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Target Catalyzed Assembly of Pyrene Labelled Hairpins for Exponentially Amplified Biosensing

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ABSTRACT: Rapid and sensitive detection of nucleic acids is vital for disease diagnosis. This work designed an enzyme-free isothermal strategy for rapid exponential signal amplification through target-triggered catalytic hairpin assembly (CHA) to induce the spatially sensitive fluorescent signal of pyrene excimer. Functionally, this system consisted of three pyrene labelled hairpins (H1, H2 and H3) and one catalyst DNA C. In the presence of C, the CHA was activated to generate intermediate I, which contained a single-stranded region identical to C sequence for initiating the second cycle of CHA to obtain 2I, and thus achieved the exponential formation of I along with the switching of pyrene excimer. The fluorescent signal of pyrene excimer could be further enhanced via the inclusion of γ-cyclodextrin and showed the linear increase upon increasing logarithm of C concentration. Through the introduction of a help hairpin H4 containing C sequence and a region specific to target, this strategy could be extended to realize the quick and sensitive detection of different analytes. Using dengue virus RNA as an analyte model, the proposed fluorescent method showed a linear range from 0.1 to 50 nM with a limit of detection of 0.048 nM at 3σ and good selectivity. The excellent performance and convenient operation demonstrated its promising application in clinical disease diagnosis.

Keywords: Biosensing; Catalytic hairpin assembly; Exponential signal amplification; Pyrenes; γ-cyclodextrin; Nucleic acids

INTRODUCTION

The sensitivity and specificity of nucleic acid detection have attracted considerable interest due to the extensive demand in clinical diagnosis, food safety and environmental protection.[1] A great number of amplification strategies, including enzyme- and non-enzyme-based amplification, have been developed for design of nucleic acid detection methods. The polymerase chain reaction (PCR)[2] is the most classical enzyme-based amplification technology. The exponential amplification assures the high sensitivity of this method. However, it requires expensive equipment and special detection environment to avoid false positive results. Although some isothermal enzyme-based amplification techniques such as strand displacement amplification (SDA),[3] loop mediated isothermal amplification (LAMP)[4,5] and rolling circle amplification (RCA)[6] can be performed in a tube without need of any equipment, the use of polymerases increases the cost and instability of these techniques. Therefore, non-enzyme-based amplification technologies, such as hybridization chain reaction (HCR)[7] and catalytic hairpin assembly (CHA),[8,9] possess obvious advantages in nucleic acid detection due to their simple operation, high selectivity and precision.[10-12] A toehold-mediated strand displacement (TMSD) reaction has also been presented to avoid the use of polymerase.[13,14] The CHA can be considered as one of the most typical example of TMSD reactions.

The CHA amplification can be divided into linear[15,16] and exponential[17,18] CHA (L-CHA and E-CHA). The L-CHA is usually activated by the catalyst strand to form a stable double-stranded DNA structure, which releases the catalyst strand to trigger the next round of assembly. In E-CHA the hairpins are catalyzed to assemble and generate an intermediate, which in turn activates synchronously the next round of hairpin assembly without the participation of catalyst strand. Obviously, the E-CHA possesses higher amplification efficiency and faster reaction kinetics than L-CHA,[19] just like other isothermal exponential

amplification based on SDA.[20-22] This work used three kinds of pyrene labelled hairpins (H1, H2 and H3) to design an E-CHA strategy. In the presence of catalyst DNA (C), the E-CHA could be achieved to produce spatially sensitive fluorescence signal of pyrene excimers[23,24] for isothermal and enzyme-free homogeneous detection of nucleic acids.

Pyrene is a spatially sensitive fluorescent dye. When it is labelled to the 5′ and 3′ ends of H1, the 5′ end of H2 and the 3′ end of H3 (Scheme 1a), the CHA can bring two aromatic pyrene molecules in a parallel coaxial configuration with an interplanar distance of 3-4 Å (Scheme 1b),[25,26] which forms an excimer state to enhance the fluorescence output at a substantially longer wavelength (485 nm) than the monomer emission for improving the background discrimination.[25-27] The formed intermediate I contains a single-stranded region identical to C sequence, which comes from the designed H3, and can initiate the second cycle of CHA to obtain two I. The exponential formation of intermediate I along with the switching of pyrene excimer leads to the greatly amplified fluorescence signal. Moreover, the fluorescent signal of pyrene excimer can be further enhanced via including it into the lipophilic inner cavity of γ-cyclodextrin (γ-CD) (Scheme 1c).[26-29] Through designing the 5' end of H1 in the sequence complementary to target sequence, the catalyst DNA can be replaced with target nucleic acid, which leads to a method for quick and sensitive detection of nucleic acids.

To improve the practicability and extend the detectable range of the proposed strategy, a help hairpin H4, containing C sequence and a region complementary to target sequence or an aptamer for specific target, is introduced to recognize the target analyte, which produces the free catalyst DNA sequence for triggering the E-CHA. Thus the E-CHA of three pyrene labelled hairpins can be performed for different target nucleic acids or aptamer-specific target analytes. As a proof-of-concept, the help hairpin H4 was designed to recognize dengue virus RNA-1 (DENV-1). The excellent performance of the resulting fluorescent detection

method and the advantages of isothermal, enzymatic-free and homogeneous operation along with the high amplification efficiency and universality indicated the promising application of the suggested biosensing strategy in clinical disease diagnosis.

