Article

Ratiometric fluorescence sensor for the microRNA determination by catalyzed hairpin assembly

Yi Liu, Tian Shen, Jing Li, Hang Gong, Chunyan Chen, Xiaoming Chen, and Changqun Cai

ACS Sens., **Just Accepted Manuscript** • DOI: 10.1021/acssensors.7b00313 • Publication Date (Web): 22 Sep 2017

Ratiometric fluorescence sensor for the microRNA determination by catalyzed hairpin assembly

Yi Liu, Tian Shen, Jing Li, Hang Gong*, Chunyan Chen, Xiaoming Chen, Changqun Cai*

Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, College of Chemistry, Xiangtan University, Xiangtan, Hunan 411105, China

E-mail: hgong@xtu.edu.cn (H. Gong); ccq@xtu.edu.cn (C. Cai)

ABSTRACT: A novel catalyzed hairpin assembly-based turn-on ratiometric fluorescence biosensor was constructed for the determination of microRNA-122 (miRNA-122) by using 2-aminopurine (2-AP) and thioflavin T (ThT) as detection signal sources. Hairpin DNA sequence (H1) includes the complementary strands of miRNA-122 and G-quadruplex-forming sequence. When miRNA-122 was presented, hybridization occurred between miRNA-122 and part of H1, causing a double-stranded DNA and a G-quadruplex formed. The formed double-stranded DNA significantly decreased the fluorescence intensity of 2-AP. Furthermore, after binding with ThT, the formed G-quadruplex led to the fluorescent enhancement. The hairpin DNA sequence (H2) hybridized with the unfolded H1 and displaced miRNA-122. Finally, the displaced miRNA-122 again hybridized with the H1 and initiated cycle amplification. This sensor showed a linear ranges of 0.5-50 nM and the limit of detection for miRNA-122 assay was 72 pM (with the lowest measured concentration of 500 pM) for determination of miRNA-122 when no other miRNA was present. Measurements on cell lysates from 100, 1000, and 10000 cells of three different cell lines provided increasing signal ratios, which showed the application potentiality of the sensor for miRNA determination in real samples.

KEYWORDS: *ratiometric fluorescence sensor; catalyzed hairpin assembly; MicroRNA-122; 2-aminopurine (2-AP); thioflavin T (ThT)*

Ratiometric fluorescent methods, which detect analytes at two wavelengths via measuring the changing ratios of photoluminescence (PL) intensities, are essential tools for real-time analytical sensing of biological molecules and processes in organisms and live cells [1-3]. Ratiometric fluorescent bioprobes possess intrinsic advantage in terms of increasing signal accuracy, because it can provide an intrinsic built-in correction to the environmental effects [4-6]. Thus, ratiometric fluorescent bioprobes have received intense attention recently [7,8].

MiRNAs play a crucial role in most biological processes, including proliferation, fate determination and apoptosis of cells. The current miRNA detection methods include colorimetric[9], electrochemical [10,11], and fluorescent methods [12,13]. Fluorescent miRNA assays have some advantages of great reproducibility, simple operation, low cost and fast response. However, most of fluorescent miRNA assays are focused on a single response signal for detection[14,15], which can be easily interfered by the vary of fluorescence intensity and other external factors such as experimental instrument and detection environment. The ratiometric miRNA detection is rare [16] and that more ratiometric concepts may contribute to improve that situation. Based on these, we sought to design a high-sensitivity and high-selectivity ratiometric fluorescent sensor for detecting miRNA, which can reduce the influence of the environment and equipment and improve accuracy.

Many signal amplification strategies for determination of miRNAs sensitively have been developed, including rolling circle amplification [17], hybridization chain reaction amplification [18–20], catalyzed hairpin assembly (CHA) amplification [21–23], and isothermal exponential amplification [24, 25]. Among them, CHA amplification shows

great potential in signal amplification, because CHA is a kinetics-controlled reaction, in which a cascade of hybridization reaction are triggered between two kinds of metastable DNA hairpin probes to release target without requiring any enzymes, and shows highly sensitive and selective toward the detection of target [26]. Thus, a combination of accuracy of ratiometric fluorescent methods and sensitivity of CHA amplification is an ideal candidate for the detection of miRNAs.

In this work, an enzyme-free ratiometric fluorescent sensor based on CHA is designed for high sensitivity and selectivity detection of miRNA. In this sensor, 2-aminopurine (2-AP) and thioflavin T (ThT) were selected as detection signal sources. Two DNA hairpin probes (H1 and H2) were ingeniously designed. When the target miRNA was presented, H1 was unfolded via hybridization with miRNA to trigger the autonomous cross-opening of the two hairpin probes through the toehold-mediated strand displacement reaction, and a large amount of duplex DNA chain product with G-quadruplexes were formed [27]. Due to the additional influence of base-stacking, the fluorescence intensity of 2-AP in the loop of H1 was much higher than that in double-stranded DNA [28–30], and the fluorescence intensity of ThT bound to the K^+-stabilized G-quadruplexes was enhanced [31, 32]. The enzyme-free ratiometric fluorescent strategy based on CHA amplification for sensitive miRNA assay was readily used.

