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# A label-free light-up fluorescent sensing platform based upon hybridization chain reaction amplification and DNA triplex assembly

Li Zou[a,1], Tingting Li[a,1], Ruidi Shen[a], Shaozhi Ren[b], Liansheng Ling[a,*]

[a] School of Chemistry, Sun Yat-Sen University, Guangzhou 510275, China
[b] Guangdong Pustar Adehesives & Sealants Co., Ltd., Dongguan 523600, China

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ABSTRACT

A label-free light-up fluorescent sensing strategy using hybridization chain reaction (HCR) amplification and DNA triplex assembly has been developed. Remarkably, the proposed fluorescence assay is successfully applied to the determination of avian influenza A (H7N9) virus DNA and thrombin. Herein, in the presence of targets, the target DNA/initiator triggers a cascade of hybridization events between H1 and H2 that yields nicked double helices analogous to alternating copolymers. With the additions of triplex-forming oligonucleotide (TFO) and berberine, the triplex structures form between HCR products and TFO. Then, a large amount of berberine can bind to the triplex structures and the sensing system exhibits a dramatic increase in the fluorescence intensity at 530 nm. Under optimal conditions, the label-free fluorescent sensing platform shows sensitive responses to H7N9 virus DNA and thrombin in the range of 0.2–100 nM and 0.5–200 nM, respectively. The detection limits of H7N9 virus DNA and thrombin are as low as 0.14 nM and 0.32 nM, respectively. Owing to the simplicity and low cost, the proposed strategy can be used in other biomarkers assays, providing a promising tool for clinical diagnosis and biomedical detection.

## 1. Introduction

The sensitive and selective detection of disease-related biomolecules is of great importance in biological studies, clinical diagnostics and drug discovery [1,2]. However, biomarkers of interest are typically found in trace amounts, it is essential to explore sensitive method for specific detection of biomarkers at trace levels. In recent years, DNA amplification techniques have gained comprehensive attention, such as polymerase chain reaction (PCR) [3], rolling circle amplification (RCA) [4], strand displacement amplification (SDA) [5], hybridization chain reaction (HCR) [6] and catalyzed hairpin assembly (CHA) [7]. HCR, as a novel signal amplification approach, was first introduced by Dirks and Pierce in 2004 [8]. The isothermal, enzyme-free amplification of target molecules via HCR involves two kinetically-trapped DNA hairpin probes which are triggered to hybridize with each other upon the introduction of initiator. As a result, long-nicked double helices are yielded which are analogous to alternating copolymers. HCR has been extensively utilized to construct various platforms for sensitive detection of nucleic acids, small molecules, proteins and cells [9]. Many signal transduction techniques such as colorimetric method [10], fluorescence [11], chemiluminescence [12] and electrochemical method [13], have been used in HCR amplification methods. Among them, fluorescence is particularly attractive due to its high sensitivity, easy readout, low sample volume, simple operation and feasibility of quantification. However, the labeling of the hairpin probes is necessary for most HCR-based fluorescent sensing strategies, which makes them high-cost. Recently, a label-free fluorescent strategy has been developed that used DNA-intercalating fluorescence dyes as indicators [14], which might suffer from intrinsic limitations of high background. The fluorescent ligands of duplex DNA (ds-DNA), such as SYBR Green, exhibits strong fluorescence upon binding to the hairpin probes, resulting in a relatively high background signal. Hence, it is still a great challenge to develop a feasible fluorescent method with simplicity and low cost for sensitive and selective detection of biomarkers.

Nucleic acid structure-sensitive fluorescence probes have received much interest due to their various applications in the selective identification of specific nucleic acid structures and development of high performance sensors [15–17]. In aqueous solution, the signaling probes are nonfluorescent or weakly fluorescent, but exhibit strong fluorescence upon binding to specific nucleic acid structures. The fluorescence probes are more straightforward in recognizing nucleic acids and benefit from a nucleic acid label-free manipulation. Label-free sensing

* Corresponding author.
*E-mail address:* cesllsh@mail.sysu.edu.cn (L. Ling).
[1] These authors contributed equally to this work.

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systems have been widely developed by using the interaction of single-stranded, duplex, and quadruplex DNA with their fluorescent ligands [18,19]. However, the label-free fluorescent sensing strategy is not sufficiently employed for triplex DNA system, due to the lack of an ideal triplex ligand which concurrently meet the *4 S* requirements: (1) the ligand must support a high triplex binding *selectivity* over ds-DNA and other nucleic acid structures; (2) the ligand binding can *stabilize* the triplex structure; (3) the triplex formation can be easily readout by a *switch-on* fluorescence response of the ligand that is triggered only by the recognition event; (4) the ligand has a *solely* qualified binding mode in the triplex [17]. Triplex DNA structures are formed via the sequence-selective recognition double-stranded DNA (dsDNA) at the major groove by a third strand known as the triplex-forming oligonucleotide (TFO). The triplex structure has been identified to regulate DNA-related bioactivities including gene expression, DNA damage/repair, and even diseases [20–22]. In addition, benefiting from the unique structure, the triplex DNA has been found to have various applications in molecular switches, nano-devices, and drug release [23–27]. Furthermore, the triplex structure has been employed as sensor element in the recognition and detection of nucleic acids, proteins, small biomolecules, toxic metal ions, and even cancer cells [28–31].

