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A strategy for development of electrochemical DNA biosensor based on site-specific DNA cleavage of restriction endonuclease

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ABSTRACT

A new strategy for development of electrochemical DNA biosensor based on site-specific DNA cleavage of restriction endonuclease and using quantum dots as reporter was reported in this paper. The biosensor was fabricated by immobilizing a capture hairpin probe, thiolated single strand DNA labeled with biotin group, on a gold electrode. BfuCI nuclease, which is able to specifically cleave only double strand DNA but not single strand DNA, was used to reduce background current and improve the sensitivity. We demonstrated that the capture hairpin probe can be cleaved by BfuCI nuclease in the absence of target DNA, but cannot be cleaved in the presence of target DNA. The difference before and after enzymatic cleavage was then monitored by electrochemical method after the quantum dots were dissolved from the hybrids. Our results suggested that the usage of BfuCI nuclease obviously improved the sensitivity and selectivity of the biosensor. We successfully applied this method to the sequence-selective discrimination between perfectly matched and mismatched target DNA including a single-base mismatched target DNA, and detected as low as 3.3×10^{-14} M of complementary target DNA. Furthermore, our above strategy was also verified with fluorescent method by designing a fluorescent molecular beacon (MB), which combined the capture hairpin probe and a pair of fluorophore (TAMRA) and quencher (DABCYL). The fluorescent results are consistent with that of electroanalysis, further indicating that the proposed new strategy indeed works as we expected.

1. Introduction

Methods for the sequence-specific DNA detection have attracted significant attention due to possible applications in fields ranging from virus detection to the diagnosis of genetic diseases (Heller, 2002; Balakin et al., 1998). Consequently, various techniques have been employed for the detection of DNA hybridization, such as chromosome analysis (Jorge et al., 1996), fluorescence in situ hybridization (Jilani et al., 2008) and real-time quantitative reverse transcription PCR (Rong et al., 2002). But there were some limitations in these techniques, such as time-consuming, poor precision and expensiveness. So it is very significant to develop new effective methods.

Within recent years, several inventive designs for DNA sensors based on an electrochemical readout have appeared due to the fact that electrochemical detectors are simple, reliable, cheap, sensitive and selective for genetic detection (Miao and Bard, 2003, 2004). These sensors can be prepared by immobilizing single-stranded DNA probes on different electrodes and using electroactive indicators or other methods to measure the hybridization events between the DNA probes and their complementary DNA fragments. Consequently, a variety of sensing strategies have been developed, aiming at the improvement of sensitivity and selectivity. For example, DNA hybridization detection dealing with the use of electroactive indicators interacting directly and specifically with the DNA duplex, such as intercalators, DNA groove binders, metal complexes and threading agents, have been recently studied (Demeunynck et al., 2003; Li et al., 2007; Liu and Anzai, 2004; Niu et al., 2006; Chen et al., 2008a,b). While intercalator-based DNA sensing protocols take the advantages of design simplicity and operation convenience, they often suffer from high background signals that are associated with nonspecific binding of intercalators to unhybridized ssDNA.

In an attempt to circumvent this problem, DNA hairpins were proposed and popularly employed. DNA hairpins have been found to exhibit extraordinary stability, better selectivity and higher specificity than similar assays performed using single-stranded DNA probe. Furthermore, DNA probes with hairpin structure exhibit a particularly high sensitivity to detect mismatch. Consequently, their hybridization processes can significantly enhance the specificity and improve the signal-to-noise ratio, thus enabling the

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detection of as few as picomolar DNA targets. The detection limit can be further pushed down to the femtomolar level by coupling the hairpin-type sensors with signal amplification offered by using either enzymes (Liu et al., 2008) or inorganic nanoparticles labels (Gao and Yang, 2006). These strategies greatly improved the sensitivity, however, practical application of these strategies might be hampered due to poor stability of enzymes or relatively poor specificity for the detection of single-base mismatch and single-nucleotide polymorphisms (SNPs). Moreover, it is important to develop a simple and rapid approach to realize simultaneous detection of multiple targets. Existing sensors are often not amenable to detection in complicated samples such as DNA species related to virus. So, new sensitive and selective DNA hairpin-type strategies still need to be developed.

