Full Paper

Electrochemical DNA Hybridization Detection Using DNA Cleavage

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Abstract
We report the new method for detection of DNA hybridization using enzymatic cleavage. The strategy is based on that S1 nuclease is able to specifically cleave only single strand DNA, but not double strand DNA. The capture probe DNA, thiolated single strand DNA labeled with electroactive ferrocene group, was immobilized on a gold electrode. After hybridization of target DNA of complementary and noncomplementary sequences, nonhybridized single strand DNA was cleaved using S1 nuclease. The difference of enzymatic cleavage on the modified gold electrode was characterized by cyclic voltammetry and differential pulse voltammetry. We successfully applied this method to the sequence-selective discrimination between perfectly matched and mismatched target DNA including a single-base mismatched target DNA. Our method does not require either hybridization indicators or other exogenous signaling molecules which most of the electrochemical hybridization detection systems require.

Keywords: DNA, Ferrocene, Nuclease, Electrochemistry, DPV

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1. Introduction

Nucleic acids act as the carriers of genetic information, and most organisms contain a type of enzymes called endonucleases, which can hydrolyze the phosphodiester linkages in the nucleic acid backbone [1]. These nucleases related to DNA-DNA, protein-DNA interactions play important roles in life processes, such as the replication, transcription, recombination and repair of nucleic acids. Because of their great importance, DNA-DNA and DNA-protein interactions have justifiably drawn much attention from biochemical researchers over the last half century. Specially, electrochemical research using DNA-modified electrodes or DNA biosensors has been extended to the detection of DNA damage, analysis and sequencing of specific sequences of DNA bases by hybridization techniques, and interactions between DNA and other molecules [2–5]. Fojta et al. has reported that cleavage of supercoiled DNA by deoxyribonuclease I in solution and at the surface of the mercury electrode was studied by means of AC voltammetry [2]. They showed that the kinetics of the cleavage of DNA in solution and at the electrode surface substantially differ suggesting restricted accessibility of the surface confined DNA for the interaction with the enzyme. Li et al. proposed to study DNA cleavage by *Eco*RI endonuclease using ferrocene as electrochemical indicator [3]. Ferrocene was not covalently crosslinked to the DNA molecule but co-immobilized on the gold nanoparticles with thiolated target DNA via Au-S bond. *Eco*RI-DNA interaction was monitored based on the alteration of ferrocene signal before and after digestion with *Eco*RI. Enzymatic cleavage specificity of *Eco*RI was investigated, and enzymatic cleavage at DNA-modified electrode was also monitored real-time.

Compared to the conventional methods for analyzing DNA, electrochemical methods are simple, highly sensitive, and do not require sophisticated instrumentation [6–8]. Moreover, electrochemical detection systems can be miniaturized, which make them ideal for the development of a portable bioanalytical microdevice for the simple, rapid, and high-throughput analysis of a small amount of DNA. Electrochemical methods for the detection of DNAs are attractive because they provide a direct electrical readout, which reduces the complexity of the assay.

Here, we report an electrochemical method for detecting a single-base mismatch in the DNA sequence. The detection of single-nucleotide polymorphisms (SNPs), for example, involves conversion of a molecular genotyping reaction into a measurable physical process [9, 10]. Detection of SNPs is of increasing interest because many human diseases are associated with specific changes in the DNA sequence. This strategy takes advantage of S1 nuclease, which cleaves ss-DNA but not double-stranded DNA (ds-DNA) [11, 12]. Thiolated ss-DNA labeled with an electroactive ferrocene group (Fc ss-DNA) was immobilized on a gold electrode as the capture probe DNA. The standard approach for detecting DNAs employs immobilization of a known single stranded DNA (ss-DNA), followed by hybridization with labeled unknown target DNA [13, 14]. This approach,

however, requires target labeling, which is expensive, time-consuming, and introduces the potential for errors due to the need for additional steps. We used ferrocene because it is electrochemically well behaved and because its derivatives are widely used in electrochemical applications due to their stability in solution and their rapid response to a wide variety of electroactive substances [15, 16]. After immobilization of the capture probe DNA, it was hybridized with DNA containing complementary and noncomplementary sequences. Nonhybridized ss-DNA was then cleaved using S1 nuclease. The electrochemical behavior of the Fc ss-DNA was then measured by cyclic voltammetry (CV) and differential pulse voltammetry (DPV), which is much more sensitive than conventional sweep techniques for detecting very low concentrations of a redox probe. In contrast to other electrochemical systems for detecting SNPs, our simple strategy does not require the use of hybridization indicators or other exogenous signaling molecules.

