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A sensitive fluorescence method for sequence-specific recognition of single-stranded DNA by using glucose oxidase

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A sensitive fluorescence method was developed for sequence-specific recognition of single-stranded DNA on the surface of silver-coated glass. Oligonucleotide 5′-HS-$(T)_{18}$-CGT CGC ATT CAG GAT-3′ (Oligo-1) was designed to assemble on the surface of silver-coated glass and acted as capture DNA, and Oligonucleotide 5′-TCT CAA CTC GTA GCT-$(T)_{18}$-biotin-3′ was designed as signal DNA (Oligo-2). Upon addition of target DNA (5′-AGC TAC GAG TTG AGA ATC CTG AAT GCG ACG-3′, Oligo-3), signal DNA could bind on the surface of silver-coated glass because of DNA hybridization. The biotin groups on Oligo-2 are then coated with streptavidin, and biotin labeled glucose oxidase (biotin-GOx) is added to bind to streptavidin. The quantity of GOx immobilized in this way is directly related to the quantity of target DNA bound on the surface. Following cleavage of the aptamer with DNase I, glucose is added and oxidized by GOx to yield H_2O_2. Horseradish peroxidase is added and causes the oxidation of 3-*p*-hydroxyphenylpropanoic acid to yield a fluorescent product. The intensity of the fluorescence is directly related to the target DNA concentration in the range of 25 pM to 5500 pM, and the detection limit was 7 pM. The assay had good sequence selectivity.

A Introduction

Deoxyribonucleic acid (DNA) is not only the carrier of genetic information, but also the main genetic material in organisms. Variations in DNA sequences are found to be the origin of several genetic diseases and individual differences in drug metabolism, and the DNA is usually of low abundance in the early stage of the above-mentioned diseases.[1–5] Because of the above-mentioned reasons, it is highly desirable to develop ultrasensitive methods for DNA detection. Therefore, a lot of DNA biosensors have been developed by using surface plasma resonance,[6–8] atomic force microscopy,[9,10] chemiluminescence,[11–13] electrochemical reactions,[14–16] fluorescence,[17–19] *etc.* Significant progress has been achieved in the diagnosis of genetic diseases using nanomaterials,[20] such as AuNPs,[21] magnetic nanoparticles,[22] silica nanoparticles,[23] carbon nanomaterials,[24] quantum dots,[25] *etc.*

Enzymes are widely employed in biosensors as recognition and signaling elements for the detection of specific molecules due to their high sensitivity and good selectivity.[26] Glucose oxidase (GOx) is one of the cheapest and most stable redox enzymes, which could catalyze the oxidation of glucose. So far, GOx has been used to construct electrochemical,[27–30] fluorescence[31–33] and colorimetric[34] sensors for glucose on one hand. On the other hand, GOx was conjugated to recognition biomolecules and acted as an amplifying label, which was used to develop electrochemical sensors for protein[35–37] and DNA[38] successfully. Herein we explore the possibility to develop a fluorescence sensor for sequence-specific recognition of ssDNA with GOx modified oligonucleotides.

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B Experimental

Reagents

Quartz glass slides (10 mm × 10 mm × 1 mm) were purchased from Guangliang High-tech Co. (Jiangsu, China). All oligonucleotides were synthesized and purified by Sangon Bioengineering Technology and Services Co. Ltd. (Shanghai, China). They were dissolved in PBS buffer, followed by cooling in ice prior to use (Table 1). Streptavidin (SA), horseradish peroxidase (HRP), glucose oxidase (GOx) and DNase I were obtained from Sangon Bioengineering Technology and Services Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA) and tri-(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich (USA). 3-(*p*-Hydroxyphenyl)-propanoic acid (HPPA), silver nitrate, ammonia (25%) and glucose were purchased from Sinopharm chemical Reagent Co., Ltd. (Beijing, China). An EZ-link Sulfo-NHS-Biotinylation Kit was purchased from Thermo Fisher Scientific Inc (USA). All chemicals were of analytical grade.

Phosphate buffered solution (PBS) (pH 7.4, 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, and 0.1 M $NaNO_3$)[39] and Tris–HAc buffer

Table 1 Sequences of oligonucleotides

Probes	Sequence
Oligo-1	5′–HS–$(T)_{18}$-CGT CGC ATT CAG GAT-3′
Oligo-2	5′-TCT CAA CTC GTA GCT-$(T)_{18}$-biotin-3′
Oligo-3	5′-AGC TAC GAG TTG AGA ATC CTG AAT GCG ACG-3′
Oligo-4	5′-AGC TAC GCG TTG AGA ATC CTG AAT GCG ACG-3′
Oligo-5	5′-AGC TAC GCG TTG AGA ATC CTG ACT GCG ACG-3′
Oligo-6	5′-AGC TAC TCG TTG AGA ATC CTG AAT GCG ACG-3′

(10 mM, pH 7.5, 2.5 mM $Mg(NO_3)_2$, 0.5 mM $Ca(NO_3)_2$) were prepared for research.

