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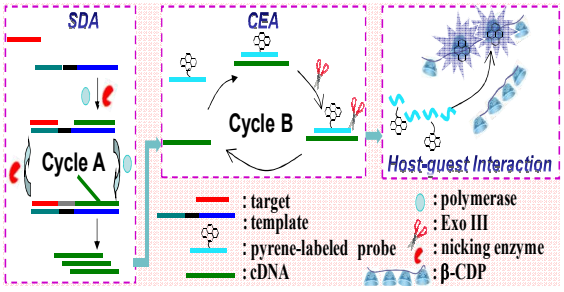
Graphical abstract

The proposed multiple amplification strategy based on the excellent fluorescence enhancement capability of β-CDP for pyrene is facile, sensitive and rapid.



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PAPER

A Multiple Amplification Strategy for Nucleic Acid Detection Based on Host-guest Interaction between β-Cyclodextrin Polymer and Pyrene

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A multiple amplification strategy has been developed for nucleic acid detection based on host-guest interaction between β-cyclodextrin polymer (β-CDP) and pyrene. Briefly, the detection system consists of three parts: the polymerase and nicking enzyme-assisted isothermal strand displacement amplification (SDA) activated by a target DNA or microRNA; the Exonuclease III-aided cyclic enzymatic amplification (CEA); the fluorescence enhancement effect based on host-guest interaction between β-CDP and pyrene. This strategy showed a good positive linear correlation towards target DNA concentrations in the range from 75 fM to 1 pM with a detection limit of 41 fM. Significantly, our amplification platform was further validated and evaluated successfully by assaying miRNA-21 in human serum. The proposed assay has great potential as a nucleic acids quantification method for use in biomedical research, clinical analysis and disease diagnostics.

Introduction

The detection of nucleic acids is fundamental for studying their functions and development of molecular diagnostics, such as detection of viruses and cancer genes in samples from patients which can be critical to decisions that impact the recovery of patients.[1-2] At present, many ingenious fluorescent probes have been designed for the label-free, rapid and convenient detection of nucleic acids and other nucleic acid-related analytes.[3-8] Besides, the analyses of most nucleic acids are usually performed by amplifying trace amounts of specific sequence to a detectable level with various emerging enzyme-assisted amplification strategies.[9-13] Despite this, sensitivity is still a challenge to detect disease-related genes in low abundance, such as microRNA (miRNA) and circulating tumor DNA, which is not only at low concentration but also susceptible to degradation.[14-16] What's more, with the recent emergence of miRNAs as crucial post-transcriptional regulators of gene expression, sensitive, specific and quantitative detection of miRNAs is imperative to better understand the functions and potential clinical applications of miRNAs.[17]

In the past few decades, polymerase chain reaction (PCR), rolling circle amplification (RCA) and strand displacement amplification (SDA) are used as the typical target sequence amplification methods.[18-21] Among these methods, PCR is the most widely used technique for nucleic acids amplification by which trace amounts of nucleic acids can be amplified to detectable levels through the exponentially reaction proceeding.[22-23] However, it has some rigid restrictions on its use, such as the requirement of thermal cycling instrument and identification of PCR products.[24-25] RCA has also been applied to nucleic acids detection due to its good specificity and high sensitivity, but it is limited by many weaknesses, like low ligation efficiency and slow reaction kinetics. Besides, some sequences in the RCA such as circular template, padlock probe and short primer are sophisticated to design and the cost is high.[26-28] To overcome these drawbacks, Weizmann and Zuo et al have developed many simple enzymes-aided target recycling methods for rapid, low cost, sensitive and selective detection of nucleic acids.[29] Unfortunately, most of the methods are limited by its unsatisfactory sensitivity and applicability in RNA detection.[30-31] At the same time, it should not be overlooked that many new signal amplifying methods based on nanomaterials and supramolecular polymers have sprung up.[32-33] In particular, biomedical applications of supramolecular systems based on host-guest interactions have attracted considerable attention on the horizon of material science because of their remarkable and unique properties.[34] Hence, a new and simple signal amplifying method based on the host-guest interaction of supramolecular polymer might surmount the difficulty of probe design and achieve further improvement in sensitivity for nucleic acids detection. Our group has developed a fluorescence analytical method based on host-guest interaction between epichlorohydrin cross-linked β-cyclodextrin polymer (β-CDP) and pyrene. Compared with β-CD, this kind of β-CDP is far more soluble in water and could achieve more excellent fluorescence enhancement effect for pyrene.[35]

