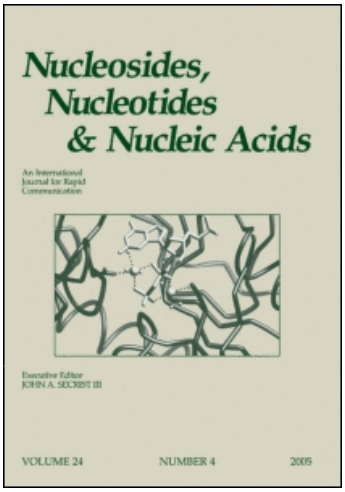

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AN ELECTROCHEMICAL ASSAY OF β-1,3-GLUCANASE GENE FROM TRANSGENIC CAPSICUM USING ASYMMETRIC PCR

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□ *5′-Thiol-derivatized specific DNA probes were added to the single primer polymerase chain reaction (asymmetric PCR) solution. In the PCR process, the DNA probes extended in the presence of target; the extended probes were then immobilized on a glassy carbon electrode (GCE) via gold nanoparticles. Finally, methylene blue and the extended probes were combined and the electrochemical signals were measured. This signal was higher than that of the GCE modified only by the original probe. When there was no target in PCR solution, the probe did not extend and the signal did not increase. The specific sequences of the β-1,3-glucanase gene were detected successfully from three targets with different length: oligonucleotide, molecule clone vector DNA, and total genome DNA of transgenic capsicum. The detection limits of* 2.6×10^{-13}, 7.8×10^{-13}, *and* 9.1×10^{-13} *moll*$^{-1}$ *for oligonucleotide, molecule clone vector DNA, and total transgenic capsicum genome DNA were estimated.*

Keywords Probe extension; DNA assay; β-1,3-glucanase gene; genetically modified organism; methylene blue

1. INTRODUCTION

In plant gene transformation studies, DNA detection is involved in all of the processes including gene clone, construction of vector, and gene expression. As more attention has been paid to the security of the genetically modified organism, a highly sensitive method becomes a concern. The electrochemical detection method based on hybridization is now favored

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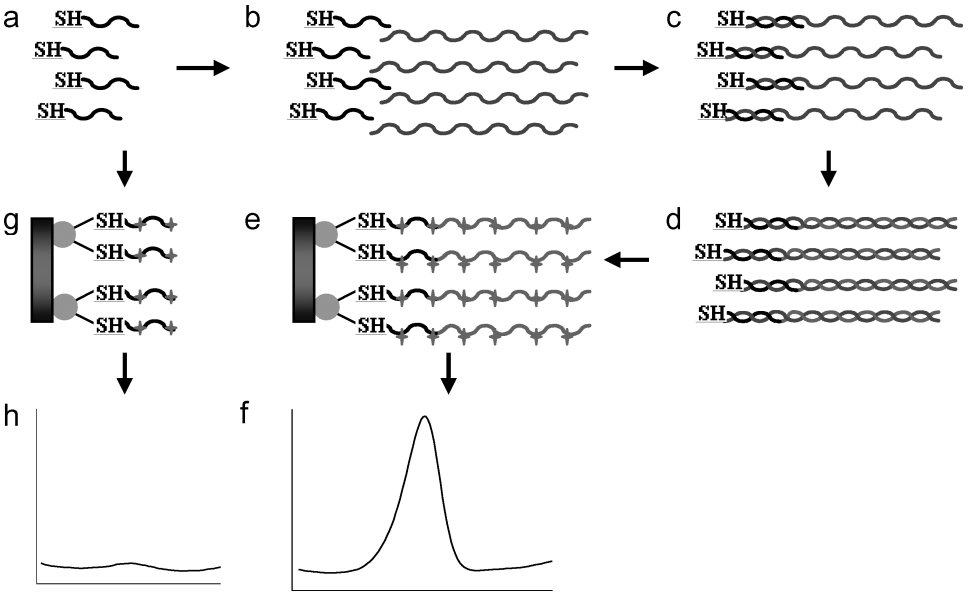
as a good alternative for the inherent advantages of low cost, high sensitivity, miniaturization, and capability of producing portable electrochemical sensors.[1–5] In many reports, the DNA hybridization events have been detected based on the different combinations of indicators and single strand DNA (ssDNA) or double strand DNA (dsDNA). Moreover, the specific DNA has been detected only based on the short hybrids region (20∼40 mer). In fact, many redox indicators, such as methylene blue (MB),[6–9] combine with both ssDNA and dsDNA.

