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ORIGINAL PAPER

Electrochemical detection of the neomycin phosphotransferase gene (*NPT-II*) in transgenic plants with a novel DNA biosensor

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Abstract A novel DNA electrochemical biosensor is described for the detection of neomycin phosphotransferase gene (*NPT-II*), a selection marker for transgenic plants. A thiol-modified capture probe immobilized onto the surface of a gold electrode and a biotinylated signaling probe were designed to be complementary to target regions of *NPT-II* flanked by PCR primers to eliminate false-positive signals from non-specific PCR products. The electrochemical assay of hybrids on the electrode surface was evaluated by means of both cyclic voltammetry (*CV*) and square wave voltammetry (*SWV*) after the coupling of biotinylated catalase with streptavidin-modified hybrids based on dendritic signal magnification and subsequent formation of polymerized aniline (PAn). It has been revealed that the sensor showed a linear increase within the target concentration ($1.0–100 \times 10^{-6}$ mmol L^{-1}). The limit of detection was about 0.2×10^{-6} mmol L^{-1} and the specificity was enhanced significantly.

Keywords DNA electrochemical biosensor · *NPT-II* · Transgenic plant · Polymerized aniline (PAn)

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1 Introduction

With the development of transgenic plants on a large scale, there are increasing concerns about their security, especially their ecological safety faced by people all over the world [1]. An important aspect in solving this problem is the establishment of reliable, accurate, sensitive, rapid methods for the detection of emerging transgenic plants. Routine protocols include polymerase chain reaction (PCR) and Southern blotting hybridization. PCR is relatively fast [2], but its failure to provide any sequence information in relation to the amplified DNA fragment might lead to unreliable results [3–10]. The presence of false positive products, which can result from non-specific amplification of approximately the same size of the expected target fragment, would potentially confuse the authenticity and validity of the assays [2, 11]. On the other hand, although Southern blotting hybridization can satisfy the requirement of sequence information and provide more reliable and accurate experimental results due to the high specificity of base pairings, the process is very complicated and time-consuming and requires a substantial amount of genomic DNA of transgenic plants.

In contrast, biosensors based on molecular hybridization between any two complementary DNA sequences hold promising advantages in the detection of transgenes [12–14]. The system combines biomolecules containing specific recognition elements for target nucleic acid with a transducer that converts hybridization events into acoustic, optical or electrochemical signals [15–17]. Acoustic transducers require expensive materials and bulky instruments, which is not generally adaptable for ordinary labs. Fluorescence-based optical biosensors are extraordinarily sensitive, but their popularity is low due to high incidents of false positive signals, expensive laboratory instrument

requirements, and low labour-efficiency. By comparison, biosensor detection based on electrochemical transduction is extremely practical for the detection of target DNA sequences due to its high sensitivity and selectivity, rapid response, stable performance and inexpensive laboratory equipment requirements.

The detection of a specific DNA sequence based on electrochemical analysis has been widely used to identify trace amounts of pathogenic bacteria in food and beverage [18, 21, 22], as well as for the diagnosis of human hereditary diseases [13, 19, 20]. Based on electronic signals obtained from hybridization between DNA sequences and subsequent enzyme-linked catalytic reaction, the existence of specific sequences can be accurately confirmed. However, only a few studies have been published on the detection of specific genes integrated into transgenic plants using this protocol. Lucarelli et al. developed two sets of electrochemical biosensors, which were successfully used in the analysis of genetically modified soybean plants. An interesting aspect of the aforementioned sensors is the combination of PCR and DNA hybridization, where both sensitivity and selectivity for sequence-specific detection were greatly improved [23, 24]. Chaumpluk et al. demonstrated the use of 5′ thiol-modified forward primers and 5′ biotinylated reverse primers in the amplification of trace target DNA from food samples, in which binding of ferrocene-streptavidin to biotin-labeled PCR products allows direct detection of target DNA sequences [21]. Although this method eliminates the need of immobilization of relevant probes onto surface of the electrode, the optimization of PCR parameters is indispensable for minimal non-specific amplification.

In plant genetic engineering, T-DNA delivery mediated by *Agrobacterium tumefaciens* has been utilized extensively, and most vectors use the *NPT-II* gene conferring kanamycin resistance as selectable marker. Detection of *NPT-II* can be used as an indirect confirmation that a linked gene of interest gene has been integrated into the plant genome. In our present study, we have established a novel transgene detection system using a combination of PCR and electrochemical analysis. A specific fragment of *NPT-II* was first amplified from genomic DNA of transgenic plants by PCR and used as target sequence for a specific hybridization process. Two ssDNA probes, a thiol-modified electrode capture probe and a biotinylated signaling probe were designed to be complementary to target regions downstream of the forward PCR and reverse PCR primers, respectively. After hybridization, the biotinylated signaling probe can be efficiently labeled with streptavidin followed by linking of biotinylated catalase to DNA hybrids on the electrode surface to form dendritic structures [25]. Finally, the amount of catalase binding to the electrode surface will be increased several-fold, which can magnify the signal strength and effectively improve the detection sensitivity.

2 Materials and methods

2.1 Reagents and probes

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide solution (EDC) and *N*-hydroxysuccinimide (NHS) were produced by Sangon Biotech (Shanghai, China). Streptavidin and biotin were purchased from Sigma (USA). *Taq* polymerase was obtained from Takara (Dalian, China), Ultra-purified water was used throughout the experiment. All chemicals used in this study were analytical grade.

Oligonucleotides were synthesized and purified by Sangon Biotech (ShangHai, China). Immobilized capture probe, signaling probe and other oligonucleotides were designed with the program marked OLIGO-6. Detailed sequences are listed in Table 1.

All oligonucleotides were dissolved with 10 mmol L^{-1} TE buffer into stock solutions and stored at −20 °C. They were diluted with 0.5×10^3 mmol L^{-1} phosphate buffer to appropriate concentrations prior to use. Thiol-modified capture probes were treated overnight with 0.05×10^3 mmol L^{-1} DTT at the room temperature in the dark to reduce the S–S bonds before experiment.

