Double-signal Mode based on Metal-Organic Frameworks Coupling Cascaded Nucleic Acid Circuits for Accurate and Sensitive Detection of Serum Circulating miRNAs

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Experimental section

Chemicals and materials

HPLC purified miRNAs and RT-PCR kit were obtained from Takara Biotechnology (Dalian, China). All other oligonucleotide sequences were synthesized and purified by HPLC at Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequence of oligonucleotides were showed in **Table S1.** Streptavidin magnetic beads (MBs, Mean diameter 1.5 μm) were purchased from Bangs Laboratories Inc. (Fishers, IN). 2-Amino-benzenedicarboxylic acid (NH_2-BDC, 98%) was purchased from Aladdin (Shanghai, China). N, N-dimethylformamide (DMF, 99.5%) were purchased from Fuyu Fine Chemical Co.,Ltd (Tianjin, China). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, 99%) were supplied by Fengchuan Chemical Reagent Science And Technology Co., Ltd (Tianjin, China). O-phenylenediamine(OPD), glucose oxidase (GOx, 100 U/mg), D-(+)-glucose (Glu, 99.5%), maleimidobenzoic acid N-hydroxy-succinimide ester(MBS, 95%) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 99%) were all obtained from Sangon Biotech Co., Ltd (Shanghai, China). The stock solution of OPD was freshly prepared daily with ethanol and the other solutions were prepared using ultra-pure water. 10 mM glucose were prepared in 0.1 M phosphate buffer solution (PBS, pH 7.4). All other reagents were of analytical reagent grade.

Tabel S1 Sequence of oligonucleotides

Name	Sequence(5'-3')
hairpin 1 (H1)	biotin-5'-TTTTTTTTTTTTTTTATCGAATAGTCTGACTACAACTTCAACACCTCTAAACGCGCCTACCAGTTGTAGTCAGACTATTCGAT-3'
hairpin 2 (H2)	5'-TGCTCTTGATGTTGACCATCCGCGCAATAGCTTATCAGACTGATGTTGAAGTTGTGGAGATTTGCGCGGATGGTCAACATCAGTC -3'
hairpin 3	HS-5'-TTTTTTTTTTGCGCGGATGGTCAACATCAAGAGCACCTATCTCCTGCTC

(H3)	TTGATGTTGACCATCC -3'
hairpin 4 **(H4)**	5'- TGCTCTTGATGTTGACCATCCGCGCAATTCGGATGGTCAACATCAAGAG CAGGAGATTTTTTTTTTT -3'-SH
miRNA-21	5'-UAG CUU AUC AGA CUG AUG UUG A-3'
miRNA-122	5'-UGG AGU GUG ACA AUG GUG UUU G-3'
let-7a	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

Apparatus

The fluorescence spectra were measured on a Hitachi F-7000 spectrofluorometer (Hitachi, Japan). The excitation wavelength was 410 nm, and the spectra are recorded between 435 and 650 nm. The excitation and emission slit widths were 5 nm and 10 nm, respectively. PMT detection voltage was 700 V. The images of gel electrophoresis were scanned by the Gel Image Analysis System (Bio-Rad, USA). The water (18.2 MΩ cm) used throughout the experiments was pre-treated with Milli-Q (Millipore, Inc., USA). Scanning electron microscopy (SEM) was carried out with a Hitachi S-4800 spectrometer. The phase identification was accomplished by powder X-ray diffraction (XRD) employing a Philips X'-pert X-ray diffractometer with Cu Kα radiation (λ =1.5418 Å). Fourier transform infrared (FT-IR) spectra were acquired with a Nicolet-5700 infrared spectrometer (Shanghai, China).

Preparation of NH_2-MIL-101 MOFs

MOFs were prepared based on previous reported work with some minor modifications. Briefly, $FeCl_3 \cdot 6H_2O$, NH_2-BDC, and DMF with a molar ratio of 2:1:156 were mixed at 110 °C for 24 h, and synthesized NH_2-MIL-101 by hydrothermal treatment of the mixture. The synthesized NH_2-MIL-101 MOFs were then collected by centrifugation, and washed with DMF and ethanol several times.

