A Simple, Rapid, and Highly Sensitive Electrochemical DNA Sensor for the Detection of α - and β -Thalassemia in China

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Background: Because of the life-consuming treatment and severe consequences associated with thalassemia, it is more effective to prevent than cure thalassemia. Rapid and sensitive detection is critical for controlling thalassemia. In this study, we developed a rapid and accurate test to genotype nondeletional α - and β -thalassemia mutations by an electrochemical DNA sensor. **Methods:** Screen-printed electrodes were used as electrochemical transducers for the sensor, in which the capture probe DNA was attached to the golden surface of the working electrode via an S–Au covalent bond, which is highly suitable for immobilizing the biological element. In addition, two types of ferrocene with varying redox potentials for modified signal probe DNA were adopted. The hybridization signal is detected by alternating current voltammetry when the capture probe and signal probe hybridize with the target DNA. **Results:** With this technique, 12 types of nondeletional

α - and β -thalassemia mutations were detected, which constitute more than 90% of all the nondeletional types of thalassemia mutation determinants found in China, including the CD142 (TAA>CAA) Constand spring, CD125 (CTG>CCG) Quonsze, CD122 (CAC>CAG) Weastmead, –28 (A>G), Cap+1 (A>C), initiation codon (ATG>AGG), CD17 (AAG>TAG), CD26 (GAG>AAG), CD31(-C), CD41-42 (-CTTT), CD71-72 (+A), and IVS-II-654 (C>T) mutations. Concordance levels were 100% within the 20 blood samples of homozygous wild-type individuals and 238 blood samples of heterozygous mutant individuals. **Conclusions:** The electrochemical DNA sensor developed here can be applied for rapid genotyping of thalassemia or other clinical genotyping applications and is useful for early screening of thalassemia in high-risk groups by minimizing the time and investment cost. J. Clin. Lab. Anal. 30:719–726, 2016. © 2016 Wiley Periodicals, Inc.

Key words: thalassemia; DNA sensor; electrochemical sensor; rapid genotyping

INTRODUCTION

Thalassemias are currently the world's most common, autosomal recessive blood disorders (1). People with thalassemia cannot make enough hemoglobin. This is highly problematic because when there is a lack of hemoglobin, hypoxia will occur in parts of the body. The organs and tissue in the body are unable to receive enough oxygen from the blood and cannot maintain healthy body functions.

α - and β -thalassemia are the two major types of thalassemia. Moreover, the α and β gene clusters that make up normal hemoglobin are located on chromosome 16 and chromosome 11, respectively (2). α -Thalassemia is mainly caused by the deletion or mutation of the α -globin gene (*HBA*). Deletional α -thalassemia is mainly caused by the deletion of one ($-\alpha^{3.7}$, $-\alpha^{4.2}$; termed α -thalassemia-

2) or two ($-\alpha^{SEA}$, $-\alpha^{THAI}$; termed α -thalassemia-1) of the two functional α -globin genes. Nondeletional α -thalassemia results from a mutation of the α -globin gene, which can be induced by a point mutation of the *HBA* gene (CD142 [TAA>CAA] Constand spring mutation

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[CS], CD125 [CTG>CCG] Quonsze mutation [QS], and CD122 [CAC>CAG] Weastmead mutation [WS]) (3). Deletional α -thalassemia is much more prevalent than nondeletional α -thalassemia. However, nondeletional α -thalassemia could result in a more severe expression than deletional α -thalassemia. A previous study found that 27 of the 59 cases (45.8%) of hemoglobin H diseases were diagnosed as nondeletional thalassemia in Guangxi, China (4). A second type of thalassemia is β -thalassemia, which is primarily caused by the occurrence of point mutations in the β -globin gene (*HBB*) and is a subset of the β -hemoglobinopathies that are characterized by hereditary anemia with a wide phenotypic spectrum and can have significant morbidity and mortality (5). To date, over 34 *HBB* gene mutations have been identified in the Chinese population; however, only four mutations (CD41-42 [-CTTT], IVS-II-654 [C>T], CD17 [AAG>TAG], and -28 [A>G]) account for approximately 90% of cases (6).

There are several means of combatting thalassemias, but each method has its limitations. For example, to maintain a sufficient supply of hemoglobin for normal life, regular blood transfusions and adequate chelation treatment are required for conventional thalassemia treatments (7). Furthermore, bone marrow transplant could be used to cure thalassemias, but a perfect matched is required and the treatments are expensive (8). Limited treatment options combined with a high occurrence of the disease (southern China reports high frequencies of thalassemia) have caused thalassemia to become a major problem of public hygiene and a social economic burden in southern China (9).