Cymbidium mosaic virus (CymMV) is one of the most prevalent and economically important orchid viruses (Wong et al., 1994). It infects numerous commercially important orchid genera and has attained a worldwide distribution (Zettler et al., 1990). CymMV cause flower colour breaking, size reduction and stunted growth, thus reducing the quality of the orchids and greatly affecting the economy of the orchid industry. Therefore, it is very significant to develop a new effective method for detection of CymMV. Herein, we reported a new strategy for development of electrochemical DNA biosensor for detection of DNA species related to virus (cymbidium mosaic virus, CymMV, in this case) using site-specific DNA cleavage of restriction endonuclease (REN) – BfuCI. A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded or single-stranded DNA at specific recognition nucleotide sequences known as restriction sites (Kessler and Manta, 1990). In this case, BfuCI is able to specifically cleave only double strand DNA, but not single strand DNA. It have been widely used in life processes, such as the replication, transcription, recombination and repair of nucleic acids, however using BfuCI in electrochemical biosensors have not been reported. In our strategy, the capture probe, thiolated hairpin DNA sequence labeled with biotin group at the other end, was immobilized on a gold electrode through S–Au bonding (Fig. 1). The stem of hairpin DNA is a special double-stranded DNA that contains a BfuCI REN recognition site (-mer sequence: 5′-GATC-3′) (Shizuka et al., 2008). The electrode was then blocked with 2-mercaptoethanol (MCH) to form a mixed monolayer. MCH has been used as a spacer to minimize nonspecific binding and maximize the efficiency of hybridization of capture and target probes (Cheng et al., 2007). Next, the immobilized biotin hairpin probes were hybridized with various target DNA sequences, then the hybridized electrode surface was incubated with REN to discriminate between perfectly matched target DNA (PM, complementarity with the loop part of hairpin probe) and mismatched target DNA (MM), including a single-base mismatched (SM) target DNA.

2. Experimental

2.1. Chemicals and apparatus

All oligonucleotides were synthesized by TaKaRa biotechnology Co., Ltd. (Dalian, China), and their base sequences are illustrated in Table 1. BfuCI buffer were obtained from BoCai Biotechnology Co. Ltd. (Shanghai, China). Avidin-QDs (with a CdSe/ZnS core–shell structure about 10 nm) were obtained from Wuhan Jiayuan Quantum Dots Co., Ltd. (Wuhan, China). Tris-(hydroxymethyl) aminomethane was purchased from Cxbio Biotechnology Co. Ltd. (Denmark). Ethylenediaminetetraacetic acid (EDTA), mercaptohexanol (MCH) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma–Aldrich (USA). The buffer solutions are as follows: hybridization buffer was the mixture of 100 mM NaCl and 10 mM TE (pH 8.0), buffers for DNA immobilization buffer are the mixture of 10 mM TE, 10 mM TCEP, 100 mM NaCl and 10 mM $MgCl_2$ (pH 7.4), $MgCl_2$ was added into the electrolyte to induce the formation of the hairpin structure of cDNA assembled onto the electrode surface, as reported previously (Dai et al., 1998; Dao et al., 1992; Gao et al., 1999; Ramsing et al., 1989). Washing buffer was the mixture of 0.1 M NaCl and 10 mM PB (pH 7.4). Solution contained 0.1 mol/L KCl and 2 mmol/L $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ was used for electrochemical impedance spectroscopy (EIS) characterization. All solutions were prepared with MilliQ water (18.2 MΩ/cm resistivity) from a Millipore system.

The electrochemical measurements for electrochemical impedance spectroscopy (EIS), stripping voltammetry and differential pulse voltammetry (DPV) were carried out on a CHI 660C electrochemical working station (CH Instrument Company, USA) using a three-electrode system consisted of a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and a 2-mm diameter Au disk electrode as working electrode (for EIS) or a glassy carbon electrode as working electrode (for stripping voltammetry and DPV). The spectra and intensity of fluorescence were measured with a Eclipse spectrofluorometer (Varian).