MB has been reported to combine specifically with the guanine bases[9–11] and thus a lower current signal is observed upon hybridization since less MB can bind to dsDNA. The reason is the inaccessibility of the guanine residues in dsDNA. This is the basic principle on which DNA detection depends when using MB as the indicator. Kelly 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 on the gold electrode surface.[8] Erdem has also investigated the interaction of DNA and MB using a carbon paste electrode,[2,9,12] a gold electrode,[13] and a self-assembled alkanethiol monolayer on gold electrodes.[11] Their group has also performed the electrochemical detection of hybridization based on peptide nucleic acid probes with MB as the electroactive label on carbon paste and SAM-modified gold electrodes.[14]In these reports, the decrease in the magnitude of the voltammetric signal 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. After hybridization, there would be a long single strand DNA residue. This single strand DNA residue can also combine with MB and lead the signals of MB to increase, which would counteract the aforementioned reduction and thus the sensitivity would be limited in the long DNA sequence detection.

In this article, the potential shortcoming in the detection of long DNA sequences is overcome by using probe extension. The electrochemical signal comes from the extended region of probe rather than from the short hybrid region. The SH-ssDNA probes (Scheme 1a) are added to the polymerase chain reaction (PCR) solution (Scheme 1b) and hybridized with the template (Scheme 1c). These probes are the primer of PCR and extend in PCR (Scheme 1d). After PCR, the dsDNA is denatured and the extended probe is immobilized on the gold nanoparticle (AuNPs) modified GCE and the indicator molecules combine with the immobilized probe (Scheme 1e); at last, electrochemical detections are achieved (Scheme 1f). In the same way, the original probe treated by PCR in the absence of dNTP is also immobilized on a GCE (Scheme 1g) and serves as the reference (Scheme 1h). The test of the length of the probe depends on the magnitude of the electrochemical indicator, that is, MB. If the target is longer than the probe,



SCHEME 1 The principle of experiments: a) the SH-ssDNA probe; b) the probe is added to asymmetric PCR solution; c) the probe hybridize with the template; d) the probe is extended by PCR; e) the extended probe is immobilized on gold nanoparticles modified GCE after dsDNA denaturalizaton and template elimination and the indicators are combined with the DNA; f) the high signal of electrochemical detection; g) the original probe is immobilized on GCE in same way as panel e; and h) the low signal of electrochemical detection.

the probe will extend and the MB molecule combining with the probe will increase, and thus a high peak current (i_p) will be obtained. On the contrary, if there is no target DNA, if the target is not longer than the probe, or if the target is mismatched with the probe at the 3′ end, the probe will not extend.

In general PCR, sequence information from both sides of the desired target locus is known to design appropriate primers. The two primers used are complementary to the target DNA strands, and their 3' ends are oriented toward each other. In this study, only one primer is designed according to the sequence of the β-1, 3-glucanase gene (Glu). The PCR in the study is actually an asymmetrical PCR, and only a single flank primer is applied (it is abbreviated here as spPCR). Because the PCR system is conducted with thermal cycling, some probes can always be extended in each thermal cycle (including denaturation, annealing, and extension) even if only a trace amount of template exists in the PCR solution. In each cycle, some copies of probe preferentially anneal to the target and extend by a heat-stable DNA polymerase. After one phase of synthesis (elongation or extension phase), the reaction mixture is heated again (94°C) to dissociate the strands and then cooled to allow reannealing (65°C) of the target with the excess probe. Then the excess probes also extend and the incompletely extended probes

in the preceding extend again (72°C). When the number of cycles reaches a certain point, the probes will completely extend.

In DNA electrochemical biosensor design, a major challenge is how to immobilize enough probe onto the electrode. The adequately immobilized probe can amplify the detection of the duplex generated between the analyzing primer and the target DNA. In prior research, the adoption of AuNPs has shown high efficiency in improvement of probe immobilization and detection sensitivity.[15–21] AuNPs can bind with the amido of an organic compound tightly through noncovalent electrostatic adsorption and can form a powerful Au-S covalent bond with hydrosulfuryl.[22] In this way, the AuNPs can integrate with the biologically active components. A probe modified by AuNP can be used in detection of the biological system. In DNA sequence detection, the AuNPs can act not only as the immobilizing carrier to increase the amount of probe immobilized on substrate, but also serve as the signal particle to magnify the detection signal and enhance the accuracy of detection. Nucleic acid-functionalized metallic AuNPs used as catalytic labels can amplify electrochemical[23] detection of DNA. In our experiments, AuNPs were taken as the immobilizing carrier by which only the 5′-thiol probe was immobilized on the electrode and thereby the extended probes were isolated from the compound of PCR. The aim was to develop a DNA biosensor to detect the β-1,3-glucanase gene (Glu) in transgenic plants and transgenic foods.

