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Short communication

Enzyme-enhanced fluorescence detection of DNA on etched optical fibers

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ABSTRACT

A novel DNA biosensor based on enzyme-enhanced fluorescence detection on etched optical fibers was developed. The hybridization complex of DNA probe and biotinylated target was formed on the etched optical fiber, and was then bound with streptavidin labeled horseradish peroxidase (streptavidin-HRP). The target DNA was quantified through the fluorescent detection of bi-*p*,*p*′-4-hydroxyphenylacetic acid (DBDA) generated from the substrate 4-hydroxyphenylacetic acid (*p*-HPA) under the catalysis of HRP, with a detection limit of 1 pM and a linear range from 1.69 pM to 169 pM. It is facile to regenerate this sensor through surface treatment with concentrated urea solution. It was discovered that the sensor can retain 70% of its original activity after three detection-regeneration cycles.

1. Introduction

DNA sequence recognition was generally based on the hybridization between immobilized oligonucleotide probes and the targets. The massive practice of DNA hybridization detection requires the development of easy-to-use, fast, inexpensive and miniaturized analytical devices. Various protocols have been used to detect DNA hybridization, including fluorescence, electrochemical, chemiluminescence and colorimetric detection (Wu et al., 2008; Shiddiky et al., 2007; Zhang et al., 2008; Cao et al., 2005). Of these techniques, the fluorescence detection has attracted particular attention because it provides a faster, more accurate, more sensitive and selective platform than others (Yamada et al., 2008; Stevens et al., 2008; Ryu et al., 2008; Chiang et al., 2008). In a traditional DNA fluorescent sensor, a fluorophore was always attached to the target sequence, and the fluorescence was generated and detected when the hybridization event occurred. Molecular Beacons (MB) technique emerged in recent years (Li et al., 2008a; Miranda-Castro et al., 2007; Mahajan et al., 2008; Jin et al., 2007; Dubertret et al., 2001; Bratu et al., 2003; Kostjukov et al., 2007; Du et al., 2005; Mao et al., 2008; Wu et al., 2008; Angelatos et al., 2007), involving the use of a hairpin DNA probe with a fluorophore and a quencher at each end. The conformation of the probe changes when hybridization happens, and the fluorescence was generated and detected.

The sensitivity of DNA detection can be improved using target or signal amplification strategies (Schweitzer and Kingsmore, 2001; Feng et al., 2008; Campbell et al., 2002). PCR technique is often employed to amplify the target, but its approach always requires tedious, time-consuming and expensive operation. The enzyme bound to DNA hybridization complex through biotin–streptavidin interaction has been established as a representative system for signal amplification (Mao et al., 2008; Alfonta et al., 2001; Riccardi et al., 2006). Traditionally, enzyme-labeled DNA sensor was constructed through the binding of enzyme on the hybridization complex, and the signal was electrically generated in the redox reaction of the products of the enzyme-catalyzed reactions. Recently, novel enzyme-labeled electrochemical DNA sensors were developed. As a typical example, Patolsky et al. demonstrated that enzyme labels, such as peroxidase and alkaline phosphatase, induce the biocatalyzed precipitation on electronic transducers and the amplification of signal (Patolsky et al., 2001).

It was considered that the combination of enzyme-amplification and the fluorescence detection in the design and construction of a DNA sensor can lead to better performance. In this work, a novel fluorescent DNA biosensor with enzyme-amplification was developed. The probe was immobilized onto the modified fiber surface through a biotin–streptavidin binding, and was then hybridized with the target. After streptavidin labeled horseradish peroxidase (streptavidin-HRP) was bound to the hybridization complex, DNA was quantified through fluorescent detection of

Abbreviations: Streptavidin-HRP, streptavidin labeled horseradish peroxidase; DBDA, bi-*p*,*p*′-4-hydroxyphenylacetic acid; *p*-HPA, 4-hydroxyphenylacetic acid; NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate; 3-APTS, (3-aminopropyl) triethoxysilane; PBS, sodium phosphate-buffered saline; B–R, Britton–Robinson; HRP, horseradish peroxidase.