Finally, the MOFs were dried thoroughly at 60 °C.

Conjugation of GOx to DNA.

For GOx conjugation, to 20 μL of 1 uM thiol-DNA (H3 or H4) were firstly added and mixed 31 μL 10 mM sodium phosphate buffer (10 mM, pH=5.5) and 4 μL 10 mM TCEP at room temperature for 1 hour to reduce disulfide bonds. Then, 40 uL 5.0 mg/mL GOx was mixed with 5 μL DMSO solution containing 6.4 mM MBS for 1 h at room temperature on a shaker to activate GOx. Finally, MBS-activated GOx was reacted with thiol-DNA (H3 or H4) at room temperature for 24 h. To remove unreacted thiol-DNA (H3 or H4), the solution was purified by Amicon-100K 5 times using PBS buffer.

Fabrication of Double-signal Mode based on Metal-Organic Frameworks Coupling Cascaded Nucleic Acid Circuits for miRNA-21 detection

Firstly, 2 μL of streptavidin-functionalized magnetic beads (MBs) (10 mg/mL) was rinsed with PBS buffer for three times. The MBs were then resuspended in 100 μL of PBS buffer. Subsequently, to the MBs solution was added 10 μL biotinylated DNA probe (H1, 1 uM), followed by shaking and reaction for 40 min at room temperature. The obtained MB-H1 conjugates were rinsed for three times with PBS buffer. Next, to the MB-H1 conjugates was added and incubated different concentration miRNA-21 for 30 min at room temperature, followed by washing three times with PBS buffer by magnetic separation. Next, To the aforementioned solution were added and incubated H2 (0.5 μM) for CHA reaction at 37℃for 1 h , followed by washing three times with PBS (10 mM, pH=5.5) to remove free H2 by magnetic separation. Then, DNA(H3/H4)-GOx conjugates (1.5 mg mL^{-1}) were added and reacted at room temperature for HCR reaction 1.5 h, followed by washing three times with PBS by magnetic separation to remove free DNA(H3/H4)-GOx. Finally, 100 μL of glucose (10 mM), 50 μL 200 μg/mL NH_2-MIL-101 and 125 μL 100 μM OPD were

added to the above attained MB, and incubated for 60 min at 37 ℃, 430-650 nm fluorescence spectra (410 nm excitation) were recorded.

Gel electrophoresis analysis

A 12% native polyacrylamide gel was prepared to analyze the products of H3-GOx and H4-GOx, which was performed in the 5× TBE buffer. The sample containing 10 μL each reaction sample and 2 uL 6× loading buffer was subjected to polyacrylamide gel electrophoresis (PAGE), running about 2 h at a current of 80 V. Subsequently, the gels were stained with ethidium bromide, and finally photographed with Gel DocXR+ imaging system.

After electrophoresis, the resulting gel board was further used for coomassie brilliant blue analysis. Briefly, the gel was firstly stained in 0.025% (w/v) coomassie brilliant blue R-250 dye for 1 h, and then destained in a solution containing 45% ethanol and 45% acetic acid for 1.5 h at room temperature, and finally destained overnight in a aqueous solution of ethanol.

Sample analysis.

Human plasma samples were obtained from Liaocheng People's Hospital. Circulating miRNA-21 in serum were extracted using the miRNeasy RNA isolation kit. The miRNeasy spike-in control (*C. elegans* miR-39 miRNA mimic) was used as an internal control for miRNA recovery and reverse transcription efficiency. Reference values of miRNA were obtained by qPCR and normalized according to the parallel recovery of Cel_miR-39. For direct profiling of circulating miR-21 in serum, each 10 μL of serum sample from patients was diluted with PBS to a final volume of 50 μL (1:5 dilution), heated at 95 °C for 5 min, and then cooled rapidly on ice for 5 min. Then, the heat-denatured serum lysates were centrifuged at 15000 g for 20 min at 4 °C. Finally, 20 μL of the supernatant (equal to 4 μL of serum) was added to each

double-signal mode sensor based on NH_2-MIL-101 MOFs coupling cascaded nucleic acid circuits.