2.2. Electrode preparation

The whole fabrication process of this biosensor is outlined in Fig. 1. A gold disk electrode (GE) was firstly polished to obtain mirror surface with 0.05 μm alumina powder, followed by sonication in ethanol and water for 5 min, respectively. Then, the GE was electrochemically cleaned to remove any remaining impurities (Fan et al., 2003). After drying with nitrogen, the electrode was immediately used for DNA immobilization. Firstly, 5 μL of S1 solution was first spread on the pre-cleaned gold electrode surface for 12 h in the 100% humidity. Next, this electrode was immersed in 1 mmol/L MCH for 2 h to remove the nonspecific DNA adsorption and optimize the orientation of the capture probes to make hybridization easier (Zhang et al., 2006). Then, the S1-immobilized electrode was immersed in hybridization buffer containing complementary T1, single-base mismatch T2 or uncomplementary T3, respectively. The hybridization was allowed for 30 min with stirring at 45 °C. Afterwards, the sensor was then immersed in a solution of BfuCI reaction buffer (0.067 units/μL) in NEBuffer 41× (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) and rinsed with wash buffer. Prior to electrochemical measurement, the modified electrode was incubated with 3 μL of Avidin-QDs for 25 min at room temperature and washed with wash buffer to remove the physical adsorption of QDs and dried under a stream of nitrogen.

2.3. Electrochemical detection

The Au electrode modified with hybrids labeled with biotin–avidin-system was immersed into a colorimetric tube containing 200 μL of 0.2 M nitric acid solution for 5 min. The dissolved Cd^{2+} solution was transferred into 2.0 mL of the 0.1 M pH 5.3 HOAC-NaAC buffer supporting electrolyte solution containing 10 μg/mL $HgCl_2$. Stripping voltammetric measurements of the dissolved Cd^{2+} were performed using an in situ plated mercury film on a glassy carbon electrode following a pretreatment at 0.6 V for 1 min, and a accumulation at −1.4 V for 5 min. The positive (DPV) scan was performed after a 15-s rest period from −0.8 to −0.5 V (vs. Ag/AgCl), with pulse amplitude of 50 mV and pulse width of 50 ms. The anodic stripping peak current i_p, a located at about −0.68 V was taken as the analytical response.

Electrochemical impedance experiments were performed in the presence of 2 mM $[Fe(CN)_6]^{4-/3-}$. The biased potential was 0.22 V (vs Ag/AgCl) and the amplitude was 5.0 mV. The electrochemical

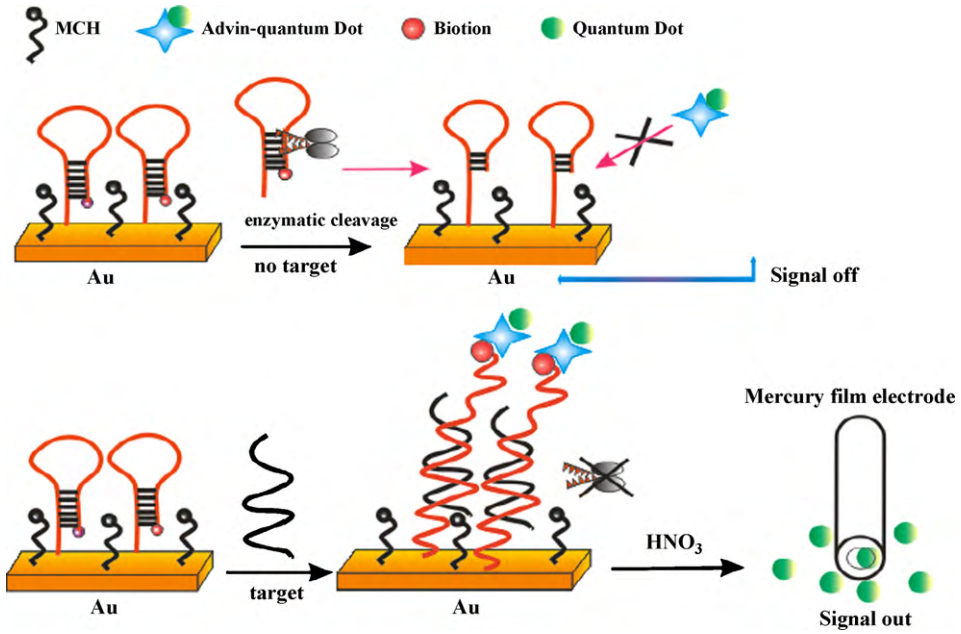

Fig. 1. Diagram of the procedure for the fabrication of DNA biosensor.

impedance spectra (EIS) were recorded in the frequency range of 0.1–10^5 Hz with a sampling rate of 12 points per decade. A Nyquist plot (Z_{re} vs Z_{im}) was drawn to analyze the impedance results.