EXPERIMENTAL SECTION

Chemicals and materials. The 2-AP probe, DNA oligonucleotides (HPLC-purified), miRNA (HPLC-purified) and deionized water (diethylpyrocarbonate (DEPC)-treated), 1× TE buffer (1 mM EDTA and 10 mM Tris, pH 8.0), and 10× TM buffer (pH 7.5, 500 mM Tris and 80 mM $MgSO_4$) were obtained from Shanghai Sangon Co., Ltd. (Shanghai, China). Tioflavin T (ThT) was obtained from J&K

Scientific Ltd. (Beijing, China), Potassium chloride (KCl) was obtained from Comio Chemical Reagent Co., Ltd. (Tianjin, China), which was of analytical grade and used without further treatment or purification. Before used, miRNAs were diluted in DEPC-treated water to appropriate concentrations. The DNA hairpin probes were diluted with 1× TE, to give the stock solutions. The oligonucleotides sequences were presented in Table 1.

Fluorescent miRNAs assay. Before used, the 2-AP -labeled oligonucleotide H1 and oligonucleotide H2 were incubated in TM buffer (250 mM Tris and 40 mM $MgSO_4$, pH 7.5) for 10 min at 95 ^oC, and cooled down to room temperature slowly. The miRNA assay was performed in 250 μL of reaction mixture containing 200 nM H1, 200 nM H2, 10 mM KCl solution, 2 μM ThT and the different concentrations of target miRNA. The CHA reaction was conducted at 40 ^oC for 80 min. Fluorescence spectra of the resultant reaction solution were measured by using a RF-5301 PC fluorescence spectrophotometer (SHIMAPZU, Japan). The slit widths of excitation and emission were both kept at 10.0 nm and the excitation wavelength was at 300 nm. The fluorescence signal of 2-AP was observed at 367 nm, whereas the fluorescence of ThT was measured with an emission of 485 nm. All experiments were repeated three times.

Circular dichroism (CD) analysis. CD spectra were recorded on a CD chiroptical spectrometer (Chirascan, Applied Photophysics, UK) at room temperature. The H1 (2 μM) and H2 (2 μM) was prepared in 10× TM buffer (100 mM Tris, 16 mM $MgSO_4$, pH 7.5). The target miRNA was added to the reaction mixture (500 nM). CD spectra were measured between 220-320 nm, the scan speed was 100 nm/min, the bandwidth and the response time was 1.0 nm and 0.5 s, respectively. Each tube was measured three times and spectra were averaged from 3 scans.

Gel Electrophoresis Analysis. The CHA reaction sequences and the products were resolved using 2% agarose gel in 1 × TBE buffer (88 mM Tris-acetic acid and 2 mM EDTA, pH 8.0), and then the electrophoresis was performed at 110 V for 40 min. The gels were visualized via Bio-Rad Laboratories (USA) gel image system.

RESULTS AND DISCUSSION

Principle of the sensor. As illustrated in **Scheme 1** is the principle of the CHA-amplified homogeneous ratiometric fluorescent bioprobe for sensitive detection of miRNA. It consists of two hairpin structures (H1 and H2). In H1, the sequence was labeled with one replacement of 2-AP molecule for adenine and includes the encoded sequence at the 3′ end of the stem region, which consists of the G-quadruplex sequence. Hairpin H2 includes the segments of the partially complementary sequences to the hairpin H1. When the target miRNA is absent, both H1 and H2 maintain their hairpin structures in the solution. Hence, the probe fluorescence was high at 367 nm because the fluorescence of the 2-AP in the single-stranded DNA was significantly higher than that in the double-stranded DNA, and was low at 485 nm because the G-quadruplex-forming sequences associated with ThT locked in the hairpin structures of H1. Hairpin H1 includes the recognition sequence for the target miRNA (miRNA-122). When the target miRNA is present, the red part of H1 completely hybridizes with the target miRNA and unfolding the hairpin structure of H1. Then the H2 hybridizes with the unfolded H1 and the target miRNA was displaced based on the mechanism of strand displacement. The H1 sequence again hybridizes with the displaced target miRNA and the cycles of strand displacement was initiated, causing the 2-AP in the double-stranded DNA, and massive active G-quadruplex were generated at the 3′ end of H1. The emission spectrum of 2-AP

(367 nm) was finally weakened. Furthermore, fluorescence emission peak of ThT at 485 nm was significantly enhanced in the presence of target miRNA.

CD assay. CD spectroscopy was used to confirm the above-mentioned principle. As shown in **Figure 1**, a weak positive peak and a negative peak emerged at approximately 265 nm and 240 nm respectively (**Figure 1, curve 1**) by H1 reaction solution. In the closed H1, the G-quadruplex-forming sequence cannot form the structure of G-quadruplex. Upon the addition of miRNA-122, the spectrum intensity increased and exhibited a positive peak at around 265 nm (**Figure 1, curve 2**); this result indicates that the hairpin H1 was unfolded, and the G-quadruplex structures were formed. The peak in the same position was presented at the H1 and H2 solutions (**Figure 1, curve 3**). When target miRNA was added to this solution, the peak intensities around 240 and 260 nm were increased obviously (**Figure 1, curve 4**). These results suggest that target miRNA initiates the target recycling amplification cycle.

Gel Electrophoresis Assay. To further confirm the above-mentioned principle, the CHA reaction sequences and the products were resolved using 2% agarose gel. As shown in **Figure 2**, the base number of H1 (lane1) derived from electrophoresis was consistent with our design. Without the addition of miRNA-122, only a small amount of H1:H2 duplex could be formed via the hybridization between H1 and H2 (land 2). The miRNA-122 could successfully unfold H1 in lane 3. However, when miRNA-122 was presented, the bands of amplification product were more obvious (lane 4), indicating the successful CHA reaction.