RT-PCR experiment

According to the manufacturer's instructions, the total RNA content in serum was extracted from human blood using the Trizol Reagent (Invitrogen). To quantify the expression level of miRNA-21, the following procedure was carried out. 1.5 μg of total RNA was first reverse-transcribed to cDNA by AMV reverse transcriptase and looped antisense primers. The resulted cDNA was then quantified by RT-PCR. The reaction are described as follows: 95 °C for 5 min, followed by 40 cycles with a 15 s interval at 95 °C and a 1 min interval at 60 °C. All reactions were performed in triplicate, and U6 was used as the internal control. Notably, all primers used for these assays are listed as follows: miR-21 forward primer: 5'-ACA CTCCAG CTG GGT AGC TTA TCA GAC TGA-3'; miR-21 reverse primer: 5'-CTCAAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TC-3'; U6 forward primer: 5'-CTC GCT TCG GCA GCA CA-3'; miR-21 reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'.

Results and discussion

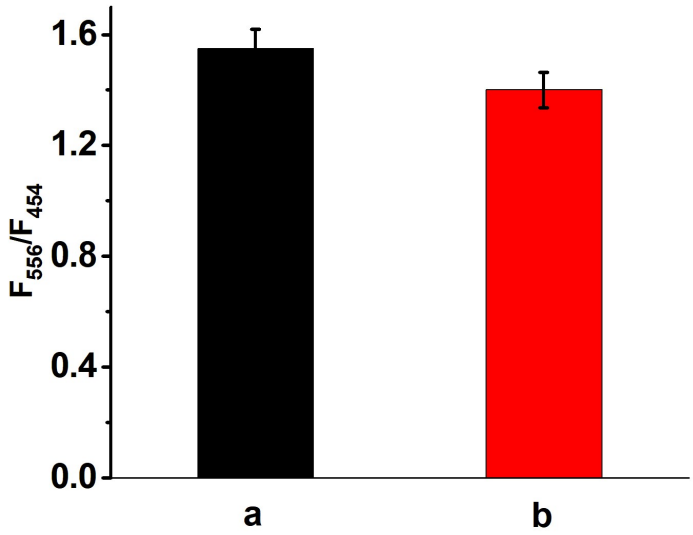
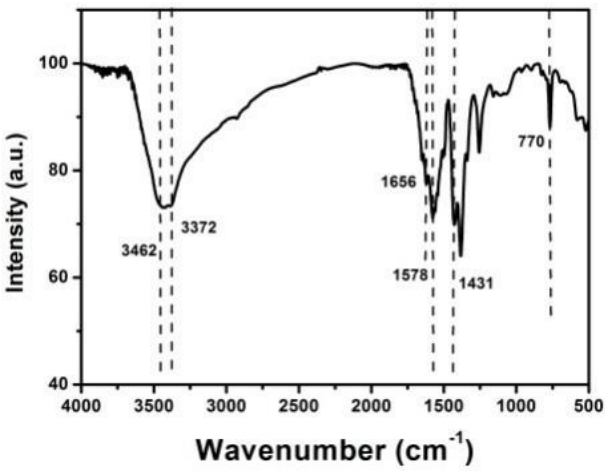


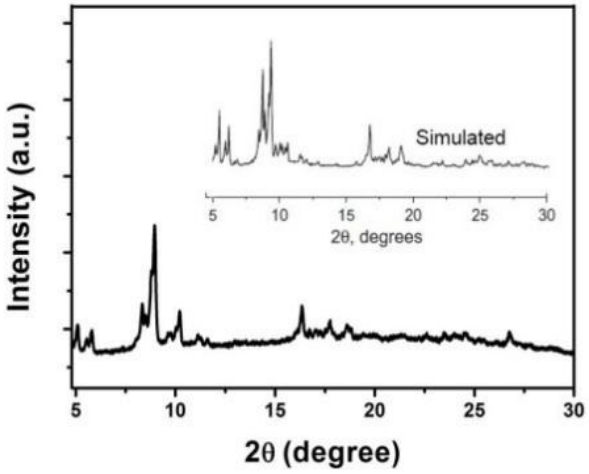
Fig S1. The activity of **(a)** GOx in free solution **(b)** the conjugated GOx. The concentration of GOx in free solution and the conjugated GOx are 0.2 mg/mL.

