Analytical Methods

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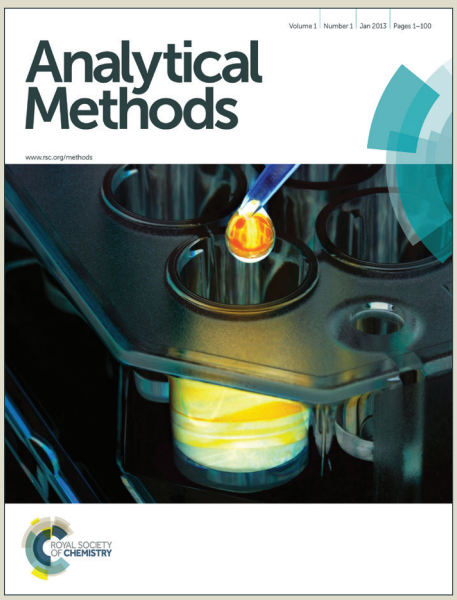

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Sensitive fluorescent method for sequence specific recognition of single-stranded DNA by using Glucose Oxidase

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Abstract A sensitive fluorescent method for sequence-specific recognition of single-stranded DNA was developed 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 from 25 pM to 5500 pM, and the detection limit was 7 pM. The assay had good sequence selectivity.

Keywords: ssDNA; fluorescence; Glucose oxidase; Silver - coated glass

A Introduction

Deoxyribonucleic acid (DNA) is not only the carrier of genetic information, but is the main genetic material in the organism as well. 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 low-abundant 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 biosensor have been established by using surface plasma resonance [6-8], atomic force microscopy [9, 10], chemiluminescence [11-13], electrochemical reactions [14-16], and fluorescence [17-19] et al. 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] and Quantum Dots[25] et al..

Enzymes are widely employed in biosensors as recognition and signaling elements for the detection of specific molecule due to its 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 was 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 biomolecule and acted as amplifying label, which was used to establish electrochemical sensors for protein [35-37] and DNA [38] successfully. Herein we explore the possibility to develop a fluorescent sensor for sequence-specific recognition of ssDNA with GOx modified Oligonucleotides.

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. 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), 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). EZ-link Sulfo-NHS-biotinylation Kit was purchased by Thermo Fisher Scientific Inc (USA). All chemicals were 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 $NaNO_3$), Tris-HAc buffer (10 mM, pH 7.5, 2.5 mM $Mg(NO_3)_2$, 0.5 mM $Ca(NO_3)_2$) were prepared for research .

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'

Apparatus

Centrifugation experiment was carried out on Anke GL-20G-II centrifuge (Anting Scientific Instrument Factory, Shanghai, China). Fluorescence experiment was carried

out on RF-5031PC (SHIMADZU, Japan). UV-vis absorption spectra were obtained on a TU-1901 double-beam spectro-photometer (Beijing Purkingje General Instrument Co. Ltd, China).

Preparation of silver-coated glass slide

Silver-coated glass was prepared using the traditional silver mirror reaction[45]. The quartz glass slides must be stringently cleaned under ultrasound using following substance one by one: detergent, sodium hydroxide, dilute nitric acid, ultrapure water, ethanol. Finally, glass slides were dried in air prior to use. To get Ag $(NH_3)_2OH$ solution, 1% ammonia was added dropwise in 5.0 mL of 3% silver nitrate with gentle swinging to form a transparent yellow solution. Each of glass slide was immersed in the mixture at room temperature for 5 minutes, and the ratio of $Ag(NH_3)_2OH$ solution and 10% glucose was 1:2. 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 [39-42] with a little modification. Each of the silver-coated glass slide 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 by 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: Firstly, 50 μl 10 mM Sulfo-NHS-biotin was injected into 2.0 mL of 1 mg/mL GOx in PBS solution (pH 7.4) and incubated at room temperature under gently stirring for 2.0 h. Excess sulfo-NHS-biotin was washed off using an Amicon filtration device 50,000 cutoff, 1.96 biotin molecules was modified on per GOx molecule, which was calculated according to the instructions of 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 concentration of target DNA (Oligo-3) and incubated at 25 ^{o}C for 2 hours. Then, the glass was dipped into 1.1 mL PBS buffer containing 10 nM Oligo-2 at 25 ^{o}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 SA solution was added into each tube and incubated for 15 minutes at room temperature. Then, 1.1 mL of 100 μg/mL biotin-GOx solution was added into each tube and incubated for 15 minutes at room temperature. The slide was washed twice, and then was immersed in 1.1 mL Tris - HAc buffer that containing 50 U of DNase I for 1 hour at 37 ^{o}C. Then, 0.8 mL of the mixture was added into 0.2 mL of 50 mM glucose for 2 hours at 37 ^{o}C to yield H_2O_2. 3-(p-Hydroxyphenyl)-propanoic acid

