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Single-nucleotide polymorphism genotyping using a novel multiplexed electrochemical biosensor with nonfouling surface

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ABSTRACT

A novel electrochemical DNA biosensor for single-nucleotide polymorphism (SNP) analysis was developed. In this work, an oligonucleotide-incorporated nonfouling surface (ONS) was constructed to resist nonspecific absorption. The biosensor was developed using a 16-electrode array for high-throughput SNP analysis. The proposed strategy was primarily based on specific oligonucleotide ligation. Fully matched target DNA templated the ligation between a capture probe assembled on gold electrodes and a tandem signal probe with a biotin moiety that could capture avidin–horseradish peroxidase and sequentially generate a catalysed amperometric signal. A pre-core mutation in the hepatitis B virus (HBV) genome at G1896A and two adjacent polymorphisms in the human CYP2C19 genome at C680T and G681A were analysed. Polymerase chain reaction (PCR) products were used as real-life samples and analysed. Our results showed that 10% of a single-mismatched mutant gene was clearly distinguished with a current signal 16 times higher than that of the blank sample, demonstrating the selectivity and practicability of the multiplexed electrochemical DNA biosensor.

1. Introduction

Single-nucleotide polymorphisms (SNPs) are prevalent in all types of genetic variations. Reliable SNP analysis provides excellent markers for identifying heritable polymorphisms (Herbert et al., 2006), genetic disorders and pathogen drug resistance (Halushka et al., 1999; Nordborg and Tavare, 2002), which is needed for the genetic diagnosis of diseases and subtle genetic risk factors (Chen et al., 2005; Ho-Pun-Cheung et al., 2006; Ng and Liu, 2006).

The most frequent hepatitis B virus (HBV) SNP occurs at nucleotide 1896, where G is replaced by A (HBV G1896A), preventing HBeAg synthesis by introducing a premature stop codon (Amini-Bavil-Olyaee et al., 2005). G1896A has drawn a great deal of interest because it is usually accompanied by weaker antiviral immune response to persistent infection. G1896A is also associated with hepatocellular carcinoma (Yang et al., 2008), fulminant hepatitis (Panassie et al., 2011) and HBeAg seroconversion time (Liu et al., 2004).

Analysis of adjacent SNPs presents greater challenges in both probe design and the throughput of the biosensors. An important pair of adjacent SNPs in the CYP2C19 genome occurs at C680T and G681A (Nakamoto et al., 2007). This pair has been demonstrated to affect the drug-metabolising function of the CYP2C19 enzyme. Genotyping of adjacent SNPs in CYP2C19 has potential applications in clinical pharmacogenomic tests and personalised medicines (Wang et al., 2011).

Electrochemical DNA sensors provide excellent methods for SNP genotyping (Fan et al., 2003; Kelley et al., 1999; Lao et al., 2005; Lubin et al., 2006; Wan et al., 2009; Xiao et al., 2009; Zhang et al., 2006), with the advantages of high sensitivity and minimal power/cost/mass requirements (Drummond et al., 2003; Wang et al., 2003). Despite the remarkable progress in recent years, however, electrochemical DNA sensors still face challenges in specificity because their signal-to-noise ratio is often hampered by nonspecific adsorption, especially when applied to real-world samples (e.g., serum or PCR products, etc.). Moreover, the analysis efficiency is also limited by the low-throughput of most of the electrochemical sensors. To simultaneously investigate all possible functional variants of SNPs in complex biological samples, there is a pressing need for a highly specific and sensitive genotyping platform with high-throughput and relatively low cost.

Oligo(ethylene glycol) (OEG) is well-known to be protein-resistant when co-immobilised with thiolated DNA probes for the construction of a biosensor interface (Zhang et al., 2008). The oligonucleotide-incorporated nonfouling surfaces (ONS) repel nonspecific adsorption in real-world samples such as serum and PCR amplicons. Because of this advantage, a significantly higher signal-to-noise ratio can be achieved in either hybridisation detection or ligase based biosensors (Wan et al., 2010).

