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A label-free fluorescent biosensor based on a catalyzed hairpin assembly for HIV DNA and lead detection†

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Herein, a label-free fluorescent signal amplification system based on a catalyzed hairpin assembly (CHA) is reported. In this system, two hairpin probes, H1 and H2, were well-designed in which G-quadruplex sequences were integrated into H2. The CHA reaction was triggered by target/trigger DNA and G-quadruplex sequences were released, which can bind the fluorescent amyloid dye thioflavin T (ThT) to provide fluorescence signals. At the same time, target/trigger DNA was released from the product of the CHA reaction (H1–H2), which continued to initiate the next CHA cycle, and the signal was eventually amplified. This signal amplification approach has been successfully used to develop a label-free fluorescent sensing platform for sensitive detection of human immunodeficiency virus (HIV) DNA and Pb^{2+}.

Introduction

With the development of the field of life science, nucleic acid analysis is widely used in biological research, clinical diagnosis, environmental analysis, food safety and forensic analysis.[1–3] Polymerase chain reaction (PCR) is the most widely used amplification technology for the amplification and detection of trace amounts of nucleic acids.[4,5] However, PCR technology has disadvantages such as time consuming, complex operation, and the requirement of specific temperature control instruments for thermal cycling. Enzyme-linked immunosorbent assay (ELISA) is a technique used to detect and quantify molecules in liquid samples.[6] It is widely used in clinical diagnosis and environmental monitoring.[7] However, ELISA has low sensitivity and cannot meet the requirements of complex biological sample analysis.[8] To overcome these problems, some isothermal signal amplification techniques, which can be performed at a constant and relatively low temperature, have been designed and applied in DNA-sensing applications. This includes strand-displacement amplification (SDA),[9,10] rolling circle amplification (RCA),[11,12] nucleic acid sequence-based amplification (NASBA),[13] and loop-mediated isothermal amplification (LAMP),[14,15] *etc.* Nevertheless, these enzyme-based amplification techniques may have some disadvantages such as complex experimental conditions and enzyme instability. Therefore, in order to overcome the above disadvantages, several enzyme-free nucleic acid signal amplification techniques have been developed, such as hybridization chain reaction (HCR),[16,17] entropy-driven circuit (EDC)[18] and catalyzed hairpin assembly (CHA).[19–21] Among them, the CHA is a programmable signal amplification DNA circuit, which has the advantages of low background, high sensitivity and efficient signal amplification.[22] Currently, it has been widely used in the detection of nucleic acids,[23] small molecules,[24] metal ions[25] and proteins.[26]

In a sensing system, label-free directly reflects the interaction between the target molecule and the sensing unit. It commonly requires well-designed DNA probes, which can interact with aptamers, complementary strands, targets and signal probes.[27] Label-free technology avoids the expensive labeling process and reaction, and can maintain the highest activity and affinity of the recognition element.[28–30] Recently, several label-free fluorescent biological indicators, such as SYBR Green, thiazole orange (TO) and thioflavin T (ThT), have attracted much attention. ThT is a water-soluble fluorescent probe and a highly sensitive sensing agent; the fluorescence intensity can be significantly enhanced by the electrostatic interaction between ThT and the G-quadruplex structure.[31]

In this work, taking advantage of the CHA and ThT, a label-free fluorescent CHA signal amplification system was developed. The target/trigger DNA initiated the CHA, opened the hairpin probe H2, and the G-quadruplex sequence was released, which bonded to ThT and emitted fluorescence. The recycled target/trigger DNA retriggered a new CHA reaction, which

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amplified the fluorescence signal. Finally, a sensitive detection system for HIV DNA and Pb^{2+} detection was developed.

Experimental

Materials and methods

Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), sodium chloride, ethidium bromide, potassium chloride, magnesium chloride, sodium chloride, and Thioflavin T (ThT) were bought from Sigma (St. Louis, MO). Lead acetate trihydrate, nickel acetate tetrahydrate, zinc acetate dihydrate, cupric acetate monohydrate, cobalt acetate tetrahydrate, and barium acetate were of analytical grade and were bought from Sigma. All of the DNA sequences purified by HPLC were obtained from Sango Biotechnology Co. Ltd. (Shanghai, China) and are listed in Table S1.† Water used in all of the experiments was prepared using a Milli-Q water purification system and displayed a resistivity of ≥18.2 MΩ cm^{-1}. The human serum samples were obtained from Wangjiang Hospital, Sichuan University, and river water was obtained from Jinjiang River. DNA fluorescence intensity was measured using a F-7000 fluorescence spectrophotometer (Hitachi, Co. Ltd., Japan). Photographs of the electrophoresis gel were obtained using a GenoSens 1800 Gel Image Analysis System (Clinx Science Instruments Co. Ltd., China).