Herein, we reported a multiple amplification strategy for nucleic acids assay based on host-guest interaction between β-CDP and pyrene by taking merits of the target-triggering SDA and the Exonuclease III (Exo III)-aided cyclic enzymatic amplification (CEA). Under the joint actions of polymerase and nicking enzyme, SDA enabled one-pot amplification of target sequences efficiently at a mild reaction temperature. The Exo III-aided CEA avoids complex handling procedures and decreases time-cost of detection required by other methods like RCA. In addition, linear reporter mono-pyrene-labeled probe in this detection system is easier to design, synthesize, purify and convenient for usage. Importantly, the excellent fluorescence enhancement capability of β-CDP for pyrene makes it to be a new material for fluorescent assay. Our multiple amplification strategy showed high sensitivity and good selectivity toward target DNA detection. Meanwhile, the proposed method was

further successfully applied to detect miRNA-21 in complex biological matrix, such as human serum samples.

Experimental section

Materials

Klenow fragment polymerase (exo$^-$), Nb.BbvCI endonuclease, Deoxynucleotide (dNTP) and 10×NEB buffer (100 mM Tris-HCl, 100 mM $MgCl_2$, 500 mM NaCl and 10 mM DL-dithiothreito, pH 7.9) were purchased from New England Biolabs (NEB, UK). Exonuclease III, DNA marker and loading buffer were purchased from TaKaRa Bio Inc. (Dalian, China). SYBR Gold was purchased from Invitrogen (USA). RNase inhibitor was purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China). β-CD (97 %) was purchased from Sigma-Aldrich Co. All other chemicals were of analytical grade. All the solutions were prepared with ultrapure water obtained from a Millipore water purification system (>18.2 MΩ•cm). In RNA related experiments, solutions, tips and tubes were treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved to protect from RNase degradation. The RNA sequences and pyrene-labeled probe were synthesized by TaKaRa Bio Inc. (Dalian, China) and purified by HPLC. All the DNA sequences were synthesized and purified through HPLC by Shanghai Sangon Biotechnology Co. (Shanghai, China). The sequences used in this work are listed in Table S1 and the mass spectra and HPLC characteristic spectra for the pyrene-labeled probe were shown in Fig. S1 (see ESI).

Apparatus

All fluorescence spectra were measured using a Hitachi F-7000 fluorescence spectrometer (Hitachi Ltd., Japan) controlled by FL Solution software equipped with aqueous thermostat (Amersham) accurated to 0.1 ^oC. Excitation and emission slits were all set for a 5.0 nm band-pass with a 700 V PMT voltage. The excitation wavelength was set at 345 nm and the emission spectra from 365 to 480 nm were collected with a 0.2×1 cm^2 quartz cuvette containing 100 μL of solution. The fluorescence intensity at 380 nm was used to evaluate the performance of the proposed assay strategy.

Preparation of β-CDP

The β-CDP can be easily synthesized *via* a reaction of β-CD and epichlorohydrin in a sodium hydroxide solution according to the procedure reported previously.[36] In a typical experiment, 10 g (8.81 mmol) of β-CD was dissolved at first in 15 mL of a 15 wt % aqueous sodium hydroxide solution by stirring for at least 2 h at 35 ℃. Subsequently, 2 mL (8.81 mmol) of toluene was added. After an additional 2 h of stirring at 35 ℃, 3.8 mL epichlorohydrin was added to the mixture by drop. After 3 h of stirring, the mixture was further treated with 200 mL of isopropanol and the precipitate was filtered. The raw product was dissolved in water, neutralized with dilute hydrochloric acid and dialyzed for 7 days (MWCO 5000~8000). Finally, the product was isolated *via* lyophilization. The purified β-CDP powder was re-dispersed in aqueous solution and stored at 4 ℃ for use. The structure of β-CDP was confirmed by FTIR spectra and ^1H NMR spectra (Fig. S2). The molecular weight of β-CDP (Mn~94,400) was measured by using gel permeation chromatography (GPC, waters-515).