Optimization of detection system. Some related factors, including the concentrations of ThT, K^+, Mg^{2+}, and H2, reaction temperature, and reaction time were optimized to achieve better detection results (**Figure S1-S6)**.

Sensitivity of miRNA assay. To evaluate the analytical performance of the proposed sensor, under the optimum condition, the different concentrations of target miRNA (miRNA-122) were added into the reaction solution. As shown in **Figure 3a**, with the increasing concentration of miRNA-122 from 0 nM to 200 nM, the PL intensity at 367 nm of the 2-AP molecule for adenine was continuously decreased, and the PL intensity of ThT at 485 nm was gradually increased. Therefore, PL intensity ratio $(I_{485}/I_{367})/ (I_{485}/I_{367})_0$ and miRNA concentration in the range of 0.5−50 nM have good linear relationship (shown in **Figure 3b**). The regression equation for the relationship between target miRNA and fluorescence intensity was determined as $y= 0.039\ c + 1.052$ ($R^2 = 0.990$), here y represents the value of $(I_{485}/I_{367})/(I_{485}/I_{367})_0$ and c is the concentration of miRNA-122. The obtained detection limit for miRNA-122 assay was 72 pM (with the lowest measured concentration of 500 pM). These results indicated that sensitive analysis of miRNA was achieved via the desired enzyme-free and ratiometric fluorescence technique based on CHA.

Selectivity of miRNA assay at 10nM and 3nM miRNA concentrations. To further investigate the selectivity of this strategy, the same concentration of miRNA-122, miRNA-21, miRNA-26a, miRNA-144, and miRNA-199 were added into the reaction solution, respectively. **Figure 4** shows that miRNA-122 induced a prominent fluorescence ratio $(I_{485}/I_{367})/ (I_{485}/I_{367})_0$ increment. By contrast, a smaller change in fluorescence ratio occurred when other miRNAs were added because these added miRNAs were not complementary to the DNA oligonucleotides of H1, causing less chance of opening the hairpin structures of H1. The results indicated that the proposed sensor has characteristic of high sensitivity for miRNA-122 over other competitive miRNAs.

Real sample analysis. The application of the designed sensor in real samples was analyzed the lysates from a normal hepatic cell (L-02) and two cancerous cell lines, containing the hepatocellular carcinoma cell (SMMC-7721) and human breast (MCF-7) by monitoring miRNA-122. **Figure 5** shows that the lysates with an increasing number of SMMC-7721, MCF-7 and L-02 cells (from 100 to 1,000 to 10,000) lead to a gradually elevated fluorescence response of the probe solution. The weaker increase for the SMMC-7721 cells suggests a lower content of miRNA-122 in the SMMC-7721 cells compared to the MCF-7 and L-02 cells. These preliminary results on real samples show that the developed method may become useful to monitor miRNA biomarkers from cancer cells.

CONCLUSIONS

A simple, enzyme-free, and dual-emission ratiometric fluorescent probe for the miRNA determination based on CHA signal amplification has been developed. This probe exhibits good sensitivity and good selectivity of miRNA-122 against four other miRNA targets because of CHA signal amplification and dual-emission peak, in which an outside signal is enhanced and a built-in reference signal is quenched, are used. Furthermore, analytical applicability of the ratiometric fluorescent miRNA sensor was tested on human cell lysates. The proposed strategy is accurate, simple, selective and sensitive. This strategy for probe construction has the application potentiality in miRNA-related biochemical research and clinical diagnostics.

ASSOCIATED CONTENT

Optimization of detection conditions, including the concentrations of ThT, K^+, Mg^{2+}, and H2, reaction temperature, and reaction time etc.

AUTHOR INFORMATION

Corresponding Authors *Tel.: (86)15273219560. Fax: +86 7315292251. E-mail: cai_mao3@hotmail.com (C. Cai)

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (No. 217755132, 21402168, and 21505112), Scientific Research Foundation of Hunan Provincial Education Department (No.16A204, 15B232), and Hunan 2011 Collaborative Innovation Center of Chemical Engineering & Technology with Environmental Benignity and Effective Resource Utilization.

REFERENCES

(1) Zhang, X.; Xiao, Y.; Qian, X. A ratiometric fluorescent probe based on FRET for imaging Hg^{2+} ions in living cells. *Angew. Chem. Int. Ed.* **2008**, *47* (42), 8025-8029.

(2) Xu, Z.; Baek, K. H.; Kim H. N.; Cui, J.; Qian, X.; Spring, D. R.; Shin, I.; Yoon, J. Zn^{2+}-triggered amide tautomerization produces a highly Zn^{2+}-selective, cell-permeable, and ratiometric fluorescent sensor. *J. Am. Chem. Soc.* **2010**, *132* (2), 601-610.

(3) Yang, Z. R.;Wang, M. M.; Wang, X. S.; Yin, X. B. Boric-acid-functional lanthanide metal-organic frameworks for selective ratiometric fluorescence detection of fluoride ions. *Anal. Chem.* **2017**, *89* (3), 1930-1936.

(4) Liu, B.; Zeng, F.; Wu, S.; Wang, J.; Tang, F. Ratiometric sensing of mercury(II) based on a FRET process on silica core-shell nanoparticles acting as vehicles. *Microchim. Acta.* **2013**, *180* (9), 845-853.

