Anal. Chem. **2009,** *81,* 1982–1987

Electrochemical DNA Biosensor Based on the Proximity-Dependent Surface Hybridization Assay

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This paper describes a novel electrochemical DNA (E-DNA) biosensor for simple, rapid, and specific detection of nucleic acids based on the proximity-dependent surface hybridization assay. This E-DNA biosensor was constructed by self-assembly of a 3′ short thiolated capture probe on the gold electrode. DNA detection was realized by outputting a remarkable redox current of the 5′ ferrocene (Fc) tail labeled probe. When the target DNA was introduced into the system, it was complementary to the 5′ Fc labeled probe at the one-half-segment and complementary to the 3′ short thiolated capture probe at the other half-segment, resulting in forming a stable duplex complex. As a result, the Fc probe was proximate to the electrode surface, and the Faradaic current was observed. This E-DNA biosensor was proved to have a low detection limit (1 fM) and a wide dynamic range (from 1 fM to 1 nM) due to the stable hybridization mode. In addition, the sensing system could discriminate the complementary sequence from mismatch sequences, with high sensitivity, stability, and reusability.

Recently considerable efforts have been made to develop the rapid, simple, and highly specific detection of DNA sequence due to its important applications in many ranges, such as gene identification, molecular diagnostic, drug research, forensic science, environmental protection.[1−3] Various approaches of DNA detection have been described in the previous works, including optical,[4−8] electrochemical,[9−13] acoustic,[14,15] and gravimetric techniques.[16,17] Among these techniques, electrochemical methods have attracted significant attention and become popular for their simplicity, reliability, sensitivity, and selectivity as well as compatibility with microfabrication technology.[18−20] Some papers have reviewed the development of electrochemical DNA biosensors.[21−23]

Various labeled E-DNA biosensors have been developed to improve the nucleic acid detection, where the sensor tags can be enzyme,[9] electroactive moiety,[10,24] an interactive substance (a groove binder, or an intercatalator),[11,25] nanoparticles,[12,26] cantalytic oxidation of guanine,[13] and so on. Among these labels, the Fc has attracted broad attentions and various Fc derivatives have been used to promote the detection performance,[27] owing to their redox reversibility and synthetical versatility. Hence the Fc derivatives are considered as excellent probes in electrochemical DNA assays[28,29] because they can

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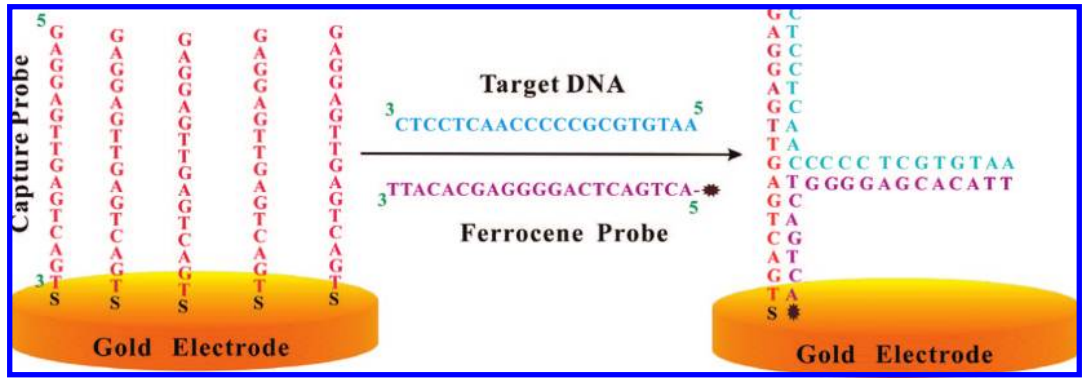
10.1021/ac802512d CCC: $40.75
Published on Web 01/27/2009

Scheme 1. Schematic Presentation of the E-DNA Biosensor Principle[a]



[a] In the presence of added target DNA, the ferrocene-tagged detection probe is annealed on the target sequence to form a stable duplex with residual parts of the target DNA and detection probe drawn in close proximity. This promoted proximity-dependent hybridization of the complementary sequences of the ferrocene-labeled probe and target DNA with the capture probe, bringing the ferrocene moiety close to the electrode with a strong redox current obtained.

successfully characterize the hybridization efficiency and the DNA interaction with other analytes.[30−32]