2.4. Investigation of specificity by fluorescence

Under the optimum condition, the molecular beacons were added in NEBuffer 41× (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) containing BfuCI (0.067 units/μL), complementary DNA or complementary DNA + BfuCI (0.067 units/μL), respectively. The hybridization was allowed for 30 min with stirring at 45 °C. Afterwards, in the determined excitation and emission wavelengths (λ_{ex} = 521 nm, λ_{em} = 586 nm), the fluorescence intensity of molecular beacons and the fluorescence intensity of hybridization buffer were detected individually. Compare the change of the fluorescence intensity and investigate the specificity.

3. Results and discussion

3.1. Strategy for design of this electrochemical DNA biosensor

In the absence of PM target, the capture probe exits predominantly in the hairpin form (loop–stem structure, at certain concentrations and buffer conditions). So it can be easily cleaved by REN. Thus, avidin labeled QDs (Avidin-QDs) cannot bind to the capture probe through the biotin–avidin-system. However, in the presence of the PM target, the loop part of the hairpin probe was hybridized with the target strand to form the matched duplex DNA. Meanwhile, the stem is opened to form single strand and resistant to the cleavage of REN. Thus the Avidin-QDs can then bind to the capture probe. So, the hybridization events can be monitored by stripping voltammetry after the QDs were dissolved from the hybrids. In contrast to prior electrochemical DNA detection schemes based on DNA hairpins (Immoos et al., 2004; Fan et al., 2003), this strategy generates an electrochemical signal upon recognition of the target DNA (i.e., signal "on" device).

3.2. EIS of different modified electrodes

Electrochemical impedance technique was employed to characterize the fabrication in whole process. Fig. 2 shows the EIS changes for surface-modified process. Compared with the bare Au electrode (Fig. 2a), the probe S1 modified and MCH treated Au electrode (S1/MCH/GE) shows a larger eT resistance (Fig. 2b), mainly due to the electrostatic repulsion between negative charges of the DNA backbone and the $Fe(CN)_6^{3-/4-}$ probe. After the probe modified electrode was immersed into the BfuCI reaction buffer solution, the eT resistance (Fig. 2c) decreased. The resistance decrease could be attributed to the fact that the stem of the hairpin probe structure contains a cleavage site for BfuCI and was subsequently cleaved by this enzyme. The cleavage resulted in the decrease of the negative charges of DNA on the GE surface. Under optimal condition, the S1 can hybridize with target DNA T1 and become double-stranded DNA (S1–T1/MCH/GE). At this time, the monolayer of DNA on the electrode surface became denser, and the negative charges on the electrode surface increased. Thus the electron-transfer resistance is enhanced further, and the value of Ret increases (Fig. 2d). When

Table 1
Details of the DNA sequences.

Capture probe (S1)[a]	5′-HS-AAA A GA TCT T CA AAC TGC GGA CAT T AA GAT C-3′
Molecular beacon[b]	5′-TAMRA-GATCTT CAA ACTGCGGACATT AAGATC-DABCYL-3′
Target (T1)	5′-AAT GTC CGC AGT TTG-3′
Single-base mismatch (T2)	5′-AAT GTC CAC AGT TTG-3′
Noncomplementary (T3)	5′-CCG TCA ATT AAG CCA-3′

[a] The program was used to predict the solution-phase conformation of this sequence. This program predicts that under the hybridization conditions adopted for the analysis, only one secondary structure (the hairpin one) is thermodynamically stable, confirming the suitability of the chosen sequence (Zuker, 2003).
[b] Tetramethoxyl Rhodamine (TAMRA) as fluorophore, 4-(2-methyl-on-amino-azobenzene) benzoate (DABCYL) as quencher.

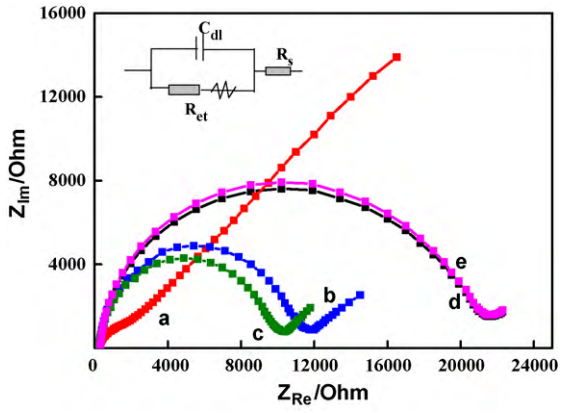

Fig. 2. Impedance spectra (Nyquist plot) of bare GE (a), the S1/MCH modified GE (b), the S1/MCH modified GE after being immersed into the BfuCI in reaction buffer solution (c), the S1–T1/MCH modified GE (d) and S1–T1/MCH modified GE after being immersed into the BfuCI in reaction buffer solution (e) in the presence of 2 mM $[Fe(CN)6]^{4-/3-}$. The biased potential was 0.22 V (vs Ag/AgCl) in the frequency range of 0.1–10^5 Hz and the amplitude was 5.0 mV.