(5) Wang, X.; Yu, J.; Kang, Q.; Shen, D.; Li, J.; Chen, L. Molecular imprinting ratiometric fluorescence sensor for highly selective and sensitive detection of phycocyanin. *Biosens. Bioelectron*. **2016**, *77*, 624-630.

(6) Zhang, K.; Yu, T.; Liu, F.; Sun, M.; Yu, H.; Liu, B.; Zhang, Z.; Jiang, H.; Wang, S. Selective fluorescence turn-on and ratiometric detection of organophosphate using dual-emitting Mn-doped ZnS nanocrystal probe. *Anal. Chem.* **2014**, *86* (23), 11727-11733.

(7) Zhang, K.; Zhou, H.; Mei, Q.; Wang, S.; Guan, G.; Liu, R.; Zhang, J.; Zhang, Z. Instant visual detection of trinitrotoluene particulates on various surfaces by ratiometric fluorescence of dual-emission quantum dots hybrid. *J. Am. Chem. Soc.* **2011**, *133* (22), 8424-8427.

(8) Liu, F.; Bing, T.; Shangguan, D.; Zhao, M.; Shao, N. Ratiometric fluorescent biosensing of hydrogen peroxide and hydroxyl radical in living cells with lysozyme–silver nanoclusters: lysozyme as stabilizing ligand and fluorescence signal unit. *Anal. Chem.* **2016**, *88* (21), 10631−10638.

(9) Wen, Y.; Xu, Y.; Mao, X.; Wei, Y.; Song, H.; Chen, N.; Huang, Q.; Fan, C.; Li, D. DNAzyme-based rolling-circle amplification DNA machine for ultrasensitive analysis of microRNA in drosophila larva. *Anal. Chem.* **2012**, *84* (18), 7664-7669.

(10) Wang, M.; Fu, Z.; Li, B.; Zhou, Y.; Yin, H.; Ai, S. One-Step, Ultrasensitive, and electrochemical assay of microRNAs based on T7 exonuclease assisted cyclic enzymatic amplification. *Anal. Chem.* **2014**, *86* (12), 5606-5610.

(11) Liu, Q.; Ma, C.; Liu, X. P.; Wei, Y. P.; Mao, C. J.; Zhu, J. J. A novel electrochemiluminescence biosensor for the detection of microRNAs based on a DNA functionalized nitrogen doped carbon quantum dots as signal enhancers. *Biosens. Bioelectron.* **2017**, *92*, 273-279.

(12) Yin, B. C.; Liu, Y. Q.; Ye, B. C. One-step, multiplexed fluorescence detection of microRNAs based on duplex-specific nuclease signal amplification. *J. Am. Chem. Soc.* **2012**, *134* (11), 5064-5067.

(13) Jin, Z.; Geißler, D.; Qiu, X. K.; Wegner, D.; Hildebrandt, N. A rapid, amplification-free, and sensitive diagnostic assay for single-step multiplexed fluorescence detection of MicroRNA. *Angew. Chem., Int. Ed.* **2015**, *54* (34), 10024-10029.

(14) Liao, R.; He ,K.; Chen, C.; Chen, X.; Cai, C. Double-strand displacement biosensor and Quencher-free fluorescence strategy for rapid detection of MicroRNA. *Anal. Chem.* **2016**, *88* (8), 4254.

(15) Degliangeli, F.; Kshirsagar, P.; Brunetti,V.; Pompa, P. P.; Fiammengo, R. Absolute and direct microRNA quantification using DNA–gold nanoparticle probes, *J. Am. Chem. Soc.* **2014**, *136*, 2264-2267.

(16) Qiu, X.; Hildebrandt, N. Rapid and Multiplexed MicroRNA Diagnostic Assay Using Quantum Dot-Based Förster Resonance Energy Transfer, *ACS Nano*. **2015**, *9* (8), 8449-8457.

(17) Liu, H.; Li, L.; Duan, L.; Wang, X,; Xie, Y.; Tong, L.; Wang, Q.; Tang, B. High specific and ultrasensitive isothermal detection of microRNA by padlock probe-based exponential rolling circle amplification. *Anal. Chem.* **2013**, *85* (16), 7941-7947.

(18) Yang, L.; Liu, C.; Ren, W.; Li, Z. A Graphene Surface-anchored fluorescence sensor for sensitive detection of MicroRNA coupled with enzyme-free signal amplification of hybridization chain reaction. *ACS Appl. Mater. Inter.* **2012**, *4* (12), 6450-6453.

(19) Yang, C.; Shi, K.; Dou, B.; Xiang, Y.; Chai, Y.; Yuan, R. In situ DNA-templated synthesis of silver nanoclusters for ultrasensitive and label-free electrochemical detection of microRNA. *ACS Appl. Mater. Inter.* **2015**, *7* (2), 1188-1193.

(20) Hou, T.; Li, W.; Liu, X.; Li, F. Label-Free and Enzyme-Free homogeneous electrochemical biosensing strategy based on hybridization chain reaction: a facile, sensitive, and highly specific MicroRNA assay. *Anal. Chem.* **2015**, *87* (22), 11368-11374.

(21) Wu, H.; Liu, Y.; Wang, H.; Wu, J.; Zhu, F.; Zou, P. Label-free and enzyme-free colorimetric detection of microRNA by catalyzed hairpin assembly coupled with hybridization chain reaction. *Biosens. Bioelectron.* **2016**, *81*, 303-308.