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In the present work, we propose a novel SNP electrochemical biosensor aimed at highly specific and high-throughput SNP analysis by the construction of ONS onto the surface of a high-throughput 16-electrode array.

2. Experimental

2.1. Reagents

Tris (hydroxymethyl) aminomethane was purchased from Cxbio Biotechnology Ltd. Ethylenediaminetetraacetic acid (EDTA), mercaptohexanol (MCH), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO). Ethylene glycol-terminated thiol ($HS–(CH_2)_{11}–EG_2–OH$, OEG) was purchased from Prochimia (Poland). TMB substrate (TMB, 3,3′,5,5′-tetramethylbenzidine; Neogen K-blue low-activity substrate, H_2O_2 was added) was purchased from Neogen (U.S.). Avidin–horseradish peroxidise conjugate (avidin–HRP) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. DNA oligonucleotides

DNA oligonucleotides were purchased from Sangon Inc. (Shanghai, China). Capture probes and signal probes were designed for electrochemical analysis. The capture probes have 5′-SH groups and 10 deoxyadenosine residues as spacers, followed by nucleotides complementary to the corresponding SNP target sequences. The signal probe, containing 29 nucleotides with a sequence adjacent to the SNP nucleotide, was synthesised with a biotin at its 3′ end for detection and a phosphate at its 5′ end for ligation.

2.3. The buffer solutions

The PCR buffer contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM of each dNTPs. The hybridisation buffer contained 1 M NaCl and 10 mM TE buffer (pH 7.4). The buffer for electrochemical DNA quantification contained 10 mM Tris–HCl solution (pH 7.4). The DNA immobilisation buffer contained 10 mM Tris–HCl, 1 mM EDTA, 10 mM TCEP (pH 7.4), and 1 M NaCl. The enzyme was diluted in a 0.1 M PBS buffer with 0.5% casein (pH 7.2). The ligation reaction buffer contained 20 mM Tris–HCl, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, and 0.01% Triton X-100 (pH 8.3). All solutions were prepared with Milli-Q water (18 MΩ cm resistivity) from a Millipore system.

2.4. PCR amplification

PCR amplification of genomic DNA fragments (HBV or human) was performed on a Bio-Rad PCR cycler (PTC-100). A pair of asymmetric primers (forward primer/reverse primer = 1:100) was employed to generate the single-strand DNA targets that could be directly sensed by the single stranded probes. The amplification was performed in 20 μL of PCR buffer containing 2.0 units of Taq DNA Polymerase (Invitrogen). The thermal cycler condition is as follows: 4 min at 94 °C, 30 cycles of 55 s at 94 °C, 60 s at 60 °C, 60 s at 72 °C, and then a final extension at 72 °C for 5 min. PCR fragments were confirmed by agarose gel electrophoresis.

2.5. Formation of the ONS on a gold electrode

3 μL of thiolated capture probes at a concentration of 5 μM was added to each electrode and incubated at room temperature for 1 h. Electrodes were then incubated with 2 mM OEG for 2 h to obtain mixed SAMs (self-assembled monolayer). The electrode was then rinsed with Milli-Q water and dried with N_2 after each step.

2.6. SNP detection

100 μL of mixed solutions containing 1 nM target (or 20 μL PCR product), 100 nM signal probe, 2 U ligase, and 0.5% BSA in ligation reaction buffer was preannealed at 50 °C for 5 min and then added to each working electrode with mixed SAMs. As the employed ligase is relatively thermostable, the ligation was performed at 55 °C with a constant humidity. After 20 min of incubation, a denaturation step was followed by treatment with 100 mM NaOH at 70 °C for 10 min. After being washed, electrodes were treated with 1% BSA, followed by the addition of avidin–HRP (0.5 U/mL in 0.1 M PBS buffer with 0.5% casein). After 15 min of incubation at room temperature, electrodes were washed with 0.1 M NaCl and 10 mM PBS buffer (pH 7.4) and subsequently subjected to electrochemical measurements.

