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Fabrication and application of a new DNA biosensor based on on-substrate PCR and electrochemistry

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ABSTRACT

DNA probes immobilized on a gold electrode (AuE) were employed as the primers of asymmetric PCR on the AuE. In the asymmetric PCR process, the DNA probes extended in the presence of target strands in the PCR solution. After PCR the dsDNAs were denaturalized and the target DNAs were eliminated and only the extended probes maintained on the AuE. At last the electrochemical indicator of methylene blue combined to the extended probes and the electrochemical signal of indicator was measured. This signal was higher than that of the AuE modified only by original probe. When there was no target in the PCR solution, the probe did not extend and the signal did not increase. The specific sequences of chitinase gene were detected successfully from four sorts of target with different length: oligonucleotide acid, PCR products, molecule cloning vector DNA and total genome DNA of transgenic capsicum, and the estimated detection limit were 7.3×10^{-12}, 3.2×10^{-11}, 5.4×10^{-11} and 4.1×10^{-10} mol l^{-1} respectively. The regeneration of the biosensor was also tested and the results indicated that its half life was 6 times.

1. Introduction

Among the DNA biosensors, the electrochemical DNA biosensor is able to offer a simple, rapid yet accurate, low-cost method for testing of selected DNA sequences and this has been the topic of considerable interest to many researchers [1–4]. Different forms of electrochemical DNA biosensors have been developed which transduce DNA hybridization using a redox active molecule [5–9]. In many reports, the DNA hybridization events have been detected based on the selective combinations of indicators and single strand DNA (ssDNA) or double strand DNA (dsDNA). However, the specific DNA has been detected only based on the short hybrids region (20–40 mer). In fact, many redox indicators can bind to both ssDNA and dsDNA, such as methylene blue (MB) [10–17].

MB has been reported to combine specifically to the guanine bases and thus a lower current signal is observed upon hybridization since less MB can bind to dsDNA. It is due to the inaccessibility of MB to the guanine bases in dsDNA. This is the basic principle that the DNA detection depend on when using MB as the indicator. Kelley et al. [11] has reported a strategy for the electrochemical detection of single-base mismatches in oligonucleotides, based on charge transport from the intercalated MB through self-assembled monolayers of oligonucleotides immobilized onto the gold electrode surface. Erdem et al. have also investigated the interaction of DNA and MB using carbon paste electrode [13,14,17], gold electrode and also self-assembled alkanethiol monolayer on gold electrodes [15]. Kerman et al. have also performed the electrochemical detection of hybridization based on peptide nucleic acid probes with MB as an electroactive label on carbon paste and SAM modified gold electrodes [15,17]. Our group once studied the DNA detection on a probe DNA/Au nanoparticles modified glass carbon electrode, using calf thymus DNA as dispersant of nanoparticles and MB as the indicator [18]. In these reports, the decrease in the magnitude of the voltammetric reduction signals of MB reflects the extent of the hybrid formation. However, in any real case of genome detection, the target is much longer than the probe. So in hybridizing, a long ssDNA residue is often introduced to the hybrid dsDNA. This ssDNA residue can also combine with MB and lead the signals of MB to increase. This increase would counteract the aforementioned reduction and thus the sensitivity would be limited in the long DNA sequence detection.

In this article, the shortcoming in detection of long DNA sequence is overcome by using a novel strategy of probe extension. That is the electrochemical signal comes from the extended region of the probe rather than from the short hybrid region. The principle is described as: The SH-ssDNA probe is immobilized on a gold electrode (AuE) firstly; The modified AuE is immersed in PCR solution containing target; The probes hybridize with the target and act as the primers of polymerase chain reaction (PCR) on the AuE; The probes extend in the PCR and the dsDNAs are denaturalized and the templates are eliminated and only the extended probes maintain on the AuE and combine with the indicator; A high signal at extended

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probe modified AuE in electrochemical detection is obtained. The length of the probe is tested according to the signal. It is that if the target is longer than the probe, the probe will extended, the MB molecule combining with the probe will increase and a high peak current (i_p) will be obtained. Contrarily, if target DNA absents, or if the target is not longer than the probe, or if the target mismatches with the probe at 3′ end, the probe will not extend, as a result, only a low signal is obtained at this unextended probe modified AuE. Here the target detection does not depend traditionally on the MB's different combination with ssDNA and dsDNA, but originally on the quantity of MB molecules combined with original probe DNA and extended probe DNA.

In general PCR, sequence information from both sides of the desired target locus must be known so as to design appropriate primers. The two primers used are complementary to the target DNA strands, and their 3′ ends orient toward each other. In this study, only one primer is designed according to the sequence of chitinase gene (Chi) and the PCR is actually asymmetric PCR. Because the PCR system is treated with thermal circle (including denaturation, annealing and extension), there always some probes can extend in each thermal cycle even if only the trace amount template exists in the PCR solution. In each cycle, there are certain copies of probe anneal to the target and extend. After one phase of synthesis (elongation or extension), the reaction mixture is heated again (94 °C to dissociate the strands and then cooled to allow the target annealing (65 °C) again with the excess probe. Then the excess probes extend too. When the number of cycles reaches to a certain times, all the probes will completely extend. In our design, the PCR is achieved on the surface of a substrate. It is also named as on-substrate PCR. It is similar as the on-chip PCR in the reports [19–26].

2. Materials and methods

2.1. Instrumentation

Electrochemical systems were performed with an electrochemical workstation CHI660 (USA). A standard three-electrode system containing an Ag/AgCl (saturated KCl) reference electrode, a platinum wire auxiliary electrode, and the modified AuE working electrode was used. The spPCR reagents were purchased from Takara Biotechnology Co. (Dalian, China) and the amplification reactions were performed in a thermal cycler (MyCycler, BIO-RAD, USA).