EXPERIMENTAL SECTION

Materials and Reagents. All DNA strands and pyrene labelled hairpins in this study were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China) and purified with HPLC. The selected dengue virus RNA sequences (DENV-1, -2, -3 and -4) were synthesized by Genepharma Biotechnology Co. Ltd. (Shanghai, China). The detailed sequences and secondary structures were shown in Table S1 and Figure S1, respectively. All oligonucleotides were diluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA-2 Na, 12.5 mM $MgCl_2$, pH 8.0) to give stock solutions of 10 μM. A 20-bp DNA ladder, loading buffer (6×) and Gel Red nucleic acid dye were acquired from Thermo Fisher Scientific. Co., Ltd. (Shanghai, China).

Tris(hydroxymethyl) aminomethane (Tris), EDTA-2Na and $MgCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). γ-CD was obtained from BoKa chemical technology Co., Ltd. (Shanghai, China). Human serum samples were provided by The Second Affiliated Hospital of Hainan Medical University. The selection of target RNA was mainly based on the 3′ untranslated region (3′UTR) gene sequence of (DENV-1, -2, -3 and -4) with Genbank: NC_001477.1, NC_001474.2, NC_001475.2, NC_002640.1.[30,31] The specificity of target RNA sequence was confirmed by blast analysis through the database of National Center for Biotechnology Information (NCBI, USA).[32,33] Ultrapure water from a Millipore water purification system (Milli-Q, Millipore) was used throughout the work.

Apparatus. All oligonucleotide solutions were quantified with the absorbance at 260 nm, which was determined using Cary 100 ultraviolet-visible (UV–vis) spectrophotometer

(Agilent). Native polyacrylamide gel electrophoresis (PAGE) analysis was carried through an electrophoresis analyzer (Bio-Rad, USA) and photographed with Bio-rad ChemDoc XRS (Bio-Rad, USA). Fluorescence spectra was recorded on a F-7000 fluorescence spectrometer (HITACHI, Japan) equipped with an aqueous thermostat (GE Healthcare, Amersham Biosciences, Sweden).

PAGE Analysis. The preparation of native PAGE was divided into the following steps: Firstly, 8% PAGE was prepared by mixing 1.6 mL of 30% acrylamide/bis-acrylamide gel solution (29:1), 1.2 mL of 5×TBE buffer, 3.2 mL water, 48 μL of 10% ammonium persulfate (APS) and 6 μL of N, N, N′, N′-tetramethylethylenediamine (TEMED). After polymerization at 37 ℃ for 15 min, the prepared gel was soaked in 1×TBE buffer (pH 8.0). Then 10 μL of the sample was mixed with 2 μL of loading buffer (6×) and loaded onto the gel. Gel electrophoresis analysis was performed at a constant voltage of 100 V for 50 min, afterward the gel was stained with UltraPower DNA dye for 20 min and imaged by a Molecular Imager Gel Doc XR.

Fluorescence Measurements. Prior to experiments, each hairpin (10 μM in TE buffer containing 10 mM Tris–HCl, 1 mM EDTA-2Na, 12.5 mM $MgCl_2$, pH 8.0) was heated to 95 °C for 5 min, and then slowly cooled down to room temperature for at least 2 h. After target catalyzed assembly of 500 nM pyrene labelled hairpins for 50 min in 100 μL samples containing 100 nM of H4, and then reaction with 1 mM γ-CD for another 5 min, the emission spectra of pyrene excimers were recorded at the excitation wavelength of 340 nm. The excitation and emission slits were both set at 5.0 nm with a PMT voltage of 800 V.

RESULTS AND DISCUSSION

Biosensing Principle. The proposed biosensing platform consisted of three pyrene labelled hairpins (H1, H2 and H3) and one catalyst DNA (C). In the absence of C, three

pyrene-labelled hairpins could coexist stably because the complementary areas were enclosed in the hairpin stems. In contrast, the addition of C into the mixture of H1, H2 and H3 triggered firstly the quick hybridization of C with H1 in a, x, b and y regions via a TMSD reaction, which opened H1 to expose the domains of z*, c*, y*, b* and x* for hybridization with the single-stranded toehold region x of H2 and then b, y, c, and z through TMSD. The formed complex C•H1•H2 exposed the x*, a*, z*, c*, y* and b* domains of H2, in which the c* domain could recognize the toehold domain c of H3 to facilitate the binding of H3 to the C•H1•H2 complex through another TMSD to generate the intermediate I and release C (Scheme 1b). The released C could trigger the next assembly of H1, H2 and H3 to achieve classic CHA (CHA1). Meanwhile, the formed I contained a single-stranded region a*-x*-b*-x* identical to C sequence, which could synchronously initiate another cycle of CHA (CHA 2) to generate 2I. So, with the synchronization of CHA1 and CHA2, the intermediate grew in the form of 2^n exponential amplification along with the switching of parallel coaxial pyrene excimer. The switched pyrene excimer could emit the fluorescence signal at 485 nm, which was further enhanced by the inclusion of γ-CD (Scheme 1c) for quick and sensitive biosensing of C related analytes.

Optimization of Assay Conditions. To achieve the sensitive detection, several experimental parameters were firstly optimized with the highest signal-to-noise ratio, where the fluorescence intensity obtained in the absence of C was considered as the noise.