Apparatus

The centrifugation experiment was carried out on an Anke GL-20G-II centrifuge (Anting Scientific Instrument Factory, Shanghai, China). The fluorescence experiment was carried out on an RF-5031 PC (Shimadzu, Japan). UV-vis absorption spectra were obtained on a TU-1901 double-beam spectrophotometer (Beijing Purkinje General Instrument Co. Ltd, China).

Preparation of the silver-coated glass slide

Silver-coated glass was prepared using the traditional silver mirror reaction.[40] The quartz glass slides must be stringently cleaned under ultrasound using the following substances one by one: detergent, sodium hydroxide, dilute nitric acid, ultrapure water, and ethanol. Finally, glass slides were dried in air prior to use. To obtain $Ag(NH_3)_2OH$ solution, 1% ammonia was added dropwise into 5.0 mL of 3% silver nitrate with gentle swinging to form a transparent yellow solution. Each of the glass slides was immersed in the mixture at room temperature for 5 minutes, and the ratio of $Ag(NH_3)_2OH$ solution to 10% glucose was 1 : 2. A shining silver film was formed on the surface of glass.

Immobilization of capture DNA on the silver coated glass slide

Immobilization of capture DNA (Oligo-1) on the silver film was based on the reported methods[41–44] with a little modification. Each of the silver-coated glass slides was placed in PBS buffer with Oligo-1 and deprotected by TCEP at room temperature for 12 hours. The final concentration of Oligo-1 was 1.5 μM. Then, the modified silver-coated glass was washed twice using the same buffer to remove excess Oligo-1.

Preparation of biotin-GOx

Based upon the conjugation of sulfo-NHS-biotin to GOx, the biotin-GOx was prepared as follows: first, 50 μl 10 mM sulfo-NHS-biotin was injected into 2.0 mL of 1 mg mL^{-1} GOx in PBS solution (pH 7.4) and incubated at room temperature under gentle stirring for 2.0 h. Excess sulfo-NHS-biotin was washed off using an Amicon filtration device 50 000 cutoff, and 1.96 biotin molecules were modified on each GOx molecule, which was calculated according to the instructions of the EZ-link Sulfo-NHS-Biotinylation Kit. Biotin-GOx was redispersed in PBS buffer (2 mL, pH 7.4) for the research.

Fabrication of the sensor

The Oligo-1 modified silver-coated glass was immersed in 1.1 mL PBS buffer containing different concentrations of target DNA (Oligo-3) and incubated at 25 °C for 2 hours. Then, the glass was dipped into 1.1 mL PBS buffer containing 10 nM Oligo-2 at 25 °C for another 2 hours. The silver-coated glass was immersed in 3% bovine serum albumin of PBS buffer for 30 minutes to block possible remaining active sites and avoid the nonspecific adsorption of streptavidin and biotin-GOx. 1.1 mL of 500 ng mL^{-1} SA solution was added into each tube and incubated for 15 minutes at room temperature. Then, 1.1 mL of 100 μg mL^{-1} biotin-GOx solution was added into each tube and incubated for 15 minutes at room temperature. The slide was washed twice, and then immersed in 1.1 mL Tris–HAc buffer containing 50 U of DNase I for 1 hour at 37 °C. Then, 0.8 mL of the mixture was added into 0.2 mL of 50 mM glucose for 2 hours at 37 °C to yield H_2O_2. 3-(*p*-Hydroxyphenyl)-propanoic acid (HPPA) and horseradish peroxidase (HRP) were added into 1.0 mL of the above-mentioned solution. The final concentrations of HPPA and HRP were 50 μM and 20 ng mL^{-1}, respectively, and the mixture was kept in darkness for 30 minutes at 37 °C. After that, the fluorescence spectra of the oxidative product of HPPA were recorded with an excitation wavelength of 320 nm. The fluorescence intensity at 415 nm was used for quantitative analysis of target DNA.[45]

