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Integrating PDA microtube waveguide system with heterogeneous CHA amplification strategy towards superior sensitive detection of miRNA

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ABSTRACT

Catalytic hairpin assembly (CHA) is a typical enzyme-free amplification strategy, in which the target can catalyze two hairpin probes to form a duplex and yield multiple outputs signal. However, the non-specific hybridization of two hairpin probes in CHA circuit usually occurred even in the absence of target, causing significant background leakage and impeding its practical applications in trace miRNA analysis. Herein, we proposed a novel heterogeneous CHA (hetero-CHA) design integrating with PDA microtube waveguide system, offering the advantages to enhance the target signal, but suppress the background leakage simultaneously. In hetero-CHA strategy, single-stranded targets are enriched nearby the surface of PDA microtube, facilitating the target-triggered CHA amplification and strand displacement reactions. In contrast, double-stranded DNA complexes formed by uncatalyzed hybridizations are isolated from PDA microtube, impeding the leakage signal. By combination with condensing enrichment effect, the proposed hetero-CHA probe exhibited high selectivity and sensitivity to miRNA target, giving a detection limit as low as 3.3 fM. More importantly, the proposed hetero-CHA probe can be applied directly to distinguish the expression of miRNA-21 in clinical serum of cancer patients (including lung, breast and pancreatic) from those of healthy human beings, favoring the cancer diagnosis and therapeutic evaluation.

1. Introduction

Unique profiles of cancer-related mRNAs or miRNAs biomarker based on differences in relative expression levels provide vital information about tumor progression and risk estimation of decease in clinical settings, and allow the doctor to personalize the anticancer treatment plan for patients (Henry and Hayes, 2012; Spiller et al., 2010; Tyagi, 2009). However, the inadequate signal output of biomarkers, especially the mRNAs or miRNAs with low expression levels, challenges the fast, simple and accurate detection, analysis and quantitation of nucleotide sequence in early clinical diagnosis prior to disease treatment. Traditional polymerase chain reaction (PCR)(Saiki et al., 1988) methods, and recent developed isothermal enzymatic methods, such as loop-mediated isothermal amplification (LAMP)(Notomi et al., 2000), strand displacement amplification (SDA)(Walker et al., 1992), and rolling circle amplification (RCA)(Ali et al., 2014; Zhao et al., 2008), have advanced our knowledge of miRNA in regulating the cellular metabolism and have showed the potential use of miRNA in clinical diagnostics. However, some drawbacks including the use of enzymes, easy contamination, high cost, and lack of portability limit the quantitative assay conditions of nucleic acid target assay and complicate the assay procedures (Kim et al., 2016). Thus molecular engineering of nucleic acid based probe without the participation of enzyme for low abundant nucleotide biomarkers, especially for clinical samples and single cells, is of great demand.

Catalyzed hairpin assembly (CHA), is a typical enzyme-free DNA amplification technique developed by Pierce, Yin, and co-workers (Yin et al., 2008), in which one target can catalyze two hairpin probes to form a duplex and yield multiple signal outputs (Wu et al., 2015; Wu et al., 2017; Zheng et al., 2012). Unfortunately, the non-specific hybridization of two hairpin probes in CHA circuit usually occurred even in the absence of target, causing significant background leakage and preventing its practical applications in ultralow concentration miRNA analysis (Ma et al., 2012; Yuan et al., 2017). To reduce background

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leakage in CHA circuit, some methodologies including mismatched nucleotides (Jiang et al., 2014), multi-arm junctions (Kotani and Hughes, 2017), locked nucleic acids (Olson et al., 2017) have been proposed. However, some drawbacks, e.g. lower CHA reaction rate and poor detection sensitivity caused by mismatch designs for the hairpin probes, impeded their practical applications. Since the non-specific hybridization of two hairpin probes in CHA circuit is inevitable. Herein, we proposed single Polydiacetylene (PDA) microtube waveguide system integrated with heterogeneous CHA (hetero-CHA) designs, to simultaneously enhance the target signal but suppress the background leakage. In hetero-CHA strategy, single-stranded (ss-) target miRNA can be enriched onto the surface of PDA microtube due to strong attraction (cation–π interactions between the nucleobase in ss-miRNA and amine cation within the surface of PDA microtube), facilitating the target-triggered CHA and strand displacement reactions within the surface of PDA microtube. While the double-stranded (ds-) DNA complexes intermediates generated by the non-specific hybridization of two hairpin probes in the absence of target remain in the solution (isolated from PDA microtube due to the electrostatic repulsion), offering the advantage to suppress the background leakage signal. By combination with condensing enrichment effect, the detection limit of the proposed hetero-CHA probe is 3.3 fM, which is sufficiently sensitive to distinguish the abnormal expression of miRNA-21 in clinical serum. It is noteworthy that the proposed hetero-CHA probe could be applied directly to discriminate the clinical serum between the cancer patients (including lung, breast and pancreatic) and healthy human beings without further separation, enrichment or pre-amplification process, which may pave the way to develop novel minimally invasive, portable clinical diagnostic equipment for cancer diagnosis and therapeutic evaluation. This work not only provides novel CHA probes toward practical discrimination of miRNA expression in clinical serum but also is of great fundamental value for rational design of non-enzymatic nucleic acid circuits.

