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Design of Electrochemical Biosensor Systems for the Detection of Specific DNA Sequences in PCR-Amplified Nucleic Acids Related to the Catechol-*O*-methyltransferase Val108/158Met Polymorphism Based on Intrinsic Guanine Signal

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Psychiatric disorders are common and complex diseases that show polygenic and multifactorial heredity. A single nucleotide polymorphism (Val108/158Met) in the catechol-*O*-methyl transferase (COMT) gene is related to many psychiatric disorders such as schizophrenia, alcoholism, bipolar disorder, and obsessive-compulsive disorder. Schizophrenia is a complex disorder and a single nucleotide polymorphism (Val108/158Met) at the COMT gene is related to schizophrenia susceptibility. A novel hybridization-based disposable electrochemical DNA biosensor for the detection of a common functional polymorphism in the COMT gene from polymerase chain reaction (PCR) amplicons has been described without using an external label. This developed technology combined with a disposable carbon graphite electrode and differential pulse voltammetry was performed by using short synthetic oligonucleotides and PCR amplicons in length 203 bp to measure the change of guanine oxidation signal obtained at ~+1.0 V after DNA hybridization between probe and target (synthetic target or denatured PCR samples). COMT-specific oligonucleotides were immobilized onto the carbon surface with a simple adsorption method in two different modes: (a) Guanine-containing targets were attached or (b) inosine-substituted probes were attached onto an electrode. By controlling the surface coverage of the target DNA, the hybridization event between the probes and their synthetic targets or specific PCR products was optimized. The wild-type or polymorphic allele-specific probes/targets were also interacted with an equal amount of noncomplementary and one-base mismatch-containing DNAs in order to measure the sensor selectivity. The decrease or appearance in the intrinsic guanine signal simplified the detection procedure and shortened the assay time because protocol eliminates the label-binding step. The nonspecific binding effects were minimized by using sodium dodecyl sulfate with different washing methods. The Val108/158Met COMT genotype detection were performed with real samples containing wild-type (healthy controls), polymorphic (mutant type), and heterozygous PCR products. The detection limit (S/N = 3) of the biosensor was 2.44 pmol of target sequence in the 30-μL samples. Analytical performance of the sensor is described, along with future prospects.

The brain neurotransmitters dopamine, epinephrine (adrenaline), and norepinephrine (noradrenaline) belong to a group of catecholamines that also have, besides their activator and inhibitor roles in the peripheral nervous system, important roles in the central nervous system like stimulating respiration and increasing psychomotor activity. Since catecholamines cannot pass the blood−brain barrier, they are locally synthesized in the brain from phenylalanine and tyrosine amino acids and cleared from the synaptic cleft after fulfilling their functions.[1] For example, the function of dopamine in the synaptic cleft is terminated by its reuptake into the neuron via a carrier protein in the presynaptic membrane. Because dopamine carrier proteins are expressed in lower amounts in the prefrontal cerebral cortex, the catechol-*O*-methyltransferase (COMT) enzyme is responsible for its inactivation by *S*-adenosylmethionine-dependent methyl conjugation in this part of the brain.[2] In addition to catecholamines, COMT is also responsible for the metabolism of catechol-containing drugs that are used in the treatment of hypertension, asthma, and Alzheimer's and Parkinson's disease.[3−6]

The COMT enzyme exists in two forms; a soluble (S-COMT) and a membrane bound (MB-COMT) form, with the later being

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50 amino acids longer in its N-terminus.[7] Although both forms are encoded from a single COMT gene localized at chromosome 22q11 in the genome, their expressions are controlled from two different promoter regions.[8−12] In many tissues, two different COMT mRNAs, one long and the other short, are transcribed. The long mRNA can either code for the MB- or the S-COMT form, since it contains both transcription initiation sites; but the short mRNA can only code for the S-COMT form and is highly expressed in all tissues besides the brain. In the brain, the widely expressed form is MB-COMT.[12,13] Subcellular localizations of both COMT forms also differ, with MB-COMT preferentially localizing to the endoplasmic reticulum and nuclear membrane,[14] and S-COMT to the cytoplasm and nucleus.[15]

In humans, the enzyme activity of COMT is genetically polymorphic. This is mainly due to the presence of the COMT Val108/158Met polymorphism that arises from a guanine to adenine transition at positions 158 in MB- and 108 in S-COMT, resulting in a valine to methionine amino acid change (rs4680 [10,16]). While the wild-type 108/158Val allele generates a thermostable and highly active (H) COMT form, the polymorphic 108/158Met allele constitutes a thermolabile and lower active (L) COMT form. Taken together, two alleles (Val-H and Met-L) and three genotypes (Val/Val−H/H, Val/Met−H/L, and Met/Met−L/L) can be distinguished for this polymorphism.[17] The activity of the COMT enzyme encoded from the polymorphic 108/158Met allele is decreased by 3−4-fold, corresponding to only 25% of its wild-type counterpart. As a result of this, the L/L genotype with low enzyme activity slows the catecholamine metabolism down, whereas the H/H genotype accelerates catecholamine metabolism via its high enzymatic activity. Catecholamine levels of individuals with the L/L genotype are therefore higher than in individuals with the H/H or H/L genotypes.[18]

Changes in the catecholamine metabolism due to different COMT genotypes can give rise to the development of neuropsychiatric disorders. Some COMT-related psychiatric disorders are schizophrenia, alcoholism, bipolar disorder, obsessive-compulsive disorder, and anxiety, and it may also influence the pathogenesis of aggressive and antisocial behaviors and metabolic changes of catechol-containing drugs.[18−20]

For all these reasons, identification of the COMT Val108/158Met polymorphism is important in the determination of an individuals' enzyme genotype. Usually a PCR-based restriction fragment length polymorphism (RFLP) method is used for this purpose.

