; Gitan et al., 2002). Bisulfite treatment of DNA has been the most popularly used approach to detect DNA methylation (Frommer et al., 1992). The basic principle of this method is that the cytosines are completely deaminated to uracil, whereas the 5-methyl cytosines remain unmodified; the sequences under investigation are then amplified by PCR, especially with MSP, using a set of primers specific for the converted sequence. The specificity of MSP is higher than that of other methylation detection techniques due to specific primer design.

Alterations of PCR samples, including DNA methylation, can be analyzed by agarose gel electrophoresis. However, the classical detection of PCR samples by agarose gel electrophoresis

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is labor intensive, redundant, and involves the use of hazardous elements such as ethidium bromide and ultraviolet light.

Electrochemical DNA biosensor applications offer a highly sensitive and promising method for the detection of hybridization, genetic polymorphisms, and mutations, as well as for methylation analysis, because of their short assay time, miniaturization, portability, and inexpensiveness. The main principle of electrochemical DNA biosensors is based on the conversion of hybridization events to analytical signals via a transducer. There are many aspects of DNA hybridization analysis; for instance, the most easy and rapid way is the direct detection of DNA oxidation signals of guanine and adenine bases through voltammetry techniques (Topkaya et al., 2010). Specific DNA hybridization events can also be monitored through indirect DNA detection using selective redox indicators (Jelen et al., 2002), by utilization of PNA sequences instead of DNA (Gao and Tansil, 2009) and using nanomaterial (Tamiya et al., 2008) or enzyme tags (Rochelet-Dequaire et al., 2009) for signal amplification. Electrochemical impedance spectroscopy (EIS) is another simple and powerful method for probing the interfacial reaction mechanisms of modified electrodes, providing a rapid and very sensitive label-free detection of affinity interactions of biomolecules; it can also detect hybridization events between probe and target sequences (Zhang et al., 2008).

Recently, an efficient electrochemical method for the analysis of DNA methylation was proposed on the basis of direct electrocatalytic oxidation of DNA bases (Dai et al., 2010). Another electrochemical approach for the assay of methyltransferase activity and the detection of DNA methylation of specific CpG sites was based on the voltammetric response of the electroactive label ferrocene acetic acid (Cai et al., 2011).

In the present study, we describe a label-free electrochemical genosensor for the detection of GSTP1 hypermethylation via hybridization event using DNA oligonucleotides and PCR samples as an alternative to agarose gel electrophoresis which is the classical method to identify the alterations of PCR samples. Sequences related to methylation or unmethylation can hybridize their complementary and this interaction between them cause guanine oxidation or resistance changes and can be monitored by electrochemical methods. To our knowledge, there have not yet been any reports about the rapid electrochemical detection of this hypermethylation based on the decrease of guanine signal with DPV and resistance changes with EIS.

The results of our study demonstrate great promise for practical applications in the development of clinical assay techniques. The analytical characterization of these sensors is discussed in the following sections, with the obtained results confirmed by agarose gel electrophoresis.

2. Materials and methods

2.1. Apparatus

Differential pulse voltammetry (DPV) and electrochemical impedance spectrometry (EIS) experiments were performed using the AUTOLAB PGSTAT-10-FRA electrochemical analysis system. A Techne-512 Thermal Cycler was used for denaturation of the PCR samples, and a Thermo Electrophoretic Gel System/UV transilluminator was used for electrophoresis. The 3-electrode system consisted of a pencil graphite electrode (PGE) as the working electrode, a reference electrode (Ag/AgCl), and a platinum wire as the auxiliary electrode.

2.2. Chemicals

Stock solutions of DNA were prepared in triple-distilled water and were stored at −20 °C. Dilute solutions of the oligonucleotides were prepared daily with 0.05 M phosphate buffer containing 20 mM NaCl (PBS, pH 7.4). Tris–HCl buffer solution (20 mM, pH 7.00) was also used in optimization experiments.

2.3. Real samples and synthetic oligonucleotides

Cell-free DNA extraction: 12 ml of venous blood was collected from all cases in EDTA-containing tubes and centrifuged at 2000 rpm for 10 min at 4 °C to separate the plasma from the blood cells. Cell-free DNA from the plasma was extracted with the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) according to the protocol provided by the manufacturer.

