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An Enzyme-Based E-DNA Sensor for Sequence-Specific Detection of Femtomolar DNA Targets

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Abstract: In this work, we report an enzyme-based E-DNA sensor for the sequence-specific detection of nucleic acids. This DNA sensor employs a "stem−loop" DNA probe dually labeled with biotin and digoxigenin (DIG). The probe is immobilized at an avidin-modified electrode surface via the biotin−avidin bridge, and the DIG serves as an affinity tag for the enzyme binding. In the initial state of the sensor, the probe adopts the stem−loop structure, which shields DIG from being approached by a bulky horseradish peroxidase-linked-anti-DIG antibody (anti-DIG-HRP) due to the steric effect. After hybridization, the probe undergoes a significant conformational change, forcing DIG away from the electrode. As a result, the DIG label becomes accessible by the anti-DIG-HRP, and the target hybridization event can be sensitively transduced via the enzymatically amplified electrochemical current signal. By using this new strategy, we demonstrate that the prototype E-DNA sensor has been able to detect as low as femtomolar DNA targets with excellent differentiation ability for even single mismatches.

Introduction

The sequence-specific detection of DNA hybridization has attracted considerable interest in a wide range of areas including molecular diagnostics, environmental monitoring, and antibioterrorism.[1,2] Consequently, various optical, piezoelectric, and electronic transduction techniques have been employed for the development of DNA sensors.[3−9] Among them, electrochemical DNA sensors have been well recognized to be a promising solution for the point-of-care diagnostics due to the fact that electrochemical detectors are simple, portable, and inexpensive.[10,11]

In 1993, Millan and Mikkelsen developed a prototype sensor to electrochemically discriminate hybridized, double-stranded (ds-) DNA from single-stranded (ss-) one by using exogenous, redox-active hybridization indicators (double-helix intercalators),[12,13] a method resembling the widely employed fluorescent hybridization indicators (e.g., ethidium bromide, EB). While these intercalator-based DNA sensors take advantage of design simplicity and operation convenience, they often suffer from high background signals that are associated with nonspecific binding of intercalators to unhybridized ssDNA.[4,12,13] In an attempt to circumvent this problem, the sandwich-type strategy was proposed and popularly employed, which involved a pair of DNA probes (capture and redox-labeled reporter probes) that flanked the target DNA sequence.[14,15] Such dual hybridization processes significantly improved the signal-to-noise ratio, thus enabling the reliable detection of as low as picomolar DNA targets (in contrast to the micromolar to nanomolar sensitivity of intercalator-based sensors).[16] The detection sensitivity could be further pushed down to the femtomolar scale by coupling the sandwich-type sensors with signal amplification offered by replacing small redox molecules (e.g., ferrocene[16]) with either redox enzymes[14,17] or inorganic nanoparticle labels.[18]

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Table 1. Oligonucleotides Employed in This Work

oligo 1 (stem−loop probe)	5′-DIG-ggccgtTACTCCCTTCCTCCCCGCacggcc-biotin-3′
oligo 2 (target)	5′-GTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATA-3′
oligo 3 (1 mismatch)	5′-GTACTTTCAGCGGGGAGGAAGGGAGTGAGGTTAATA-3′
oligo 4 (4 mismatches)	5′-GTACTTTCAGCGGGGAGGAAGGCAGAAGAGTTAATA-3′
oligo 5 (noncognate)	5′-GCAAATCCTACAAAACGAACATCAT-3′
oligo 6 (*E. coli* probe)	5′-DIG-ggccgtACTGATCGTTAAAACTGCCTacggcc-biotin-3′