In exponentially amplified experiments, background amplification due to the not fully annealed hairpins[21,34] is a major challenge. In this work, the stem at the 5′ end of H3 played a key role in the system. When the stem was too short, H3 was unstable, leading to the exposure of the catalytic strand to produce high background signal. However, if the stem was too long, in order to open the hairpin H3, the stem of H2 had to be lengthened accordingly, which increased the difficulty of CHA and thus decreased the efficiency of

signal amplification. Therefore, we designed five different hairpins, $H3_a$, $H3_b$, $H3_c$, H3 and $H3_d$, with the stem lengths of 6, 8, 10, 12 and 14 bp at the 5′ end of H3. Accordingly, five hairpins, $H2_a$, $H2_b$, $H2_c$, H2 and $H2_d$ with the stem lengths of 18, 20, 22, 24 and 26 bp at H2 stem were prepared. As shown in Figure 1(A), the signal-to-noise ratio gradually increased when the stem length at the 5′ end of H3 increased from 6 to 12 bp, and then decreased when stem length changed from 12 to 14 bp. Therefore, the stem with a length of 12 bp at the 5′ end of H3 was selected as the optimal design for application in the whole experiment.

The effect of CHA temperature on was examined at 4, 10, 15 and 20℃ (Figure 1B), which showed the maximum signal-to-noise ratio at 15 ℃. Lower temperature slowed down the hybridization and assembly rate due to the good stability of hairpins for obtaining background, thus the fluorescence signal was relatively low. However, higher temperature led to the instability of hairpins, which produced higher background signal. Therefore, 15 ℃ was the optimal temperature of the CHA system.

To demonstrate the pyrene excimer switch, the fluorescence signal was real-time measured (Figure 1C). Upon the addition of 100 nM catalyst DNA C, the fluorescence intensity of the formed pyrene excimers increased gradually and reached a plateau at 50 min (curve a), which was used as the optimal time in the following experiments. In the absence of C, however, little fluorescence could be observed (curve b). Corresponding fluorescence spectra at the time of 50 min were shown in Figure 1D. Moreover, when the Eppendorf reaction tubes were observed under the ultraviolet lamp, the detection system with the catalyst DNA C showed blue color visible to the naked eye, while only the background signal was exhibited without the C (inset in Figure 1C). Therefore, the fluorescence signal of pyrene excimers occurred upon the dynamic assembly of these hairpins in the presence of C.

Feasibility of CHA. To verify the feasibility of CHA with each branched junction, a native PAGE in 1×TBE running buffer was performed. The samples containing C+H1,

C+H1+ H2, C+H1+H2+H3, or H1+H2+H3 at the hairpin concentration of 500 nM and C concentration of 100 nM were firstly incubated at 15 °C for 50 min. The mixtures were then added into the gel for electrophoresis. As shown in Figure 2, the bands corresponding to H1, H2 and H3 could be observed in lanes 1, 2 and 3, respectively. Comparing to lane 1, the mixture of C+H1 showed a new band at higher weight, and a band for excess H1 (lane 4), indicating the formation of C•H1 complex. Similarly, the mixture of C+H1+H2 showed another new band at the weight higher than C•H1 complex, attributing to the presence of C•H1•H2 complex, while the bands corresponding to H1, H2 and little C•H1 could be observed (lane 5). Different from the mixture of H1, H2 and H3, which showed only the bands of these hairpin (lane 7), and other mixtures, the mixture of C, H1, H2 and H3 showed several new bands (lane 6), due to the formation of I and the presence of I•H1, I•H1•H2 et al., the products of step 5, step 6 and step 7, respectively (Scheme 1b). These results demonstrated the dynamic assembly process of this system.

Amplified Fluorescence Response to Catalyst DNA. To improve the sensitivity, the concentration of γ-CD was optimized to be 1 mM (Figure S2A). The mixture of three hairpins did not show any fluorescence signal, and the addition of 1 mM γ-CD caused a slight rise in the background signal (Figure S2B, curves a and b). Upon addition of the catalyst DNA C in the mixture of three hairpins, the fluorescence spectrum showed an emission peak of pyrene excimers at 485 nm, and the peak intensity increased from 1049 a.u. to 2042 a.u after incubation with 1 mM γ-CD for 5 min (Figure S2B, curves c and d). The fluorescence peak intensity increased continuously with the increasing concentration of C from 0.01 nM to 100 nM (Figure 3A). The incubation of the CHA product with γ-CD resulted in greater change of fluorescence intensity (Figure 3B, curves a and b). The plot of fluorescence intensity vs the logarithmic value of C concentration in 0.1-50 nM showed a linearity (R^2 = 0.991) with the linear equation of F = 887 + 480 lg $C_{(nM)}$ (Figure 3C). The

limit of detection for C was calculated to be 0.063 nM at 3 times standard deviation of background. These performance was comparable and even better than some enzyme-free fluorescence detection and PCR methods, and showed the fastest response among these strategies (Table 2).[27, 34-37] Moreover, the CHA could well distinguish the catalyst DNA C from one-base mismatched (C_a), three-base mismatched (C_b) and random (C_c) DNA (Figure 3D), indicating good selectivity. Thus the amplified fluorescence response could be used for quantitative biosensing of C or C related analytes.