It is more effective to prevent than cure hemoglobin disorders such as thalassemia. More specifically, genetic screening is essential to identify thalassemia carriers to reduce the birth rate of patients with severe thalassemia (10). The classic methods of detection include dot-blot and reverse dot-blot with allele-specific oligonucleotide probes, the amplification refractory mutation system, the multiplex amplification refractory mutation system, and direct DNA sequencing (11). However, with such time-consuming procedures, tedious operations, and expensive instruments, the above methods do not meet the clinical requirements for the fast and exact genetic screening of high-risk thalassemia groups.

Recently, electrochemical DNA sensors have received a great deal of interest owing to their unique advantages such as an innately high sensitivity, simple instrumentation, automation, and low cost from a number of different fields, including infectious disease detection, genetic diagnosis, environmental pollutant determination, food safety, epidemiological studies, forensic identification, and the detection and diagnosis of clinical pathogenic microorganisms (12). Moreover, while

fluorescent DNA probes have the drawbacks of expensive reagents and instrumentation, electrochemical labeling is currently booming as a result of its simplicity, cost effectiveness, and ease of miniaturization (13).

In this article, we developed an electrochemical DNA sensor capable of rapidly detecting thalassemias found in the Chinese population that are caused by *HBA* gene and *HBB* gene nondeletional mutations. Our results indicate that this method possesses great potential for application in screening nondeletional thalassemias.

EXPERIMENTAL

DNA capture probes and signaling probes were produced by Sangon Biotech (Shanghai, China). According to a previous report (14), two ferrocenes (FC), FC(I) and FC(II), were synthesized by the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (Guangzhou, China). The printed circuit board (PCB) of the sensor was produced by Shenzhen Fastprint Circuit Tech Co., Ltd. (Shenzhen, China). The DNA sensor detection device DA9100 was produced by DAAN Gene Co., Ltd. of Sun Yat-sen University (Guangzhou, China). Thiol-modifier C6 S-S was purchased from Glen Research Corp. (Sterling, VA, USA). The QIAmp DNA Blood Mini Kit was obtained from Qiagen, Inc. (Valencia, CA). dNTPs and 10 $\times$ buffer were purchased from Takara (Shiga, Japan). HotStarTaq polymerase was purchased from Qiagen GmbH (Hilden, Germany). Trizma base and betaine were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade. Capture probe immobilization buffer and signal probe hybridization buffer were proprietary.

Ethics Statement

This study was approved by the Institutional Review Board of Nanfang Hospital, Guangzhou, China. A total of 248 human whole blood samples (20 homozygous wild-type blood samples and 238 heterozygotes mutant samples, including WS [20 cases], QS [20 cases], CS [18 cases], -28 [20 cases], Cap+1 [20 cases], Int [20 cases], CD17 [20 cases], CD26 [20 cases], CD31 [20 cases], CD41-42 [20 cases], CD71-72 [20 cases], and IVS-II-654 [20 cases]) were kindly donated by patients in Nanfang Hospital.

Synthesis of DNA Capture Probes and Signaling Probes

Capture probes (Table 1) and signal probes (Table 2) were designed to hybridize *HBA* and *HBB* sequences within the distinctive region amplified by the *HBA* and *HBB* primer set. Capture probes were prepared by

TABLE 1. Sequences of Capture Probes

Capture probes	Sequence
WS-CP	5'-GCCGAGTTCACCCCTGCGG-Linker(2*C6 S-S)-3'
QS-CP	5'-Linker(2*C6 S-S)-AGTTCCTGGCTTCTGTG-AG-3'
CS-CP	5'-GTGAGCACCGTGCTGACCTCCA-Linker(2*C6 S-S)-3'
-28-CP	5'-GGAGGGCAGGAGCCAGGGC-Linker(2*C6 S-S)-3'
Cap+1-CP	5'-GGCAGAGCCATCTAT-Linker(2*C6 S-S)-3'
Int-CP	5'-CTGTGTTCACTAGCAACCTC-Linker(2*C6 S-S)-3'
CD17-CP	5'-CCTGAGGAGAAGTCTGCCGTTAC-Linker(2*C6 S-S)-3'
CD26-CP	5'-AACGTGGATGAAGTTG-Linker(2*C6 S-S)-3'
CD31-CP	5'-TGCCTATTGGTCTATTTTCCCACC-Linker(2*C6 S-S)-3'
CD41-42-CP	5'-CTACCCCTGGACCCAGA-Linker(2*C6 S-S)-3'
CD71-72-CP	5'-TGAAGGCTCATGGCAAGAAAGTGC-Linker(2*C6 S-S)-3'
IVS-II-654-CP	5'-TTCTAAAGAATAACAGTGATAATTTCTGG-Linker(2*C6 S-S)-3'