Characterization of NH_2-MIL-101

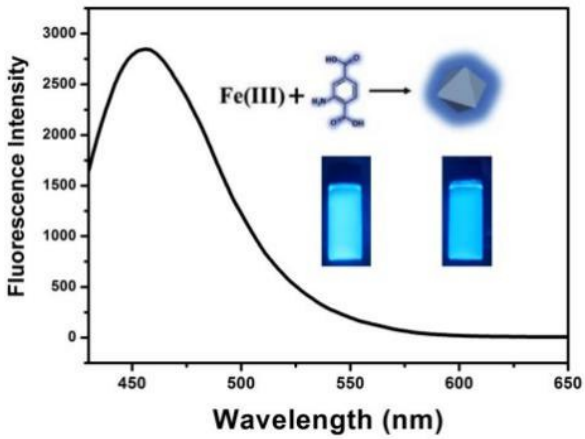
Based on the NH_2-MIL-101 chemical structure, the 770 cm^{-1} peak in the FT-IR spectrum **(Fig. S2A)**, was attributed to C–H bending vibrations in benzene. The peaks at 1431 cm^{-1} and 1578 cm^{-1} were asymmetric and symmetric vibrations of carboxyl groups in the frameworks. The 1656 cm^{-1} peak for C=O stretching was from guest DMF CONH bands. Peaks at 3462 cm^{-1} and 3372 cm^{-1} were attributed to asymmetrical and symmetrical amine stretching. The XRD pattern in **Fig. S2B** indicated that the characteristic peaks of NH_2-MIL-101 were consistent with a simulation of MIL-101;[1] a pure MOF phase was indicated by the absence of other peaks. The fluorescence spectrum of NH_2-MIL-101 (**Fig. S2C)** exhibits an intense fluorescence emission at 456 nm, which originated from its organic linker NH_2-BDC. And the corresponding energy dispersive X-ray spectroscopy showed the peaks of C, N, O and Fe elements (**Fig. S2D**), further confirming the formation of NH2-MIL-101 MOFs.


Intensity (a.u.)
100
80
60
40
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)
3462
3372
1656
1578
1431
770

(A)


Intensity (a.u.)
Simulated
5
10
15
20
25
30
2θ, degrees
2θ (degree)

(B)


Fluorescence Intensity
3000
2500
2000
1500
1000
500
0
Fe(III)+
450
500
550
600
650
Wavelength (nm)

(C)

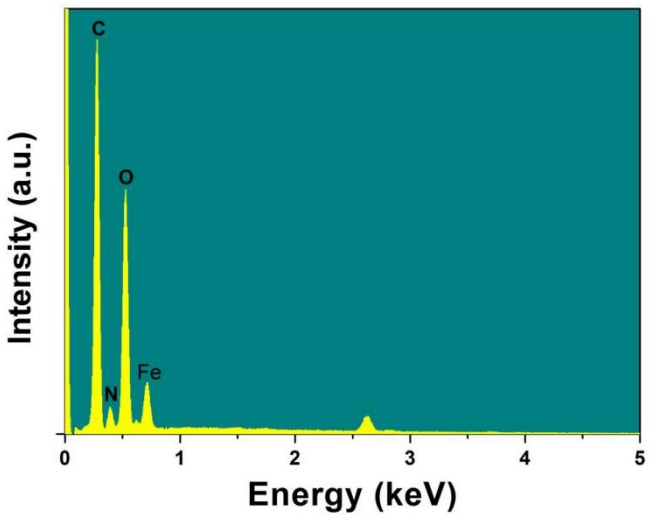
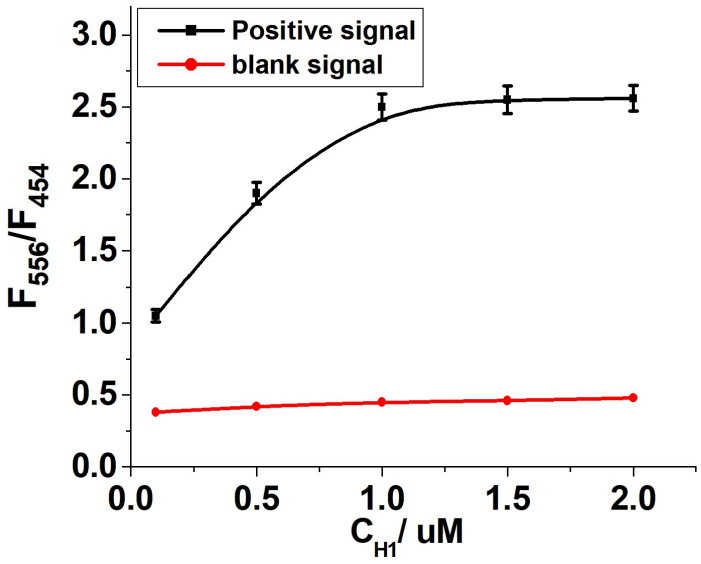