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bi-*p*,*p*′-4-hydroxyphenylacetic acid (DBDA) generated from the 4-hydroxyphenylacetic acid (*p*-HPA) substrate, catalyzed by HRP.

2. Experiment

2.1. Apparatus

A Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) equipped with 1 cm quartz cells was used for fluorescence measurements. The optical fibers (φ 3.6 mm) were purchased from Peking Glass Research Institute R&D Center. The pH of all solutions was measured by a Model pHS-3D digital acidometer (Rex Electric Chemical Products Department, Shanghai Precision Scientific Instrument Co., Ltd.).

2.2. Materials

All oligonucleotides and streptavidin labeled horseradish peroxidase (streptavidin-HRP) were purchased from Beijing Sunbiotech Co. Ltd. (Beijing, China) and were used without further purification. The oligonucleotide sequences used in this work were:

- H1 (probe): 5′-GTG AGC CAA GAC GGA AAG ACC CGC TCA C-(-3′C6)(biotin)-3′.
- T1 (H1 complementary): 5′-GGG TCT TTC CGT CTT G-(-3′C6)(biotin)-3′.
- T1N1 (non-complementary): 5′-CCC AGA AAG GCA GAA C-(-3′C6)(biotin)-3′.
- T1C1 (control DNA): 5′-(C6 fluorescein) GTG AGC CAA GAC GGA AAG ACC CGC TCA C(-3′C6)(biotin)-3′.

Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin), the streptavidin, (3-aminopropyl) triethoxysilane (3-APTS) and Tween-20 were purchased from Sigma. The 4-hydroxyphenylacetic acid (*p*-HPA) was obtained from Johnson Matthey Company (Shanghai, China). The buffer of 0.1 M sodium bicarbonate (pH 8.5), 0.07 M sodium phosphate-buffered saline (PBS, pH 7.4) and 0.2 M (pH 8.0) Britton–Robinson (B–R) were used as incubating and washing buffer. The substrate solution containing *p*-HPA was prepared by dissolving 13 mg *p*-HPA in 10 mL 0.2 M B–R (pH 8.0) buffer solution containing 0.6 mM H_2O_2, 1.3 M isopropanol. All other chemicals employed were of analytical grade.

2.3. Preparation of a molecular beacon DNA sensor

2.3.1. Pretreatment of fibers

A bunch of fibers of 3.6 mm diameter were used in a single immobilization cycle. The cladding was stripped away from the core through chemical etching in 65% HNO_3 for 30 min. The core fiber material is silica, and the cladding around each fiber is germania-doped silica, which was etched at a slower rate. According to relating reports (Rissin and Walt, 2006; Li et al., 2008b), this etching procedure would create an array of vessels with average depth of 2.9 μm and volume of 46 fL, thus facilitating the immobilization of the probes. The etched fibers were washed thoroughly with ultrapure water until neutrality before being used for the subsequent immobilization experiment.

2.3.2. Immobilization of biotinylated probes on fibers

The etched fibers were first cleaned in the following procedure: immersion in a 1:1 v/v concentrated HCl/MeOH mixture for 30 min; rinsing in water; immersion in concentrated sulfuric acid for 30 min; rinsing in boiling water for 8–10 min. The fibers were then chemical-modified by APTS through silanization through being immersed in a stirring solution consisting of 600 μL of toluene, 60 μL of Tween-20, 600 μL of ultrapure water, and 600 μL of APTS for 15 min. The APTS-modified fibers were thoroughly rinsed with ultrapure water to remove excess APTS. The modified fibers were then biotinylated through immersion in 0.1 mg mL^{-1} sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) in 0.1 M sodium bicarbonate buffer (pH 8.5) for 3 h at 20 °C. Streptavidin was bound to the fiber surface by incubating the biotinylated fibers overnight at 4 °C in 300 μL solution containing 2.8 mg mL^{-1} streptavidin and 40 mg mL^{-1} Tween-20. To test the effectiveness of this surface modification, probe DNA labeled with fluorescein (fluorescein-DNA-biotin, T1C1) was used. The streptavidin-immobilized fibers were immersed in a 1.83×10^{-6} M biotinylated probe solution (70 mM PBS, pH 7.4) overnight at 4 °C to ensure the captured probes to be immobilized on the surface. Any unreacted probes were then removed by rinsing with PBS. The biotinylated probe modified fibers were stored in 70 mM PBS at 4 °C for future use.

