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An electrochemical DNA sensor for sequence-specific DNA recognization in a homogeneous solution

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ABSTRACT

In this work, a sensitive electrochemical DNA sensor based on an avidin modified electrode and a DNA-functionalized Au nanoparticle (DFNP) was developed. The DNA-functionalized Au nanoparticle contained two kinds of DNA, one is hairpin probe DNA with a biotin at the 3′ terminal and a thiol at the 5′ terminal, the other is methylene blue (MB)-labeled linear signal DNA. Without hybridizing with the target DNA, the loop of the hairpin impeded biotin linked with avidin on electrode. However, after target hybridization, the hairpin was opened and biotin was recognized by avidin which resulted in a DNA-functionalized Au nanoparticle brought on the electrode surface. Electrochemical signals of MB bound to signal DNA were measured by differential pulse voltammetry (DPV). Taking advantage of amplification effects of the AuNP and binding specificity of the hairpin probe, this DNA biosensor greatly simplified the electrochemical DNA detection method and displayed high specificity in DNA detection. By using this new method, we demonstrate that this prototype sensor has been able to detect as low as picomolar DNA targets with excellent differentiation ability even for a single mismatch.

1. Introduction

Nucleic acids are one of the most fundamental molecules for all life forms, and as a result the specific nucleic acid sequence quantification is essential in biological and biomedical studies, such as medical diagnostics, gene expression analysis, and the detection of infectious diseases. Until now, many approaches have been successfully developed for the sequence-selective DNA hybridization examination, including fluorescence, electrochemical and colorimetric etc. methods (Huang et al., 2012; Liu et al., 2013; Shen et al., 2012; Yang et al., 2013).

The electrochemical DNA sensing technology has received particular attention mostly due to its high sensitivity, selectivity and easy operation with simple instrumentations (Wang et al., 2001a, 2001b; Cai et al., 2003a, 2003b). The DNA recognition event can be detected by using different strategies, including intrinsic electroactivity of the nucleic acid (Jelen et al., 2002; Wang et al., 2001a, 2001b), enzyme labels (Lumley et al., 1996; Alfonta et al., 2001), DNA duplex intercalators (Kara et al., 2002; Erdem et al., 2001), electroactive markers (Wang et al., 2003a, 2003b; Ihara et al., 1997), and metal nanoparticles/quantum dots (Cai et al., 2003a, 2003b; Jacob Hansen et al., 2006; Wang et al., 2003a, 2003b; Liu et al., 2005; Park et al., 2002). In recent years, the electrochemical DNA(E-DNA) sensor due to its reagentless, simple operation and regeneration have attracted much attention (Fan et al., 2003; Zhang et al., 2007; Xiao et al., 2007; Immoos et al., 2004). E-DNA sensors are comprised of an oligonucleotide probe modified on one terminus with a redox reporter and attached to an electrode at the other. Hybridization of this probe DNA to a target oligonucleotide influences the rate at which the redox reporter collides with the electrode, leading to a detectable change in redox current. This design is in fact an electrochemical analog of fluorescent “molecular beacons”, i.e., stem-loop (hairpin) probes with internally quenched fluorescence (Tyagi and Kramer, 1996; Tyagi et al., 1998). Broadly, the reagentless, single-step E-DNA sensors could be classified into “signal-on” and “signal-off” two types. In the “signal-off” sensors, target hybridization sequesters the redox moiety from the electrode, reducing the observed Faradaic current. However,“signal-off” sensors suffer from false positives when either the probe DNA or the redox tag become degraded and are limited in their signal gain (Xiao et al., 2007). In contrast, in the signal-on sensors, target hybridization enhances the rate which the redox moiety approaches the electrode, avoids false positives and leads to a current increase. However, signal-on E-DNA sensor usually bears low sensitivity (Xiao et al., 2007; Immoos et al., 2004).

For <!–td:hybridization-basCompared with the sensors with hybridization happens in homogeneous solution, hybridization-based DNA electrochemical sensors causes lower available hybridization efficiency, becausehybridization-based DNA electrochemical sensors need probe

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DNA pre-immobilization on the electrode surface. And then we are faced with the difficulty for real-time electrochemical monitoring PCR and recognizing specific sequence DNA in living cells, and the fluorescent molecular beacons have been widely used forreal-time monitoring DNA in PCR and in cells with hybridization in homogeneous solution (Ram et al., 2008; Santangelo et al., 2004; Nitin et al., 2004). Therefore, developing the electrochemical DNA sensing technology directly in homogeneous solution is still a challenge.