Table 1 Sequence of primers, probes and oligonucleotides used in different experiments

Thiol-modified capture probe	5′-SH-(CH_2)6-TGA ATG AAC TGC AGG ACG AG-3′
Biotinylated signaling probe	5′-CCG GCT ACC TGC CCA TTC GA-Biotin-3′
Complementary oligonucleotide	5′-TGT TTC GCT TGG TGG TCG AAT GGG CAG GTA GCC GGC TGC CTC GTC CTG CAG TTC ATT CAA GGC AC-3′
Non-complementary oligonucleotide	5′-TGT TTC GCT TGG TGG TCC TTA CCC GTC CAT CGG CCG GAG CAG GAG CAG TTC ATT CAA GGC AC-3′
Forward primer of *NPT-II*	5′-GTG CCT TGA ATG AAC TGC-3′
Reverse primer of *NPT-II*	5′-TGT TTC GCT TGG TGG TC-3′
Forward primer of *β*-actin	5′-TAC GTC GCC ATT CAA GCC-3′
Reverse primer of *β*-actin	5′-TGG CAA AGC ATA TCC CTC-3′

2.2 Amplification of target sequence by PCR

Genomic DNA from three plant species (*Nicotiana tabacum*, *Actinidia deliciosa*, *Gentianae macrophyllae*) transformed with T-DNA containing *NPT-II* as selectable marker and different linked genes of interest were extracted with the CTAB method [6] and used as template for the amplification of a 253-bp fragment of *NPT-II*. PCR procedures were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min. pBI121 vector DNA was used as positive control. Negative controls were as follows: (1) Genomic DNA extracted from non-transgenic tobacco plant was added into a parallel reaction system as a template; (2) a mock PCR reaction without template; (3) a 273-bp fragment of β-actin gene was amplified from non-transformed tobacco genomic DNA in order to confirm the specificity of the subsequent hybridization events. PCR products were separated on a 2.0% agarose gel and fragment sizes determined.

2.3 Assembly of DNA biosensors on the surface of gold electrode

2.3.1 Biomodification of the gold electrodes

Before immobilization of the thiol-modified capture probe, gold electrodes were immersed in a mixture of 10 mmol L^{-1} H_2SO_4 and 30% H_2O_2 (1:1) for 5 min to eliminate other substances, and then consecutively rinsed in 100% ethanol and ultra-pure water. After ultrasonic treatment for 5 min, the electrodes were rinsed with liquid nitrogen prior to use.

In order to obtain well-aligned monolayers of thiol-modified capture probes on the gold surface, electrodes were first immersed in 0.2×10^{-3} mmol L^{-1} thiol-modified capture probe solution diluted with 0.5×10^{3} mmol L^{-1} phosphate buffer [14] and incubated overnight at 16 °C in the dark to avoid oxidation of SH-groups. Electrodes were further treated with 1×10^{3} mmol L^{-1} MCH for 3 h and finally rinsed with 0.5×10^{3} mmol L^{-1} phosphate buffer.

2.3.2 Hybridization reactions on the surface of gold electrodes

PCR-amplified dsDNA was first diluted with 0.5×10^{3} mmol L^{-1} phosphate buffer containing 30% (v/v) formamide into a series of concentrations. To prepare ssDNA, samples were denatured in boiling water for 10 min and rapidly cooled in an ice-water bath for 5 min. Subsequently, 2.0×10^{-3} mmol L^{-1} of oligonucleotide signaling probe was added and thoroughly mixed. The mixtures were incubated at 75 °C for 5 min and complementary sequences were annealed by gradual cooling to 37 °C for 1 h to avoid the formation of hairpins between or inside ssDNAs. Modified gold electrodes were first treated at 55 °C in 0.5×10^{3} mmol L^{-1} phosphate buffer for several minutes to avoid the formation of hairpins inside the thiol-modified capture probe, then immersed in 500 μL mixture of target DNA and signaling probe, and hybridized for 30 min at 37 °C. After hybridization, gold electrodes were thoroughly rinsed with phosphate buffer.

2.3.3 Ligation of streptavidin to signaling probes

Hybridized electrodes were immersed for 30 min into 500 μL of 1.0×10^{-3} mmol L^{-1} streptavidin solution diluted with phosphate buffer. Streptavidin specifically binds to biotins labelled at the 3′ end of the signaling probes. The electrodes were then thoroughly rinsed with phosphate buffer.

2.3.4 Ligation of biotin-labeled catalase to streptavidin

Biotin-labelled catalase was prepared in the presence of EDC and NHS in a solution containing 100×10^{-3} mmol L^{-1} of catalase and 1.223×10^{-3} mmol L^{-1} of biotin. Hybridized electrodes were incubated in biotin-labeled catalase solution for 30 min at room temperature; biotin-labelled catalase binds to the electrode surface through affinity between biotin and streptavidin (see Fig. 1 for details).

2.4 Electrochemical measurements

After incubation, the electrode surface was rinsed thoroughly and consecutively with phosphate buffer and ultra-pure water and then incubated in aniline/H_2O_2 solution for 15 min for enzymatic reaction. The electrochemical technique used in this study was cyclic voltammetry (*CV*) and square wave voltammetry (*SWV*). All detection processes were carried out at room temperature.

3 Results and analysis

3.1 Cyclic voltammetry of polymerized aniline at SH-probe/target DNA/biotin-probe-modified electrodes

We first evaluated the response of the biosensor with 50×10^{-6} mmol L^{-1} synthetic complementary oligonucleotide. 1.0×10^{-3} mmol L^{-1} of noncomplementary oligonucleotides were used as negative control. As illustrated in Fig. 2, Curve (a) is the cyclic voltammogram of

