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Target Triggered Self-powered DNAzyme–MnO_2 Nanosystem: towards Imaging MicroRNAs in Living Cells

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Experimental section

Reagents and Materials. Human breast adenocarcinoma (MCF-7) cells, Human cervical cancer (HeLa) cells, and Human normal liver (LO2) cells were purchased from KeyGEN Biotechnology Company (Nanjing, China). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Inc. (St. Louis, MO). Manganese chloride tetrahydrate ($MnCl_2$•$4H_2O$) and tetramethylammoniam were purchased from Aladdin (Shanghai, China). Hoechst 33342, high glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Lipofectamine 2000 were bought from Invitrogen (Shanghai, China). All other reagents were analytical grade and used without further purification. All solutions were prepared using ultrapure water, which was made using ultrapure water from a Millipore purification system. All HPLC-purified oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the involved oligonucleotides are listed in table S1.

Table S1. Sequences of oligonucleotides used for this work.

Name	Sequence (5'-3')
MB1	Cy5- ATCGAATAGTCTGACTACAACTTGATAC$(T)_8$CTCTTCT CCGAGCCGGTCGAAATAGTAGTTGTAGTCA-BHQ2
MB2	Cy5- ATAACGACGAAGCGCTGCACTATrAGGAAGAGAAAAT CCCGTCGTTAT-BHQ2
target miR-21	UAGCUUAUCAGACUGAUGUUGA
anti-miR-21	TCA ACA TCA GTC TGA TAA GCT A

For selectivity tests	
One-base mismatched RNA	UAGCUUAUCAGGCUGAUGUUGA
Random RNA	CACAGCCGGACUACUCCUAGUG
miR-155	UUAAUGCUAAUCGUGAUAGGGGU
miR-141	UAACACUGUCUGGUAAAGAUGG
miR-182	UUUGGCAAUGGUAGAACUCACACU
miR-197	UUCACCACCUUCUCCACCCAGC

Apparatus. Transmission electron micrographs were obtained on JEM-2100 transmission electron microscope (JEOL Ltd., Japan). UV-vis absorption spectra and Fluorescence emission spectra were detected by a UV-vis spectrophotometer (UV-3600; Shimadzu Co., Japan) and a Shimadzu fluorescence S-3 spectrophotometer (RF-5301PC, Shimadzu Co., Japan). Confocal fluorescence images of cells were acquired with a TCS SP5 confocal microscopy (Leica, Germany). The cell viability tests were detected by a Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, USA).

Synthesis of MnO_2 Nanosheets. MnO_2 nanosheets were prepared according to previous reports.[1] 20 mL of 0.6 M tetramethylammonium hydroxide and 3.0 wt % H_2O_2 were first prepared and then added to 10 mL of $MnCl_2$ (0.3 M) quickly within 20 s. The color of this solution became dark brown immediately and stayed for 12 h with vigorously stirring. After that, the product was collected by centrifugation at 2000 rpm for 20 minutes, then washed with water and methanol for three times, followed by drying under high vacuum. Then the small-sized MnO_2 nanosheets were obtained by dispersing 10 mg of prepared bulk MnO_2 in 20 mL of water with the

process of ultrasonication.

Preparation of DNAzyme-MnO_2 nanosystem. The hairpin molecules MB1 and MB2 adsorbed on MnO_2 nanosheets were carried out by mixing 0.5 mg/mL MnO_2 nanosheets with MB1 (10 μL, 1 μM) and MB2 (10 μL, 20 μM) for 10 min at room temperature. Then 1 mL of Tris-HCl buffer (0.1 M, pH 7.4) was added and the mixed solution was stirred at room temperature for 20 min. After this procedure, the products were kept at 4 ^oC for further use.

Cell culture. MCF-7 cells, HeLa cells and LO2 cells were cultured in Dulbecoo's modification of Eagle's medium (DMEM) (HyClone, 100 U/mL penicillin and 100 μg/mL streptomycin) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 ºC in a humidified atmosphere (95% air and 5% CO_2).

Cell viability Assay. MCF-7 cells were seeded in 96-well plates at a final concentration of 1×10^5 cells per well and cultured in cell medium for 12 h. After removing the original medium, the cells were divided into three groups, which were further incubated with pure culture medium, medium containing bare MnO_2 or medium containing the DNAzyme-MnO_2 nanocomplex respectively for variant time. Following that, cells were washed with PBS several times, and then 200μL MTT solutions (0.5 mg/mL in PBS) were added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL DMSO was added to each well to dissolve the formazan crystals. Subsequent absorbance measurements were detected by measuring the absorbance at 490 nm using Thermo Scientific Varioskan Flash.

