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ARTICLE TYPE

Label-free technology for the amplified detection of microRNA based on the allosteric hairpin DNA switch and hybridization chain reaction

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By using the allosteric hairpin DNA switch, a novel assay for the detection of microRNA (miRNA) let-7a via hybridization chain reaction (HCR) was introduced. Briefly, the hairpin DNA switch probe is a single-stranded DNA consisting of a streptavidin (SA) aptamer sequence, a target binding sequence and a certain sequence that acts as a trigger of HCR. In the presence of target let-7a, the hairpin DNA switch would open and expose the stem region sequences, where a part of this sequence acts as initiator sequence strands for HCR and triggers a cascade of hybridization events that yields nicked double helices analogous to alternating copolymers, another part is the SA aptamer sequence which activates its binding affinity to SA on SA-coated magnetic particles. The hybridization event could be sensitively detected via an instantaneous derivatization reaction between a special chemiluminescence (CL) reagent, 3, 4, 5-trimethoxylphenylglyoxal (TMPG) and the guanine nucleotides within the target, hairpin DNA switch probe, and HCR helices to form an unstable CL intermediate for the generation of light. Our results show that the coupling of hairpin DNA switch probe and HCR for the amplification detection of let-7a achieves a better performance (e.g. wide linear response range: 0.1~1000 fmol, low detection limit: 0.1 fmol, and high specificity). Furthermore, this approach could be easily applied to the detection of let-7a in human lung cells, and extended to detect other types of miRNA and protein such as PDGF based on aptamer. We believe such advancements will represent a significant step towards improved diagnostics and more personalized medical treatment.

Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs (19–25 nucleotides) that are powerful transcriptional and post-transcriptional regulators of gene expression.[1] Evidence accumulated from many studies has uncovered that distinct patterns of that miRNAs play important roles in a wide range of biological processes and a number of diseases, including cancer, neurodegenerative disorders, and diabetes, and represent promising biomarker candidates for informative diagnostics.[2-4] Despite miRNAs has shown great potential in basic research as well as for clinical applications, such as diagnosis and the evaluation of drug efficiency, the development of sensitive analytical techniques for the quantitation of miRNAs has lagged behind. The sensitivity and specificity of common methods are not satisfactory because of the small size, sequence similarity, and low abundance of miRNAs. Therefore, a simple, low-cost, and highly sensitive and selective method for miRNA detection is desirable.

Molecular beacon (MB) , which is composed of a hairpin-like DNA stem-loop structure, was first proposed by Tyagi and Kramer in 1996.[5] Afterwards, MB was widely used in the recognition analysis, such as the detection of DNA and RNA, the study of the interaction between protein and DNA, the establishment of biosensors, and the fabrication of biochips. Most applications of the MB were based on the fluorescence enhancement caused by its hybridization with single stranded target DNA (or RNA). Traditional MB, however, has an intrinsic limitation on the sensitivity, as one target molecule converts only one beacon molecule to its signal form. Thus signal amplification is important for the MB detection of biological targets. Various amplification strategies for miRNA analysis have been reported to improve the sensitivity, such as the polymerase chain reaction (PCR),[6-8] enzyme amplification,[9-11] nanoparticle amplification methods,[12, 13] and rolling circle amplification (RCA). [14-16] An approach for amplifying short sequences of oligonucleotides called hybridization chain reaction (HCR) was recently reported and allowed for selective and specific extension at room temperature without enzymes.[17, 18] In HCR, two stable species of DNA hairpins coexist in solution until an initiator strand is introduced. The initiator triggers a cascade of hybridization events to yield nicked double helices analogous to alternating copolymers.