(22) Kai, Z.; Ke, W.; Xue, Z.; Xu, F.; Xie, M. Sensitive detection of microRNA in complex biological samples by using two stages DSN-assisted target recycling signal amplification method. *Biosens. Bioelectron.* **2017**, *87*, 358-364.

(23) Ouyang, W.; Liu, Z. H.; Zhang, G. F.; Chen, Z.; Guo, L.; Lin, Z.; Qiu, B.; Chen, G. Enzyme-free fluorescent biosensor for miRNA-21 detection based on MnO_2 nanosheets and catalytic hairpin assembly amplification. *Anal. Method* **2016**, *8* (48), 8492-8497.

(24) Liu, Y. Q.; Zhang, M.; Yin, B. C.; Ye, B. C. Attomolar ultrasensitive MicroRNA detection by DNA-scaffolded silver-nanocluster probe based on isothermal amplification. *Anal. Chem.* **2012**, *84*, 5165−5169.

(25) Yu,Y.; Chen, Z.; Shi, L.; Yang, F.; Pan, J.; Zhang, B.; Sun, D. Ultrasensitive electrochemical detection of MicroRNA based on an arched probe mediated isothermal exponential amplification. *Anal. Chem.* **2014**, *86* (16), 8200-8205.

(26) Yin, P.; Choi, H. M.; Calvert, C. R; Pierce, N. A. Programming biomolecular self-assembly pathways. *Nature* **2008**, *451* (7176), 318-322.

(27) Bourdoncle, A.; Estévez, T. A.; Gosse, C.; Lacroix, L.; Vekhoff, P.; Saux, T. L.; Jullien, L.; Mergny, J. L. Quadruplex-based molecular beacons as tunable DNA probes. *J. Am. Chem. Soc.* **2006**, *128* (34), 11094-11105.

(28) Somsen, O. J. G.; Hoek, V. A. Amerongen V H. Fluorescence quenching of 2-aminopurine in dinucleotides. *Chem. Phys. Lett.* **2005**, *402* (1–3), 61-65.

(29) Mitsis, P. G.; Kwagh, J. G. Characterization of the interaction of lambda exonuclease with the ends of DNA. *Nucleic Acids Res.* **1999**, *27* (15), 3057-3063.

(30) Rachofsky, E. L.; Osman, R.; Alexander, R. J. B. Probing structure and dynamics of DNA with 2-Aminopurine: effects of local environment on fluorescence. *Biochem.* **2001**, *40* (4), 946-956.

(31) Sen, D.; Gilbert, W. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature* **1988**, *334* (6180), 364-366.

(32) Williamson, J. R.; Raghuraman, M. K.; Cech, T. R. Monovalent cation-induced structure of telomeric DNA: The G -quartet model. *Cell* **1989**, *59*, 871-880.

FIGURE AND TABLE CAPTIONS

Table 1. Sequences of the oligonucleotides used in the experiments.

Scheme 1. Schematic illustration of the ratiometric probe for the detection miRNA-122.

Figure 1. CD spectra of curves (1) H1, (2) H1+ miRNA122, (3) H1+H2 and (4) H1+H2+miRNA-122.

Figure 2. Electrophoresis of CHA amplification products using 3% agarose gel.

Figure 3. (a) Fluorescence spectra of probe in the presence of different concentrations of miRNA-122. (b) The linear plot of the PL intensity ratio $(I_{485}/I_{367})/(I_{485}/I_{367})_0$ versus the concentration of miRNA-122.

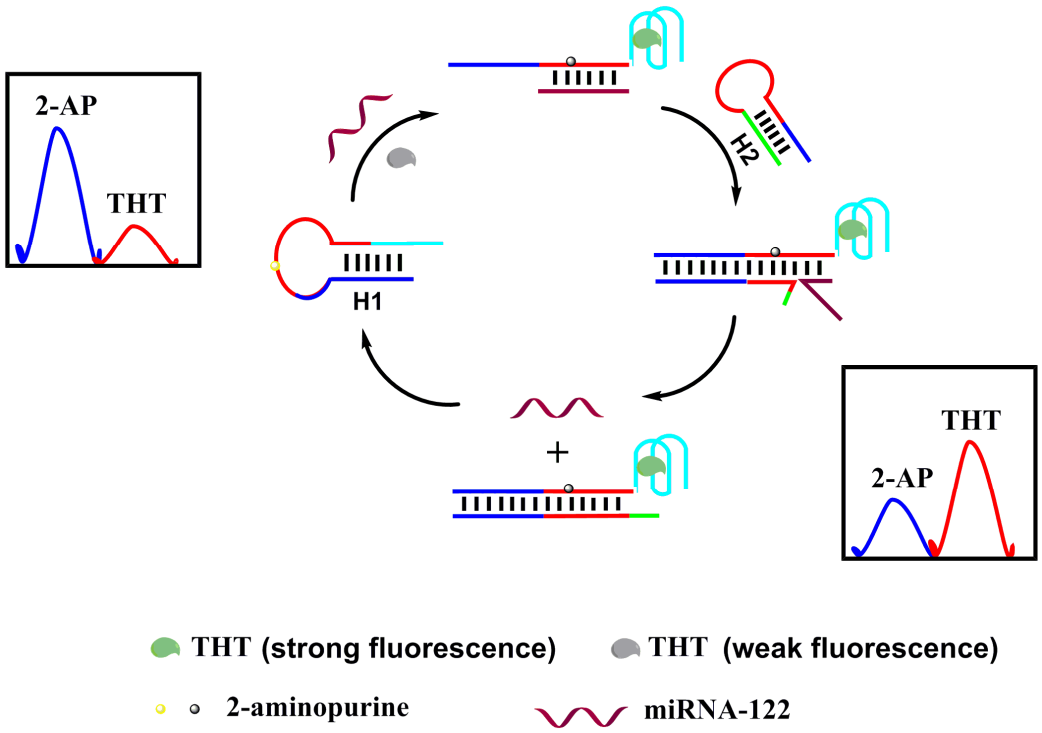
Figure 4. Specificity investigation for miRNA-122 against miRNA-21, miRNA-26a, miRNA-144, miRNA-199.