2. Material and methods

2.1. Reagents and instrumentation

10, 12-Pentacosadiynoic acid (PCDA) was purchased from Tokyo Chemical Industry Co., Ltd., and purified to remove the polymerized part before use. Ethylenediamine-substituted Pentacosadiacetylene (EPDA) were synthesized in analogy to the previous procedure (Hu et al., 2014). All other solvents and reagents were of analytical grade and used as received. Milli-Q water purified system (18.2 MΩ cm) was used in all cases. Human Serum were collected from Anhui Provincial Hospital. Oligonucleotides were quantified by UV–vis absorption spectroscopy, using a SHIMADZU UV-2550 PC spectrophotometer. Fluorescence spectra were measured by using a JY-ihR 550 spectrophotometer. Optical microscopy images were obtained in a BX-51 fluorescence microscope. FTIR experiments were performed on a MAGNA 750 FT-IR spectrometer. X-ray photoelectron spectroscopy (XPS) characterization was carried out with a VG ESCALAB MK-II photoelectron spectrometer. The Raman characterization was performed with a LABRAM-HR Confocal Laser Micro Raman Spectrometer with 514.5 nm radiation at room temperature. The transmission electron microscopy (TEM) characterization was measured by a JEOL-2000 microscope (operated at 200 kV).

2.2. DNA purification

All HPLC-purified miRNAs and DNA oligonucleotides used in this work were purchased from Shanghai Bio-Engineering Company (Shanghai, China), and the sequences of DNA and miRNA were shown in Table S1. The DNAs were dissolved in Tris-acetate/EDTA/Mg^{2+} buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 12.5 mM magnesium acetate, pH 8.0) and then quantified by UV–vis absorption spectroscopy. Hairpin probes annealed over a temperature gradient from 95 to 25 °C in 2 h before use.

2.3. The fabrication of PDA microtube waveguide system

PDA composite microtubes were prepared by a similar method (Hu et al., 2014) as illustrated in Fig. S4. Probe1 (5′-SH- GGTTCCTATATG CGAAAGTCTGA-3′) modified PDA microtubes and probe2 (5′-SH-TTC GCATATAGGAACC-3′) functionalized gold nanorods were prepared by a similar procedure described in the literature (Zhu et al., 2016).

2.4. MiRNA-21 detection experiments in buffer

The miRNA-21 detection experiments in buffer were carried out by placing as-prepared Au@PDA composite microtube into 200 μL Tris-acetate/EDTA/Mg2+ buffer (containing 100 nM H1, 400 nM H2 and the target miRNA-21 at various concentration, respectively), and further incubating at 25 °C for 2 h. After rinsing for several times, single PDA composite microtube was placed on the glass and the waveguide performance of PDA composite microtube was characterized by single-tube PL imaging method (Hu et al., 2014). A 532 nm excitation light was launched onto the body of the microtube and its out-coupled tip emission was characterized by single-tube PL imaging method, which was found to be dependent on the concentration of miRNA-21. The experimental set-up for fluorescent signal detection of single PDA microtube with imaging system was shown in Fig. S10. The control experiments using reporter-FAM/Dabcyl with the same hybridized DNA structure as the fluorescence probes with traditional homo-CHA design were carried out at the same conditions.

2.5. Condensing-enrichment effect combined with hetero-CHA amplification strategy

Firstly, a glass substrate coated with hydroxyl was immersed in an ethanol solution of octadecytrichlorosilane (OTS) for 1 h to modify with alkyl chain. The water contact angle of the OTS-modified substrate was 140° and it can help to achieve condensing-enrichment effect. Single hydrophilic AuNRs-PDA microtube was placed on the OTS coated hydrophobic substrate. Then adding 10 μL buffer solution (containing 100 nM H1, 400 nM H2 and the target miRNA-21) to Au@PDA microtube on hydrophobic substrate. Since the wettability difference between the hydrophilic microtube (water contact angle of 140.0° ± 0.3°) and its surrounding hydrophobic substrate (water contact angle of 31.3° ± 0.4°), the droplet would be pinned to the microtube in a small contact area (Fig. S7). After solvent evaporation, the volume of the droplet would gradually decreased and followed by the concentration of analytes increased. The analytes were enriched from the highly diluted solution and then anchored onto the hydrophilic microtube. Then, analytes were highly enriched during solvent evaporation and resulted in more successful collision with the reaction processed. Therefore, the effective contact frequency between the reactants and microtube would be enriched, allowing for highly sensitive detection of miRNA. The selectivity experiments and clinical experiments were carried out with condensing enrichment effect. To study sequence-specificity of the sensor, similarly sequenced miRNAs including single-base mismatched miRNA-21, three-base mismatched miRNA-21, and non-complementary sequence (miRNA-141, miRNA-199a) were considered as ideal models to demonstrate the specificity of the assay. To test anti-interference ability of the sensor, the prepared PDA microtube probes were directly applied to detect miRNA-21 in complex biological environment, e.g.