Herein, we report a novel technique called allosteric hairpin DNA switch-HCR detection to improve the sensitivity of the detection for miRNA let-7a. Our hairpin DNA switch probe is a single-stranded DNA consisting of a streptavidin (SA) aptamer sequence, a target binding sequence and a certain sequence. The introduction of target miRNA leads to open the hairpin DNA switch probe, and thus activates the SA aptamer sequence for the binding affinity to streptavidin (SA) and simultaneously triggers a

cascade of hybridization events that yields nicked double helices analogous to alternating copolymers. In the absence of target miRNA, the hairpin DNA switch is closed and thus the SA aptamer sequence is blocked, leading to the extra-low blank signal. The let 7 family of miRNAs is chosen as the model miRNA, for it is one of the most extensive miRNA, expression level of let 7 is low in many types of cancers, such as in lung, stomach and colon.[19, 20] This label-free technique uses a special chemiluminescence (CL) reagent, 3,4,5-trimethoxylphenyl-glyoxal (TMPG) which acts as the signaling molecule and an instantaneous derivatization reaction between TMPG and the guanine (G) nucleotides, rather than a labeled probe, for the amplified detection of miRNA let-7a, at a considerably reduced detection cost. The fast and isothermal hairpin DNA switch-HCR resulted in a simple and rapid assay procedure. Allosteric hairpin DNA switch could sensitively detect let-7a in human lung cells. Moreover, the approach presented herein also demonstrates remarkable generality for other types of targets including protein through aptamer.

Experimental

Materials.

All chemicals were of analytical reagent grade and were used as received. DEPC treated deionized water which was purified using a Millipore Milli-XQ system (Bedford, MA) was used in all experiments. The streptavidin-coated magnetic particles (SA-MP, 1 μm, 5 mg/mL) were purchased from Polysciences, Inc. TMPG was synthesized as described previously.[21] Oligonucleotides were acquired from Invitrogen Biotechnology Co., Ltd (Shanghai, China, Table S1). A miRNeasy Serum/Plasma Kit was purchased from QIAGEN (China). All the tubes and tips were soaked with DEPC overnight and then treated at 121 °C and 0.2 MPa for 20 min.

Apparatus.

CL detection was carried out with a BPCL CL analyzer (Beijing, China) and absorbance was determined by a Hitachi U-2900 Spectrophotometer. The high-pressure steam sterilizer was TOMY/SX-500 (Tokyo, Japan).

Assay procedures on SA-MPs.

In a typical experiment, C1 and C2 were firstly heated to 95 °C for 2 min and then allowed to cool to room temperature before use. 20 pmol C1 and C2 probes were mixed together with 10 pmol hairpin DNA in 50 μL in buffer A (20 mM Tri-HCl, pH 8.0, 0.5 M NaCl, 20mM $MgCl_2$). Different amounts of target let-7a or mismatched RNA in 50 μL in buffer A were added and the hybridization reaction was allowed to proceed for 1 h at 37°C. Secondly, 4 μL SA-MP was added and the mixture was incubated for 1 h at 37 °C. The resultant SA-MP-RNA conjugates were washed three times with 150 μL wash buffer (7 mM Tris-HCl, pH 8.0, 0.17 M NaCl, 0.05% Tween 20) through magnetic separation, and then it was transferred into 14 x 40 mm glass tubes with 90 μL of TMPG (30 mM in DMF). 9 μL of tetrabutylammonium hydroxide-phosphate buffer (pH 8.5) was added into the tube and the tube was placed in the luminescence reader. The CL signal was then integrated for 2 s.

Cell Lysis and RNA preparation.

The human lung cells (A549) were collected and centrifuged at 3000 rpm for 5 min in culture medium, washed once with PBS buffer, then spun down at 3000 rpm for 5 min. Total RNA was extracted from human lung cells using miRNeasy Kit according to the manufacturer's procedures. The RNA concentration was determined to be 1.43 ng/mL from the UV vis absorption at 260 nm. The sample of let 7a in these cells was diluted, and then analyzed with the proposed miRNA assays.

Total RNA extraction from human serum.