Bisulfite treatment: Sodium bisulfite conversion of the obtained cell-free DNA was performed as follows. Briefly, 1 μg of cell-free DNA was diluted with distilled H_2O (dH_2O) to reach a final volume of 50 μl. Then 5 μl of 2 M NaOH was added to this sample and incubated at 37 °C for 10 min. After incubation 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite (pH 5.0) – both freshly prepared – were added. The sample was mixed and after adding mineral oil, incubated at 50 °C for 16 h in the dark. After incubation mineral oil was removed with a pipette and the bisulfite treated DNA was purified with the Wizard® DNA Clean-Up Kit and System (Promega, USA) according to the protocol provided by the manufacturer. At the end of this procedure DNA was eluted from the column with 50 μl of preheated dH_2O (60–70 °C). Then 5.5 μl of 3 M NaOH was added into the sample and incubated for 5 min at RT. After incubation a mixture consisting 1 μl glycogen (5 mg/ml) and 17 μl of 10 M ammonium acetate was first added to the sample, before adding three volumes of ice cold 100% EtOH. The sample was mixed by turning it up and down, incubated at −20 °C for 1–1.5 h and than centrifuged at 14,000 rpm for 20 min at 4 °C. The overlying supernatant was than removed and the pellet washed with 300 μl of ice cold 70% EtOH. After centrifugation at 14,000 rpm for 5 min at 4 °C the supernatant was removed again and the pellet was resuspended in 20 μl of dH_2O, after drying it for 30 min at RT to get rid of all remaining EtOH.

Methyl specific primer – PCR analysis: Two consecutive PCR reactions were performed: 1.PCR reaction to pre-amplify the DNA region of interest, i.e. the GSTP1 promoter region; and the 2.PCR reaction, to analyze hypermethylation of the GSTP1 promoter region by methyl specific primer (MSP) – PCR. For this purpose 0.2 μmol of HPLC-purified synthetic oligonucleotides were obtained in the form of lyophilized powder and dissolved in nuclease-free H_2O to reach a final volume of 100 pmol/μl (Interactiva, Germany).

GSTP-External Up: 5′-GGG ATT TTA GGG YGT TTT TTT G-3′ (22-mer)
GSTP-External Down: 5′-ACC TCC RAA CCT TAT AAA AAT AAT CCC-3′ (27-mer)
GSTP-IntMe-S: 5′-TTC GGG GTG TAG CGG TCG TC-3′ (20-mer)
GSTP-IntMe-AS: 5′-GCC CCA ATA CTA AAT CAC GAC G-3′ (22-mer)
GSTP-IntUn-S: 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ (24-mer)
GSTP-IntUn-AS: 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′ (24-mer)

For the 1.PCR reaction; 4 μl of bisulfite modified DNA was added to 26 μl of PCR mix containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U FastStart Taq DNA polymerase, 1 pmol/μl of each GSTP-External Up and GSTP-External Down primers. PCR was carried out under following conditions: denaturation at 95 °C for

10 min; 35 cycles of amplification at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 8 min. The resulting amplicon of this PCR reaction was 159 bp-long. For the 2.PCR two parallel reactions were set for each sample: one reaction that could detect previously methylated cytosines by using the GSTP-IntMe-S and GSTP-IntMe-AS primer pair; and the second, that could detect previously unmethylated cytosines by using the GSTP-IntUn-S and GSTP-IntUn-AS primer pair. The 2.PCR reaction mix consisted of 2 μl of pre-amplified DNA from the 1.PCR reaction and 28 μl of PCR mix containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U FastStart Taq DNA polymerase, 1 pmol/μl of each GSTP-IntMe-S and GSTP-IntMe-AS primers or GSTP-IntUn-S and GSTP-IntUn-AS primers. PCR was carried out under following conditions: denaturation at 95 °C for 10 min; 35 cycles of amplification at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 8 min. The resulting amplicon after this 2.PCR reaction was 91 bp-long when the GSTP-IntMe-S and GSTP-IntMe-AS primer pair was used; or, 97 bp-long when the GSTP-IntUn-S and GSTP-IntUn-AS primer pair was used. All PCR products were analyzed after 2% agarose gel electrophoresis.

GSTP1 promoter region: gggactccagggcgcccctctgGCCTCCGAG-CCTTATAAGGGTGGTCCCGGGACTCCAGGGCGCCCCTCTGCGGCC-*gacg***CCCGGGGTGCAGCGGCCGCC**GGGGCTGGGGCCGGCGGGAGTC-CGCGGGACCCTCCAGAAGAGCGGCCGG**CGCCGTGACTCAGCACTGG-GGC***gg*AGCGGGGCgggaccacccttataaggctcggaggc.

