



## Ligase-based multiple DNA analysis by using an electrochemical sensor array

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### ARTICLE INFO

#### Article history:

Received 7 April 2008

Received in revised form 6 July 2008

Accepted 7 July 2008

Available online 12 July 2008

#### Keywords:

Electrochemical detection

DNA sensors array

Ligase

SNP

### ABSTRACT

We herein report an electrochemical biosensor for the sequence-specific detection of DNA with high discrimination ability for single-nucleotide polymorphisms (SNPs). This DNA sensor was constructed by a pair of flanking probes that “sandwiched” the target. A 16-electrode electrochemical sensor array was employed, each having one individual DNA capture probe immobilized at gold electrodes via gold-thiol chemistry. By coupling with a biotin-tagged detection probe, we were able to detect multiple DNA targets with a single array. In order to realize SNP detection, a ligase-based approach was employed. In this method, both the capture probe and the detection probe were in tandem upon being hybridized with the target. Importantly, we employed a ligase that specifically could ligate tandem sequences only in the absence of mismatches. As a result, when both probes were complementary to the target, they were ligated in the presence of the ligase, thus being retained at the surface during the subsequent stringent washing steps. In contrast, if there existed 1-base mismatch, which could be efficiently recognized by the ligase, the detection probe was not ligated and subsequently washed away. A conjugate of avidin–horseradish peroxidase was then attached to the biotin label at the end of the detection probe via the biotin–avidin bridge. We then electrochemically interrogated the electrical current for the peroxidase-catalyzed reduction of hydrogen peroxide. We demonstrated that the electrochemical signal for the wild-type DNA was significantly larger than that for the sequence harboring the SNP.

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### 1. Introduction

Coupled with the ability to produce a simple, accurate and inexpensive platform for patient, DNA sensors have attracted intense research interest (Broude, 2002; Drummond et al., 2003; Fan et al., 2003; Ju and Zhao, 2005; McGlennen, 2001; Wang, 1999). Many techniques have been developed toward sensitive detection of DNA either in solution (Fan et al., 2003; Taton et al., 2000) or on solid substrates (Boon et al., 2000; Broude, 2002; Hook et al., 2001). Although the hybridization reaction in solution is easy to carry out, there is an inherent disadvantage because it requires additional isolation and purification steps. On the contrary, DNA sensors on solid substrates avoid such steps and are potentially automatable. A typical solid-state DNA sensor consists of a recognition layer that is a single strand DNA capture probe immobilized at surfaces and a signal transducer which converts the binding of target DNA to optical (Fan et al., 2003; Jenison et al., 2001; Taton et al., 2000, 2001; Yamashita et al., 2001), mechanical (Hook et al., 2001) or electrochemical signals (Cai et al., 2002; Fan et al., 2005; Shipway et al., 2000). Among these strategies, electrochemical method is

well suited for DNA diagnostics because it is based on inexpensive, portable and automatable electronic readout devices (Ju and Zhao, 2005; McGlennen, 2001; Zhang et al., 2006).

Many amplification strategies have been developed to improve the sensitivity of electrochemical DNA sensors, such as enzyme-based amplification (Boon et al., 2000; Wang et al., 2002) and nanomaterials-based amplification (Zhang et al., 2006). These strategies have made significant progress toward sensitivity improvement. Selectivity is another important issue in DNA sensors, with the detection of single-nucleotide polymorphisms (SNPs) a goal of DNA sensors (Boon et al., 2000; Zhang et al., 2008; Zhong et al., 2003). Moreover, it is important to develop a simple and rapid approach to realize simultaneous detection of multiple targets (Wang et al., 2002, 2003). In this work, we reported a “sandwich-type” detection strategy for simultaneous detection of multiple DNA targets by using a 16-channel gold sensor array. The sandwich-type strategy involved a pair of DNA probes, one capture probe and the other reporter probe that flanked the target DNA sequence (Zhang et al., 2006). As a result, the dual hybridization significantly enhances the specificity and improves the signal-to-noise ratio. In order to realize SNPs detection, we further introduced ligase in our assay to improve the discrimination ability toward single-base mismatch (Zhang et al., 2008; Zhong et al., 2003).