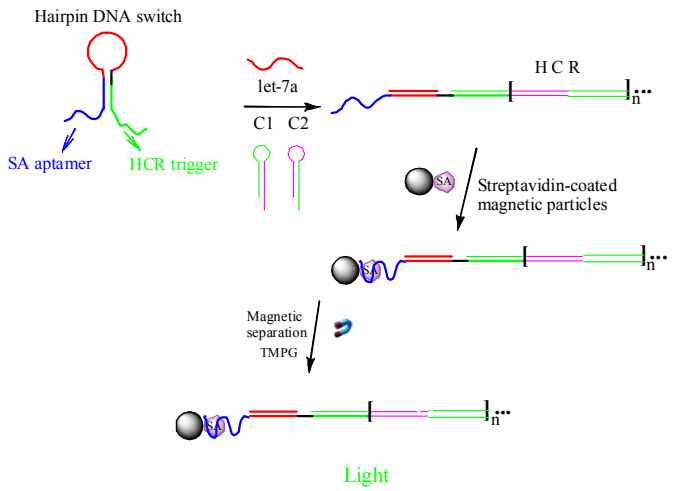
400 μL of freshly collected serum sample from each healthy donor were processed by the QIAGEN miRNeasy Serum/Plasma Kit for total RNA extraction. The extracted RNA was eluted into approximately 40 μL of H_2O.

Serum let-7a spike/recovery evaluation

Spike/recovery was evaluated by combining 10 μL of serum extracts and 40 μL of buffer A containing 1, 10 or 100 fmol of let-7a spiked in serum extracts. The spiked extracts were measured by the proposed miRNA assays described above.

Results and Discussion

This hairpin DNA switch-HCR-based miRNA technique demonstrated a label-free amplified detection in response to target miRNA (Scheme 1). In the absence of let-7a, only minimal CL signal was observed, evidencing that the stability of hairpin DNA switch probes were extremely high and the SA aptamer sequence was completely blocked. After reaction with 100 fmol target miRNA let-7a, a strong CL emission appeared with reference to that for the blank. To confirm the polymerizing hairpins, native gel electrophoresis was performed on revealing a distribution of polymer lengths. Figure 1 demonstrated that the hairpin DNA switch was opened by target let-7a both in the presence (line 1) and absence (line 2) of C1 and C2. After the HCR amplification, the majority of hairpin DNA turned into polymerization (lines 1 and 2). Note also that the C1 and C2 polymerized only in the presence of initiator hairpin DNA switch and let-7a (lines 1 and 3).



Scheme 1. Schematic representation of label-free amplified detection for miRNA let-7a based on the hairpin DNA switch and HCR.

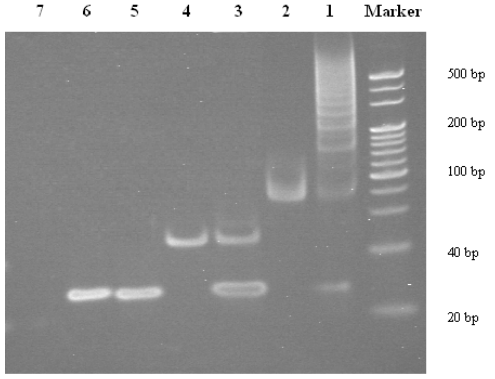


Figure 1. Native gel electrophoresis of the hairpin DNA switch-HCR. (1) 20 pmol let-7a, 10 pmol hairpin DNA switch probe, 30 pmol C1 and 30 pmol C2 in 10 μL reaction buffer; (2) 10 pmol hairpin DNA switch probe, 20 pmol let-7a in 10 μL reaction buffer; (3) 10 pmol hairpin DNA switch probe, 30 pmol C1 and 30 pmol C2 in 10 μL reaction buffer; (4) 10 pmol hairpin DNA switch probe in 10 μL reaction buffer; (5) 30 pmol C1 in 10 μL reaction buffer; (6) 30 pmol C2 in 10 μL reaction buffer; (7) 20 pmol let-7a in 10 μL reaction buffer.