(HPPA) and horseradish peroxidase (HRP) were added into 1.0 mL of above-mentioned solution. The final concentration of HPPA and HRP were 50 μM and 20 ng/mL respectively, and kept the mixture at 37 ^{o}C in darkness for 30 minutes. After that, fluorescent spectra of the oxidative product of HPPA were recorded with excitation wavelength of 320 nm. The fluorescent intensity at 415 nm was used for quantitative analysis of target DNA [43].

C Results and discussion

Strategy of the sensor

The Strategy of the sensor is demonstrated in Fig.1. Capture DNA (Oligo-1) is designed to assemble on the surface of silver-coated glass through 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 on the end of signal DNA. GOx is immobilized on the surface of silver-coated glass, and its' concentration is depended on that of target DNA. In order to avoid nonspecific adsorption of GOx to 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 the 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.

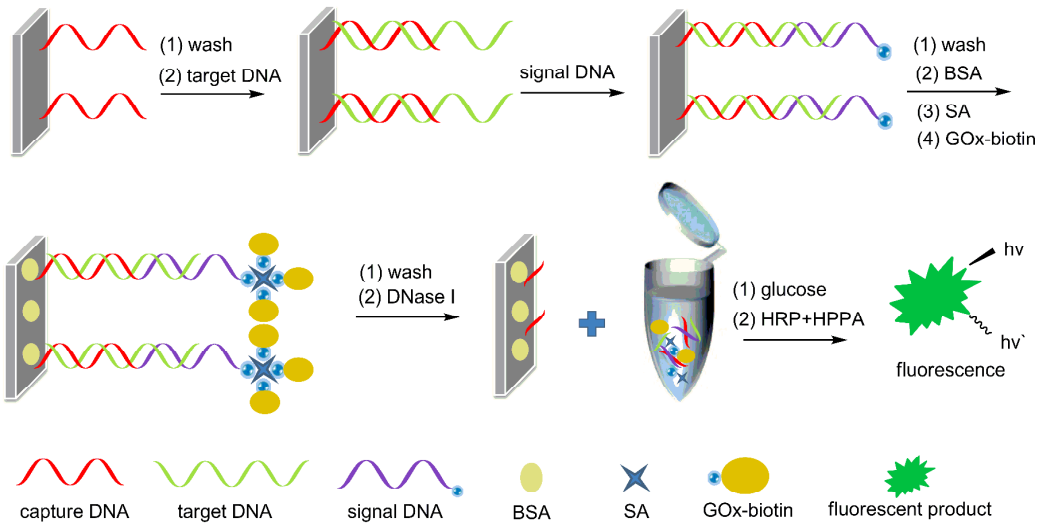

Figure 1. Scheme for fluorescent determination of target DNA by using an capture DNA immobilized on the surface of silver-coated glass and amplification by glucose oxidase that hydrolysis from the surface by DNase I

Optimization of the experiment conditions

To obtain the optimum results, the experiment conditions such as the concentration of capture DNA (Oligo-1), signal DNA (Oligo-2), streptavidin (SA), biotin-GOx, 3-(p-Hydroxyphenyl)-propanoic acid (HPPA), horseradish peroxidase (HRP) are optimized. The optimal conditions were selected by obtaining the maximum change of fluorescence intensity (ΔI). ΔI was 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 careful investigation, as shown in Fig. 2, 1.5 μM of capture DNA, 10 nM of signal DNA, 500 ng/mL of SA, 100 μg/mL of biotin-GOx, 50 μM HPPA and 20 ng/mL of HRP are the optimum conditions for the research.