To further widen its application in DNA-based sensors, several fluorophores including derivatives of indoloquinoline, 4-amino-naphthalimide and Ru-dipyridophenazine (Ru-dppz) complexes have been selected out as potential triplex ligands for developing the label-free methods [32–34]. Berberine, an isoquinoline alkaloid from plants, is almost nonfluorescent in aqueous solution, but exhibits strong fluorescence upon binding to triplex DNA [35]. Herein, based on the HCR amplification and DNA triplex assembly, we construct a label-free light-up fluorescent sensing platform for the sensitive and selective detection of avian influenza A (H7N9) virus DNA and thrombin using cost-effective berberine as the triplex indicator. In the absence of targets, the HCR process cannot be triggered, and the triplex structures cannot form between the hairpin probes (H1 and H2) and TFO, which results in a relatively low background signal. In the presence of targets, the HCR can occur between H1 and H2, then DNA triplex assembly forms between HCR products and TFO. The triplex structures are easily recognized by berberine, resulting in a dramatic increase in the fluorescence intensity at 530 nm.

## 2. Experimental

### 2.1. Materials and chemicals

All DNA oligonucleotides used in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences were listed in Table S1. Bovine serum albumin (BSA), lysozyme, fibronectin, platelet-derived growth factor BB (PDGF-BB) and human thrombin were purchased from Sigma-Aldrich. Berberine and spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) were obtained from Aladdin Reagent (Shanghai, China). Ultrapure water (18.2 MΩ cm) obtained from a Milli-Q water purification system (Millipore Co., USA) was used in all experiments. Concentrated DNA stock solutions were prepared in a buffer and stored in a fridge at 4 °C. Other reagents were all of analytical reagent grade and used as received. Healthy human real serum samples were supplied by donors from the local hospital (Guangzhou, China).

### 2.2. Apparatus

All fluorescence spectra were recorded on a RF-5301 fluorescence spectrophotometer (Shimadzu, Japan), with the excitation wavelength at 365 nm and the emission spectra from 450 nm to 700 nm. The fluorescence intensity at 530 nm was utilized to evaluate the performance of the proposed strategy. The images of gel electrophoresis were scanned by the Gel Image Analysis System (JY02S, Beijing, China). Circular dichroism (CD) spectroscopy was carried out using a J-810-150S spectropolarimeter (JASCO International CO. Ltd, Japan). All pH values were measured with a model pHs-3e meter.

### 2.3. Procedures for H7N9 virus DNA detection

Two DNA hairpin probes (H1 and H2) were individually heated at 95 °C for 5 min and then gradually cooled to room temperature for 1 h before use. Different concentration of H7N9 virus DNA was mixed with H1 (1.0 μM) and H2 (1.0 μM) in the reaction buffer (10 mM PBS, 0.1 M NaCl, pH = 6.0) and then were incubated at 37 °C for 2 h to trigger HCR and form a long-nicked duplex DNA. Subsequently, TFO (3.0 μM) and spermine (0.2 mM) were added into the above reaction mixture and the solution was further incubated for 30 min at room temperature to form triplex DNA. Finally, berberine (5.0 μM) was added into the mixture to recognize the triplex structures, after incubating for about 10 min at room temperature, the fluorescence spectra were recorded.

### 2.4. Procedures for thrombin detection

In this assay, a mixture containing aptamer of thrombin (TBA) and Initiator at an equimolar level (0.2 μM) was initially dispersed into 10 mM PBS buffer (pH 6.0, 0.1 M NaCl), and then incubated for 30 min at room temperature to form TBA-initiator duplex. Subsequently, different concentrations of thrombin was added and reacted for another 30 min at room temperature to release the initiator. Then, a mixture containing 1.0 μM H1 and 1.0 μM H2 was added and incubated for 2 h at 37 °C to trigger HCR and form a long-nicked duplex DNA. 3.0 μM TFO and 0.2 mM spermine were added into the above reaction mixture and the solution was further incubated for 30 min at room temperature to form triplex DNA. Finally, 5.0 μM berberine was introduced to recognize the triplex structures, after incubating for about 10 min at room temperature, the fluorescence spectra were recorded.

### 2.5. Gel electrophoresis analysis

Polyacrylamide gel electrophoresis was carried out to confirm the presence of the HCR event. Hairpin probes were separately heated at 95 °C for 5 min and then allowed cooled to room temperature for 1 h before use. Different mixtures of oligonucleotides were incubated at 37 °C for 3 h. A 12% polyacrylamide gel electrophoresis analysis of the products via the HCR was carried out in 0.5 × Tris-borate-EDTA (TBE) buffer at 120 V constant voltages for about 75 min, stained with 4S Red Plus Nucleic Acid Stain, visualized under UV light and finally photographed with a digital camera.

### 2.6. CD spectroscopy measurement

To demonstrate the formation of triplex DNA, circular dichroism spectroscopy of mixtures of oligonucleotides was investigated. Hairpin probes were separately heated at 95 °C for 5 min and allowed cooled to room temperature for 1 h before use. Then, target DNA was incubated with hairpin probes for 16 h at 37 °C to trigger the HCR. Subsequently, TFO was added into the system and the final mixture was further incubated at room temperature for 1 h. Finally, the circular dichroism spectroscopy was recorded on a spectropolarimeter under the following conditions: room temperature, wavelength from 200 to 320 nm, path length 0.1 cm, response time 1.0 s, bandwidth 1.71 nm. The sample was measured three times.

## 3. Results and discussion

### 3.1. The principle of fluorescent sensing strategy

Firstly, single stranded DNA (sequence in Table S1, ESI†) derived from hemagglutinin (HA)-encoding sequences from H7N9 virus is