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A DNA electrochemical biosensor based on homogeneous hybridization for the determination of *Cryptococcus neoformans*

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ABSTRACT

The sensitive analysis of DNA is of extreme importance in the field of clinical diagnosis, targeted therapeutics, gene therapy, and a variety of biomedical studies. Even though significant achievements have been made in the detection of single-stranded DNA (ssDNA), facile strategy for the detection of double-stranded DNA (dsDNA) is still lacking. In this study, we have constructed an electrochemical DNA sensor based on homogeneous hybridization. Target dsDNA is thermal denatured and hybridized with thiolated capture probe and biotin-labeled reporter probe in solution phase. Excessive amount of the probes and precise control of hybridization temperature ensure the predominant formation of sandwich SH-dsDNA-biotin rather than reannealing product of the target dsDNA. A bovine serum albumin (BSA)-modified gold surface is designed to improve self-assembly capability of the sandwich SH-dsDNA-biotin structure in the presence of excessive SH-ssDNA probe. Thus, the target dsDNA can be successfully detected by using enzyme-linked amperometric amplification. By employing this strategy, dsDNA can be detected in a linear range from 5 pM to 1 nM with a detection limit down to 800 fM (S/N = 3). *Cryptococcus neoformans* in clinical real samples from cryptococcal meningitis patients can be discriminated successfully by the proposed method. Our research would make the electrochemical biosensor be an excellent candidate for pathogens detection in clinical diagnosis and prognosis.

1. Introduction

Cryptococcus neoformans is a globular recessive infectious pathogen. It forms a yeast-type colony when developed in a few days, but is different from yeast and candida. *Cryptococcus neoformans* can break through the blood-cerebrospinal fluid barrier and infect the central nervous system, causing fatal cryptococcal meningitis. The disease is characterized by the nonspecific clinical and laboratory findings associated with long treatment period, high morbidity and mortality, and difficulty in early diagnosis [1–3]. Furthermore, in recent years, it has been paid high attention to pulmonary cryptococcosis induced by *Cryptococcus neoformans*. *Cryptococcus neoformans* can attack the lungs rapidly when patients have some immunocompromised diseases, such as organ transplant, malignant tumor and HIV infection, and then developed into acute respiratory distress syndrome (ARDS) [4–7]. At present, the common laboratory methods for identification of *Cryptococcus neoformans* are microbiological culture, smear and serologic antigen test. Among these methods, microbiological culture identification is considered classic method but it is time-consuming for 4 days at least. Smear by ink dyeing is fast and simple, but it needs to be over checked to find the positive result and the positive rate is non-rewarding. Antigen detection is commonly accepted for the diagnosis because of the higher specificity but it has been found that there was a cross-reaction between the cryptococcal capsular polysaccharide and sera from the trichosporum and some other bacterias in ELISA [8]. In addition, various factors, such as rheumatoid factor and antinuclear antibodies, can test positive in the latex agglutination test and cause a false positive result for the cerebrospinal fluid antigen [9]. Therefore, these methods can not accurately reflect the virulence and vitality of *Cryptococcus neoformans* and it is necessary and very important for the detection of *Cryptococcus neoformans* in higher specificity and sensitivity.

DNA is one of the key biomolecules that is especially attractive for bioscience, because it carries out many biological functions such as

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storing and transmitting genetic information, decoding information in cells, and regulating biochemical activities [10]. Over the past few decades, biologists and chemists have devoted considerable efforts to the development of sensitive and effective DNA probing methods [11–17]. The DNA hybridization event between single-stranded DNA (ssDNA) probe and its complementary ssDNA target to form a DNA double helix has become the main principle for the construction of DNA biosensors. The optical, electrochemical, piezoelectric, or thermal transducer can convert the result of this recognition into a quantified signal for detection. Among these approaches, electrochemical DNA biosensor is particularly attractive for development of clinical point-of-care platform for DNA analysis because of its unique merits such as amenable to miniaturization, fast response, sensitive, simple operation, and low cost [18–33].

Electrochemical DNA biosensors usually rely on the immobilization of ssDNA probe onto an electrode, which permits them to capture its complementary DNA target sequence by heterogeneous hybridization occurring on the interface between solution and electrode [34]. Using the electrochemical biosensors based on this protocol, satisfactory results can be obtained for the detection of synthetic ssDNA. However, only a few studies have focused on the detection of dsDNA in clinical samples. The key problem is how to effectively make the dsDNA in the sample suitable for the hybridization system in the biosensor. High temperature denaturation is the simplest way to obtain ssDNA from dsDNA. However, the complementary sequence of the target ssDNA in the solution severely competes with the capture probe on the electrode surface to hybridize with the target, which leading to low hybridization efficiency on the biosensor surface [35,36]. Other methods, such as asymmetric PCR and Lambda exonuclease digestion, have been used to prepare single-stranded target DNA required for electrochemical DNA sensors [37–41]. Although these methods work better than high temperature denaturation for electrochemical DNA sensor, the increased procedure and detection time are inevitable.