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## 2. Materials and methods

### 2.1. Materials

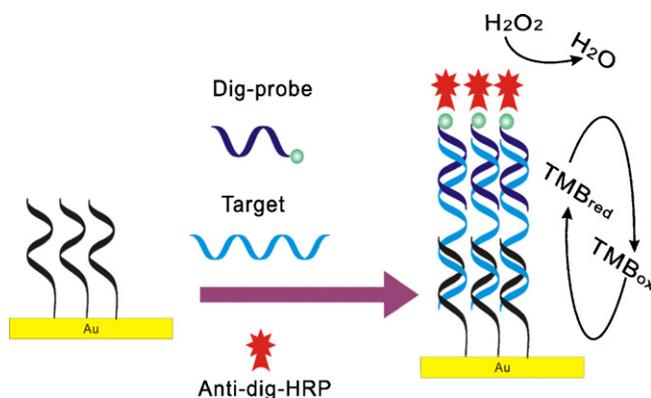
All oligonucleotides (Table S1 of the Supporting information) were synthesized and purified by Sangon Inc. (Shanghai, China). Their concentrations were quantified by OD260 based on their individual absorption coefficients. There were 15 capturing probes DNA, 4 targets DNA and 4 reporter probes for DNA detection. Capturing probes 1–15 were thiolated with a  $-(\text{CH}_2)_6^-$  spacer at the 3' end. Reporter probes 1–4 had a 5'-Digoxigenin as an affinity label. Target DNA 1–4 were 40-base sequences that contained complementary sequences to one of the capturing probes on one end and reporter probes 1–4 on the other end, respectively. For SNP detection, four capture probes and a single reporter probe are designed. Capture probes 16–19 were 50-base sequences which were thiolated at their 5' end with a 10 "T"s and a  $-(\text{CH}_2)_6^-$  as a spacer. They differ only at their 3'-terminal nucleotide. Reporter probe 28 had a 3'-biotin as an affinity label and phosphate at its 5' end for ligation. Target 5 contained complementary sequences to reporter probe 5 on one end and to capturing probe 1 on the other end. Target 6 also contained a complementary sequence to reporter probe 5 but include a single-nucleotide mismatch to capturing probe 1 and could complement to capturing probe 2.

TMB substrate (TMB = 3, 3', 5, 5' tetramethylbenzidine; Neogen K-blue low activity substrate) was purchased from Neogen (U.S.). Anti-Dig-HRP (horseradish peroxidase) and avidin-HRP was purchased from Roche Diagnostics (Mannheim, Germany). Ampligase and 10 $\times$  reaction buffer were bought from Epicentre biotechnologies. Ethylenediaminetetraacetic acid (EDTA), mercaptohexanol (MCH), and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were from Sigma. Tris (hydroxymethyl) aminomethane was from Cxbio Biotechnology Ltd. All solutions were prepared with MilliQ water (18 M $\Omega$  cm) from a Millipore system.

### 2.2. Electrochemical measurements

Electrochemical measurements of electrode were performed with a CHI 620 electrochemical work station (CH Instruments Inc., Austin) and a conventional three-electrode configuration was employed all through the experiment, which involved a gold working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Cyclic voltammetry (CV) was carried out at a scan rate of 50 mV/s, and the voltage of amperometric current versus time was fixed at 150 mV and the electroreduction current was measured at 100 s after the HRP redox reaction reached steady state.

Electrochemical sensor arrays (Figure 1S) were provided by GeneFluidics (Monterey Park, CA), each sensor in the 16-sensor array consisted of a working electrode in the centre surrounded by



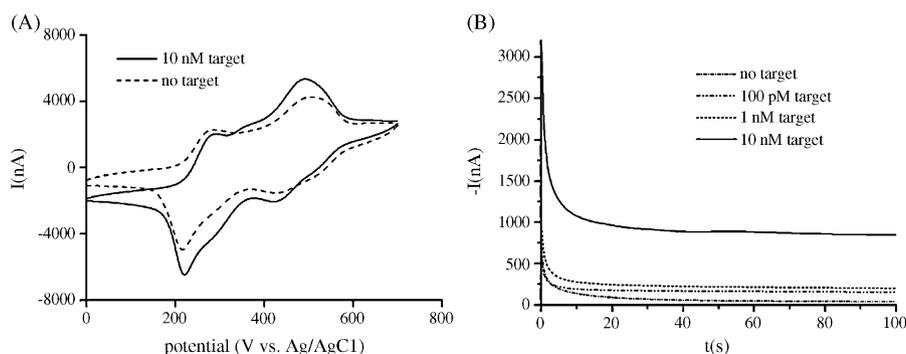
**Fig. 1.** Scheme for the "sandwich-type" detection strategy: in the presence of target DNA, Dig-labeled reporter probe formed "sandwich" complexes with target and capture probe anchored at the electrode surface.

an auxiliary electrode and a reference electrode. Measurements on DNA array were performed at room temperature using a PM3000 workstation (genefluidics Monterey Park, CA). PM3000 16-channel electrochemical sensors were purchased from genefluidics (Monterey Park, CA). Amperometric current versus time was measured using a multichannel potentiostat (GeneFluidics). The voltage was fixed at  $-100$  mV (versus gold reference), and the electroreduction current was measured at 60 s.