With the need for simple, rapid, inexpensive, and portable testing devices for molecular detection of nucleic acids, many electrochemical DNA biosensors (genosensors) have been developed for the detection of DNA hybridization and DNA polymorphisms recently.[21−23,27−30] The specific DNA hybridization[24−28] can be monitored based on the oxidation signal of guanine or by mostly DNA intercalators such as some metal coordination complexes, antibiotics, etc.,[27−31] or by using some metal tags such as gold or silver nanoparticles.[26,32]

In the current study, we describe a novel label-free electrochemical genosensor for the detection of the COMT Val108/158Met polymorphism as an alternative to RFLP for the first time. There have not yet been any reports about the rapid electrochemical detection of this polymorphism from PCR-amplified samples based on the decrease or appearance in the guanine signal by using a disposable carbon graphite electrode in connection with differential pulse voltammetry (DPV). The main features of the analytical procedure are discussed in the following sections.

EXPERIMENTAL SECTION

Electrochemical Apparatus. An Autolab 30 electrochemical analysis system and the GPES 4.8 software package (Eco Chemie) were used to obtain the differential pulse voltammograms. A conventional three-electrode system consisted of a disposable carbon graphite electrode (CGE) as the working electrode, the reference electrode (Ag/AgCl), and a platinum wire as the auxiliary electrode. A Noki pencil model 2000 was used as a holder for the 0.5-mm graphite lead Tombo. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part of the pencil. The pencil lead was held vertically with 13 mm of the lead extruded outside (10 mm of which was immersed into the measurement buffer). The convective transport was provided by a magnetic stirrer.

Chemicals and Solutions. The 20-mer synthetic oligonucleotides of the wild-type (WT) and mutant-type (MT) COMT-specific probes and their complementary targets (as lyophilized powder)

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were purchased from TIB Molbiol with purification by HPLC. The base sequences of the oligonucleotides were as follows: wild-type COMT specific target (WTT), 5′-TTC GCT GGC ***GTG*** AAG GAC AA-3′; wild-type COMT specific probe (WTP), 5′-TTI TCC TTC **ACI** CCA ICI AA-3′; mutant-type COMT specific target (MTT), 5′-TTC GCT GGC ***ATG*** AAG GAC AA-3′; mutant-type COMT specific probe (MTP), 5′-TTI TCC TTC **ATI** CCA ICI AA-3′.

The oligonucleotide stock solutions (1000 μg/mL) were prepared with Tris EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.00, TE) and stored in a −20 °C freezer until use. More dilute solutions of oligonucleotides were prepared using 0.05 M potassium phosphate buffer solution containing 20 mM NaCl (pH 7.40, PBS).

The inosine (I)-substituted wild-type capture probes had the complementary sequence as a region of the common functional polymorphism in the wild-type COMT gene (Val/Val−H/H). Similarly, the inosine-substituted mutant-type capture probe had the complementary sequence as the same region of the one-base mismatch containing COMT gene (Met/Met−L/L).

A 20-mer wild-type target oligonucleotide collinear with the portion of the human genome for COMT region was designed to serve as a wild-type COMT specific target (WTT). A second target containing a single G-to-A transition at position 10 from the 5′ end of the oligonucleotide was used as the mutant-type COMT specific target. Single-base mismatch, from G to A at the 67th position of the amplified PCR region, carries the mutation responsible for the amino acid substitution from valine to methionine Val108/158Met causing the common functional polymorphism. Capture probes were perfect complements to WT or MT COMT gene alleles.

The 203-bp fragment of the COMT gene harboring the polymorphic site of interest was amplified by using following primers: forward 5′-CTCATCACCATCGAGATCAA-3′ and reverse 5′-GATGACCCTGGTGATAGTGG-3′. The PCR reaction mix contained 25 mM $MgCl_2$, 100 μM concentration of each primer, 2 mM dNTP mix, 10× PCR buffer, 1 unit of *Taq* DNA polymerase, and 50 nmol of genomic DNA in a final volume of 50 μL. PCR conditions were set as 1-min initial denaturation at 94 °C, followed by 40 cycles of 20-s denaturation at 94 °C, 30-s annealing at 56 °C, 30-s elongation at 72 °C, and a final extension of 7 min at 72 °C. The resulting PCR product was purified from the undesired small side products (<100 bp) with use of the High Pure PCR Product Purification Kit (Roche Applied Science). The 10 μL of purified PCR product was control digested with 1 unit of *Nla*III (MBI Fermentas) for 4 h at 37 °C and analyzed after running on a 12% polyacrylamide gel (29:1) stained with ethidium bromide. The presence of the guanine nucleotide at codon 108/158 (GTG/ Val/ high COMT enzyme activity) resulted in three fragments of 87, 62, and 54 bp after digestion. But, when an adenine nucleotide was present at the same codon instead (***A***TG/ Met/ low COMT enzyme activity), four fragments of 69, 62, 54, and 18 bp were observed. The amplicons were characterized as homozygous WT, homozygous MT, or heterozygous (Val/Met−H/L). In the current study,a total of five real PCR samples were analyzed and each genotype was studied.

Sodium dodecyl sulfate (SDS) was purchased from Merck. Other chemicals (buffers, etc.) were of analytical reagent grade and supplied by Merck and Sigma. Ultrapure water was used in all solutions. All experiments were conducted at room temperature (22.0−23.0 °C).