Agarose electrophoresis analysis

A 3% agarose gel was prepared by using 0.5× tris-borate-EDTA (TBE) (pH 8.0). SYBR gold was used as the DNA stain and mixed with the samples. The gel was run at 100 V for 45 min at room temperature with loading of 10 μL of sample into each lane, and then photographed in Gel Imaging (Tanon 2500 R, Tianneng Ltd., Shanghai, China).

DNA and miRNA-21 detection procedures

Before measurement, the reaction solution was firstly prepared as follows: target DNA at different concentrations, 50 pM template, 0.15 U/μL Klenow fragment polymerase (exo$^-$), 0.1 U/μL Nb.BbvCI endonuclease, 250 μM dNTPs and 1 ×reaction buffer in a total volume of 90 μL.

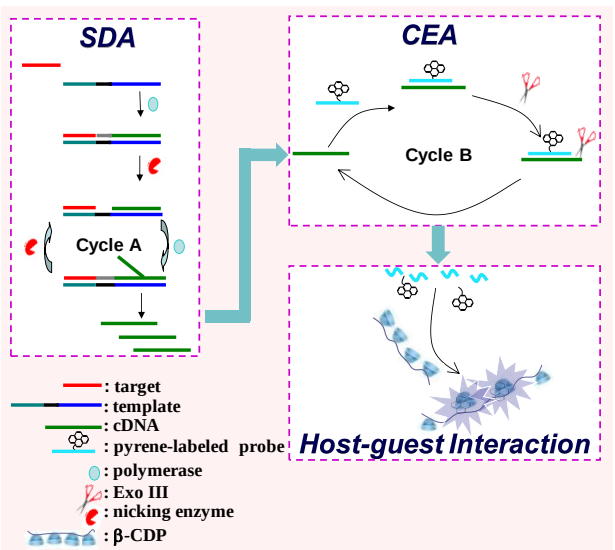
Initially, the prepared solution was incubated for 1.5 h at 37 ℃ for the isothermal polymerization reaction and then blocked by heating to 95 ℃.[37-38] Following that, the resultant mixture was cooled to 0 ℃ quickly. Afterward, 150 nM pyrene-labeled probe and 0.05 U/μL Exo III were added for incubation about 25 min. Subsequently, the resultant mixture was annealed and mixed with 2 mg/mL β-CDP for the fluorescence measurements.

The assay procedure for miRNA-21 was similar with that for DNA detection. (Note: Additional 0.4 U/μL ribonuclease inhibitor should be added to create an RNase-free environment, and the human serum sample was diluted 10 times with ultrapure water). Unless noted otherwise, all the experiments for measurements were repeated three times at least in this study.

Results and Discussion

Design strategy for nucleic acids detection

As shown in scheme 1, this multiple amplification system consists of three parts: the target-triggering SDA, the Exo III-aided CEA and the fluorescence enhancement effect of β-CDP for pyrene based on host-guest interaction.



Scheme 1 Schematic illustration of the enzyme-aided multiple amplification strategy based on host-guest interaction between β-CDP and pyrene. SDA: strand displacement amplification; CEA: cyclic enzymatic amplification; Host-guest interaction: host-guest interaction between β- CDP and pyrene.

In the first part, SDA is initiated in the presence of target DNA. Target DNA can hybridize with its complementary

sequence at 3' terminus of the template and then extend along the template to form dsDNA in the presence of polymerase. The extension product generates a double-stranded nicking enzyme recognition site in the middle of dsDNA and would be cleaved by nicking enzyme. The upper cleaved DNA strand will extend again, and cDNA will be displaced and released continuously. Thus, repeated extension, cleavage and strand displacement could produce numerous cDNAs, and result in Cycle A for target sequence amplification.