This detection contains only two steps: firstly, hairpin DNA switch probe, let-7a, C1 and C2 were mixed and incubated for 1 h at 37 ^{o}C. Secondly, SA-MP was added for another 1 h with continuous shaking to capture the hairpin DNA switch probe opened by the target let-7a and then TMPG was injected and CL signal was achieved for the let-7a quantitation. To further simplify the detection procedures, we also tried to carry out the above-described assay in one step, i.e. hairpin DNA switch probe, let-7a, C1, C2 and SA-MP were mixed and incubated for 1 h at 37 ^{o}C in one step, and then TMPG was injected for CL detection. As shown in Figure 2, CL intensity for the one-step assay was decreased obviously and thus its detection limit was 10 times poorer than that of the two-step one. The main reason for this phenomenon attributes to the immobilization of hairpin DNA switch probe opened by let-7a on the surface of SA-MP prior to the initiation of HCR. This may indicate that the immobilized hairpin DNA switch probe is not so effective to initiate HCR, weakening the effect of the HCR amplification and thus leading to a lower CL signal and sensitivity. Furthermore, we carried out those non-HCR experiments in parallel (yellow curve, Figure 2). Note that, although the HCR amplification efficiency for the one-step assay is lower than that for the two-step assay, it is still much better than the non-HCR method. This further confirmed that the HCR amplification indeed took place even for the one-step assay, although it is obviously poorer than that for the two-step assay. Therefore, we decided to employ the above-described two-step assay for subsequent work.

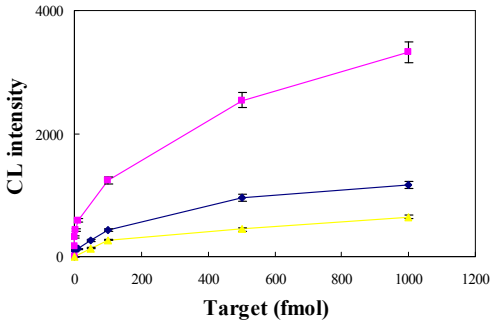


Figure 2. CL intensity vs different detection schemes, the non-HCR method (yellow), the one-step assay (blue) and two-step assay (pink). Experimental conditions: SA-MP was 20 μg, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, 20 mM Mg^{2+} ions were in BA, respectively. The detection procedure was carried out as described in the Experimental section.

Note that the location of HCR trigger on the hairpin DNA switch probe needs to be redesigned to improve the sensitivity and selectivity of the let-7a. It is well known that the stability of the nucleic acid hybridization events is controlled by two essential parameters: base pairing between complementary strands and stacking between adjacent bases. Base stacking proved to be the main stabilizing factor in the DNA double helix.[22, 23] As described above, each target let-7a can open one hairpin DNA switch probe to trigger a chain reaction of hybridization events between C1 and C2 to form a long nicked DNA polymer, with a high CL signal and sensitivity. When a gap formed between target let-7a and C1 on DNA/RNA helixes, possibly resulting in a marked decrease in the HCR polymer length, thus the CL intensity would decrease. At the same time, the steric hindrance effect caused by SA-MP is also a key issue to be considered, since a gap could reduce steric hindrance and promote the resultant hairpin DNA switch-let-7a-HCR helixes to bind with SA-MP. To balance the base stacking and steric hindrance, three kinds of hairpin DNA switch probes were designed with a different base gap. As shown in Figure 3, CL intensity and CL ratio of Model B with 5-base gaps are significantly better than Model A with no gap and Model C with a 13-base gaps. Notes also that background signal gradually decreased when the gap was lengthen from 0 bases to 13 bases. In Model A, the steric hindrance plays a major role as compared to base stacking, and its CL intensity and ratio are thus lower than Model B. With a 5-base gap, both CL intensity and CL ratio was greatly increased. However, further lengthening the gap from 5 bases to 13 bases, both CL intensity and CL ratio decreased. Thus Model B was employed in the following study.