Different buffers were used in this work: pretreatment buffer containing 0.50 M acetic acid and 20 mM NaCl (pH 4.8, ACB); immobilization and hybridization buffers containing 0.01 M KH_2PO_4, 0.04 M K_2HPO_4 (pH 7.4, PBS); washing buffer 1 diluted from 2× sodium saline citrate (SSC), which contains 300 mM NaCl, 30 mM sodium citrate (pH 7.0), and different ratios of SDS; washing buffer 2 containing 0.01% SDS in water. Stock solutions of the oligonucleotide were prepared in 10 mM Tris-HCl, 1 mM EDTA (pH 8.00, TE). The pH was adjusted with either NaOH or HCl solution.

Methods. The electrochemical detection procedure consists of two different approaches and is illustrated in Scheme 1.

Method 1 (Detection of Synthetic Olionucleotides Based on Signal Decrease). Target Immobilization onto CGE Surface. Before all the immobilization of DNA, the carbon working electrodes were pretreated by applying a potential of +1.4 V for 60 s in 4 mL of blank acetate buffer solution (0.50 M, pH 4.8). A 30-μL aliquot of target solution, which contains 8 μg/mL target in 50 mM potassium phosphate buffer with 20 mM sodium chloride (pH 7.4), was immobilized onto the pretreated CGE. For this reason, CGE was dipped into the target sample containing vials using a wet adsorption method,[28,33] and the target immobilization continued for 15 min. The electrode was then rinsed with phosphate buffer (pH 7.4) for a short time (5 s).

Hybridization with Inosine-Substituted Probes. The target-immobilized CGEs were dipped into the 12 μg/mL concentration of capture probe containing PBS (pH 7.4) solution. The hybridization was allowed to proceed for 20 min. The following washing steps were applied onto the electrode surface in order to minimize the nonspecific adsorption. The hybrid modified lead was dipped into the water containing SDS (1−5%) vials from 1 to 5 min and then immediately dipped into blank PBS for 10 s. with stirring.[25,34]

Washing was also done with 1× SSC, 0.1% SDS once for 10 min, 0.1× SSC, 0.1% SDS twice for 10 min, and 0.1× SSC, twice for 10 min[35]

The same protocol was also applied to one-base mismatch containing and noncomplementary oligonucleotides in order to control selectivity of this biosensor.

Voltammetric Transduction for the Detection of Immobilized DNA. A freshly prepared CGE surface was used for each electrochemical measurement. The oxidation signal of guanine was measured by using DPV in blank PBS by scanning from +0.75 to +1.40 V with an amplitude of 50 mV at 16 mV/s scan rate. The raw voltammograms were treated by using the Savitzky and Golay filter (level 2) included in the General Purpose Electrochemical Software (GPES) of Eco Chemie with moving average baseline correction, as described in the literature,[25,36] using a "peak width" of 0.01 V. Repetitive measurements were

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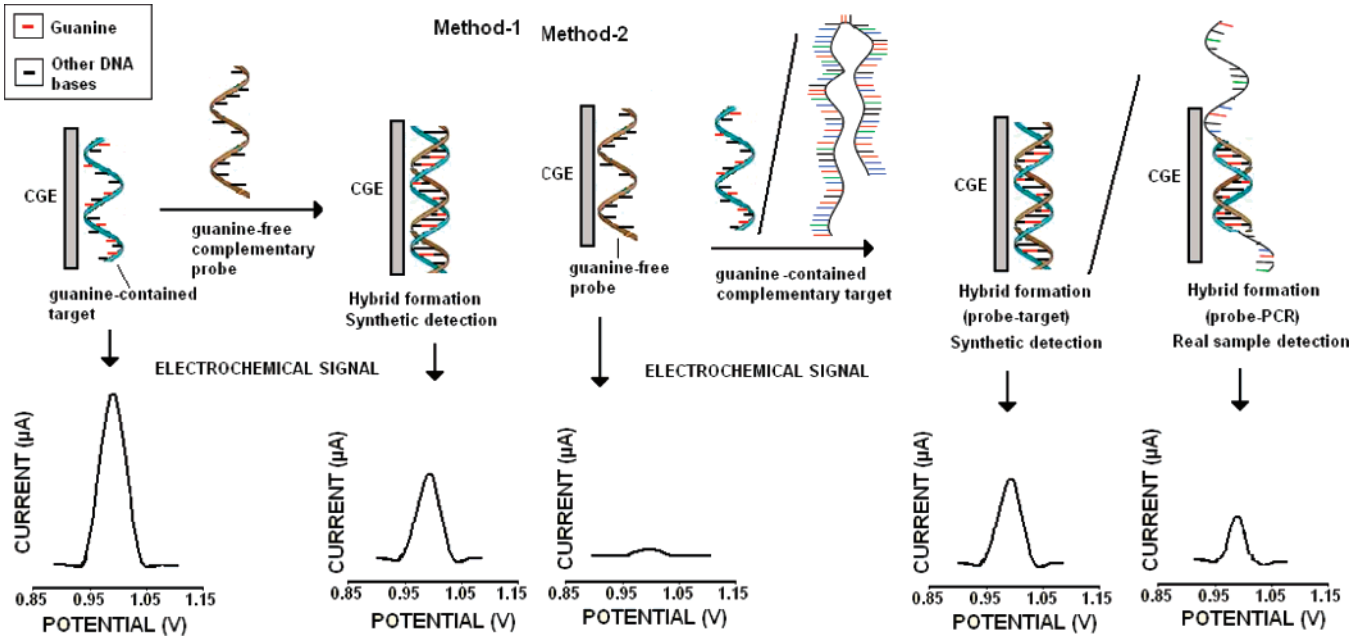
Scheme 1. Detection of Hybridization Using Guanine-Containing Target (Method 1) and Guanine-Free Probe (Method 2) as Beginning Materials of the Procedure



carried out by renewing the surface and repeating the above assay format by using a DNA-modified CGE.