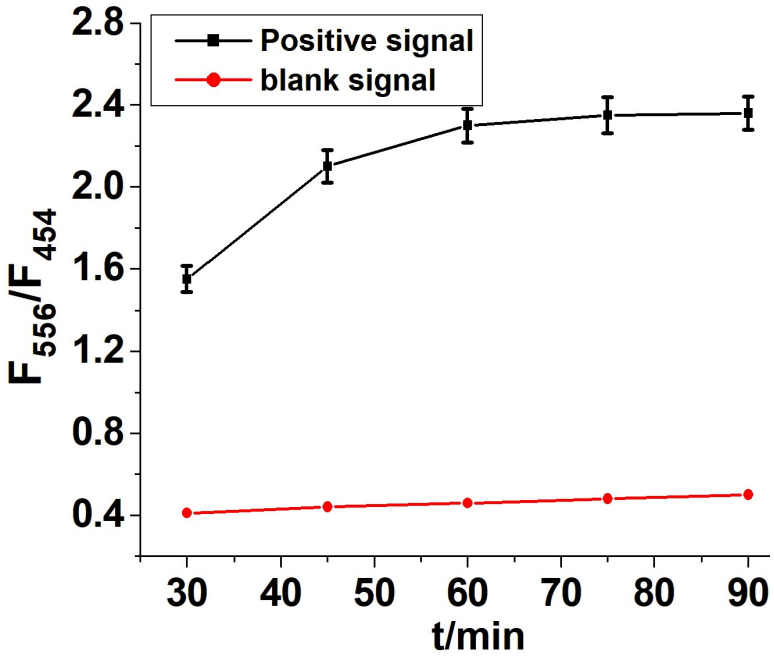
Fig S2. (A) FT-IR spectrum of NH_2-MIL-101. **(B)** XRD profiles of NH_2-MIL-101 and simulated MIL-101. **(C)** Fluorescence spectrum of NH_2-MIL-101 (Inset: schematic of fluorescent NH_2-MIL-101 synthesis; photos of NH_2-BDC and NH_2-MIL-101 under UV excitation). (D) The energy dispersive X-ray spectroscopy of NH_2-MIL-101.

