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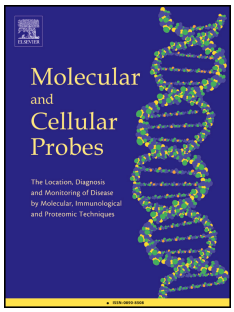

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Colorimetric detection of microRNA based on DNAzyme and nuclease-assisted catalytic hairpin assembly signal amplification

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Abstract

Accurate and quantitative analysis of microRNA (miRNA) expression is critical for the diagnostics and theranostics of a disease. Herein, a proof-of-concept of a colorimetric horseradish peroxidase-mimicking DNAzyme (HRP-DNAzyme) biosensor for miRNA assay based on nuclease-assisted catalytic hairpin assembly (CHA) signal amplification was demonstrated. *Duplex-specific nuclease* (DSN) was employed to cleave the single-stranded DNA (ssDNA) chimeric probe (CP) on the magnetic bead (MB) surface via hybridization of the CP and target miRNA. The regenerated miRNA can cleave a large number of ssDNA CP to produce CHA initiator sequence fragments. The CP consists of two main regions: a target miRNA recognition DNA sequence at the 5' end and a CHA initiator (CI) sequence at the 3' end. The catalyzed assembly process of CHA produces a large amount of G-rich DNA. In the presence of hemin, the G-rich DNA forms G-quadruplex/hemin complex and mimics the horseradish peroxidase activity, which catalyzes a colorimetric reaction. For the proof-of-concept, microRNA-21 (miR-21) was selected as the model target to authenticate this strategy as a versatile assay platform. The proposed strategy allowed quantitation of the sequence specificity of miRNA-21 with a detection limit of 9.2 fM in a dynamic range from 10 fM–1 nM, with an excellent ability to discriminate the differences in miRNAs. Additionally, the miRNA assay in real samples was satisfactory, thereby confirming its applicability. Therefore, this method exhibited a great potential as a miRNA quantification method in biomedical research and clinical diagnosis.

Keywords: MicroRNAs detection; *Duplex-specific nuclease*; Catalytic hairpin assembly; DNAzyme;

Introduction

MicroRNAs (miRNAs) are a group of small, nonprotein coding RNAs with the length of 18–25 nucleotides (nt)[1, 2] found in eukaryotic cells. Accumulating evidence has proved that the abnormal expression of certain miRNAs is closely related to diverse pathological processes, such as cancers, rheumatic diseases, myocardial injury, and neurological disorders[3, 4]. Thus, miRNAs have been regarded as potential therapeutic targets, as well as, improved diagnostic biomarker panels[5]. Despite promising application, the detection of miRNAs is challenging due to the unique characteristics, such as small size, sequence similarity (single nucleotide difference) among family members, and trace-amounts in total RNA samples[6]. Therefore, strategies for sensitive and selective detection of miRNAs are essential, especially for biomedical research and early clinical diagnosis.

Several conventional methods, such as Northern blot, microarrays, and quantitative reverse transcription polymerase chain reaction (qRT-PCR), have been widely applied as gold standard approaches for miRNA analysis[7]. However, Northern blot and microarrays methods have some intrinsic limitations, such as low sensitivity and poor specificity. The qRT-PCR method- relies on reverse transcription and has improved sensitivity of miRNA detection. A number of strategies, such as locked nucleic acid technology, have been used to combine with these traditional methods for low levels of miRNAs detection [8]. These traditional methods pose some limitations, such as tedious procedure, a prolonged duration of the assay, and dependence on laboratory-oriented sophisticated instruments, which severely hamper their further practical application, especially under stringent conditions. Currently, a variety of novel methods have been developed for miRNA detection, including colorimetric[9-11], fluorescence-based[12-14],

bioluminescence-based[15-17], electrochemiluminescence-based[17-18], surface plasmon resonance[19], mass spectrometric[20] assays. Among them, colorimetric assay offers a rapid and convenient option for miRNA detection without any requirement of advanced instruments.