Native PAGE analysis

The hairpin probes H1 and H2 were annealed at 95 °C for 5 min and then cooled down slowly to room temperature for 2 h. The solution containing certain concentrations HIV DNA were mixed with H1 (400 nM), H2 (400 nM) in 50 mM Tris–HCl (pH 7.0) buffer solution containing 10 mM NaCl, 30 mM $MgCl_2$ and 20 mM KCl. The solution mixture was incubated for 40 min at room temperature. The gel was run in 12% acrylamide solution (Acr : Bis = 29 : 1) with 1× TBE buffer, at 100 V voltage for 1 h. The gel was stained with ethidium bromide for 10 min to obtain the image of the position of DNA, which was obtained under visible light with a UV imaging system.

Fluorescence spectroscopy

H1 and H2 hairpin probes were prepared as above. For the fluorescence detection, different concentrations of HIV DNA and ThT (4 μM) were mixed into 200 μL of Tris–HCl buffer solution (50 mM, 10 mM NaCl, 30 mM $MgCl_2$, 20 mM KCl, pH 7.0) and then incubated for 40 min at room temperature. The fluorescence spectra were recorded using a Hitachi F-7000 fluorescence spectrophotometer with excitation at 443 nm and the emission wavelength in the range from 463 nm to 650 nm at room temperature. Both excitation and emission slit widths were set at 5.0 nm.

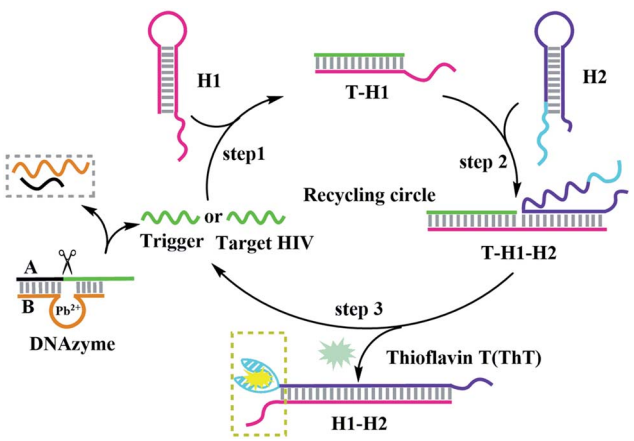
Detection of Pb^{2+}

First, the substrate strand (A), enzyme strand (B), H1 and H2 were respectively prepared in Tris–HCl buffer solution (50 mM, 10 mM NaCl, 20 mM KCl, 30 mM $MgCl_2$, pH 7.0). Hairpin probes H1 and H2 were separately annealed at 95 °C for 5 min, and then slowly cooled to room temperature for 2 h. Subsequently, the A strand was incubated with the B strand at a molar ratio of 2 : 1 to form Pb^{2+}-dependent GR-5 DNAzyme. In addition, solutions of Pb^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} of certain concentrations were prepared respectively and mixed with GR-5 DNAzyme in Tris buffer solution and incubated at room temperature for 20 min. Then H1, H2 and ThT were added into the cleavage reaction solution, mixed by centrifugation and incubated for 40 min at room temperature. Finally, fluorescence measurements were carried out.

Results and discussion

Principle of the label-free fluorescent biosensor

Scheme 1 shows the response system for HIV DNA and Pb^{2+} detection by using a signal amplified label-free fluorescence assay based on the CHA reaction. In this system, two hairpin probes H1 and H2 were designed to complement each other (DNA sequences are listed in Table S1†), and a G-rich sequence was added to H2. The specific secondary structure formed by the G-rich sequence can be folded to form a G-quadruplex structure promoted by K^+ ions.[32] Since the complementary sequences were located in the stems, the spontaneous hybridization between H1 and H2 was blocked by kinetics.[33] For Pb^{2+} detection, trigger DNA sequences were integrated into GR-5 DNAzyme, which is made up of a substrate strand (A) and an enzyme strand (B). In the presence of target HIV DNA/Pb^{2+}, trigger DNA was released after GR-5 DNAzyme was cleaved at the ribonucleoside adenosine (rA) position. The target/trigger DNA can hybridize with H1 through a toehold-mediated strand displacement reaction (step 1), and H2 will hybridize with the newly exposed sticky end of H1 and form the T–H1–H2 complex (step 2). This short-period complex is unstable that the target/trigger DNA can be displaced by H2 through spontaneous decomposition. Then, the released target/trigger DNA can trigger a new CHA reaction, and a large number of H1–H2 complexes were formed (step 3). At the same time, the G-quadruplex sequence was