Optimization of the reaction conditions

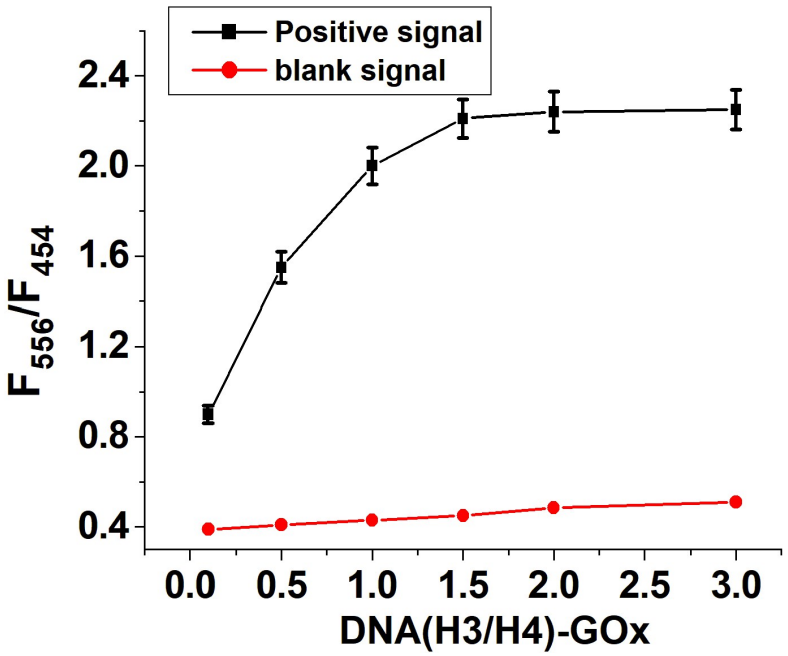
To obtain the best performance, some experimental conditions were optimized in the proposed biosensor. Since the H1 recognition and CHA reaction was a prerequisite for signal transduction, the concentration of H1 probe and CHA reaction time were firstly investigated, respectively(**Fig. S3A-B**). Moreover, HCR and NH_2-MIL-101 MOF catalytic reaction were also critical factors for signal response. Thus, the concentration of invertase-H3 or invertase-H4 probe, HCR reaction time, the concentration of OPD and NH_2-MIL-101 MOF catalytic reaction time were investigated(**Fig. S3C-F**).


Positive signal
blank signal
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F_{556}/F_{454}
0.0
0.5
1.0
1.5
2.0
C_{H1}/ uM

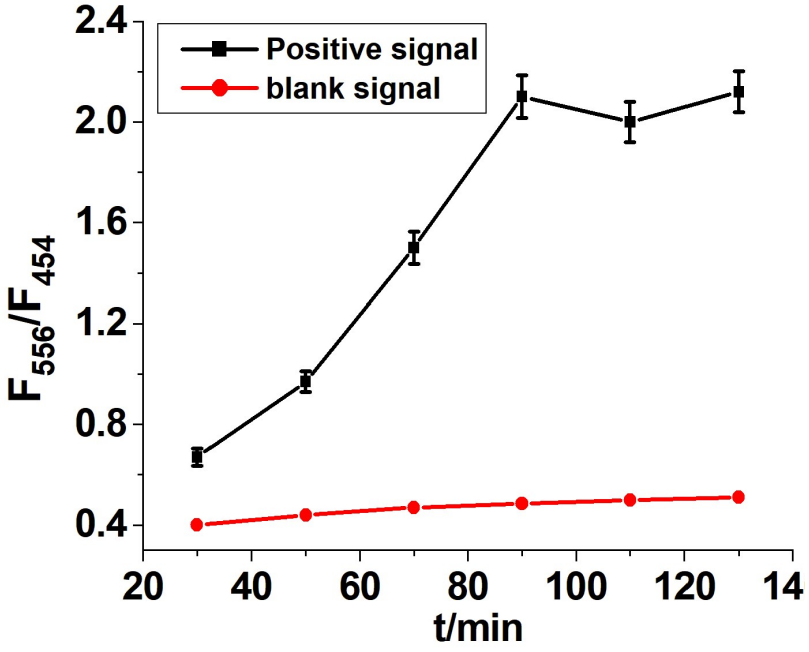
(A)


Positive signal
blank signal
2.8
2.4
2.0
1.6
1.2
0.8
0.4
F_{556}/F_{454}
30
40
50
60
70
80
90
t/min