Control experiments were performed with a noncomplementary and a one-base mismatched oligonucleotides. The WT target was interacted with the one-base mismatched mutant-type capture probe, and the MT target was interacted with the one-base mismatched wild-type capture probe.

Method 2 (Detection of Synthetic Olionucleotides and Real Samples Based on Signal Appearance). Inosine-Substituted Probe Immobilization onto CGE Surface. The pretreated CGEs were immersed into the 30-μL aliquot of inosine-substituted capture probe solutions that include 8 μg/mL probe in PBS (pH 7.4) in order to probe binding onto the carbon surface for 15 min. The electrode was then rinsed with PBS for 5 s.

Hybridization with Synthetic Targets. The inosine-substituted capture probe immobilized CGEs were dipped into the 12 μg/mL COMT specific target solution. The hybridization was allowed to proceed for 20 min. The electrode was then rinsed with washing buffers as explained in method 1 to decrease the nonspecific adsorption.

Hybridization with Real PCR Samples. The real samples obtained from the PCR amplification had an concentration of ~300 ng/μl and were diluted with PBS (dilution rate is 1:30); the diluted samples was then placed in a vial and denatured by heating in a water bath at 95 C for 6 min.[37] The capture probe-covered electrodes were immersed into denatured PCR samples rapidly. The hybridization was carried out for 20 min at room temperature. During this period, there is a competition between the renaturation process and hybridization reaction; however, thanks to the high temperature, which was provided at the beginning of hybridization, more single-stranded sample molecules were available for hybridization. The electrode was then rinsed with water, which contained 0.01% SDS, for 5 min and 4 mL of stirred phosphate buffer solution for 30 s (pH 7.4).

Voltammetric Transduction. The oxidation signal of guanine was measured as explained in method 1.

RESULTS AND DISCUSSION

Study of the COMT Polymorphism (Single-Base Mismatch) Detection Using Synthetic Oligonucleotides. The label-free detection of COMT polymorphism by using synthetic oligonucleotides or PCR products is illustrated in Scheme 1. In this study, an electrochemical DNA biosensor was developed that relies on the DPV transduction of the hybridization reaction between the wild or polymorphic COMT allele and probes, by using the renewable sensor, CGE, in a 1:30 fold diluted PCR product or 12 μg/mL concentration of target in 20 min of hybridization time. In method 1, the guanine-containing target is immobilized onto the electrode and then interacted with inosine-substituted probe. The detection of hybridization is accomplished with the decrease in the magnitude of the guanine oxidation peak,[38,39] whereas the highest guanine signal is obtained from the target-covered electrode. Alternatively, In method 2, the inosine-substituted probe was immobilized on the CGE, and after hybridization between probe and its synthetic target, the appearance of the guanine signal thus reflects the analytical signal of hybrid formation (yes/no system).[25,37,40] The amplified amplicon is also diluted (1:30) in the hybridization buffer and then introduced onto the probe immobilized electrode. The hybridization procedure was completed as the synthetic oligonucleotides by DPV.

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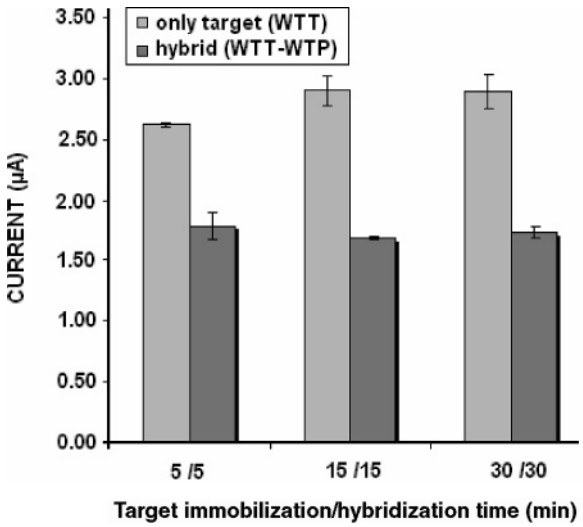

Figure 1. Effect of different target immobilization and probe hybridization times, 5, 15, and 30 min, on the hybridization reaction based on the guanine signal at WTT (light gray columns) and synthetic hybrid (dark gray columns) modified CGE surfaces. A 10 μg/mL target immobilization on pretreated CGE surface by simple wet adsorption method for 5, 15, and 30 min; 15 μg/mL probe immobilization on target-modified CGE surface in the vial for 5, 15, and 30 min; DPV measurement, scanning between +0.75 and 1.40 V in PBS with 20 mmol/L NaCl.

Figure 1 displays histograms obtained from the guanine oxidation signals at a WTT-coated electrode before and after hybridization with the WTP. The different immobilization and hybridization times were applied in the range between 5 and 30 min. The only 10 μg/mL target modified CGEs showed the highest guanine peaks (light gray columns) because of the opening guanine bases for oxidation. When hybridization occurred on the CGE surface, the low guanine signals were observed using WTT-modified electrodes with the 15 μg/mL concentration level of WT inosine-substituted probe (dark gray columns). The concentrations of target and probe were chosen from our previous reports.[25,31] In these conditions, only target immobilized electrodes showed approximately the same guanine peak heights. There was obtained better and more reproducible discrimination between single strand (target) and double strand (hybrid) applying 15-min hybridization time. These data, summarized in Figure 1, showed that excellent results can be obtained after making calibration experiments such as the probe and target concentration studies.