Amplification Efficiency of E-CHA. To demonstrate the high amplification efficiency of the proposed E-CHA strategy, the control L-CHA was carried out using H1 and the designed H2* and H3* (Figure S3). Obviously, the fluorescence intensity of L-CHA product was much lower than E-CHA product in whole concentration range of catalyst DNA C (Figure 3B), indicating the higher amplification efficiency of E-CHA. Moreover, the fluorescence intensities of L-CHA product at different reaction times were also lower than E-CHA product, and the E-CHA showed faster reaction kinetics than L-CHA (Figure S4). At the C concentration of 100 nM, the signal amplification capacity of E-CHA was 2.87 times that of L-CHA, calculated with $(F_{\text{E-CHA(100 nM)}} - F_{\text{E-CHA(0 nM)}})/(F_{\text{L-CHA(100 nM)}} - F_{\text{L-CHA(0 nM)}})$, which led to better analytical performance of E-CHA (Figure S5). In the absence of γ-CD enhancement, the E-CHA showed a linear response to catalyst DNA C in the concentration range of 0.5-50 nM, while it was 10-500 nM for L-CHA. Therefore, the proposed E-CHA was more suitable for sensitive detection of C related analytes.

Detection of DENV-1 RNA. Considering the inherent amplification effect of the proposed strategy, a hairpin probe containing the catalyst DNA sequence and a region complementary to target nucleic acid was designed as a help hairpin H4 to perform the function of catalyst DNA C. Using dengue virus-1 RNA (DENV-1) as the target analyst, the sequence of H4 was shown in Table S1. Dengue is an endemic infectious disease in tropical

and subtropical regions, which is transmitted by mosquito bites.[38,39] The DENV strains can be divided into four dengue virus serotypes (DENV-1, -2, -3 and -4) according to plaque reduction neutralization test.[40] Among these serotypes, DENV-1 is most widespread in Hainan province, China. In the mixture of DENV-1 with H1, H2, H3 and H4, DENV-1 firstly recognized the complementary loop region h to open H4, which exposed single-stranded C region (a*-x*-b*-y*) to trigger the E-CHA of H1, H2 and H3 (Scheme 2). Similarly, the mixture of H1, H2, H3 and H4 showed very low background in the absence of DENV-1, while the fluorescence intensity of pyrene excimer included in γ-CD increased continuously with the increasing concentration of DENV-1 from 0.01 nM to 100 nM (Figure 4A and 4B), which led to a linear plot of fluorescence intensity vs the logarithmic value of DENV-1 concentration in 0.1-50 nM (R^2 = 0.995) with the linear equation of F = 935 + 508 lg $C_{(nM)}$ (Figure 4C). The limit of detection was calculated to be 0.048 nM at 3 times standard deviation of background.

To examine the specificity of the proposed detection method for DENV-1 RNA, three other dengue virus serotypes (DENV-2, -3 and -4 RNA) were selected as interfering nucleic acids. At the same concentration of 100 nM, these RNA showed almost negligible fluorescence response, compared to DENV-1 RNA (Figure 4D), demonstrating the excellent selectivity of the proposed method for DENV-1 RNA detection.

To demonstrate the practical application of the proposed strategy, different concentrations of DENV-1 RNA were added to diluted human serum samples. The detection results were listed in Table 1. Satisfactory recoveries and acceptable relative standard deviations (RSD) indicated the reliability of this method for target DENV-1 detection in real biological samples.

CONCLUSIONS

A target-catalytic hairpin assembly strategy has been constructed for enzyme-free exponential signal amplification and amplified biosensing by combining with the emission-switching property of pyrene and its inclusion enhancement. After the CHA is triggered, an intermediate can be quickly produced to activate the next cycle of CHA, and thus achieves 2^n amplification. This strategy possesses at least five advantages: 1) CHA is an enzyme-free isothermal amplification technology, and can be performed in a homogeneous tube, which greatly simplifies the operation and improves the detection precision; 2) the reaction can be completed in 50 min, which greatly improves the detection throughput; 3) the exponential signal amplification greatly improves the sensitivity of the proposed biosensing method; 4) the optimally designed catalyst DNA and three hairpins ensure the specificity; 5) the introduction of help hairpin greatly extends the analyte range. The help hairpin can be designed to contain different aptamer sequences for detection of a wide range of analytes, such as nucleic acids, proteins and other species. The excellent biosensing performance for dengue virus RNA detection demonstrates the promising application of the suggested biosensing strategy in clinical disease diagnosis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Oligonucleotide sequences and secondary structures of hairpins; schematic illustration of traditional linear CHA; optimization of γ-CD concentration; comparison of reaction kinetics, amplification capacity and detection performance E-CHA with L-CHA (PDF).

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Notes

The authors declare no competing financial interest.

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FIGURE CAPTIONS:

Scheme 1. (a) Secondary structures of catalyst DNA C and hairpins H1, H2 and H3. (b) Reaction pathway of the E-CHA process triggered by catalyst DNA C for CHA1 and CHA2. (c) Introduction of γ-CD for fluorescence enhancement. Letters marked with * are complementary to the corresponding unmarked ones and each letter-labeled fragment is six nucleotides in length.

Figure 1. Optimization of (A) the stem length at 5′ end of H3, and (B) temperature for E-CHA based on the optimized stem of H3. (C) Real-time fluorescence change of the mixture of 500 nM hairpins in presence (a) and absence (b) of 100 nM catalyst DNA C. The inset shows the fluorescent imaging under UV light. (D) Fluorescence spectra of (C) at assembly time of 50 min. The lines through the data points are guides to the eyes.

Figure 2. Native PAGE for H1 (1), H2 (2), H3 (3), C+H1 (4), C+H1+H2 (5), C+H1+H2+H3 (6), H1+H2+H3 (7) and 20 bp DNA ladder (8). The concentration of each hairpin is 500 nM, and the concentration of C is 100 nM. The reactions are performed at 15 °C for 50 min.