More recently, we and others developed a new class of reagentless, sensitive, and selective E-DNA sensors,[5,19,20] which integrated the capture probe and the reporter probe (redox labels) by using a single surface-confined stem−loop DNA structure.[5,8] This design is in fact an electrochemical analogue of fluorescent "molecular beacons", i.e., stem−loop (hairpin) probes with internally quenched fluorescence.[21,22] Significantly, stem−loop probes inherently possessed high specificity due to their conformational constraints,[23] and thus were superior to linear ones for DNA detection. The E-DNA sensor exploited the hybridization-induced conformational change of stem−loop probes, which could be electrochemically interrogated on the basis of the distance-dependent electron transfer property of the coupled redox molecule.[5] The E-DNA sensor represents an important progress in the area of electrochemical DNA detection; however, the original design was a signal-off sensor that is susceptible to false-positives. Efforts have been taken to design signal-on E-DNA sensors,[20,24] but most previously reported ones employed complicated structural design of capture probes. Recently, a colorimetric method for DNA detection with stem−loop probes was developed,[25] which exploited variation in the steric effect due to the hybridization-induced conformational change. Inspired by this observation, we herein reported an enzyme-based signal-on E-DNA sensor that employed stem−loop structured probes and featured ultrahigh sensitivity up to low femtomolar.

Materials and Methods

Materials. All oligonucleotides were synthesized and purified by Sangon Inc. (Shanghai, China), and their sequences are shown in Table 1. The stem−loop probe oligonucleotide (oligos 1 and 6) has a 5′-digoxigenin (DIG) affinity label and a 3′-biotin. Oligo 1 is the probe for the model system, while oligo 6 is the probe for *Escherichia coli*. It has six complementary bases at its 5′ and 3′ ends (five of them are G-C pairs), which will form the stem at appropriate ionic strength. The sequence of the target (oligo 2) is perfectly matched to the loop sequence of the probe; oligo 3 contains a one-base mismatch, while oligo 4 has four one-base mismatches. Oligo 5 is a random sequence unrelated to the probe sequence.

Cyclic voltammetry (CV) measurement and amperometric measurement were performed at room temperature using a PM3000 multichannel potentiostat (Genefluidics, Monterey Park, CA). TMB (3,3′,5,5′ tetramethylbenzidine) was purchased from Neogen (Lexington, KY) in the format of a ready-to-use reagent (K-blue low-activity substrate, H_2O_2 included). This K-blue is a substrate designed for immunoassays, which offers better sensitivity than our homemade TMB substrate solution. Anti-DIG-HPR was purchased from Roche Diagnostics (Mannheim, Germany). Sensor test solution was 0.5 mM $K_3Fe(CN)_6$ in 0.1 M KCl.

Electrochemical electrode arrays consist of 16 sensors (Genefluidics, Figure 1-S), each having a gold working electrode (2.3 mm in diameter) in the center surrounded by a gold auxiliary electrode (circle) and a gold reference electrode (small square).

Preparation of streptavidin-coated electrode surfaces. A solution of 1 mM 11-mercaptoundecanoic acid (MUA) and 3 mM 11-mercapto-1-undecanol (MU) in ethanol was drop cast to cover gold electrodes, which were incubated overnight. The sensor was then rinsed with MilliQ water (18 MΩ·cm, Millipore) and dried with nitrogen. The activation solution (200 mM EDC and 50 mM NHS in MilliQ water) was then cast on the surface to activate the carboxyl group for 10 min at room temperature. The activated electrode surfaces were incubated with EZ-biotin (5 mg/ml biotin-PEO-amine in 0.1 M MES buffer, pH 5.0) for 10 min. Then the sensors were incubated with 1 M ethanolamine for 10 min to block the residual activated carboxyl groups. The biotinylated sensors were incubated in 0.5 mg/ml of streptavidin in 0.1 M PBS for 10 min. After being washed and dried in nitrogen, the sensor was stored in a desiccator before use. Test solutions consisting of 0.5 mM $K_3Fe(CN)_6$ and 0.1 M KCl were applied to characterize each sensor.