Figure 5. Detection of miRNA-122 from different cancer cell lysates.

Table 1. Sequences of the oligonucleotides used in the experiments[α].

Name	Sequence (from 5′ to 3′)
H1	5’-TA**C CAC CCA TGG AG**A TC*C AAA C<u>A</u>C CAT TGT CAC A**CT CCA TGG GTG G**GT GGG TGG G-3’
H2	5’- **A TGG AGA TCC AAA CACC** TGG AGT GTG ACA A**TG GTG TTT GGA TCT CCA T**GG GTG G-3’
miRNA-122	5'-UGGAG UGUGA CAAUG GUGUU UG-3'
miRNA-26a	5'-UUCAA GUAAU CCAGG AUAGG CU-3'
miRNA-21	5'-UAGCU UAUCA GACUG AUGUU GA-3'
miRNA-199	5'-ACAGU AGUCU GCACA UUGGU UA-3'
miRNA-144	5'-CAUCU UCCAG UACAG UGUUG GA-3'

[α] The double underlined base of H1 indicates the 2-AP substitution. In H1 and H2, the boldface letters represent the sequences complementary to each other to form the stems of the hairpin probes, respectively.



Scheme 1. Schematic illustration of the ratiometric probe for the detection miRNA-122.

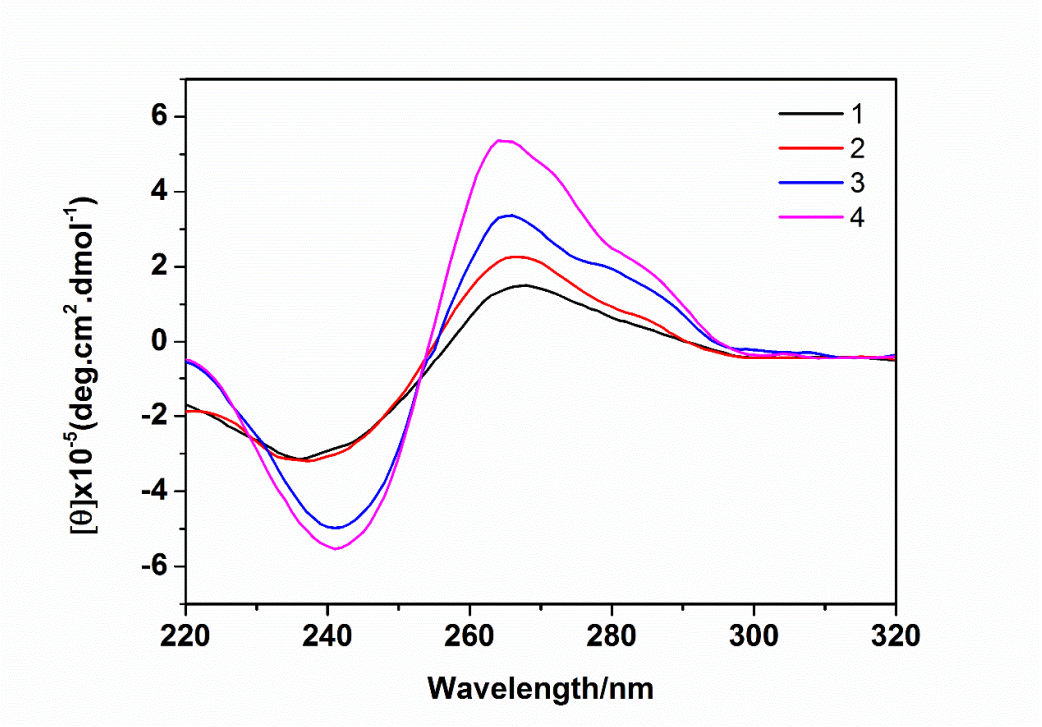


Figure 1. CD spectra of curves (1) H1, (2) H1+ miRNA122, (3) H1+H2 and (4) H1+H2+miRNA-122. Experimental conditions: C_{H1}: 2 μM, C_{H2}: 2μM, $C_{miRNA\text{-}122}$: 500nM.