C Results and discussion

Strategy of the sensor

The sensing mechanism of the sensor is shown in Fig. 1. Capture DNA (Oligo-1) is designed to assemble on the surface of silver-coated glass through the Ag–S bond. Upon addition of target DNA (Oligo-3) and signal DNA (Oligo-2), they can bind on the surface of silver coated glass. After addition of streptavidin (SA)-biotin-GOx, it can bind to biotin at the end of signal DNA. GOx is immobilized on the surface of silver-coated glass, and its concentration is dependent on that of target DNA. In order to avoid nonspecific adsorption of GOx onto silver-coated glass, DNase I was used to cleave the bound GOx from the surface of silver-coated glass. Then, the concentration of target DNA can be transduced into that of H_2O_2 in the presence of glucose. H_2O_2 can oxidize HPPA into a fluorescent product under the catalysis of HRP. The concentration of target DNA could be estimated with the fluorescence intensity of oxidized HPPA in the end.

Optimization of the experimental conditions

To obtain optimum results, the experimental conditions such as the concentrations of capture DNA (Oligo-1), signal DNA (Oligo-2), streptavidin (SA), biotin-GOx, 3-(*p*-hydroxyphenyl)-propanoic acid (HPPA), and horseradish peroxidase (HRP) are optimized. The optimal conditions were selected by obtaining the maximum change of fluorescence intensity (ΔI). ΔI is defined as $I_{target} - I_{blank}$, I_{target} represents the fluorescence intensity in the presence of target DNA, while I_{blank} denotes the fluorescence intensity in the absence of target DNA. After

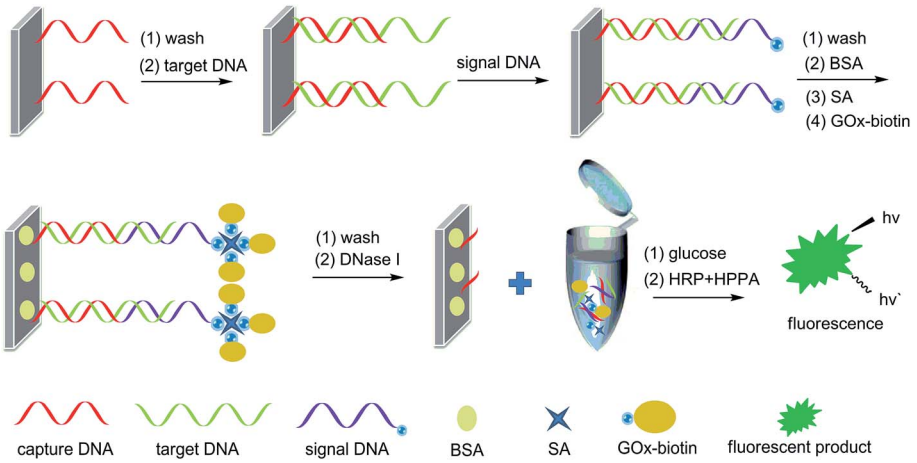

Fig. 1 Scheme for fluorescence determination of target DNA by using a capture DNA immobilized on the surface of silver-coated glass and amplification by glucose oxidase that hydrolyses from the surface by DNase I.