Fabrication of E-DNA sensors. Biotinylated stem−loop probes were immobilized streptavidin-coated sensors. To each working electrode, 4 μL of probes (1 μM) in 1 M PBS buffer (100 mM phosphate, pH 7.4 and 1 M NaCl) was added and incubated overnight at 4 °C. Two μ L of target samples in 1 M PBS buffer were added to each working electrode, and incubated for 30 min at 37 °C. After being washed with ice-cold washing buffer (0.1 M PBS buffer containing 0.5% tween), the sensors were incubated with 40 μl of PEG (0.05% polyethylene glycol 3350 in 0.1 M PBS) to prevent any possible nonspecific binding. The sensors were again washed with washing buffer, and then 4 μl of anti-DIG-HPR (0.5 U/mL in 0.1 M PBS buffer with 0.5% casein) was added and incubated for 10 min at room temperature. The sensors were washed with MilliQ water and dried with nitrogen, to which 40 μl of the TMB substrate (K-Blue aqueous TMB; Neogen, Lexington, KY) was added. Measurements were immediately made by using the potentiostat. The voltage was fixed at −100 mV (vs gold reference) that was experimentally proven to provide best response, and steady-state amperometric currents were measured at 60 s.

Detection of *E. coli* genomic DNA. In order to test the real applicability of our new E-DNA sensor, we challenged it with PCR amplicons for *E. coli* genomic DNA. We chose a 250-bp region (uidA gene,[26] Supporting Information) in the genomic DNA as the target, which was detected by the oligo 6 probe immobilized at the electrode surface. *E. coli* was cultured overnight, and its genomic DNA was isolated by using a Bacteria Genomic DNA Isolation Kit (Sangon, Shanghai). The PCR amplification was performed in a Bio-Rad PCR cycler (PTC-100). A pair of asymmetric primers (primer 1/primer 2 = 100:1) was employed in order to generate the ssDNA target.[19,27] Of note, the use of asymmetric PCR ensured that the product was in the single-

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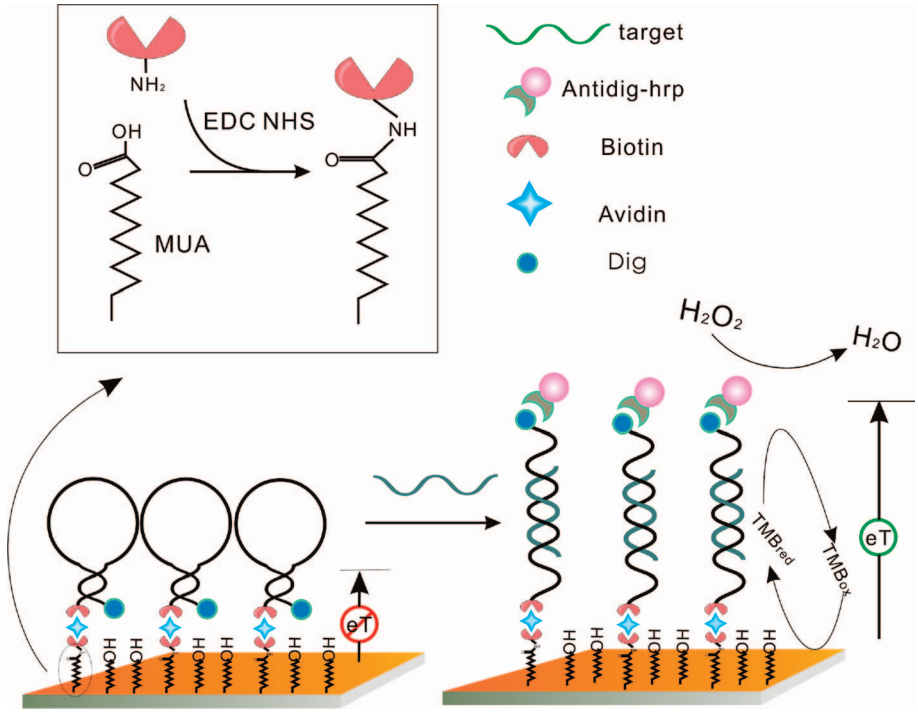

Figure 1. Scheme for the enzyme-based E-DNA sensor. In the closed conformation the DIG label is sterically shielded and thus inaccessible to the reporter enzyme. Upon target binding, the disruption of the stem−loop and the formation of the duplex make the DIG label accessible for HRP binding, which catalyzes the electrochemical reduction of hydrogen peroxide.

stranded format that could be directly sensed by the single-stranded probe. The amplification protocol is as follows, 2 min at 95 °C followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The reaction system was further incubated for 5 min at 72 °C to extend any incomplete products. The PCR products were diluted by 10 times and then subjected to electrochemical sensing as described above.