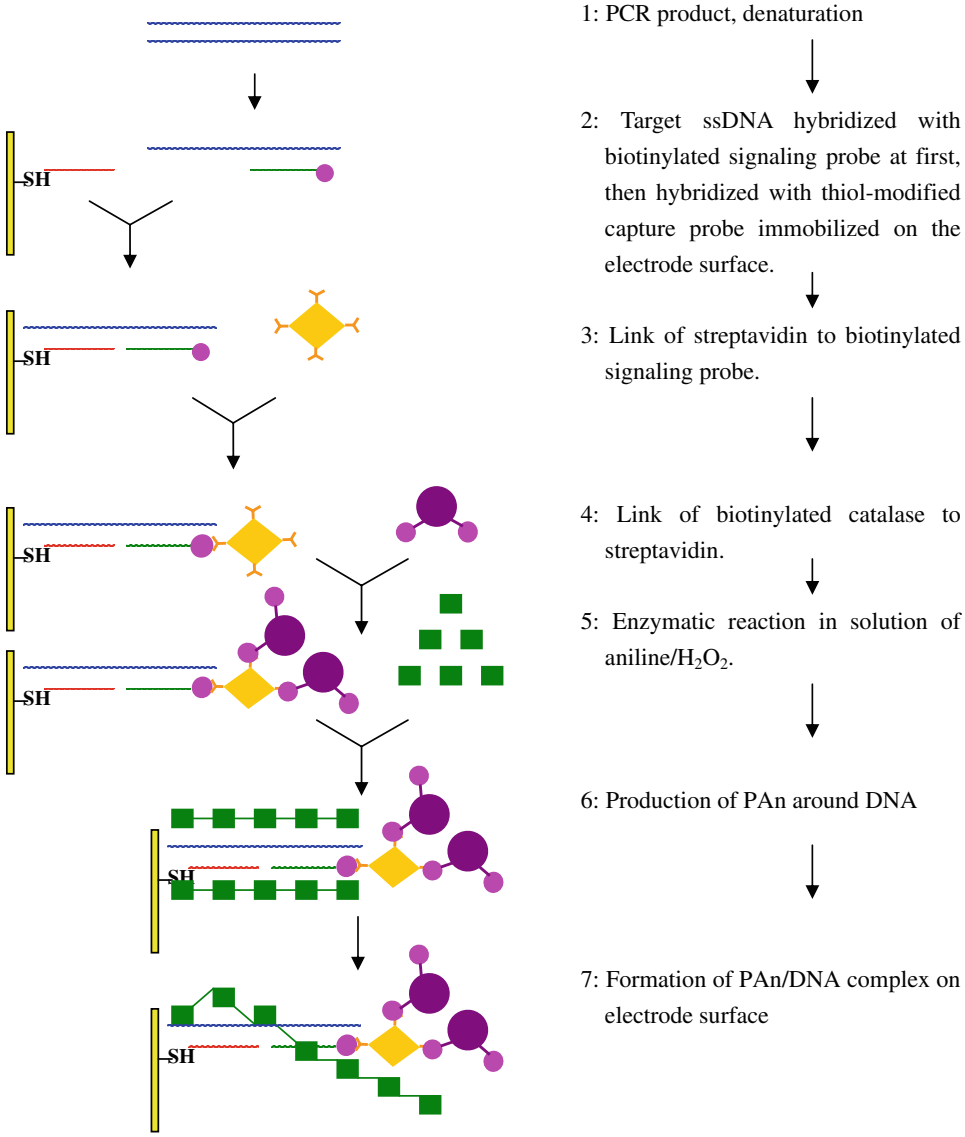

Fig. 1 Schematic representation of the biosensor fabricated in the present work. ● biotin; ✣ streptavidin; ● catalase; ■ aniline

polymerized aniline (PAn) using noncomplementary oligonucleotide as target. Redox peaks cannot be observed in the potential range −0.2 to 0.60 V, indicating that no PAn was formed on the gold electrode surface owing to failure in hybridization. Curve (b) is the cyclic voltammogram of PAn with complementary oligonucleotides as target, which showed a pair of well-defined redox peaks with the highest current of 0.495 μA. This is attributable to the good electrical conductivity of PAn formed on the electrode surface. It is expected that, when the hybridized biosensor is incubated in the mixture of aniline/H_2O_2 at pH 4.3, the protonated aniline molecules (p*K*a 4.6) will be concentrated and aligned around the hybridized DNA strands through electrostatic interaction between the protonated aniline molecules and negatively charged phosphate groups of DNA. The high proton concentration around DNA provides a local environment of high acidity that permits polymerization of aniline in a much less acidic medium than that in conventional electrochemical and chemical approaches. This facilitates a predominantly head-to-tail coupling of aniline and deters parasitic branching during the polymerization reaction, offering the much desired structure for high conductance. The PAn/DNA complex, in which PAn wraps around the DNA template, is utilized for target DNA sensing. As shown in the inset of Fig. 2, almost symmetric SWV curves were obtained and no other peaks were observed.

3.2 Optimization of variables in assembly of the biosensors

The influence of the capture probe coverage on hybridization efficiency was investigated as a critical factor for assembly of the biosensor. Electrodes were first immersed

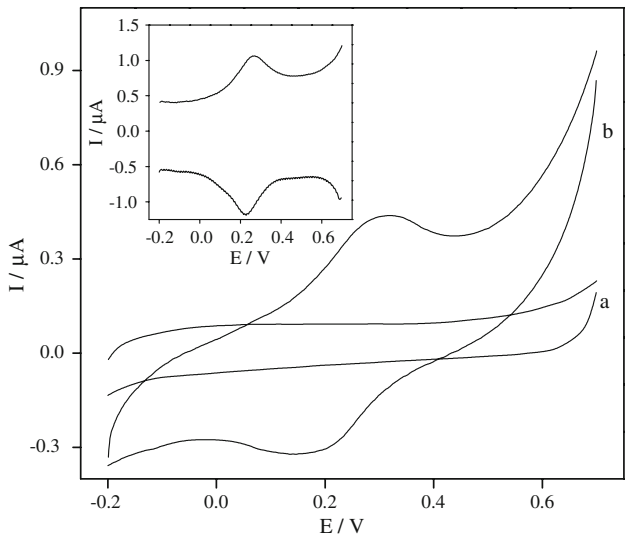

Fig. 2 Cyclic voltammograms of PAn at the surface of thiol-modified electrode in aniline/H_2O_2 solution. **a** noncomplementary oligonucleotide, **b** complementary oligonucleotide. The inset shows the peak currents (b) plotted as a function of the scan rate

into a series of capture probe solutions (0.05, 0.1, 0.2, 1.0 and 2.0×10^{-3} mmol L^{-1} in 0.5×10^{3} mmol L^{-1} phosphate buffer) overnight. In subsequent experimental steps, 0.1×10^{-3} mmol L^{-1} synthetic complementary and non-complementary oligonucleotides were used as target sequences. Based on their ideal theoretical values, other variables were set as follows: 1.0–2.0×10^{-3} mmol L^{-1} signal probe, 1.0–2.0×10^{-3} mmol L^{-1} streptavidin, 1.0×10^{-3} mmol L^{-1} biotin and 100×10^{-3} mmol L^{-1} catalase. In Fig. 3a, signal ratios of specific vs. non-specific voltammetric data are shown. The most favorable ratio was obtained with the 0.2×10^{-3} mmol L^{-1} capture probe. This clearly showed that the density of the electrode capture probes on the surface was essential for the enhancement of biosensor performance, and only a small quantity of biotinylated hybrids were formed when the