In Vitro miR-21 Detection. The target molecule miR-21 (from 0 to 200 pM) were added to the mixture of DNAzyme-MnO_2 and GSH (1 mM), and then incubated at 37 °C for another 2 h. Fluorescence measurements of the reaction solution were excited at 630 nm, and the resulting spectra were recorded between 650 nm and 750 nm.

Polyacrylamide gel electrophoresis. The reaction products were analyzed by 4%-15% polyacrylamide gel. The gel was placed in 1×electrophoresis Tris-borate-EDTA (TBE) buffer at 120 V for 0.5 h and stained with Gel Green for 10 min. Gel imaging was performed using a Bio-rad fluorescence gel imaging system.

Imaging of miRNA in Living Cells. Cells were seeded on confocal dishes at 37 °C overnight. After that, these cells were incubated with the 0.5 mg/mL of MB1/MnO_2, MB2/MnO_2, DNAzyme-MnO_2 nanosystem, respectively. After incubation for 4 h, PBS (0.1 M, pH 7.4) was used to wash cells for three times, and the fresh medium was added into each dish, then cells were performed for fluorescence imaging experiments under a Leica TCS SP5 confocal laser scanning microscopy.

Quantification of miR-21 by RT-PCR. The relative expression levels of miRNA-21 in LO2 cells, MCF-7 cells and HeLa cells were conducted by qRT-PCR analysis. The primers used for gene expression analyses in this study were designed and synthesized by Sangon (Shanghai, China) and their sequences were listed in Table S2. Total RNA was isolated by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocols. The first-strand cDNAs were reverse-transcribed and synthesized from 0.5 μg total cellular RNA with random hexamers. Briefly, the RT-PCR cycles were

conducted to 40 cycles for amplification of miR-21. Fluorescent dye used in RT-PCR reactions was SYBR Green PCR Master Mix. Following the PCR cycling program was set for one cycle of pre-denaturation at 95 ˚C for 30 s, 60 ˚C for 30 s, 72 ˚C for 30 s, melting curve from 55-95 ˚C read every 0.2 ˚C, holding for 1 second between reads. All qRT-PCR reactions were performed in triplicate. The relative expression of miRNA was calculated using the $2^{-\Delta CT}$ method, in which $\Delta CT = CT_{miRNA} - CT_{U6}$. The experiment was repeated three times.

Table S2. The primers used for gene expression analyses in this study.

Primer name	Primer sequences (5'-3')
miR-21 forward primer	ACACTCCAGCTGGGTAGCTTATCAGACTGA
miR-21 reverse primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTG AGTCAAC ATC
U6 forward primer	CTC GCT TCGGCA GCA CA
U6 reverse primer	AAC GCT TCA CGA ATT TGC GT

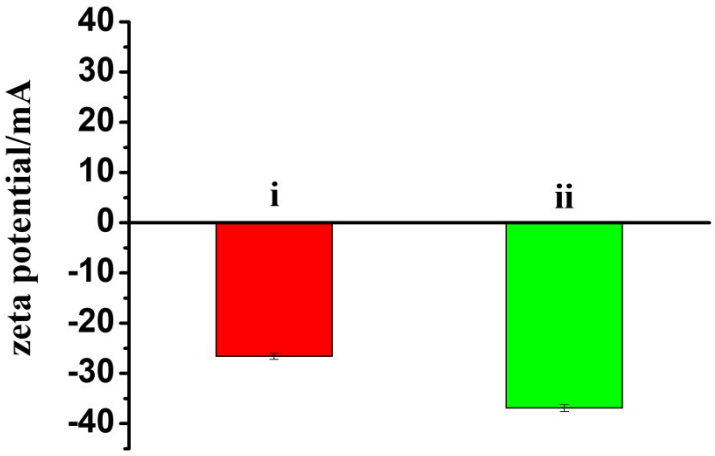

Fig.S1 Zeta potential of MnO_2 (i), DNAzyme-MnO_2 nanosystem (ii).