2.4. Hybridization of target DNA to the immobilized probes on fiber surface and linking the streptavidin-HRP

The sensor surface was washed with 70 mM PBS (pH 7.4). The biotinylated probe modified fibers were incubated for 2 h at 38 °C in 2 mL of PBS containing different concentrations of biotinylated target DNA and 1 mM $MgCl_2$, followed by a thoroughly washing with PBS to remove unbound target DNA. Then the fibers were immersed in 300 μL ultrapure water containing 20 μg mL^{-1} streptavidin-HRP for 35 min at 4 °C. After being rinsed with PBS to remove unbound streptavidin-HRP, the fibers were soaked in PBS at 4 °C prior to quantification.

2.5. Procedure of fluorescence quantification

To perform the fluorescence detection, the modified fibers were incubated at room temperature with 10 mL of substrate solution (the concentration of which was a parameter to be examined, see Section 3.3) in the black plastic cells for 1 h. 2 mL reacted solution was transferred into 1.0 cm quartz cells and the fluorescence measurements were carried out on a Hitachi F-4500 fluorescence spectrophotometer in the wavelength range from 340 nm to 520 nm at a scan speed of 1200 nm min^{-1} with excitation wavelength at 320 nm. Background fluorescence had been subtracted for each value.

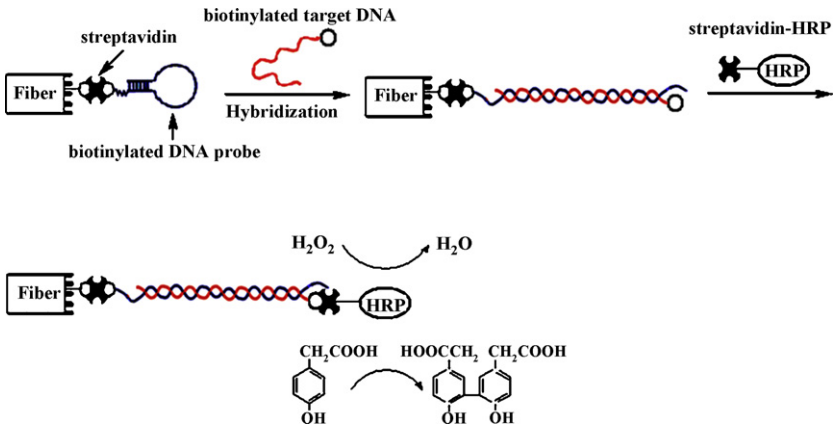
3. Results and discussion

3.1. Immobilization of the probes onto the fiber surface

The widely used streptavidin–biotin binding protocol was employed in this study to immobilize the probe on the sensor. The immobilization consisted of two steps: APTS modification through silanization which added amine groups (–NH_2) to the surface; conjugation between the amine groups and the NHS-ester of biotin. The streptavidin served as a bridge between the surface-immobilized biotin and biotinylated probe (Liu and Tan, 1999). Probe DNA labeled with fluorescein (fluorescein-DNA-biotin) was used to examine the modification. Fluorescence was observed on the surface only when fluorescein-DNA-biotin was immobilized (see Supplementary Materials, Figure S1), indicating that the biotinylated probe can be immobilized onto the optical fiber surface.