### 2.3. Multi-target detection using the DNA array

To test the feasibility of the proposed enzyme-amplified "sandwich-type" detection strategy, two gold electrodes were prepared following a literature procedure (Zhang et al., 2007), and then capturing probe 2 was immobilized. DNA immobilization buffer: 10 mM Tris-HCl, 1 mM EDTA, 10 mM TCEP, and 1 M NaCl (pH 7.4). 4  $\mu$ L of 5  $\mu$ M probe 2 was added to each electrode for 1 h. Then the DNA modified electrode was further treated with 1 mM MCH for 1 h to obtain well aligned DNA monolayer. The electrode was rinsed with MilliQ water and dried lightly with N<sub>2</sub> after each step. 100 nM target DNA 1 was mixed with 100 nM reporter probe 1 in 1 M phosphate buffered saline (PBS, pH 7.4) and then pre-annealed at 75  $^{\circ}$ C for 5 min. 4  $\mu$ L of this solution was placed onto each electrode with DNA SAMs for 0.5 h. After washing with MilliQ water, 4  $\mu$ L of the anti-Dig-Fabfragment-HRP (0.5 U/mL in 0.1 M PBS buffer with 0.5% casein) was added. Following the incubation for 20 min at room temperature, the electrode was washed with MilliQ water and dried lightly with N<sub>2</sub> prior to electrochemical characterization.

In the detection of multiple DNA targets, 15 probes were immobilized on a chip following the steps of immobilization on gold



**Fig. 2.** (A) Cyclic voltammograms for no target (dashed line) and 10 nM target (solid line) and (B) amperometric curves of target DNA with a series of concentrations (0, 100 pM, 1 nM and 10 nM) in TMB substrate solution. Scan rate of CV: 100 mV/s. Potential of amperometry: 100 mV.

electrodes. In order to prevent non-specific adherent, 20  $\mu\text{L}$  of PEG 3350 (0.05% polyethylene glycol 3350 in 0.1 M PBS) was added to cover all three electrodes of the chip. DNA sample was mixed solution containing four targets. The mixed solution was then incubated with four reporter probes in 1 M phosphate buffered saline and then pre-annealed at 75  $^{\circ}\text{C}$  for 5 min. 2  $\mu\text{L}$  of the solution was placed onto each working electrode of the sensor with DNA SAMs for 0.5 h. After washing with MilliQ water, pipette 20  $\mu\text{L}$  of BSA (1% bovine serum albumin in 0.1 M PBS) to cover all three electrodes. The chip was washed again and 2  $\mu\text{L}$  of anti-Dig-HRP was added. Following an incubation for 20 min at room temperature, the chip was washed with 0.1 M NaCl and 10 mM PB buffer (pH 7.4) and subjected to electrochemical measurements.

