Catalytic Hairpin Assembly Actuated DNA Nanotweezer for Logic Gate Building and Sensitive Enzyme-Free Biosensing of MicroRNAs
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Supporting Information

ABSTRACT: A target-switched DNA nanotweezer is designed for AND logic gate operation and enzyme-free detection of micro-RNAs (miRNAs) by catalytic hairpin assembly (CHA) and proximity-dependent DNAzyme formation. The double crossover motif-based nanotweezer consists of an arched structure as the set strand for target inputs and two split G-rich DNAs at the termini of two arms for signal output. Upon a CHA, a small amount of binary target inputs can switch numerous open nanotweezers to a closed state, which leads to the formation of proximity-dependent DNAzyme in the presence of hemin to produce a highly sensitive biosensing system. The binary target inputs can be used for successful building of AND logic gate, which is validated by polyacrylamide gel electrophoresis, surface plasmon resonance and the biosensing signal. The developed biosensing system shows a linear response of the output chemiluminescence signal to input binary miRNAs with a detection limit of 30 fM. It can be used for miRNAs analysis in complex sample matrix. This system provides a simple and reusable platform for logic gate operation and enzyme-free, highly sensitive, and specific multianalysis of miRNAs.

Recently, a variety of complex static or dynamic DNA-based architecture systems,1−5 such as tweezers,6−8 gears,9 walkers,10−14 metronome operations,15 logic gates, and circuits,16−18 have been successfully constructed to perform mechanical operations. Activated by diverse external stimuli, including pH, metal ions, or fuel DNA strands, some DNA nanodevices have been suggested for various potential applications in sensing fields,20,21 nanotransporing,22 logic gate operations,23−27 and nanomedicine.28,29 Among these nanodevices, DNA tweezers have gained much attention for applications in cascade reaction and interaction research5,30 since the first report in 2000.31 The molecular tweezer can be regulated by target for fluorescent detection.31 To improve the rigidity and variability of the tweezers, a DNA tweezer based on two DNA double crossover (DX) motifs has recently been constructed for protein holding and releasing.32 By introducing a hairpin loop motif at the internal connecting position, the spatial distance between its two arms can be better modulated, which provides a sensitive switch for biosensing. Thus, this work designed an arched structure as both the set strand to hybridize the regulatory oligomer for opening the DNA tweezer and the recognition probe to capture the binary targets for closing the tweezer (Figure S1 in SI). The designed DX motif-based tweezer could be used for both sensitive detection of binary targets, using microRNAs (miRNAs) as multianalytes, and AND logic gate operation.

MiRNAs are an emerging class of short noncoding RNAs that play significant roles in regulating gene expression. They are involved in various cellular processes, including differentiation, proliferation, apoptosis and stress response,33 and the aberrant expression of miRNAs is closely associated with the pathogenesis of human diseases.34−36 Thus, miRNAs are potentially useful biomarkers for early diagnosis of human diseases. However, the small size, low abundance, and sequence homology make miRNAs profiling a challenging task.37,38 Although some well-established methods, including real-time quantitative PCR (RT-qPCR), microarray, and Northern blot have been developed for profiling the expression of miRNAs, they still suffer from some shortcomings in practical

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applications. For instance, RT-qPCR, a gold standard for gene expression analysis, is an enzyme-required technique and restricted by the complicated design of PCR primers.39 Current microarray technologies necessitate a high amount of starting material and display only a limited dynamic range for quantification.42 Northern blot is semiquantitative, laborious and time-consuming.43,44 In recent years, some improved biosensing techniques have been developed for sensitive detection of miRNAs by using various amplification strategies, including DNA isothermal amplification and nanoparticles-based amplification. However, the development of simple, specific, sensitive, and convenient methods for multianalysis of miRNAs is still urgently needed.