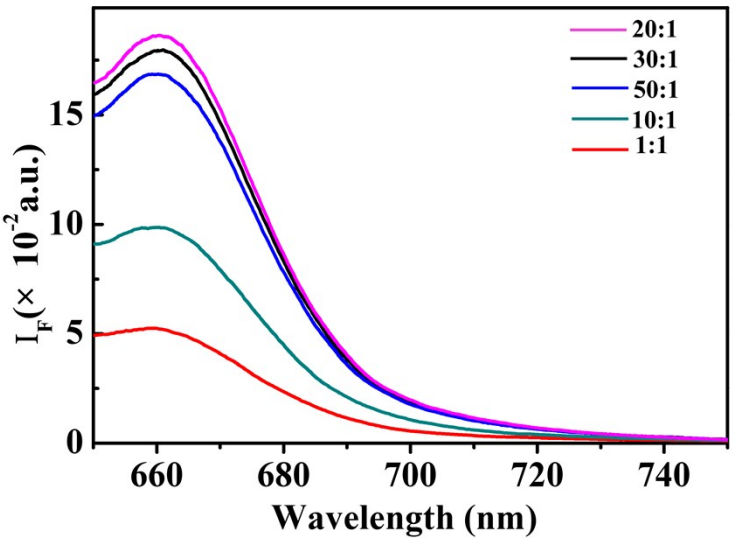

Fig. S2 Fluorescence measurements for optimizing the ratio of the MB2 to MB1 in preparation of the DNAzyme-MnO_2 nanosystem.

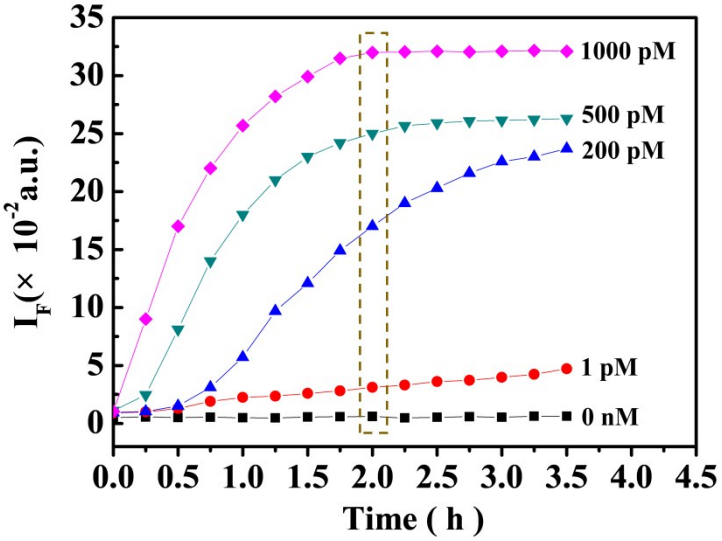

Fig. S3 Time-dependent fluorescence changes of DNAzyme-MnO_2 nanosystem upon addition of different concentrations of target miR-21, [GSH] = 1 mM.

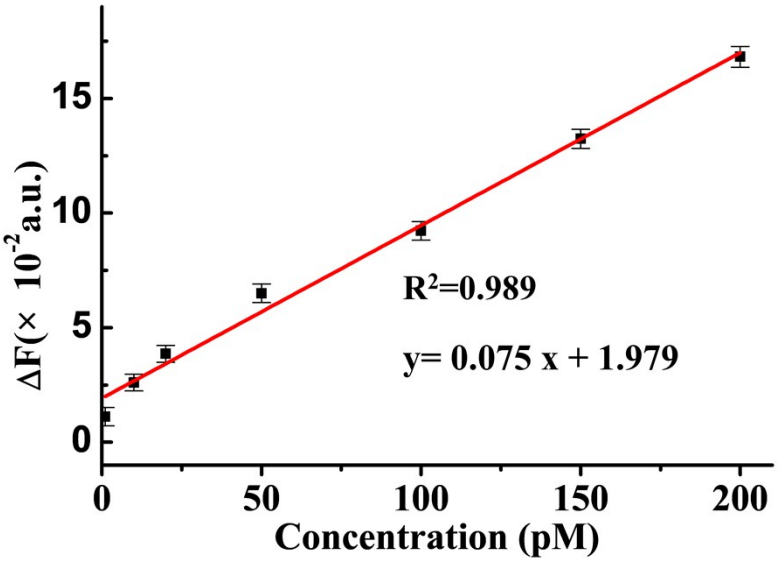

Fig. S4 Calibration curves of the fluorescence intensities versus corresponding miR-21 concentrations ranged from 1 to 200 pM. Error bars were estimated from three replicate measurements.