2. MATERIALS AND METHODS

2.1. Instrumentation

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

2.2. Reagents and Materials

The following solutions were used: potassium ferricyanide solution (0.1 $moll^{-1}$ $K_3Fe(CN)_6$ + 0.1 M $K_4Fe(CN)_6$ + 0.1 $moll^{-1}$ KCl), Tris–HCl buffer (10 $mmoll^{-1}$+ 0.1 $moll^{-1}$ NaCl, pH 8.0), TE buffer (10 $mmoll^{-1}$ Tris–HCl buffer +1.0 $mmoll^{-1}$ ethylenediaminetetraacetic acid (EDTA), pH8.0), PCR buffer (200 $mmoll^{-1}$ Tris-HCl+500 $mmoll^{-1}$ KCl, pH 8.0) and DNA extraction buffer (100 $mmoll^{-1}$ Tris–HCl pH 8.5, 100 mM NaCl, 50 $mmoll^{-1}$ EDTA, 2% SDS). Aurichlorohydric acid ($HAuCl_4.4H_2O$) was

purchased from Shanghai Rare Metal Research Institute (Shanghai, China). Other chemicals used were analytical reagent grade. All chemicals were used without further purification and all solutions were prepared with doubly distilled water. The DNAs as the following were employed:

- 5′-thiol specific probe sequence: 5′SH-ATGGCTGCTATCACACT-3′.
- sequence complementary with the probe: 5′-AGTGTGATAGCAGCCAT-3′ (cDNA).
- cDNA with five cytidine nucleosides added to the 5′ end: 5′-CCCCC AGTGTGATAGCAGCCAT-3′ (acDNA).
- acDNA with one mismatched nucleoside: 5′-CCCCCTGTGTGA TAGCAGCCAT-3′ (macDNA, the underlined base is the mismatched base).
- DNA of the molecule clone vector $pETGlu^+$ (containing the full length of Glu) and $pETGlu^-$ (without Glu).
- genomes DNA from the Glu transgenic capsicums.

All the oligonucleotides were purchased from Sangon Bioengineering (Shanghai, China). Both the molecule clone vector and the Glu transgenic capsicums were provided by Gansu Agricultural University, China. The capsicums had been identified as the Glu transgenic capsicums.[24]

2.3. Experiments

2.3.1. DNA Extraction

DNA was isolated using SDS extraction buffer as described by Edwards et al.[25] and purified with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform before PCR. Approximate amounts of isolated DNA were determined by loading 5 μl aliquots onto 0.8% agarose gel run in TAE buffer.[26] DNA purity in the solution was checked by measuring the UV 260/280 nm absorption ratio.[27] These ratios of capsicum leaf extracts were in the range of 1.8 to 2.0, indicating that the DNA samples were pure for subsequent PCR analysis.

2.3.2. PCR Protocol

For each PCR experiment, the 100 μl mixture contained 10 μl PCR buffer, 2.0 $moll^{-1}$ $MgCl_2$, 1.0 $mmoll^{-1}$ dNTPs, 0.8×10^{-7} $moll^{-1}$ probe (the primer), 0.5 gl^{-1} bovine serum albumin, proper magnitude of DNA target and 6 units Taq DNA polymerase. The following thermal cycling in a thermal cycler was used: initial denaturation at 95°C for 4 minutes, 30 cycles of denaturation at 94°C for 30 seconds, anneal at 65°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 1 hour. After the PCR reaction, the tube was immersed in 94°C water for 5 minutes, and then cooled in ice-water bath promptly to denature dsDNA to

ssDNA. Here the single strand extended probe was shortened as $ssDNA_{ep}$ (the original probe DNA was shortened as $ssDNA_p$).

2.3.3. Electrode Modification and Electrochemical Detection

The AuNPs modified GCE (AuNP/GCE) was obtained according to the literature.[28] AuNPs were deposited on the surface of a GCE by electrodeposition from a gold solution. The procedure involves applying a constant potential of −200 mV for 60 seconds in a solution of 1.2 μmoll^{-1} $HAuCl_4$. The solution was prepared in distilled water and deoxygenated by purging with N_2 for approximately 15 minutes. Prior to modification, the GCE was cleaned and dried according to the following sequence: polish with 0.3 μm alumina powder, wash ultrasonically for 10 minutes in distilled water, rinse with distilled water, and dry under a nitrogen stream.

The DNA modified electrode was obtained by immersing the AuNP/GCE in 5′-thiol $ssDNA_p$ or 5′-thiol $ssDNA_{ep}$ solution. The solution was stirred at room temperature for 120 minutes,[18] followed by washing the electrode with distilled water. Thus, the ssDNA was immobilized on the AuNP/GCE. The DNA modified AuNP/GCEs were referred to as DNA_p/AuNP/GCE and DNA_{ep}/AuNP/GCE, respectively.

The surface of the electrode was characterized by cyclic voltammetry (CV) at room temperature in ferricyanide potassium solution and Tris–HCl buffer. The three electrodes of the modified work electrode, the Ag/AgCl reference electrode and the platinum auxiliary 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. In the detection of MB in Tris–HCl buffer, the potential was between −0.6 and −0.1 V and, before each detection, the modified electrode was immersed into 20 mM MB solution for 5 minutes and thoroughly rinsed.[9,10,29,30]

The detection of DNA extension was carried out by CV and differential pulse voltammetry (DPV) in Tris–HCl buffer as above at a scan rate of 100 mV/s.