Inspired by the advantages of DX motif-based tweezers, this work integrated a catalytic hairpin assembly (CHA) with DNA nanotweezer to design an amplification strategy for enzyme-free and highly sensitive miRNAs biosensing via proximity-dependent DNAzyme formation (Scheme 1A). In the DX motif-based nanotweezer, the arched set strand could be dismantled from the regulatory oligomer by binary target inputs, which led to a closed state of the nanotweezer and the formation of proximity-dependent DNAzyme (Scheme 1B). In all experiments, nanotweezers at a final concentration of 250 nM were used for the absorbance measurements of ABTS$^\ddagger$ and the CL readout of luminol. Before the formation of DNAzyme, a mixture containing 250 nM opened nanotweezer, 250 nM assistant strand, and different concentrations of target inputs was incubated at 25 °C for 1 h. Afterward, 600 nM hemin was added and incubated at RT for another 30 min to form DNAzyme. Absorbance measurements were conducted in a cuvette that included 150 μL of the DNAzyme solution and 150 μL of the DNAzyme substrate solution that included 4 mM ABTS$^\ddagger$ and 0.14 mM H$_2$O$_2$. The absorbance of the mixture was then measured using a UV−vis absorption spectrophotometer in the wavelength range from 500 to 400 nm against a blank. CL assays were carried out in a cuvette that included 20 μL of the DNAzyme solution and 4 μL of CL substrates. Soon after the addition of the CL substrates, the light emission intensity was measured immediately.

Scheme 1. (A) Designed Strategy for Target Detection and Logic Gate Operation;46 (B) Truth Table of “AND” Logic Gate

![Scheme 1](image-url)

“Arrows drawn on DNA strands represent 3’ end and numbers represent base numbers.

### EXPERIMENTAL SECTION

#### Materials and Reagents

Target miRNAs were obtained from TaKaRa Biotech. Inc. (Dalian, China). DNA oligonucleotides were synthesized and purified by Sangon Inc. (Shanghai, China). All oligonucleotides were dissolved in tris-ethylendiaminetetraacetic acid buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA) and stored at −20 °C, which were diluted in appropriate buffer prior to use. Hemin and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^\ddagger$) were obtained from Sigma-Aldrich (St Louis, MO, U.S.A.). Chemiluminescence (CL) substrate was purchased from Advansta (California, U.S.A.). All other reagents were of analytical grade, and Millipore-Q water (≥18 MΩ) was used in all experiments.

**Apparatus.** A UV−visible spectrophotometer (UV-2550, Shimadzu, Japan) was used to record the absorbance spectra. The CL signal was collected with IFFM-E luminescent analyzer (Remax, China). Biacore XTM analytical system (Biacore AB, Sweden) were used in SPR measurement. A Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, U.S.A.) was used to record the FL signal. The gel electrophoresis was performed on DYY-6C electrophoresis analyzer (Liuyi Instrument Company, China) with 6% native polyacrylamide gel in 1X TBE buffer (89 mM Tris−boric acid, 2 mM EDTA, pH 8.3) at 110 V for 40 min at 4 °C and imaged on a Biorad ChemDoc XRS (Bio-Rad, U.S.A.).

**Preparation of DNA Nanotweezer.** DNA nanotweezer was prepared with 0.5 μM of DNA strands 1−9 and set strand in 1X TAE/Mg$^{2+}$ buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA and 7.5 mM magnesium acetate, 20 mM potassium acetate, pH 8.0). All samples were annealed in a T100TM Thermal Cycler (Bio-Rad, U.S.A.). The temperature steps in the annealing protocol were described in Table S2.

**SPR Experiment.** Thiolated DNA probe was first immobilized on gold sensor chip via Au−S binding, which was blocked with MCH to decrease nonspecific adsorption. The chip surface was preconditioned with running buffer (30 mM sodium phosphate containing 450 mM NaCl, 3 mM EDTA, and 0.25% Triton x100 (pH 7.4)) to establish a stable baseline. CHA product was injected over the chip surface with a flow rate of 5 μL min$^{-1}$ at 25 °C. At the end of each cycle, the chip surface was regenerated with 50 mM NaOH for next detection. The sensorgrams were analyzed using BIA 4.1 software (Biacore).

**Detection Protocol.** In all experiments, nanotweezers at a final concentration of 250 nM were used for the absorbance measurements of ABTS$^\ddagger$ and the CL readout of luminol. The chip surface was preconditioned with running buffer (30 mM sodium phosphate containing 450 mM NaCl, 3 mM EDTA, and 6.5% Triton X100 (pH 7.4)) to establish a stable baseline. CHA product was injected over the chip surface with a flow rate of 5 μL min$^{-1}$ at 25 °C. At the end of each cycle, the chip surface was regenerated with 50 mM NaOH for next detection.
RESULTS AND DISCUSSION