2.4. SNP detection using the DNA array

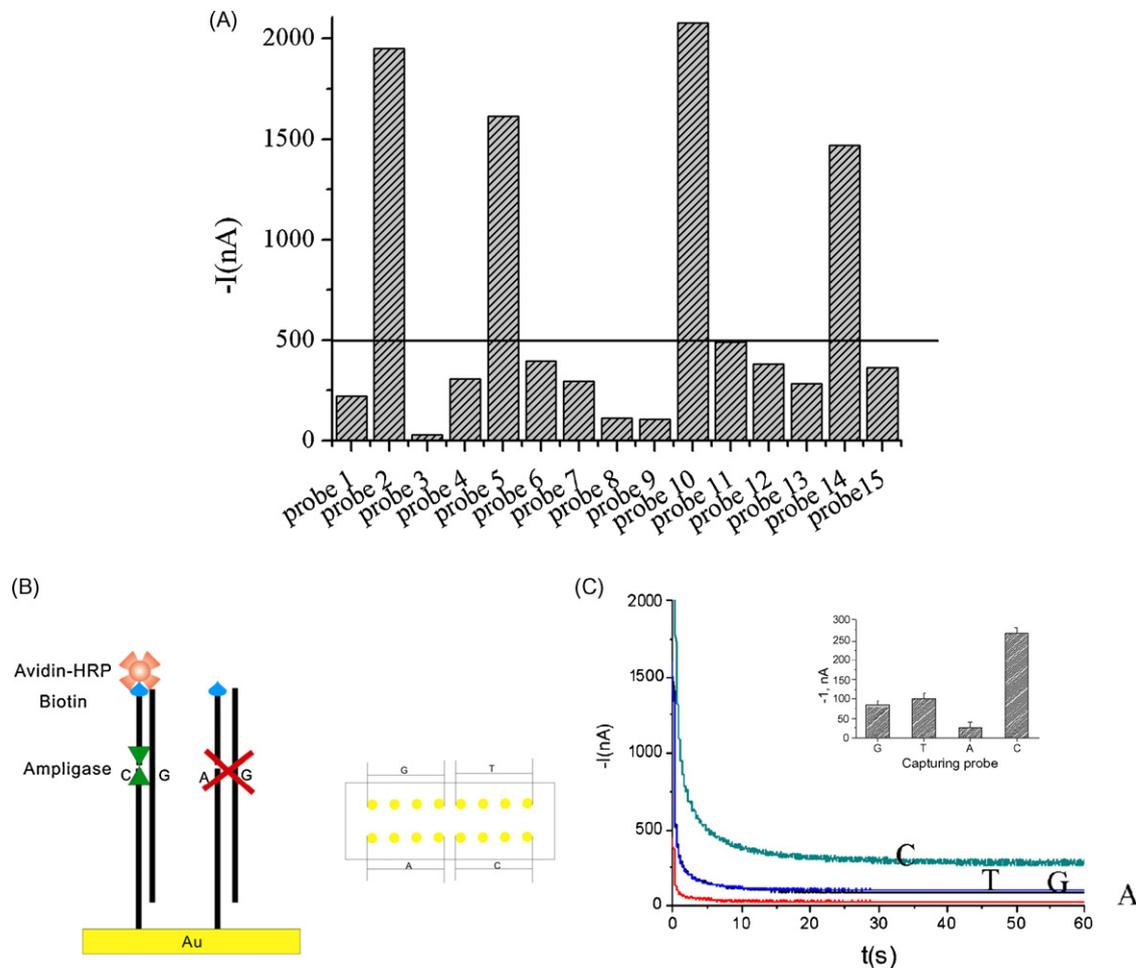
In the detection of SNP, four probes were immobilized on the chip as described above; each probe used four working electrodes. 2  $\mu\text{L}$  of 10  $\mu\text{M}$  each probe in the immobilization buffer with 2 M NaCl was added to a working electrode of the sensor respectively and the plate incubated for 1 h.

2  $\mu\text{L}$  of the mixed solution containing 10 nM target, 100 nM reporter probe, 1 U ampligase and 0.5% BSA in reaction buffer was pre-annealed at 55  $^{\circ}\text{C}$  for 5 min and added to each working electrode of the chip. Then the chip was incubated at 55  $^{\circ}\text{C}$  for 0.5 h

in Constant temperature and humidity Incubator. After washing, it was also treated with 1% BSA and then the avidin-HRP (0.5 U/mL in 0.1 M PBS buffer with 0.5% casein) was added. Following the incubation for 20 min at room temperature, the chip was washed with 0.1 M NaCl and 10 mM PB buffer (pH 7.4) and subjected to electrochemical measurements.

3. Results and discussion

We employed a “sandwich-type” detection strategy (Fig. 1), which involved a capturing probe DNA self-assembled at gold electrodes and a reporter probe DNA labeled with Dig, both of which flank the DNA target sequence, respectively. When the capturing probe, target and the reporter probe formed a sandwich complex, the anti-Dig-HRP could bind to the Dig-labeled reporter probe and catalyzed TMB substrate, producing significantly amplified electrochemical current signals. In our assay, the capturing probe 2 was self-assembled through “Au-S” bond on the gold working electrode. Then pre-annealed solution of the reporter probe 1 and the target DNA 1 was pipetted to the surface of the electrode. After the affinity binding of the anti-Dig-HRP and the reporter probes, the amperometric method was employed to interrogate the hybridization process, with a reduction potential of TMB at 100 mV (versus Au pseudo-reference electrode).



