Multiplexed Electrochemical DNA Sensor for Single-Nucleotide Polymorphism Typing by Using Oligonucleotide-Incorporated Nonfouling Surfaces

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Received: January 29, 2010; Revised Manuscript Received: March 26, 2010

In this work, we report a multiplexed electrochemical DNA sensor for highly specific single-nucleotide polymorphism (SNP) detection by using oligonucleotide-incorporated nonfouling surfaces (ONS). A typical "sandwich" scheme was employed to perform the SNP assay. The presence of the target DNA templated the ligation between the capture probe DNA anchored on gold electrodes and the tandem reporter probe tagged with a biotin moiety, which could be transduced to peroxidase-based catalyzed amperometric signals. This method could effectively differentiate SNP sites with only one-base mismatch. Importantly, the differentiation ratio was significantly higher than that with surfaces without the nonfouling property, which clearly demonstrated the superiority of the ONS strategy.

Introduction

Analysis of DNA sequence variation has important implications in molecular genetics, such as the linkage and association analysis of disease genes, genetic vulnerability toward diseases, and legal medical applications to identify criminals.[1] Single-nucleotide polymorphisms (SNPs) are the most prevalent and stably inherited types of sequence variations in the human genome[2] and have been widely regarded as promising disease markers.[3,4] Therefore, great efforts have been taken to develop convenient, sensitive, and selective genotyping methods for the detection of SNPs,[5–7] with the hope of rapid and reliable genetic analysis of diseases and subtle genetic risk factors.[7–11]

Several elegant strategies for SNP interrogation have been proposed,[6] including allele-specific oligonucleotide (ASO) hybridization,[12] oligonucleotide ligation assays (OLA),[8,13–17] and primer extension assays,[10,11] relying on various physical readout such as optical,[12–14,18] electrochemical,[15,19–22] and mechanical techniques.[23] Because of their inherent attractive properties of high sensitivity with minimal power/cost/mass requirements,[24–26] electrochemical DNA sensors have attracted significant research interest and shown great promise in SNP genotyping.[27–34]

A typical electrochemical DNA sensor involves an electrode anchored with capture probe DNA. Hybridization of the capture probe DNA with target DNA can be associated with signaling elements that are transduced to electrochemical currents. Whereas the DNA−DNA hybridization is highly specific because of the strong base pair recognition properties, electrochemical DNA sensors are often hampered from real-world applications because of nonspecific adsorption of proteins (e.g., in the matrix) to the electrode surface.[35] We recently developed a highly selective electrochemical DNA sensor by using oligonucleotide-incorporated nonfouling surfaces (ONS).[35] Oligo(ethylene glycol) (OEG) is well-known to be protein-resistant and popularly employed to prepare nonfouling surfaces. In our previous study, we have demonstrated that gold electrode surfaces self-assembled with both OEG-terminated thiols (SH-OEG) and thiolated DNA probes (SH-DNA) retains the nonfouling property and repels nonspecific adsorption of proteins, leading to an electrochemical DNA sensor that can effectively identify DNA targets with high sequence specificity even in the presence of complicated biological fluids (e.g., human sera). In this work, we aim to develop a ligase-based strategy for SNP detection with this ONS-based electrochemical DNA sensor.

Experimental Section

Materials. DNA oligonucleotides were purchased from Sangon Inc. (Shanghai, China) with their sequences listed in Table 1. For SNP detection, four capture probes and a single-reporter probe were designed as follows: Capture probes 1−4 (CP 1−4) are of 50-base sequences with thiol modification at their 5′-terminal with 10 "T"s-spacer and a $-(CH_2)_6-$ alkyl chain. They differ only at their 3′-terminal base. Reporter probe 5 (RP 5) has a biotin at its 3′-end as an affinity label and a phosphate group at its 5′-end for ligation. Target 6 (T6) contains sequences complementary to both RP 5 and CP 1.