Design of DNA Nanotweezer. The DNA nanotweezer was constructed by self-assembly of oligonucleotides with the sequences listed in Table S1 in the SI. All these DNA single strands were mixed and then annealed according to the program given in Table S2 in the SI to form an opened DNA nanotweezer with two ~14 nm arms. At the end of two arms, two split G-rich strands were linked at the 5′- and 3′-end of oligomer 8 and oligomer 9, respectively. In the middle of the designed DNA nanotweezer, a 32-nt single-stranded DNA (ssDNA in red) in oligomer 7 acted as a structural regulatory element to control the state of the nanotweezer. Here, the regulatory oligomer was 7-nt longer than that in a previous report for increasing the spatial distance between two arms, which could in turn increase the signal-to-noise ratio. In the initial opened state, a rigid double helix structure was formed by the hybridization of the regulatory oligomer with a complementary set strand to separate the split G-rich strands, which blocked the formation of complete G-quadruplex. A 6-nt toehold at 3′ end of the set strand enabled it to hybridize with 5′ end of miRNA-21 to efficiently release half of the set strand, and the released arced sequence then acted as another toehold to hybridize with 5′ end of miRNA-155 to release the whole set strand from the DNA nanotweezer. The structure of inputs: set strand contained a 5-nt toehold at 5′ end of miRNA-21 to each other due to the spatial blocking of bulge loop to 5′ end of the set strand, thus this could hybridize with 3′ end of assistant strand to actuate the CHA process, which led to the formation of numerous closed nanotweezers due to the presence of a "GGGT" stem-loop hairpin structure in regulatory oligomer 7 (Figure S2 in the SI). The designed system could be stable at pH 8.0, which was in agreement with the previous reports, in which the DNA tweezers were stable at pH 5.2 to 8.0.

CHA-Actuated Formation of Proximity-Dependent DNAzyme. In the absence of target inputs, the set strand and the assistant strand were kinetically impeded from "reacting" each other due to the spatial blocking of bulge loop to 5′ end of the set strand and the binding of two bases with regulatory oligomer. Upon the binary inputs of miRNA-21 and miRNA-155, the released inputs: set strand was attacked by assistant strand via the 5′-toehold to dissociate the double-stranded complex, which released miRNA-21 and miRNA-155 to trigger the CHA, resulting in continuous removal of set strand away from the regulatory oligomer to switch the opened tweezer to closed state. At the closed state, the split G-rich strands at the termini of two arms could bind hemin to form a horseradish peroxidase (HRP) mimic DNAzyme. The formation of proximity-dependent DNAzyme further produced a CL readout by DNAzyme catalyzed luminol-H₂O₂ system. Obviously, the CL signal depended on the simultaneous presence of two targets, thus it could define as an AND logic gate (Scheme 1B).

Characterization of Assembled DNA Nanotweezer. A native polyacrylamide gel electrophoresis (PAGE) experiment was carried out to characterize the assembly of DNA nanotweezer (Figure 1A and Figure S3 in the SI). After the assembly process without presence of set strand, the mixture showed an obvious single band of DNA with more than 500 bases (lane 1), indicating the formation of single DNA nanostructure containing DNA 1–9. The presence of set strand in the mixture produced a single band with more bases (lane 2), indicating that the set strand was further assembled in the DNA nanostructure. The two nanostructures could be attributed to the formation of closed and opened DNA nanotweezers, respectively, which were further verified with UV–vis absorption spectra (Figure 1B). Here the colorless ABTS− could be catalytically oxidized in the presence of H₂O₂ and formed DNAzyme to produce ABTS•+ with a characteristic absorption peak at 418 m. In the presence of set strand, the reaction mixture did not show the formation of ABTS•+, while the absence of set strand led to an obvious absorption of ABTS•+, indicating the formation of DNAzyme in the closed state of the nanotweezer. This result further proved the successful construction of DNA nanotweezer and the formation of proximity-dependent DNAzyme for catalyzed signal readout.

Verification of AND Logic Gate Operation. The binary inputs of miRNA-21 and miRNA-155 (inputs 1 and 2) to the designed nanotweezer could simultaneously switch the output of 1, while single input of miRNA-21 or miRNA-155 produced the output of 0 (Scheme 1B). The AND logic gate operation was characterized using native PAGE (Figure 2) and surface plasmon resonance (SPR; Figure 3). The presence of miRNA-21 led to slightly slower migration of the opened nanotweezer (lanes 1 and 3), indicating the hybridization of set strand with miRNA-21. However, the presence of miRNA-155 did not change migration of the opened nanotweezer (lanes 1 and 4),
thus, miRNA-155 could not hybridize with the loop-stated toehold. 0.5-fold amount of binary target inputs resulted in four bands (lane 5), which corresponded to miRNA-21 bound nanotweezer, opened nanotweezer, closed nanotweezer, and formed inputs:set strand (lane 7). In the presence of assistant strand, two bands in lane 5 disappeared, and the bands of closed nanotweezer and inputs:set strand became stronger (lane 6), while the assistant strand alone did not change the opened nanotweezer (lane 9). Here, assistant strand located between 40 and 20 bp showed much weaker bands (lanes 8 and 9) due to lower DNA concentration. These results certificated the specific building of the AND logic gate. The disappearance of opened nanotweezer in the presence of binary inputs and assistant strand demonstrated the CHA process.