2. Experimental

2.1. Chemicals and Reagents

S1 nuclease was purchased from Promega. All buffer salts and other inorganic chemicals were obtained from Sigma or Aldrich unless otherwise stated. All chemicals were used as received. All oligonucleotides employed in this work were synthesized and purified by high-performance liquid chromatography by GenoTech (Daejon, Korea). Capture probes (CP) were modified with 5′-dithiol-terminated 6-carbon spacers ($-(CH_2)_6-$) and 3′-amine-terminated. The targets of hybridization were designed to be perfect-matched (PM), noncomplementary (NC), and single-base mismatched (SBM) (Table 1).

The DNA deposition buffer (D-BFR) consisted of 0.5 M KH_2PO_4 and 0.5 M K_2HPO_4, pH 7. The rinsing buffer (R-BFR) was composed of 10 mM NaCl and 5 mM Tris buffer, pH 7.4, and the hybridization buffer (H-BFR) consisted of 1.0 M NaCl, 10 mM Tris buffer, and 1 mM ethylenediaminetetraacetic acid, pH 7.4.

2.2. Preparation of Ferrocenyl Oligonucleotide

The purified 3′-aminohexyl linked oligonucleotides (26 nmol) were dissolved in 20 μL of 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) and mixed with 6 μL of *N*-hydroxysuccinimide ester (1.3 μmol) of ferrocenecarboxylic acid in dimethyl sulfoxide. After sonication for 10 min, the suspension was stirred at room temperature overnight. Next, the solution was diluted to 1 mL with 0.1 M triethylammonium acetate (pH 7.0) and chromatographed on a NAP 25 column. The obtained material was purified by high performance liquid chromatography. The synthesis and purification were carried out as previously reported [12, 13]. The product was characterized by matrix-assisted laser desorption time-of-flight mass spectroscopy using a Voyager System 4095 (PE Biosystems).

Table 1. Sequence of modified oligonucleotide strands.

Oligonucleotide	Sequence ($5' \rightarrow 3'$)
Capture probe (CP)	GCG TAA TGC GCT CGC
Perfect-matched target (PM)	GCG AGC GCA TTA CGC
Noncomplementary target (NC)	GCG AGT ATG CCG TAT
Single-base mismatched target (SBM)	GCG AGC GTA TTA CGC

2.3. Instruments

Electrochemical experiments were carried out using a BAS 100B electrochemical analyzer (Bioanalytical Systems, Inc.). The three-electrode electrochemical cell consisted of a modified Au electrode, a Pt wire counter electrode, and a Ag/AgCl reference electrode (3 M KCl). The cell was filled 0.1 M $HClO_4$ solution deoxygenated with argon.

2.4. Electrode Preparation

Gold electrodes were prepared by electron-beam evaporation of 40 nm of Ti followed by 150 nm of Au on Si (100) wafers. The electrode was cleaned in piranha solution (H_2SO_4 : 30% $H_2O_2 = 3:1$), rinsed with water, and dried under a stream of nitrogen gas.

The electrode for the DNA sensor was constructed as follows. Mixed monolayer surfaces containing thiolated Fc ss-DNA probe and 6-mercapto-1-hexanol (MCH) were prepared by immersion of the cleaned gold substrate in a 1.0 μM of probe oligonucleotide in 1.0 M D-BFR for 1 h, followed by rinsing with R-BFR for 5 s, immersion for 1 h in 1.0 mM MCH in deionized water, and, finally, rinsing with R-BFR for 5 s and drying under a stream of nitrogen. Hybridization with target DNA was carried out by incubation at 37 °C for 1 h in H-BFR. The concentration of PM, NC, and SBM was 10 nM. After annealing at room temperature for 2 min, the electrode was rinsed with R-BFR and dried under a stream of nitrogen. Finally, the modified electrode was immersed in a solution of S1 nuclease in reaction buffer (50 mM sodium acetate, 280 mM NaCl and 4.5 mM $ZnSO_4$, pH 4.5) at 25 °C, 37 °C, or 50 °C and then rinsed with R-BFR and dried under a stream of nitrogen.