The sequence of the GSTP1 promoter region which was amplified to detect hypermethylation (5′ → 3′) is shown above. The sequences to which the GSTP-External Up and GSTP-External Down primers are binding to after bisulfite treatment are shown by lowercase letters at the 5′- and 3′-ends, respectively. Meanwhile, the sequences to which the GSTP-IntMe-S (sense) and GSTP-IntMe-AS (antisense) primers binding to are shown by bold uppercase letters. Since the GSTP-IntUn-S and GSTP-IntUn-AS primers are just four and two nucleotides longer at their 5′-ends, respectively, than the GSTP-IntMe-S (Sense) and GSTP-IntMe-AS (Antisense) primers; these adjacent extra-nucleotides are shown by italic lowercase letters. Methylated cytosines that are detected by methyl specific primer (MSP) – PCR analysis are indicated by underlined bold uppercase letters; whereas, cytosines that are also methylated but not detected by MSP-PCR since they are located at the 5′- and 3′-ends are indicated by underlined italic lowercase letters.

Synthetic sequences used for methylated specific (M), unmethylated (U) GSTP1 promoter and noncomplementary (NC) were:

5′-TTC GGG GTG TAG CGG TCG TC-3′ **(M-prob)**
5′-GAC GAC CGC TAC ACC CCG AA-3′ **(M-target)**
5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ **(U-prob)**
5′-AAC AAC CAC TAC ACC CCA AAC ATC-3′ **(U-target)**
5′-TGG AGT ATT GAA GCT TTT GCC GAA GGT-3′ **(NC-target)**

NC sequences were selected from the HA gene of B/Kobe/1/94 (GenBank accession no. D38646). PCR amplification of 273-bp of the influenza B virus region was performed at the Department of Microbiology in the Faculty of Medicine, Dokuz Eylul University.

2.4. Procedure

Electrochemical measurements (DPV and EIS) and agarose gel electrophoresis were used for determining hybridization events and noncomplementary discrimination. The procedure is illustrated in Scheme 1.

A. Hybridization on the electrode surface

The procedure involves the following steps after optimization conditions:

1. **Electrode pretreatment**: The PGE was pretreated by applying +1.4 V for 30 s in 0.5 M acetate buffer solution containing 20 mM NaCl (pH 4.8) (ACB) without stirring.
2. **Probe immobilization**: 100 μl of DNA probe solution (5 μg/ml), prepared in PBS, was placed in vials. The pretreated PGE was placed into the probe solution. The probe was subsequently immobilized on PGE by adsorption for 20 min. Then, the electrode surface was washed once with blank PBS solution to remove the unbound DNA.
3. **Hybridization with synthetic targets**: Probe–target hybridization was performed by dipping the probe-modified PGE into a solution of 7 μg/ml target prepared in PBS for 20 min.

   Hybridization with real PCR samples: The real samples obtained from PCR amplification were diluted with PBS (dilution rate, 1:10); the diluted samples were then placed in vials and denatured by heating at 95 °C for 5 min and then ice shocking for 1 min. The probe-covered electrodes were quickly immersed into the vials of denatured PCR samples. Hybridization was carried out for 20 min at room temperature. The electrodes were then rinsed once with PBS.
4. **Measurements**

   Voltammetric transduction with DPV: The oxidation signals of guanine bases were measured by DPV at 50 mV amplitude scanning from +0.75 to +1.4 V in ACB. Repetitive measurements were carried out by repeating the assay.

   Impedimetric transduction with EIS: Electrochemical impedance spectroscopy was used to detect DNA hybridization/specific discrimination and was performed with PBS in the presence of 0.5 mM $Fe[CN_6]^{3-/4-}$ at +0.24 V; a frequency range of 10 kHz to 50 mHz and an AC amplitude of 10 mV were applied. The impedance data were fitted to an equivalent circuit.