The CHA played an essential role in the AND logic gate operation and could be specially characterized with SPR at a thiolated-capture probe modified gold chip (Figure 3A). The capture probe was complementary to the regulatory oligomer. In the presence of inputs miRNA-21 and miRNA-155, the regulatory oligomer was cyclically freed from the complex of regulatory oligomer and set strand by toehold-mediated CHA. Afterward, the regulatory oligomer could specifically hybridize with the capture probe to produce SPR signal. The SPR response corresponding to sole miRNA-21 or miRNA-155 at the concentration of 10 nM and 1 nM was as small as that of blank control, respectively. However, the SPR response significantly increased upon simultaneous addition of miRNA-21 and miRNA-155 (Figure 3B,C), indicating the release of a large number of single-stranded regulatory oligomer. These

Figure 3. (A) Scheme of SPR biosensing. (B) SPR sensorgrams with inputs (1, 1), (1, 0), (0, 1), and (0, 0) at 10 nM (left) and 1 nM (right). (C) SPR characterization of AND logic gate operation for sensing target inputs at 10 and 1 nM.

Figure 4. Optimization of (A) Mg$^{2+}$ concentration for nanotweezer assembly, (B) CHA reaction temperature, (C) poly(T) linker length in strand 9, and (D) hemin concentration (nM). When one parameter changed the others were under their optimal conditions.
results suggested that the CHA actuated DNA nanotweezer could successfully implement the amplified AND logic gate operation. Here the SPR technique was used for the characterization of CHA in the AND logic gate operation, thus the signal leakage did not affect the developed biosensing system.

**Optimization of Experimental Conditions.** To achieve the excellent assay performance, some experiment conditions were optimized with an equal amount of two external inputs at 100 pM. The signal-to-noise ratio was used to evaluate the performance of this system. The Mg\(^{2+}\) concentration used in the assembly of DNA nanotweezer was first optimized to be 7.5 mM (Figure 4A). The signal-to-noise ratio reached the maximum value at a reaction temperature of 25 °C for CHA (Figure 4B), which was used in all subsequent experiments. To achieve efficient formation of proximity-dependent DNAzyme, a single-stranded polythymidine (T) linker was used to attach split G rich DNA to oligomer 9. The 6 T linker with a length of 6 showed the maximum signal-to-noise ratio (Figure 4C), therefore, 6 T was used for preparation of oligomer 9. In addition, under these conditions the concentration of hemin was optimized to be 600 nM (Figure 4D).

**Regulatory Cycling to Actuate Signal Switch.** The CHA actuated signal switch for reusable AND logic gate operation and biosensing application of the nanotweezer was further demonstrated with native PAGE and Förster resonance energy transfer (FRET; Figure 5).\(^{51}\) The reversible conversion of nanotweezer between open state and closed state could be achieved by in turn addition of assistant strand and set strand. After scant amount of targets were incubated with opened nanotweezer, besides the opened nanotweezer, two new bands corresponding to the closed nanotweezer and inputs:set strand were observed (Figure 5A, lane 4). After assistant strand was added, the opened nanotweezer disappeared, and the lane showed only the bands of closed nanotweezer and inputs:set strand (Figure 5A, lane 5), indicating that the CHA could be triggered by inputs and assistant strand. Upon the further addition of set strand, the closed nanotweezer was opened again, leading to the disappearance of its band (Figure 5A, lane 6). The following conversion showed the outputs of 1 and 0 (Figure 5A, lanes 7–10). With the increasing cycle, the intensity of band located near 40 bp increased due to the increased amount of assistant:set complex, indicating a recyclable AND logic gate operation.