Fredriksson and co-workers have developed a method for the detection of proteins, termed as proximity ligation assay.[32] The proximity ligation assay depends on simultaneous recognition of a target molecule by a pair of affinity probes. This brings the tail sequences of the affinity probe pair in close proximity to hybridize together with a connector oligonucleotide followed by a ligation-based amplification and detection. We have recently reported a reusable electrochemical aptasensor for single-step and sensitive detection of proteins based on a proximity-dependent surface hybridization assay.[33] The proximity-dependent surface hybridization assay relies on simultaneous recognition of a target molecule by a pair of affinity probes followed by the hybridization of the proximate affinity probes with surface-tethered oligonucleotide strands. Considering the great significance of DNA detection in analytical chemistry, here we proposes a novel strategy based on the principle of the proximity-dependent surface hybridization assay for DNA detection. This strategy can magnify the detection signals and offers a promising technique for the development of sensitive and selective DNA biosensors.

A simple, single-step, signal "on" electrochemical biosensor for the nucleic acids detection is reported for the first time in the present work. This E-DNA biosensor was constructed by self-assembly of a 3′ short thiolated capture probe on the gold electrode for the first step (as shown in Scheme 1). Because of the specific hybridization among the capture probe, Fc-probe, and target DNA as well as the predesigned melting temperature, the surface proximity assay was realized. The signal from the redox of ferrocene was corresponding to the target DNA detection. This E-DNA biosensor was proved to be sensitive, specific, and selective with a low detection limit and a wide linear dynamic range. Moreover, it was successfully used in distinguishing the complementary sequence from the mismatching sequences with stability and regeneracy.

Table 1. Sequence of Synthesized Oligonucleotides Probes Used in This Work[a]

oligonucleotide	sequence (5′ → 3′)
capture probe	GAG GAG TTG AGT CAG T-$(CH_2)_6$-SH
complementary target	AAT GTG CTC CCC CAA CTC CTC
noncomplementary target	AAC GTG TGA ATG ACC CAG TAC
single-base mismatch target	AAT GTG **G**TC CCC CAA CTC CTC
four-base mismatch target	AAT GT**C** CTC **T**CC C**G**A CT**T** CTC

[a] The boldface portion is the mutation base.

EXPERIMENTAL SECTION

Chemicals and Reagents. The DNA oligonucleiotides used in this paper (sequences shown in Table 1) were synthesized by Takara Biotechnology Co. Ltd., Dalian, China. *N*-Hydroxysulfosuccinimide (Sulfo-NHS), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), and ferrocenecarboxylic acid were purchased from Sigma Aldrich Chemical Co. All other reagents were of analytical-reagent grade. Ultrapure water (electric resistance >18.3 MΩ) was obtained through a Nanopure Infinity Ultrapure Water System (Barnstead/Thermolyne Corp., Dubuque, IA).

Preparation of Ferrocene-Labeled Oligonucleotides. The ferrocene label was conjugated to the 3′ NH_2-moiety probes by the succinimide coupling (EDC-NHS).[34] Briefly, 100 μL of 10 μM detection probe and 100 μL of 10 mM PBS (pH 7.4) containing 10 mM ferrocenecarboxylic acid, 1 mM EDC, and 5 mM sulfo-NHS were mixed together and then incubated at 37 °C for 2 h. The conjugate was dialyzed against 10 mM PBS (500 mL) for 12 h to remove excessive ferrocenecarboxylic acid. Finally, the mixture was stored in the refrigerator at −20 °C. Note that degraded or unlabeled DNA after the conjugation reaction only caused a slight loss of the sensitivity but had no effect on the specificity of the biosensor.

DNA Immobilization and Hybridization. The gold electrodes (99.99% polycrystalline gold, ∼2 mm diameter, i.e., 3.14 mm^2 area) were cleaned in piranha solution (30% H_2O_2 and 70% H_2SO_4 in volume) for 2 h twice, polished on a microcloth (Shanghai Chenhua Inc., China) with a 0.05 μm alumina slurry for 2 min, and rinsed with ultrapure water and ethanol. The electrodes were then ultrasonicated in ultrapure water for 5

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min and rinsed thoroughly with ultrapure water and dried in nitrogen. The sensing interface was fabricated as follows: 15 μL of 1 μM thiolated oligonuleotide solution was injected into 10 mM PBS (pH 7.4, 1 M NaCl), and the pretreated gold electrode was incubated in the mixture for 12 h at room temperature. The electrode surface was then rinsed with the buffer at 50 °C for 20 min and dried under a stream of nitrogen. The resulting thiolated oligonucleotide functionalized electrode was incubated in 10 mM PBS (pH 7.4, 0.3 M NaCl) containing the 0.1 μM ferrocene-tagged detection probe and 80 μL of target solutions of different concentrations at 52 °C for 2 h.