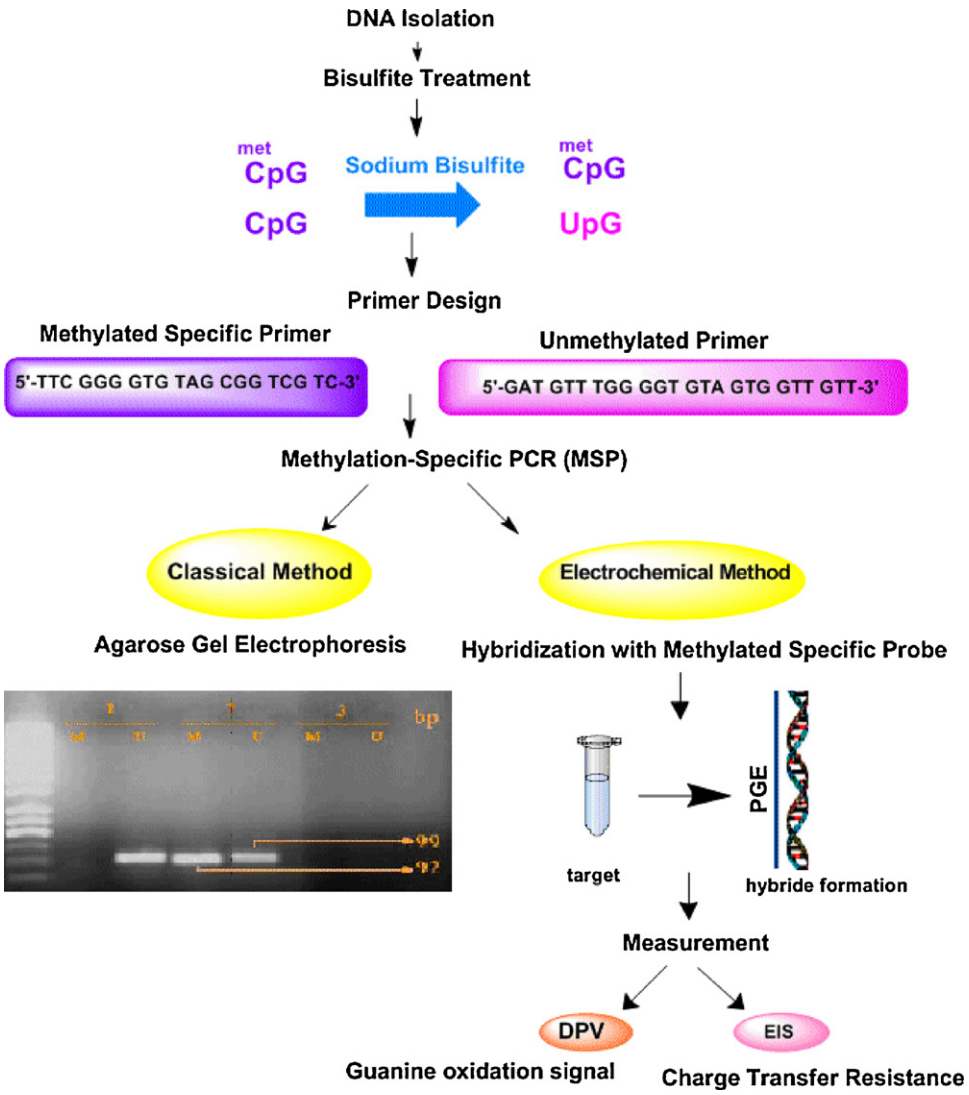
   The same protocol was done using a non-complementary sequence instead of the target sequence in order to control for biosensor selectivity.

B. Hybridization in solution

Hybridization with synthetic targets: Unlike in the adsorption procedure, probe/target and probe/noncomplementary target couples were mixed in solution. First, the electrodes were pretreated by applying +1.4 V for 30 s in ACB without stirring. The probe (2.5 μg/ml) and target/noncomplementary target (3.5 μg/ml) were mixed in PBS and put into vials. The vials were stirred up for 30 min at 25 °C at a mixing speed of 600 rpm. After gentle mixing, the probe, hybrid, and non-complementary solutions were put into the vials in a volume of 100 μl each; electrodes were placed into these solutions for 20 min. The electrodes were then rinsed once with PBS. Measurements were obtained using DPV and EIS.

Hybridization with real PCR samples: The real samples obtained from the PCR amplification were diluted with PBS (dilution rate, 1:10); the diluted samples were then placed into vials and denatured by heating at 95 °C for 5 min and then ice shocking for 1 min. Probe solution was added to samples (target and noncomplementary), and the vials were stirred for 30 min at 25 °C at 600-rpm mixing speed. After gentle mixing, the probe, hybrid, and noncomplementary solutions were put into the vials in a volume of 30 μl each; electrodes were placed into these solutions for 20 min. The electrodes were then rinsed once with PBS. Measurements were obtained using DPV and EIS.