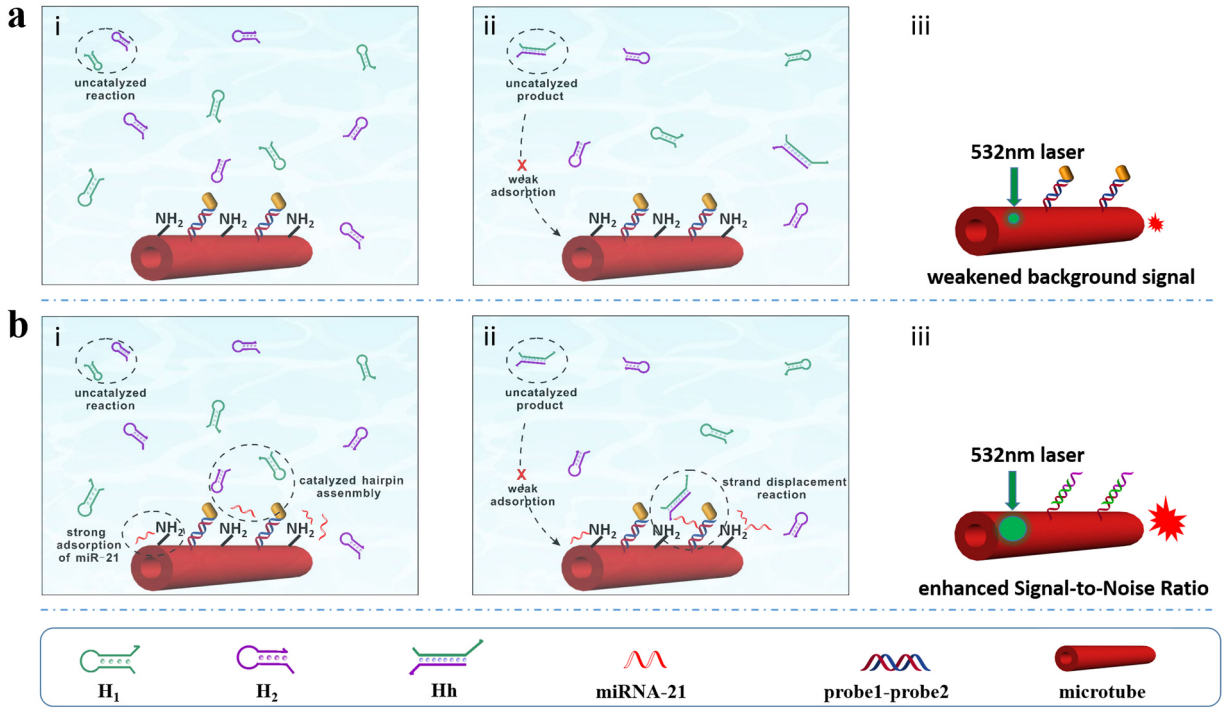
mixture containing 50% human serum in buffer. In order to prove practicality of this method, we measured miRNA-21 in human blood serum from three types of cancer patients, including breast cancer, pancreatic cancer, and gastric patients, and then compared results from healthy people.

3. Results and discussion

3.1. Analytic framework and probe design

A simple amplification circuit based on the CHA model is depicted in Fig. S1. In this homogenous CHA (homo-CHA) system, a pair of incubated hairpins (H1 and H2) are initially in metastable states. Characterized by the initial burst of signal followed by a slow but steady uncatalyzed hybridization of H1 and H2 species, the CHA system is found to execute non-specifically and present a severe background leakage (dark blue curve in Fig. 1a). This background leakage can roughly classified into two categories, initial leakage and asymptotic leakage (Chen et al., 2013b; Jiang et al., 2014). Initial leakage can be attributed to the sample impurities (Qian and Winfree, 2011) and the misfolding of nucleic acids (Chen et al., 2013a), while asymptotic leakage corresponds to the formation of Hh duplex from perfectly formed H1 and H2 in the absence of target because of the end "breathe" of kinetically trapped hairpin substrates (Frank-Kamenetskii, 1987). To facilitate the target miRNA-triggered CHA reactions and suppress background leakage, herein, a conceptually new design based on PDA microtube waveguide system with a heterogeneous CHA amplification strategy was proposed (Fig. S2), in which the reporter complexes are grafted on the surface of PDA microtube (isolated from bulk buffer containing the hairpins H1 and H2). Unlike homogenous solution, there are two subsystems including bulk solution (B) and PDA microtube based reporter system (R) in our proposed hetero-CHA system (Fig. S3).