We herein reported a non-immobilizing E-DNA sensor with hybridization which occurred in a homogeneous solution that employed DNA-functionalized Au nanoparticle (DFNP) and avidin modified electrode. In the present study, we have developed a DNA-functionalized Au nanoparticle, integrated DNA recognition; signal amplification and specific biotin–avdin link functional section. They are used as probe for DNA detection and featured high sensitivity up to low picomolar.

In the present study, we have developed a DNA-functionalized Au nanoparticle, integrated DNA recognition; signal amplification and a specific biotin–avdin link functional section, as a probe for DNA detection and featured high sensitivity up to low pmtomolar. In target DNA detection, as shown Fig. 1, when the probe DNA of DFNP is under stem-loop state, a comparatively huge bulk of Au nanoparticle and the loop of probe DNA would prevent biotin on the probe DNA being captured by the avidin on the electrode for the spatial effects. After the DFNP solution is incubated in a buffer solution containing target DNA, and then target DNA would combine probe DNA by forming double strand with complementary sequences, which caused hairpin open and eliminate steric hindrance to biotin. Afterwards, when avidin modified electrode was immersed in a solution after the hybridization event, the biotin of the DFNP could have attached to avidin on the electrode and thus was captured on the electrode. The electrochemical signals of MB of signal DNA were measured by differential pulse voltammetry (DPV). Taking advantage of amplification effects of the Au nanoparticle (AuNP) and binding specificity of the hairpin probe, this biosensor greatly simplifies the electrochemical detection method of DNA and displays high specificity.

2. Experimental section

2.1. Materials

All the probes and target oligonucleotides were purchased from the Takara Biotechnology Company. The oligonucleotides were purified via the C18 reversed-phase HPLC and polyacrylamide gel electrophoresis (PAGE) and their sequences are shown in Table 1. The stem-loop probe oligonucleotide (oligo 1) has a 5′-HS label and a 3′-biotin, which will form the stem at appropriate ionic strength. The MB-labeled signal DNA (oligo 2). The sequence of the target (oligo 3) is perfectly matched to the loop sequence of the probe; oligo 4 contains a one-base mismatch, while oligo 5 has three-base mismatches. Oligo 6 is a random sequence unrelated to the probe sequence.

Unless otherwise noted, all chemicals were purchased from Dingguo Biotechnology Inc. (Shanghai, China) and were of analytical reagent grade. The biotin and avidin were purchased from Sangon Biotechology Inc. (Shanghai, China). All of the solutions were prepared with ultrapure water from a Millipore Milli-Q system.

2.2. Apparatus

All voltammetric experiments were performed using a CHI 660 electrochemical analyzer (CHI Instrument Inc, USA). Electrochemical experiments were carried out in a 3 ml electrochemical cell at room temperature (25 °C) by using three electrode configurations. A platinum wire served as a counter electrode and an Ag/AgCl as reference electrode with saturated KCl solution.

2.3. Preparation of Au nanoparticles

Au nanoparticles were prepared by citrate reduction of $HAuCl_4$ according to the literature (Doron et al., 1995). In brief, 100 mL of 0.01 g $HAuCl_4$ solution was brought to reflux with stirring, and 2.5 ml of 1% sodium citrate solution was introduced into this $HAuCl_4$ solution. The mixture solution was kept boiling for another