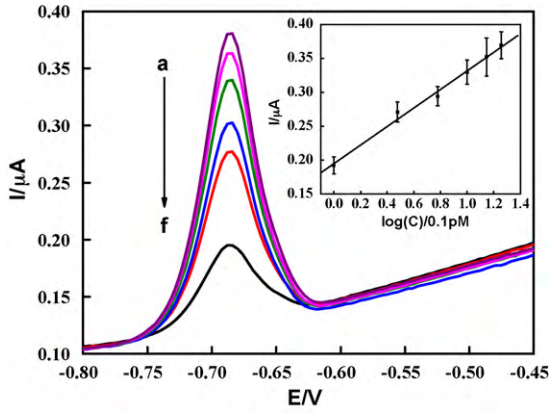

Fig. 3. DPV for different target concentrations of T1 sequence ($\times 10^{-13}$ mol/L): (f–a) background, 1.0, 3.0, 6.0, 10.0, 14.0 and 18.0. Inset: plot of *I* vs logarithm of concentration. Error bars = ±relative standard deviation.

the S1–T1/MCH/GE was directly combined with BfuCI, no significant difference of eT resistance was observed (Fig. 2e). It may be that the formation of S1–T1 structure essentially does not contain the REN cleave site. Therefore, it cannot be cleaved by BfuCI, which indicated that the hybridization events could be monitored by employing this novel electrochemical strategy.

3.3. Sensitivity of the DNA biosensor

The sensitivity of the DNA biosensor was detected as shown in Fig. 3. The results showed that the peak current value of DPV increased with the concentration of the target DNA. The linear range for logarithm of target DNA was $1.0 \times 10^{-13} \sim 1.8 \times 10^{-12}$ M with the equation of I (μA) = 0.1366 log C (0.1 pM) + 0.1953 (I was the current intensity, and C was the concentration of target DNA, r = 0.9956, n = 6). The relative standard deviations (RSD) of the sensor for 10 replicate determination of 1.0×10^{-12} M target DNA was 2.8%. The detection limit of 3.3×10^{-14} M target DNA could be estimated using 3σ. This detection limit is lower than some existing electrochemical biosensors based on quantum dots as reporter (Sun et al., 2008; Wang et al., 2003; Zhu et al., 2004).

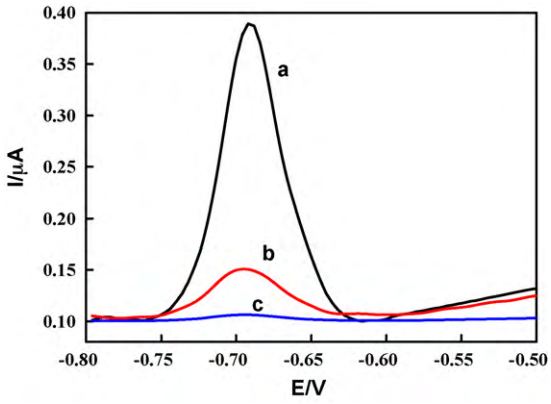

Fig. 4. DPV of hybridization with different kinds of target DNA: (a) complementary sequence; (b) one-base mismatched sequence; (c) noncomplementary sequence. All the concentrations of target DNA used were 1.8×10^{-12} M.

3.4. Selectivity of the DNA biosensor

The selectivity of the present biosensor was investigated by using the DNA probe (S1) to hybridize with the same concentration of complete complementary target DNA sequence (T1), the single-base mismatched DNA sequence (T2) and the noncomplementary DNA sequence (T3), respectively. As shown in Fig. 4, a well-defined peak current signal of Cd^{2+} was obtained for the complementary sequence (Fig. 4, curve a). It can be also known that the present biosensor has high hybridization specificity, it can easily discriminate the complementary from single-base mismatch target DNA. In the presence of oligonucleotide containing a single-base mismatch, significantly decreased voltammetric signal can be observed (Fig. 4, curve b), which indicates that the complete hybridization is not accomplished due to the base mismatch. In addition, as expected, nearly no response of peak current can be observed for the capture probe hybridization with noncomplementary sequence oligonucleotide (Fig. 4, curve c), since no successful hybridization occurs due to the sequence mismatch between the capture probe and the noncomplementary sequence.