Figure 3. (A) Fluorescence spectra of E-CHA system at different concentrations of catalyst DNA C after addition of 1 mM γ-CD. (B) Fluorescence intensity change for: (a) E-CHA after addition of gamma-CD, (b) E-CHA alone and (c) L-CHA alone. (C) Plot of fluorescence intensity for E-CHA after addition of γ-CD system vs C concentration. (D) Fluorescence intensity corresponding to 500 nM H1+H2+H3 (a), (a)+C (b), (a)+C_a (c), (a)+C_b (d), (a)+C_c (e) after 50-min reaction and addition of 1 mM γ-CD. C_a, C_b and C_c are one-base mismatched, three-base mismatched and random C at 100 nM. Error bars are derived from three independent measurements. The lines through the data points are guides to the eyes.

Scheme 2. Schematic illustration of E-CHA for amplified detection of target T using help hairpin H4 and three hairpins along with the addition of γ-CD. Letters "h" and "h*" represent twenty-six nucleotides in length.

Figure 4. (A) Fluorescence spectra of the mixture of 500 nM H1, H2, H3 and 100 nM H4 for E-CHA at different concentrations of target DENV-1 RNA after addition of 1 mM γ-CD. (B) Fluorescence intensity change upon increasing concentration of target DENV-1 RNA. (C) Plot of fluorescence intensity vs DENV-1 RNA concentration. (D) Fluorescence intensity corresponding to 500 nM H1+H2+H3+100 nM H4 (a), (a)+100 nM DENV-1 RNA (b), (a)+100 nM DENV-2 RNA (c), (a)+100 nM DENV-3 RNA (d), and (a)+100 nM DENV-4 RNA (e) after 50-min reaction and addition of 1 mM γ-CD. Error bars are derived from three independent measurements. The lines through the data points are guides to the eyes.

Table 1. Detection of Target DENV-1 RNA in Diluted Serum Samples.

sample no.	added (nM)	found (nM)	recovery (%)	RSD [a] (%)
1	0.10	0.11	110.0	4.52
2	1.00	1.14	114.0	7.11
3	10.0	10.25	102.5	7.21
4	100	104.9	104.9	3.92

[a] Means three parallel experiments.

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Table 2. Comparison of proposed assay with previous methods which based on enzyme-free and traditional PCR amplification for nucleic acid detection.

Strategies	Time	LOD	Working range	Ref.
Target-triggered catalytic hairpin assembly	3h	10 pM	0.1-400 nM	[25]
Target-induced catalytic hairpin assembly	100 min	8.3 pM	0.02-1 nM	[34]
MiRNA-triggered dynamically self-assembly	360 min	2.5 nM	2.5-50 nM	[32]
Real-time PCR assay	3h	-	-	[35]
MiRNA-triggered hybridization chain reaction	2 h	5.9 pM	0.1-100 nM	[33]
Target-triggered catalytic hairpin assembly for exponential amplification	50 min	63 pM	0.1-50 nM	This work