Agarose gel electrophoresis: 10 μl of real PCR samples was loaded on agarose gel. DNA fragments were separated on 3.5% agarose in Tris–borate–EDTA (TBE) buffer by electrophoresis at 120 V for 30 min and detected with a UV transilluminator after staining with ethidium bromide (10 mg/ml).



Scheme 1. Schematic procedure for the detection of *GSTP1* hypermethylation by electrochemical and electrophoretic methods.

3. Results and discussion

GSTP1 hypermethylation was detected by comparing the voltammetric transduction of the hybridization reaction between probe and target DNA sequences. In the electrochemical approach, the differences between the guanine oxidation signals determined by DPV and resistance changes analyzed by EIS before and after hybridization. Hybridization was detected by the decrease in the magnitude of the guanine oxidation peak as measured by DPV (Kerman et al., 2003; Meric et al., 2002), with the highest guanine signal obtained from the probe-covered electrode. An impedimetric system was proposed for direct electrochemical detection of *GSTP1* hypermethylation via DNA hybridization by comparing the resistance before and after hybridization. The electrochemical results were also confirmed by agarose gel electrophoresis, which is the classical detection method for PCR products.

Fig. 1 shows the DPV signals of guanine at the electrode surface of the probe (a) and hybrid (b). Methylation-specific and unmethylated probes (Fig. 1A and B, respectively) were modified onto the transducer surface. After probe–target hybridization, no substantial difference was found between the electrochemical signals of only probe-covered and hybrid-formed surfaces (Fig. 1A), but a significant difference was observed when sequences related to the unmethylated DNA region were used (Fig. 1B). In other words,

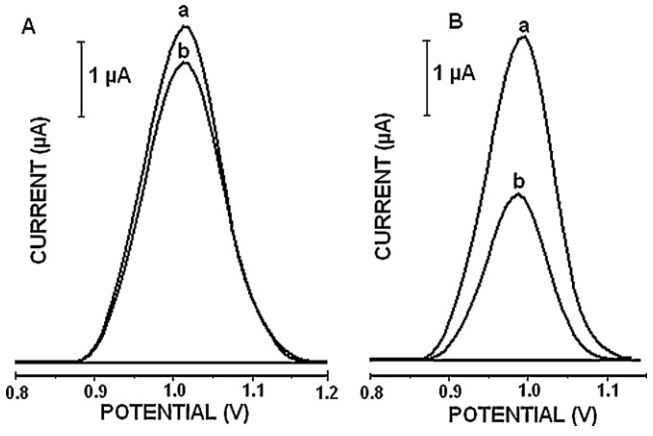


Fig. 1. Differential pulse voltammograms of guanine oxidation signals for probe (a) and hybrid (b) of methylation-specific (A) and unmethylated (B) *GSTP1* synthetic oligonucleotides. The experimental conditions were as follows: PGE pretreatment, +1.4 V for 30 s without stirring; immobilization with 5 μg/ml probe for 20 min; hybridization with 7 μg/ml of target sequence for 20 min by adsorption; and DPV measurement by scanning between +0.75 and +1.4 V in ACB.

detection of hybridization was based on guanine signals using unmethylated oligonucleotides. Fig. 1B shows a dramatic decrease in the peak current of guanine due to the hybridization reaction between the guanine-containing probe and the guanine-free target sequences. Thus, all guanines in the probe DNA were partly closed to oxidation after the hybridization. A series of 3 repetitive measurements of the oxidation of guanine resulted in reproducible results with a relative standard deviation (RSD) of 8.1% and 8.2% for methylation-specific *GSTP1* sequences of synthetic probe (a) and hybrid (b), respectively (Fig. 1A). A series of 3 repetitive measurements of the oxidation of guanine resulted in reproducible results with an RSD of 5.1% and 8.3% for unmethylated *GSTP1* sequences of synthetic probe (a) and hybrid (b), respectively (Fig. 1B). The detection limits estimated from S/N = 3 correspond to 5 μg/ml for the probe and 7 μg/ml for the target with unmethylated *GSTP1* sequences after the optimization experiments.

3.1. The control study of hybridization for synthetic oligonucleotides

An impedimetric system was also proposed for the detection of direct electrochemical hybridization of methylation-specific and unmethylated *GSTP1* sequences. EIS spectra of the DNA-based electrochemical sensor before and after probe–target and probe–noncomplementary target oligonucleotide hybridization were shown (Supplementary information, Fig. S1). Hybridization did not occur with methylation-specific sequences (Supplementary information, Fig. S1A); the results were similar to those of DPV analysis (Fig. 1A). In comparison with the signals of the unmethylated probe-modified electrode (Supplementary information, Fig. S1B-a), the charge transfer resistance (Rct) of the hybrid-modified electrode increased (Supplementary information, Fig. S1B-b). The resistance of noncomplementary sequences was between these values, as expected, and close to the probe resistance value as a proof of specific hybridization (Supplementary information, Fig. S1B-c). The EIS results were confirmed by the obtained voltammetric results.