Tris(hydroxymethyl)aminomethane was from Cxbio Biotechnology Ltd. Ethylenediaminetetraacetic acid (EDTA), MCH, and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO). Ethylene glycol-terminated thiol ($HS-(CH_2)_{11}-EG_2-OH$, OEG) was purchased from Prochimia (Poland). TMB substrate (TMB = 3,3′,5,5′-tetramethylbenzidine; Neogen K-blue low-activity substrate) was purchased from Neogen (U.S.). Avidin-HRP (horseradish peroxidase) was from Roche Diagnostics (Mannheim, Germany). The buffer solutions involved in this study are as follows: The hybridization buffer was 1 M NaCl and 10 mM TE buffer (pH 7.4). The buffer for electrochemical DNA quantification was 10 mM Tris-HCl solution (pH 7.4) and that for hybridization measurement was TMB substrate. The DNA immobilization buffer was 10 mM Tris-HCl, 1 mM EDTA, 10 mM TCEP (pH 7.4), and 1 M NaCl. Enzyme was diluented to

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10.1021/jp100871u
Published on Web 04/23/2010

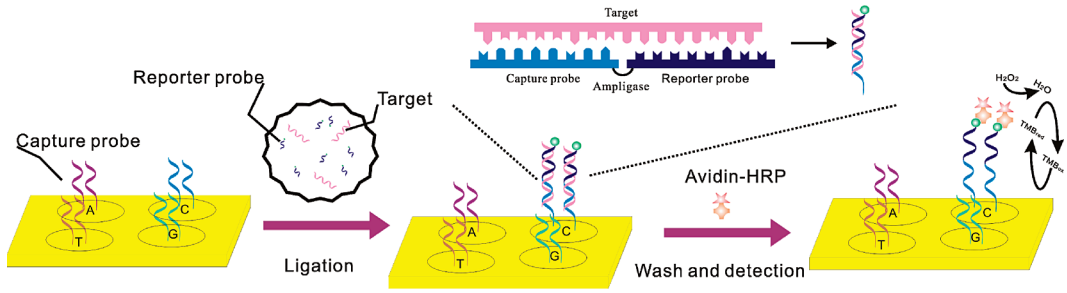

Figure 1. Schematic demonstration for the ligation- and ONS engineering-based electrochemical SNP typing strategy. Four capture probes that differ only at their 3′-terminal base are immobilized onto the gold electrodes of the array. The target DNA and the reporter probe forms a "sandwich" complex with the capture probe. In the presence of the ligase, the target DNA templates the ligation between the capture and the reporter probe, leading to permanent localization of the biotin tag that can be transduced to the catalytic amperometric readout of avidin-HRP. The reporter probe is washed off the electrode surface if a single-base mismatch exists.

TABLE 1: Sequences of Oligonucleotides

capture probe 1	5′-SH-TTTTTTTTTTTGAAAGACAATATAGTTCTTGGAGAAGGTGGA ATCACAC
capture probe 2	5′-SH-TTTTTTTTTTTGAAAGACAATATAGTTCTTGGAGAAGGTGGA ATCACACA
capture probe 3	5′-SH-TTTTTTTTTTTGAAAGACAATATAGTTCTTGGAGAAGGTGGA ATCACACT
capture probe 4	5′-SH-TTTTTTTTTTTGAAAGACAATATAGTTCTTGGAGAAGGTGGA ATCACACG
reporter probe5	5′-P-TGAGTGGACGTCAACGAGCAA -biotin
target 6	5′-TTG CTC GTT GAC GTC CAC TCA *G*TG TGA TTC CAC CTT CTC CAA GAA CTA TAT TGT CTT TCA

a 0.1 M PBS buffer with 0.5% casein (pH 7.2). All solutions were prepared with Milli-Q water (18 MΩ·cm resistivity) from a Millipore system.