Scheme 1

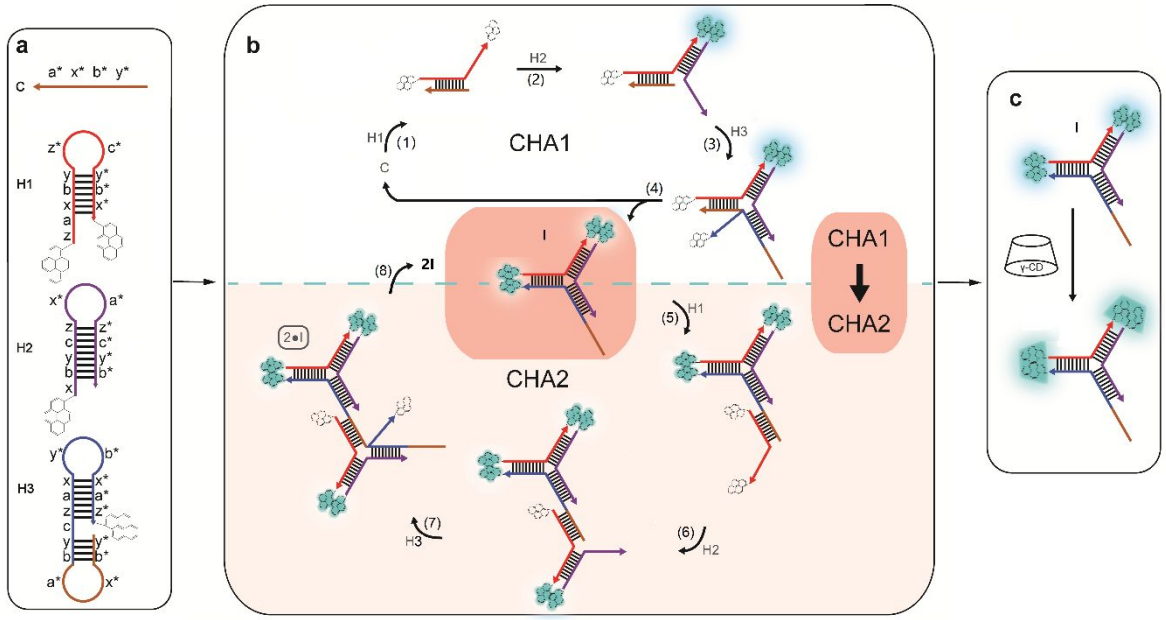


Figure 1

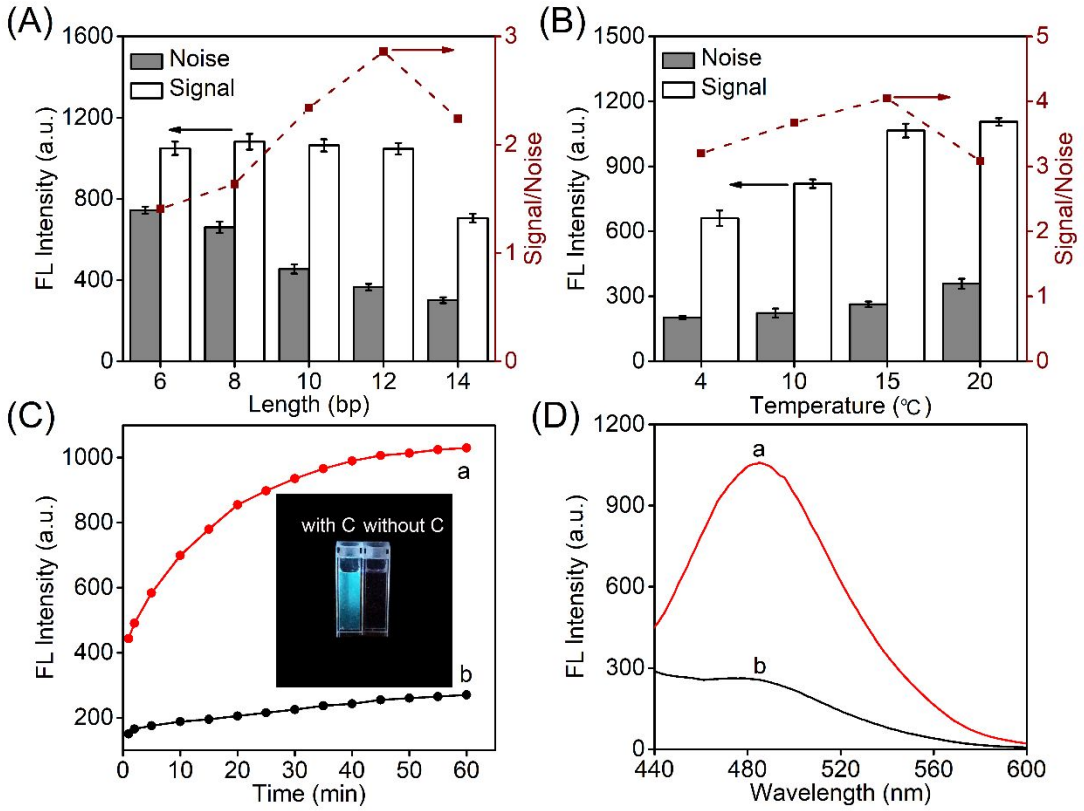


