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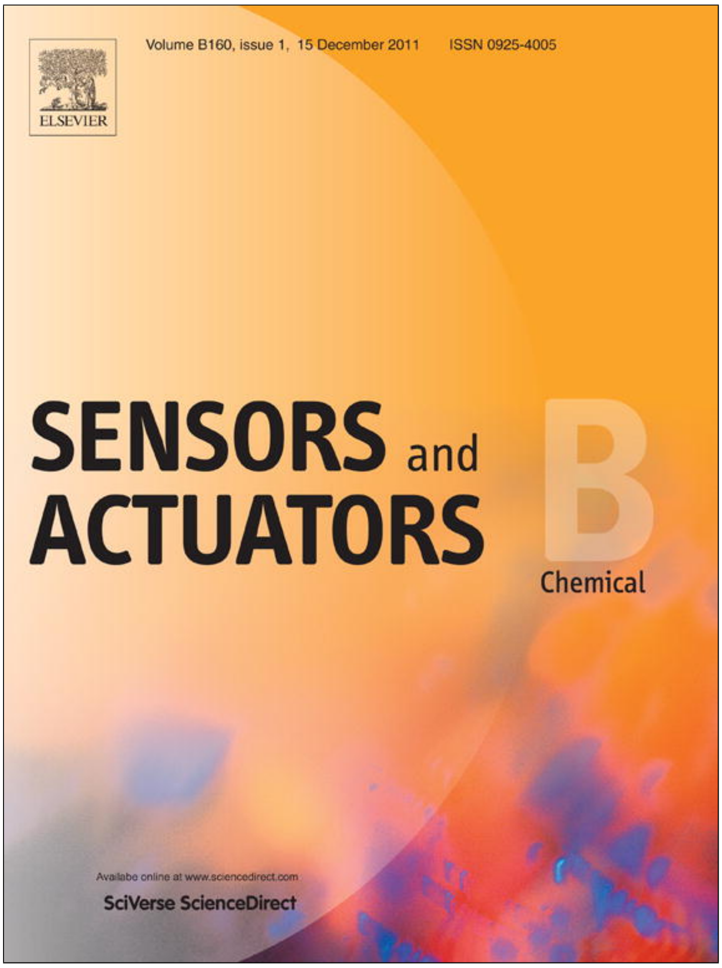
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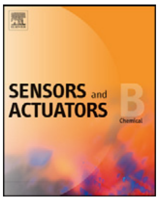

Fabrication and application of a new DNA biosensor based on on-substrate PCR and electrochemistry

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A B S T R A C T

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.

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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′-CCCCC$\underline{T}$ 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, 1.6×10^{-7} mol l^{-1} primer (each 0.8×10^{-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$_{ep}$.

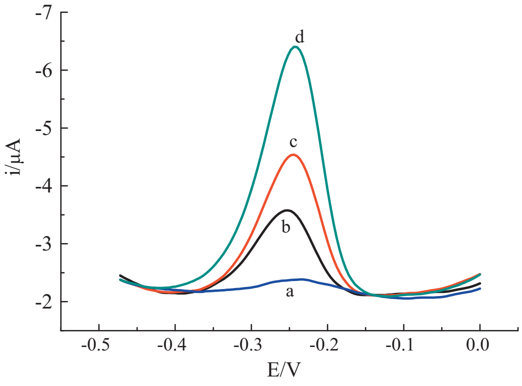


Fig. 1. Differential pulse voltammetry curves of MB in Tris–HCl buffer obtained at (a) bare AuE, (b) the original probe modified AuE, (c) the dsDNA modified AuE after the probe extension, and (d) the ssDNA modified AuE after the probe extension. The scan rate was 100 mV s^{-1}. The target was oligonucleotide acid with concentration of 17 μmol l^{-1}.

Here the extended probe DNA modified AuE was referred to as DNA$_{ep}$/AuE.

The surface of the electrode was characterized by cyclic voltammetry (CV) at room temperature in ferricyanide potassium solution and Tris–HCl buffer containing 20 mmol l^{-1} MB, using acDNA as the target. The three electrodes of modified work electrode, Ag/AgCl versus electrode and platinum reference electrode were immersed in ferricyanide potassium solution, and scanned between the potential −0.1 and 0.6 V at a scan rate of 100 mV s^{-1}. The detection of DNA extension was carried out by differential pulse voltammetry (DPV) in MB-free Tris–HCl buffer at a scan rate of 100 mV s^{-1}. Before each detection in Tris–HCl buffer, the modified electrodes were immersed into 20 mmol l^{-1} MB solution for 5 min and thoroughly rinsed [13,16], then the same three electrodes were immersed in Tris–HCl buffer and was scanned between the potential −0.6 and 1.0 V at a scan rate of 100 mV s^{-1}.