Our calculations demonstrated that simply extracting the reporter complex from the bulk solution and concentrating it in a smaller space near the surface of PDA microtube (R space) would not affect the reaction kinetics, therefore contribute negligibly to the leak suppression (see the Supplementary material "Probe design principle" for details). The interactions between bulk species and PDA microtube determine the adsorption kinetics of bulk species onto microtube, which is a critical step of hetero-CHA system in detecting target strand. The incorporation of positively charged amino group into the orderly aligned conjugate polymer plays an essential role in the interactions between DNA and microtube because of the strong cation-π interactions between the amino and aromatic pyrimidine ring of nucleobase, in combination with the electrostatic attractions between the amino and phosphates of DNA. As suggested by the Langmuir isotherm of surface hybridization (Gong and Levicky, 2008; Hekstra et al., 2003; Peterson et al., 2002), we have established: i) strong enrichment of single stranded target oligomer on microtube surface; ii) slight enrichment of H1 and H2, therefore a constant concentrations of H1 and H2 in R phase is guaranteed at the initial stage of reaction; iii) strong repulsion of uncatalyzed Hh duplex from PDA microtube (see the Supplementary Material "Probe design principle" for details). Specifically, the strong attraction including cation-π and electrostatic interactions drive ss-target miRNA strongly move onto the surface of PDA microtube and result in a high coverage. The enrichment of ss-target miRNA on the microtube surface therefore greatly facilitates the target miRNA-triggered CHA reactions. In contrast, the nucleobases in uncatalyzed ds-DNA are effectively shielded within the densely negatively charged phosphate, and only a limited amount of uncatalyzed ds-DNA are near the surface of PDA microtube due to strong repulsion between uncatalyzed ds-DNA and PDA microtube, which offers an effective way of eliminating background leakage signal. In contrast to homogeneous CHA circuit with all DNA species in free states, there are two merits in our hetero-CHA amplification strategy: firstly, the target miRNAs are enriched into a high concentration in the local space adjacent to PDA microtube, facilitating CHA reactions; secondly, most uncatalyzed ds-DNAs stay far from the surface of PDA microtube, suppressing the background signal effectively (Scheme 1).



Scheme 1. (a) Uncatalyzed products (dsDNA) are away from PDA microtube surface, resulting in weakened background signal. (b) Single-strand miRNA-21 are enriched on the surface of PDA microtube, resulting in improved performance of CHA reaction on the PDA microtube surface.

3.2. Probe performance and reaction kinetics

To implement the proposed hetero-CHA probe, the Au@PDA composite microtubes with an outer diameter of 1–3 μm were prepared in analogy to the previous procedure (Hu et al., 2014), and the preparation process was shown in Fig. S4. As mentioned above, there are two types of functional units on the surface of PDA microtube, including the amino groups, and the hybridized ds-DNA complex of the complementary probe 1 and probe 2 strands (listed in Table S1) which is attached onto the Au nanorods. The Au@PDA composite microtubes were incubated with miRNA-21 at various concentrations in the buffer solution (containing H1, and H2 hairpin probes) at room temperature. As a reference sample, similar experiments were performed for the fluorescence probes with the homo-CHA design (Reporter-FAM/Dabcyl). For the homo-CHA probes (Fig. 1a), the background leakage signal was too severe to even overwhelm the positive signal when the concentration of target miRNA is low, and a discernable fluorescence enhancement could only be detected when the concentration of target miRNA-21 was higher than 0.1 nM. In contrast, an obvious enhancement of the tip emission of PDA microtube can be detected with the presence of miRNA-21 at 0.01 nM (Fig. 1b). More impressively, the rate of the catalytic reaction was obviously enhanced in the hetero-CHA system, especially at ultralow trigger concentration.

target miRNA signal while keeps background signal at a manageable level, especially at ultralow trigger concentration. Rather than the homogeneous reactions in homo-CHA probes, it is speculated that the ss-target miRNA-21 moves onto the surface of PDA microtube through adsorption because of the strong attraction between them. The enrichment of miRNA-21 on PDA surface facilitates the CHA amplifier reactions, while simultaneously promotes the formation of complex Hh with a non-protected single stranded domain, which can specifically reacts with the reporter probes on PDA microtube, resulting in the tip-emission recovery of PDA microtube. The uncatalyzed ds-DNA would be far from PDA microtube surface due to strong repulsion, resulting in weakened background leakage effect. In order to prove above hypothesis, the absorption experiments were performed between the amino-functionalized PDA microtube and the quencher-labeled (BHQ3) ss-DNA probe 3 or ds-DNA probe 4 (probe 3 hybridized with its complementary strand), respectively (Fig. S5). It is apparent that the quencher-labeled ds-DNA presented a much weaker signal than its ss-DNA counterparts, strongly supports our speculation. Moreover, when incubates with the mixture of H1 and single-strand miRNA-21, the tip-emission of PDA microtube was much stronger than those upon incubating directly with the pre-hybridized H1/miRNA-21 helix (Fig. S6), indicating the distinct execution mechanism in hetero-CHA system from that in homo-CHA system.