2.7. Electrochemical measurement

The 16-electrode sensor arrays were provided by GeneFluidics (Monterey Park, CA), with each consisting of a working electrode (2.3 mm in diameter) in the centre surrounded by an auxiliary electrode and a reference electrode. Cyclic voltammetric (CV) and amperometric (I–t) measurements of DNA arrays were performed at room temperature using a 16-channel PM3000 workstation (Genefluidics). For I–t measurements, the voltage was fixed at −100 mV (relative to the gold reference electrode), and the reduction current was measured at 60 s.

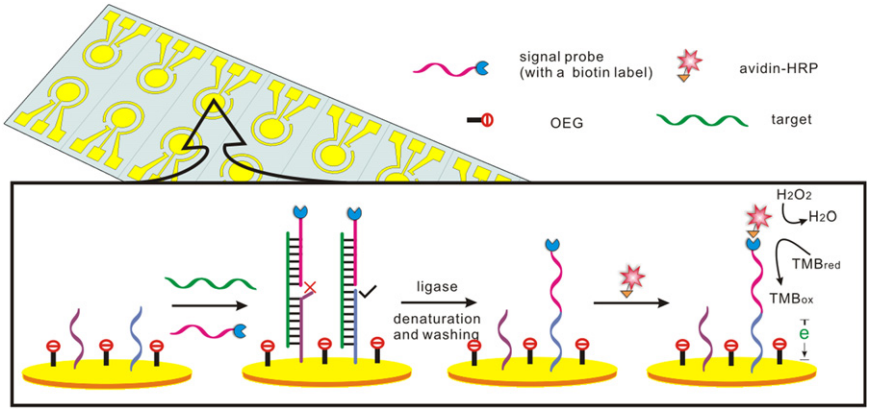
3. Results and discussion

3.1. Strategy of SNP analysis

Our DNA-templated ligation SNP analysis was performed on a gold electrode surface with ONS SAM, constructed via the co-assembly of 5′-thiolated capture DNA probes and SH–OEG. Next, a sandwich-like DNA structure was formed by specific hybridisation of the target DNA to the capture probe and subsequent combination of a signal probe DNA to the other end of the target, adjacent to the capture probe. DNA sequences of the capture probes differed from each other only at the SNP sites. As a result, they hybridised to the target DNA with either full complementation or one mismatch. Only when the sequence of the target DNA is fully complementary to the capture probe, ligation between the tandem capture probe and the signal probe can be performed using the target DNA as template under the effect of a ligase (Wang et al., 2011). A stringent denaturation and wash step was carried out to challenge the DNA sandwich structure. Without ligation, the hybridisation would be broken and the signal probe would be removed from the electrode surface. However, after ligation, the signal probe covalently ligated to the capture probe would survive. After the wash step, avidin–HRP was incubated with the surface, which combined to the biotin label at the end of the signal probe to catalyse the reduction reaction of hydrogen peroxide, providing an amperometric signal. A 16-electrode sensor array was employed to perform the electrochemical detection to realise a high-throughput platform for multiplexed SNP analysis (Scheme 1).

3.2. SNP analysis of HBV G1896A and optimisation of PCR condition

Hepatitis B virus (HBV) is a blood-borne pathogen and a major public health problem. Thus, analysis of HBV G1896A SNP (Carman et al., 1989) is of important clinical significance. To provide a simple, specific, and low cost analysis method that can be used in routine diagnostic laboratories, we focused our strategy on the analysis of the G1896A SNP in the HBV genome. A capture probe and a signal



Scheme 1. Schematic illustration of the ligation-based electrochemical DNA biosensor for SNP analysis on an ONS electrode. Capture probes (cps) assembled on the electrode surface contained different end bases at 3′ terminals. Ligation between two probes could be performed and anchor the signal probes only when perfect hybridisation occured. Avidin–HRP was then added to catalyse the redox of the TMB substrate.