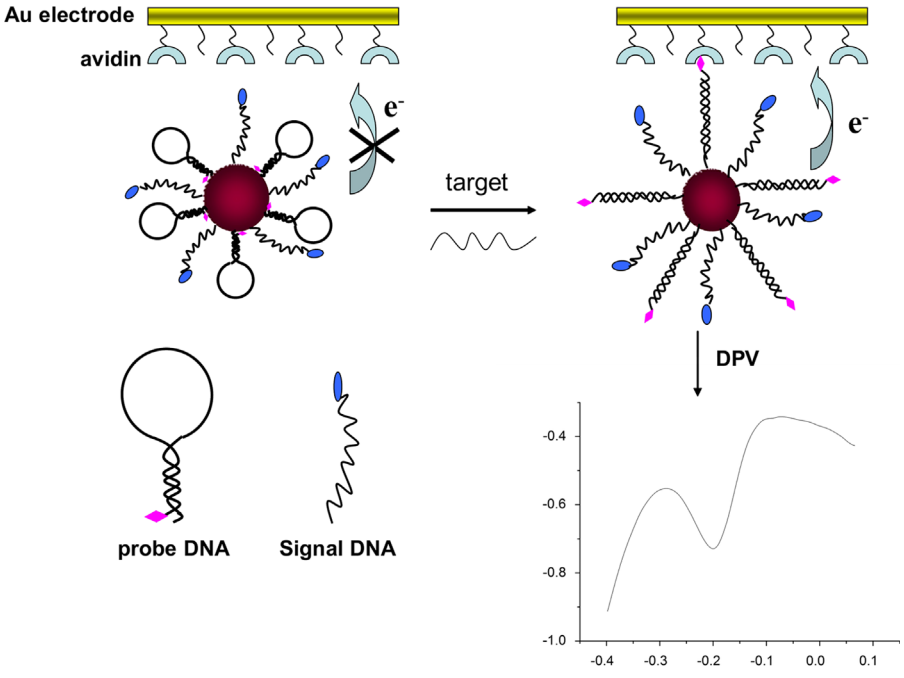

Fig. 1. Scheme for the DNA-functionalized Au nanoparticle-based E-DNA sensor.

Table 1
Oligonucleotides employed in this work.

oligo 1 (stem-loop probe)	5′-SH-CCACGCTTGTGGGTCAA-CCCCCGTGG-Biotin-3′
oligo 2 (linear signal DNA)	5′-SH-GGTGGAGGGACGAGG-MB-3′
oligo 3 (target)	5′-GGGGTTGAC CCACAAG-3′
oligo 4 (one base-mismatched DNA)	5′-GGGGTCGACCCACAAG-3′
oligo 5 (three base-mismatched DNA)	5′-GGGGTCTTCCCACAAG-3′
oligo 6 (non-complementary DNA)	5′-TTCGGCTCTATCAATC-3′

30 min and left to cool to room temperature. The diameter of such-prepared Au nanoparticle was determined ca. 16 ± 3 nm by using atomic force microscopy scanning with line scan mode and taking the tip convolution effects into account (The AFM image is in the Supporting Information Fig. S1). The obtained Au nanoparticles were then stored in a brown glass bottle at 4 °C for further use.

2.4. Preparation of the DNA-functionalized Au nanoparticles

The process of probe DNA and signal DNA labeling was performed according to paper (Taton et al., 2000) as follows: The mixture of 5.6 nM probe DNA and 2.8 nM signal DNA was activated with acetate buffer (pH 5.2) and 1.5 μL of 10 mM Tris(2-Carboxyethyl)Phosphine Hydrochloride(TCEP) for 1 h, then added to 1 mL of freshly prepared Au nanoparticles, and shaken gently overnight. Over the course of 16 h, the DNA-AuNP conjugates were aged in salts (0.1 M NaCl, 10 mM acetate buffer) for another 24 h. Excess reagents were removed by centrifuging at 16,000 rpm for 30 min. The red precipitate was washed and centrifuged repeatly for three times. The resulting nanoparticles were dispersed into a buffer solution (0.1 M, containing 0.3 M NaCl and 3 mM Mg^{2+}, pH 8.1) and stored at 4 °C.

According to the method in previous reports (Zhang et al., 2008; Demers et al., 2000), it was found that there were 94 single strands per AuNP, consisting of 21 probe DNA and 73 signal DNA on each AuNP (see Supporting information (SI) for details).

2.5. Preparation of avidin-coated electrode surfaces

The gold electrode was exposed to an ethanolic 5 mM solution of 3,3′-dithiodipropionic acid for 30 min, followed by water rinsing. 5 μl of 100 mg mL^{-1} EDC solution was then placed on the surface, followed immediately by 5 μL of 100 mg mL^{-1} NHS solution. These solutions were allowed to interact with the electrode with 3,3′-dithiodipropionic acid modification for 30 min in a 100% humid environment to prevent solution evaporation. The surface was then rinsed with water and immersed in an aqueous 0.2 mg mL^{-1} avidin solution for at least 120 min, after which the surface was rinsed again. The electrode was then exposed to a 1 mM 2-aminoethanol solution (pH 8.0, adjusted using HCl) for 60 min, rinsed, and used in hybridization detection.