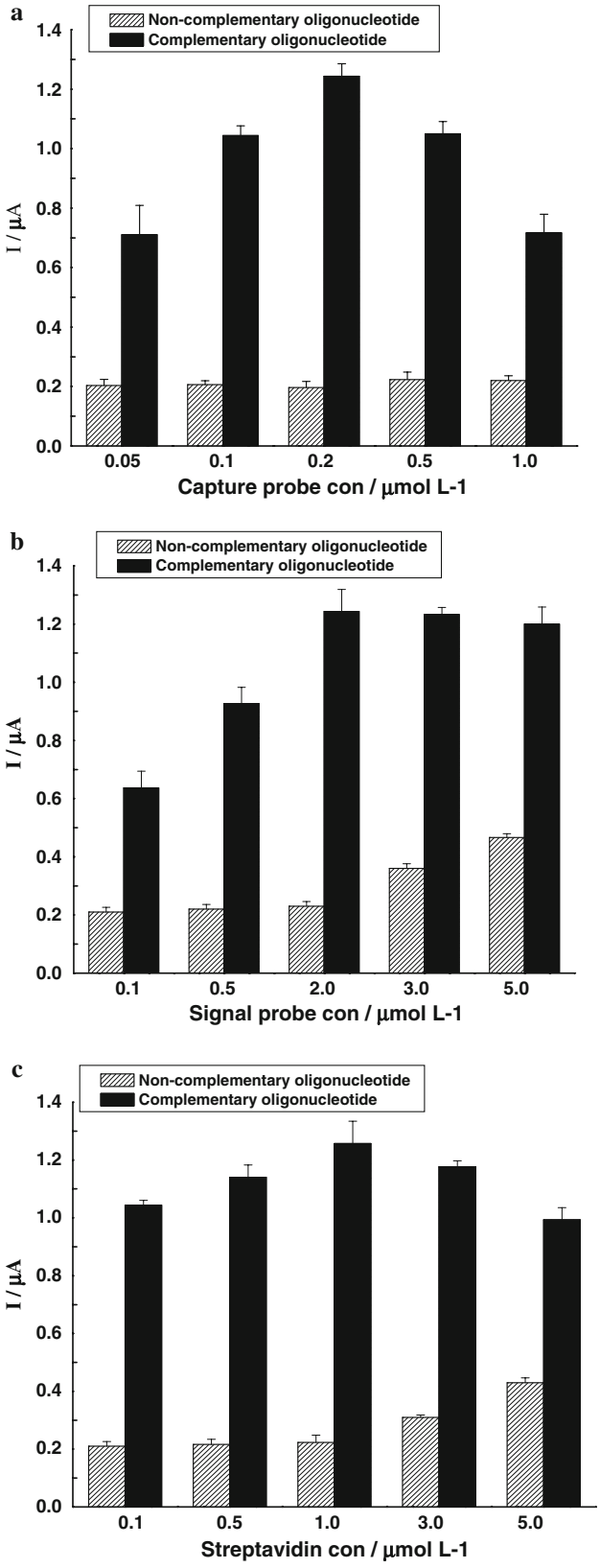

Fig. 3 Optimization of variables. **a** Influence of capture probe concentration on biosensor performance. Electrodes modified by a series of capture probe solutions (0.05, 0.1, 0.2, 1.0 and 2.0×10^{-3} mmol L^{-1} in 0.5×10^{3} mmol L^{-1} phosphate buffer) overnight were immersed in 500 μL mixtures of target (synthetic complementary or non-complementary oligonucleotide) (0.1×10^{-3} mmol L^{-1}) and signaling probe (2.0×10^{-3} mmol L^{-1}) to hybridize for 30 min. Then the hybridized electrodes were immersed into 500 μL of streptavidin solution (1.0×10^{-3} mmol L^{-1}) for 30 min, followed by Incubation in 500 μL of biotin-labeled catalase solution (biotin: 1.0×10^{-3} mmol L^{-1}, catalase: 100×10^{-3} mmol L^{-1}) for 30 min. Each experiment was repeated at least three times. **b** Influence of signaling probe concentration on biosensor performance. The electrodes modified by 0.2×10^{-3} mmol L^{-1} capture probe were immersed in 500 μL of mixtures of target DNA (0.1×10^{-3} mmol L^{-1}) and a series of signaling probe solutions (0.1, 0.5, 2.0, 3.0 and 5.0×10^{-3} mmol L^{-1}) to hybridize. Other conditions were the same as Fig. 3a. **c** Influence of streptavidin concentration on biosensor performance. The electrodes modified with optimized concentration of capture and signaling probe were immersed into a series of streptavidin solutions (0.1, 0.5, 1.0, 3.0 and 5.0×10^{-3} mmol L^{-1}). Other conditions were the same as in Fig. 3a

Table 2 Effect of concentrations of catalase and biotin on biosensor performance (specific signal/nonspecific signal)

Biotin con ($\times 10^{-3}$ mmol L^{-1})	Catalase con ($\times 10^{-3}$ mmol L^{-1})			
	10	50	100	200
0.5	0.423/0.201	0.5/0.192	0.61/0.1883	0.63/0.233
1.0	0.653/0.205	0.7/0.20	1.0/0.195	1.0266/0.216
1.223	0.546/0.196	1.076/0.201	1.31/0.189	1.313/0.208
2.0	0.543/0.213	0.966/0.207	1.20/0.193	1.236/0.211

Electrodes modified with optimized concentration of capture and signal probes in the presence of synthetic complementary or non-complementary oligonucleotides (0.1×10^{-3} mmol L^{-1}) were immersed into streptavidin solutions (1.0×10^{-3} mmol L^{-1}), followed by incubation in a series of biotin-labeled catalase solutions (biotin: 0.5, 1.0, 1.223 and 2.0×10^{-3} mmol L^{-1}; catalase: 10, 50, 100 and 200×10^{-3} mmol L^{-1}). Other conditions were the same as Fig. 3a

concentration of capture probe was too low due to incomplete coverage on the electrode surface. On the other hand, if probe concentrations were aberrantly high, a compact monolayer would form, resulting in small quantities of biotinylated hybrids on the electrode surface. The 0.2×10^{-3} mmol L^{-1} capture probe was therefore adopted in subsequent experiments.

The concentration of the biotinylated signaling probe also plays an important role in the performance of the biosensor itself. Different concentrations of signaling probes (0.1, 0.5, 2.0, 3.0 and 5.0×10^{-3} mmol L^{-1}) were tested for their influence on the hybridization reaction while the concentration of strepavidin, biotin and catalase were kept constant. As shown in Fig. 3b, the specific signals increased gradually with the higher signaling probe concentrations and leveled off around 2.0×10^{-3} mmol L^{-1}. Meanwhile, non-specific signals began to increase as the concentration of the signaling probe exceeded 2.0×10^{-3} mmol L^{-1}. This may be mainly ascribed to non-specific binding between capture probes immobilized on the electrode surface and signaling probes. Because the most favorable ratio of specific versus non-specific signaling was achieved at 2.0×10^{-3} mmol L^{-1}, this concentration of signal probe was selected in all subsequent experiments.