3. RESULTS AND DISCUSSION

3.1. The Modification of Electrode

Figure 1 shows CV curves in $[Fe(CN)_6]^{3-/4-}$. As can be seen, the bare GCE electrode showed a pair of $[Fe(CN)_6]^{3-/4-}$ diffusion peaks (Figure 1, curve a). After Au colloid was deposited on the GCE electrode, the comparable peaks were also obtained (Figure 1, curve b), and the peak currents of both cathodic and anodic peak increased compared with that of bare GCE. This is attributed to the large surface area of the gold nanoparticles. The deposit of Au colloid on GCE enlarged the area of the electrode. The real surface area of AuNP/GCE in our experiment was calculated to

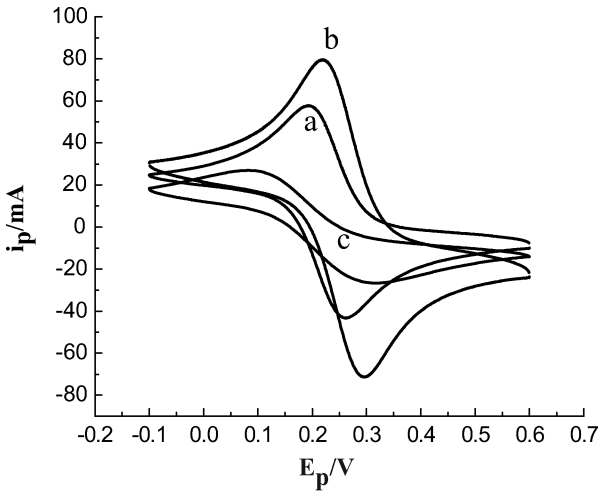


FIGURE 1 Cyclic voltammetry curves in 0.1 $moll^{-1}$ potassium ferricyanide in 0.1 $moll^{-1}$ KCl solution, obtained at: a) bare GCE; b) gold nanoparticals modified GCE; and c) probe/gold nanoparticles modified GCE. Scan rate is 0.1 mVs^{-1} (vs. Ag/AgCl).

reach 0.346 cm^2 according to the Randles-Sevcik equation $i_p = 2.69 \times 10^5 n^{3/2} A D_0^{1/2} v^{1/2} C_0$ (i_p, peak current; n, electron number for transfor; A, area of electrode; D_0, diffusion coefficient; v, scan rate; C_0, concentration of $[Fe(CN)_6]^{3-/4-}$). However, the real surface area of the bare GCE was 0.126 cm^2. Here the diffusivity of ferricyanide potassium was estimated according to Bard and Faulkner.[31] It was obvious that the AuNP/GCE had a large surface area for gold nanoparticles immobilization. At the probe-modified AuNP/GCE (Figure 1, curve c), the corresponding redox peaks decreased greatly. As both the phosphate group of the probe and $[Fe(CN)_6]^{3-/4-}$ have negative charges, it is likely that electrostatic repulsive interactions between the phosphate group of the modified electrode and $[Fe(CN)_6]^{3-/4-}$ dominate limiting the approaching of $[Fe(CN)_6]^{3-/4-}$ to the electrode.

To demonstrate that MB was bound to the probe DNA but not other components of the electrode, the electrochemical response to MB was measured by CV in Tris–HCl buffer. Before modification, the probe was treated with PCR with the absence of dNTP. CV curves of MB on the DNA_p/AuNP/GCE were obtained at different scanning rates (see Figure 2). At every scan rate, the CV response was stable in successive cycling. With the increase of scan rate, the peak current of MB increased. The inset of Figure 2 showed the slope of the peak currents vs. the square root of the scan rate. The relationship between them is linear in the range of 20 to 160$(mVs^{-1})^{1/2}$, with correlation coefficient $r = 0.995$, which illustrates that the process of the electrode reaction is controlled by the diffusion of MB. It was a typical characteristic curve of immobilized species.[31,32] The purpose

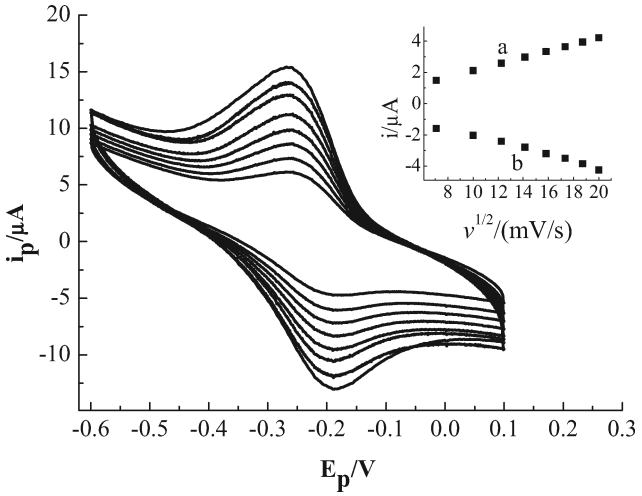


FIGURE 2 Cyclic voltammogrmns of the MB on modifled electrode in Tris-HCl buffer at different scan rates of 50, 100, 150, 200, 250, 300, 350, 400 mV/s (from inside to outside). The inset is a plot of anodic (a) and cathodic (b) peak currents versus square root of scan rate ($v^{1/2}$).