Figure 2

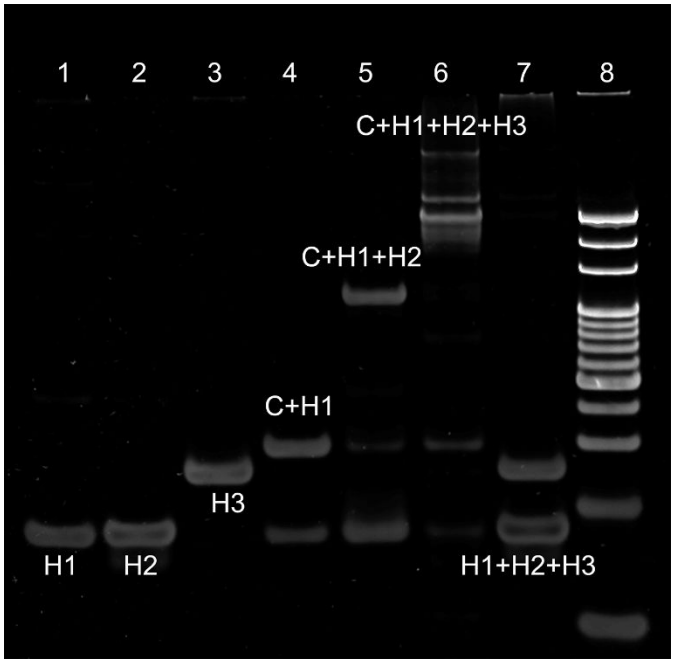
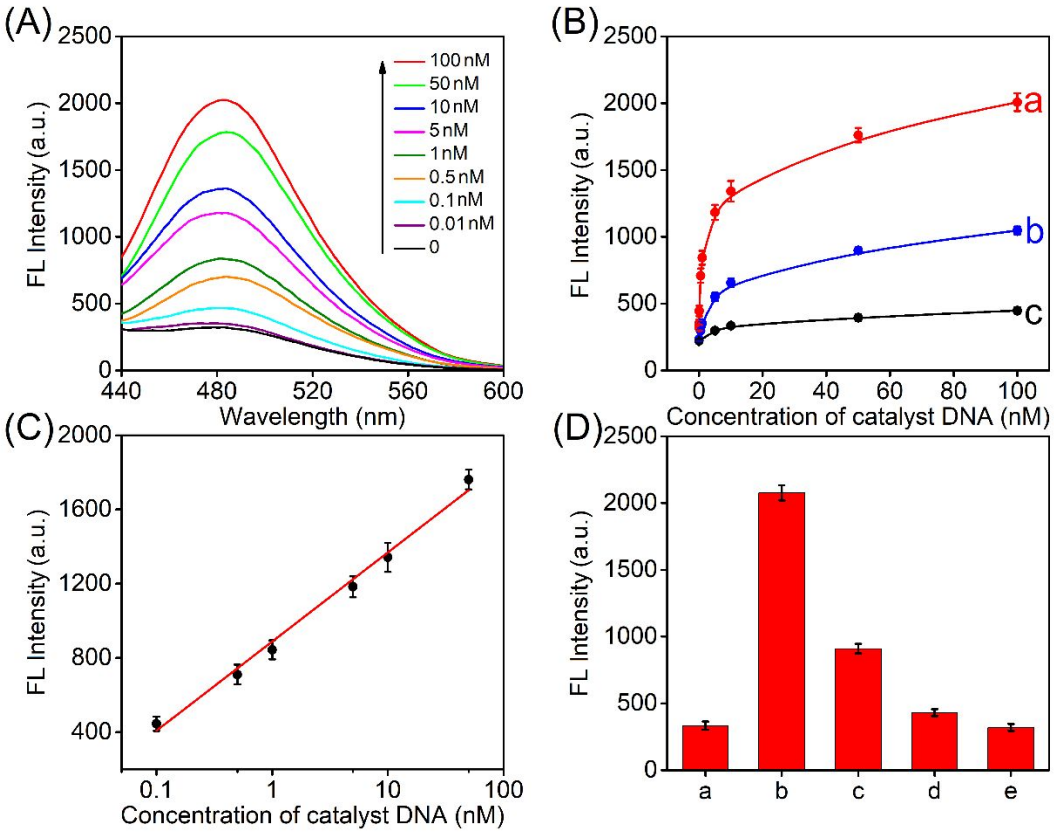