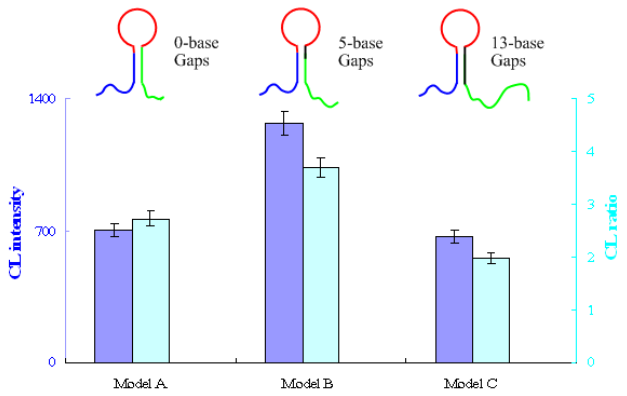


Figure 3. CL intensity and CL ratio vs Model A, B and C. Experimental conditions: SA-MP was 20 μg, let-7a was 100 fmol, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, 20 mM Mg^{2+} ions were in BA, respectively. The detection procedure was carried out as described in the Experimental section.

It is generally recognized that both the addition of $MgCl_2$ in the reaction buffer and the composition of the buffer have great impact on the binding property of the aptamer.[24-26] Divalent cations such as Mg^{2+} ions thus typically play more than just a backbone charge compensation role and are important in the stabilization of the aptamer complex's stereo-structure. In addition, Mg^{2+} ions also affect DNA duplex stability and thus DNA hybridization kinetics,[27] therefore, Mg^{2+} could help the hairpin DNA switch to keep closed in the absence of let-7a and thus reduce the blank signal. However, too much Mg^{2+} would make the binding ability of SA aptamer strong enough to unfold the hairpin DNA switch probe even without target let-7a and reduces its net CL intensity. Thus, the concentration of Mg^{2+} ions should be optimized to balance its influence on aptamer and DNA duplex and give the highest CL intensity. Hence, the effect of the reaction buffer was monitored by varying the concentration of Mg^{2+} ions (Figure 4). CL intensity in the presence of $MgCl_2$ was obviously higher than that in the absence of $MgCl_2$. CL intensity increased with the increase of Mg^{2+} ions at the beginning, and however, too high Mg^{2+} ions significantly reduced CL intensity.

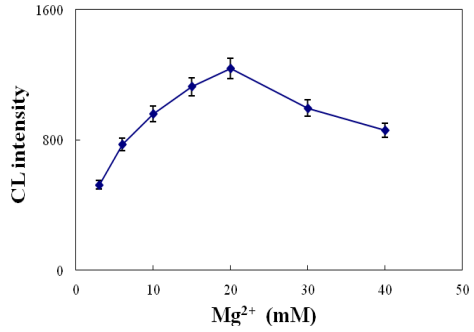


Figure 4. CL intensity vs concentration of Mg^{2+} ions. Experimental conditions: let-7a was 100 fmol, SA-MPs were 20 μg, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, respectively. The detection procedure was carried out as described in the Experimental section.

Optimization of reaction parameters.

Several parameters were investigated systematically to establish optimal conditions for the CL detection of the miRNA let-7a, including the amounts of SA-MPs, hairpin DNA switch probe, C1 and C2.

As shown in Figure S1, with the increase of the amount of SA-MP, CL intensity increased and reached a maximum at 20 μg and then decreased, possibly due to the CL quenching by an inner filter effect of an excess of black SA-MP. Hence, subsequent work employed 20 μg of SA-MP. The effects of different amounts of hairpin DNA switch probe on CL intensity were shown in Figure S2. CL intensity was observed to increase over the range of 2-20 pmol of hairpin DNA switch probes and then decreased gradually, possibly due to that the binding sites on the SA-MP were progressively saturated and excess hairpin DNA switch probes brought a much higher blank. Thus, 20 pmol of hairpin DNA switch probe was selected for subsequent experiments. The effects of the amounts of C1 and C2 were subsequently examined and optimized. CL signal intensity increased with increasing amount of C1 sequence over the range of 10 to 20 pmol, and then slowly decreased in the range of 20-40 pmol (Figure S3). The CL intensity increased in the range of 5 to 15 pmol of C2 and then slowly decreased (Figure S4). Thus, 20 pmol of C1 and C2 sequence were selected for the use in further studies.