No hybridization was observed with methylation-specific *GSTP1* oligonucleotides on the electrode surface (Figs. 1A and S1A). This could have occurred because the carbon solid surface was unsuitable for hybridization because of the design of the methylation-specific *GSTP1* oligonucleotides. It is more difficult to monitor hybridization on a solid surface than hybridization in solution because a solid surface involves additional factors such as probe density, surface heterogeneity, nonspecific adsorption, and steric hindrance. For example, probe density-dependent kinetic or steric limits may change the stability or selectivity of probe–target binding for surface-immobilized oligonucleotides (Peterson et al., 2002). Also, strong nonspecific DNA interactions contacts with the surface can lead to lower probe densities and more flat strand orientation. Probe film characteristics have steric and orientational consequences, which may reduce target binding on actual sensor surfaces (Wolf et al., 2004).

3.2. Hybridization in solution

Another hybridization method, which was named 'hybridization in solution', was performed as described in Section 2.4B. Fig. 2 shows the voltammograms (A) and histograms (B) obtained from the guanine oxidation signals at probe-coated electrode (Fig. 2A-a), hybrid (Fig. 2A-b), and noncomplementary (Fig. 2A-c) with synthetic methylated oligonucleotides in solution phase. A mixture containing the probe and its complementary target was prepared in the same solution and incubated for 20 min. A decrease of approximately 50% was observed in the guanine signal and thus reflected

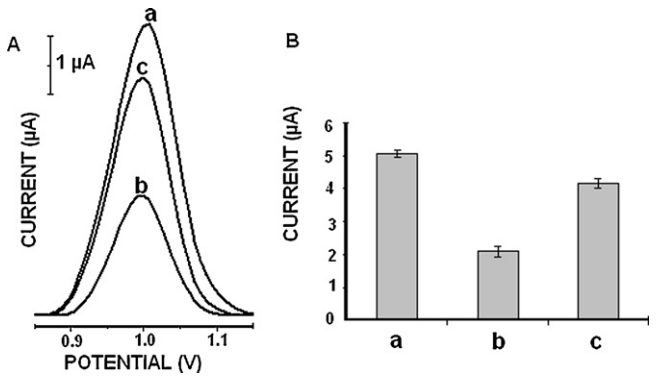


Fig. 2. Differential pulse voltammograms (A) and histograms (B) of oxidation signals of methylation-specific *GSTP1* synthetic oligonucleotides: probe (a), hybrid (b), and noncomplementary (c) in solution phase.

the extent of hybridization between the probe and its complementary target. Hybridization was achieved with that methodology may be due to the target DNA can easily be selected according to its best orientation which is necessary for the hybridization (Nasirizadeh et al., 2011). Hybridization also occurred with the unmethylated oligonucleotides in solution phase (not shown). As shown in Fig. 2, the detection limit was calculated as 2.92 pmol in a 100-μl reaction volume.

3.3. Optimum analytical performance of the sensor

To identify the conditions for optimal analytical performance, various parameters were tested. As summarized in Table 1, excellent results could be obtained with calibration of probe and target concentration, hybridization and adsorption times, and hybridization buffer types.

As shown in Table 1, probe/target concentrations in ranges of 0.5–2.5, 2.5–3.5, 3.5–6, 5–7, 1–15, 5–15, and 7–15 μg/ml were applied. Taking into account the p/h ratio, an optimum probe concentration of 2.5 μg/ml and a target concentration of 3.5 μg/ml were chosen for further experiments; PBS was chosen as the hybridization buffer. The effect of hybridization time on sensor selectivity was investigated. The nc/h ratio increased with increasing hybridization time between 5 and 30 min and then decreased; therefore, 30 min was chosen as the optimum hybridization time. Different adsorption periods were applied for DNA immobilization, between 10 and 30 min, for designing a rapid biosensor. A better and more reproducible discrimination was obtained between probe and hybrid with a 20 min adsorption time.