of the 5′-thiol modified on probe was to immobilize the probe onto AuNP covered GCE by S-Au bond. Figures 1 and 2 strongly support the fact that the probe is immobilized on the surface of the electrode.[11,22,33]

3.2. The Detection of Oligonucleotide Sequence

The three targe DNAs in this experiment are an oligonucleotide, molecule clone vector DNA and the total genome DNA of transgenic capsicum. The oligonucleotide includes 17 bases matched with Glu gene and five cytidine nucleosides. The molecule clone vector DNA is composed of the Glu gene (about 1.5 kb) and vector DNA (about 4.1 kb). The total genome DNA of transgenic capsicum is the total plant DNA with the Glu gene integrated. To investigate the effect of the PCR in detection of Glu, the CV response to MB was employed while using the oligonucleotide as the initial target. Figure 3 shows CV curves of MB on different electrodes. At bare electrode (Figure 3, curve a), no distinct redox peak current was found. However, clear pairs of the redox peak current were found at modified electrodes. Figure 3, curve b, is the electrochemical response of the AuNP/GCE. The i_p increase was attributed to the adsorption of MB on the AuNP surface. The MB cation that adsorbed electrostatically on the surface of the electrode by AuNP produced well-defined signals.[22] Figure 3, curve c, is the electrochemical response of the DNA_p/AuNP/GCE, at which higher redox peak currents than that at the AuNP/GCE were found. This is because after the immobilization of the ssDNA, many more free guanine bases of DNA molecules were introduced to the electrode which had a

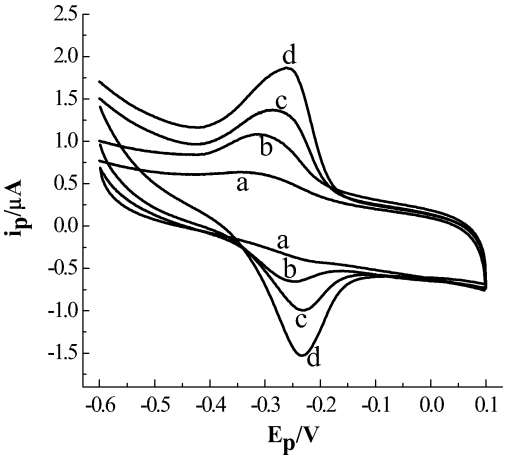


FIGURE 3 Cyclic voltammetry curves of 20 mM MB in Tris–HCl buffer (10 $mmoll^{-1}$+ 0.1 $moll^{-1}$ NaCl, pH 8.0), obtained at: a) bare GCE; b) the AuNP modified GCE; c) the original probe/AuNP modified GCE; and d) the extended probe/AuNP modified GCE in detection of oligonucleotide acid. Scan rate is 0.1 Vs^{-1} (vs. Ag/AgCl). The PCR cycling number is 30.

strong affinity with MB at the ionic concentration of the experiment.[11,33] Thus, the redox peak currents of MB at the DNA_p/AuNP/GCE were enhanced significantly. Figure 3, curve d is the electrochemical response of the DNA_{ep}/AuNP/GCE, on which the redox peak currents were much higher than those at the DNA_p/AuNP/GCE. The only difference between DNA_{ep}/AuNP/GCE and DNA_p/AuNP/GCE was the length of DNA, so the increase of peak currents came from nothing but the extended region of the DNA probe. In this process, because of the presence of the five cytidines at the 5′ end of the target, DNA polymerase replicated the probe in terms of the G-C pairing principle. Consequentially, five guanines were added to the 3′ end of the probe, and as a result, more MB combined to the probe. This led to the increase of the redox peak currents at DNA_{ep}/AuNP/GCE.

The mean values of surface MB concentration were also quantitively calculated according to the equation $Q = nAF\Gamma$ (Q, integral electrical quantities of the peak; n, electron number for transform; A, area of electrode; F, Faraday constant; Γ, surface concentration). The surface concentrations of 2.1×10^{-10}, 3.8×10^{-9}, 5.7×10^{-9}, and 6.6×10^{-9} $molcm^{-2}$ were obtained at bare GCE, AuNP/GCE, DNA_{ep}/AuNP/GCE, and DNA_p/AuNP/GCE, respectively. Comparing DNA_{ep}/AuNP/GCE with DNA_p/AuNP/GCE, an increase of 9.0×10^{-10} $molcm^{-2}$ MB was obtained.