Results and Discussion

We employed a stem−loop probe dually labeled with DIG and biotin at the 5′- and the 3′- end, respectively, which could be facilely immobilized at avidin-coated electrode surfaces via the biotin−avidin bridge and transduced to electrochemical signals by using a horseradish peroxidase-linked-anti-DIG antibody (anti-DIG-HRP). The detection strategy is demonstrated in Figure 1. Initially, the immobilized stem−loop probe was in the "closed" state in the absence of the target, which shielded DIG from being approached by the bulky anti-DIG-HRP conjugate due to the steric effect. After hybridization, the loop sequence (18-base) formed a rigid duplex with the target, breaking the relatively shorter stem duplex (6-base). Consequently, DIG was forced away from the electrode and became accessible by the anti-DIG-HRP. Of note, one HRP enzyme brought about by one hybridization event could efficiently catalyze thousands of reduction reactions of hydrogen peroxide (H_2O_2), leading to significantly amplified electrochemical current signals.

The sensor array employed in this work consists of 16 microfabricated sensors, each having a set of working, reference, and counter electrodes (Figure -S of Supporting Information). This sensor array thus allows simultaneous detection of multiple targets. Gold working electrodes were first coated with a mixed self-assembled monolayer (SAM) that was composed of thiolated 11-MUA/11-MU, where carboxylic 11-MUA served as the conjugation site and 11-MU as the dilution molecule. We used ferricyanide as a redox probe to characterize the SAM and found it nearly electrochemically silent (Figure 2-S of Supporting Information), suggesting that the SAM was densely packed and effectively repelled ferricyanide.[28] Such high-quality packing of SAM provided low background that was critical for the sensor detection.[29] Amine-modified biotin (EZ-biotin) was then attached to the conjugation site of the SAM (carboxylic groups of 11-MUA) with the help of EDC/NHS. After that, streptavidin (a modified version of avidin) was coated on the biotinylated surface. Since streptavidin is a tetrametric protein containing four binding sites for biotin with extraordinarily high affinity ($K_d = 10^{-15}$), this configuration allows site-specific orientation of streptavidin and provides an ideal way to subsequent immobilization of biotinylated oligonucleotides.[29]

We immobilized the dually labeled stem−loop probe at the streptavidin-coated surface, which was expected to form the stem−loop structure and adopt the "close" state at high ionic strength. We then incubated the probe-modified electrode with the anti-DIG-HRP. Of note, HRP does not directly exchange electrons with the electrode due to the fact that its redox site is shielded within insulating peptide backbones.[30] In order to circumvent this problem, we employed a small redox molecule, TMB, as an electron shuttle that can diffuse in and out of the redox site of the macromolecule, thus coupling the catalytic reduction of H_2O_2 with the redox reaction of TMB at the