Apparatus and Electrochemical Detection. The electrochemical sensors were measured via cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (ESI) with a CHI Instruments model 760B electrochemical analyzer (CHI Inc.) in the three-electrode system, which was comprised of a KCl saturated calomel reference electrode (SCE), a platinum counter electrode, and the working electrode. All electrochemical measurements were performed in 10 mM PBS (pH 7.4) containing 0.1 M $KClO_4$ at room temperature. The DPV curves scanned from 0 to 0.5 V were background-subtracted using ORIGIN 7.0 (Micirocal Software, Northampton, MA) through extrapolation to the baseline in the regions far from the peaks. The surface coverage of the ferrocene-labeled probes under conditions of saturated electrochemical response was determined according to the integration of the area under either the anodic or the cathodic peak corrected from the background current.[35]

RESULTS AND DISCUSSION

Scheme of the E-DNA Biosensor. Scheme 1 shows the designed elements of the E-DNA biosensor based on the proximity-dependent surface hybridization assay. Two oligonucleotide probes are designed based on the melting temperatures in our system according to the target DNA sequence. One is the thiolated capture probe, and the other is the ferrocene-tagged detection probe. The melting temperatures for the ferrocene-tagged probe and target sequence are designed to be higher than the reaction temperature, while melting temperatures for the capture probe and target sequence as well as the Fc-tagged probe and capture probe are both lower than the reaction temperature. This design allows the hybridization of the ferrocene-tagged probe with a target sequence under the reaction conditions, while annealing of the ferrocene-tagged probe to capture probe is precluded under these conditions, thus ensuring a low background in the absence of the target sequence. To fabricate the E-DNA biosensor, the thiolated capture probe was covalently attached to the gold electrode via well-established self-assembled monolayer chemistry. In detecting the target sequence, the modified electrode was directly dipped into the analytical samples containing different concentrations of the target DNA and ferrocene-tagged detection probes at a prescribed reaction temperature. Cyclic voltammetry and differential pulse voltammetry were used to characterize the hybridization efficiency, and DPV was found to provide excellent resolution of the response. In the absence of the target DNA, the ferrocene-tagged detection probes cannot hybridize with the thiolated oligonucleotide at the reaction temperature because of the predesigned lower melting temperature. (The melting temperature of the duplex corresponding to the Fc detection probe and thiolated capture probe was calculated as ~10.0 °C by using the Zuker program.[36]) When the target DNA was introduced, the ferrocene-tagged detection probe was annealed on the target sequence to form a stable duplex complex. (Melting temperature of the duplex corresponding to the ferrocene-tagged detection probe and target DNA was calculated as ~52.7 °C by using the Zuker program.[36]). Then, the residual parts of the target DNA and detection probe were drawn in close proximity, which promoted cooperative hybridization of the residual complementary sequences of the ferrocene-labeled probe and the target DNA with the capture probe. As a result, the ferrocene moiety is brought close to the electrode surface (as shown in Scheme 1) and a strong Faradaic current was obtained.