Horseradish peroxidase-mimicking the DNAzyme (HRP-DNAzyme)-based biosensors have attracted great attention and interest in recent years. Thus, HRP-DNAzyme is frequently used in catalytic labels for biorecognition and biosensing events[21]. It contains a complex of hemin and single-stranded (ss) guanine-rich nucleic acid, which can catalyze the oxidation of 2,2' -azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{2-}$) by H_2O_2 to produce a colorimetric signal that can be visualized by the naked eye. However, the sensitivity of these biosensors is not accurate due to the low catalytic activity of the HRP-DNAzyme. Some highly sensitive HRP-DNAzyme biosensors have been developed via different amplification approaches, such as hybridization chain reaction (HCR)[22], entropy-driven catalysis[23], and target catalyzed hairpin assembly (CHA)[21]. The CHA is an enzyme-free and nucleic acid isothermal amplification method that has been widely used for developing biosensors. As an isothermal nucleic acid amplification reaction, CHA is easily tractable as compared to PCR used for the amplification of nucleic acid analytes in point-of-care applications. Briefly, in a CHA amplification reaction, two stable species of DNA hairpins (H1 and H2) coexist in solution until an initiator strand is introduced. A target DNA, used as the initiator, can trigger a cascade of hybridization events to yield H1-H2 double helices. CHA has been adopted as an enzyme-free signal amplification strategy via transduction of the analyte recognition event to optical detection modalities. CHA can not only amplify the signal but also reduce the costs and simplify the detection conditions. A G-enriched DNA fragment is engineered within the hairpin motif. This DNA fragment can be

exposed upon CHA amplification reaction, further forming the G-quadruplex/hemin DNAzyme that catalyzes a colorimetric reaction.

Recently, duplex-specific nuclease signal amplification (DSNSA), an isothermal technique simplifying the operations and reducing the instrument requirement, is a promising and attractive strategy for miRNA detection. DSN, a nuclease isolated from the hepatopancreas of the Kamchatka crab, cleaves the ssDNA probe only when it is hybridized to complementary RNA, and if the conjugate is a DNA-RNA hybrid, it leaves the original RNA intact so that it can bind to another immobilized ssDNA. DSN is practically inactive on ssDNA, or single- or double-stranded RNA. An improved sensitivity could be achieved by the introduction of amplification methods into short output DNA fragments from the ssDNA probe by DSN cleavage in the first round of amplification[24].

Herein, we established a colorimetric detection of miRNA based on HRP-DNAzyme and DSN-assisted CHA signal amplification. The proposed strategy allowed a quantitative sequence-specific evaluation of miRNA-21 with a detection limit of 9.2 fM in a dynamic range from 10 fM to 1 nM, with an excellent ability to discriminate the differences in miRNAs. Additionally, the performance of the developed method for miRNA assay in real samples was excellent, thereby confirming its applicability. Therefore, this method harbors great potential as a miRNA quantification method for use in biomedical research and clinical diagnosis.

Materials and methods

Materials and reagents

DNA probes were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The miRNAs were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). The sequences of the synthetic oligonucleotides and miRNAs are listed in S1 Table 1. RNase inhibitor and DEPC-treated water

were obtained from Sangon Biotech Co., Ltd (Shanghai, China). DSN was obtained from Evrogen (Moscow, Russia). Streptavidin-coated magnetic beads (MBs, 0.8mm in diameter, 10mg/mL) were purchased from Themo Fisher Scientific Co., Ltd (Norway). All chemicals were from Sigma-Aldrich, Inc. (Saint Louis, MO, USA) and used without further purification. The buffers were prepared with 0.1 % DEPC treated water and autoclaved. All tips and tubes used were RNase-free and did not require any pretreatment to inactivate the RNases.