3. Result and Discussion

Our method for detecting an analyte DNA using S1 nuclease is depicted schematically in Figure 1. To produce a mixed monolayer surface, Fc ss-DNA capture probes and MCH were immobilized on an Au electrode. MCH has been used as a spacer to minimize nonspecific binding and maximize the efficiency of hybridization of capture and target probes [17, 18]. Next, the immobilized Fc ss-DNA capture probes

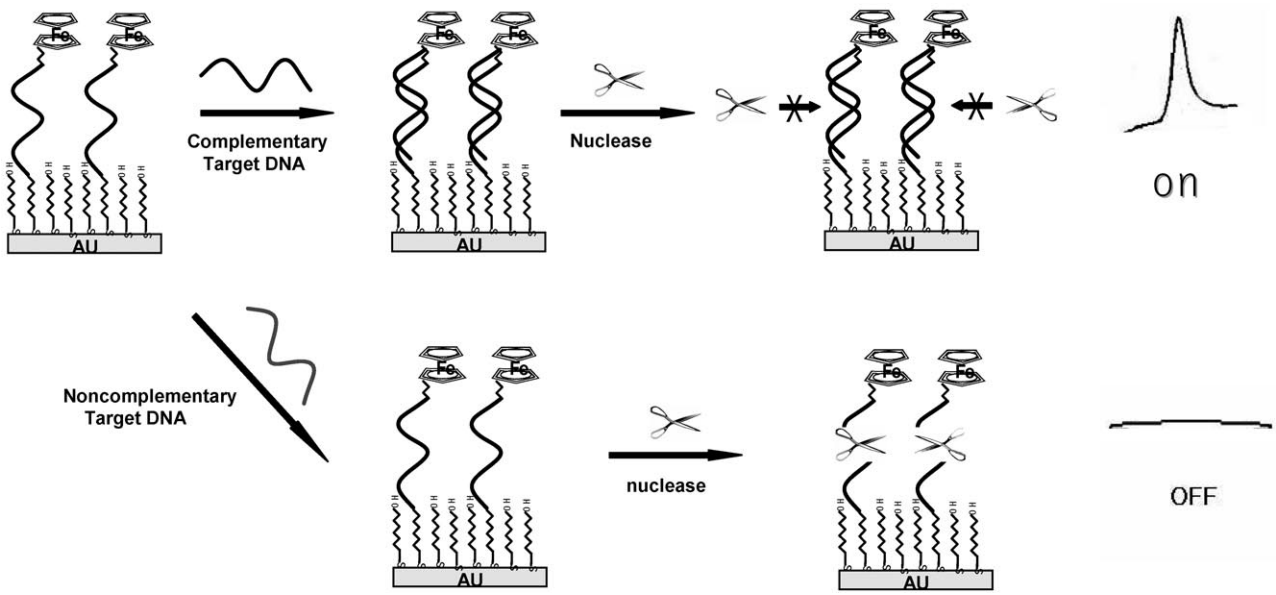

Fig. 1. Schematic illustration of DNA detection using S1 nuclease.

were hybridized with various target DNAs, after which the hybridized electrode surface was incubated with S1 nuclease to discriminate between PM, NC, and SBM target DNA. Nonhybridized Fc ss-DNA probe was cleaved and electrochemical response of ferrocene was disappeared. The electrochemical behavior of the immobilized Fc ss-DNA capture probes upon hybridization with target DNA was measured by electrochemical method. Thus, our method generates an electrochemical signal upon recognition of the target DNA.