Analytical Performance of the Sensor. Factors affecting the probe immobilization, hybridization, and nonspecific adsorption were optimized to obtain a more sensitive and rapid assay.

To evaluate the analytical performance of the genosensor, calibration experiments were carried out first. The influence of target concentration on the sensor surface coverage was observed by using guanine signals (Figure 2A). Voltammetric signals increased gradually with the target concentration up to 8 μg/mL and then started to level off between 8 and 10 μg/mL.

The effect of probe concentration on the hybridization signals was also obtained in Figure 2B. The guanine-containing target concentration was kept constant at 8 μg/mL, and the WT probe concentrations were increased from 2 to 16 μg/mL. The hybridization response decreases with increasing probe concentration up to 12 μg/mL and then it leveled off. Thus, an optimum target concentration of 8 μg/mL and probe concentration of 12 μg/mL were chosen for further experiments. According to the obtained results, the surface coverage of the CGE with the target and the saturation of the target hybridization sites on the target-immobilized CGE surface were reached.

The reproducibility of the measurements (evaluated as relative standard deviation over four results) was 7.7% for optimum target concentration (Figure 2A) and 3.2% for optimum probe concentration in hybrid structure (Figure 2B).

Table 1 displays the influence of the SDS (5%)[37] washing time when the synthetic 8 μg/mL WT target and 12 μg/mL probe/noncomplementary/mismatch containing oligonucleotides were used for the hybridization. A 3-min washing time was found as the optimal time to obtain better discrimination among probe, noncomplementary, and mismatch oligonucleotides after the hybridization with WTT-coated CGEs, but this difference was not enough to design a mismatch-sensitive biosensor.

In Figure 3, the effects of SDS ratio in washing buffer on the guanine (Figure 3) signals were observed. The washing time was kept constant at 3 min (according to Table 1), and the percentage of SDS was increased from 1 to 5%. The SDS ratio of 2.5% was detected as the optimum ratio in order to observe better hybridization between target and inosine-substituted probe. However, the indication of the decrement of the guanine signal was not enough for the formation of the reliable hybrid structure onto the CGE surface.

In Figure 4, voltammograms (Figure 4A and C) and histograms (Figure 4B and D) show the guanine oxidation signals obtained when the 8 μg/mL synthetic WT target (Figure 4A and B) or WT probe (Figure 4C and D) was modified on the CGE surface. In these studies, we have chosen a special washing procedure[35] (1× SSC, 0.1% SDS once for 10 min, 0.1× SSC, 0.1% SDS twice for 10 min, 0.1× SSC, twice for 10 min) to define and improve biosensor selectivity. The highest guanine signals were obtained from the synthetic target immobilized electrode (Figure 4A-a and Figure 4B-a). A decrease of approximately half was observed in the magnitude of the guanine peak and thus reflected the extent of the hybrid formation between target and probe (Figure 4A-b and Figure 4B-b). When the target-covered electrode was immersed into a solution containing the 12 μg/mL concentration level of synthetic noncomplementary sequence for hybridization, the obtained guanine signal showed an increase as a signal obtained from only the target-covered electrode (Figure 4A-c and Figure 4B-c). This clear difference between full match and noncomplementary indicated that the complete hybridization was not accomplished with a noncomplementary sequence. Mismatch-containig synthetic oligonucleotide was also hybridized with target-modified CGEs and the high guanine peaks were obtained again (Figure 4A-d and Figure 4B-d). Through the use of a several SDS washing steps for 50 min, all of the nonspecific adsorption effects grow out of mismatch and noncomplementary synthetic oligonucleotides were minimized.

The voltammetric signals obtained with the single-stranded DNA-modified CGEs (Figure 4A-a,c,d and Figure 4B-a,c,d as probe, noncomplementary, and mismatch, respectively) were higher than the ones obtained with the hybrid-modified CGE

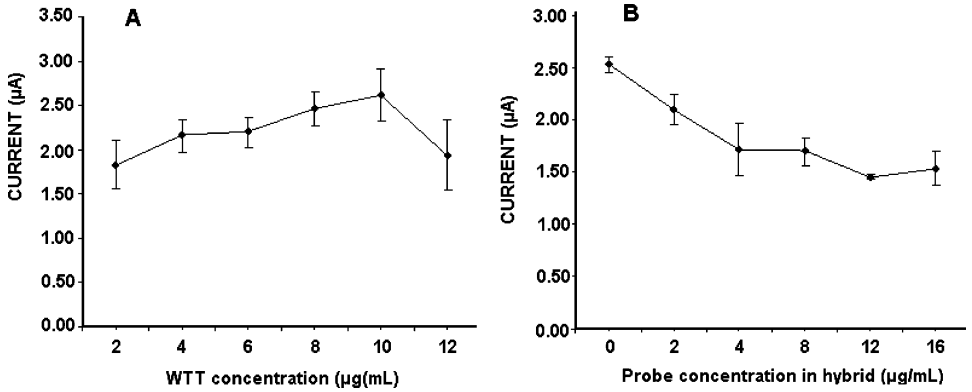

Figure 2. Calibration plot for synthetic oligonucleotides based on the oxidation signal of guanine. (A) Increasing concentration of the only WTT-immobilized CGE and (B) WTT-modified sensor were exposed to increasing concentrations of the probe. Each point is the mean of three measurements, and the error bars correspond to the standard deviation. The target immobilization on CGE by a simple wet adsorption method for 15 min; probe immobilization on target-modified CGE surface by adsorption in the vial for 15 min; 10-s washing with PBS; DPV measurement, scanning between +0.75 and 1.40 V in PBS with 20 mmol/L NaCl.