Preparation procedures for MBs

The functional MBs were prepared according to the standard method. Briefly, 100μL of the streptavidin-coated microspheres was rinsed 2 times with 200 μL TTL buffer (100 mM Tris-HCl pH 8.0, 0.1% Tween 20, 1 M LiCl). Next, the MBs were resuspended in 20 μL TTL buffer, and 5 μL biotinylated chimeric probe (CP; 100 μM) was added to the tube. The mixture was incubated at room temperature (25 °C) for 15 min with gentle agitation to allow the completion of the reaction. Subsequently, the probe/microsphere conjugates MB-CPs were washed in 0.15 M NaOH for the removal of any nonspecifically bound CPs. Next, the complexes were rinsed 2 times in TT buffer (250 mM Tris-HCl pH 8.0, 0.1% Tween 20) and resuspended in TTE buffer (250 mM Tris-HCl pH 8.0, 0.1% Tween 20, 20 mM Na_2EDTA), and incubated at 80°C for 10min. Finally, any unstable biotin/streptavidin complexes were removed and the functional MBs resuspended in 100 μL 1× DSN buffer (50 mM Tris-HCL pH 7.0, 10 mM $MgCl_2$, 10 μM DTT) for storage at 4°C until further use.

Protocol for miRNA detection

First, the miRNA was detected in a 15 μL amplification reaction mixture containing 0.2 U DSN (solubilized in 25 mM Tris-HCl, pH 8.0; 50% glycerol), 20 U RNase inhibitor, 3 μL prepared

functional MBs, 1×DSN buffer, and different concentrations of target miRNA at 60°C for 30min. Subsequently, the MBs were separated from the reaction solution using a magnetic separation rack and the solution transferred to a new centrifuge tube. This was followed by the addition of H1 (5 μL, 2 μM) and H2 (5 μL, 2.5 μM) that were heated to 95 °C for 5 min, and gradually cooled to room temperature before use (diluted with HEPES buffer: 8 mM $MgCl_2$，20mM KCl，200mM NaCl, pH 7.0). Subsequently, the reaction mixture was incubated at 37 °C for 2 h. Then, hemin (2 μL, 5 μM) was added to the above reaction mixture and incubated at room temperature for 60 min. Finally, 10 μL ABTS (2 mM) and 5 μL H_2O_2 (40 mM) were added to the mixture to yield a total volume of 37 μL that was incubated for 5 min. The absorbance of the resulting samples was measured.

Absorbance measurements

Absorbance measurements were recorded with a Nano Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence intensity at 414 nm was chosen as the optimal wavelength for data analysis.

Results and discussion

Principle of the miRNA assay

Here we have developed a dual signal amplification strategy for visual miRNAs detection. The overall working principle of this dual amplified biosensor is depicted in Fig 1. CPs were modified onto MBs. The CP consists of two main regions: a target miRNA recognition DNA sequence at the 5’ end and a CI sequence at the 3’ end. The first step of the amplification strategy was initiated by the hybridization of target miRNA with the recognition region of the CP to form a capture probe/miRNA duplex. After the DNA/RNA heteroduplex was formed, the target recognition

sequence of the CP was selectively hydrolyzed by DSN. As a result, the CI sequence fragments were released, and the target miRNA strands were recycled, thereby resulting in a target-recycling amplification through which, thousands of CI sequence fragments were cleaved off CPs by one target miRNA strand during hybridization/DSN incubation. Eventually, one miRNA can lead to the release of numerous CI sequence sections. Next, a magnetic field was applied to separate the MBs from the solution after the completion of this enzyme-assisted amplification. The CI sequence section in cleaved probes would unfold H1 and expose its stem of H1. This exposed sequence was complementary to another hairpin DNA (H2), thereby forming the H1-H2 double helices. Then, the CI sequence section was replaced and dissociated from H1. The CI sequence section in the cleaved probes triggered a cascade of hybridization events. Consequently, the G-enriched DNA fragment of H2 was exposed to form an activated HRP-mimicking DNAzyme upon the binding of G-quadruplex with hemin. In the presence of H_2O_2, the HRP-DNAzyme catalyzes the oxidation of colorless $ABTS^{2-}$ to produce a green colored $ABTS^{-}$. Therefore, a dual-amplification strategy of the colorimetric signal can be obtained for the ultrasensitive visual detection of target miRNAs. The facile, label-free, ultrasensitive, and reusable visual biosensor nanoplatform has wide applications: the detection of pathogens to the diagnosis of genetic diseases.