Quantification of target let-7a.

Under the optimized experimental conditions, the quantitative behavior of the method was assessed by monitoring the dependence of the CL intensity upon the concentration of the target let-7a. A calibration graph in the amount range of 0.1-1000 fmol showed an approximately linear correlation (R^2 = 0.9882, Figure 5) between the amount of target miRNA and the CL intensity (represented by LgI= 0.3292LgC + 2.4829, where I is the CL intensity and C is the amount of target miRNA). In addition, at amounts higher than 1000 fmol, the CL intensity leveled off and deviated from the calibration curve (Figure 5). A readily achieved detection limit was found to be 0.1 fmol, which is equal or better than most previous methods for miRNA detection (Table S2).

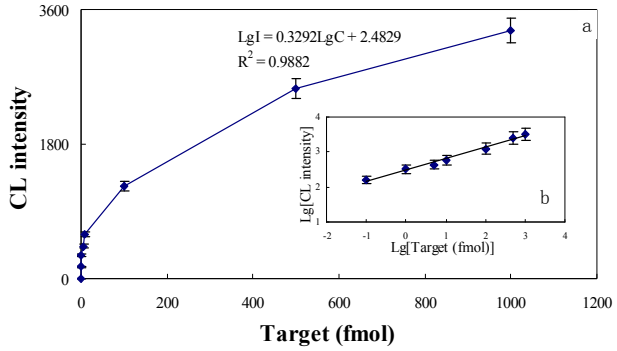


Figure 5. Calibration plot (a) and Log-Log calibration data (b) for the target miRNA let-7a. Experimental conditions: SA-MPs were 20 μg, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, 20 mM Mg^{2+} ions were in BA, respectively. The detection procedure was carried out as described in the Experimental section.

Meanwhile, we also carried out the one-step assay and non-

HCR assay in parallel. As shown in Figure 6, the one-step assay shown a linear correlation (R^2= 0.9903, Figure 6) in the concentration of 5–1000 fmol between the amount of target RNA and the CL intensity (represented by lgI = 0.4707lgC + 1.6728, where I is the CL intensity and C is the amount of target miRNA). The detection limit was estimated to be 5 fmol (3s, n = 5), which is 50 times poorer than that of two-steps assay. We assumed that this was associated with assembly of hairpin DNA switch probe and C1 and C2. In two-steps, all of the DNA and RNAs' reaction was in homogeneous phase, and its hybridization kinetics is much more efficient. Without HCR amplification, the LOD of this assay was 50 fmol (3s, n = 5) under its optimal conditions (Figure S5~S6). Thus, the two-steps were adopted in this assay.

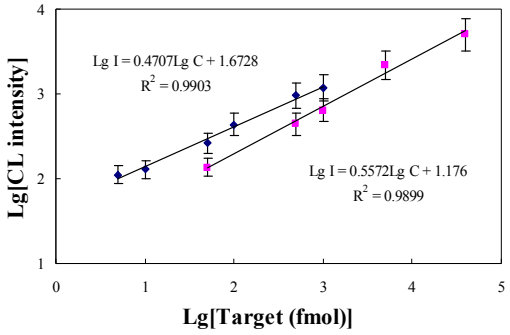


Figure 6. Log-Log calibration data for the target miRNA let-7a. Experimental conditions (◆ one-step assay): SA-MPs were 20 μg, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, respectively. Experimental conditions (■ assay without amplification) : SA-MPs were 25 μg, hairpin DNA switch probe was 10 pmol, respectively. The detection procedure was carried out as described in the Experimental section.

Discrimination of complementary RNA sequences from mismatched sequences.