solid-state synthesis using C6 S-S as the linker at the 5' end or 3' end of the capture probes. Signal probes (Table 2) containing a ferrocene substitution on the ribose ring were tagged with six FCs at the 5' end or 3' end of the signal probes.

TABLE 2. Sequences of Signal Probes

Signaling probes	Sequence
WSW-SP	5'-6*FC(I)GCACGCCTCCCTGG-3'
WSM-SP	5'-6*FC(II)GTGCAGGCCTCCCTG-3'
QSW-SP	5'-CACGCCTCCCTGGACA-6*FC(I)-3'
QSM-SP	5'-CACGCCTCCCGGACA-6*FC(II)-3'
CSW-SP	5'-6*FC(I)ATACCGTTAAGCTGGAG-3'
CSM-SP	5'-6*FC(II)AATACCGTCAAGCTGGA-3'
-28W-SP	5'-6*FC(I)TGGGCATAAAAGTCAGG-3'
-28M-SP	5'-6*FC(II)TGGGCATAGAAGTCAGG-3'
Cap+1W-SP	5'-6*FC(I)TTGCTTACATTGCTT-3'
Cap+1M-SP	5'-6*FC(II)TTGCTTCCATTGCTTC-3'
IntW-SP	5'-6*FC(I)AGACACCATGGTGCA-3'
IntM-SP	5'-6*FC(II)AGACACCAGGGTGCA-3'
CD17W-SP	5'-6*FC(I)CCCTGTGGGGCAAGGTG-3'
CD17M-SP	5'-6*FC(II)CCCTGTGGGGCTAGGTGA-3'
CD26W-SP	5'-6*FC(I)GTGGTGAGGCCCTGG-3'
CD26M-SP	5'-6*FC(II)GTTGGTGTAAGGCCCTGG-3'
CD31W-SP	5'-6*FC(I)CTTAGGCTGCTGGTG-3'
CD31M-SP	5'-6*FC(II)CTTAGGTGCTGGTG-3'
CD41-42W-SP	5'-6*FC(I)GGTCTTTGAGTCCTTT-3'
CD41-42M-SP	5'-6*FC(II)GGTTGAGTCCTTTGGGA-3'
CD71-72W-SP	5'-6*FC(I)TCGGTGCCTTTAGTGAT-3'
CD71-72M-SP	5'-6*FC(II)TCGGTGCCTTTAAGTGAT-3'
IVS-II-654W-SP	5'-6*FC(I)GTAAAGGCAATAGCAAT-3'
IVS-II-654M-SP	5'-6*FC(II)GTAAAGGTAATAGCAAT-3'

TABLE 3. PCR Primer Sequences

Primer	Sequence
HBA-F (forward)	5'-ACTGCCTGCTGGTGACCCT-3'
HBA-R (reverse)	5'-GCTGCCCACTCAGACTTTATT-3'
HBB-1F (forward)	5'-CTGTCATCACTTAGACCTCACCCTG-3'
HBB-1R (reverse)	5'-GCCCAGTTTCTATTGGTCTCCTT-3'
HBB-2F (forward)	5'-GGGTTTCTGATAGGCACTGACTCTC-3'
HBB-2R (reverse)	5'-AGGTTGTCCAGGTGAGCCAG-3'
HBB-3F (forward)	5'-ACTTTCCTAATCTCTTTCTTTTCAG-3'
HBB-3R (reverse)	5'-ATGAAACCTCTTACATCAGTTACAA-3'

Immobilization of DNA Capture Probes

The electrochemical DNA sensors (45 × 35 × 1 mm diameter) with 48 exposed gold-modified electrodes as a working electrode, one reference electrode (Ag/AgCl), and one auxiliary electrode were produced and manufactured using the PCB technology. The diameter (1.0 mm) of the electrodes was defined by the solder mask, which covers the lead to the connector. Capture probes were deposited on the gold electrodes mixed with the constituent so as to form a self-assembled monolayer after the rapid damage-free plasma processing on the surface of the exposed gold electrodes. After deposition, the electrochemical DNA sensors were rinsed with sterile water, dried at 37°C and controlled humidity below 40%, and placed into a plastic casing with a vessel for adding the mixed sample solution for hybridization.