Table 1
Sequences of probes for the HBV G1896A analysis.

HBV sp (signal probe)	5′-P-AAAGCCACCCAAGGCACAGCTTGGAGGCT-biotin-3′
HBV cp-W (wild-type capture probe)	5′–SH-TTTTTTTTTTCGGGTCAATGTCCATGCCCC-3′
HBV cp-M (mutant capture probe)	5′–SH-TTTTTTTTTTCGGGTCAATGTCCATGCCCT-3′

probe were designed to flank the 1896 nucleotide. The sequences are listed in Table 1.

First, PCR amplification of a commercial HBV reference plasmid (wild-type) was performed. For analysis based on the sandwich-type structure at the solid/liquid interface, the hybridisation efficiency is important for the final analysis of the sensor. Therefore, the PCR primer design was optimised before SNP analysis to determine the length of the target and the hybridisation position. We used three different pairs of primers to determine the best PCR product. The sequences of the primers are listed in Table S1. As shown in Fig. S1, all three pairs of primers produced the anticipated targets under the same PCR conditions.

We analysed the three different PCR products with the sandwich-type strategy as shown in Fig. S2A. The aim was to find the largest current signal before the ligation and stringent washing steps, which would result in the best hybridisation between the targets and the probes. The fully matched wild-type capture probe (cp-W) was assembled on the electrode, forming a sandwich-like structure with the signal probe and the corresponding PCR product targets via hybridisation. The avidin–HRP was then added to catalyse the electrochemical redox of the TMB substrate. As shown in Fig. S2B, the current signal increased as the length of the PCR products decreased, possibly due to a higher molecular diffusion speed and lower steric hindrance for the affinity enzyme label. We took HBV RP1 and HBV FP as the best pair of primers for the HBV G1896A SNP analysis. The length of the corresponding PCR product was 190bp. Considering the high similarity between the wild-type and mutant HBV genomes, we can easily anticipate that the optimisation would also be the same for cp-M.

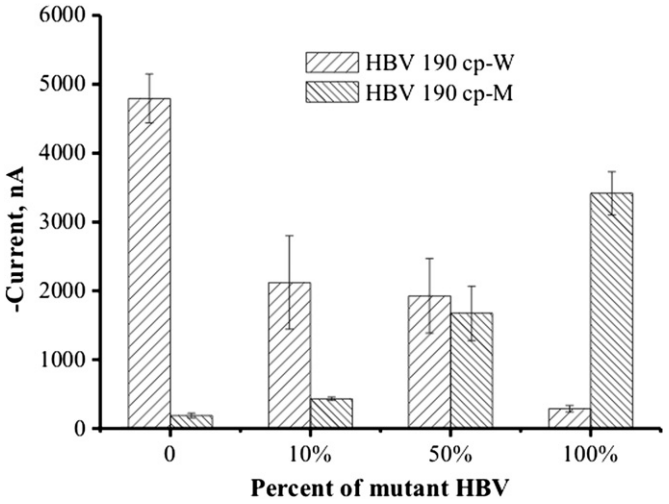


Fig. 1. Result of the multiplexed electrochemical analysis of the 190bp PCR amplicons from mixed samples containing different percentages of the mutant HBV genome.