Figure 3



Scheme 2

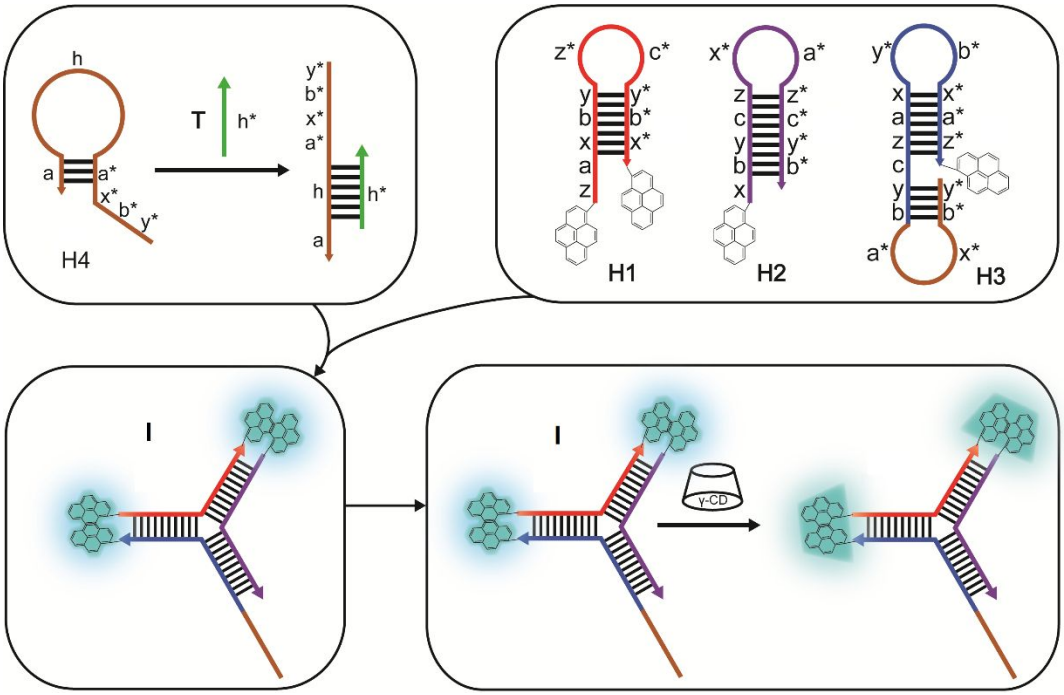
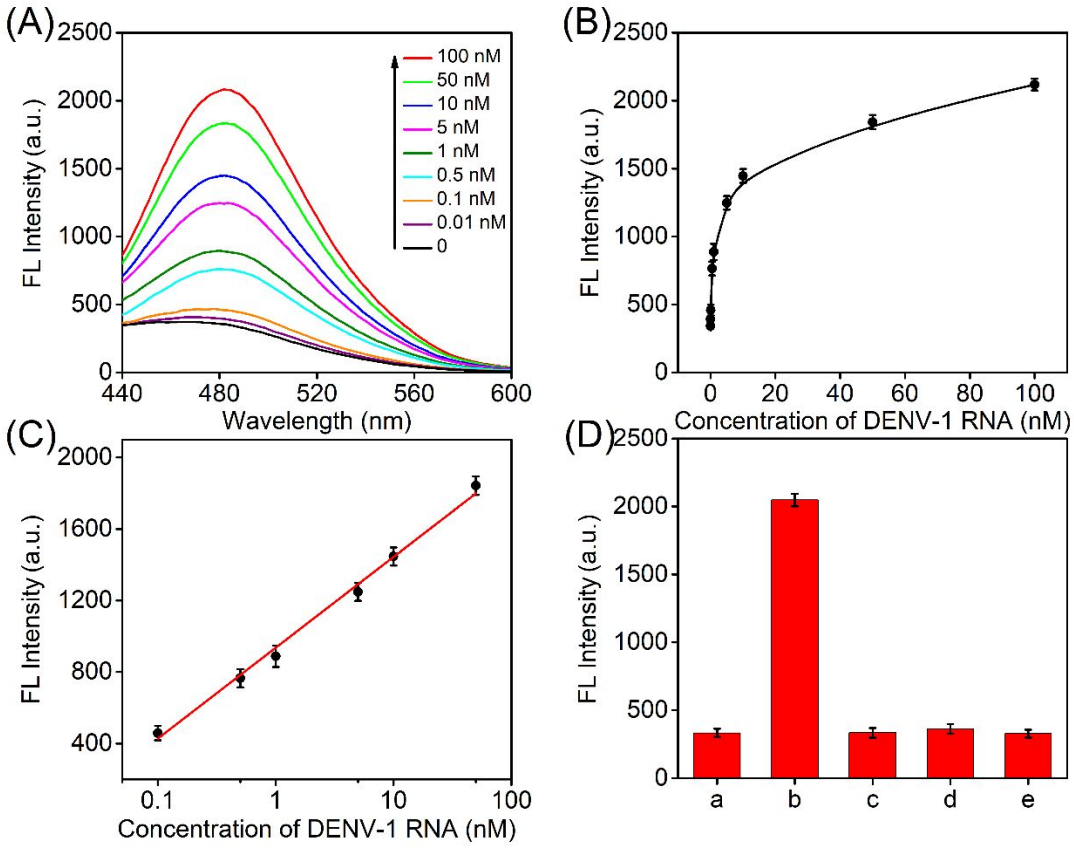
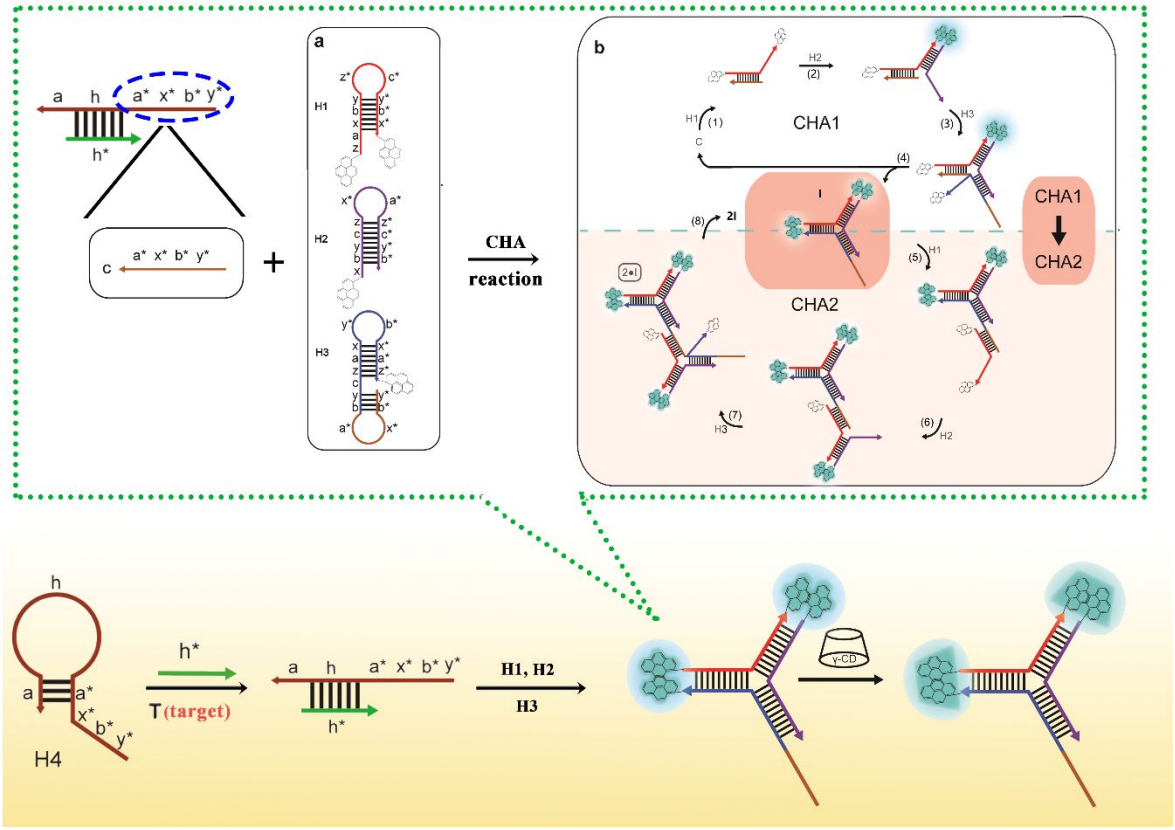


Figure 4



For TOC only


a
h
a* x* b* y*
h*
c
a* x* b* y*
H1
H2
H3
CHA
reaction
b
CHA1
CHA2
CHA1
CHA2
h
a
a*
x*
b*
y*
H4
h*
T (target)
a h a* x* b* y*
h*
H1, H2
H3
γ-CD