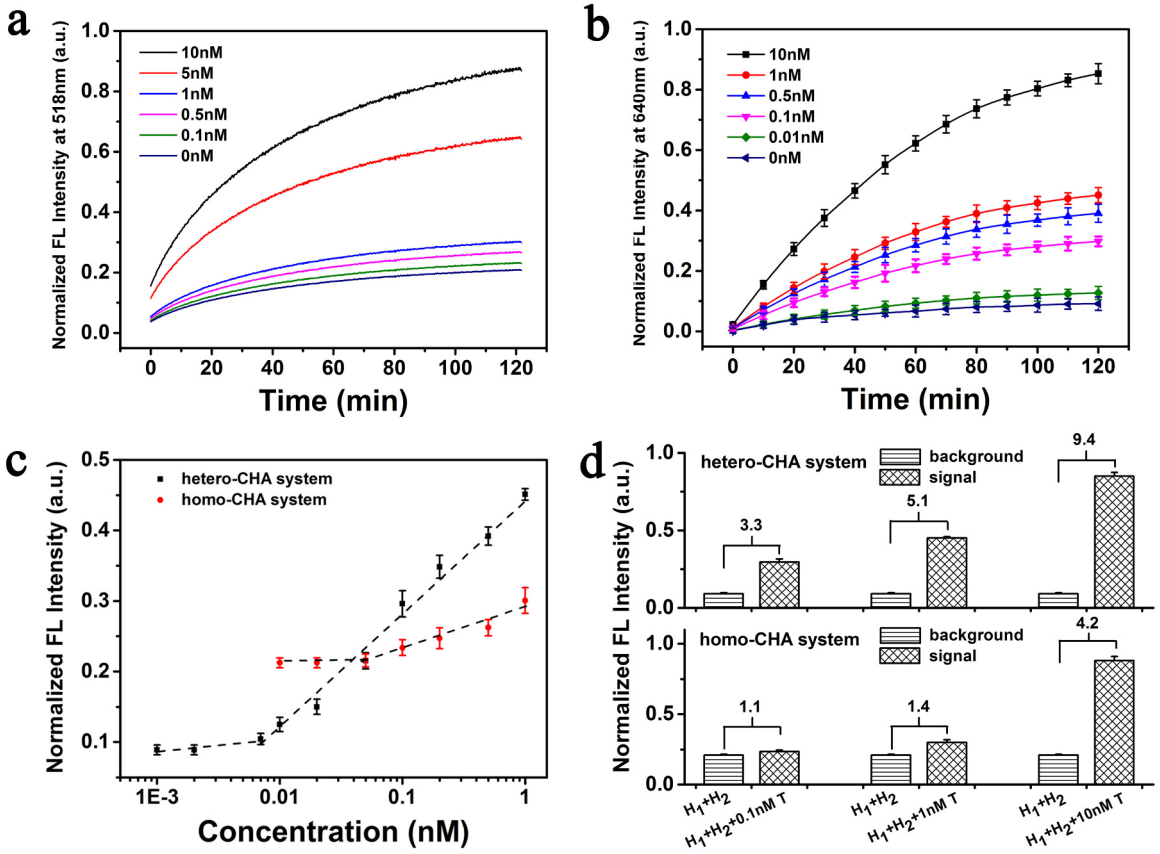


Fig. 1. (a) Fluorescence kinetics of homo-CHA system with various concentrations of miRNA-21. (b) Fluorescence kinetics of hetero-CHA system with various concentrations of miRNA-21. (c) Plot of fluorescence-intensity of hetero-CHA system (black) and homo-CHA system (red) upon gradual addition of miRNA-21 (ranging from 1 pM to 1 nM). (d) Signal-to-background ratios for two systems as a function of concentration of miRNA-21.

We evaluated the limits of detection (LOD) based on an IUPAC methodology ($3 < S/N < 5$) and the signal-to-background ratio (the catalyzed reaction relative to the uncatalyzed background) for the homo-CHA and hetero-CHA design. The calculated LOD for the homo-CHA system is 50 pM, while only 7 pM for the hetero-CHA system (Fig. 1c). It is apparent that the proposed hetero-CHA probe amplifies

The non-specific binding and crosstalk among hairpin probes may slow down the effective rate of individual reactions as well as cause background leakage signal. The initial leakage can be characterized using initial burst of fluorescence signal after the start of reaction. As shown in Fig. 1a, an evident early burst of hybridization between hairpins H1 and H2 could be detected in the homo-CHA system, and the

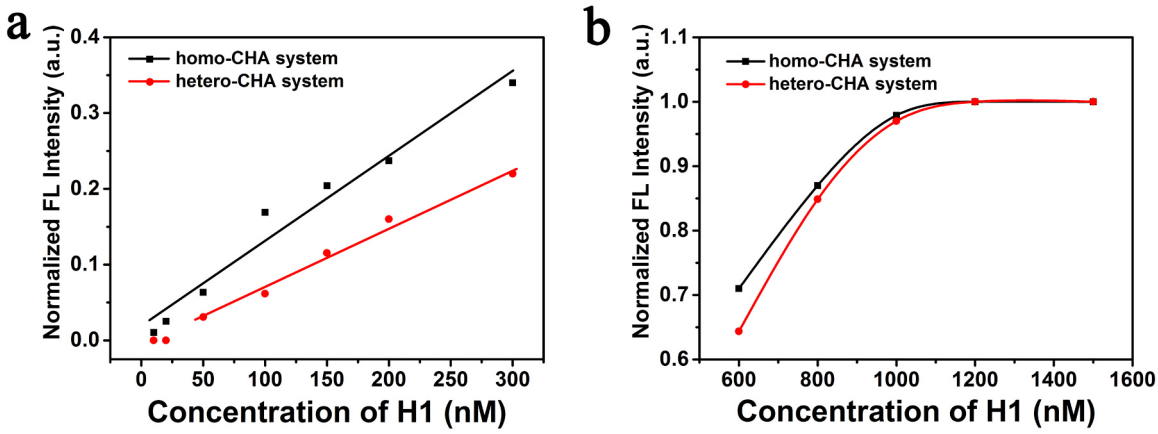


Fig. 2. Fluorescence intensity of hetero-CHA system and homo-CHA system as a function of the concentration of hairpin H1, at the low (a) and high (b) concentration region. The concentration ratio between H1 and H2 is set to a constant 1:4 in these experiments.