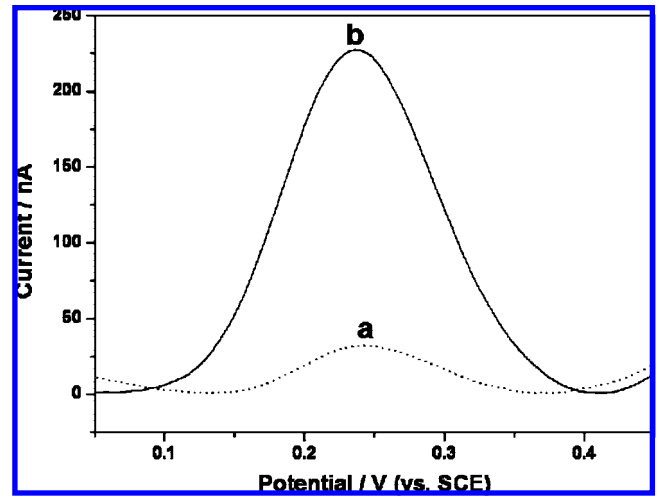

Figure 1. Differential pulse voltammetry responses of the E-DNA biosensor recorded in 10 mM PBS (pH 7.4) containing 0.1 M $KClO_4$ before (a) and after (b) hybridization with 500 pM target DNA. Reaction conditions: 10 mM PBS (pH 7.4, 0.3 M NaCl) incubation at 52 °C for 2 h. DPV, pulse amplitude, 50 mV; pulse period, 0.2 s.

From the differential pulse voltammograms (Figure 1), the E-DNA biosensor shows obvious reduction peaks at 256 mV vs SCE recorded in 10 mM PBS (pH 7.4) containing 0.1 M $KClO_4$. This electrochemical signal was from Fc reduction on the gold electrode because the Fc was brought close the electrode surface when the capture probe and the Fc-probe simultaneously recognized the target DNA. Moreover, the reduction peak with 500 pM target DNA (Figure 1, curve b) is more than 200 nA, about 10 times more than the blank ground (Figure 1, curve a). The background signal was attributed to nonspecific adsorption of Fc-tagged probes. Actually, in a control experiment using the bare Au electrode in the same solution, no background current was observed. The apparent signal enhancement of current demonstrated this E-DNA biosensor was capable of target DNA detection. Impedance spectroscopy is an effective method for probing the features of surface-modified electrodes. Thus, electrochemical impedance spectroscopy (EIS) was carried out to study the gold electrodes modified by different procedures. The impedance plot for the bare gold electrode (Figure 2, curve a) exhibits an almost straight line, as characteristics of an

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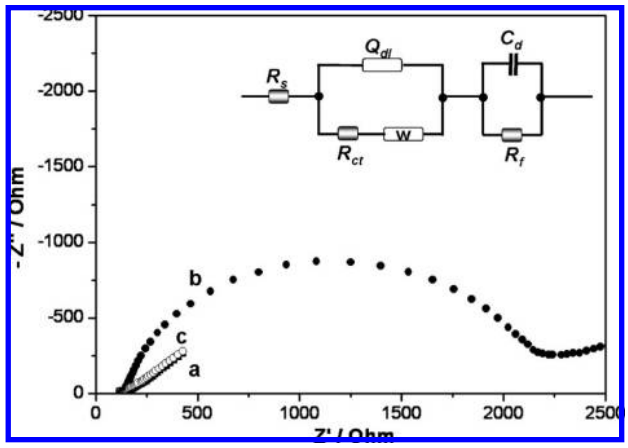

Figure 2. Nyquist plots in PBS buffer (10 mM, pH 7.4) containing 5 mM $Fe(CN)_6^{3-/4-}$ at 240 mV for (a) a bare gold electrode, (b) the thiolated DNA modified gold electrode, (c) the modified gold electrode after hybridization with 0.1 μM FC-probes and 100 pM target DNA. Frequency range, 0.1−100 kHz; ac amplitude, 5 mV. The insert was the equivalent circuit: R_s, solution resistance; R_{ct}, charge-transfer resistance; Q_{dl}, constant phase element related to double-layer capacitance; W, Warburg impedance; C_d, film capacitance; R_f, film resistance.