Using the optimised primers, we performed PCR amplification with a mixed sample containing both the wild-type and mutant HBV genomes. In this work, we used an asymmetric PCR protocol (Lai et al., 2006; Poddar, 2000) to obtain ssDNA targets of 190bp that could be directly sensed. Amplicons from mixed templates were then analysed by our biosensor based on the ONS electrode array. The analysis was performed on both the wild-type capture probe (HBV cp-W) and the mutant capture probe (HBV cp-M). The results are shown in Fig. 1. The 100% mismatched sample showed only a negligible signal, because of minimal nonspecific adsorption of the enzyme at the ONS which has been proven to be highly protein-resistant and compatible with electrochemical measurements in our previous work (Zhang et al., 2008). When the percentage of the fully matched template increased, the amperometric signal also increased accordingly. Even the current signal was lower than the PCR optimisation experiment (without ligation steps) for the much more complex reaction system and the challenge of the ligation/washing steps, we found that this sensor could achieve a more than 16-fold signal distinction between the fully matched target and the SNP target PCR amplicons with both capture probes. 10% of the mutant HBV genome could be selectively identified.

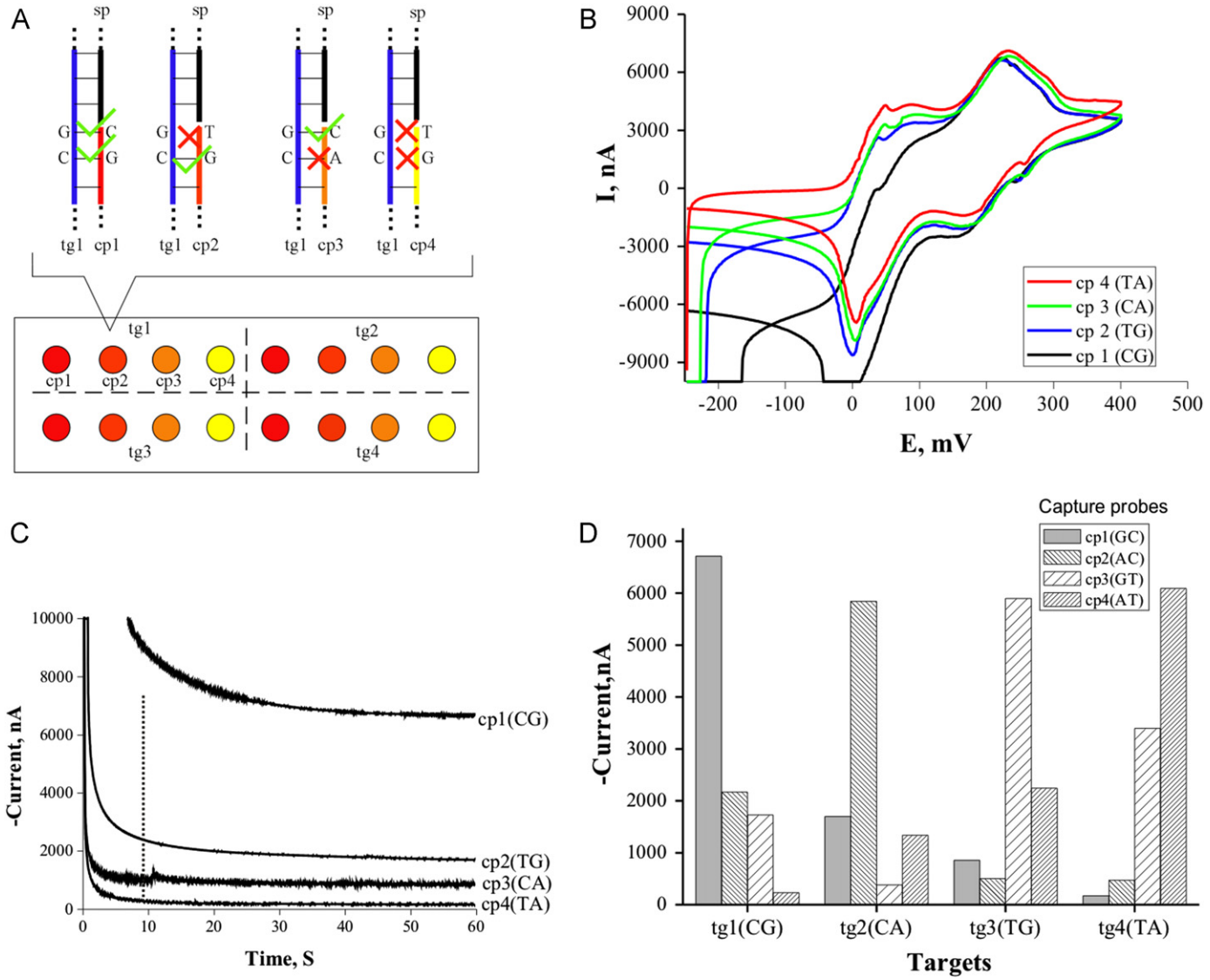


Fig. 2. Analysis results of four simultaneous DNA targets of the CYP2C19 C680T and G681A polymorphisms on one 16-electrode array. (A) Scheme for the ligase-based SNP detection: Each target was added onto four electrodes, the target DNA, capture probe and reporter probe formed a "sandwich" complex. The reporter probe was washed off in the presence of SNP. (B) CV analysis results of CYP-tg1 (CG) with different capture probes. (C) Amperometric curves of CYP-tg1 analysis. The current signals were recorded at 60 s, and the analysis voltage was −100 mV. (D) Detection of DNA samples with a 16-electrode DNA array immobilised with 16 capture probes (the line located at 500 nA was used as a cut-off). When the targets fully matched the capture probes, we obtained a higher current signal than the other three electrodes covered by capture probes with a SNP or two adjacent SNPs.

3.3. *Analysis of adjacent SNPs in CYP2C19*