Table 1. Effect of Different SDS Washing Times for Synthetic Analysis, 1, 2, 3, 5, and 10 min, at Target-Modified CGES after the Hybridization with Probe, Noncomplementary and Mismatch Sequence

washing method	SDS[37] (%)	washing time	comp/ noncomp discrim (%)	comp/single bp mismatch discrim rate (%)
SDS and PBS	5	10 min, 10 s	35	
SDS and PBS	5	5 min, 10 s	29	17
SDS and PBS	5	3 min, 10 s	**40**	**43**
SDS and PBS	5	2 min, 10 s	22	43
SDS and PBS	5	1 min, 10 s	36	

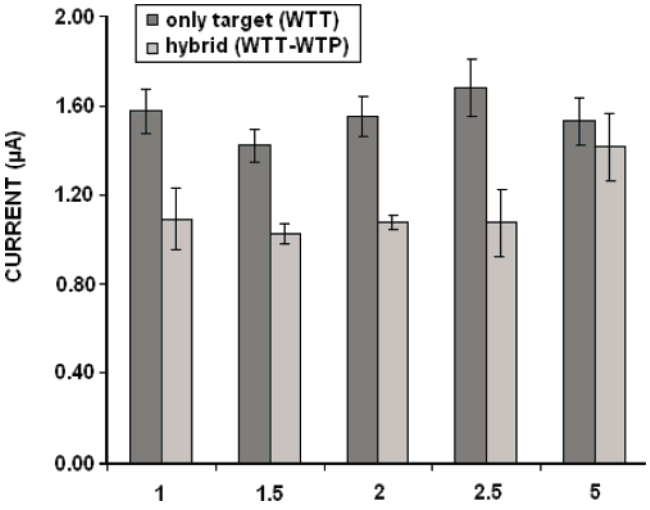

Figure 3. Peak currents (at +1.0 V) of only target-modified (dark gray columns) and hybrid-modified (light gray columns) CGEs dependence on the ratio of SDS in washing buffer. Other conditions are as in Figure 2.

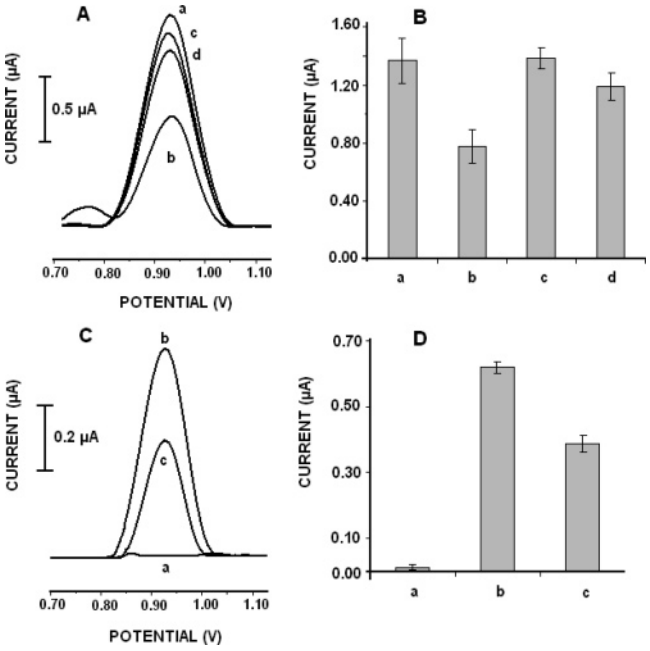

Figure 4. Voltammograms and their histograms representing the guanine signals obtained from WTT-covered (A and B) and WTP-covered (C and D) CGEs before and after hybridization with synthetic oligonucleotides. Guanine-containing wild-type target signal (A-a and B-a); guanine-free wild-type probe signal (C-a and D-a). Hybrid signals obtained after hybridization between WTT and WTP (A-b and B-b); WTP and WTT (C-b and D-b); after the hybridization between WTT and its noncomplementary sequences (A-c and B-c); after the hybridization between WTT and its one-base mismatch sequences (A-d and B-d) and WTP and its one-base mismatch sequences (C-c and D-c). The 8 μg/mL target/inosine-substituted probe immobilization on CGE surface by a wet adsorption method for 15 min; 12 μg/mL target/noncomplementary/mismatch immobilization on target/probe-modified CGE in vial for 20 min; Washing with 1× SSC, 0.1% SDS once for 10 min, 0.1× SSC, 0.1% SDS twice for 10 min, and 0.1× SSC, twice for 10 min; DPV measurement, scanning between +0.75 and 1.40 V in PBS with 20 mmol/L NaCl.

(Figure 4A-b and Figure 4B-b) because of available guanine bases for oxidation. Due to the binding of guanine bases to complementary cytosine bases in hybrid structure, the electrochemically active groups of guanine were only partly available for oxidation; and the peak heights obtained from the hybrid-modified CGEs showed a decrease of ~56.2%.