In the second part, CEA is performed. As shown in Cycle B, the shorter pyrene-labeled probe is complementary with the produced longer cDNA. Exo III specifically cleaves duplex DNA from blunt or recessed 3'-termini, while it is less active on single-stranded DNA or 3' protruding termini of double-stranded DNA. In the presence of cDNA, pyrene-labeled probe is rapidly digested by Exo III yielding pyrene-labeled mononucleotides. At the same time, the intact cDNA would initiate a new cleavage process leading to enzymatic signal amplification.

In the third part, β-CDP serves as the host component for host-guest interaction between β-CDP and pyrene. Because of weak steric hindrance of mononucleotides, the pyrene-labeled mononucleotides would be easily trapped into the hydrophobic cavity of β-CDP, and accompanied with prominent fluorescence enhancement.

In the absence of target DNA, the target-triggering SDA could not happen and cDNA would not be produced. Thus, the pyrene-labeled probe could not digested by Exo III and pyrene labeled in the middle of the probe was difficult to enter the cavity of β-CDP because of steric hindrance and weak-binding interaction, which will lead to a weak fluorescent signal.

Verification of the multiple amplification method

To confirm the enzyme-aided SDA and CEA, we utilized gel electrophoresis to monitor the polymerization, nicking and digestion reaction.

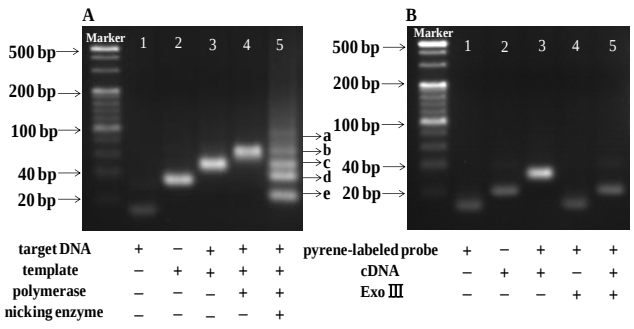


Fig. 1 (A) Gel electrophoretic analysis of the products by SDA. Target DNA (Lane 1, 3, 4): 500 nM; target DNA (Lane 5): 100 nM; template: 500 nM; polymerase: 0.15 U/μL; nicking enzyme: 0.1 U/μL; (B) Gel electrophoretic analysis of the products by CEA. Pyrene-labeled probe (Lane 1, 3, 4): 500 nM; pyrene-labeled probe (Lane 5): 2 μM; cDNA: 500 nM; Exo III: 0.05 U/μL. Both the gels were run at 100 V for 45 min. (+ means in the presence of, - means in the absence of).

The SDA reaction was initiated by the 22-base target DNA. As shown in Fig. 1A, when polymerase was alone added, a band higher than that in Lane 3 was formed (Lane 4), suggesting that successful extension of target DNA generated dsDNA. Lane 5 showed the target DNA-triggering SDA, in which the ratio of the target and template was 1:5. In the presence of polymerase and nicking enzyme, several bands in Lane 5 were formed. The last band e in Lane 5 should be the SDA product (a theoretical value of 35 bases), which appeared close to the 20-bp band of the ladder (Lane Marker). The upper bands d, c and b in Lane 5 derived from excess template, target DNA-template hybridization and excess dsDNA generated from polymerization, respectively. The blurred band a in Lane 5 should be the complex of SDA product and dsDNA generated from polymerization, which was due to incomplete strand displacement. Fig. 1A showed that a target DNA-triggering SDA occurred successfully.

As shown in Fig. 1B, Lane 4 exhibited similar intensity to Lane 1. Lane 5 showed the same band with Lane 2 but no hybridized band (Lane 3) was observed. These results revealed that pyrene-labeled probe could be digested by Exo III in the presence of cDNA but no digestion in the absence of cDNA. Furthermore, the pyrene-labeled probe was digested completely when its concentration (2 μM) was far higher than that of the cDNA (500 nM) in Lane 5. These gel results in Fig. 1B demonstrated that the Exo III-aided CEA was carried out as what has been expected.