Scheme 1 Principle of the label-free signal amplification CHA system and its application for HIV DNA and Pb^{2+} detection.

released from H2, which bonds to ThT and the fluorescence signal was finally amplified.

Feasibility of this system

To prove this method, the fluorescence intensity changes of the label-free CHA system were measured before and after the addition of the HIV DNA (50 nM). As shown in Fig. 1A, we can see that without target HIV DNA, the fluorescence intensity is relatively low, when 50 nM HIV DNA was added, the fluorescence intensity of the system increased by almost three fold. The fluorescence result showed that the CHA method is very effective for signal amplification. In addition, gel electrophoresis also confirmed that there are more H1–H2 complexes generated under the same experimental conditions (Fig. 1B), which was consistent with the fluorescence results. The above results indicated that the label-free signal amplification CHA system was truly realized.

Optimization of experimental conditions

To achieve the optimal sensing performances of the proposed detection method, several experimental conditions were optimized, such as the K^+ concentration, hairpin probe concentration and ThT concentration (Fig. S1†). With the increase of the concentration of K^+, hairpin probes and ThT, the value of F/F_0 increased gradually and reached a maximum value at 20 mM, 400 nM and 4 μM, respectively. As the concentration of K^+ increased further, the background signal of the CHA reaction increased, which was caused by the excess G-quadruplex production. Then F/F_0 decreased with the further increase of their concentration. In the following experiments, the above optimized experimental conditions were used.

Application in target HIV DNA detection

As shown in Fig.2A, the fluorescence intensity value gradually increased with the increase of the target HIV DNA concentration. Therefore, we can quantitatively detect the concentration of target HIV DNA using fluorescence intensity values at 490 nm. According to Fig.2B, it has a good linear relationship in the range of 500 pM to 20 nM. The detection limit is 56 pM (S/N = 3), which is comparable or superior to the previously reported methods[34–38] (Table S2†).

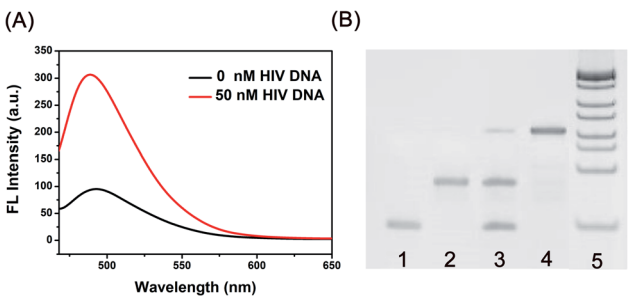


Fig. 1 (A) Fluorescence intensity at 490 nm of the system with or without target HIV DNA. H1 and H2 concentration: 400 nM. (B) Gel electrophoresis image of the sensing system. Lane 1: H1; Lane 2: H2; Lane 3: H1 + H2; Lane 4: H1 + H2 + HIV DNA; Lane 5: DNA ladder. Concentration of H1 and H2: 400 nM; HIV DNA concentration: 50 nM. All measurements were performed in Tris–HCl buffer (50 mM, 10 mM NaCl, 30 mM $MgCl_2$, 20 mM KCl, pH 7.0).

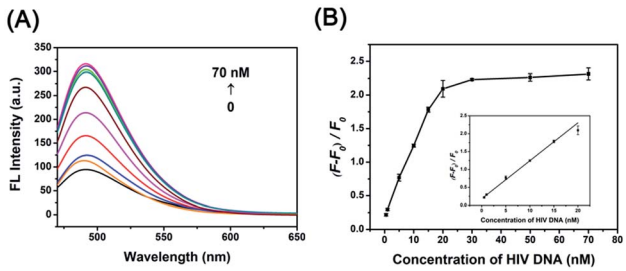


Fig. 2 (A) Fluorescence spectra of the system in the presence of HIV DNA with different concentrations (from bottom to top: 0, 0.5, 1, 5, 10, 15, 20, 30, 50 and 70 nM). (B) Relative fluorescence intensity of target HIV DNA at different concentrations. The inset shows the fluorescence responses to HIV DNA at low concentrations from 0.5 to 20 nM. F and F_0 correspond to the fluorescence intensity of the sensing platform in the presence and absence of HIV DNA, respectively. The excitation wavelength was set at 443 nm, and emission wavelength was set in the range from 463 nm to 650 nm. The concentration of H1 and H2: 400 nM.