In Figure 4C and D, nearly no signal was observed from the guanine-free WT probe-modified CGEs (Figure 4C-a and Figure 4D-a). After hybridization between probe and 12 μg/mL guanine-containing target (complementary), the highest guanine signals

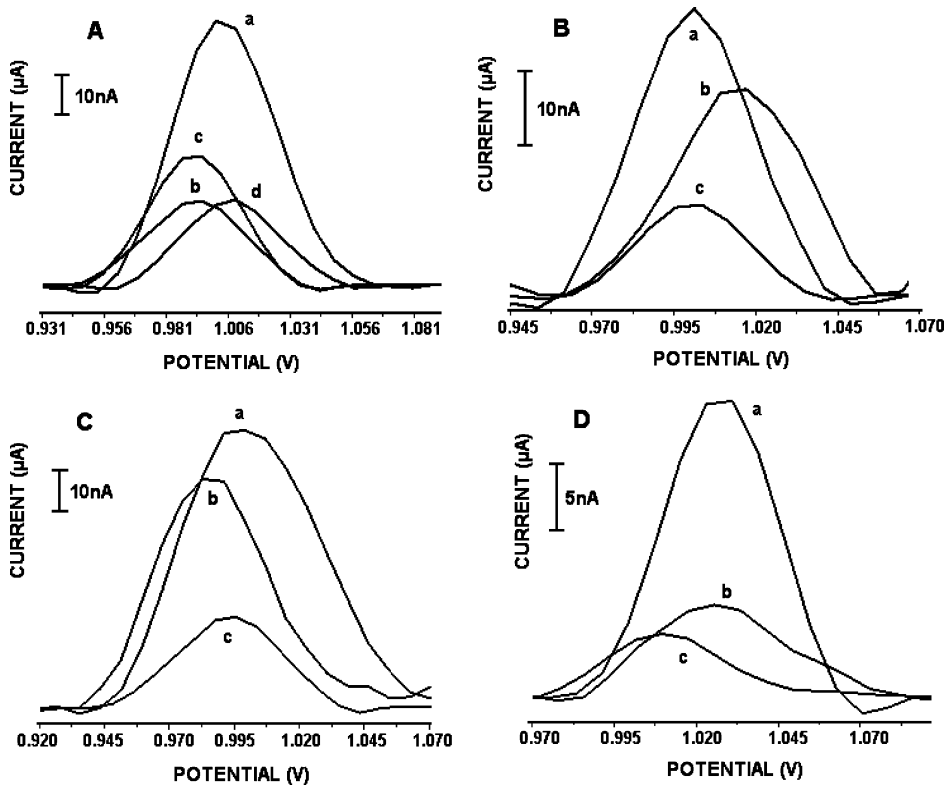

Figure 5. Hybridization detection between inosine-substituted WT or MT capture probes and the real samples (PCR products). The appearance of the guanine signal obtained with the PCR product, which contained the related DNA sequence, confirmed hybridization. The guanine-free WTP signal (data not shown) and guanine-free MTP signal (D-c), Hybrid signals obtained after hybridization between WTP and WTT (A-a, B-a, C-a), MTP and MTT (B-b, C-b, D-a); after hybridization between probes and noncomplementary oligonucleotides (A-c, A-d, B-c, C-c); and after hybridization between probes and their one-base mismatch sequences (A-b, D-b). The 8 μg/mL inosine-substituted probe immobilization on CGE surface by a wet adsorption method for 15 min; the 1:30-fold-diluted real sample immobilization onto probe-modified CGE in the vial for 20 min; washing with water containing 0.01% sodium dodecyl sulfate for 5 min and stirred PBS for 30 s. and DPV measurement, scanning between +0.75 and 1.40 V in PBS. (pH: 7.4).

were observed (Figure 4C-b and Figure 4D-b). When the WTP interacted with mismatch-containing synthetic oligonucleotide, a decrease of about half was observed on the guanine signal (Figure 4C-c and Figure 4D-c).

A series of three repetitive measurements of the oxidation of guanine gave reproducible results with relative standard deviations (RSD) of 15.5% for the hybrid (Figure 4B-b), 7.5% for the mismatch (Figure 4B-d), and 5.0% for the noncomplementary (Figure 4B-c). The guanine signal obtained from the hybridization between inosine-substituted WT capture probe and its guanine-containing target gave a RSD value of 3.1% (Figure 4D-b). The guanine signal gave a RSD value of 6.5% after the hybridization with MT target for WT capture probe-modified CGE (Figure 4D-c). The detection limit was estimated to be 2.44 pmol of complementary sequence (S/N = 3) in the 30−μL samples.

The synthetic mismatch oligonucleotides can hybridize strongly with the related probes because of their short length and one-base difference from complementary target. Therefore, the removal of these oligonucleotides from the probe-covered CGE surface was difficult. When the experiments were performed by using denatured PCR products, the discrimination of hybrid signal from mismatch signal was observed clearly.

For the real sample analysis, denatured PCR amplicon was first immobilized on the electrode as a target and then inosine-substituted probe was added for hybridization. However, the PCR section that is not bound to probe is free for oxidation. Therefore, the measurement of the guanine signal did not decrease as expected in contrast to the probe immobilized system (data not shown). For this reason, method 2 was used for the detection of PCR amplicons instead of method 1.

In Figure 5, the lowest signals were observed with guanine-free wild-type probe (data not shown) and guanine-free mutant-type probe (Figure 5D-c). The wild-type denatured amplicon was shown the highest guanine signal after the hybridization with wild-type probe at the electrode surface (Figure 5A-a). When the WT capture probe-immobilized electrode immersed into mutant-type or noncomplementary samples, a half of decrease was shown in the voltammetric signal (Figure 5A-b and c). When the MT capture probe-modified electrode interacted with noncomplementary samples, a decrease was observed similar to that of WT capture probe immobilized electrode (Figure 5A-d). With the help of a SDS and PBS washing step for 5 min, the effects of nonspecifically adsorbed PCR products as noncomplementary and samples from homozygous mutant patients (Figure 5A-d) were minimized.