To investigate the effect of streptavidin concentration, electrodes modified with optimized concentration of capture and signaling probes were immersed into a series of streptavidin solutions (0.1, 0.5, 1.0, 3.0 and 5.0×10^{-3} mmol L^{-1}), while the concentrations of biotin and catalase were kept constant. As shown in Fig. 3c, peak currents increased gradually with streptavidin concentration ranging from 0.1–1.0×10^{-3} mmol L^{-1}, and non-specific signals remained relatively lower. However, when 3.0 or 5.0×10^{-3} mmol L^{-1} of streptavidin were used, non-specific signals increased, while specific signals decreased to some extent. This suggests that excessive streptavidin concentrations might increase the chance of non-specific binding and interfere with the coupling of streptavidin to biotinylated hybrids. Based on those results, 1.0×10^{-3} mmol L^{-1} of streptavidin was used in all subsequent experiments.

Influences of biotin and catalase on the electrochemical signal were investigated to further optimize the analytical performance. To minimize the quantity unlabeled biotin which would compete for binding to streptavidin with catalase-labeled biotin molecules, catalase in high concentration (10, 50, 100 and 200×10^{-3} mmol L^{-1}) was labelled with biotin in low concentration (0.5, 1.0, 1.223 and 2.0×10^{-3} mmol L^{-1}) in the presence of EDC (0.125 g L^{-1}) and NHS (0.25 g L^{-1}). The optimum ratio of specific to non-specific signal could be achieved with 100×10^{-3} mmol L^{-1} of catalase and 1.223×10^{-3} mmol L^{-1} of biotin (Table 2).

3.3 Determination of biosensor specificity by square wave voltammetry

Experiments were performed to examine the selectivity of the biosensors for hybridization with target DNA sequences. To investigate the specificity of the hybridization reaction using plant samples, a 253-bp fragment of *NPT-II* was PCR-amplified from genomic DNA of three individual transgenic plants (for details see Sect. 2). As shown in Fig. 4, non-specific bands were observed together with the target fragment. Interestingly, a non-specific band of almost the same size as the target fragment was amplified from non-transformed tobacco plant (lane 4), indicating that a PCR approach without subsequent hybridization assays would be inconclusive.

The detection specificity of biotinylated signaling probes to 100×10^{-6} mmol L^{-1} PCR product of *NPT-II* was validated by exposing the modified electrode to an aniline/H_2O_2 solution. Negative controls (NC) included pure buffer (NC1), 0.2×10^{-3} mmol L^{-1} of the signaling probe alone (NC2), amplified fragments of the β-actin gene (NC3), non-specific PCR products from untransformed tobacco plants (NC4), and a PCR reaction without DNA

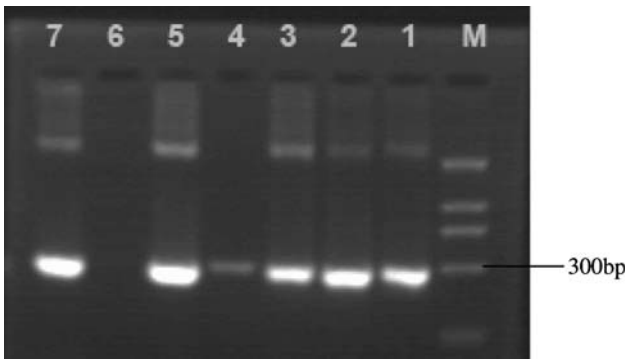

Fig. 4 Confirmation of the quality of PCR products. Lane 1, 2 and 3—PCR products of transgenic *Nicotiana tabacum*, *Actinidia deliciosa*, *Gentianae macrophyllae* respectively with the primers of *NPT-II*, Lane 4—PCR product of non-transformed *Nicotiana tabacum* with the primers of *NPT-II*, Lane 5—PCR product of pBI121 (positive control) with the primers of *NPT-II*, Lane 6—PCR blank (without template), Lane 7—PCR product of β-actin gene

template (NC5). In addition, the selectivity of synthetic complementary and the non-complementary oligonucleotides of *NPT-II* were also investigated.

The biosensor produced a strong response to PCR-amplified target sequence and the synthetic oligonucleotide complementary to *NPT-II*. The square wave voltammetry (*SWV*) signal obtained from the hybridization of the probes with the synthetic oligonucleotide complementary to *NPT-II* reached 1.05 ± 0.20 μA, while hybridization with PCR products from experimental samples of three individual transgenic plants gave average signals of 0.55 ± 0.05, 0.56 ± 0.06 and 0.54 ± 0.05 μA at the 100×10^{-6} mmol L^{-1} concentration, respectively. Poor responses to all negative controls and non-complementary oligonucleotide were observed (Table 3). This is in agreement with the performance of noncomplementary oligonucleotides (Fig. 2), indicating that almost no hybridization occurred.

Non-specific PCR products containing target-specific primers may generate false-positive signals if the capture and signaling probes are complementary to the primer sequences. To eliminate such false-positive signals, a thiol-modified capture probe and a biotinlyated signaling probe were designed to be complementary to regions downstream of the *NPT-II* primers used in this work. As expected, a significant signal enhancement was observed when PCR products of *NPT-II* hybridized to the modified gold electrode surface. Curve (b) in Fig. 5 represents *SWV* of PCR fragments from transgenic tobacco plants transformed with *NPT-II*. An increase in oxidation anodic peak current from 0.0053 ± 0.001 μA to 0.55 ± 0.05 μA (mean of four replicates ± a 95% confidence level) was produced compared to PCR products of the β-actin gene (NC3) [Curve (b) in Fig. 5]. These results indicated that PCR amplified target sequences can be investigated effectively with the novel probes designed in this study, because hybridization occurred only between probes and specific target DNA, but not between probes and nonspecific PCR products, or partially complementary DNA sequences.