Optimization of experimental conditions

To achieve the best performance of the proposed method for specific miRNA detection, several pivotal experimental conditions affecting the colorimetric HRP-DNAzyme assay for miRNA, such as the volume of beads, the concentration of dithiothreitol (DTT), the concentration of K^+, the hybridization time of CHA and the pH value, were optimized. The ratio of I/I_0 was employed to

evaluate the performance of the test parameters. I and I_0 represent the fluorescence intensity in the presence and absence of miR-21, respectively.

Firstly, we investigated the effect of the concentration of MBs on the signal of the color system (DNAzyme-ABTS-H_2O_2 oxidation reaction). As shown in Fig. 2A, the signal was dependent on the concentration of MBs and tended towards stability in the concentration of MBs $\geqslant$3 μM. Accordingly, 3 μM was chosen as the optimal concentration of MBs for the colorimetric biosensor. The pH exerts a considerable influence during the formation of G-quadruplexes. The basic unit of a G-quadruplex is a guanine tetrad, composed of a planar arrangement of four guanine bases stabilized by Hoogsteen hydrogen bonding. The pH value can influence the hydrogen bonds. Thus, to enhance the reaction efficiency, we optimized the pH value. Since the I/I_0 value increased as the pH value increased from 5.5 to 7, and followed by a decrease when the pH value was >7, pH 7 was used as the I/I_0 was highest, accompanied by a low background signal, at this pH value (Fig. 2B). G-quadruplexes are cation-dependent and stabilized by alkaline-earth cations. K^+ and Na^+ are prevalent in the cell, and thus, have received maximum attention. The G-quadruplexes prefer to bind K^+ over Na^+. This selectivity is often attributed to the better fit of K^+ into the G-quadruplexes cage. This sensing system requires K^+ for the activity. Hence, we next optimized the concentration of K^+ using a variety of concentrations. As shown in Fig. 2C, the I/I_0 value increased as the concentration of K^+ increased from 0 mM to 20 mM, as a result, the I/I_0 curves tended to increase steadily. Upon the addition of excessive K^+, including K^+ from samples, no DNA fragment is induced to forming the G-quadruplex/hemin DNAzyme [25, 26]. Thus, 20 mM of K^+ was used in subsequent experiments. The reducing property of DTT could influence the color development of DNAzyme owing to the DNAzyme-ABTS-H_2O_2 oxidation reaction. But DTT as an enzyme

stabilizing agent can maintain the DSN activity in the supplied reaction buffers [27]. Then, we optimized the concentration of DTT in the color system (DNAzyme-ABTS-H_2O_2 oxidation reaction). the maximum performance of the color system was gained when DTT was used at 0-10 μM, for the sake of activity of DSN, the optimum concentration of DTT was chosen at 10 μM (Fig. 2D). Besides, the enzymatic reaction temperature of DSN and the reaction time of CHA also play a key role in guaranteeing the high sensitivity and good selectivity in the assay. We carried out experiments under the optimized conditions to ascertain the enzymatic reaction temperature of DSN and the reaction time of CHA. As a result, the optimal enzymatic reaction temperature of DSN and the reaction time of CHA were found to be 60 ℃ (Fig. S1) and 120 min (Fig. S2), respectively.

Sensitivity

To demonstrate the sensitivity of the developed sensing system, the target miR-21, at different concentrations (0, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM), was measured under the optimal experimental conditions. As shown in Fig. 3B, the variance of absorption intensity was proportional to the concentration of the target miRNA. When the concentration of the target miRNA increased, a gradual increase was clearly observed in the peak at 414 nm. In addition, the relative change in the signal exhibited a good linear correlation with the concentration of miR-21 within the range of 10 fM to 1 nM. The regression equation is $I/I_0=0.5182+0.4936 \log_{10}C$ with a correlation coefficient of 0.972. The detection limit was calculated as 9.2 fM (3α/S, where α is the standard deviation of the blank solution and S is the slope rate), and the detection limit of colorimetric signal for naked eye is 1 pM in our assay (Fig. 3A). Furthermore, the proposed method was comparable to some other miRNA detection methods based on different signal

amplification strategies (S2 Table 2). Compared to the other miRNA detection methods, the present method exhibited excellent sensitivity.