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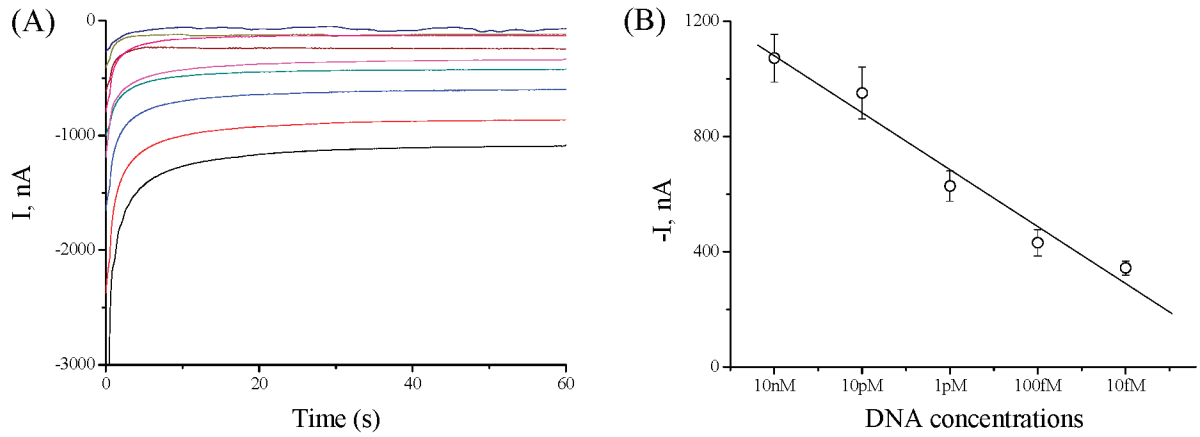

Figure 2. (A) Amperometric measurements for a range of targets. From top to bottom: background; 10 nM noncognate DNA; 1 nM 4-base mismatched DNA; 1 nM 1-base mismatched DNA; 10 fM, 100 fM, 1 pM, 10 pM, and 10 nM perfectly matched DNA. (B) Plot for concentration of perfectly matched DNA vs amperometric current. Error bars show the standard deviations of measurements taken from independent experiments with at least three distinct sensors.

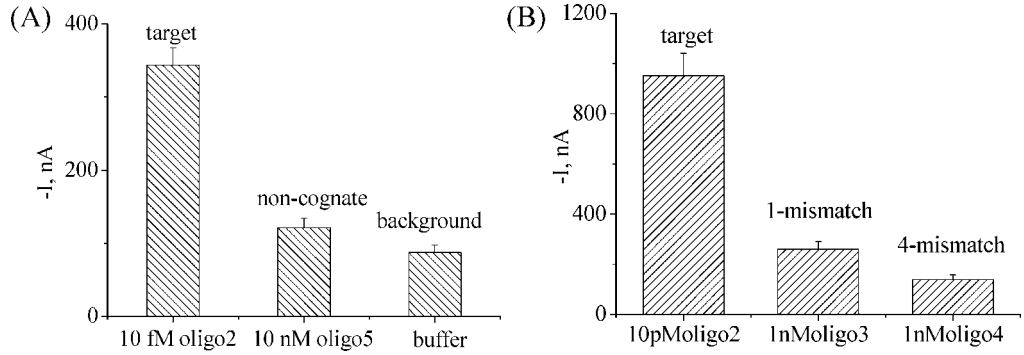

Figure 3. (A) Evaluation of the detection limit for the enzyme-based E-DNA sensor. The signal intensity for the 10 fM target DNA (oligo 2) is significantly higher than those for the background (in pure buffer) and the 10 nM noncognate DNA (oligo 5) (>3SD). (B) Selectivity for the enzyme-based E-DNA sensor. Comparison for the signal intensity for sensors hybridized with 10 pM perfectly matched target DNA (oligo 2), 1 nM 1-base mismatched DNA (oligo3), 1 nM 4-base mismatched DNA (oligo4). Error bars show the standard deviations of measurements taken from at least three independent experiments with at least three distinct sensors.

electrode surface.[31,32] Also of note, TMB is a hydrophobic molecule; thus, it can penetrate the SAM and facilely exchange electrons with the underlying electrode, which is in direct contrast with the hydrophilic ferricyanide probe (Figure 2-S of Supporting Information). Amperometric technique provides a rapid and direct measure of the current associated with the HRP-catalyzed electrochemical process. We held the potential at the catalytic reduction potential for H_2O_2 (−100 mV vs the Au reference electrode). A decay curve for current (I) vs time (t) was observed instantly after the onset of the potential, which rapidly reached a plateau (steady-state current) within ∼60 s (Figure 2A).