Specificity of the fluorescence assay

Furthermore, the specificity of this system was investigated *via* the addition of 50 nM one base mismatch (1MT), two-base mismatch (2MT), deleted (DT) or perfectly matched target DNA (PT) as the target, respectively. As shown in Fig. S2,† only a significant fluorescence intensity increase was observed for the PT, indicating that PT could trigger CHA signal amplification and the system exhibited a high selectivity. In addition, this method has been successfully applied for the target HIV DNA detection in real serum samples, and the human serum samples were diluted 100 times before use. Satisfactory recovery values and relative standard deviations (RSDs) were obtained for different concentrations of target HIV DNA (Table S3†).

Feasibility and application in Pb^{2+} detection

In order to prove the versatility of this detection system, we analyzed this assay for the Pb^{2+} detection. Pb^{2+}-dependent DNAzyme, GR-5 DNAzyme is a catalytic nucleic acid which hydrolyzes the internal single RNA splicing site in DNAzyme with Pb^{2+} as a cofactor.[39–43] In our previous work, we constructed a rapid and colorimetric biosensor for the detection of Pb^{2+} based on the GR-5 DNAzyme.[44] Herein, we also used the GR-5 DNAzyme to detect Pb^{2+} by using the label-free CHA system we proposed in this work. As shown in Fig.3A, after the addition of Pb^{2+}, the fluorescence intensity was significantly enhanced, indicating that the A strand in DNAzyme was cut by Pb^{2+}. Due to the lack of base pair stability of the remaining DNA fragments in DNAzyme, they were dissociated in the solution and finally trigger DNA was released, which triggered the CHA reaction and the fluorescence signal was amplified (Fig.3A). The optimization of the ratio of A to B strand is shown in Fig. S3.† In Fig.3B, the gel electrophoresis image also verified that the result is consistent with the fluorescence

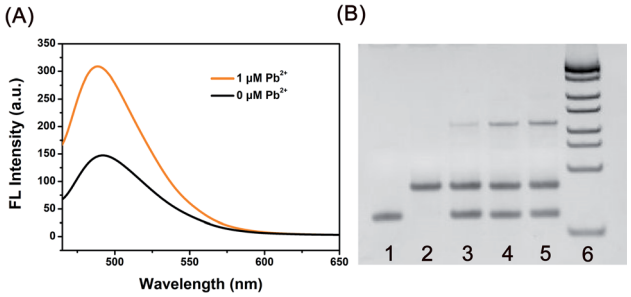


Fig. 3 (A) Fluorescence intensity at 490 nM of the system with and without 1 μM Pb^{2+}. The H1 and H2 concentration: 400 nM. (B) Gel electrophoresis image for the sensing system. Lane 1: H1; Lane 2: H2; Lane 3: H1 + H2; Lane 4: H1 + H2 + DNAzyme; Lane 5: H1 + H2 + DNAzyme + Pb^{2+}; Lane 6: DNA ladder. The concentration of H1 and H2: 400 nM; Pb^{2+} concentration: 1 μM. All of these measurements were performed in Tris–HCl buffer (50 mM, 10 mM NaCl, 30 mM $MgCl_2$, 20 mM KCl, pH 7.0).