Specificity

A great challenge for an excellent miRNA assay is its ability to distinguish the miRNA family members based on their small size and high sequence homology. To investigate the specificity of the colorimetric biosensor, several miRNA sequences, including the target miR-21, M1 (one-base-mismatched), M2 (two-base-mismatched), M3 (three-base-mismatched), and Let-7a were selected as random sequences. Fig. 4 showed the altered signal responding to various miRNAs at the same concentration (10 nM). Compared to the target miR-21, the change in the signal was induced by these similar sequences and the random sequence miRNAs was much lower. The results suggest that the proposed method is a specific miRNA assay.

Real sample assay

The proposed assay was utilized for the determination of the miRNA-21 in biological samples. The different concentrations of miRNA-21 added to HeLa cell lysates were investigated with the present biosensor. The results were shown in Fig. 5. The miRNA-21 could be quantified over a concentration range from 1×10^{-13} to 1.0×10^{-10} M. The regression equation was $Y = 0.3845\lg X + 0.1528$ (Y was the I/I_0; X was the concentration of miRNA in M); the correlation coefficient was 0.9931.

Conclusions

In summary, we developed a naked-eye and highly sensitive HRP-DNAzyme biosensor for the detection of miRNA by DSN-assisted CHA signal amplification. First, the signal amplification can greatly increase the sensitivity and lower the limit of detection to as low as 9.2 fM. Second, the

assay exhibits an excellent discriminatory ability for the differentiation of highly similar miRNA sequences with only a single base difference. Furthermore, the signal generation in the proposed method involves a cost-effective G-quadruplex-hemin-ABTS-H_2O_2 system. Thus, this approach is a promising application in biological research and clinical diagnosis.

Competing financial interests

The authors have declared that no competing interests exist.

Acknowledgements

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Figure Captions

Figure.1. Schematic representation of DNAzyme biosensor for specific detection of miRNA based on DSN-assisted CHA. The explanations of all acronyms were listed as follows: MB, magnetic bead; CP, chimeric probe; CI, CHA initiator; Micro RNA, target miRNA-21; DSN, *Duplex-specific nuclease*; H1, Hairpin probe 1; H2, Hairpin probe 2; ABTS, 2,2' -azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

Figure.2. Effect of the quantity of beads (A) the pH value (B) the amount of K^+ (C) and the amount of DTT (D), respectively. The reaction was performed with 1×reaction buffer, 0.2 U DSN, 5 μL probe complex and the concentration of mRNA was 1 nM for both. Error bars are the standard deviation of three repetitive experiments.

Figure.3. Sensitivity of the proposed method by testing different concentrations of miR-21. (A) Photographic images of the assay performance. (B) I and I_0 represent the fluorescence intensities in the presence and absence of miR-21, respectively. Inset: I/I_0 as a function of the concentration of miR-21 from 10 fM to1 nM.

Figure.4. Selectivity of the proposed method by assessing different miRNAs targets. The concentration of each miRNA sample is 10 nM. Error bars indicate the standard deviation of three experiments.

Figure.5. The linear relationship between the absorbance I/I_0 and the concentration of

miRNA-21 in HeLa cell lysates. Error bars are the standard deviation of three experiments.

Supporting information

Figure S1. The performance of the assay was evaluated by I/I_0 at different temperature, where I_0 and I were the fluorescence signals in the absence and the presence of miR-21, respectively. The error bar indicates the standard deviation of three repetitive experiments.

Figure S2. Effect of the CHA reaction time on the I/I_0 value in this assay, where I_0 and I are the fluorescence signals in the absence and the presence of miR-21, respectively. The error bar indicates the standard deviation of three repetitive experiments.

Figure S3. Effect of the DTT concentration in this assay. The error bar indicates the standard deviation of three repetitive experiments.

S1 Table 1: Sequence of nucleic acids in this study. The sequences used are as follows (5'-3'). CP: chimeric probe; CI: CHA initiator; miR-21: target miRNA; M1: one-base-mismatched; M2: two-base-mismatched; M3: there-base-mismatched; Let-7a: as a random sequence; the red base pairs indicate the mutation location.

S2 Table 2: Detection of miRNA based on different signal amplification strategies. LOD: limit of detection.