3. Results and discussion

3.1. The DPV detection of oligonucleotide sequence

Before DPV detection, the response of the modified electrode to Fe$^{3+/2+}$ and MB was tested and the results indicated that the sensors were fit for DNA detection. The nucleotide acids were taken as the targets firstly with DPV technique. The anodic peak currents (i_ps) were recorded in every experiment. The results are shown in Fig. 1. Curve a was at the bare electrode and on it hardly any response to MB was found. Curve b was at the electrode modified with the original probe (DNA$_p$/AuE), on it a clearly increased i_p was found. Curve c was at the dsDNA modified electrode on which the asymmetric PCR process was achieved but the process of denaturalization was not carried out. On it, though the probe was extended, the immobilized DNA was still dsDNA, and because the guanines of dsDNA region were concealed, so the increment of i_p was not tremendous. Curve d was at the extended probe (ssDNA) modified electrode (DNA$_{ep}$/AuE), on which the target was eliminated and only extended probe DNA was maintained, and the maximum i_p was obtained. Comparing curve d and curve b, the immobilized DNAs were all ssDNA. The only difference was none but the length of DNA chain. The clearly increased i_p was attributed to nothing but the longer DNA chain on DNA$_{ep}$/AuE than that on DNA$_p$/AuE. The same experiment was also done by using the oligonucleotide acids of cDNA and macDNA as the target. A series of three repetitive measurements resulted reproducible results. The results indicated the sensor was efficacious in identifying acDNA from cDNA, macDNA and the others.

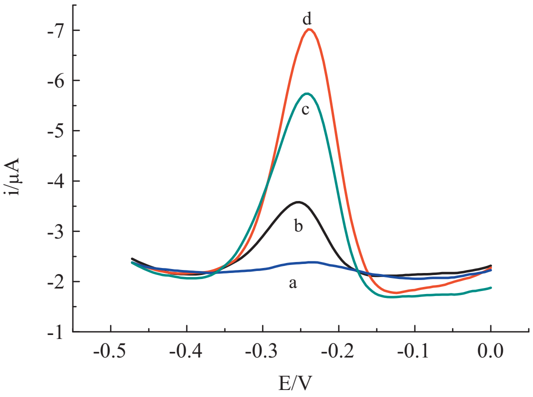


Fig. 2. Differential pulse voltammetry curves of MB in Tris–HCl buffer obtained at (a) bare AuE, (b) the original probe modified AuE, (c) the dsDNA modified AuE after the probe extension, and (d) the ssDNA modified AuE after the probe extension. The scan rate was 100 mV s^{-1}. The target was PCR products.

3.2. The DPV detection of PCR products and molecule cloning vector DNA

The PCR products were the whole sequence of Chi with the length of about 1300 bp. The molecule cloning vector DNA (pETChi$^+$, length of 5946 bp) contains the whole sequence of Chi. Both targets were much longer than the oligonucleotide acid. In the detection of them (Fig. 2 for PCR products and Fig. 3 for pETChi$^+$), similar trends of the i_p change similar as Fig. 1 were obtained, indicating the method was also efficacious in detecting long chain target DNA. But because both targets were much longer than acDNA, after PCR the dsDNA region and the extended portion were much longer than that of Fig. 1. As a result, much more increased i_p was obtained compared to the corresponding curve of Fig. 1. In gene transformation studies, the molecule cloning vector DNA was involved in many processes including gene cloning, gene expression and gene transformation. The detection of special DNA sequence from a molecule cloning vector was absolutely necessary. In the experiments, Chi gene was integrated with the DNA of its molecule cloning vector. The detection of Chi from the pETChi$^+$ was much more approaching to the real case of genome detection. To investigate the selectivity of the sensor, the same cloning vector DNA without Chi (pETChi$^-$) was employed as the reference, and this time, a similar magnitude of i_p at DNA$_{ep}$/AuE as that at DNA$_p$/AuE was obtained (not illustrated). Under this situation, DNA hybridization event did not occur for the absence of the complementary sequence. Therefore, the probe