3.4 Effect of target DNA concentration

To investigate the sensitivity of the biosensor, a calibration experiment was carried out with synthetic complementary oligonucleotide ranging from 0–100×10^{-6} mmol L^{-1}. Current values in *SWV* response were recorded with three repetitive measurements. As shown in Fig. 6, the current peak was found at 1.05 μA after hybridization with 100×10^{-6} mmol L^{-1} of complementary oligonucleotide, and the voltammetric signal increased almost linearly with the concentration of complementary oligonucleotide up to 0.18×10^{-3} mmol L^{-1}. When the concentration of complementary oligonucleotides increased up to

Table 3 Specificity assessment of the biosensor by SWV

Sample	Concentration (nmol L^{-1})	Current signal (μA)
Buffer alone (NC1)	–	0.0
Biotinylated signaling probe alone (NC2)	200	0.0
PCR product of β-actin gene (NC3)	100	0.0053 ± 0.001
PCR product of non-transformed tobacco plant (NC4)	100	0.0015 ± 0.001
PCR blank (without template) (NC5)	–	0.0010 ± 0.001
PCR product of *NPT-II* (1)	100	0.55 ± 0.05
PCR product of *NPT-II* (2)	100	0.56 ± 0.06
PCR product of *NPT-II* (3)	100	0.54 ± 0.05
Synthetic complementary oligonucleotide	100	1.05 ± 0.20
Synthetic noncomplementary oligonucleotide	100	0.0

Thiol-modified electrodes were immersed in 0.5×10^{3} mmol L^{-1} phosphate buffer containing 30% (v/v) formamide for 30 min in the presence of biotinylated signaling probe. NC1-pure buffer, NC2-biotinylated signaling probe, NC3-PCR product of tobacco β-actin gene, NC4-PCR product of non-transformed tobacco plant with the primers of *NPT-II*, NC5-PCR blank (no template in PCR), (1) to (3)—thermal denatured *NPT-II* PCR product of transgenic *Nicotiana tabacum*, *Actinidia deliciosa*, *Gentianae macrophyllae* respectively

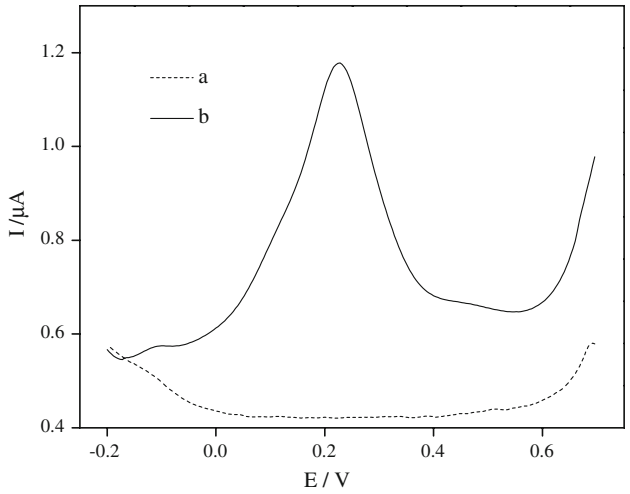

Fig. 5 SWV of the thiol-modified electrode after hybridization with PCR product of β-actin gene (100×10^{-6} mmol L^{-1}) (**a**) and PCR product of *NPT-II* (100×10^{-6} mmol L^{-1}) from transgenic *Nicotiana tabacum* (**b**) in 0.5×10^{3} mmol L^{-1} phosphate buffer containing 30% (v/v) formamide for 30 min in the presence of biotinylated signaling probe

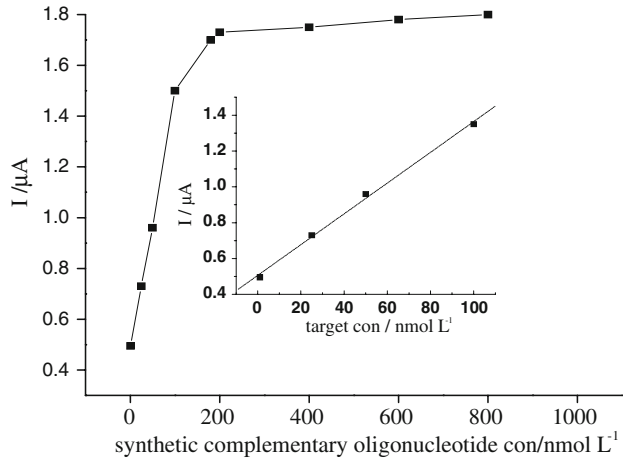

Fig. 6 Calibration plot of the thiol-modified electrode hybridizated with synthetic complementary oligonucleotide ranging from 0–1000×10^{-6} mmol L^{-1} in 0.5×10^{3} mmol L^{-1} phosphate buffer containing 30% (v/v) formamide for 30 min in the presence of biotinylated signaling probe. Inset shows the linear plot of current peak height to concentration of synthetic complementary oligonucleotide in the range of 1.0–100×10^{-6} mmol L^{-1}

0.2×10^{-3} mmol L^{-1}, the signal leveled off gradually, implying that 0.2×10^{-3} mmol L^{-1} of complementary oligonucleotide was a saturation concentration of hybridization. These results indicated that the signal strength depended not only on target concentration, but also on the quantity of available probes on the electrode surface. When the concentration was below 0.1×10^{-6} mmol L^{-1}, no obvious current peak was observed.

Hybridization of probes to PCR amplified fragment of *NPT-II* was performed in the same way, and three repetitive

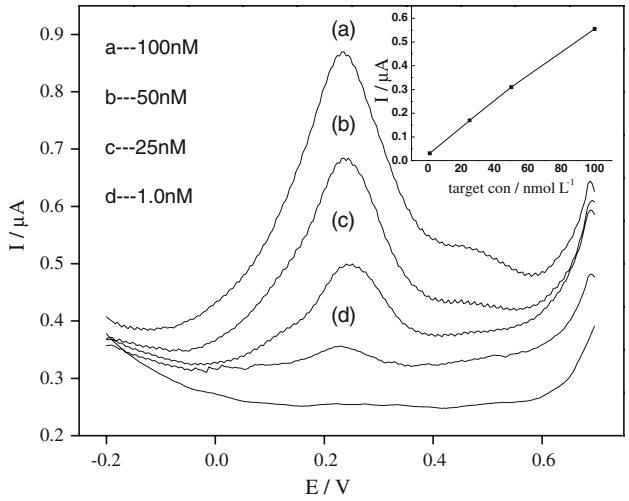

Fig. 7 Effect of the concentration of the amplified fragment of *NPT-II* (1.0, 25, 50, and 100×10^{-6} mmol L^{-1}) on electrochemical response. Values are mean of three replicates ± a 95% confidence level. Inset shows linear plot of current peak height to target concentration in the range of 1.0–100×10^{-6} mmol L^{-1}

measurements yielded reproducible results. *SWV* curves of the electrode using PCR products of transformed tobacco plants (1.0–100×10^{-6} mmol L^{-1}) are shown in Fig. 7. The current peak values decreased with lower levels of PCR products ($0.55 \pm 0.05, 0.34 \pm 0.021, 0.17 \pm 0.01, 0.031 \pm 0.01$ μA for 100, 50, 25, 1.0×10^{-6} mmol L^{-1} PCR products, respectively), indicating a dependence on concentration of the target DNA. There was a linear increase in voltammetric signal for PCR product in the range 1.0–100×10^{-6} mmol L^{-1} (inset of Fig. 7). It should be noted that no obvious current peak was observed when the DNA concentration was below 0.2×10^{-6} mmol L^{-1}, slightly higher than that of synthetic complementary oligonucleotide.