Indeed, in the "close" state we only found a relatively small and stable background amperometric current (∼100 nA, Figure 2A). Importantly, as we challenged the sensor with 10 nM target DNA, which was expected to open the stem−loop, a large amperometric signal (>1000 nA) was observed, with a signal gain of more than an order of magnitude. This high signal gain is an inherent advantage of signal-on sensors as compared to signal-off ones. It is worthwhile to point out that our enzyme-based E-DNA sensor achieved even higher gain than the previously reported strand displacement-based signal-on sensor (∼7 fold).[20]

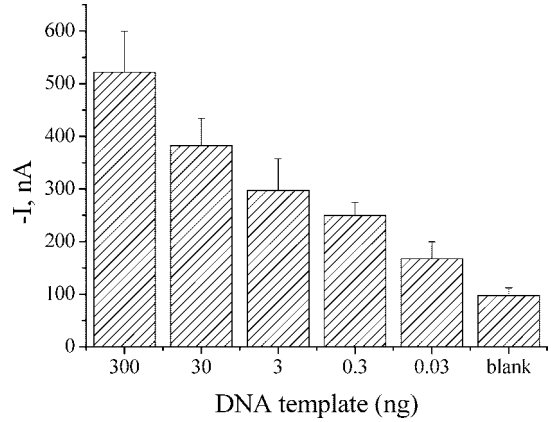

Figure 4. Concentration profile for the detection of *E. coli* genomic DNA by using the enzyme-based E-DNA sensor coupled with an asymmetric PCR protocol. The DNA template was the genomic DNA isolated from *E. coli* and serially diluted to the required amount.

We found that the amperometric signal was logarithmically related to the target concentration across the range of 10 fM to

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Table 2. Comparison of Electronic DNA Detection Schemes

methods	transduer	detection limit	SNP differentiation	sensor mode
enzyme-based E-DNA sensor (this work)	electrochemical	10 fM	good	turn-on
E-DNA: ferrocene-tagged DNA stem−loop probe[5]	electrochemical	10 pM	not reported	turn-off
E-DNA: artificial DNA−PEG−DNA triblock probe[38]	electrochemical	200 pM	not reported	turn-on
displacement-basedE-DNA sensor[20]	electrochemical	400 fM.	not reported	turn-on
E-DNA: MB-tagged DNApseudoknot probe[24]	electrochemical	2 nM	good	turn-on
nanocompartment-based detection[39]	electrochemical	not reported	good	turn-on
ligase-mediatedE-DNA sensor[40]	electrochemical	1 pM	good	turn-on
stem−loop probes withenzymatic amplification[25]	colorimetric	60 pM	good	turn-off
stem−loop-based opticaldetection with electricalpotential control[41]	fluorescent	1 nM	good	turn-on
surface-immobilizedmolecular beacons[42,43]	fluorescent	10 nM	good	turn-on

10 pM (Figure 2B), spanning a response region of at least 4 orders of magnitude. The detection limit was experimentally found to be smaller than 10 fM (>3 SD, Figure 3A). This ultrahigh sensitivity reflected the high signal amplification of the enzyme and the improved signal gain as a signal-on sensor.

We selected the detection of PCR amplicons from the uidA gene of *E. coli* to test the real applicability of our new E-DNA sensor. By using an asymmetric PCR protocol, we obtained ssDNA targets of 250 bp that could be directly sensed by the stem−loop probe. Of note, while the asymmetric PCR is usually much less efficient in amplification than normal PCR due to the fact that the depletion of one primer will lead to a linear, nonexponential, amplification region, it generates DNA target in the single-stranded format that avoids the denaturing step during subsequent probe−target hybridization.[19,27] Interestingly, our sensor performed equally well despite the fact that this target is much longer than the model system as described above. We found that, by coupling asymmetric PCR, this sensor could selectively identify as few as 30 picograms *E. coli* genomic DNA (Figure 4). Importantly, this enzyme-based E-DNA sensor was 3 orders of magnitude more sensitive than normal electrophoretic analysis of PCR products (30 ng), which suggested that the E-DNA sensing was a promising method for the rapid detection of *E. coli* (Figure 3-S in the Supporting Information).