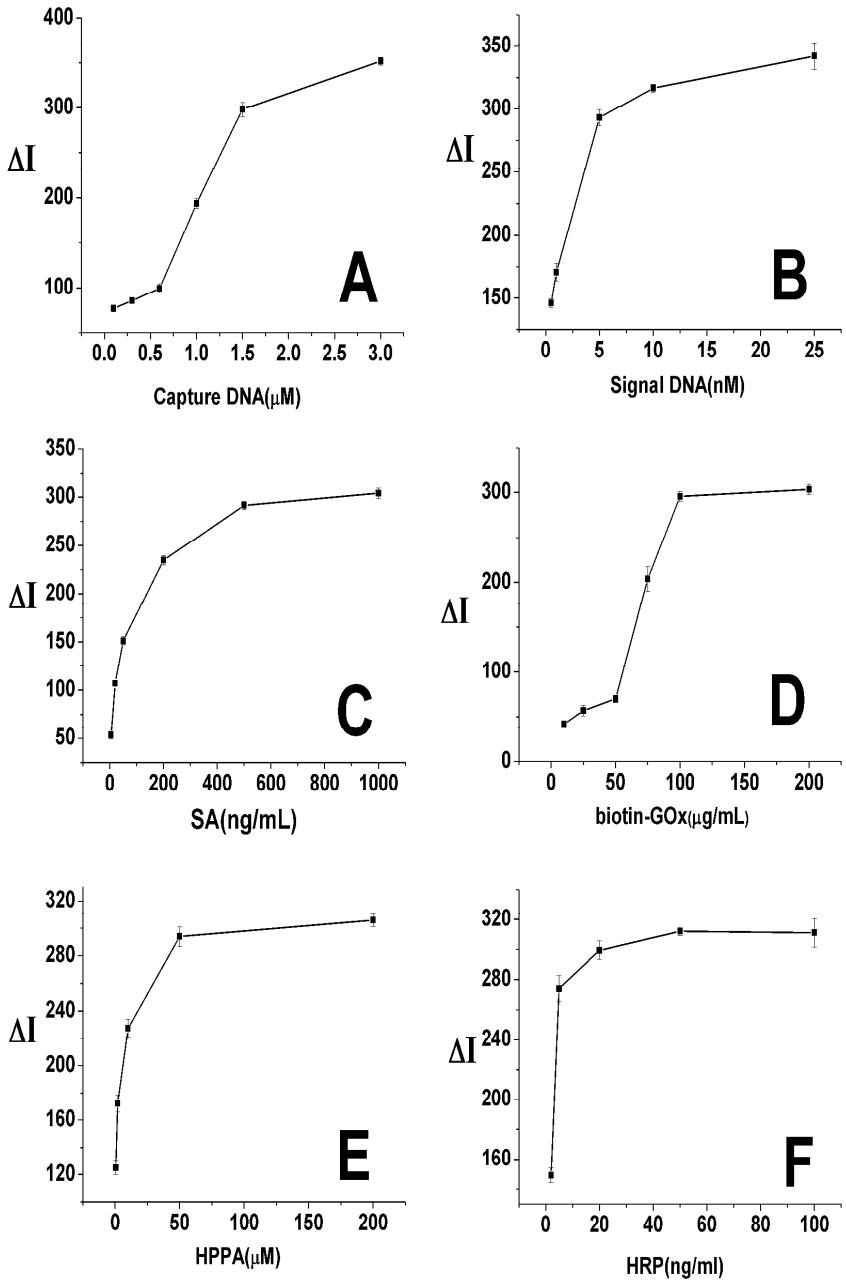

Figure 2. (A) Effects of capture DNA concentrations, (B) Effects of signal DNA concentrations, (C) Effects of SA concentrations, (D) Effects of biotin - GOx concentrations, (E) Effects of HPPA concentrations, (F) Effects of HRP concentrations . $\Delta I = I_{target} - I_{blank}$. The concentration of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

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 from 25 pM to 5500 pM, with a linear regression equation of I = 0.123 C + 41.9 (C: pM, r = 0.995, C was the concentration of target DNA, I was the fluorescence intensity) and a detection limit of 7 pM, which was obtained from the equation of DL = 3 б / slope. The reproducibility for 5.0pM target DNA was 3.2%.