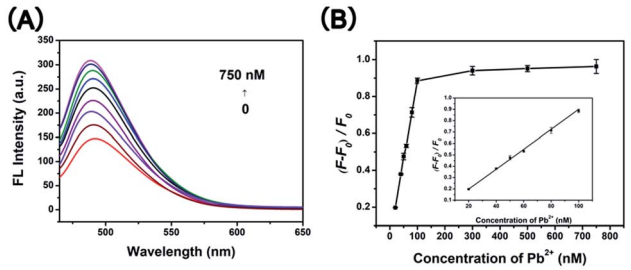
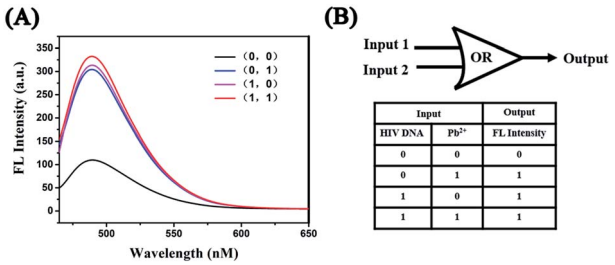


Fig. 4 (A) Fluorescence intensity of the strategy for the detection of different concentrations of Pb^{2+} (0, 20, 40, 50, 60, 80, 100, 300, 500 and 750 nM). Concentration of H1 and H2: 400 nM. (B) Linear relationship between the intensity of fluorescence and the Pb^{2+} concentration. The inset shows the fluorescence response to the concentration of Pb^{2+} in the range from 20 to 100 nM.

experiment. Fig.4A depicts that the fluorescence intensity at 490 nm was increased with the increase of the Pb^{2+} concentration. It can be observed from Fig.4B that a linear relationship between the fluorescence intensity and the Pb^{2+} concentration from 20 to 100 nM was obtained, and the detection limit was calculated to be 1.5 nM (S/N = 3). The sensitivity of this system is comparable to those of the previously reported methods for Pb^{2+} detection[44–48] (Table S4†).

Selectivity of the Pb^{2+} detection system

To verify the selectivity of this label-free Pb^{2+} detection system, several metal ions such as Ba^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Co^{2+} were examined as controls. In Fig. S4,† only Pb^{2+} induced a significant enhancement of fluorescence intensity compared to other metal ions, indicating that Pb^{2+} could trigger the system and has high selectivity. In addition, to demonstrate the application of the Pb^{2+} fluorescence assay in practical analysis, five repetitive experiments for Pb^{2+} detection in Jinjiang River samples were carried out and RSDs of 4.2%, 2.5% and 2.2% were obtained, respectively (Table S5†), suggesting that this method has good reproducibility.



Input		Output
HIV DNA	Pb^{2+}	FL Intensity
0	0	0
0	1	1
1	0	1
1	1	1

Fig. 5 (A) The fluorescence spectra of the strategy depicting the "OR" logic gate. (B) The truth table depicting the "OR" logic gate.

Verification of "OR" logic gate operation

Using HIV DNA and Pb^{2+} as inputs, the "OR" logic gate was fabricated. As shown in Fig.5. The absence and presence of each of them is defined as "0" and "1", respectively. In the absence of both HIV DNA and Pb^{2+} inputs (0, 0), no signal reporter releases, and the fluorescence change can be negligible (defined as output 0). In the presence of either HIV DNA (1, 0) or Pb^{2+} input (0, 1), or both of the HIV DNA and Pb^{2+} inputs (1, 1), the hybridization of the target/trigger with the hairpin probes sequence can expose the G-quadruplex. When combined with ThT, the G-quadruplex/ThT produces a fluorescence response (defined as output, Fig.5A). Fluorescence spectra were applied to demonstrate the mechanism of the "OR" logic gate system. The obvious absorbance changes at 490 nm were observed with addition of HIV DNA, Pb^{2+}, or a mixture of HIV DNA and Pb^{2+} to the system, which indicates that the target/trigger DNA triggered the CHA reaction. As shown in Fig.5B, only in the absence of both inputs output 0 is obtained, while in the presence of either or both of the targets, output 1 can be obtained.

Conclusions

In summary, a label-free CHA signal amplification system for HIV DNA and Pb^{2+} detection was proposed. In this system, target HIV DNA and Pb^{2+} triggered the CHA reaction and many G-quadruplex structures were produced. Then the fluorescent probe ThT binds to the G-quadruplexes and the signal was amplified by the recycling target HIV DNA or trigger DNA. In addition, this detection system has also been applied for river water and human serum sample detection and satisfactory results were obtained. Given the attractive feature of flexibility in the probe design, this assay can be extended to the detection of other targets only by modifying the corresponding identification domain of the detection system.

Author contributions

Yu Xiong: data curation and writing original draft; Jianyuan Dai: reviewing and editing; Yujiao Zhang: conceptualization; Cuisong Zhou: reviewing; Hongyan Yuan: supervision; Dan Xiao: funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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