leakage is too severe to even overwhelm the positive signals when the concentration of target miRNA is low. In contrast, the fluorescence intensity starts from zero in the hetero-CHA system, indicating a negligible early burst. In the absence of target miRNA, the fluorescence intensities increase by ~0.2 for the homo-CHA designs, and ~0.05 for hetero-CHA designs after 2 h incubation (Figs. 1a, 1b), respectively, confirming a slower kinetics of asymptotic leakage in the hetero-CHA system. These results fully verify our theoretical speculation that the isolation of hetero-CHA probes from the bulk hairpins benefits the resistance of leakage and the improvement of overall kinetics (see the Supplementary material "Probe design principle C and D"). Moreover, the leakage behaviors caused by the non-specific reaction between two hairpin substrates in the absence of target miRNA-21 as a function of bulk hairpin probe concentration were characterized in detail (Fig. 2), with the concentration ratio of H1: H2 being set to a constant 1:4. At either the low concentration region (< 300 nM, Fig. 2a) or the high concentration region (> 600 nM, Fig. 2b), the leakage of circuit is significantly larger for the homo-CHA system than the hetero-CHA counterpart. When the concentrations of bulk hairpin probes approach 1.0 μM, the differentiation of leakage in the homo-CHA or hetero-CHA systems became negligible. This result can be understood using the Langmuir isotherm. A larger concentration of bulk specie benefits its binding onto PDA surface, therefore provides a larger production yield of complex Hh, as well as an enhanced enrichment of hairpins and complex Hh in the spaces of PDA microtubes, resulting in a larger leakage background.

Summarily, the proposed hetero-CHA probe exhibits an enhanced amplification performance and a weakened background leakage effect at the same time. It's noteworthy that the present detection limit is still far higher than the typical level of miRNA in serum, and further efforts are needed to enhance the sensitivity.

3.3. Condensing-enrichment effect

It has been reported that the sensitivity of PDA microtube sensor can be greatly enhanced (Xu et al., 2015) by placing the microtube on the hydrophobic substrate due to the condensing-enrichment effect. It's expected that the analysts will be enriched on the PDA microtube surface due to different wettability between the hydrophilic PDA microtube surface and the hydrophobic substrate. As shown in Fig. S7a, single Au@PDA composite microtube was placed on an OTS modified glass substrate, then a drop of buffer solution (10 μL) containing H1 (100 nM), H2 (400 nM), and target miRNA-21 was injected onto the surface of PDA composite microtube. After incubating at 25 °C and 40% relative humidity for 120 min, the droplet of the buffer solution concentrated, covering a very small area. After rinsing with PBS buffer for 3 times, tip emission of PDA microtube was characterized by single-tube photoluminescence imaging method. The target miRNA-21 catalyzed the hairpin assembly sequentially, spontaneously released the gold nanorods from the PDA microtube surface. Gradual addition of miRNA-21 enhanced the fluorescence intensity of the out-coupled tip emission of PDA microtube (Fig. 3a). To verify the reproducibility of our designed sensor system, we repeated the examinations with miRNA-21 at a concentration of 5 pM for six times (Fig. S8). The tip emission of PDA microtube was very sensitive to the concentration of miRNA-21, and discerned emission enhancement could be easily observed even at a very low concentration (10 fM) (Fig. 3b). The monotonic increase and linear variation of tip emission at 640 nm has been observed at the miRNA-21 concentration range of 10–100 fM, indicating that PDA microtube can be directly applied to accurate detection of miRNA-21 at ultralow concentration. The calculated LOD was approximately 3.3 fM, which is about 3000-fold enhancement compared to normal strand displacement reaction without hetero-CHA design (Zhu et al., 2016). Nevertheless, compared with homo-CHA system, the leakage effect maintains at a very low level.

3.4. The target selectivity

The specificity of our hetero-CHA probe was tested using four additional sets of target analogs and non-complementary miRNAs, besides the perfect target miRNA-21 (Fig. 3c). It was showed that a negligible fluorescence enhancement was exhibited for the non-complementary sequences at a high concentration (50 p.M.), while a significant signal change was provided in the presence of miRNA-21 even at ultralow concentration (50 fM). The single base change could greatly reduce the response of tip emission for the spurious strand of miRNA-21, specifically, the fluorescence is approximately three times stronger for perfect miRNA-21 than the spurious counterpart at a concentration of 50 pM. We then tested the ability of resistance to the interference from other miRNAs with their sequences of high similarity to miRNA-21. It was observed that the strength of fluorescence is three times higher for miRNA-21 than the mixture of the four analogous miRNAs (Fig. 3d), demonstrating the robustness of hetero-CHA probe to the interference of analogous sequence in the environment. Our results indicated that the PDA based hetero-CHA assay possesses a high specificity in the detection of target against other analogous miRNAs.