CYP2C19 is a member of the cytochrome-P450 enzyme superfamily and is a monooxygenase that metabolises many drugs. CYP2C19 C680T and G681A are a pair of adjacent SNPs existing in the CYP2C19 genome that produce allelic proteins exhibiting a lower enzyme activity than the common protein (Blaisdell et al., 2002; De Morais et al., 1994a, 1994b; Ferguson et al., 1998).

The adjacent SNPs in the CYP2C19 genome were analysed with our system. Four capture probes (cp1-4) (Table S2) differing in the last 2 nucleotides were assembled on a 16-electrode sensor array (Fig. 2A). Only the perfectly matched target will anchor the signal probe through the ligation and generate an obviously detectable current signal; one or two mismatched nucleotides will hinder the ligation and only generate low electrochemical signals from nonspecific absorbance or incorrect ligation.

As mentioned above, we employed a 16-electrode microfabricated sensor array, with each having a set of working, reference and counter electrodes (Fig. 2A). Sensors were divided into four groups, and electrodes in each group were separately covered with four capture probes (cp1-4). In this way, the sensor array allowed the simultaneous detection of four targets with four different capture probes.

Taking target 1 (CYP-tg1) as an example, when 5 nM of CYP-tg1 was added onto 4 ONS electrodes in the first group of sensors, only CYP-cp1 completely hybridised to the target sequence and formed a "sandwich" conformation with a signal probe (CYP-sp). Under the effect of a ligase, the signal probe with a biotin label is kept on the electrode and generates a current signal. Fig. 2B reveals a typical CV curve for TMB with two pairs of characteristic redox peaks. Adjacent SNPs (cp4) lead to a significantly low background signal, while the HRP catalysed reduction peak was significantly increased when the mismatched sites was reduced to one (cp2 and cp3) or zero (cp1). The sensor showed the ability to distinguish SNPs when we recorded the amperometric signal at 60 s (Fig. 2C), and a detection voltage of −100 mV was used which was experimentally proven to provide best response (Zhang et al., 2008). The current signal from the electrode covered

with cp1 was more than 3 times higher than that from electrodes covered with either cp 2 and cp 3 (one SNP), and more than 28 times higher than that from the electrode covered with cp4 (two adjacent SNPs) (Fig. 2D).