2.6. DNA sequence sensing performance

The DNA assay procedure was initiated in 10 mM phosphate buffer (PBS) containing 3.0 mM Mg^{2+} by adding 10 μL of 0.43 μM DFNP to target DNA and stirring the mixture for 30 min at 37 °C. During the process, the target DNA hybridized with probe DNA, and then one avidin-modified electrode was immersed in the solution for capturing DFNP-target DNA hybridization. After 40 min, the avidin-modified electrode was washed with a 0.1% sodium dodecyl sulfate (SDS) phosphate buffer (pH 7.3) to remove the non-specific adsorption.

The electrochemical investigation was carried out in a 3 ml electrochemical cell with the resulted gold working electrode, an Ag/AgCl reference electrode and the platinum wire counter electrode by DPV. The electrochemical signal of the MB was obtained in the 10 mM PBS buffer (pH 8.0) at about −0.20 V (versus Ag/AgCl).

2.7. Optimization of testing conditions

Many experimental parameters, such as hybridization temperature, hybridization time, and Mg^{2+} ion strength, capture time, can affect the detection sensitivity of the assay. A series of optimizations of these parameters were carried out to improve the sensitivity and selectivity of the DNA assay. Supporting information Fig. S4 shows the effect of the capture time on the signal of DNA detection.

In this study, a 1.8×10^{-11} M complementary DNA target was incubated with DFNP for 30 min at a series of temperatures ranging from 25 to 45 °C. As shown in Supporting information Fig. S5, the highest signal is obtained at 37 °C. Therefore, a hybridization temperature of 37 °C was selected in the following experiments. The effect of the hybridization time has also been investigated. The target DNA was incubated with DFNP at 37 °C for different times from 10 to 50 min. The signal responses of the assay to the different hybridization times are shown in Supporting information Fig. S6. It can be seen from this figure that the signal increases rapidly as the hybridization time increases from 10 to 30 min and then increases slightly from 30 to 50 min, indicating that the hybridization reaches equilibrium at 30 min. As a result, a reaction time of 30 min was selected for the DNA hybridization.

The cation Mg^{2+} plays an important role in DNA hybridization. For a hairpin DNA probe-based DNA assay, there is hybridization competition between intramolecular stem part hybridization and intermolecular target DNA-probe hybridization. Therefore, Mg^{2+} concentration in the hybridization buffer should be balanced. Although a high concentration of Mg^{2+} can enhance target DNA hybridized to the loop of the structure, it may also strengthen the binding of the stem part of the probe, which may adversely affect the performance of the assay. Supporting information Fig. S7 shows the effect of Mg^{2+} concentration in the hybridization buffer on the response signal of the assay. As shown in this figure, the assay produces the highest signal when the Mg^{2+} concentration is 3 mM. Accordingly, a 3 mM Mg^{2+} concentration was used throughout the experiment to obtain the highest signal.

3. Results and discussion

We employed a stem-loop DNA probe dually labeled with HS and biotin at the 5′- and the 3′- end, respectively, which could be facilely immobilized at Au nanoparticle surfaces via the Au–S bridge and hybridized with target DNA. The MB-labelded signal DNA with HS at 5′ end could provide the electrochemical signal. Two kinds of DNA have been immobilized at Au nanoparticle to construct DFNP.

The detection strategy is demonstrated in Fig. 1. Before the hybridization, the DFNP remained in the stem-loop structure, which forced the biotin to close to the Au nanoparticle. Due to the steric effect of the Au nanoparticle, the biotin was prevented from conjugating with the avidin on the electrode and resulting in that the DFNP could not be captured by the electrode. After being hybridized with the target DNA, the DFNP′s loop-stem structure opened and then the biotin molecule was easily bound to avidin modified electrode, resulting in the DFNP′s being captured by the electrode. The capture efficiency was proportionate with the concentration of the target DNA.