3.5. Clinical RNA biomarker

MiRNA-21 is an important serum biomarker in the clinical diagnosis of multiple cancers (Kumarswamy et al., 2011; Pan et al., 2010; Shen et al., 2011). Cancer has become one of the main factors that hinder the

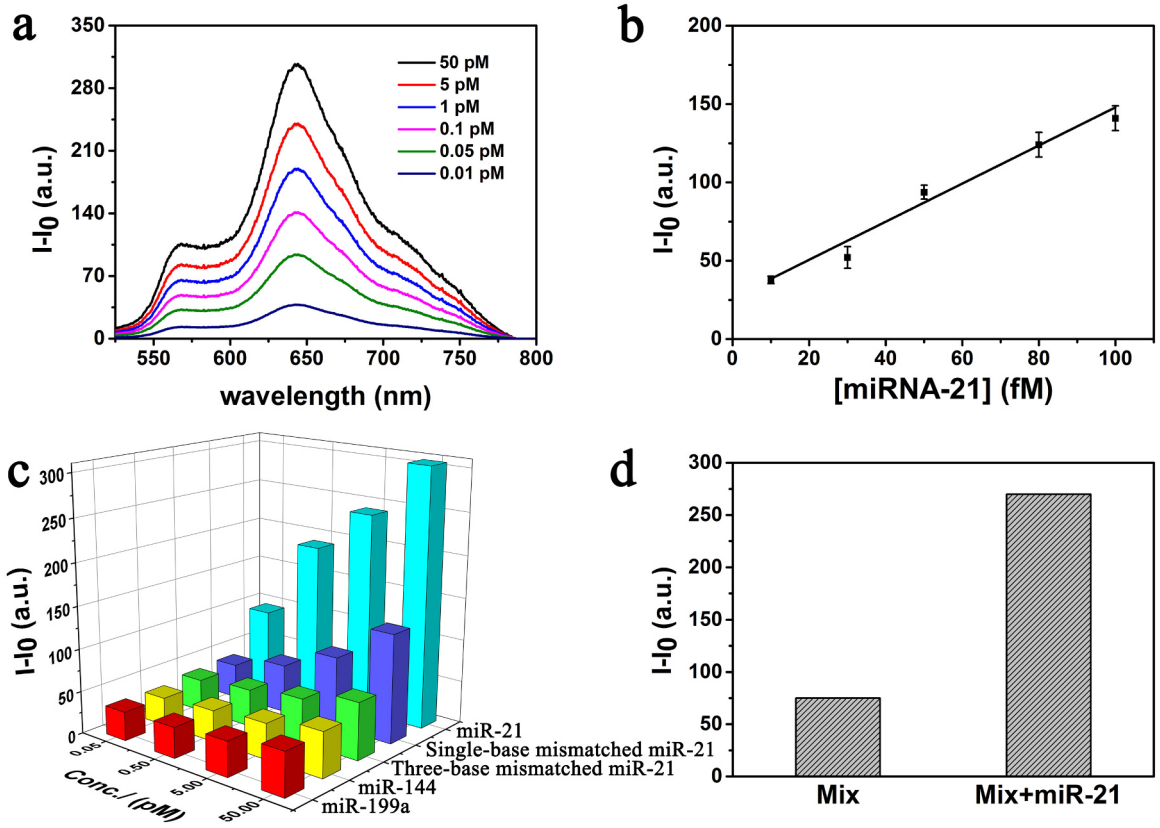


Fig. 3. (a) The fluorescence enhancement in tip emission of PDA microtube upon gradual addition of miRNA-21. (b) The fluorescence enhancement in tip emission of PDA microtube at 640 nm upon gradual addition of miRNA-21 (ranging from 10 fM to 100 fM). I_0 and I represent the tip emission intensity of PDA microtube at 640 nm before and after displacement reaction with miRNA-21, respectively. (c) The fluorescence enhancement in tip emission of PDA microtube at 640 nm with miRNA-21 and its analogous. (d) The fluorescence enhancement in tip emission of PDA microtube at 640 nm upon addition of a mixture of four analogous miRNAs (mix: miRNA-21, single-base mismatched miRNA-21, three-base mismatched miRNA-21, miRNA-144, miRNA-199a, each 5 pM) before and after addition of miRNA-21 (5 pM) to the mixture solution.