3.2. Fluorescent characterization of the DNA sensors

The working principle of the enzyme-amplified detection DNA was schematically represented in Scheme 1. It involved a DNA hybridization reaction between the immobilized probe and target DNA, binding of streptavidin-HRP and fluorescent detection of the



Scheme 1. The procedure of the enzyme-enhanced fluorescence detection of DNA.

enzyme-generated products. It was based on the fluorescence generated in the HRP-catalyzed reaction, which took place when the DNA hybrid complexes were formed on the fiber surface. The probe H1 was designed in a hairpin pattern, that is, the six bases on each of the two ends were complementary, just like a traditional molecule beacon without the fluorescein and quencher; according to several reports, this design could improve the hybridization specificity and sensitivity (Mahajan et al., 2008; Wang et al., 2008; Jin et al., 2007). In the presence of hydrogen peroxide and horseradish peroxidase (HRP), 4-hydroxyphenylacetic acid is very efficiently transformed into bi-*p*,*p*′-4-hydroxyphenylacetic acid. To evaluate the selectivity of the assay, the interaction of two oligonucleotides with the probe immobilized on the fiber surface was investigated. Fig. 1 showed the representative fluorescence intensity obtained in B–R buffer solution (pH 8.0) using bare fiber (curve a), non-complementary target DNA T1N1 (curve b), and target DNA T1 (curve c). There was no fluorescent peak at 406 nm for the bare fiber and the non-complementary target DNA, indicating the hybridization did not take place under the addition of T1N1. Fluorescent peak appeared after the fiber were incubated with complementary DNA (T1, curve c). The streptavidin-HRP was bound to the hybridization complex, resulting in enzymatic amplification.

3.3. Optimization of experimental conditions

3.3.1. DNA hybridization time

The fluorescent signal was monitored as a function of hybridization time. It was shown in Figure S2 A (see Supplementary Materials) that fluorescent signals increase with time up to 3 h after which the fluorescent intensity remains virtually unchanged. Therefore, 3 h was selected throughout our experiments.

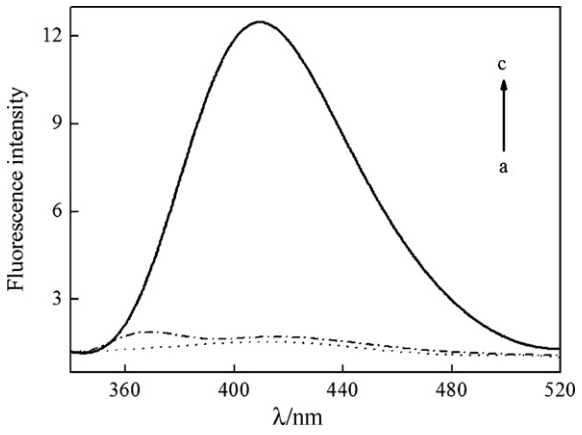


Fig. 1. Fluorescence response versus different optical fibers in substrate solution containing 0.2 M B–R buffer, 0.6 mM H_2O_2, 1.3 M isopropanol, 7.42 mM HPA. (a) Bare fiber; (b) H1 probe modified fiber, incubated 3 h in the presence of 1.69 pM non-complementary target DNA; (c) H1 probe modified fiber, incubated 3 h in the presence of 1.69 pM target DNA T1.

3.3.2. Enzyme incubation time

It was found the fluorescent intensity increased with an increase of the incubation time until the time up to 1 h (data not shown). So 1 h was chosen as the time of incubation in the enzyme conjugate solution for further studies.

To further optimize the analytical performance of the sensor, the influence of the hybridization temperature on the fluorescence intensity was also investigated (see Supplementary Materials, Figure S2 B). Since lower temperature leads to poor efficiency and higher temperature leads to denaturation, 38 °C was the optimal hybridization temperature.

3.3.3. The optimal enzymatic substrate concentration

The optimal enzymatic substrate concentration was also assessed (see Supplementary Materials, Figure S2 C). The response of the sensor was dependent only on the concentration of enzyme on the surface, which was directly related to the amount of hybridized biotinylated target. The fluorescence responses for the target sequence were examined against the concentration of HPA. Fluorescence intensity increased with the substrate concentration up to 7.42 mM; therefore, this value was used for all experiments.