3.1. Characterization of Ferrocenyl Oligonucleotide

We investigated the electrochemical properties of the gold electrode modified Fc ss-DNA probe before hybridization and enzymatic cleavage. Figure 2 displays the typical CV features of the surface-bound molecules. The peak currents linearly increase with scan rate up to 400 mV/s. The full width at half-maximum is ~103 mV, indicating a large formal peak separation, whereas the ideal value for reversible responses is 90 mV. The CV of a gold electrode modified with Fc ss-DNA was as expected theoretically for a (Fc/Fc^+) surface-confined redox system exhibiting a nernstian behavior [19]. Therefore, the area under the anodic (or cathodic) peak corrected for background current, represents the Faradaic charge Q required for the full oxidation (or full reduction) of the grafted layer. The total amount of bound-Fc on the electrode surface $A\Gamma$, can then be quantitatively derived from the integrated charge:

$$Q = nFA\Gamma$$

n being the number of electrons transferred = 1, F the Faraday constant (C/equiv), and A the effective surface area (cm^2). From CV, integration of the background-subtracted peak currents provided a surface coverage of 2.3×10^{12} molecules/cm^2, which is in agreement with results reported by others [20]. This indicates that Fc ss-DNA form well-defined self-assembled monolayers on the Au surface of the electrode.

3.2. Time Dependence of the Enzymatic Cleavage

The interactions of Fc ss-DNA and S1 nuclease was investigated, which was monitored by detecting the change of ferrocene signal with the increasing of immersed time. Fc ss-DNA is immobilized on an Au electrode surface. After adding S1 nuclease, the change of signal is inquired to hydrolyze the free ss-DNA. Figure 3a shows DPV signal of remaining Fc ss-DNA, which Fc ss-DNA was cleaved by S1 nuclease. The peak current was decreased with the increasing time, indicating that enzyme cleavage occurred. The enzyme reaction time was ranged form 0 min to 120 min. The peak height increased linearly up to 60 min (Fig. 3b). ss-DNA was cleaved by S1 nuclease more than 90% after 60 min of enzyme reaction time. Such curvature reflected the saturation of enzyme interaction above 60 min.

3.3. Sequence Specific DNA Detection by Cleavage

We used DPV to examine the association of the target DNA and S1 nuclease with Fc ss-DNA modified electrode surface. S1 nuclease degrades singles strand DNA endonucleolytically to yield 5′-phosphoryl terminated products and consequently electrochemical activity due to Fc label is lowered. Namely, S1 nuclease cleaves ss-DNA but not ds-DNA. We have optimized the temperature effect on the sequence discrimination between PM and SBM. The best result was obtained when the combination of hybridization

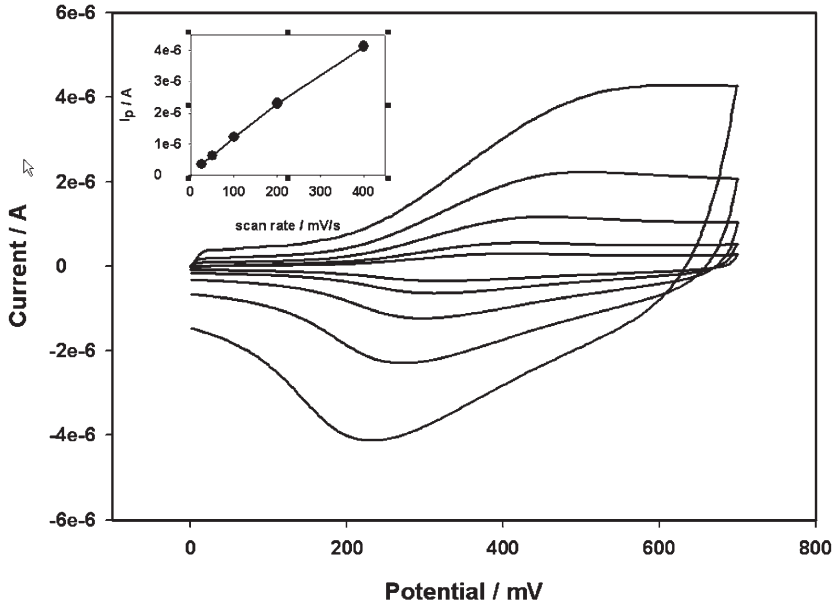

Fig. 2. Cyclic voltammogram of Fc ss-DNA modified electrode dependent on a scan rate from 25 to 400 mV/s in 0.1 M $HClO_4$. Inset: Plot of anodic peak currents to potential sweep rates.