Multiplex Asymmetric Polymerase Chain Reaction (PCR) Procedures

DNA samples were taken from homozygous wild-type blood samples and heterozygous mutant blood samples diagnosed by DNA sequencing with α - or β -thalassemia mutations. The multiplex asymmetric PCR was performed in a 25 μ l volume consisting of 2.5 μ l 10× buffer; 0.05 μ M primers HBA-F, HBB-1F, HBB-2F, and HBB-3F; 0.6 μ M primers HBA-R, HBB-1R, HBB-2R, and HBB-3R; 200 μ M dNTPs; 4.0 μ l 5 M betaine; 1.0 U HotStarTaq polymerase; and 2 μ l DNA template. The reaction was carried out as follows: initial denaturation at 94°C for 15 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec; and 10 min at 72°C for the final extension. Four pairs of primers were designed (Table 3) for the multiplex asymmetric PCR that produced one fragment of the *HBA* gene and three fragments of the *HBB* gene, and all 12 mutations of interest were flanked (Fig. 1).

Hybridization and Electrochemical Analysis

After the PCR amplification, the single-stranded target DNA acquired by the multiplex asymmetric PCR as

Fig. 1. The region amplified by primers HBA-F and HBA-R contain three loci of interest. HBB-1F and HBB-1R contain five loci of interest. HBB-2F and HBB-2R contain three loci of interest. HBB-3F and HBB-3R contain one locus of interest.

a mixed solution with signal probes was then added to the cartridge of the electrochemical DNA sensor. The electrochemical DNA sensor was then inserted into the DNA sensor detection device (DA9100) for hybridization at 42°C for 45 min. The electrochemical DNA sensor was scanned in the DA9100 by the alternating current voltammetry (ACV) technique from -50 to $+550$ mV to detect and interpret the electrochemical signal generated during hybridization. In addition, the signal probes were labeled with FCs with different redox potentials. When all of the constituents were hybridized, the single-stranded target DNA hybridized to the signal probes and capture probes coinstantaneously. A faradaic current was detected when the interfacial electron was transferred from the FC to the gold electrode, and the resultant current was detected by ACV. By using two different FC-modified signaling probes with different redox potentials, different ACVs

were obtained. No electrochemical signal will be generated if the signal probes do not match the target DNA.

RESULTS AND DISCUSSION

In the electrochemical DNA sensor, the gold electrode is functionalized with the DNA probe sequence, the oligophenylmethyl molecular wires, and the polyethylene glycol insulator molecules. The target is captured on the electrode, and it hybridizes to a second reporter sequence. It is then labeled with FC, which is called the signal probe (15). As shown in Figure 2, there are two different FC substitutions that are used as modified signal probes. In this technique, a conjugated FC modified analog was connected at the 5' or 3' end of the signal probe so that it behaved like a "wire-like" electrochemical probe. FC was used as an electron transfer between the gold electrode and FC label (16). In addition, the capture and signal probes were designed to be matched with specific regions of target DNA. Thus, the target DNA is not labeled; instead, it is "sandwiched" by the capture probe and the signal probe (Fig. 3). The electrochemical signal was recorded when the target DNA was hybridized to the capture probe and the signal probe; then, the FC label touched the self-assembled monolayer of the gold electrode (17). By using two types of FC-labeling signal probes with two redox potentials that are coupled to an electrochemical reader, different ACVs are obtained when the target DNA is detected (18).

As a model for the development and validation of the method, we detected 12 of the most common nondeletional types of α - and β -thalassemia sequence variants, including the most common mutations: CD41-42 (41.6%), IVS-II-654 (21.8%), CD17 (18.0%), -28 (8.0%), WS, QS, CS, Cap+1, Int, CD26, CD31, and CD71-72 (19).