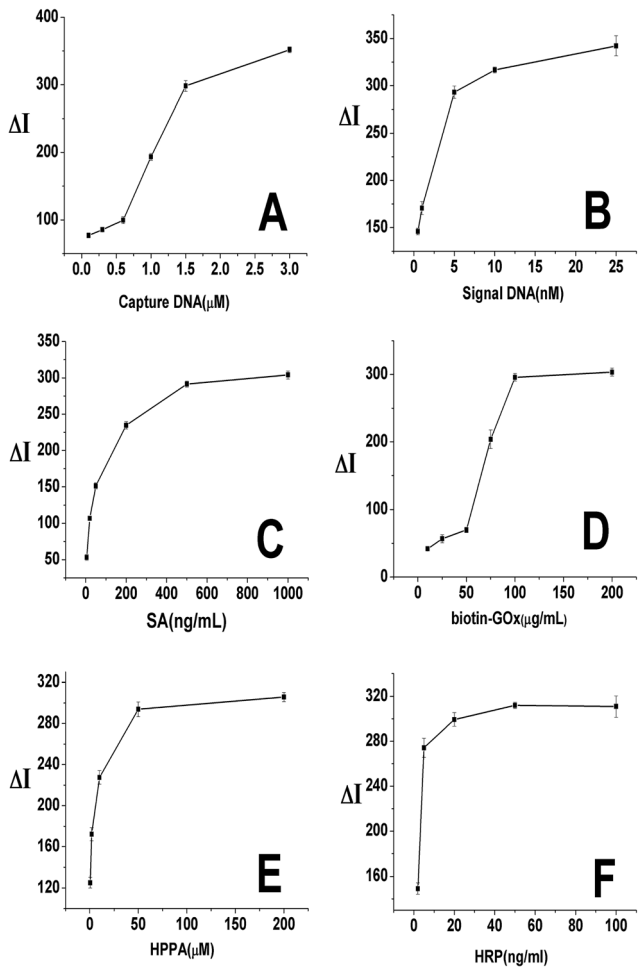

Fig. 2 (A) Effects of capture DNA concentrations, (B) effects of signal DNA concentrations, (C) effects of SA concentrations, (D) effects of biotin-GOx concentrations, (E) effects of HPPA concentrations, and (F) effects of HRP concentrations. $\Delta I = I_{target} - I_{blank}$. The concentration of target DNA was 2500 pM. Every point is the mean of three measurements. The error bar represents the standard deviation.

careful investigation, as shown in Fig. 2, 1.5 μM of capture DNA, 10 nM of signal DNA, 500 ng mL^{-1} of SA, 100 μg mL^{-1} of biotin-GOx, 50 μM HPPA and 20 ng mL^{-1} of HRP are the optimum conditions for the research.

Under the optimum conditions for the research, the effect of the concentration of target DNA on the fluorescence intensity was investigated. As shown in Fig. 3, the intensity increased with the concentration of target DNA over the range of 25 pM to 5500 pM, with a linear regression equation of $I = 0.123C + 41.9$ (C: pM, $r = 0.995$, C was the concentration of target DNA, and I was the fluorescence intensity) and a detection limit of 7 pM, which was obtained from the equation of DL = 3σ/slope. The reproducibility for 5.0 pM target DNA was 3.2%.

Sequence selectivity of the assay

In order to investigate the sequence selectivity of the assay, the fluorescence intensity of the sensor was measured upon addition of different sequences. As shown in Fig. 3, the intensity for target DNA was about 690, while those for the single-base mismatched strand (Oligo-4) and the two-base mismatched strand (Oligo-5 and Oligo-6) decreased to 212, 157 and 139

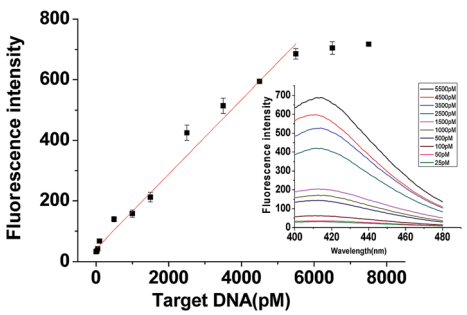

Fig. 3 Calibration curve for target DNA and the fluorescence spectrum of different concentrations of target DNA. Each point is the mean of three measurements. The error bars represent the standard deviation.

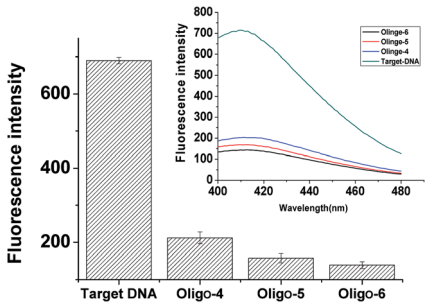

Fig. 4 Sequence selectivity of the assay and the fluorescence spectrum of target DNA, Oligo-4, Oligo-5 and Oligo-6. The concentrations of target DNA, Oligo-4, Oligo-5 and Oligo-6 were 5500 pM. Every point is the mean of three measurements. The error bar represents the standard deviation.

respectively, which indicated the good sequence selectivity of the assay (Fig. 4).

D Conclusions

In summary, a fluorescence method for sequence-specific recognition of ssDNA was established by using the amplification properties of GOx. Capture DNA was assembled on the surface of silver-coated glass through the Ag–S bond, and signal DNA was designed to conjugate with GOx through the interaction of biotin–SA, and then the GOx could be immobilized on the surface of silver-coated glass upon addition of target DNA. The concentration of target DNA controlled the number of bound GOx, which could be detected using the fluorescence of oxidized HPPA. Under the conditions of 1.5 μM of capture DNA, 10 nM of signal DNA, 500 ng mL^{-1} of SA, 100 μg mL^{-1} of biotin-GOx, 50 μM HPPA and 20 ng mL^{-1} of HRP, the fluorescence intensity increased linearly with the concentration of target DNA over the range of 25 pM to 5500 pM, with a detection limit of 7 pM. Moreover, single-base mismatched and two-base mismatched sequences could be distinguished. The advantage of this technique is high sensitivity, and the disadvantage is that it is time consuming.

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