electrochemical diffusional limiting process. Faraday impedance increased significantly because of the thiolated DNA capture probe modification (Figure 2, curve b). Electron transfer became more difficult because of the capture probe modification resulting in negative charge enhancement on the gold electrode surface. Interestingly, hybridization between target DNA and the Fc-probe induced decreased electrochemical impedance (curve c). The equivalent circuit (shown as an inset in Figure 2) was used to fit the EIS results. R_s was the solution resistance and was found to be identical during the experiments (126 Ω cm^2). Other elements in the equivalent circuit were shown as followed: R_{ct}, charge-transfer resistance; Q_{dl}, constant phase element related to double-layer capacitance; W, Warburg impedance; C_d, film capacitance; and R_f, film resistance. The self-assembly of the thiolated capture probe onto the electrode surface could be expected to increase the polarizability of the intervening medium with increasing dielectric constant. The monoelectron transfer rate constant, k^θ, could be evaluated from $R_{ct} = RT/i_0F$ (eq 1) and $i_0 = Fk^\theta\Gamma$ (eq 2), where $R = 8.31$ J mol^{-1}, T is the temperature (K), F is the Faraday constant, and Γ is corresponding to the total amount of bound Fc on the electrode surface.[37,38] R_{ct} is the charge transfer resistance obtained by computer fitting of the experimental spectra using the equivalent circuit and found to be 189 Ω cm^2 for the capture DNA modified electrode (Figure 2, curve b) and 1.93 Ω cm^2 for the capture DNA modified electrode after hybridization with target DNA (Figure 2, curve c). As a result, k^θ of the two states were figured out, respectively, as $k_b{}^\theta = 0.261$ s^{-1} (for curve b) and $k_c{}^\theta = 25.6$ s^{-1} (for curve c). The above results gave immediate evidence for surface confinement of the ferrocene labels that exhibited facilitated electron transfer kinetics when the target DNA hybridized with the other two probes.

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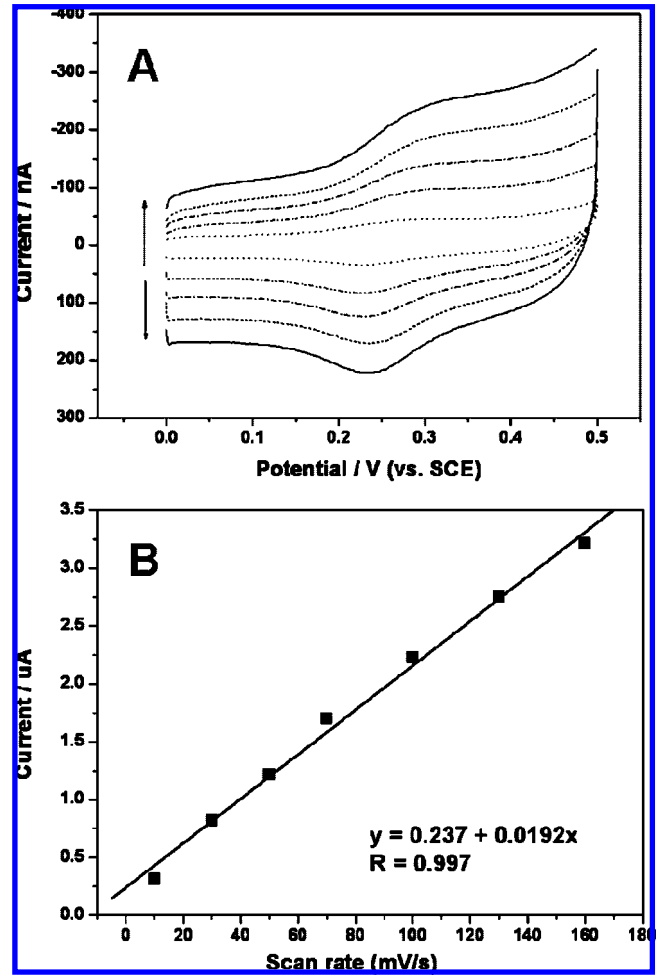

Figure 3. (A) Cyclic voltammograms of the Fc-target-Au electrode at various scan rates, 10, 30, 50, 70, 100 mV/s, in 10 mM PBS (pH 7.4) containing 0.1 M $KClO_4$. (B) The plots of oxidation peak currents vs scan rate. Reaction conditions: 10 mM PBS (pH 7.4, 0.3 M NaCl) incubation at 52 °C for 2 h.

The formal potential E^o of Fc redox was found to be 215 mV according to the cyclic voltammograms as shown in Figure 3A. E^o was estimated as $(E_{p,a} + E_{p,c})/2$, where $E_{p,a} = 256$ mV was the anodic peak potential and $E_{p,c} = 174$ mV was the cathodic peak potential, respectively. To study the controlled factor of the electrochemical process on the electrode surface, we investigated the effect of scan rate on the peak current by cyclic voltammetry. At scan rates in the ranging from 10 to 160 mV/s, the reduction peak currents of the E-DNA sensor increased linearly with the scan rate, indicating that the process is controlled by the surface reaction. Figure 3B shows the linear relationship between the peak currents and scan rates, with the equation, $y = 0.237 + 0.0192x$ ($R = 0.997$), where y and x stand for the peak current (microamps) and scan rate (millivolts per second) respectively.