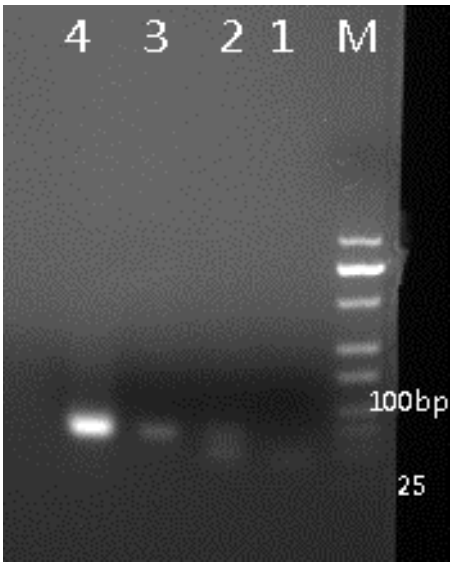


Figure 2. Electrophoresis of CHA amplification products using 2% agarose gel. Lane M: marker; Lane 1: H1; Lane 2: H1 + H2; Lane 3: H1 + miRNA-122; Lane 4: H1 + H2 + miRNA-122. Experimental conditions: C_{H1}: 2 μM, C_{H2}: 2μM, $C_{miRNA\text{-}122}$: 2 μM.

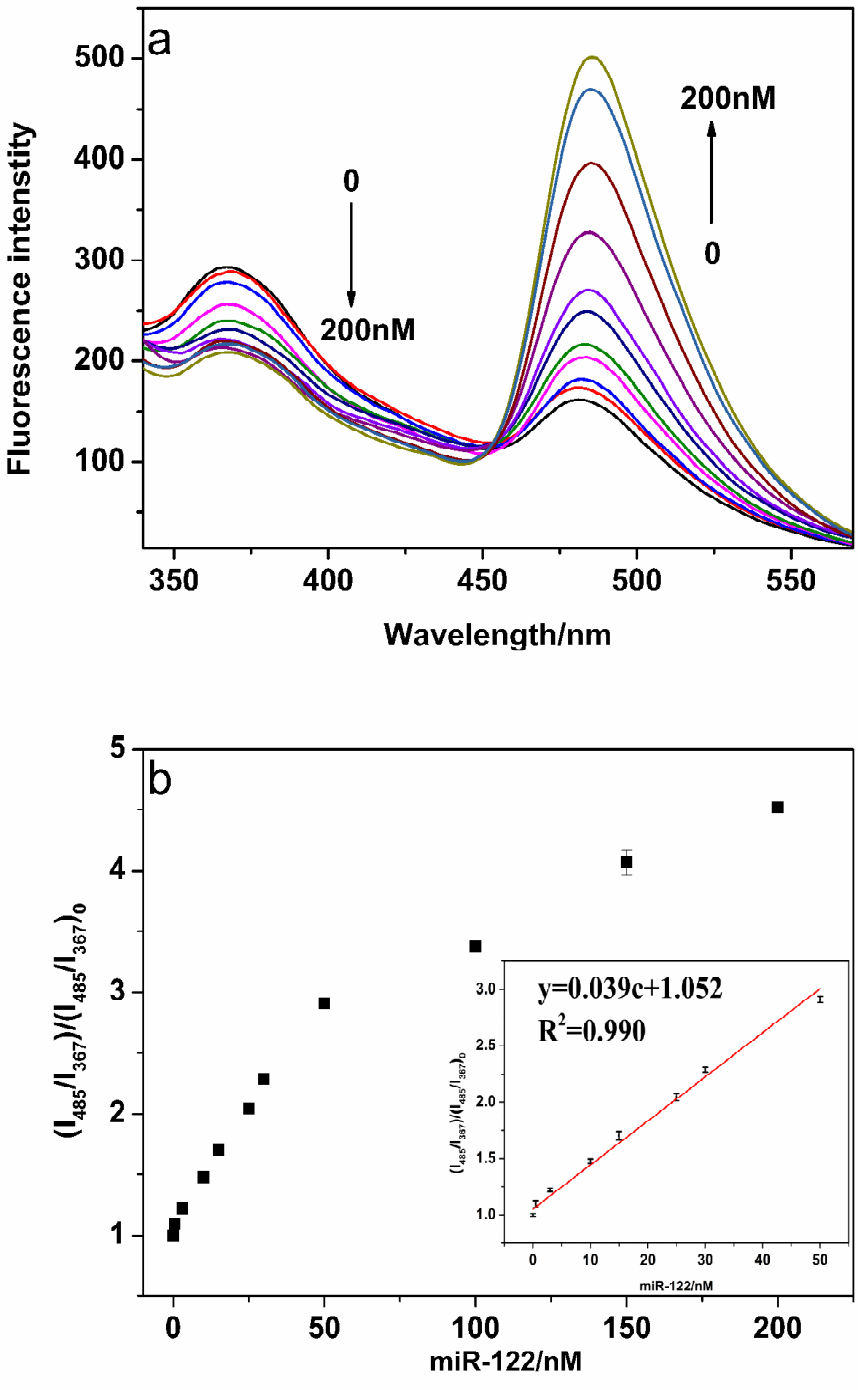


Figure 3. (a) Fluorescence spectra of probe in the presence of different concentrations of miRNA-122. The concentrations of miRNA-122 were 0, 0.5, 3, 10, 15, 25, 30, 50,

100, 150 and 200 nM respectively. (b) The linear plot of the PL intensity ratio $(I_{485}/I_{367})/ (I_{485}/I_{367})_0$ versus the concentration of miRNA-122. $(I_{485}/I_{367})_0$ and (I_{485}/I_{367}) were the PL intensity ratio of the probe in the absence and presence of miRNA-122, respectively. The error bars were calculated from three parallel experiments.