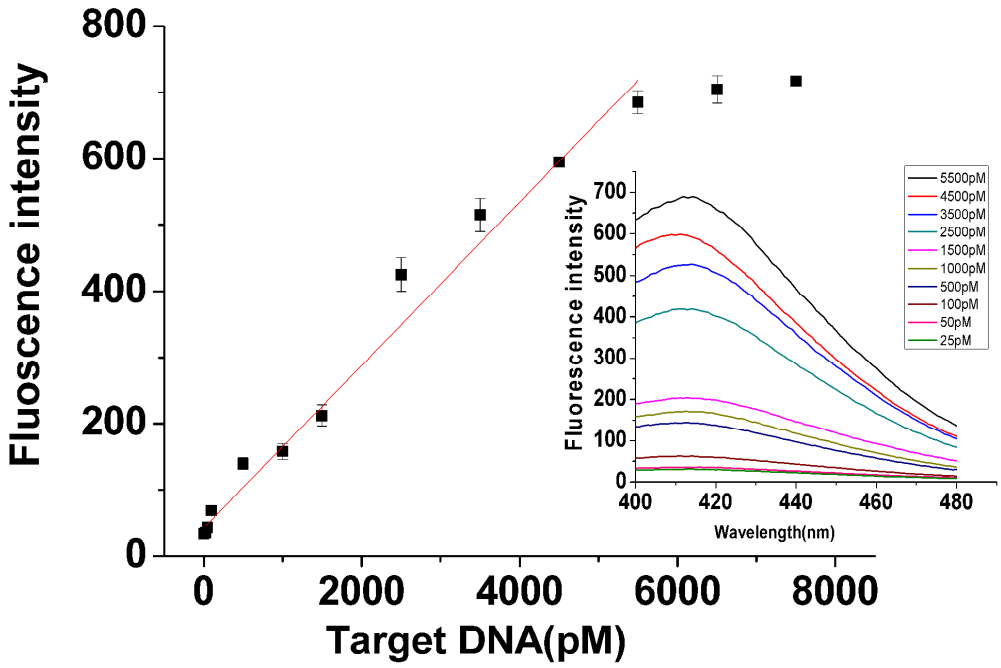

Figure 3. Calibration curve for target DNA and the fluorescence spectrum of different concentration of target DNA. Each point was the mean of three measurements. The error bars are the standard deviation.

Sequence selectivity of the assay

In order to investigate the sequence selectivity of the assay, the fluorescence intensity of the sensor were measured upon addition of different sequence. As shown in Fig.3,

the intensity for target DNA was about 690, while that for single-base mismatched strand (Oligo-4) and two-base mismatched strand (Oligo-5 and Oligo-6) were decreased to 212, 157 and 139 respectively, which indicated good sequence selectivity of the assay.