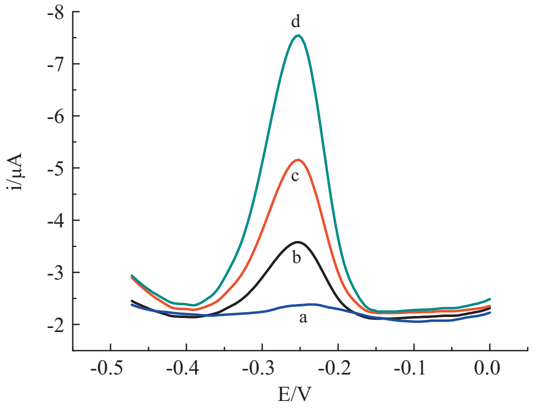


Fig. 3. Differential pulse voltammetry curves of MB in Tris–HCl buffer obtained at (a) bare AuE, (b) the original probe modified AuE, (c) the dsDNA modified AuE after the probe extension, and (d) the ssDNA modified AuE after the probe extension. The scan rate was 100 mV s^{-1}. The target was molecular cloning vector.

Table 1
The mean DPV values of ten data for each target at different modified electrodes in detection of transgenic plants' genome DNA.

Target DNA	i_p (μA)		
	Probe	Polymerase reaction (dsDNA)	Denaturalized (ssDNA)
No. 1	1.61 ± 0.14	3.77 ± 0.22	5.32 ± 0.24
No. 2	1.61 ± 0.16	3.54 ± 0.21	5.32 ± 0.21
No. 3	1.62 ± 0.16	3.67 ± 0.24	5.38 ± 0.23
No. 4	1.63 ± 0.15	3.56 ± 0.21	5.39 ± 0.22
Control	1.61 ± 0.15	1.55 ± 0.13	1.54 ± 0.15

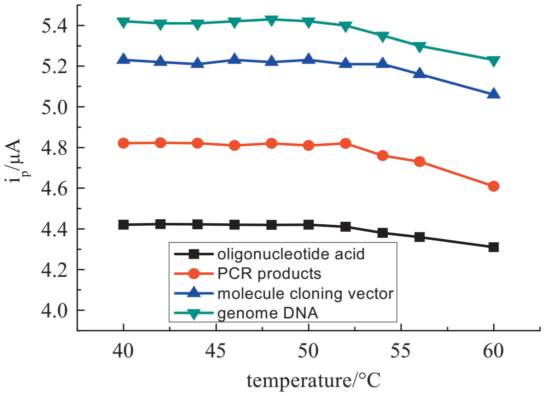


Fig. 4. The relationship of DPV peak current and the annealing temperature. The target was PCR products.

was not elongated in the following PCR. All of above experiments indicated that the DNA chain elongation had enhanced the MB accumulation, and led to increment of DPV signal, meaning that the target DNA (the template) was recognized selectively. The results agreed with the results of CV.

3.3. *The DPV detection of Chi from transgenic plant genome DNA*

The detection of a specific DNA sequence from a genome is a very important work in study of clinic, quarantine and genetically modified organism. The aim of our study is to establish a sensitive method to detect the Chi from genetically modified plants and foods. In this experiment five genomes from four transgenic plants and one controlled plant were extracted and employed as the targets. The DPV results were shown in Table 1. As shown in the table, for the transgenic plant genome DNA (No. 1–4), the i_ps of the dsDNA after PCR were all increased compared to that of the original probe. The deductive reason was that the long dsDNA region formed in the PCR process combined much more MB. After the dsDNA denatured and the targets DNAs were eliminated, all the i_ps increased clearly. The results indicated that in each plant genome, there was the Chi integrated, and the plants were all transgenic plants. Meanwhile the control did not show any i_p increase in every process.

In our tests, each experiment was held three times (except for the tests of Table 1 and Supplementary Table 1, in which each experiment repeated ten times), and the data showed a good consistency.

3.4. *Optimization of experimental conditions*

In order to obtain the best experiment effect, a series of experiments had been done to optimize the experimental conditions, including annealing temperature, annealing time and concentration of dNTP.