The selectivity and specificity of the hairpin DNA probe was also studied by using fluorescence method, which was valuable in optimization of the hairpin DNA probe design. The fluorescent molecular beacons (MB) were designed by a combination of DNA hairpin structure and a pair of fluorophore (TAMRA) and quencher (DABCYL). The ends of the stem of the hairpin were modified by fluorophore and quencher, individually. Before hybridization, the structure of DNA probe kept hairpin, and the quencher was close to the fluorophore. At this time, the efficiency of fluorescence quenching played a key role. So there were little fluorescent signals on the DNA probe (Fig. 5, curve 1). After hybridization with the complementary DNA, with the hybridization of DNA and the change of the hairpin structure, the distance between the fluorophore and the quencher was increased. So the efficiency of fluorescence quenching between the fluorophore and the quencher was decreased. Thus the fluorescence intensities were increased (Fig. 5, curve 3). It is worth mentioning that the fluorescence intensities were significantly increased after MB was directly incubated with BfuCI nuclease (Fig. 5, curve 2). It could be attributed to the fact that when the MB is incubated with solutions containing BfuCI, the BfuCI carries out catalytic reactions to cleavage of the MB at the cleave site. Then the strand is broken into two pieces and dissociated from the MB. As a result, the amount of the quencher decreases, resulting in higher fluorescence intensities. However, after hybridization with the PM target DNA, the stem of the MB was opened, meanwhile, the new double helix structure of the DNA system does not contain the REN cleave site. Therefore, it cannot be cleaved by REN, the

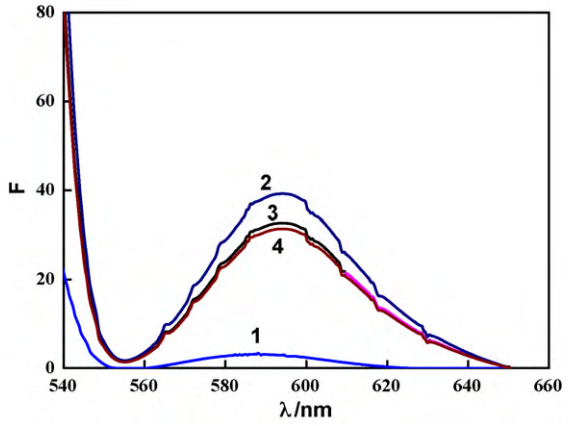

Fig. 5. Fluorescent graph of MB hybridizing with different gene fragments. Samples are MB (curve 1); MB after incubated in REN (curve 2); MB + complementary DNA (curve 3); MB + complementary DNA after incubated in REN (curve 4). Buffer solution: NEBuffer 4 1 × (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9). λ_{ex} = 521 nm, λ_{em} = 586 nm.

obtained fluorescence intensity was not significantly changed (see Fig. 5, curve 4). The results were in accordance with those obtained by electrochemical method. Of course, the performance of the hairpin DNA probe can also be monitored with fluorescent method. However, fluorescent method needs complex process of fluorescent labeling and purification, which are time-consuming, labor intensive and cost high. Compared to fluorescent method, the proposed electroanalysis has many advantages such as inexpensive instrument, lower detection limit and simplicity due to ease of obtaining electrical signal.

4. Conclusions

In summary, we have introduced here a novel strategy for development of electrochemical DNA biosensor based on hairpin probe and site-specific DNA cleavage of restriction endonuclease. This biosensor was used to detect DNA species related to cymbidium mosaic virus. The results illustrated that the proposed sensor has the advantages of higher sensitivity and lower background current. Given the simplicity in design of the proposed electrochemical sensor, it is fairly easy to generalize this strategy to detect a spectrum of targets. Furthermore, this design could be also used to construct novel optical DNA biosensors. So, it might have a promising future for investigation of DNA hybridization and would play the potential predominance in diagnosis of virus or diseases.

Acknowledgements

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