The surface coverage of the ferrocene-labeled probes under conditions of saturated electrochemical response was determined from the area of the cyclic voltammetric peaks under either the anodic or the cathodic peaks corrected from the background current. The total amount of bound Fc on the electrode surface Γ on the electrode can be calculated quantitatively from $\Gamma = Q/nFA$, where n is the number of electrons transferred ($n = 1$), F the Faraday constant (coulombs per equivalent), Q is peak area of

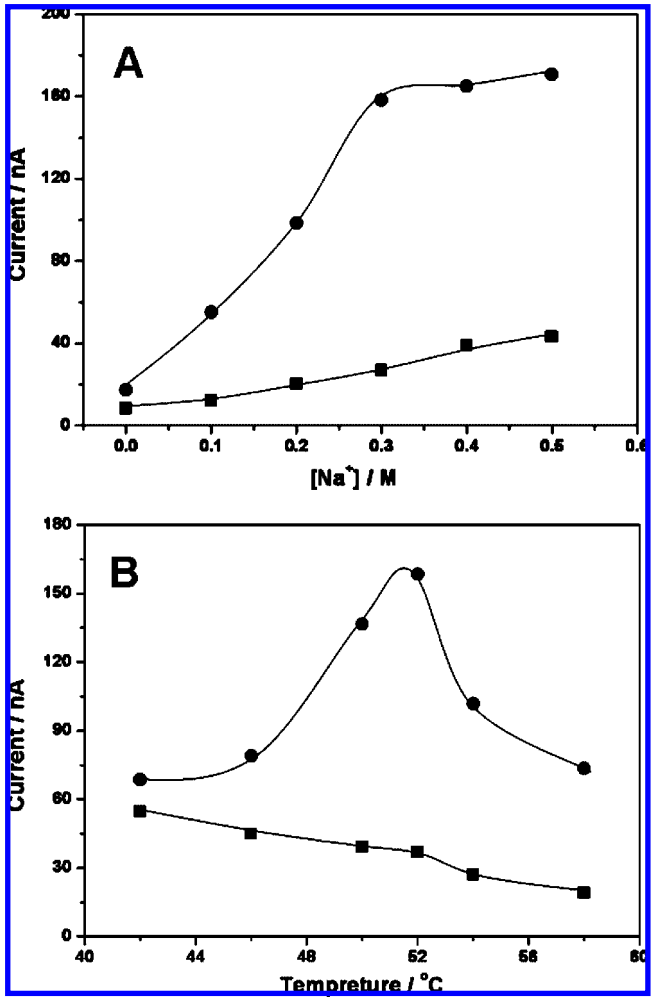

Figure 4. (A) Effects of the Na^+ concentration (from 0 to 0.4 M) for 10 pM target DNA (●) detection and blank (■). Reaction conditions: 10 mM PBS (pH 7.4) incubation at 52 °C for 2 h. (B) Effects of temperature (from 42 to 58 °C) for 10 pM target DNA (●) detection and blank (■). Reaction conditions: 10 mM PBS (pH 7.4, 0.3 M NaCl).

the immobilized layer in coulombs, and A is the effective surface area (square centimeters), thus found Γ to be 5.1×10^{13} molecules/cm^2.[39] Such a surface coverage was close to the value reported for the self-assembled monolayer of short oligonucleotides.[33] This might be attributed to the fact that the target DNA and Fc-DNA are both very short such that there is no significant increase in the volume for their complex as compared with linear DNA strands.

Optimum of Na^+ Concentration and Hybridization Temperature. The hybridization efficiencies were strongly influenced by the assay conditions, such as ion concentration or experimental temperature. So the effects of Na^+ concentration and hybridization temperature were investigated in the next work. Figure 4A shows the effect of Na^+ concentration from 0 to 0.5 M on the electrochemical readout of the DNA biosensor. The ratio of the signal and the blank increased significantly as the Na^+ concentration increased up to 0.3 M, reflecting improved hybridization performance. However, no signal enhancement could be observed when the Na^+ concentration was up to 0.4 M. Next, the effect of hybridization temperature was examined, as shown in Figure 4B. The peak current ratios of target and blank signals increased from 43 to 52 °C and then went down with more heating. Therefore, 0.3 M and 52 °C were chosen as the optimum salt ion concentration and hybridization temperature for this E-DNA biosensor, respectively.