Figures

Figure.1.

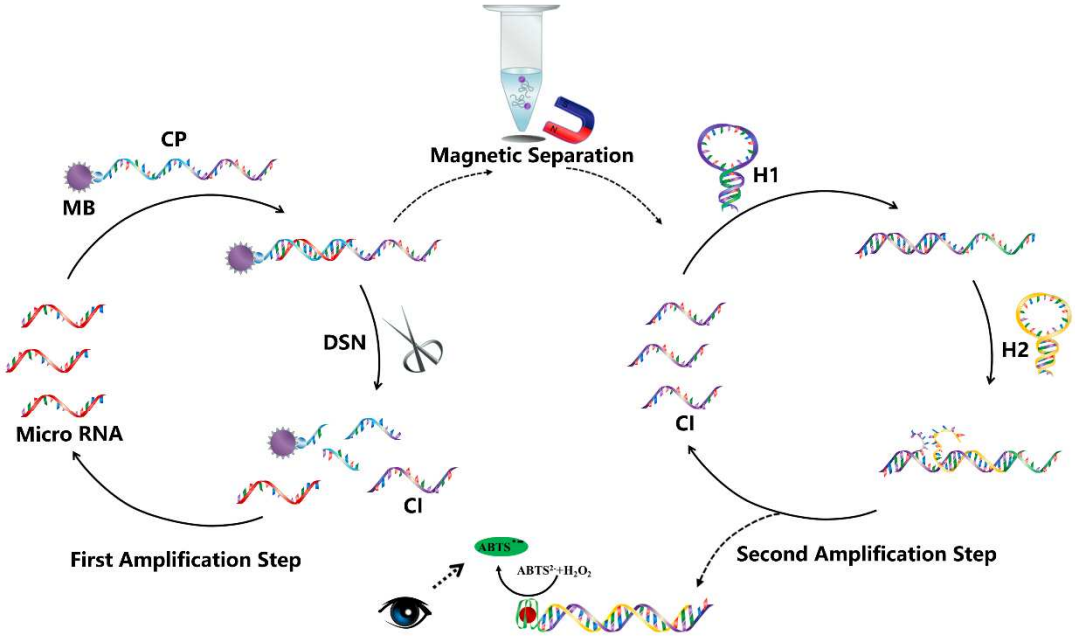


Figure.2.