To further explore the amplification effect of different methods, we evaluated their performance with control experiments under the same target DNA concentration of 10 pM. In the presence of β-CDP, a mild increase in fluorescence intensity was observed with the assistance of CEA (Fig. 2A (b)). However, a significant increase in fluorescence intensity was obtained by combining SDA with CEA (Fig. 2A (a)). These results showed that a successful SDA reaction could provide higher sensitivity. As shown in Fig. 2B, the fluorescence intensity enhanced greatly with the increasing concentration of β-CDP in the presence of 10 pM target DNA. In the absence of target DNA, the fluorescence intensity kept very faint with the increasing concentration of β-CDP due to the steric hindrance of pyrene-labeled probe. As the basis of our strategy, the excellent fluorescence enhancement effect of β-CDP for pyrene was therefore proved.

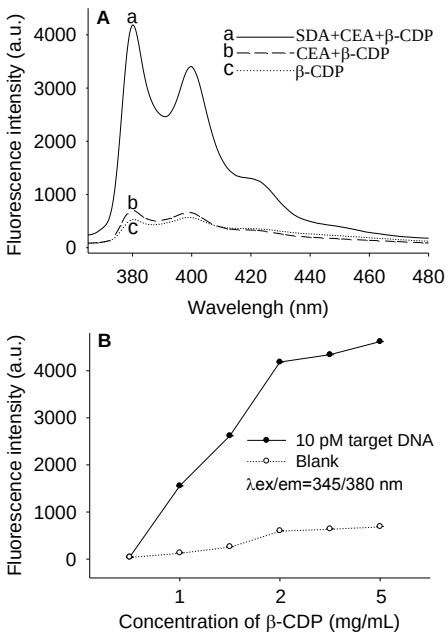


Fig. 2 (A) Fluorescence spectra of different amplification methods. (a) Target DNA + template + polymerase + nicking enzyme + pyrene-labeled probe + Exo III + β-CDP; (b) cDNA + pyrene-labeled probe + Exo III + β-CDP; (c) Pyrene-labeled probe + Exo III + β-CDP. The concentrations of target DNA, cDNA, template, polymerase, nicking enzyme, pyrene-labeled probe, Exo III and β-CDP were 10 pM, 10 pM, 50 pM, 0.15 U/μL, 0.1 U/μL, 150 nM, 0.05 U/μL and 2 mg/mL, respectively. (B) Fluorescence

enhancement of various concentrations of β-CDP: 0, 1, 1.5, 2, 3 and 5 mg/mL. The concentrations of target DNA, template, polymerase, nicking enzyme, pyrene-labeled probe and Exo III were 10 pM, 50 pM, 0.15 U/μL, 0.1 U/μL, 150 nM and 0.05 U/μL, respectively.

Optimization of experimental conditions

To ensure a better performance, several parameters were investigated to establish optimal conditions for our detection system including concentrations of pyrene-labeled probe, Exo III, template and the reaction time of CEM.

The pyrene-labeled probe plays an important role in the performance of this assay. On the one hand, sufficient amount of probe needed to be added to carry out enough rounds of cycles, on the other hand, an obvious background signal would be observed at a relatively high concentration of the pyrene-labeled probe, which could be resulted from the intrinsic fluorescence of the pyrene-labeled probe and a bit of digestion by Exo III. Therefore, concentrations of pyrene-labeled probe were firstly studied towards the detection of 10 pM target DNA. As shown in Fig. S3 (A), 150nM pyrene-labeled probe could achieve the best signal-to-noise ratio.

Next, the amount of employed Exo III was also optimized to avoid unwanted background signal mainly resulted from the non-specific cleavage for pyrene-labeled. The result has been shown in Fig. S3 (B).

It was worth noting that the concentration of template has a crucial effect on the efficiency of amplification. Since amplification efficiency of SDA depends on the ability of target DNA to hybridize with the template, low concentration of template might adversely affect the hybridization efficiency. In contrast, excessive template might hybridize with the amplification product suffering a decline in amplification productivity. We then investigated the template for five concentrations of 10 pM, 50 pM, 100 pM, 500 pM and 1000 pM. As shown in Fig. S3 (C), it was clear that the signal-to-noise ratio was strongly dependent on the initial concentration of template. To achieve good sensitivity, we chose 50 pM template which might be lower than usual but with the highest signal-to-noise ratio in our detection system.