Electrochemical Measurements. The 16-electrode sensor arrays (Figure 1S) were provided by GeneFluidics (Monterey Park, CA), each consisting of a working electrode in the center surrounded by an auxiliary electrode and a reference electrode. Cyclic voltammetric (CV) and amperometric (i−t) measurements of DNA arrays were performed at room temperature using a 16-channel PM3000 workstation (Genefluidics). In i−t measurements, the voltage was fixed at −100 mV (versus the gold reference electrode), and the reduction current was measured at 60 s.

Formation of the ONS at Gold Electrode Surfaces. Thiolated capture probes at appropriate concentrations of 3 μL were added to each electrode for 1 h at room temperature. The probe density was modulated by varying the concentration of capture probes (0.2, 0.5, 1, 5, and 10 μM). Then electrodes were incubated with either 2 mM MCH or 2 mM OEG for 2 h to obtain mixed SAMs, i.e., SH-DNA/MCH or SH-DNA/OEG SAMs. The electrode was then rinsed with Milli-Q water and dried with N_2 after each step. For the detection of SNP, 4 capture probes were immobilized on the chip, each probe occupying four working electrodes.

SNP Detection. Mixed solutions containing 1 nM target, 100 nM reporter probe, 1 U ampligase, and 0.5% BSA in reaction buffer were preannealed at 55 °C for 5 min and then added to each working electrode. As the employed ampligase is a relatively themostable ligase, the ligation was performed at 50 °C in a constant temperature and humidity incubator. After a constant time of incubation, a denaturation step was followed by treating with 10 mM NaOH at 70 °C for 10 min. After being washed, electrodes were treated with 1% BSA and then the avidin-HPR (0.5 U/mL in 0.1 M PBS buffer with 0.5% casein) was added. After 20-min incubation at room temperature, electrodes were washed with 0.1 M NaCl and 10 mM PBS buffer (pH 7.4) and subjected to electrochemical measurements.

Results and Discussion

Our system for SNP detection employs a sandwich assay on gold electrode surfaces with both DNA-templated ligation and ONS engineering strategies (Figure 1). In a typical assay, gold electrodes are first decorated with SH-DNA/SH-OEG self-assembled monolayers (SAMs). The SH-DNA functions as the capture probe to hybridize with the target (T6), with the 5′-thiolated end bound to the surface and the 3′-terminal base either complementary (CP 1) or mismatched (CP 2−4) to the SNP site in the target DNA. A 3′-biotinylated oligonucleotide serves as the reporter probe that carries a 5′-phosphate, which is complementary to the target at a position adjacent to the capture probe. If the target is fully complementary, the target DNA acts as a template to initiate the highly specific ligation reaction between the tandem capture/reporter probes with the aid of ligase. This templated ligation leads to permanent covalent linkage of the reporter DNA to the surface. In contrast, if the target is not fully complementary (harboring a SNP site), the reporter probe cannot be ligated and does not survive from subsequent stringent wash. After the ligation, avidin-HRP is incubated with the surface, which catalyzes the reduction reaction of hydrogen peroxide and provides an amperometric readout for the SNP detection. To realize simultaneous detection of different possible mismatches, we employ a 16-electrode sensor array to perform multiplexed SNP typing.

Our previous study has proven that ONS is highly protein-resistant and compatible with electrochemical measurements.[35] Figure 2A reveals a typical CV curve for TMB, which exhibits two pairs of characteristic redox peaks. In the presence of the fully complementary target of 1 nM, the capture probe and the detection probe were in tandem upon being hybridized with the target and the ligase specifically ligated the two tandem sequences, leading to a significantly enhanced HRP-catalyzed reduction peak. When there was a mismatched site, the reduction peak was not significantly increased because of the absence of ligation and wash off of the reporter probe. The background