3.3.4. Addition of isopropanol

The addition of a small quantity of isopropanol enhanced enzyme activities and had sensitization effect on the fluorescence of DBDA, which greatly amplified the hybridization signals (Tang et al., 2006). Different amount of isopropanol was added into the B–R buffer solution in the enzymatic reaction, respectively. The results (see Supplementary Materials, Figure S2 D) demonstrated that fluorescence intensity reached a maximum when the concentration of isopropanol was 1.3 M, then decreased with the increasing of concentration. The decrease may be attributed to the fluorescence of DBDA was quenched by the isopropanol, or the yield of DBDA decreased.

3.4. Regeneration of the biosensor

Reusability has always been a desired feature for biosensors in practical applications. Usually the regeneration of a DNA sensor was achieved by either thermal or chemical method. Considering that the streptavidin layer might be destroyed at high temperature, a chemical method was used to regenerate the biosensor. Specifically, after the first quantification process, the fibers were immersed in 8 M urea solution with continuous stirring for 30 min at 50 °C. The

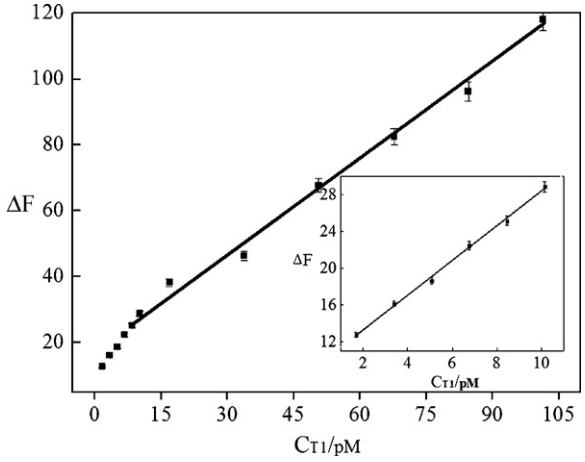


Fig. 2. Calibration plot, fluorescence response versus concentration of T1.

fibers were rinsed by ultrapure water for 15 min and rapidly cooled in an ice PBS solution for 1 min. After regeneration, the hybridization complex was dehelixed and the sensor returned to its original state. The regenerated sensor produced a similar response when treated with the same procedure described above, demonstrating the regenerability and stability of the biosensor. The responses in three regenerative cycles of the same sensor were tested. The fluorescence intensity decreased along with repeated regenerative cycles, decreasing by 16% (cycle 2), 30% (cycle 3), respectively.

3.5. The sensitivity of the biosensor

Figure S3 (see supplementary materials) showed representative fluorescent responses when increasing concentrations of target DNA hybridized under optimal experimental conditions. The signal increase with the increase of target concentration in the range from 1.69 pM to 169 pM with a detection limit of 1 pM as estimated by 3σ rule. The calibration curve is shown in Fig. 2. The regression equation was $F = 9.55 + 1.88(C_{T1}/\text{pM})$ (F was the fluorescence intensity of DBDA, background fluorescence has been subtracted. C_{T1} was the concentration of the target DNA) and the correlation coefficient r was 0.9993.

3.6. The application potential in real system

The detection protocol that was described here would be readily developed into a practical system after a few minor modifications. The physical size of the core apparatus would be small, including only a 20 mL plastic cell with φ 3.6 mm pre-etched fibers, which facilitate its porting and installing. The favorable regeneratability of this sensor make it somewhat recyclable and this ability would be securely expected to be improved in the future study. Thus this detection protocol promised a hopeful future in the practical field.

4. Conclusion

A novel DNA biosensor based on the fluorescence generated in the enzyme-catalyzed reaction was developed through binding HRP to the hybridization of target and probe. When hybridization occurred, DBDA was generated in the oxidation of *p*-HPA catalyzed by HRP, and the target DNA was quantified through the measurement of the fluorescence of DBDA. The concentration detection limit of the sensor for complementary target was 1 pM. This sensor was facile to regenerate through the treatment using urea at high concentration. It could retain 70% of original activity after three detection-regeneration cycles. It could be expected that this sensor has a hopeful future in the field of real detection system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.02.022.

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