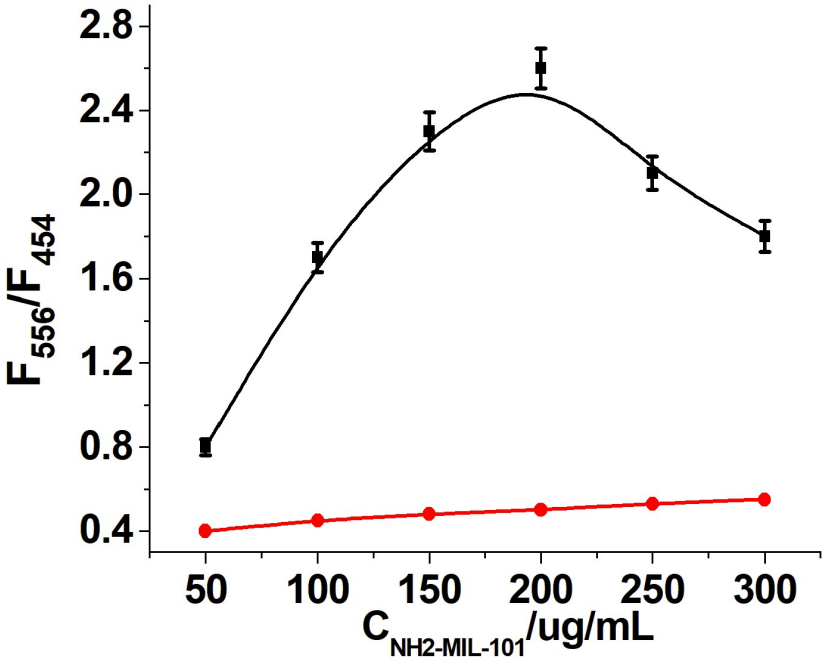
(B)


Positive signal
blank signal
2.4
2.0
1.6
1.2
0.8
0.4
F_{556}/F_{454}
0.0
0.5
1.0
1.5
2.0
2.5
3.0
DNA(H3/H4)-GOx

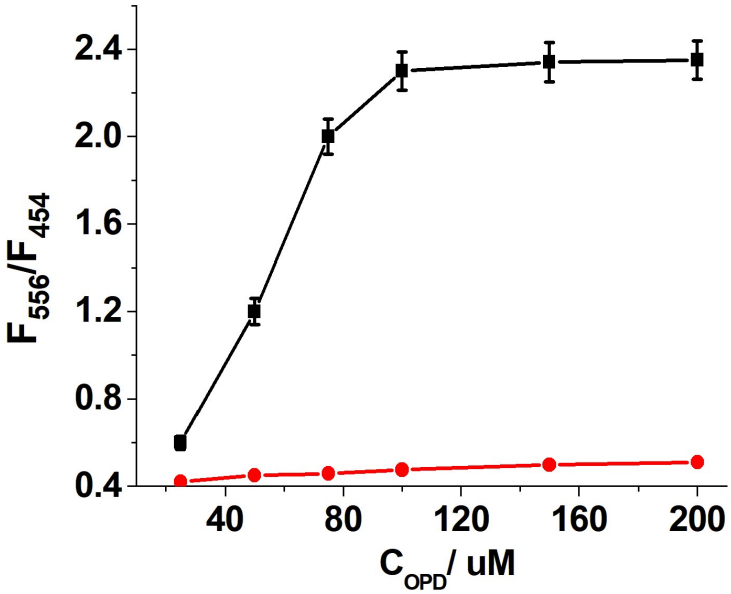
(C)


Positive signal
blank signal
2.4
2.0
1.6
1.2
0.8
0.4
F_{556}/F_{454}
20
40
60
80
100
120
14
t/min

(D)



(E)



(F)

Fig S3. Optimization of experimental conditions. **(A)** the concentration of H1 probe, **(B)** CHA reaction time, **(C)** the concentration of H3-GOx probeH3, **(D)** HCR reaction time, **(E)** the concentration of NH_2-MIL-101, and **(F)** the concentration of OPD. Error bars indicate the standard deviations of three experiments.

Table S2. Comparison of Our Ratiometric fluorescence sensor for miRNA assay with Other Ratiometric Detection Methods.

Analytical method	Target	Detection strategy	Detection limit	Ref.
ECL	miRNA	ratiometric	0.5 fM	2
PEC	miRNA	ratiometric	52 aM	3
ECL	miRNA	ratiometric	9.4 aM	4
Fluorescence	miRNA	ratiometric	0.68 nM	5
Fluorescence	miRNA	ratiometric	72 pM	6
Fluorescence	DNA	ratiometric	0.5 nM	7
SERS	miRNA	ratiometric	3.5 fM	8
Electrochemistry	miRNA	ratiometric	33 aM	9
Fluorescence	miRNA	ratiometric	0.8 aM	This work

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