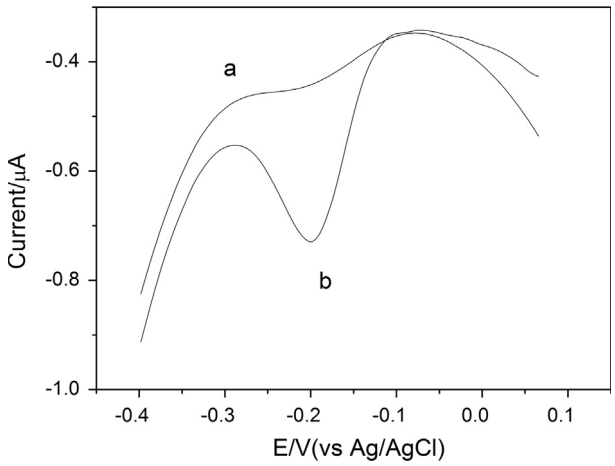

Fig. 2. The DPV response of MB when the DFNP was incubated in phosphate buffer (a) and target DNA (b).

The target hybridization event can be sensitively transduced via detecting the electrochemical reduction current signal of MB at the DFNP. Of note, a single DFNP is loaded with about 70 signal DNA strands; this offers a significant amplification for DNA detection.

Indeed, in the stem-loop state we only found a relatively small and stable background current (Fig. 2 curve a). As we challenged the sensor with 1.2×10^{-11} M target DNA, which was expected to open the stem-loop, a large signal (Fig. 2 curve b) was observed. We found that as the concentration of the complementary target DNA sequence continuously increased (Fig. 3A), which was logarithmically related to the target protein concentration from 2.3×10^{-12} to 2.3×10^{-9} M (Fig. 3B). The equation for the resulting calibration plot was calculated as $y = 0.199 \log x + 0.143$ (x is the concentration of target DNA divided by pM, y is the DPV peak current value) with correlation coefficient of 0.9953 and detection limit of 1.7×10^{-12} M (> 3 SD). This sensitivity reflected the high signal amplification of this method and the improved signal gain as a signal-on sensor used MB as indicators.

In order to confirm the binding specificity of the DFNP to the target DNA, control experiments were performed by using different DNA sequences to hybridize with 0.43 μM DFNP, including complementary DNA, one base-mismatched DNA, three base-mismatched DNA and non-complementary DNA. As shown in Fig. 4 the non-complementary DNA produced a neglectable DPV signal (signal (d)), which was similar to the background signal (signal (e)), and when compared to the complementary sequence (signal (a)), the three base-mismatched DNA produced a very low insignificant DPV signal (signal (c)), and the one base-mismatched DNA generated a one third signal (signal (b)). We propose that this sequence specificity of the DFNP is mainly due to the intrinsic recognition ability of DNA bases, and also to the reason that even if the probe is hybridized with mismatched target, the probe DNA's hairpin structure is partially opened, an obvious steric effect still exists for such "hybridizer" to contact the electrode.

In order to evaluate the applicability of using this method in real-sample detection, we tested the sensor for their ability to detect DNA targets in the presence of biological fluids and PCR environment respectively.

As demonstrated in Fig. S10, while the signals obtained in sera (both diluted and undiluted) were slightly suppressed compared to those obtained in pure buffer, we could still easily differentiate the target from a large excess of noncognate DNA. This clearly showed that our DFNP could selectively identify DNA targets even in complexes such as undiluted serum. Compared with the signal obtained from the hybridization event in buffer, the signal obtained from the hybridization event in undiluted serum is reduced by one sixth. We think that the signal decrease is due to the protein adsorption on surface of DFNP which leads to reducing efficiency of hybridization in serum.