3.4. Real sample analysis

It is important to obtain hybridization with real samples to detect hypermethylation as a biomarker for prostate cancer. For this purpose, denatured PCR amplicon was mixed with the probe in solution phase. The results obtained from EIS were also confirmed by agarose gel electrophoresis.

Fig. 3A shows the EIS spectra of real DNA samples related to the hypermethylation of the *GSTP1* promoter region before and after hybridization between the probe and target (denatured PCR sample). To determine hybridization, we evaluated the difference in resistance before and after hybridization. Immobilization of probe sequences on the graphite electrode changed the electrical properties of the sensor surface. Compared to bare electrodes, the modification with probe resulted in an increased impedance. The resistance signal obtained from the methylation-specific probe (Fig. 3A-a) was lower than that obtained from the methylated hybrid (Fig. 3A-b), which indicates hybridization. When the probe

Table 1
Summary of optimization studies for hybridization detection containing probe/target concentrations, hybridization and adsorption times, hybridization buffer types.

Concerations p/t (μg/ml)	Signal ratio p/h	Hybridization buffer	Signal ratio p/h	Hybridization time (min)	Signal ratio nc/h	Adsorption time (min)	Signal ratio p/h
0.5/2.5	1.17	ACB	2.07	5	1.25	10	1.20
2.5/3.5	**1.89**	TBS	2.14	10	1.34	15	1.25
3.5/6.0	1.15	PBS	**2.41**	20	1.43	20	**2.01**
5.0/7.0	1.36			30	**1.54**	30	1.38
5.0/15.0	1.50			60	1.47		
7.0/15.0	1.33			75	1.25		
10.0/15.0	1.44						

The optimized conditions were presented as bold. *Abbreviations*: hybridization with a complementary target (h), hybridization with a noncomplementary target (nc), probe (p), and target (t).

was hybridized with its complementary target, the resistance value increased further to a much larger value (Fig. 3-b). The possible reason was that the probe could make difficult the diffusion of $Fe[CN_6]^{3-/4-}$ toward the electrode surface. After hybridization, there were more negative charges on the surface and the DNA-modified layer became more intense. These factors led to the further increase of the resistance value (Zhang et al., 2011).

To determine sensor specificity, nonspecific binding with non-complementary PCR product was evaluated under the same hybridization conditions with full-match PCR samples. As expected, lower resistance was obtained with non-complementary samples than with fully matched samples. The same hybridization protocol was also applied to unmethylated DNA samples. The resistance obtained from unmethylated hybrids (Fig. 3B-b) was higher than that obtained from both unmethylated probe (Fig. 3B-a) and probe–noncomplementary PCR (Fig. 3A-c). These results prove that the hybridization observed in this analysis was very specific. To test the cross reactivity, methylation-specific probe/unmethylated target (Fig. 3A-d) and unmethylated probe/methylated target (Fig. 3B-d) couples were investigated. As expected, their resistance value was lower than the hybrids (Fig. 3A-b and B-b) as a proof of specific interaction.

Fig. 4 shows the analysis of complementary and non-complementary PCR products based on guanine signal by DPV. Fig. 4A presents the results of methylated-specific sequences and Fig. 4B shows the results of unmethylated sequences. Only the probe DNA-modified surface gave similar peaks with non-complementary (Fig. 4A-a and c and B-a and c) sequences while complementary gave dramatic decreases (Fig. 4A-b and B-b). In other words, it was found that positive amplicons showed low voltammetric signals. The developed methodology provided an effective discrimination between the positive and non-specific PCR amplicons.

The results obtained from EIS and DPV experiments for hybridization detection were complemented with agarose gel electrophoresis which is the gold standard methodology for real samples (PCR). The data obtained from amplicon analysis based on the luminescence signal was showed in Supplementary information, Fig. S2. After methyl specific primer (MSP) – PCR the resulting amplicons of the two reactions set for each case, either with primers that recognize the methylated (M) or unmethylated (U) state of the analyzed *GSTP1* promoter region, were run side by side on a 2% agarose gel by electrophoresis. Since the primers used to amplify the M state of the *GSTP1* promoter region were shorter in length than that for the U state, the resulting amplicons were 91 bp or 97 bp long, respectively; and showed a slight shift of migration on the gel. *GSTP1* promoter hypermethylation will never be 100% in cell-free DNA, which not only represents DNA of the prostate but also of other tissues. Therefore cases with *GSTP1* promoter hypermethylation will have PCR products from both reactions. 1: Case negative for *GSTP1* promoter hypermethylation; 2: case positive for