Finally, we investigated the kinetics of Exo III-aided CEA with increasing concentration of target DNA. We found that fluorescence intensity increased as the concentration of targets increased, and the CEA could reach 95% of the maximum in 900 seconds (Fig. S4).

Analytical performance of DNA detection

Under appropriate design and optimized concentrations, sensitivity and specificity were assessed with the proposed multiple amplification method.

As shown in Fig. 3A, fluorescence spectra were recorded at different target DNA concentrations. A gradual increase in the fluorescent peaks at 380 nm and 400 nm were clearly observed in the target DNA concentrations ranged from 0 to 100 pM. Fig. 3B illustrates that fluorescence intensity exhibited a good positive linear correlation towards target DNA in the dynamic concentrations from 75 fM to 1 pM with a detection limit of 41 fM (three times the standard deviation of the blank solution). The CEA method coupled with β-CDP could only achieve a detection limit of 3 pM (Fig. S5), while the detection limit of our multiple amplification method is about 2 orders of magnitude lower with the assist of SDA.

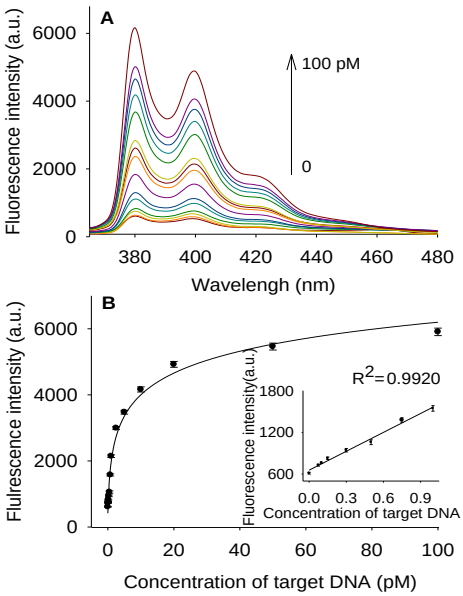


Fig. 3 (A) Fluorescence spectra of the multiple amplification method over a range of target DNA concentrations. (B) Plot of target DNA *vs* fluorescence intensity. Inset is the calibration curve for the concentrations of target DNA from 0 to 1 pM. The concentrations of template, pyrene-labeled probe, polymerase, nicking enzyme, Exo III and β-CDP were 50 pM, 150 nM, 0.15 U/μL, 0.1 U/μL, 0.05 U/μL and 2 mg/mL, respectively. Error bars indicated the standard deviations of three experiments.

In addition, our detection strategy was also investigated to achieve good specificity without sacrificing its low detection limit. To validate the specificity, four kinds of DNAs including target DNA, single-base mismatched DNA (1MT-DNA), two-base mismatched DNA (2MT-DNA) and Random DNA were chosen to investigate the selectivity of this detection system. The fluorescence increasing factor was $(F-F_0)/F_0$, where F and F_0 were the fluorescence intensities of the detection system with and without target DNA respectively. As shown in Fig. 4, when concentrations of the targets were relatively low, $(F-F_0)/F_0$ ratios were also very low due to poor hybridization efficiency. With increasing concentration of targets, $(F-F_0)/F_0$ ratio of the 1 MT-DNA increased slightly to be 2.0 and correspondingly $(F-F_0)/F_0$ ratio of the target DNA was observed to be 6.0. These results clearly indicated that our detection method has good differentiation ability for single nucleotide polymorphisms.

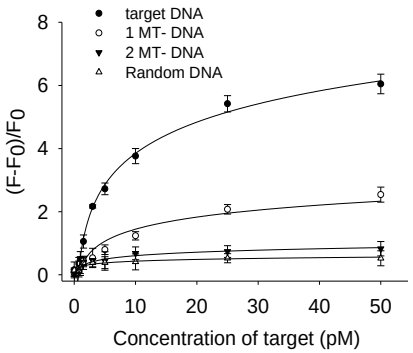


Fig. 4 $(F-F_0)/F_0$ ratios *vs* different concentrations of targets, including target DNA, single-base mismatched DNA (1MT-DNA), two-base mismatched DNA (2MT-DNA) and Random DNA from 0 to 50 pM. The concentrations of template, pyrene-labeled probe, polymerase, nicking enzyme, Exo III and β-CDP were 50 pM, 150 nM, 0.15 U/μL, 0.1 U/μL,