The DNA samples that were extracted from the homozygous wild-type and heterozygous mutant blood samples were serially diluted as follows: 20, 10, 8, 5, 2.5, and

Fig. 2. Molecular structures of FC(I)/FC(II). FC(I) ($E_{1/2}$ of 0.180 V vs. Ag/AgCl, with a low redox potential) and FC(II) ($E_{1/2}$ of 0.350 V vs. Ag/AgCl, with a higher redox potential) can be modified for different signal probes for genotypes.

Fig. 3. Scheme of electrochemical “sandwich” assays for thalassemia. The target is captured on an electrode with the capture probe and hybridizes to the signal probe labeled with FC. FC(I) was connected at the 5' or 3' end of the wild-type signal probe, whereas FC(II) was connected at the 5' or 3' end of the mutant signal probe. Different ACV was obtained when the different target DNA was detected.

1 ng/ μ l. The established PCR detection system and electrochemical DNA sensor were used to determine the limit of detection of the sensor detection system. When a sample's DNA concentration is lower than the reagent's minimum detectable quantity, a negative result is given because the electrochemical signals on the corresponding site cannot be interpreted. From the results in Table 4, it can be seen that when the DNA concentrations were 5 ng/ μ l or greater, an accurate genotype was determined for all of the samples. However, except for the 2.5 ng/ μ l DNA sample of the homozygous wild-type blood samples, when the DNA concentrations were 2.5 ng/ μ l or less, part of the corresponding site could not be determined; therefore, a negative result was given. To ensure that all 12 of the corresponding sites could be accurately determined, 5 ng/ μ l was selected as the minimum detectable concentration. The within-run precision was acquired by measuring each concentration ten times. Coefficients of variation (CVs) for each concentration were less than 6%.

The repeatability of the electrochemical DNA sensor was studied by repetition. The DNA samples extracted from the homozygous wild-type and compound heterozygous mutant blood samples were tested ten times at the following concentrations: 20, 10, 8, and 5 ng/ μ l. The intra- and interassay CVs were 0.47–3.54% ($n = 10$) and 3.64–6.78% ($n = 40$), respectively. Both of these ranges were acceptable for applications in clinical genetic screening.

The electrochemical DNA sensors were sealed after production. Every month for 15 months, 20 pieces of sensor for 20 samples of homozygous wild-type were used for electrochemical analysis. The results (Fig. 4) show that, initially, the average electrochemical signals of the site of WS steadily underwent a mild decline. After 12 months, the decline shifted to a sharp descent. And it also shows the similar trends on the other genotype sites. Therefore, acceptable stability is guaranteed within the first 12 months.

TABLE 4. Detection Results at Different DNA Concentrations

	20 ng/ μ l	10 ng/ μ l	8 ng/ μ l	5 ng/ μ l	2.5 ng/ μ l	1 ng/ μ l
Homozygous wild-type	Accurate	Accurate	Accurate	Accurate	Accurate	All negative
WS.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
QS.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CS.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
–28.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
Cap+1.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
Int.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CD17.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CD26.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CD31.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CD41/42.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
CD71/72.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative
IVS-II-654.M/W	Accurate	Accurate	Accurate	Accurate	Partly negative	All negative

Fig. 4. Stability test of the developed method. The average electrochemical signals were acquired by measuring 20 samples of homozygous wild-type signals at the WS site at each time point. It shows that the electrochemical signals mildly decline for the first 12 months; thereafter, the trend becomes a sharp decline. And it also shows the similar trends on the other genotype sites.

Twenty homozygous wild-type blood samples and 238 heterozygous mutant blood samples with known genotypes, including 20 cases of WS, 20 cases of QS, 18 cases of CS, 20 cases of -28, 20 cases of Cap+1, 20 cases of Int, 20 cases of CD17, 20 cases of CD26, 20 cases of CD31, 20 cases of CD41-42, 20 cases of CD71-72, and 20 cases of IVS-II-654, were genotyped by our electrochemical DNA sensor.