In the same way, the other 3 targets (tg2–4) with different allele genotypes were also successfully analysed (Fig. 2D) on the same array. We obtained prominent current signals on electrodes with fully complementary capture probes, while the signals were clearly lower for those with a SNP or two adjacent SNPs. These results demonstrate the effect of our sensor and the advantage of the ligase system and the ONS surface. Owning to the 16-electrode array, we were capable of distinguishing the adjacent SNPs in one single test. Thus the assay efficiency has been improved greatly.

The PCR products of CYP2C19 were analysed to further demonstrate the applicability of our system. PCR amplification of the wild-type CYP2C19 genome was first performed with a pair of primers that were optimised as described above (data not shown). Agarose gel electrophoresis (Fig. 3A) suggests that we successfully obtained the anticipated 212bp products. As described above (Fig. 2A), a 16-electrode array with four different capture probes assembled on the surface was employed for the analysis. The average signals of the *I–t* curves clearly suggest that the fully complementary capture probe ("CYP-cp1") produced much higher signals than those with either 1-base or 2-bases mismatched (Fig. 3B).

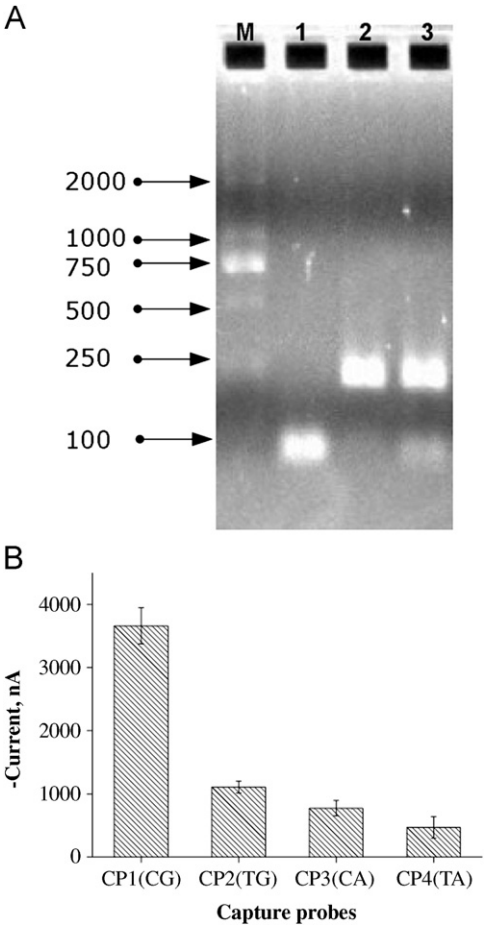


Fig. 3. (A) Agarose gel electrophoresis pattern of the CYP2C19 genome: M, DNA size marker; lane 1: negative control; lanes 2, and 3: PCR products from the wild-type CYP2C19 genome. (B) Electrochemical analysis result of the PCR product of wild-type CYP2C19 with four different captures on the same 16-electrode array, with all other experimental conditions the same.

4. Conclusion

We have developed a multiplexed electrochemical biosensor with an ONS surface for SNP detection. After optimisation of the experimental conditions, our sensor was successfully utilised for the analysis of SNPs in HBV A38 (G1896A) and the adjacent SNPs in CYP2C19 (C680T and G681A). Furthermore, analysis of their PCR products also exhibited sharp differences between the fully matched target and the sequences with 1 or 2 SNPs. Because of the advantages of the ONS and the ligation, 10% of mismatched DNA was successfully found. The 16-electrode sensor array provides a multiplexed platform for the simultaneous detection of allelic genes. And the prepared array showed a good stability even after 5-day storage in 4 °C (Fig. S3). The proposed sensor is a specific, high-throughput and cost-effective method that could easily be established in any common diagnostic laboratory, in contrast to sequencing-based methods.

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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.2012.11.005.

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