0.05 U/μL and 2 mg/mL, respectively. Error bars indicated the standard deviations of three experiments.

Analytical performance of miRNA-21 detection

MiRNA-21 is one of the early discovered miRNAs in human cells and the expression of miRNA-21 is significantly upregulated in many kinds of tumors.[39-40] Generally speaking, the expression of miRNA-21 in human serum always severs as an ideal biomarker for cancer progression.[41] To explore the feasibility for miRNA detection with our proposed method, we selected miRNA-21 as a model to study the performance over a range of target miRNA-21 concentrations in buffer shown in Fig. S6 in ESI. Compared with target DNA detection, poorer sensitivity was achieved for miRNA-21 detection. It might be owing to two reasons: (1) miRNA is susceptible to degradation by RNase; (2) the recognition ability of DNA polymerase is relatively weaker when the primer was miRNA instead of DNA, leading to relatively poor polymerization and less SDA products. For complex matrix, we employed diluted human serum as the interference system which didn't contain miRNA-21 in detectable quantity. In this experiment, different concentrations of miRNA-21 at detectable levels were added in buffer and diluted human serum samples respectively. Although complex composition of the diluted serum interfered the system signal slightly shown in Fig. 5A, this multiple amplification method could be performed in complex biological samples passably. The results show that our method holds great potential as a promising clinical diagnosis technology for direct and sensitive detection of miRNA-21 in serum of cancer patients.

Due to high sequence homology of miRNA, specificity is of great value to better understand the biological functions of individual miRNAs. Hence, we challenged this system with four miRNA sequences including miRNA-21, single-base mismatched miRNA-21 (1MT-RNA), three-base mismatched miRNA-21 (3MT-RNA) and Random RNA. As shown in Fig. 5B, the results show that our strategy could easily distinguish single-base mismatch in miRNA-21 detection.

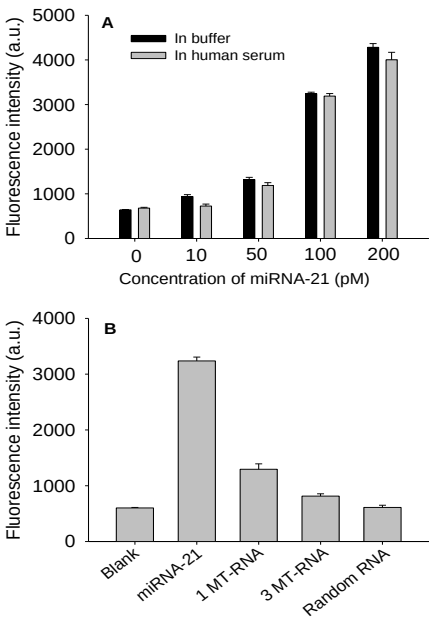


Fig. 5 (A) Detection of miRNA-21 in buffer and human serum (diluted 10 times). (B) Specificity evaluation of the proposed method for miRNA-21 detection (100 pM), 1MT-RNA, (1 nM), 3MT-RNA (1 nM) and Random RNA (1 nM). The concentrations of template, pyrene-labeled probe, polymerase, nicking enzyme, Exo III and β-CDP were 500 pM, 150 nM, 0.15 U/μL, 0.1 U/μL, 0.05 U/μL and 2 mg/mL, respectively. Error bars indicated the standard deviations of three experiments.

Conclusions

In summary, we have described a facile, sensitive and rapid multiple amplification method for nucleic acid detection based on host-guest interaction between β-CDP and pyrene, which offered several unique advantages. First, the pyrene-labeled reporter probe is just single-labeled, which is very convenient to design and use. Second, the host-guest interaction between epichlorohydrin cross-linked β-cyclodextrin polymer and pyrene makes it to be a new and simple fluorescence enhancement mode. Last, this homogeneous detection system not only exhibits competitive sensitivity compared with previously reported multiple amplification methods (Table S2 in ESI), but also has universal applicability in complex biological matrix for miRNA detection.

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Notes and references

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