The peak faradaic current signal was recorded by the ACV that was built into the DA9100, and the electrochemical signal occurred when the particular target DNA hybridized to the capture probe and signal probe that was labeled with either FC(I) or FC(II). Genotypes of each mutation were determined by the signal ratio of FC(I) (label of wild-type signal probes) to FC(II) (label of mutant signal probes). The target DNA was extracted from genomic DNAs that were diagnosed by DNA sequencing using multiplex asymmetric PCR. In addition, 24 working electrodes were collided with target DNA from the genomic DNAs of homozygous wild-type blood samples (20 cases) and heterozygous mutant blood samples (238 cases). By using the orthonormal signal output, the target DNA could be simply identified. The target DNA from the 13 different genotypes leads to different ratios of electrode signals from wild-type to mutant (20). The signal ratio was computed by dividing the FC(I) that was measured for the reaction of the wild-type signal probes (W) to the FC(II) gauged for the reaction of the mutant signal probes (M). The signals were accepted if the peak faradaic current signal was greater than 10 nA, which was the cutoff value. Samples with $M < 10$ nA and $W/M > 4$ were assigned as wild-type for the particular mutation,

Fig. 5. (A) For the 20 homozygous wild-type samples, the peak faradaic current signals of FC(II) M were less than 10 nA in each electrode type, and the peak faradaic current signals of FC(I) W were more than five times that of M. (B) For the 238 cases of heterozygotes mutant samples, all the peak faradaic current signals of FC(I) W and FC(II) M were greater than 10 nA, and the values of W/M were focused between 0.9 and 1.2 in each electrode type.

whereas when W and $M > 10$ nA and $0.5 < W/M < 2$, the sample was deemed as heterozygous for the particular mutation.

In Figure 5, the homozygous wild-type data (Fig. 5A) and the heterozygous mutant data (Fig. 5B) generated from DA9100 are summarized. The values were obtained and computed from different sites on the electrochemical DNA sensor of the 24 working electrodes and the reference electrode (Ag/AgCl). After comparing the Ag/AgCl reference electrode to the gold electrodes and standardizing the mean of each working electrode of particular genotypes on the electrochemical DNA sensor, the mean and standard deviation of the peak currents were determined. For all 20 homozygous wild-type samples (Fig. 5A), the peak faradaic current signals of FC(II) M were less than 10 nA with each electrode type, and the peak faradaic current signals of FC(I) W were more than five times that of M. For the 238 cases of heterozygous mutant samples (Fig. 5B), all of the peak faradaic current

signals of FC(I) W and FC(II) M were greater than 10 nA, and the values of W/M were focused between 0.9 and 1.2 with each electrode type. In this model assay, different genotypes of target DNA mimics were synthesized for the preliminary optimization of the capture and signal probe sequences and testing protocol (data not shown). Test results show this electrochemical DNA sensor system is effective because it accurately identified all of the blood samples of the different thalassemia genotypes within the 20 homozygous wild-type blood samples and 238 heterozygous mutant blood samples (data not published). Therefore, this work indicates the potential clinical use of the electrochemical DNA sensor system after optimizing PCR and hybridization conditions to approach 100% consistency with DNA sequencing.

While conducting this research, it was discovered that the density of capture probes deposited on the surface of the gold electrodes has a great impact on the signal value output of the electrochemical DNA sensor system (21). Therefore, we conducted an experiment while depositing the capture probes by varying the surface coverage of the concentration of the capture probes applied during the electrochemical DNA sensor preparation step. The optimal signal value output was obtained with a surface coverage of 35 ± 2 pmol/cm², and the signal output decreases at both lower ($81 \pm 3\%$ at 5 ± 2 pmol/cm²) and higher ($120 \pm 4\%$ at 65 ± 5 pmol/cm²) densities (data not shown). One reason for this phenomenon may be the consequence of two rivalrous factors. The stereo-hindrance effect impacts the probes, which form a self-complementary construction that augments the background signal output when the density of the capture probe is high. However, when the capture probe density is low, suitably folded signal probes may collide with the gold electrode surface, which, consequently, enhances the background signal output and reduces the output of the regular signal at the same time. Additionally, the cross-hybridization among the DNA target and the capture and signal probes could be readily resolved by introducing mismatched base pairs.

CONCLUSION

The electrochemical DNA sensor detection system introduced here includes a simple compact electrochemical DNA sensor and electrochemical analyzer. It was first applied to rapidly detect thalassemias found in the Chinese population caused by *HBA* and *HBB* gene non-deletional mutations. The first-generation electrochemical detection system DA9100 equipped with ACV will accelerate the development of cost-effective, high-throughput nucleic acid testing and has potential to be clinically popularized and applied. The electrochemical DNA sensor described here can be applied to rapid genotyping

of thalassemia or other clinical genotyping applications. Moreover, the described system can also be used for point-of-care testing for infectious diseases, genetic diagnosis, environmental pollutant determination, and food safety.

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