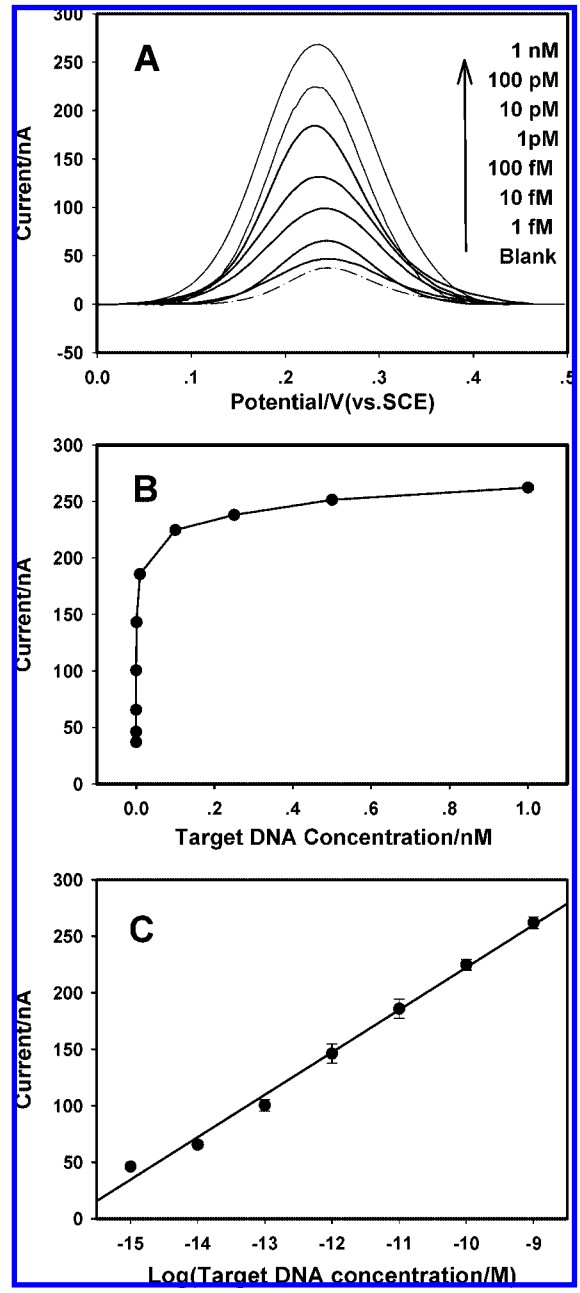

Figure 5. (A) Differential pulse voltammetry response curves of the E-DNA biosensor at various target DNA concentrations, from 1 fM to 1 nM. DPV, pulse amplitude, 50 mV; pulse period, 0.2 s. Reaction conditions: 10 mM PBS (pH 7.4, 0.3 M NaCl) incubation at 52 °C for 2 h. (B) The plots of peak currents vs the target DNA concentrations. Error bars are the standard deviation of four repetitive experiments. (C) Linear relationship between the peak current and the logarithm of the target DNA concentration.

Target Detection of the E-DNA Biosensor. DPV is a pulse technique that allows much higher sensitivity than conventional sweep techniques when detecting very low concentrations of a

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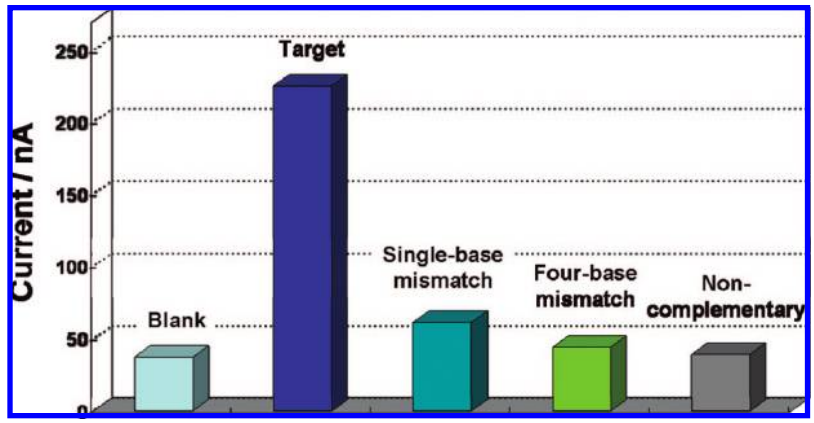