The DPV technique is more sensitive than CV, so in order to investigate all the steps of detecting the oligonucleotide target, the DPV technique in Tris–HCl buffer (10 mmol l^{-1} + 0.1 $moll^{-1}$ NaCl, pH8.0) was also employed. The results are shown in Figure 4, where curve a was at the

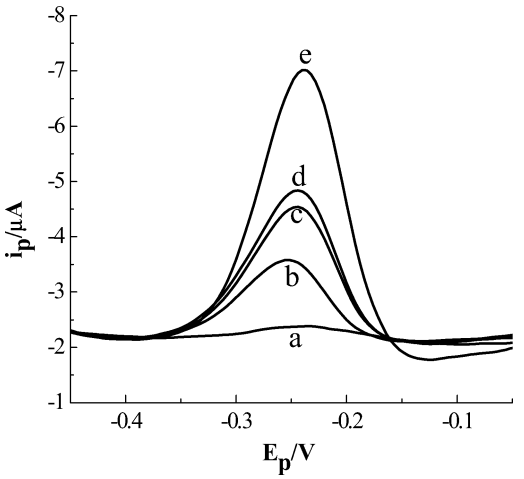


FIGURE 4 Differential pulse voltammetry curves of 20 m $moll^{-1}$ MB in Tris–HCl buffer (10 $mmoll^{-1}$ + 0.1 $moll^{-1}$ NaCl, pH 8.0) obtained at: a) bare GCE; b) the AuNP modified GCE; c) the original probe/AuNP modified GCE; d) the extended dsDNA/AuNP modified GCE; and e) the extended ssDNA/AuNP modified GCE in detection of oligonucleotide acid. The PCR cycling number is 30.

bare electrode and almost no response to MB was found on it; Figure 4, curve b was at AuNP/GCE without any DNA and on it a lower i_p was found; Figure 4, curve c was at the electrode modified with the SH-ssDNA probe (DNA_p/AuNP/GCE) and a clearly increased ip was found compared to curve a and curve b. This increase resulted from the powerful combination of ssDNA and MB cation aforementioned.[11,33] Figure 4, curve d was at the dsDNA modified electrode after spPCR process on which the spPCR process was achieved but the process of denaturalization was left out. A slight increase of the i_p was obtained compared with Figure 4, curve c. On this dsDNA-modified electrode, though the probe was extended, the immobilized DNA was still dsDNA because of the absence of a denaturation process. The guanine residues of the dsDNA were concealed, so only a slight increase of the i_p was obtained compared with Figure 4, curve c. Figure 4, curve e was at the extended probe (ssDNA) modified electrode (DNA_{ep}/AuNP/GCE), on which the target was eliminated and only extended probe DNA was immobilized; the maximum i_p was obtained in Figure 4, curve e. Though the lengths of ssDNA and dsDNA were the same, the guanine residues of the ssDNA were exposed outside of the DNA chain, so they had a stronger affinity with MB than the guanine residues of dsDNA had. Therefore, the i_p of curve e was higher than that of Figure 4, curve d. In the experiments, the most valuable result was the difference between curve e and curve c. Comparing the DNA_{ep}/AuNP/GCE (Figure 4, curve e) with the DNA_{ep}/AuNP/GCE (Figure 4, curve c), the immobilized DNAs were all ssDNA. The only difference was the length of DNA chain.

TABLE 1 The mean DPV values at different modified electrodes in detection of oligonucleotide

	i_p (μA)			
Target DNA	Probe	Hybrid (dsDNA)	Polymerase reaction (dsDNA)	Denaturalized (ssDNA)
acDNA	2.17 ± 0.08	2.10 ± 0.13	2.68 ± 0.08	5.1946 ± 0.25
cDNA	2.16 ± 0.113	2.11 ± 0.13	2.10 ± 0.11	2.09 ± 0.15
mcaDNA	2.17 ± 0.105	2.11 ± 0.10	2.10 ± 0.12	2.10 ± 0.18

The clearly increased i_p was attributed to nothing but the longer DNA chain on DNA_{ep}/AuNP/GCE rather than that on DNA_p/AuNP/GCE. This indicated that the target DNA sequence inducing DNA chain extension was successfully tested. The results agreed with the results of CV.

In order to obtain the best experimental effect, series of experiments were conducted to optimize the experimental conditions, such as the factors that influence asymmetric PCR, including the concentration of Mg^{2+}, annealing temperature, and concentration of primer. The final optimal conditions were chosen as follows: 2.0 $mmoll^{-1}$ $MgCl_2$, annealing temperature of 65°C, 1.2 $\mu moll^{-1}$ primer and 6 units Taq DNA polymerase.[34–36]

The same experiment was also done by using the oligonucleotides of the cDNA and macDNA as the target. A series of three repetitive measurements produced reproducible results. The average values are shown in Table 1. With regard to the hybrids, the dsDNA regions of the three hybrids (probe/cDNA, probe/acDNA, and probe/macDNA) were almost the same, so their i_p values were correspondingly alike. As to the ssDNA modified electrodes, the same i_p at DNA_{ep}/AuNP/GCE as that at DNA_p/AuNP/GCE was found. This is because in the PCR process, the cDNA is as long as the probe, and the probe cannot be extended for the lack of single strand template oligonucleotide residues in the hybrid. In the experiment with macDNA, similar results as with acDNA were obtained. A very important characteristic of DNA replicating is that the probe must match with the template at the 3′ end. In the hybrid (probe/macDNA), because the 3′ end of the probe mismatches with the template, the DNA polymerase cannot polymerize nucleoside to the probe's 3′ end. As a result, the probe did not extend and the i_p did not increase. The results indicated if the target chain was not longer than the probe (cDNA), or if the target chain did not match with the probe at the 3′ end (macDNA), the i_p after denaturation would be not higher than that of the primary probe. Only a target longer than the probe (acDNA) could cause the probe to extend. From the experiment, the target that was longer than the probe was distinguished from the sequences of cDNA and macDNA. This is the difference of this experiment from the others based only on hybridization.