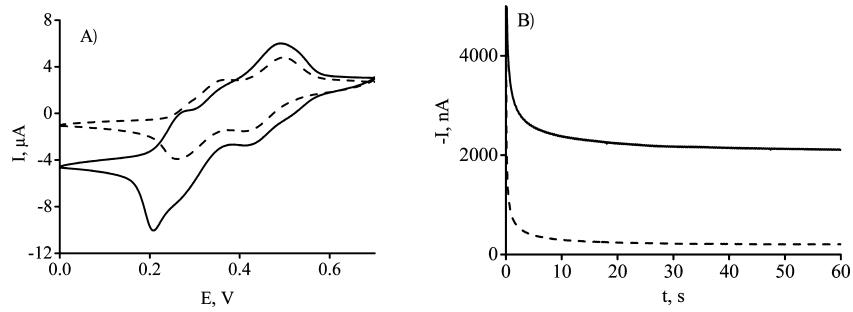

Figure 2. (A) Cyclic voltammograms and (B) amperometric curves for the detection of single-base mismatched (dashed line) and complementary probes (solid line) in the presence of the complementary target of 1 nM. Scan rate of CV: 100 mV/s. Potential of amperometry: 0.1 V.

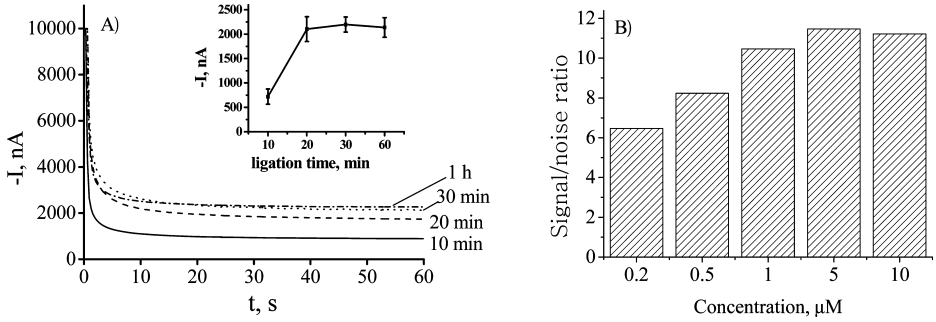

Figure 3. (A) The kinetics of the ligation at 50 °C. Amperometric curves for different ligation time. Inset: amperometric signal vs ligation time. (B) Optimization and comparison of the conditions for the sensor detection of 1 nM target DNA with different concentrations of capture probes. Plot for the signal/noise ratio vs capture probe concentration.

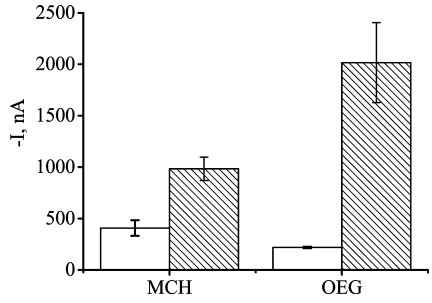

Figure 4. Comparison of the two different SAMs for SNP detection by the sensor, which shows amperometric signals for fully matched capture probe (filled space) and the mismatched one (unfilled space) with 1 nM target DNA.

signal was low because of minimal nonspecific adsorption of the enzyme at the ONS.[35] The amperometric $i-t$ curves provide a quantitative measurement (Figure 2B), which reveals that the differentiation ratio between the fully complementary target and the mutant was as high as 10-fold. We thus demonstrated that, with use of appropriate probes and ligase, unambiguous electrochemical signals could be achieved for the wild-type DNA but not the mutant sequence harboring the SNP. It is important to note that a control study with the nonphosphorylated reporter probe led to minimal signal (data not shown) since such a probe cannot be ligated.

The ligation time was critical to the performance of SNP detection. As shown in Figure 3A, the amperometric signal for the target increased along with the ligation time at first and saturated at around 20 min. We also interrogated the effect of the probe density (0.2, 0.5, 1, 5, and 10 μM) on the differentiation ability and found that the highest signal-to-noise ratio was realized at a probe concentration of 5 μM (Figure 3B). Given that signal increases, but the hybridization efficiency decreases along with the probe density, it is expected that the largest signal-to-noise ratio occurs at a medium density that coincides with the experimental observation.