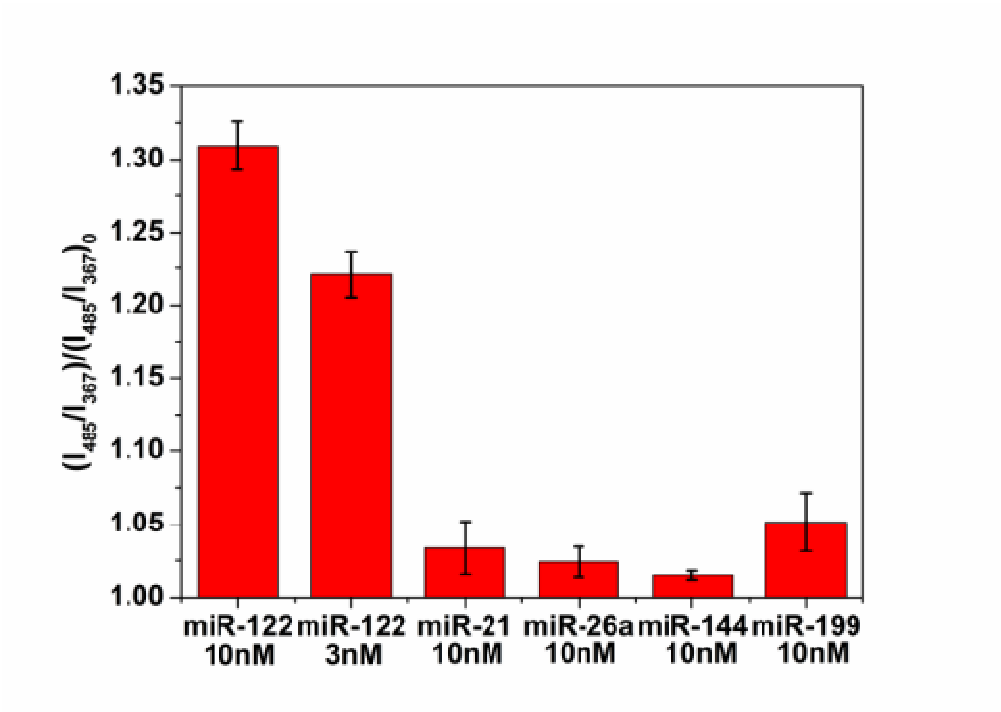


Figure 4. Specificity investigation for miRNA-122 against miRNA-21, miRNA-26a, miRNA-144, miRNA-199; (conditions: C_{H1}, C_{H2}: 200 nM, THT: 2 μM, K^+: 10 mM, Mg^{2+}: 16 mM; $C_{miRNA\text{-}122}$:3 nM and 10 nM; $C_{miRNA\text{-}21}$, $C_{miRNA\text{-}26a}$, $C_{miRNA\text{-}144}$, $C_{miRNA\text{-}199}$: 10 nM).

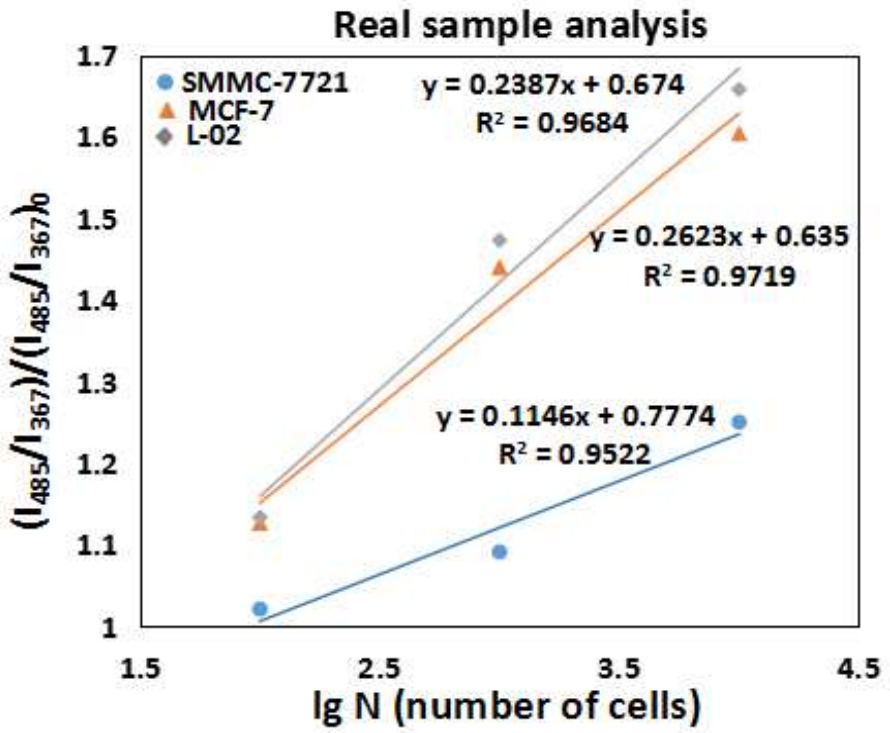
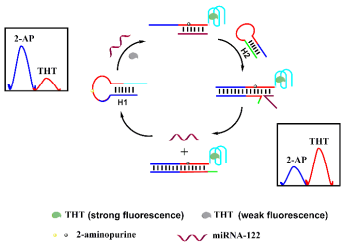


Figure 5. The corresponding calibration plot of fluorescence intensity ratio vs. logarithm of the number for SMMC-7721, MCF-7 and L-07 cells.

For TOC only


2-AP
THT
H1
H2
THT
2-AP
THT (strong fluorescence)
THT (weak fluorescence)
2-aminopurine
miRNA-122