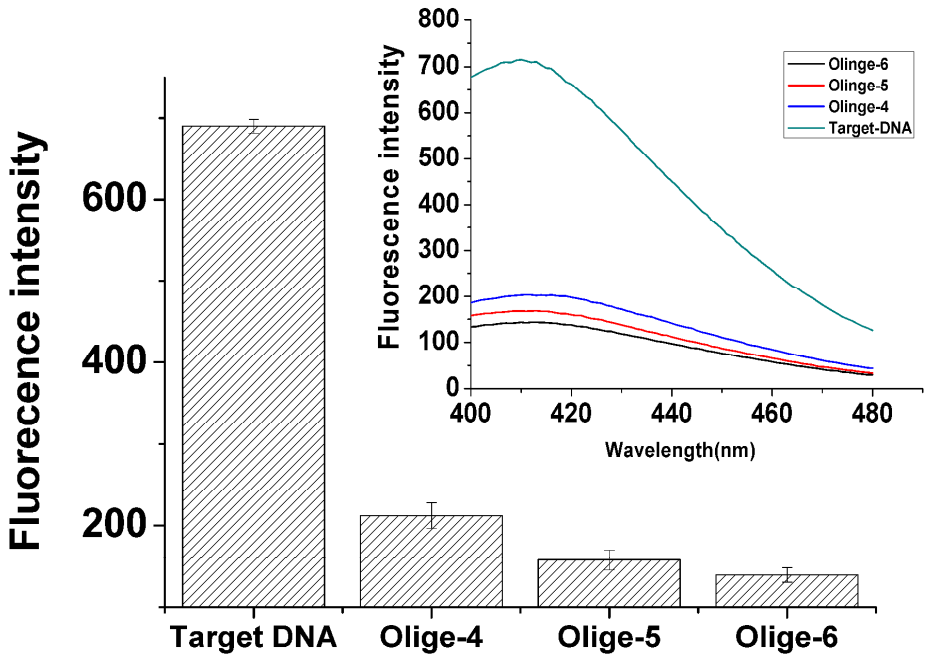

Figure 4. Sequence selectivity of the assay and the fluorescence spectrum of target DNA, Olige-4, Olige-5 and Olige-6. The concentration of target DNA, Olige-4, Olige-5 and Olige-6 were 5500 pM. Every point was the mean of three measurements. Error bar was the standard deviation.

Conclusions

In summary, a fluorescent method for sequence-specific recognition of ssDNA was established by use of the amplification property of GOx. Capture DNA was assembled on the surface of silver-coated glass through Ag-S bond, and signal DNA was

designed to conjugate with GOx through the interaction of biotin-SA, 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 with the fluorescence of oxidized HPPA. Under the conditions of 1.5 μM of capture DNA, 10 nM of signal DNA, 500 ng/mL of SA, 100 μg/mL of biotin-GOx, 50 μM HPPA and 20 ng/mL of HRP, the fluorescence intensity increased linearly with the concentration of target DNA over the range from 25 pM to 5500 pM, with a detection limit of 7 pM. Moreover, single-base mismatched and two-base mismatched sequence could be distinguished. The advantages of this technique is high sensitivity, and the disadvantage is time consuming.

Acknowledgements

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Graphical Abstract

Sensitive fluorescent method for sequence specific recognition of single-stranded DNA by using Glucose Oxidase

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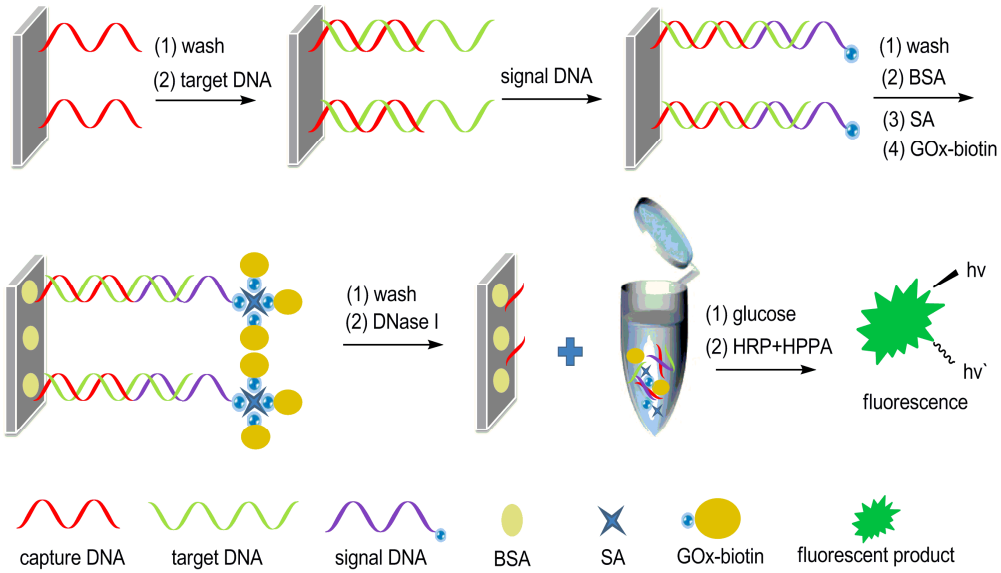

A sensitive fluorescent method for sequence-specific recognition of single-stranded DNA was developed on the surface of silver-coated glass by mean of the amplification property of glucose oxidase .