To evaluate the specificity of the proposed miRNA assay, members of the let 7 family (let-7a~g and i) were selected as a model system because of their high sequence homology (Table S1). The proposed assay was examined by detecting the CL response of perfectly complementary targets let-7a and other let 7 family miRNAs, all at an amount of 100 fmol (Figure 7). Our miRNA assay effectively discriminated between eight closely related sequences from the let 7 family of miRNAs. The ratio of the CL intensities of the eight sequences was 100:7.2:28: 8.8:1:6.1:22:6.8, respectively. The highest cross-hybridization rate was only 28% (let-7c), which is comparative to the ones previously reported let 7 family miRNA assays. In conclusion, good selectivity can be obtained using this new CL miRNA biosensor.

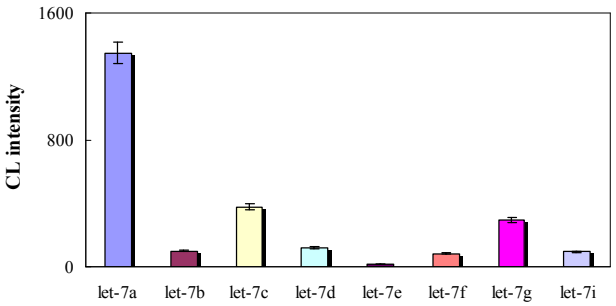


Figure 7. CL intensity vs let-7a / mismatch miRNA. Experimental conditions: SA-MPs were 20 μg, hairpin DNA switch probe, C1 and C2 sequences were 10, 20 and 20 pmol, 20 mM Mg^{2+} ions were in BA. Let-7a and other miRNA were 100 fmol, respectively. The detection procedure was carried out as described in the Experimental section.

Real sample assay.

The proposed assay was further applied to quantify the amount of let-7a miRNA in total RNA sample that was extracted from human lung cells by using the calibration method. It was calculated that the content of let-7a in human lung cell total RNA (1.43 μg/μL) was 11.9 nM. The content of let 7a in human lung total RNA sample was 5.0×10^9 copies/μg, which were in good agreement with those obtained in previous studies.[9, 28, 29,30]

Furthermore, a spike/recovery experiment was tested by adding known amount of let-7a to RNA extract from human serum. The QIAGEN RNA extraction kit is designed to purify total RNA, including miRNA and other small RNAs. The endogenous level of let-7a in human serum samples was lower than our detection limit. For spike/recovery evaluation, the synthetic let-7a was spiked at 1, 10 and 100 fmol. The recovery the recoveries of 100, 10, and 1 fmol miRNA let-7a were 76.7±3.89 %, 85.6±21.45 % and 89.6±12.65 %, respectively.

Conclusions

In conclusion, we developed a hairpin DNA switch-HCR based sensing platform for simple detection of miRNA, based on the hybridization chain reaction. The method required the opening of the hairpin DNA switch probe by target miRNA to activate its binding affinity to SA-MPs and simultaneously triggers a cascade of hybridization events that yields nicked double helices analogous to alternating copolymers. Moreover, the sensitivity of detection was greatly improved through designing the hairpin DNA switch probe which contained a sequence as a trigger of HCR which amplified the signal. This simple and rapid detection for miRNA let-7a does not require any modified DNA probes and had only two steps. Importantly, this protocol was successfully demonstrated for real sample assay in total RNA sample extracted from human lung cells. However, some challenges also remain. Release of the HCR trigger sequences resulted from any nonspecific reaction (temperature, pH, samples matrix, nonspecific hybridization, and nonspecific protein binding and so on) may cause false positive detection signals. Despite these challenges and limitations, the label-free hairpin DNA switch-HCR assay, as a new CL strategy, might create a universal technology for developing simple biosensors in sensitive and selective detection of miRNA.

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Notes and references

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† Electronic Supplementary Information (ESI) available: DNA sequences, experimental detailss.. See DOI: 10.1039/b000000x/

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