In on-substrate PCR, low temperature will enhance the hybridization but companied by the enhancement of mismatch, and high temperature will enhance the selectivity but companied by the decrease of the hybridization. In PCR, T_m (temperature at which 50% of a given oligonucleotide is hybridized to its complementary strand) is used to evaluate the annealing temperature. For a primer less than 25 bp, the equation of T_m ($^\circ$C) = 4(G + C) + 2(A + T) ± 5 is adopted to calculate the T_m. The result is shown as Fig. 4. At low temperature less than 50 °C, the peak currents for four targets were all invariable, meaning that all the probes had hybridized, including mismatch. From 50 °C to higher temperature, the peak currents started to decrease, meaning that mismatch was not happened again. From 52 °C the decrease was much more obvious, meaning that the matched hybridization had been effected by high temperature. So the other detections of experiment were carried out at annealing temperature of 52 °C.

Annealing time can also characterize the efficiency of hybridization. The peak currents were tested at annealing time of 2 min, 5 min, 10 min and 20 min using PCR products as the target. The result is shown as Fig. 5. At short annealing duration of 2 min, both the peak currents at low T_m (from 50 °C down to lower one) and high T_m (from about 50 °C up to higher one) decreased. The former was maybe because of the effect on the combination of DNA and DNA polymerase while the later was maybe because of the efficiency of matched hybridization. At low T_m, mismatched hybridization could effect on the combination of DNA and DNA polymerase. At high T_m, the efficiency of matched hybridization was affected. This is weighty supported by the results at longer annealing time: as the increase of duration, the aforementioned decrease becomes insensitive. When it up to 20 min, the invariable and highest peak currents at low T_m was obtained, meaning that all the probes had hybridized (including mismatch) and extended. The system was actually a variable balance of fully matched hybridization and mismatched hybridization. At higher T_m the peak currents were affected and decreased only in some sort, meaning that at 50 °C, the mismatched hybridization had not happened anymore, and as the increase of duration from then on, the matched hybridization was affected gradually, and as a result, the peak currents decreased correspondingly. So the other detections of experiment were carried out at annealing time of 20 min.

The concentration of dNTPs has the effect on the efficiency of extension and the stability of detection. As in Fig. 6, the relationship of peak current and concentration of dNTPs is a "S" shape. At low concentration, low peak currents were obtained, meaning there was little extension. Then in the range of from 0.4 to 0.8 mmol l^{-1} dNTPs, the peak current and dNTPs exhibited positive linear relation. From then on, the peak current was quickly up to a flat phase. Though at this phase the maxima of peak current were obtained, but the stability was lost. This can be seen from the increase of the

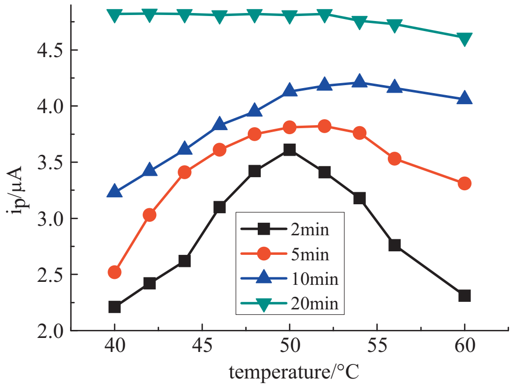


Fig. 5. The relationship of DPV peak current and the annealing time. The target was PCR products.

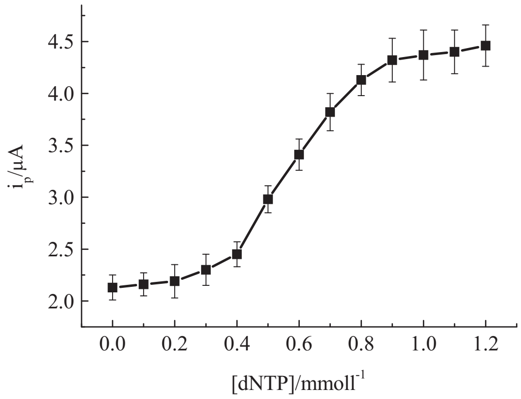


Fig. 6. The relationship of DPV peak current and the concentration of dNTPs. The target was PCR products.

standard deviation. So the detection of the experiment selected the middle dNTPs concentration of 0.6 mmol l^{-1}.