Figure 6. Bar chart of the differential pulse voltammetry responses of the blank and 100 pM complementary sequence, single-base mismatch sequence, four-bases mismatch sequence, and noncomplementary sequence.

redox probe. This is achieved by applying a small voltage pulse superimposed on the linear voltage sweep and sampling the differential current at a short time after the pulse.[39] As shown in Figure 5A, the DPV method was used for the target analyte with different concentrations. Figure 5B appears that the peak current i increased along with the increasing target DNA concentration C ranging from 1 fM to 1 nM. In Figure 5C, the peak current of the E-DNA sensor exhibited a linear correlation to the logarithm of the target concentration across the almost seven-decade range from 1 fM to 1 nM. The linear correlation coefficient was 0.997, and the detection limit was 1 fM (S/N = 3). Compared with other DNA detection methods, especially the electrochemical methods,[40−42] the proposed method was superior in sensitivity and wide linear range and it was able to discriminate a single-base difference in the DNA target with high selectivity. The Scatchard plot of C/i versus C, a variant of the Langmuir adsorption equation, was created, and a linear curve was obtained.[43] A linear fitting of the data gave an estimate of the binding coefficient as 2.9×10^{11} M^{-1}, corresponding to a Gibbs free energy of −71.3 kJ/mol.

Selectivity of the E-DNA Biosensor. Four kinds of DNA sequences (see Table 1 for details) including the complementary target DNA, the single-base mismatch DNA, the four-base mismatch DNA, and the noncDNA were chosen to study the selectivity. Figure 6 shows the comparison of the peak currents of these four target DNA sequences and the background. The same amounts of four DNA sequences (100 pM) were detected after hybridization with the Fc-probe, and the signals of peak currents were recorded. Among the four kinds of DNA sequences, the peak current of the cDNA was 225 nA, much higher than those of the other mismatch sequences whose peak currents were 61 (single-base mismatch), 44 (four-base mismatch), and 39 nA (noncomplementary), respectively, similar to the current of the blank background (37 nA). A significant current difference (Δ was about 200 nA) demonstrated that the E-DNA biosensor was able to discriminate cDNA as the target with high specificity and sensitivity.

Stability and Regeneration of the E-DNA Biosensor. Regeneration is useful for continuous monitoring of the target DNA in future research. It was found that the prepared E-DNA biosensor could be regenerated 16 times with about 15% loss of the original signal by dipping the electrode in hot (>80 °C) ultrapure water for 10 min, followed by a rapid cooling in an ice bath for 10 min. The signal attenuation seemed to be attributed to the loss of immobilized thiolated probes on the gold surface. At the same time, the stability of the E-DNA biosensor was investigated due to the significance of the practical application in DNA detection. The E-DNA biosensor was examined via DPV every 24 h after its hybridization with the cDNA and stored in deoxygenized ultrapure water at 4 °C over 6 days. Experiments demonstrated that the E-DNA biosensor had a good stability within 44 h.

CONCLUSIONS

A new electrochemical DNA biosensor based on the proximity-dependent surface hybridization assay was constructed for the simple, rapid, specific detection of a short sequence. Our strategy made use of the target DNA sequence to hybridize with the Fc probe and the thiolated probe at the same time to form a proximity assay platform. Electron transfer was enhanced because the proximity-dependent surface hybridization made the Fc probe approach to the electrode surface as close as possible. It was demonstrated that the proposed approach had a wide detection linear range from 1 fM to 1 nM with a lower limit of 1 fM. Attainment of the response was also extremely rapid with high stability and reusability. This novel electrochemical DNA biosensor architecture may prove suitable for the detection of target DNA in complex samples, even clinical analytes. Moreover, the reported sensor achieves impressive selectivity with successful discrimination of the target DNA from various mismatch sequences. Thus, the simple strategy described here may be facile to apply in DNA damage analysis and diagnostics.

ACKNOWLEDGMENT

The work was financially supported by "973" National Key Basic Research Program (Grant 2007CB310500) and NSF (Grants 20875027, 20675028, and 20775023) of China.

Received for review November 27, 2008. Accepted January 11, 2009.

AC802512D

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