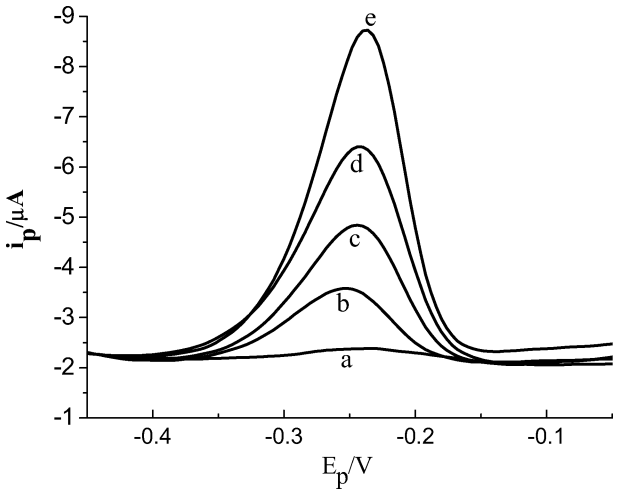


FIGURE 5 Differential pulse voltammetry curves of 20 mM MB in Tris–HCl buffer (10 $mmoll^{-1}$ + 0.1 $moll^{-1}$ NaCl, pH8.0) obtained at: a) bare GCE; b) the AuNP modified GCE; c) the original probe/AuNP modified GCE; d) the extended dsDNA/AuNP modified GCE; and e) the extended ssDNA/AuNP modified GCE in detection of molecule colon vector DNA. The PCR cycling number is 30.

3.3. The Detection of Molecule Clone Vector DNA

In gene transformation studies, the molecule clone vector DNA is involved in many processes including gene clone, gene expression, and gene transformation. The detection of a special DNA sequence from a molecule clone vector is necessary. In the experiments, the Glu gene integrates into the molecule clone vector DNA ($pETGlu^+$). The DPV results are shown as Figure 5, where a similar trend as in Figure 4 was obtained. First, the i_ps at DNA modified electrodes (Figure 5, curves c, d, and e) were higher than that at the electrodes without of DNA (Figure 5, curve a at bare electrode and curve b at AuNP/GCE). Second, the i_ps after chain extension (Figure 5, curves d and e) was higher than that at the electrodes without DNA extension (Figure 5, curve c). Third, the i_ps at the extended probe DNA modified electrode (Figure 5, curve e at DNA_{ep}/AuNP/GCE) was higher than that at the original probe DNA modified electrode (Figure 5, curve c at DNA_p/AuNP/GCE). This indicated that the method was also efficacious in detecting target DNA. However, this time the target was not oligonucleotide but the molecule clone vector DNA. It was much longer than the oligonucleotide, so after PCR the dsDNA region and the extended portion were much longer than that of Figure 4. As a result, an increased i_p (Figure 5, curve e) was obtained compared with the corresponding curve of Figure 4, curve e. It indicated that the signal for detecting long chain target was clearer than that for detecting short chain target. Taking the effect of cycle time on the DNA extension into consideration, DPVs of different cycle times were detected. The i_ps presented a typical "s" shape and steady i_p was

obtained when the cycle time reached 25. A cycle time of 30 was employed in subsequent experiments. To investigate the selectivity of the sensor, the same clone vector DNA without Glu ($pETGlu^-$) was employed as the reference, and this time, a similar magnitude of i_p at DNA_{ep}/AuNP/GCE as that at DNA_p/AuNP/GCE was obtained (not illustrated). In this situation, a DNA hybridization event did not occur because of the absence of the complementary sequence. Therefore, the probe did not extend in the PCR. All of above experiments indicated that the DNA chain elongation had enhanced the MB accumulation, and led to an increment of DPV signal, meaning that the target DNA (the template) was recognized selectively.

3.4. The DPV Detection of Glu from Transgenic Plant Genome DNA

The detection of a specific DNA sequence from a genome is important in clinical, quarantine and genetically modified organism studies. Our aim is to establish a sensitive method to detect the Glu from gene transformed 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 are shown in Table 2, where for the transgenic plant genome DNA (No.1–4), the i_ps of the dsDNA after PCR all increased compared to that of the original probe. The reason for the increases was that the long dsDNA region formed in the PCR process combined with much more MB. After the dsDNA were denatured and the targets DNAs were eliminated, the i_ps all increased clearly. The results indicated that in each plant genome there was the Glu integrated, and the plants were all transgenic plants. Meanwhile the control did not show any i_p increase.