As compared to conventional heterogeneous hybridization strategy used in electrochemical DNA sensor, the homogeneous hybridization process has been performed in solution phase, rather than in solid-liquid-phase, has faster kinetics and higher hybridization efficiency. Some new platforms such as cone-shaped Dendron [42], Au nanoparticles on horizontally aligned single walled carbon nanotubes [43], and DNA probes with tetrahedral nanostructure [44–46], have been devoted to increase target accessibility and reduce surface crowding effect, providing solution-like environment for quasi-homogeneous hybridization. Despite their many positive attributes, these approaches, however, typically require complex reagents and multistep, time-consuming processing.

In this work, a facile approach is established for dsDNA analysis based on homogeneous hybridization and competitive assembly. Two ssDNA probes are used as blocks to capture the thermal denatured target ssDNA in solution. The gold electrode is modified with bovine serum albumin (BSA) to improve the self-assembly capability of thiolated dsDNA in the competition of thiolated ssDNA. Consequently, the target dsDNA sequence can be successfully detected by using enzyme-linked amperometric amplification. By adopting such a homogeneous hybridization approach, we have realized sensitive and specific detection of *Cryptococcus neoformans* in clinical samples.

2. Experimental

2.1. Materials

BSA and streptavidin-POD were obtained from Roche Diagnostics (Mannheim, Germany). The enhanced K-blue TMB substrate solution was purchased from Neogen (Lansing, USA). The other chemicals involved were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All chemicals were of analytical grade and used when received.

All oligonucleotides were synthesized and purified by TaKaRa Biotechnology Co. Ltd. (Dalian, China). Sequences were as follows:

Capture probe (**thiol-labeled ssDNA probe**): 5′-HS-$(CH_2)_6$-T_{10} GAGAC GGGCA GAGTA ACCCA TACCG TCGAT-3′
Reporter probe (**biotin-labeled ssDNA probe**): 5′-ATCGA TCAAA TACTT GTGTC TCCAG TTCTC GCCA-Biotin-3′
Target ssDNA: 5′-TGGCG AGAAC TGGAG ACACA AGTAT TTGAT CGATA TCGAC GGTAT GGGTT ACTCT GCCCG TCTC-3′
Complementary ssDNA: 5′-GAGAC GGGCA GAGTA ACCCA TACCG TCGAT ATCGA TCAAA TACTT GTGTC TCCAG TTCTC GCCA-3′

The complementary ssDNA was complementary to the target ssDNA. The target dsDNA was formed by the target ssDNA and its complementary sequence. All DNA strands were stored in the DNA storage buffer at 4 °C.

The hybridization buffer was a 10 mM phosphate buffer solution containing 15 mM $MgCl_2$ (pH 7.4). All aqueous solutions were prepared with Milli Q water (18 MΩ cm resistivity) from a Millipore system.

All primers were synthesized and purified by Biological engineering co., LTD. (Shanghai, China). Sequences were as follows:

forward primer (F): TGATGGAATGTGGCGTTCACC
reverse primer (R): GAGACGGGCAGAGTAACCCA

2.2. Preparation of electrochemical DNA biosensor

The mixture of target dsDNA, capture probe and reporter probe in hybridization buffer (10 mM phosphate buffer solution containing 15 mM $MgCl_2$, pH 7.4) was heated to 95 °C, lasted for 10 min, then cooled to 4 °C over 10 min with a BioRad thermal cycler PTC-100 equipment. The solution was then incubated for 2 h at 46 °C.

The BSA-modified gold electrode was fabricated according to a protocol developed previously [47]. Briefly, the polished 2 mm-diameter gold electrode was incubated in 5 mg/mL BSA solution for 15 min at room temperature. Next, the BSA-modified gold electrode was thoroughly rinsed with water and dried with nitrogen.

The BSA-modified gold electrode was placed in the hybridization solution and incubated for 80 min at 45 °C. Electrode was rinsed with water and then incubated with 3 μL of streptavidin-POD (0.5 U/mL) for 15 min at room temperature. The electrode was then put in phosphate buffer solution (pH 7.4) containing 0.05% Tween-20 and stirred for 5 min. After cleaning, the sensor was extensively rinsed and subjected to the following electrochemical measurements.

2.3. Electrochemical measurements

Electrochemical experiments were conducted in a standard three-electrode electrochemical cell arrangement including a gold disk working electrode (2 mm diameter), an Ag/AgCl reference electrode, and a platinum wire counter electrode. CHI 660C Electrochemical Analyzer (CHI Instrument Inc., USA) was used for the collection of electrochemical signal. The amperometric detection was performed in TMB substrate with a fixed potential of 0.1 V (vs. Ag/AgCl) and the current was collected at 100 s after the redox reaction reaching the steady state.

2.4. PCR procedure

Bacteria solution with the concentration of 2.0 mic was centrifuged at 12,000 × *g* for 4 min. The pellet was resuspended in 100 μL water and extracted by using DNA extraction kit. Amplification was performed in a 0.2 mL tube with the instruction of the kit. PCR procedure was set as initially denatured at 95 °C for 30 s, 35 cycles of amplification (95 °C for 30 s, 55 °C for 30 s, 60 °C for 30 s), 60 °C for 5 min.

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