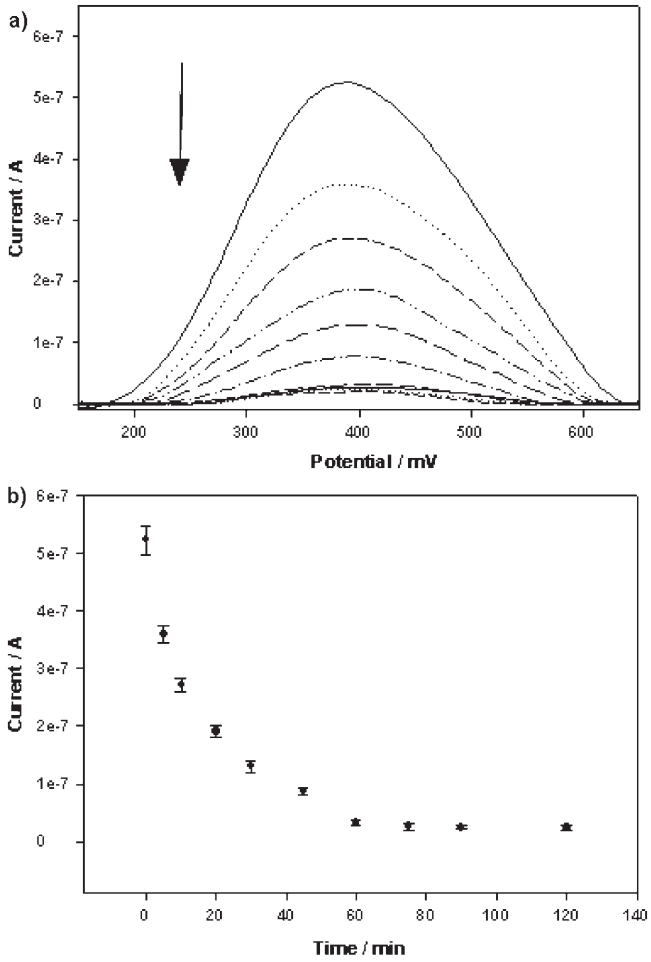

Fig. 3. The baseline-corrected DPV of Fc ss-DNA modified electrode with enzymatic reaction by S1 nuclease at different reaction time (0–120 min) (error bar is three times) (a). Plot of peak currents with the increasing time (b).The pulse amplitude was 0.05 V and the scan rate was 0.02 V/s.

and enzymatic incubation temperature were 37 and 50 °C, respectively. Figure 4 shows DPV results for the electrochemical activity of Fc ss-DNA modified electrode after enzymatic incubation at various temperatures following target hybridization reaction at 37 °C for 1 h. Figure 4a shows PM and SBM can not be discriminated at 25 °C. These results were nearly same as the value for PM and SBM. Ho et al suggested that the linear probe-based systems would be unable to discriminate the perfectly complementary target and a corresponding mismatched target containing a single base alteration [21]. At 50 °C, which is near the melting temperature (T_m) for duplex DNA, the PM duplexes can be clearly discriminated from SBM and NC duplexes (Fig. 4c). UV absorption analysis showed that the T_m values were 55 °C and 48 °C for PM and SBM duplexes, respectively. The mismatched duplex started to dissociate at a lower temperature than the perfect matched case, because the former was less stable. Thus, the system can discriminate PM and SBM duplexes because the energy of binding of a mutated sequence to the ss-DNA probe is lower than that of a complementary sequence [22].

The signal diminution of SBM, in which a single mismatched base is located in the middle of the section that binds to capture probe, due to improvement of enzymatic activity. The fact provides a possibility for discrimination between PM and SBM. Single mismatched pairs have relatively marginal effects on the stability of a duplex when the linear probes are long enough to distinguish a particular sequence. During the S1 nuclease reaction, cleavage physically separates electroactive ferrocene group form nonhybridized capture probe DNA. The structure-specific enzymatic cleavage discriminating between single strand and double strand DNA imparts high selectivity. In general principle, target DNA can be end-labeled covalently with redox indicator that produces a signal