3.5. Detection Limit

Sensitivity is the key issue for any DNA detection. In our study, the relationship between target concentration and i_p was investigated by varying

TABLE 2 The mean DPV values at different modified electrodes in detection of transgenic plants

	i_p (μA)			
Target DNA	Probe	Hybrid (dsDNA)	Polymerase reaction (dsDNA)	Denaturalized (ssDNA)
No.1	2.16 ± 0.15	4.39 ± 0.17	4.17 ± 0.23	5.62 ± 0.25
No.2	2.16 ± 0.15	4.37 ± 0.20	4.177 ± 0.21	5.592 ± 0.21
No.3	2.18 ± 0.16	4.36 ± 0.15	4.197 ± 0.25	5.58 ± 0.17
No.4	2.16 ± 0.15	4.37 ± 0.16	4.16 ± 0.15	5.59 ± 0.12
control	2.16 ± 0.14	2.37 ± 0.13	2.35 ± 0.14	2.24 ± 0.11

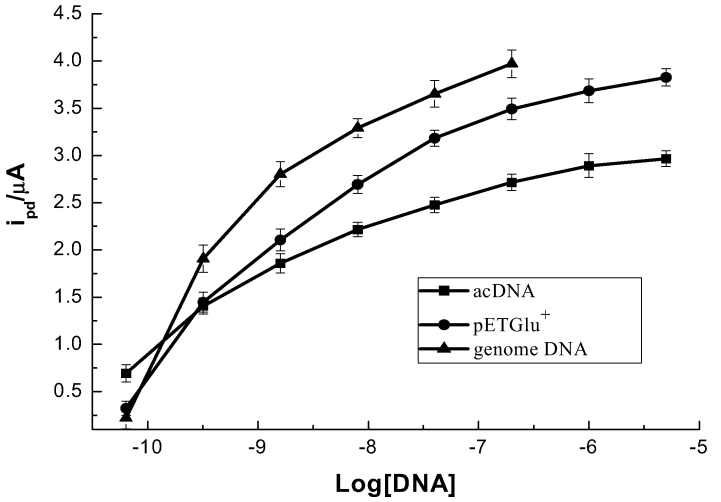


FIGURE 6 The relationship of i_{pd} and the concentration of the target DNA. i_{pd} is the difference of the peak current at the original probe/AuNP modified GCE(DNA_p/AuNP/GCE) and the extended probe/AuNP modified GCE(DNA_{ep}/AuNP/GCE). The PCR cycling number is 30.

the target concentration over the range of 6.4×10^{-11} to 5.0×10^{-6} $moll^{-1}$. The result is shown in Figure 6. 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. For 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 6.4×10^{-11} $moll^{-1}$, where the peak current for acDNA was higher than that for pETGlu$^+$. This result may be because at the low concentration, the hybrid of probe and short target chain was easier to form than that of probe and long target chain. The detection limit of 2.6×10^{-13}, 7.8×10^{-13}, and 9.1×10^{-13} mol l^{-1} for acDNA, pETGlu$^+$, 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 the reports of DNA detection based only on hybridization.[1,11,19,37] In this experiment, the detection sensitivity was improved by two means: one was magnifying the signal rather than from the short hybrid region but using the signal from other region; the other was adopting PCR, by which a trace mount DNA can be amplified to mass quantity of DNA.[38–42]

4. CONCLUSIONS

A series of stepwise experiments for the determination of DNA using chain extension are described here. Gold nanoparticles immobilized a great number of the probes on GCE owing to their large surface. PCR-based

techniques have been most frequently used for DNA sequencing and assay, by which primer-specific DNA sequence can be amplified and used for DNA qualification, sequence analysis and species identification.[43,44] In traditional PCR, two primers were needed and the two strands of a double strand DNA were all amplified. In our experiment, spPCR was used, which needed less target sequence information to be known for primer design than traditional PCR did. For another, if the traditional PCR was utilized in this assay, the target strand could also be amplified and this amplification of target would be accompanied by the enhancement of the experiment error.

The spPCR was effective in the detection of trace amounts of DNA. Specific target DNA sequences of three different lengths were detected successfully by using DNA/AuNP covered GCE. The assay combined the two main techniques with high sensitivity: PCR and electrochemical analysis. This was the advantage of the assay. The detection limits of 2.6×10^{-13}, 7.8×10^{-13}, and 9.1×10^{-13} moll^{-1} for acDNA, pETGlu^{+} and genome DNA were estimated, respectively. Other corresponding details such as the relationship between peak current and length of DNA extension have being studied in the further work. Another issue we are concerned with is how to achieve DNA chain extension directly on the surface of the electrode. The method described in this article has succeeded in transgenic plant detection and may lead to a more sensitive method in gene detection from transgenic plants and foods.

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