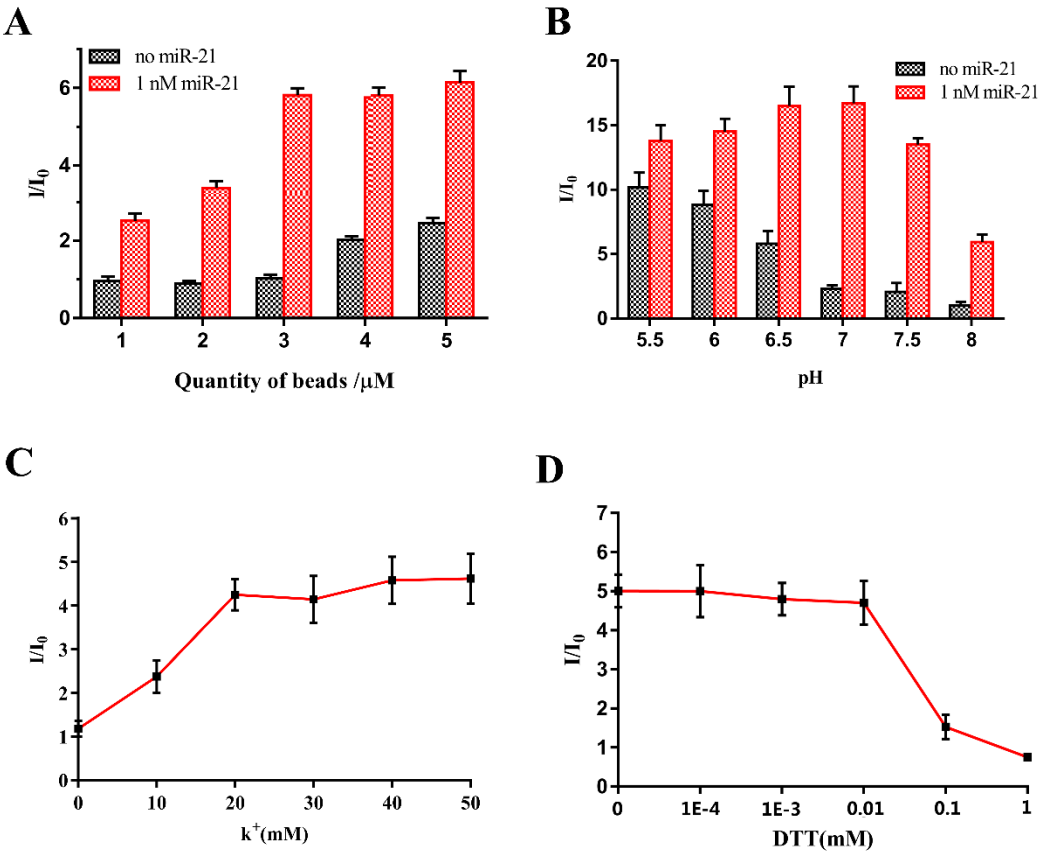
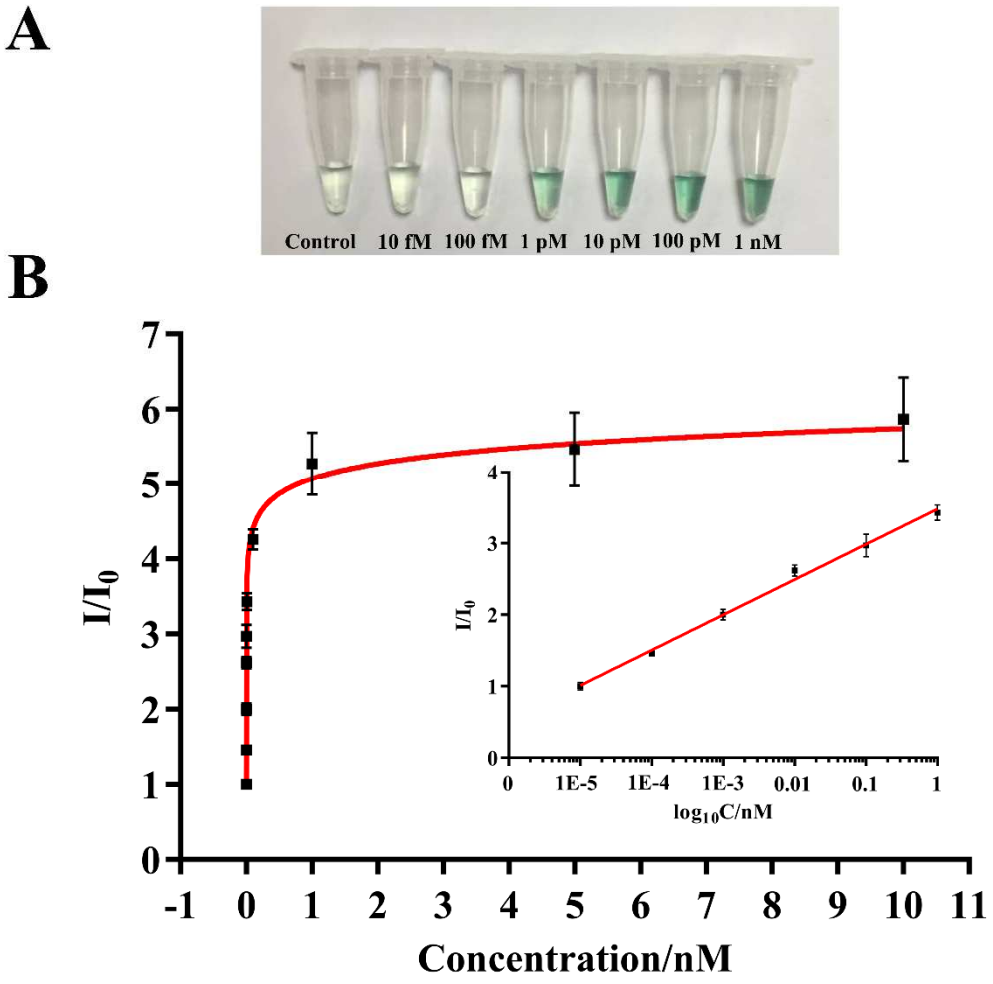


Figure.3.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I/I0
Concentration/nM
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Figure.4.