3.5. Detection limit

The sensitivity is the key issue in any DNA detection. In our study the relationship of target concentration and i_p was investigated by varying the target concentration over the range of 6.4×10^{-11} to 5.0×10^{-6} mol l^{-1}. The result was shown as Fig. 7. For all targets, enhanced peak currents were obtained at every target concentration after chain extension, and the peak currents increased with the target DNA concentration increasing. Another, the peak currents after chain extension for long chain target (pETGlu$^+$ and genome DNA) were higher than that for short chain target (acDNA) at every concentration except for at 6.4×10^{-11} mol l^{-1}, at this concentration, the peak current for acDNA was higher than that for pETChi$^+$. This maybe because that at low concentration, the probe was more easy to hybridize with short target chain than with long target chain. The detection limit of 7.3×10^{-12}, 3.2×10^{-11}, 5.4×10^{-11} and 4.1×10^{-10} mol l^{-1} for acDNA, PCR products, pETChi$^+$ and genome DNA can be estimated using 3σ (where σ is the standard deviation of a blank solution, $n = 10$). The sensitivity is superior to or similar as the reports of DNA detection based only on hybridization [5,15,32,33]. In this detection the sensitivity was ensured by magnifying the signal using probe extension. Although the sensitivity was ensured and improved in some sort, it was not improved greatly. The reason was that only a single circle extension was adopted in the experiments. If multi circle PCR is adopted, the sensitivity would be much more improved, by which, a trace mount DNA can be amplified to mass quantity of DNA [34,35].

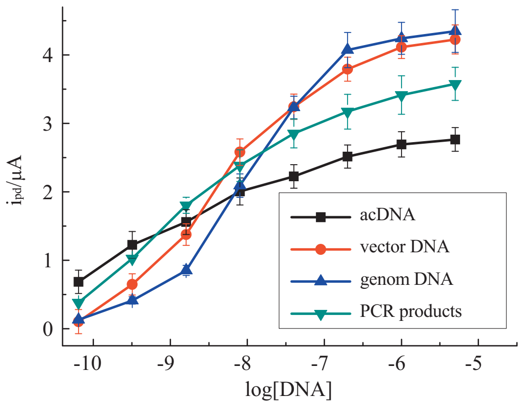
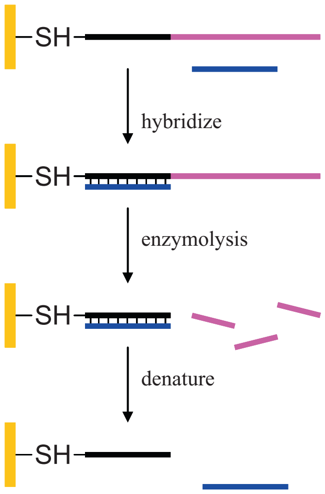


Fig. 7. The relationship of i_{pd} and the concentration of the target DNA. i_{pd} is the difference of the peak current at the original probe modified AuE (DNA$_p$/AuE) and the extended probe modified AuE (DNA$_{ep}$/AuE).



Scheme 1. The principle of the regeneration of the biosensor.

3.6. The regeneration of the biosensor

The regeneration of the biosensor was tested as Scheme 1 by using S1 nuclease and using PCR products as the target. In briefly, the biosensor after the detection was immersed into buffer containing 10 mmol l^{-1} cDNA for 30 min at room temperature, followed by immersed into buffer containing 4 units S1 nuclease for 2 h at 37 °C. Then the electrode was thoroughly rinsed and used in the target detection. The results indicated that the peak current decreased after each regeneration, and after being regenerated for 6 times, the peak current decreased to about half of the first detection, meaning that the half life of the biosensor was 6 times. The half life of the biosensor is related to the method of probe immobilization. Two main methods have been reported in probe immobilization, the one is covalent immobilization as this paper [36], the other is using biotin–avidin system [24]. In covalent immobilization, the efficiency of the hybridization between probe and target declines. It is because that the probe is tightly suppressed to the electrode. Adessi reported, adding additional nucleotide at 5′ end of probe could improve the efficiency of PCR. They obtained good effect by adding 5 repeats of G–C at 5′ end of probe in on-chip PCR [36]. The result was supported by Bier group [24] and Huber group [37,38]. However, the size of the immobilized molecule group has an effect on immobilization. In this aspect, the covalent method is superior to others.

4. Conclusions

Series of stepwise experiments of determination of DNA using on-substrate PCR are described here. The on-substrate PCR makes it reality in trace amount DNA detection. Specific target DNA sequences were detected successfully by using DNA covered AuE. The detection limit of 7.3×10^{-12}, 3.2×10^{-11}, 5.4×10^{-11} and 4.1×10^{-10} mol l^{-1} for acDNA, PCR products, pETChi$^+$ and genome DNA were estimated, respectively. The method was succeed in transgenic plant detection and was hopeful to be improved to a sensitive method in gene detection from transgenic plants and foods.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2011.08.034.

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Biography

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