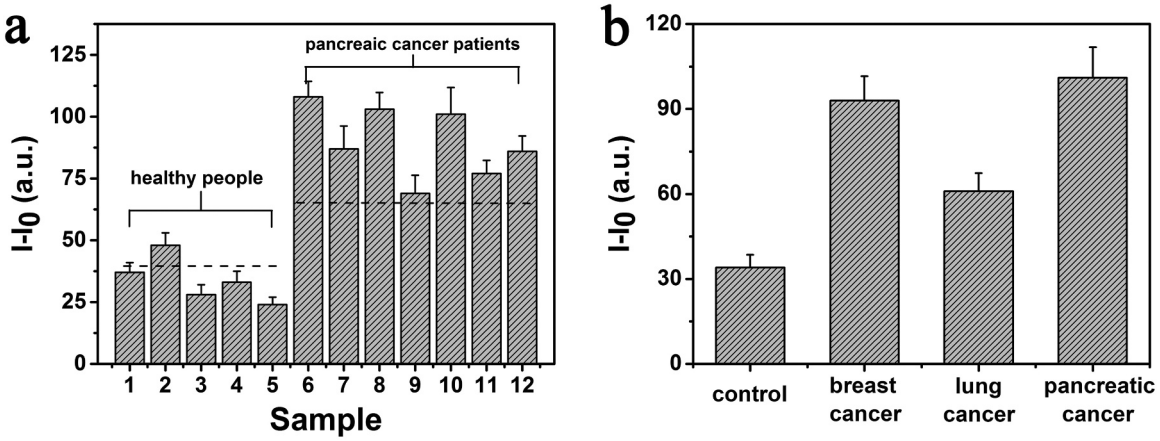


Fig. 4. (a) The fluorescence enhancement in tip emission of PDA microtube at 640 nm for miRNA-21 detection in human blood serum. Sample 1–5 were obtained from five healthy people, sample 6–12 were from seven pancreatic cancer patients. (b) The fluorescence enhancement in tip emission of PDA microtube at 640 nm with four different human blood serum samples.

further extension of human life. Therefore, the early diagnosis and treatment of cancers are of great concern (Ma et al., 2017). Given that miRNA-21 plays an oncogene role and its over-expression in serum and gastric tissues of breast cancer, lung cancer and pancreatic cancer patients (Oudeng et al., 2017), we tested the hetero-CHA probe against a RNA target like miRNA-21 which is directly extracted from the human serum. We first examined the robustness of hetero-CHA probe in complex biological environment. The analyst solution was prepared by mixing the buffer solution, which contains the miRNA-21 target and probe species, and healthy human serum, of the same amount. As shown in Fig. S9a, the florescence strength of PDA tip emission monotonically increased with the increasing of miRNA-21 concentration, and a robust enhancement in tip emission could be easily recognized at ultralow concentration (50 fM), which demonstrated the robustness of assay in complex biological environment. We note that the fluorescence signal decreased significantly in the mixture solution

of serum and buffer compared with pure buffer, which should be attributed to the interference from high-molecular-weight DNAs, plasma protein, and other substances in serum. The sequence-specificity of hetero-CHA probe in serum environment was examined (Fig. S9b), confirming its high sensitivity and specificity against the corresponding target regardless of the disturbance from serum. We then focused on whether the hetero-CHA probe can be applied in the detection of serum miRNA-21 for distinguishing cancer patients from healthy human beings. The serum samples were extracted from cancer patients or healthy people without further separation and enrichment process. 5 μL human serum was mixed with 5 μL buffer solution containing 200 nM H1 and 800 nM H2 to prepare the analyst solution. As shown in Fig. 4a, tip emission of PDA tube was enhanced by less than 40 for the serum samples of healthy people (4 of 5 cases), by contrast, an enhancement of larger than 50 was observed for the serum samples of pancreatic cancer patients (7 of 7 cases). We then assayed the serum samples extracted from lung and breast cancer patients using hetero-CHA probe. As shown in Fig. 4b, a significant larger enhancement of tip emission was exhibited for cancer samples in comparison with the serum from healthy people, although a slightly less value of fluorescence strength increment was shown by the serum of lung cancer than the other two (breast and pancreatic cancer). Albeit hetero-CHA probe has successfully distinguish cancer patients from healthy ones, there is still a long way to apply it in clinical diagnosis of cancer since the difference in expression levels of miRNA markers between individuals. Future work will focus on optimizing DNA circuits to improve the sensitivity, specificity and robustness in complex biological environment. It is anticipated that the optimized PDA microtube sensor could be integrated into optical chip to distinguish the abnormal expression of miRNA in serum, favoring the early-phase cancer diagnosis and disease prevention.

4. Conclusions

We have proposed a hetero-CHA amplification strategy integrating with PDA microtube waveguide system, which was exhibited to substantially improve the efficiency of target catalyzed hairpin assembly reaction and the ability of suppressing the uncatalyzed hybridization in comparison with the CHA probe in homogenous solution. Through utilizing the condensing enrichment effect, our hetero-CHA probe achieved a high sensitivity to target, with a LOD as low as 3.3 fM for miRNA-21 detection. The proposed hetero-CHA probe exhibited excellent performance in analyzing miRNA in human serum at an ultralow concentration without any treatment like isothermal enzymatic amplifications. However, this hetero-CHA probe could only detect specific RNA sequences, limiting its widely application (such as in the field of analyzing single base mutation or DNA methylation). It is anticipated that the structure of the composite microtube waveguide could be further optimized, which might be further integrated into the chip for early medical point-of-care applications.

CRediT authorship contribution statement

Chenlu He: Project administration. **Mengqiao Wang:** Project administration. **Xianbao Sun:** Project administration. **Yu Zhu:** Project administration. **Xiang Zhou:** Project administration. **Shiyan Xiao:** Project administration. **Qijin Zhang:** Project administration. **Funing Liu:** Project administration. **Yue Yu:** Project administration. **Haojun Liang:** Project administration. **Gang Zou:** Project administration.

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Credit author statement

G. Z. suggested the experiment. C. H., and M. W. performed the probe design with heterogeneous CHA amplification strategy. M. W. performed the miRNA-21 detection experiments. The experimental data were collected and analyzed by C. H., M. W., and G. Z. All authors discussed the results and contributed to writing and editing of the manuscript.

Declaration of interest statement

There are no interests to declare.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.bios.2019.01.003.

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