When the PCR product of *NPT-II* was used as template, the hybridization efficiency was relatively low compared to synthetic oligonucleotides. This result maybe attributed to impurity of the PCR product. The remaining polymerase, PCR primers, and nonspecific products might interfere with the binding of the target DNA to the electrode surface. On the other hand, this phenomenon might occur due to re-annealing of denatured PCR products, resulting in lower abundance of available target ssDNA [26].

3.5 Identification of PCR products by Southern blotting

To validate the results obtained by the *SWV* method, Southern blotting hybridization of PCR product was performed according to the procedure described by Ryschkewitsch et al. [27]. As shown in Fig. 8a, single bands of the correct size were observed in lanes containing PCR products from transgenic *N. tabacum*, *A. deliciosa*, *G. macrophyllae*, and the pBI121 plasmid, positive control

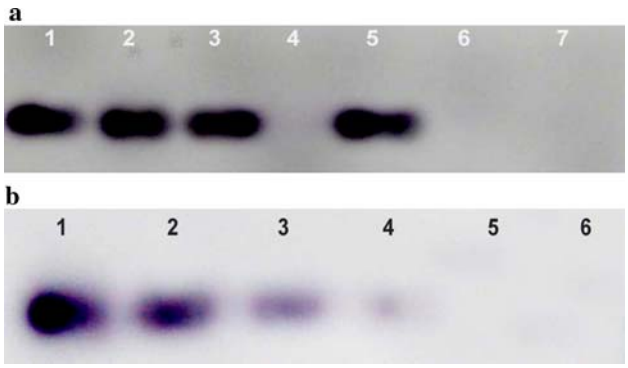

Fig. 8 Southern hybridization of PCR products using 200 bp probe of *NPT-II* labeled by Digoxin. **a** Southern-blotting of PCR products. Lane 1, 2, 3—PCR product of *NPT-II* from transgenic *Nicotiana tabacum*, *Actinidia deliciosa*, *Gentianae macrophyllae* respectively, Lane 4—PCR product of non-transformed *Nicotiana tabacum* with the primers of *NPT-II*, Lane 5—PCR product of pBI121 (positive control) with the primers of *NPT-II*, Lane 6—PCR blank (without template), Lane 7—PCR product of β-actin gene. **b** Sensitivity assessment of Southern blotting hybridization. Lane 1, 2, 3, 4, 5-amplified fragment of *NPT-II* loaded with 1.0×10^{-3}, 100×10^{-6}, 10×10^{-6}, 0.6×10^{-6} and 0.2×10^{-6} mmol L^{-1} respectively, Lane 6—PCR products of β-actin gene loaded with 1.0×10^{-3} mmol L^{-1}

(lanes 1, 2, 3, and 5, respectively). No bands were observed in lanes corresponding to non-transformed tobacco plants, PCR blank, and PCR fragment of the β-actin gene (lanes 4, 6 and 7, respectively). These results were consistent with *SWV* values (Table 3) detected by our biosensors, indicating that the biosensors generated in this study had the same specificity as Southern blotting analyses. To determine the detection limit of target DNA, PCR products of *NPT-II* amplified from transgenic *N. tabacum* were diluted to 1000×10^{-6}, 100×10^{-6}, 10×10^{-6}, 0.6×10^{-6} and 0.2×10^{-6} mmol L^{-1} and loaded into a gel to perform Southern blotting analysis with a 200-bp *NPT-II* specific probe. PCR products of the β-actin gene amplified from non-transformed *N. tabacum* at a concentration of 1.0×10^{-3} mmol L^{-1} was loaded as negative control. As shown in Fig. 8b, with the decrease of target concentration, hybridization intensity gradually became weaker. Only a faint band could be observed at 0.6×10^{-6} mmol L^{-1} (lane 4) and no hybridization could be detected at lower concentrations (lanes 5 and 7). These results were consistent with *SWV* values (Fig. 7) detected by our biosensor. Therefore, the limit of detection of was 0.2×10^{-6} mmol L^{-1} (S/N = 3).

4 Discussion

In the present study, we have demonstrated how a modelled biosensor may be applied to identify transgenic plants. In comparison with conventional methods including Southern blotting and PCR, this novel biosensor can provide a rapid, simple and low-cost DNA detection system, with no laborious or complicated operations involved. It avoids a series of time-consuming steps required for Southern blotting analysis, especially the use of radio-labelled probes. With this biosensor, target nucleic acids could gain immediate access to the capture probes immobilized on an electrode surface, which greatly enhances the efficiency of aforementioned hybridization. Once an optimized biosensor is generated, it can be utilized repeatedly and could significantly simplify the detection process. From our experience, 3 hrs were enough to finish a biosensor, compared to at least two working days for Southern blotting analyses. Moreover, no expensive instruments or chemical reagents were required for the electrochemical analysis, thus minimizing cost.

The specificity of the biosensor is a critical factor for a successful assay. In PCR, amplification of unwanted DNA fragments is consistently unavoidable. Although purification of target bands from gel electrophoresis could remove most non-specific fragments, non-specific PCR products of the same size as a specific sequence might not be completely eliminated. In this study, a capture probe and a signaling probe were designed to be complementary to target gene regions flanked by the PCR primers. Although non-specific PCR products contain the same primer sequences at both ends as specific PCR fragments, only gene specific PCR product could efficiently bind to the electrode surface for electrochemical detection. It is, therefore, not necessary to optimize PCR conditions for this biosensor assay.