After the two arms of the DNA nanotweezer were labeled with Cy3 (donor) and Cy5 (acceptor), respectively, the FRET experiments demonstrated the reusable biosensing of binary targets. The opened nanotweezer did not produce FRET signal due to the large spatial distance between two dyes (Figure 5B). The targets could switch the DNA nanotweezer to closed state, and drive the dyes to proximity close enough for efficient FRET, leading to a fluorescence decrease of Cy3 and increase of Cy5. Furthermore, this process could be recycled by in turn addition of set strand and targets (Figure 5B), proving that the CHA actuated nanotweezer could perform reusable biosensing.

**Multianalysis of MiRNA Targets.** Under the optimal experimental conditions, the analytical performance of the designed biosensing system was investigated by employing DNAzyme-catalyzed CL readout for binary miRNAs detection. As shown in Figure 6A, the CL signal increased with the increasing concentration of binary input triggers, indicating that the signal output was highly dependent on the concentration of target miRNAs. The plot of the response versus the logarithm of binary input concentration showed a good linearity in the range from 100 fM to 10 nM (Figure 6B). The limit of detection (LOD) for binary miRNAs was calculated to be 30 fM at 3σ, which was comparable to those with nanodevices for DNA detection,\(^{14,52}\) and much lower than that of single miRNA detection with duplex-based DNA tweezer.\(^{51}\) The high sensitivity of the designed biosensing system was attributed to high performance of the designed nanotweezer for target-dependent switching, cascade signal amplification of CHA and DNAzyme-catalyzed readout.

The specificity of the proposed biosensing method was evaluated by detecting five different miRNA sequences, including full-complementary target inputs (FC), single-base-mismatched target inputs (SM), double-base-mismatched target inputs (DM), noncomplementary target inputs (NC), and let 7 family miRNAs (let 7a and let 7b). The CL signals for DM, NC, and let 7a and 7b were as small as that of the blank (Figure 7), indicating that the DX-motif-based DNA nanotweezer possessed excellent specificity for the determination of target miRNA-21 and miRNA-155. The high sequence...
specificity could be attributed to the high affinity and unique specificity of the toehold-mediated strand-displacement.

To evaluate the repeatability of the developed biosensing system, the target miRNAs at 100 fM and 1 nM were detected 5 times. The coefficients of variation for both concentrations were less than 5%, showed that this method had an acceptable stability and reproducibility.

The proposed biosensing system was further used for multianalysis of target miRNA-21 and miRNA-155 in complex sample matrix. Using salmon sperm DNA as a model, which was first denatured at 95 °C for 5 min and chilled on ice for 10 min, various amounts of binary target miRNAs were spiked in the matrix and analyzed with this system. In the presence of 200 pg mL⁻¹ salmon sperm DNA, the detection of 0.5, 5.0, 50, and 500 pM targets showed the recoveries of 95% to 105% (Table 1), which indicated acceptable analysis capability of the biosensing system for complex matrix.

### Table 1. Recoveries of the Developed System via Spiking Binary Inputs in Salmon Sperm DNA Samples

<table>
<thead>
<tr>
<th>samples</th>
<th>spiked (pM)</th>
<th>measured (pM)</th>
<th>relative error (%)</th>
<th>recovery (%)</th>
</tr>
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<td>0.50</td>
<td>0.509</td>
<td>1.8</td>
<td>101.8</td>
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<td>4.8</td>
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<td>3.8</td>
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<tr>
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<td>513</td>
<td>2.6</td>
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### CONCLUSIONS

A binary targets switched nanotweezer has been developed for AND logic gate operation and enzyme-free biosensing of multianalytes. With a unique design of arched set strand, the binary miRNAs can act as the inputs to close the nanotweezer, trigger the catalytic hairpin assembly and amplify the conversion of nanotweezer from open to close state for delivering a large amount of output signals. The excellent recyclable capacity of the conversion has been demonstrated. Compared with RT-qPCR, this DNA nanotweezer-based biosensing system can analyze binary miRNAs simultaneously and avoids tedious steps, temperature cycling and housekeeping gene chosen harassment. It exhibits high sensitivity with broad dynamic range, high specificity, acceptable reproducibility, and discrimination capability. Moreover, this system is reusable and could be conveniently extended to other targets (proteins, DNA, and metal ions) by designing the recognition regions of set strand, thus it provides a promising versatile platform for multianalysis of real samples and disease diagnosis.

### ASSOCIATED CONTENT

- Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04844.

Assembly of opened DNA nanotweezer, DNA strands of closed DNA nanotweezer, native PAGE intensity of opened and closed nanotweezers analyzed with ImageJ 1.48 software, table containing sequences used in this experiment, and table for thermal annealing program for assembly of opened DNA nanotweezer (PDF).

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#### Notes

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