In order to evaluate the selectivity of this novel E-DNA sensor, we challenged the sensor with a million-fold excess of noncognate oligo (10 nM). This oligo produced signals that were statistically insignificant from those of the background (Figure 3A), suggesting that this sensor was highly selective. Solution-phase molecular beacons have been well documented to exhibit high differentiation ability toward single-nucleotide polymorphisms (SNPs).[21] This high specificity arises from the conformational constraints of the stem−loop structure; that is, the presence of the stem makes it thermodynamically unfavorable for the binding of mismatched sequence to the loop.[23] Nevertheless, in contrast to its solution-phase counterpart, immobilized stem−loop structures often show limited ability for the discrimination of single mismatches, which possibly arises from the surface-induced destabilization effect on the stem duplex.[5,19] In this work, we interrogated the sequence specificity of the sensor by using a one-base mismatched oligonucleotide (1 nM). Interestingly, we found that the signal was only 200 nA, approximately 2 times that of the background (Figure 3B). That is, the signal for the fully complementary target was at least 5 times larger than that for the SNP, suggesting that the enzyme-based E-DNA sensor has high sequence specificity toward even a single-base mismatch. We also employed a four-base mismatched oligonucleotide and found that the amperometric signal was not significantly different from the background (Figure 3B). We propose that this increased sequence specificity is attributed to the sensor configuration employed in this work. Most previously reported E-DNA sensors employed thiolated oligonucleotides with a relatively short six-carbon spacer (~1 nm) in order to realize direct, short-range transfer of electrons.[5,8,20] In contrast, the present enzyme-based signal transduction exploited a different mechanism that did not rely on direct electron transfer. As a result, the stem−loop probe stayed far away from the electrode surface (>10 nm), with underlayers including the 11-carbon MUA, streptavidin, and 6-carbon spacer (for the biotin label). This large separation reduced the surface-induced effect to a significant extent, thus rendering the stem−loop being in an environment resembling that of its solution-phase counterpart.

Our enzyme-based E-DNA sensor represents a new progress in the family of E-DNA sensors. It is a signal-on sensor with femtomolar sensitivity that excels all previously reported E-DNA sensors by several orders of magnitude (Table 2). We also note that this sensor is comparable to the state-of-the art electrochemical DNA sensors in terms of sensitivity and selectivity.[18,33]

It is worthwhile to point out that, while the femtomolar sensitivity is impressive and applicable to genetic analysis and pathogen detection in many cases, further improvement of the sensitivity is still desirable to meet high-end requirements. This may be achieved by several straightforward approaches. For example, more sophisticated passivation of the surface may lead to reduced background that increases the signal-to-noise raito;[34,35] coupling of the stem−loop with inorganic nanomaterials may lead to larger amplification than enzymes.[36,37] Another obvious advantage is the high selectivity and specificity of this sensor. We can detect the complementary target against a million-fold excess of noncognate DNA. This E-DNA sensor is also resistant to the false-positives due to its signal-on nature. More importantly, it is the first E-DNA sensor that possesses excellent SNP discrimination ability, which benefits from the use of the stem−loop probe and the employed multilayer supramolecular structure anchored at gold surfaces.

In summary, we report a novel enzyme-based E-DNA sensor that exhibits high sensitivity and selectivity. The use of electrochemistry offers the opportunity to design an integrated, portable, and low-cost device for DNA detection based on this

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proof-of-concept sensor. Simultaneous detection of many DNA targets is also feasible by using the microfabricated sensor array described in this work. These features thus make this E-DNA sensor a promising alternative to conventional fluorescent DNA detection methods.

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Supporting Information Available: Additional figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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