The response of the biosensor increased linearly with the target concentration ranging from 1.0–100×10^{-6} mmol L^{-1}, suggesting that efficient hybridization on the surface of the gold electrode occurred only at low target concentrations, when each biotinylated hybrid was sufficiently distant from the others, and intermolecular steric hindrance was minimal [28]. It has been suggested that biotinylated hybrids might agglutinate in the solution and may not effectively gain access to the electrode surface at high target concentrations. Another reason might be the length of the PCR product. After denaturation, long DNA fragments tended to form hairpins or stem-loops by intramolecular base pairing. Such structures would hide parts of the target sequences recognized by both the capture probes immobilized on the electrode surface, and the biotinylated signaling probes.

The phenotype of transgenic plants could be influenced by the copy number of transgenes and zygosity. To examine the analytical effect of the biosensor on the identification of transgene copy number, samples were amplified from tobacco plants with either one or two copies of *NPT-II* (copy number was confirmed by Southern-blotting). Negative

control samples included PCR blank and PCR products from the wild-type tobacco plant. In addition, the quantity of template and the cycle number of PCR were optimized. The results indicated that this electrochemical assay was not suitable to distinguish between one and two copies of the transgene (data not shown). This might be attributable to the amount of PCR cycles used, which probably reached saturation levels and thus did not accurately reflect the copy number of the transgene. Copy number of transgenes could be detected indirectly by the discrepancy of electrochemical signals resulting from the quantity of hybrids formed on the electrode surface, which is dependent on the amount of target DNA amplified by PCR. PCR, in turn, must be terminated before saturation and a threshold must be defined to reflect the difference of the original template number. Chaumpluk et al. found that the amplification of target genes (*Pth* and 12S rRNA genes) at 30 cycles was effective in solving the problem of similar signals caused by PCR saturation, thus successfully demonstrating the relationship between signals and gene content with the biosensor [29]. A proper cycle number of PCR in the exponential phase of amplification is a critical factor to determine copy number of transgenes in future experiments.

As for zygosity, like the case of copy number of transgene, a proper cycle number of PCR should be also defined to reflect the difference of transgene between the homozygous and heterozygous transgenic plants. German et al. developed a method named as duplex quantitative real-time PCR, which could be used in identification of zygosity of the transgenic plants in a relatively short time. In their study, fluorescence levels were directly related to the accumulation of the PCR product. Threshold cycle (Ct), a critical point of fluorescence accumulation to a significant level could be perceived by the detection system, depended primarily on the starting amounts of nucleic acid. Zygosity status of the transgene could be judged according to the magnitude of Ct [30]. However, this method required sophisticated and expensive equipment. Electrochemical assay for homozygosity and heterozygosity of transgenic tobacco plants with an optimized biosensor would be the subject of ongoing work in our laboratory and more consideration will be placed on optimization of PCR parameters, including cycle number and amount of the template.

5 Conclusions

In this study, a novel DNA electrochemical biosensor was developed and characterized for the detection of *NPT-II,* a selective marker widely used for transformation of many plant species mediated by *Agrobacterium tumefaciens*. Our biosensor possesses a feature of universal utility in the identification of transgenic plants. In our experiment, a favorable ratio of specific vs. non-specific signal was about 103.5. The specificity of the biosensor was significantly improved by using thiol-modified capture and biotinylated signaling probes complementary to target regions flanked by PCR primers.

The detection sensitivity was greatly enhanced by the dendritic enzymatic magnification of the hybridization signal. Responses of the sensor increased almost linearly with target concentrations ranging from $1.0–100 \times 10^{-6}$ mmol L^{-1}, corresponding to a maximal and minimal peak current of 0.55 ± 0.05 μA and 0.031 ± 0.01 μA, respectively. The limit of detection was 0.2×10^{-6} mmol L^{-1}. In summary, the biosensor designed in this work could provide a platform for the establishment of a reliable, accurate, sensitive and efficient detection system for emerging transgenic plants.

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References

1. James C (2005) Global status of commercialized biotech/GM crops. ISAAA Briefs 34
2. Michael S, Dipl C, Andreas M (2005) Angew Chem Int Ed 44:7842
3. Wiegel D, Glazebrook J (2002) In: Sambrook J (ed) A laboratory manual, 2nd edn. Cold Spring Harbor, New York, p 165
4. Holst-Jensen A, Rønning SB, Løvseth A et al (2003) Anal Bioanal Chem 375:985
5. Meyer R (1999) Food Control 10:391
6. Halford WP (1999) Nat Biotechnol 17:835
7. Schweitzer B, Kingsmore S (2001) Curr Opin Biotech 12:21
8. Li HK, Huang JH, Lv JH et al (2005) Angew Chem Int Ed 44:5100
9. Lopez-Andreo M, Lugo L, Garrido-Pertierra A et al (2005) Anal Biochem 339:73
10. Tajima K, Enishi O, Amari M et al (2002) Biosci Biotech Biochem 66:2247
11. Alexander TW, Reuter T, Aulrich K et al (2007) Anim Feed Sci Tech 133:31
12. Zhang J, Song SP, Zhang LY et al (2006) J Am Chem Soc 128:8575
13. Wang J (2006) Biosens Bioelectron 21:1887
14. Broude NE (2002) Trends Biotechnol 20:249
15. Gau V, Ma SC, Wang H et al (2005) Methods 37:73
16. Kelley SO, Boon EM, Barton JK et al (1999) Nucleic Acids Res 27:4830
17. Doong RA, Shih HM, Lee SH et al (2005) Sens Actuator B Chem 111–112:323
18. Mao XL, Yang LJ, Su XL et al (2006) Biosens Bioelectron 21:1178

19. Berganza J, Olabarria G, García R et al (2007) Biosens Bioelectron 22:2132
20. Palecek E (2002) Talanta 56:809
21. Chaumpluk P, Kerman K, Takamura Y et al (2007) Sci Technol Adv Mat 8:323
22. Li XM, Ju HQ, Du LP et al (2007) J Inorg Biochem 101:1165
23. Lucarelli F, Marrazza G, Mascini M et al (2005) Biosens Bioelectron 20:2001
24. Lucarelli F, Marrazza G, Masicini M et al (2004) Biosens Bioelectron 19:515
25. Lucarelli F, Marrazza G, Macini M et al (2006) Langmuir 22:4305
26. Carpini G, Lucarelli F, Marrazza G et al (2004) Biosens Bioelectron 20:167
27. Ryschkewitsch C, Jensen P, Hou J et al (2004) J Virol Methods 121:217
28. Patolsky F, Lichtenstein A, Willner I (2001) J Am Chem Soc 123:5194
29. Chaumpluk P, Chikae M, Takamura Y (2006) Sci Technol Adv Mat 7:263
30. German MA, Kandel-Kfir M, Swarzberg D (2003) Plant Sci 164:183