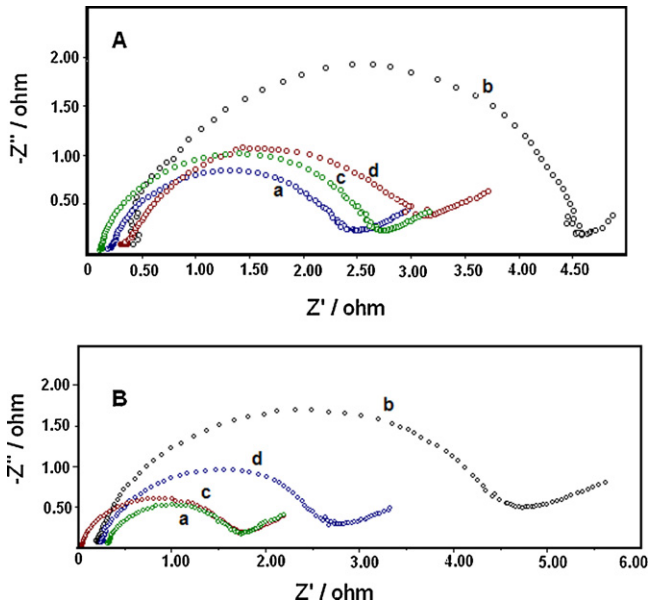


Fig. 3. Impedance spectra of for methylation-specific probe (A-a), methylation-specific probe and its complementary target (A-b), methylation-specific probe and non-complementary target (A-c), methylation-specific probe and unmethylated target (A-d), unmethylated probe (B-a), unmethylated probe and its complementary target (B-b), unmethylated probe and non-complementary target (B-c), and unmethylated probe and methylated target (B-d) of GSTP1 PCR samples. The experimental conditions were as follows: PGE pretreatment; dilution of PCR products with PBS (dilution rate, 1:10); denaturation of PCR products at 95 °C for 5 min, ice shock for 1 m; hybridization for 30 min; immobilization for 20 min; and FRA measurement.

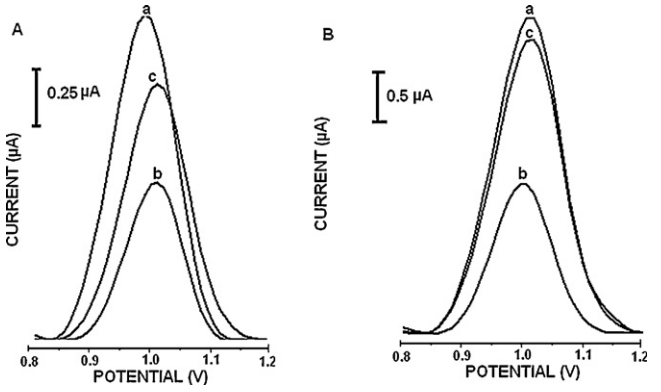


Fig. 4. Differential pulse voltammograms of guanine oxidation signals for methylation-specific probe (A-a), methylation-specific probe and its complementary target (A-b), methylation-specific probe and non-complementary target (A-c), unmethylated probe (B-a), unmethylated probe and its complementary target (B-b), and unmethylated probe and non-complementary target (B-c) of GSTP1 real samples.

GSTP1 promoter hypermethylation; 3: negative control, H_2O used instead of cell-free DNA; DNA Marker loaded far left, GeneRuler™ 50 bp DNA Ladder (Fermentas, Lithuania).

4. Conclusion

In conclusion, hypermethylation of the *GSTP1* promoter region is a highly specific marker for prostate cancer diagnosis. Because it can be studied in plasma DNA, it is a less invasive diagnostic method than biopsy and may be useful as a screening tool for people at high risk for developing prostate cancer. To our knowledge, our study is the first to use electrochemical and impedimetric methods to analyze *GSTP1* promoter region hypermethylation via hybridization. Compared with the traditional methylation screening methods, detection of *GSTP1* hypermethylation is rapid, precise, simple, and nonradioactive; thus, it is a promising method for future research on the tumorigenesis of prostate carcinomas. Moreover, the genosensor assay is cheaper than conventional agarose gel electrophoresis and optical methods. With further development, this biosensor system can detect picomole levels of target DNA sequence, which is suitable for medical diagnosis of genetic diseases and mutations. More research is needed to generate a reliable, robust and sensible microchip technology for rapid and automated nucleic acid analysis in real samples. Future efforts in our laboratory will be directed toward this goal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.11.029.

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