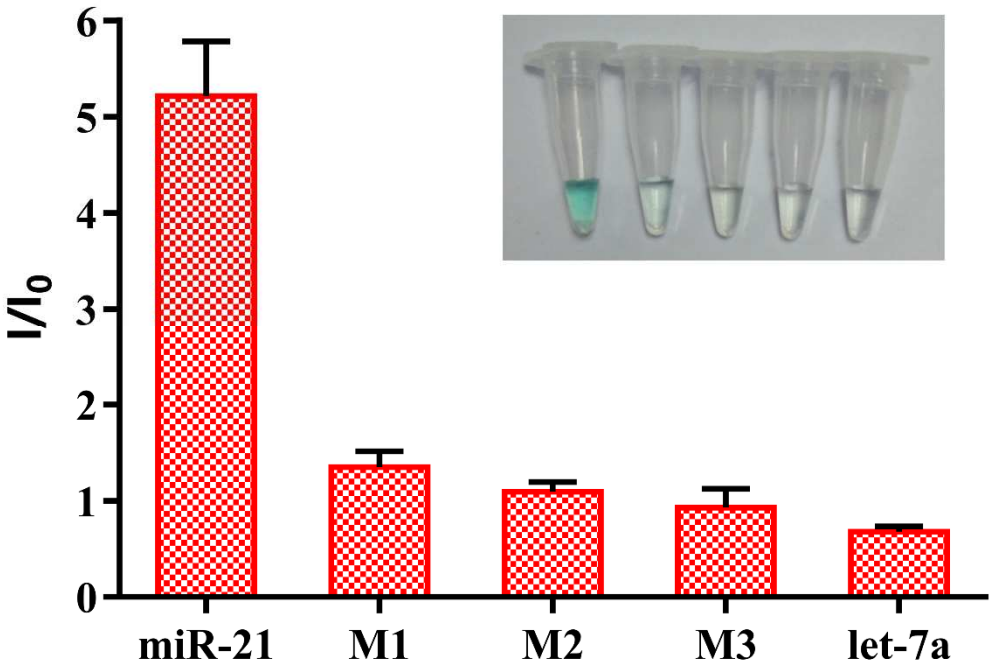
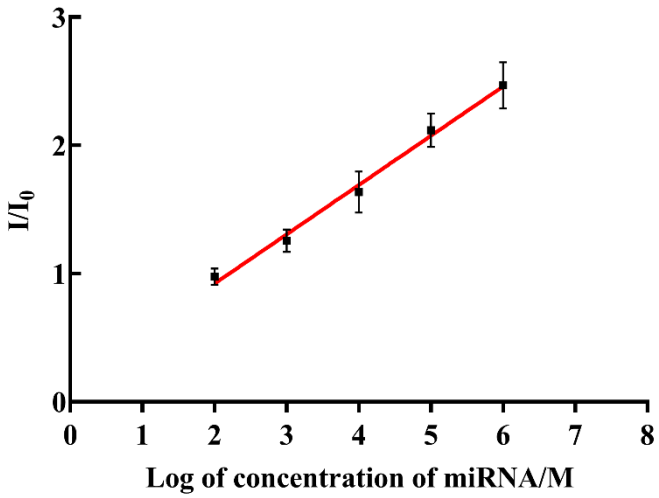


Figure.5.



Highlights

1. A proof-of-concept of a colorimetric horseradish peroxidase-mimicking DNAzyme biosensor for miRNA assay based on nuclease-assisted catalytic hairpin assembly (CHA) signal amplification was demonstrated.

2. The proposed strategy allowed quantitation of the sequence specificity with an excellent ability to discriminate the differences in miRNAs.

3. The proposed assay was utilized for the determination of the miRNA-21 in biological samples.