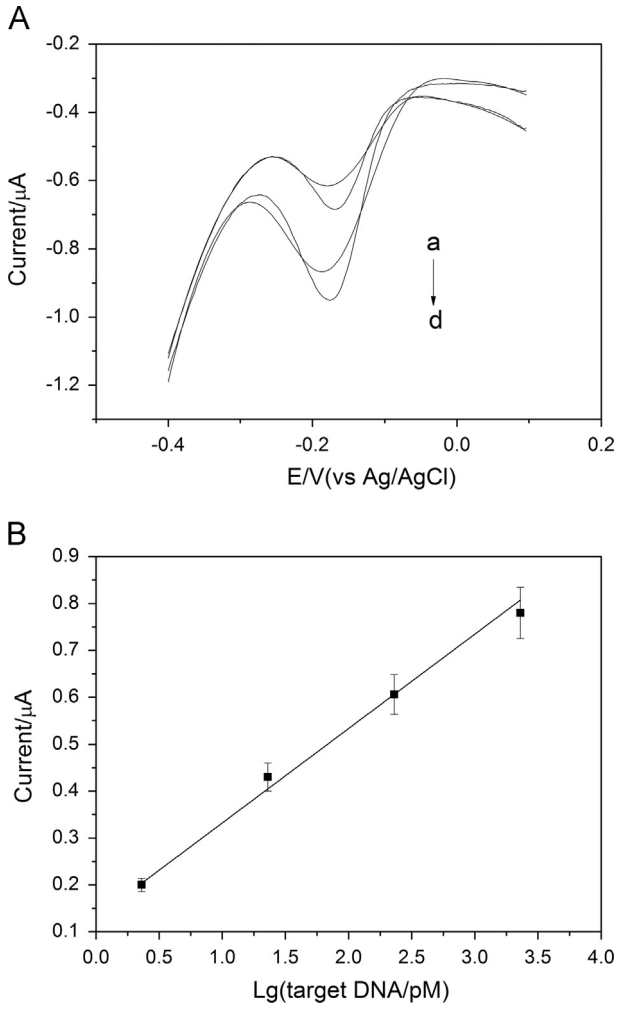

Fig. 3. (A) DPV response of DNA-functionalized Au nanoparticle-based DNA sensor in the solution containing 2.3×10^{-12} M target DNA (a), 2.3×10^{-11} M target DNA (b), 2.3×10^{-10} M target DNA (c), and 2.3×10^{-9} M target DNA (d). (B) The calibration plots of target DNA (2.3×10^{-12}–2.3×10^{-9} M).

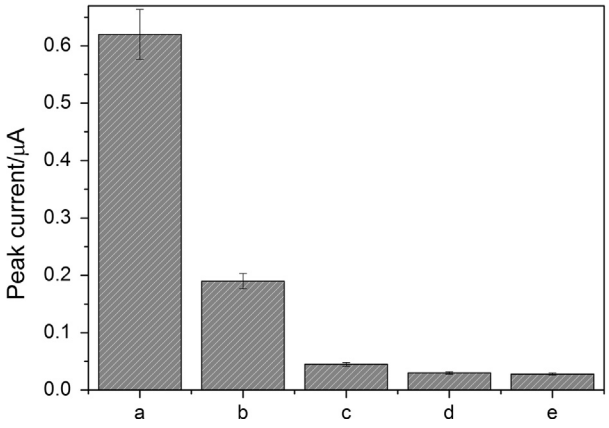

Fig. 4. The comparison of the DPV signal of MB when the DFNP is hybridized with 2.8×10^{-10} M target DNA (a), 2.8×10^{-8} M one base-mismatched DNA (b), 2.8×10^{-8} M three base-mismatched DNA (c), 2.8×10^{-8} M non-complementary DNA (d) and only the hybridization buffer containing no DNA sequences (e).

We selected the detection of PCR amplicons from the lycopene-β-cyclase (LYC) gene to test the real applicability of our new E-DNA sensor. By using PCR protocol, we obtained DNA targets of 104 bp that could be directly detected by the sensor. We found that this sensor could selectively identify as few as 4.1×10^{-11} M LYC DNA in PCR environment, which suggested that the E-DNA sensor was a promising method for the rapid detection of PCR product (see Supporting information (SI) for details).

This DNA-functionalized Au nanoparticle-based E-DNA sensor demonstrated several advantages. First, the DFNP integrated both DNA detection probe and signal carrier at the surface of AuNPs, thus obviating the use of additional capture DNA modification involved in conventional bioassays. Second, stem-loop probes inherently possessed high specificity due to their conformational constraints, and thus were superior to linear ones for DNA detection. Morever, comparing to pervious "signal-on" E-DNA sensor (Cheng et al., 2010; Tennico et al., 2010; Cho et al., 2008) that utilizes the oligonucleotide probe covalently linked with a redox label and one hybridization event could bring only one signal reporter. The present sensor has a lower detection limit due to the amplification effect of AuNP,which has a large surface, with which one hybridization event could bring multiple signal molecules, leading to larger signal amplification.

4. Conclusion

In summary, we completed a non-immobilizing E-DNA sensing strategy, which allowed the hybridization between DNA probe and target DNA which occured in a homogeneous solution. This assay protocol is simple, convenient and cost-effective and by using this novel method, we could conveniently detect as few as 1.7×10^{-12} M target DNA. We propose that it might be a promising approach to perform DNA-based diagnostics where resources are limited, such as small clinics in developing countries or field detection.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.12.027.

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