2.2. Reagents and materials

The following solutions were used: potassium ferricyanide solution ($0.1\,M\,K_3Fe(CN)_6 + 0.1\,M\,K_4Fe(CN)_6 + 0.1\,mol\,l^{-1}$ KCl), Tris–HCl buffer ($10\,mmol\,l^{-1} + 0.1\,mol\,l^{-1}$ NaCl, pH 8.0), TE buffer ($10\,mmol\,l^{-1}$ Tris–HCl buffer + $1.0\,mmol\,l^{-1}$ ethylenediaminetetraacetic acid (EDTA), pH 8.0), PCR buffer ($200\,mmol\,l^{-1}$ Tris–HCl + $500\,mmol\,l^{-1}$ KCl, pH 8.0) and DNA extraction buffer ($100\,mmol\,l^{-1}$ Tris–HCl pH 8.5, $100\,mmol\,l^{-1}$ NaCl, $50\,mmol\,l^{-1}$ EDTA, 2% SDS). Aurichlorohydric acid ($HAuCl_4{\cdot}4H_2O$) was purchased from Shanghai Rare Metal Research Institute (Shanghai, China). Other chemicals used in the experiments were of analytical reagent grade. All chemicals were used without further purification and all solutions were prepared with distilled water. The DNAs as the following were employed:

- The probe sequence: 5′SH-ATG TTGGGTTTCCTCGGAAAATCC-3′.
- The sequence complementary with the probe: 5′-GGATTTTCCGAGGAAACCCAACAT-3′ (cDNA).
- The cDNA with five cytidine nucleosides added to 5′ end: 5′-CCCCC GGATTTTCCGAGGAAACCCAACAT-3′ (acDNA).
- The acDNA with one mismatched nucleoside: 5′-CCCCCT GATTTTCCGAGGAAACCCAACAT-3′ (macDNA, the underlined base is the mismatched base).
- The DNA of the molecule cloning vector pETChi$^+$ (containing the full length of Chi) and pETChi$^-$ (without Chi).
- The PCR products of Chi from pETChi$^+$.
- The genomes DNA from the Chi transgenic capsicums.

All the oligonucleotides were purchased from Sangon Bioengineering (Shanghai, China). Both the molecule cloning vector and the Chi transgenic capsicums were provided kindly by Gansu Agricultural University, China. The capsicums had been identified as the Chi transgenic capsicums [27].

2.3. Experiments

2.3.1. Plant genome DNA extraction

DNA was isolated using sodium dodecyl sulfate (SDS) extraction buffer as described by Edwards et al. [28] and purified with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform. Approximate amount of isolated DNA were determined by loading 5 μl aliquots onto 0.8% agarose gel run in Tris acetate–EDTA (TAE) buffer [29]. DNA purity in the solution was checked by measuring the $UV_{260/280nm}$ absorption ratio [30]. These ratios of capsicum leaf extracts were in the range of 1.8–2.0, indicating that the DNA samples were pure for subsequent PCR analysis.

2.3.2. Preparation of PCR products

The PCR products of Chi were obtained by PCR using molecule cloning vector as the template. For each PCR, 50 μl mixture contains 5 μl PCR buffer, $2.0\,mol\,l^{-1}$ $MgCl_2$, $1.0\,mmol\,l^{-1}$ dNTPs, $1.6 \times 10^{-7}\,mol\,l^{-1}$ primer (each $0.8 \times 10^{-7}\,mol\,l^{-1}$), $0.2\,g\,l^{-1}$ bovine serum albumin, proper magnitude of DNA target and 2 units Taq DNA polymerase. The reaction was subjected to the following thermal cycling in a thermal cycler: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 94 °C for 60 s, anneal at 65 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 1 hour. After PCR reaction, the tube was immersed in 94 °C water for 5 min, and then cooled in ice-water bath promptly to denature dsDNA to ssDNA. This ssDNA was employed as one of the targets for biosensor detecting. Before denaturation the PCR products were tested by agarose gel electrophoresis. Other ssDNA targets were all prepared like as the treatment of PCR products.

2.3.3. Electrode modification, on-substrate PCR and electrochemical detection

The DNA modified electrode was obtained by immersing the AuE in 5′-thiol $ssDNA_p$ solution. The solution was stirred at room temperature for 120 min [31], followed by washing the electrode with distilled water. Prior to modification, the AuE was cleaned and dried according to the following sequence: polish with 0.3 μm alumina powder, wash ultrasonically for 10 min in distilled water, rinse with distilled water, and dry under a nitrogen stream. Here the original probe DNA modified AuE was referred to as DNA_p/AuE.

The on-substrate PCR was carried out as the following: the probe modified AuE was immersed upstraightly in 100 μl on-substrate PCR mixture containing 10 μl PCR buffer, $2.0\,mol\,l^{-1}$ $MgCl_2$, $0.6\,mmol\,l^{-1}$ dNTPs, $0.5\,g\,l^{-1}$ bovine serum albumin, proper magnitude of ssDNA target and 4 units Taq DNA polymerase in a tube. Then the tube was incubated at suitable temperature for 20 min, and followed by incubated at 72 °C in water bath for 20 min. Then the AuE was immersed in 94 °C water for 5 min, and then cooled it in ice-water bath promptly to denature dsDNA to ssDNA. Here the single strand extended probe was shortened as $ssDNA_{ep}$.