The two different heterozygous amplicons were interacted with both WT and MT probe-covered electrodes. Each strand of the heterozygous amplicons contained a sequence complementary to one of the probes, thus giving rise to high guanine signals, as shown by Figure 5B and C (a and b). The selectivity of the biosensor was also measured by analyzing noncomplementary

Table 2. Data on Ten Different Washing Procedures for the Detection of Target/Point Mutation Discrimination for Real PCR Samples

washing procedure	height of guanine oxidation signal	single bp mismatch (polymorphism) detection[a]
1 s in PBS	very high (~170 nA)	no discrimination
5 s. in PBS	very high (~90 nA)	no discrimination
30 s. in stirred PBS	high (~45 nA)	no discrimination
SSC−SDS washing procedure (explained in Figure 4)	very low (~8 nA)	no discrimination
target (PCR) preparation with 0.05% SDS	very low (~6 nA)	no discrimination
target (PCR) preparation with 0.01% SDS	low (~20 nA)	discrimination rate ~30%
5 min in 0.01% SDS and 5 s in PBS	low (~24 nA)	discrimination rate ~24%
10 min in 0.01% SDS and 5 s in PBS	very low (~11 nA)	discrimination rate ~63%; detection performed without reproducibility
5 min in 0.01% SDS and 15 s in stirred PBS.	high (~40 nA)	discrimination rate ~65%; detection performed without good reproducibility
5 min in 0.01% SDS + 30 s in stirred PBS	high (~50 nA)	discrimination rate ~100%; detection performed with good reproducibility (~19%)

[a] Complementary/single bp mismatch discrimination.

amplicons, and they interacted with WT (Figure 5C-c) and MT (Figure 5B-c) probe-covered CGEs. The electrochemical signals of guanine decreased dramatically with these unrelated PCR products after the washing steps.

The mutant-type (polimorphic) amplicon was shown with the highest guanine signal after hybridization with its complementary probe (Figure 5D-a). Both of the strands of the homozygous mutant patient contained the point mutation. Thus, none of the strands of the PCR product was complementary to the WT probe, and no hybridization signal was observed (Figure 5D-b). The lowest guanine signal was obtained from the inosine-substituted MT probe-modified CGE (Figure 5D-c).

The specific detection of the target sequences was performed with 30-fold dilution of the PCR-amplified products. The reproducibility of the analysis results for different PCR amplicons (evaluated as relative standard deviation with four results) was ~19%. The genosensor selectivity was also measured: both of the probes even as low as 12 μg/mL responded to the synthetic target and 30-fold-diluted target amplicons. The difference of an average signal for the target that was ~3 times greater than that obtained for the noncomplementary and 2 times greater than that obtained for the single-base mutation-containing sequence.

These data (see Table 2) show that after hybridization with the PCR products, the 5-min wash in 0.01% SDS and 30 s in stirred PBS was found more effective for the discrimination between the target and point mutation-containing sequence with the best reproducibility. A longer or concentrated SDS washing step removed all material onto the surface whereas a washing step without SDS cannot discriminate the complementary ones and mismatched ones. The mutant-type amplicon showed the highest guanine signal with the MTP-covered electrode (complementary sequence), and it showed a half decrease of the signal with the WTP-covered electrode (mismatch sequence). These data proved that the patient had single-base mutation (polymorphism). Similarly, the wild-type amplicon showed the highest peak with only the WT probe-immobilized electrode.

CONCLUSIONS

The decrease or the appearance of the intrinsic guanine oxidation signal enables the monitoring of the COMT Val108/158Met polymorphism detection based on DNA hybridization in a short time with these genotyping methods. We have made significant improvements related to label-free DNA sequence detection, particularly the guanine signal decrease-based electrochemical approach. In this strategy, the guanine-containing DNA target was first immobilized onto the working electrode and the surface coverage of the DNA was evaluated based on the guanine signal. After hybridization between the inosine-substituded probe and target, the guanine signal should show a decrease to prove the presence of formed hybrid structure on the surface. Both detection schemes are also able to discriminate between target (complementary), noncomplementary target, and one-base mismatch-containing target but the signal decrease-based method is of importance for proving the "DNA hybridization". There have not yet been many reports about the signal decrease-based electrochemical detection for DNA sequence analysis.[38,39]

In terms of the detection of COMT Val108/158Met polymorphism from PCR-amplified amplicons based on the guanine signal change, these studies provide simple, rapid, inexpensive, and sensitive electrochemical determination of mismatch analysis performed without any external labels such as carcinogenic antitumor drugs, intercalators, metal complexes, organic dyes, or thiol/amino-terminated (labeled) DNA.[27−30,41−44] These label-free electrochemical biosensing protocols also eliminate the use of toxic chemicals, such as ethidium bromide, which was used in the PCR-based RFLP method in COMT Val108/158Met polymorphism analyses and advanced surface modification. Using these 40-min continued real sample detection schemes, our genosensor is also able to detect 2.44 pmol of target DNA in the 30 μL of sample volume. The device with this low detection limit can be used for clinical diagnosis of mutations. Furthermore, this study also contains comprehensive experimental conditions for the design of the biosensor.

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It should be taken into account that the use of disposable carbon graphite electrodes has important advantages for microarray technology such as its ease-of-use and portability. They also improve the reproducibility of the analysis results. Future work may be achieved by using two different probes, from the wild-type 108/158Val allele and the polymorphic 108/158Met allele regions of the COMT DNA, in connection with a multielectrode system and multiple hybridization facts. The developed DNA-based biosensor may be used for the detection of other single nucleotide polymorphisms.

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