We further compared the performance of SNP detection at the ONS with that at the SH-DNA/MCH surface without the nonfouling property. These two SAM structures were both amenable to electrochemical SNP detection; however, the ONS engineering strategy clearly exhibited superiority in protein-

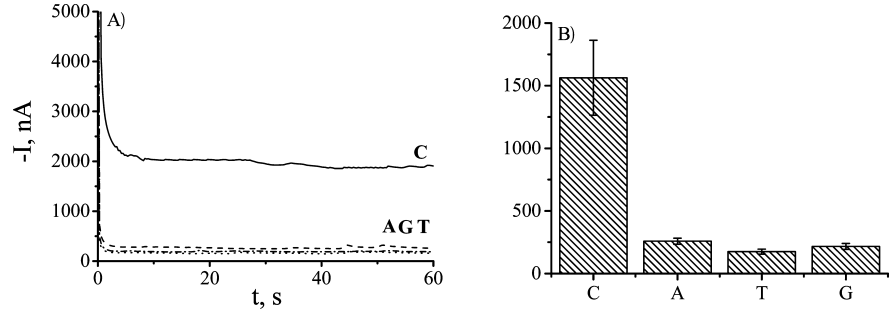

Figure 5. (A) Amperometric curves for a typical SNP detection with the DNA sensor array. The capture probe with a 3′-terminal nucleotide "C" was perfectly matched with the target (1 nM). (B) Amperometric signals for four capture probes in one sensor array for a typical SNP detection.

involving detection systems (e.g., ligases and HRP). We found that the SNP signal with the ONS was significantly lower than that at the SH-DNA/MCH surface (Figure 4), which arose from the low background current due to minimal HRP adsorption, whereas the signal for the wild type was significantly increased, possibly because of high ligation activity of the ligase at such a nonfouling condition. These two factors significantly increased the differentiation ratio by threefold.

To demonstrate the ability to simultaneously detect various SNPs, we then employed a 16-electrode sensor array to perform multiplexed SNP detection. Four capturing probes that differ only at their 3′-terminal bases were immobilized to the electrodes, with each probe occupying four electrodes. The data showed that the perfectly matched capturing probe could be efficiently ligated to the reporter probe whereas mismatched ones could not. As shown in Figure 5, signals from this chip are reproducible with relatively small electrode-to-electrode variation, and the signals of $i-t$ curves clearly showed that the fully complementary target ("C") produced much higher signals than one-base mismatched ones (either "A", "T", or "G"), with a differentiation ratio of approximately ninefold, which was significantly improved as compared to that in our previous study employing the SH-DNA/MCH SAMs (the signal-to-noise ratio was approximately threefold).[30] The greatly improved differentiation ability for SNP typing might arise from two important properties of ONS. First, ONS greatly prevents the nonspecific adsorption of avidin-HRP, which largely eliminates background signals.[35] Second, ONS provides a "waterlike" environment that is beneficial for the maintenance of enzymatic activity (e.g., ligation at the surface).[36,37]

Conclusion

In summary, we have reported ONS- and ligation-based electrochemical DNA sensor for highly specific SNP detection. We demonstrate that the use of the ONS leads to high differentiation ability toward SNPs because of its protein-resistant property that is highly beneficial for the protein-involving detection system. The 16-electrode sensor array also provides a multiplexed platform for simultaneous detection of different SNPs. Therefore, we expect that the present system might be a promising tool for the selective and inexpensive analysis of disease-associated SNPs.

Acknowledgment. This work was supported by the National Natural Science Foundation (20725516, 90913014), National Basic Research Program of China (2007CB936000), Major S&T program (2009ZX10004-301), and Shanghai Municipal Commission for Science and Technology (0952 nm04600).

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JP100871U