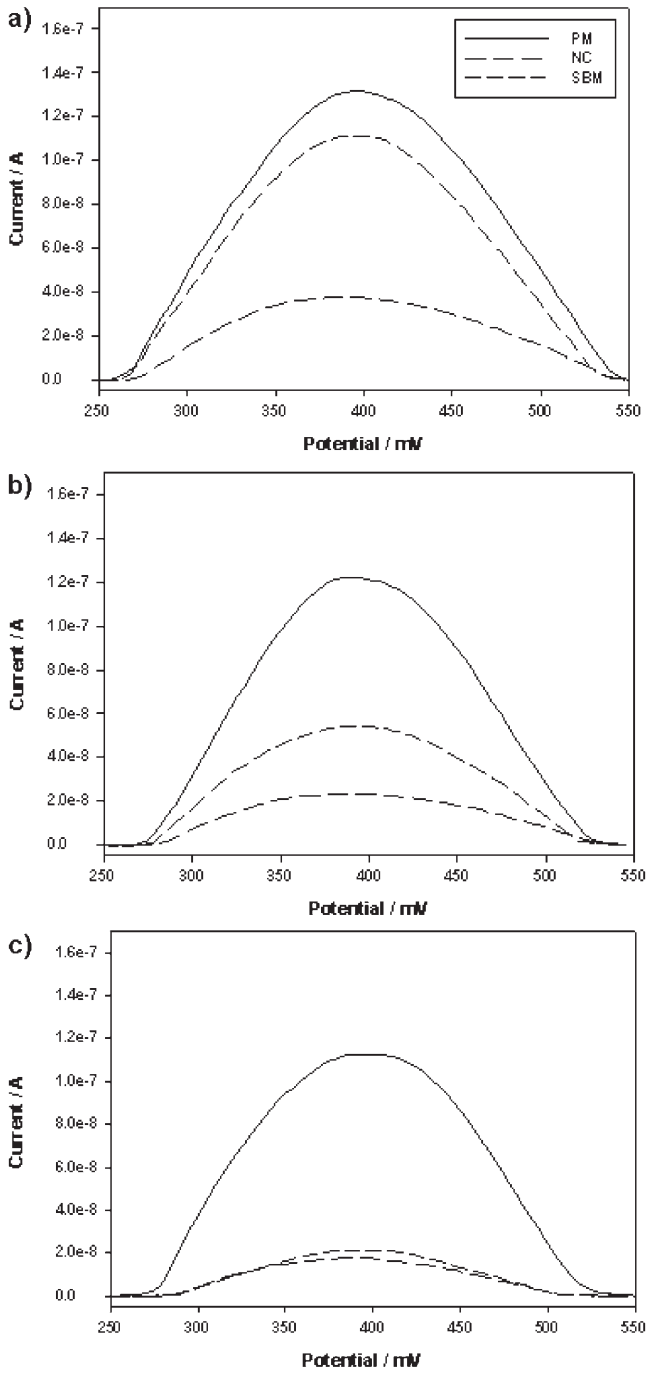

Fig. 4. The baseline-corrected DPV response of electrodes with enzymatic reaction by S1 nuclease at a) room temperature, b) 37 °C, and c) 50 °C after three different target hybridization on the gold electrode modified with Fc ss-DNA probe. The pulse amplitude was 0.05 V and the scan rate was 0.02 V/s.

when a probe binds. This is rather inconvenient, because each target DNA has to be labeled. However, our system solved a problem through attached electrochemical indicator to capture probe and allowed detection of various target DNAs. The ability to discriminate between PM and SBM improved greatly the selectivity and sensitivity for detection using thermally denatured DNA.

4. Conclusions

In this study, we demonstrated a new strategy for electrochemical detection of DNA that offers highly sensitive discrimination of SBM target DNA. This approach takes advantage of S1 nuclease and allows the detection of various target DNAs without the addition of indicators or other exogenous signaling molecules after the hybridization process. Thus, the strategy can be used for 'on – off' detection of oligonucleotides. If this method is used in a multi-electrode array, it should produce a large difference in current, producing an on/off signal. Our results also show that inclusion of S1 nuclease in surface array methods should allow the highly sensitive discrimination of SNPs.

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