**Fig. 3.** (A) Detection of DNA sample with a 16-electrode DNA array immobilized with 15 capture probes (the line located at 500 nA was used as a cut-off). The signals were the currents of amperometry detection at 100 s. (B) Scheme for the ligase-based SNP detection strategy: the target DNA, capture probe and reporter probe formed a “sandwich” complex. The reporter probe could be washed off the electrode surface in the presence of a single-base mismatch. (C) Amperometric curves for four different capture probes that were complementary to the target DNA with only 1-base variation. Inset: SNP detection with the DNA sensors array: the signals were the amperometric currents at 60 s.

As shown in Fig. 2, CV was employed to characterize the enzyme-based electrocatalytic process. Two pairs of peaks were observed which was ascribed to TMB, the electroactive co-substrate of HRP. In the presence of DNA targets, anti-Dig–HRP could bind to the electrode surface and catalytic peaks were observed, characteristic of the increase of the reduction peak at about 200 mV and diminution of the oxidation peak. Of note, since HRP cannot exchange electrons directly with the electrode, TMB serves as an electron shuttle that diffuse in and out the redox site of HRP.

Amperometry offers a more convenient way to characterize the HRP-catalyzed electrochemical process than CV. As demonstrated in Fig. 2, a decay curve for current ( $I$ ) versus time ( $t$ ) was observed instantly after the onset of the potential, which reached a plateau (steady-state current) within  $\sim 100$  s. We found that the amperometric current ( $\sim 650$  nA) for 10 nM target was significantly larger than that in the absence of the target, which produces a stable background ( $< 50$  nA).

In order to test the multiplexibility of our electrochemical DNA sensor array, we employed 15 probes for the detection of four different targets. In these 15 probes, only four of them are complementary to the corresponding targets. All the probes were self-assembled on a 16-sensor array, which consists of 16 microfabricated sensors, each having a set of working, reference and counter electrodes. This sensor array thus allowed simultaneous detection of multiple targets. The DNA sample containing four targets was mixed with four reporter probes, and then added to the work electrodes on the array. After hybridization, as shown in Fig. 3A, there are four prominent signals that are significantly larger than the background signals ( $> 3S.D.$ ). Of note, the signal for the sensor array was reproducible, with an electrode-to-electrode variation of  $\sim 7.5\%$  and array-to-array variation of  $\sim 12\%$  (data from five independent assays). The relatively large background observed in multi-target assays might be associated with partial pairing between probe and improper targets, which reflected the importance of probe design in array-based DNA assays.

We further tried to perform SNP detection with the electrochemical DNA sensor array. Various strategies have been developed in the discrimination of SNP including restriction-enzyme digestion, allele-specific hybridization, and primer extension by nucleotide incorporation. Ligase is well known to possess very high sequence specificity, thus we couple it with our electrochemical DNA sensor array. As shown in Fig. 3B, the thiolated capturing probe and the biotin labeled reporter probe formed a “sandwich” with the target, the ampligase could ligate the capturing probe and the reporter probe in the absence of mismatches. As a result, when both probes were complementary to the target, they were ligated by the ligase, thus retained at the surface during the subsequent stringent washing steps. In contrast, if there existed one point mismatch, which could be efficiently recognized by the ligase, the reporter probe was not ligated and subsequently washed off in the denaturation process.

We designed four capturing probes, the sequences of which differ only at their 3'-terminal nucleotide, and immobilize them to the sensors, each probe occupying four working electrodes. The

perfectly matched capturing probe could be ligated to the reporter probe while others not. As shown in Fig. 3C, the average signals of  $I-t$  curves clearly showed that the fully complementary target (“C”) produced much higher signals than 1-base mismatched ones.

#### 4. Conclusion

In summary, we reported an electrochemical protocol for the detection of multiple DNA targets with an electrode array, which relied on the “Sandwich-type” detection strategy and the enzyme-based amplification. In order to achieve SNP discrimination, we employed ligase to improve the sequence specificity toward mismatches. We found that the fully complementary target produced significantly higher signal than 1-base mismatched ones in the ligase-based electrochemical DNA assays.

#### Acknowledgements

This work was supported by National Natural Science Foundation (60537030, 20704043), Shanghai Municipal Commission for Science and Technology (0652nm006, 0652nm016, 06ZR14106, 06ZR14136, 0752nm021), National Basic Research Program of China (2006CB933000, 2007CB936000), and Chinese Academy of Sciences.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.07.004.

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