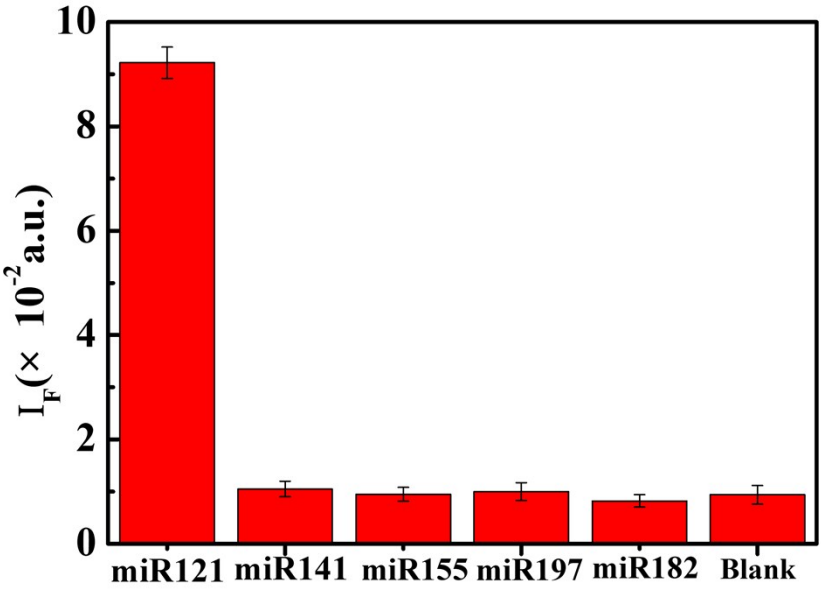

Fig. S5 Investigation of the specificity of the DNAzyme-MnO_2 nanosystem upon the treatment with other analogues from miRNAs family.

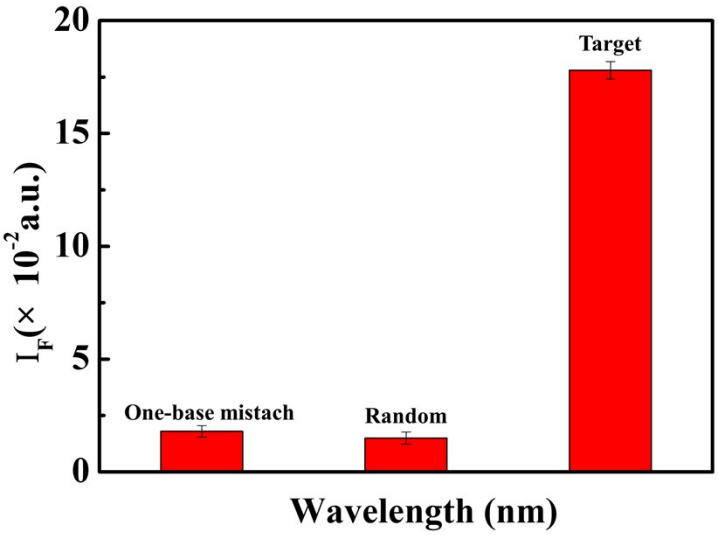

Fig. S6 Fluorescence intensities of the DNAzyme-MnO_2 nanosystem reacted with 200 pM target miR-21, one-base mismatched RNA or Random RNA, respectively.

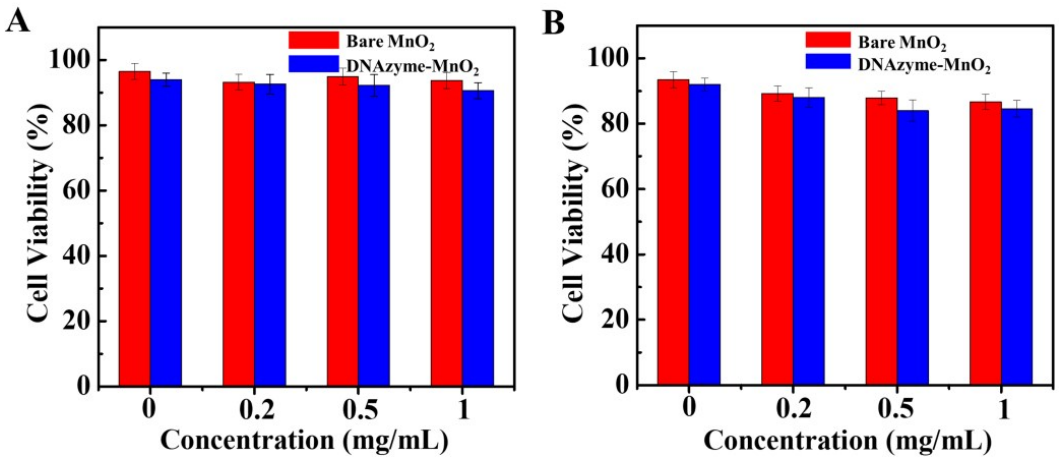

Fig. S7 Cell viability of MCF-7 cells after incubated with different concentrations of bare MnO_2 or the DNAzyme-MnO_2 nanosystem for 24 h (A) and 48 h (B). Error bars represent the standard deviation of three replicates.

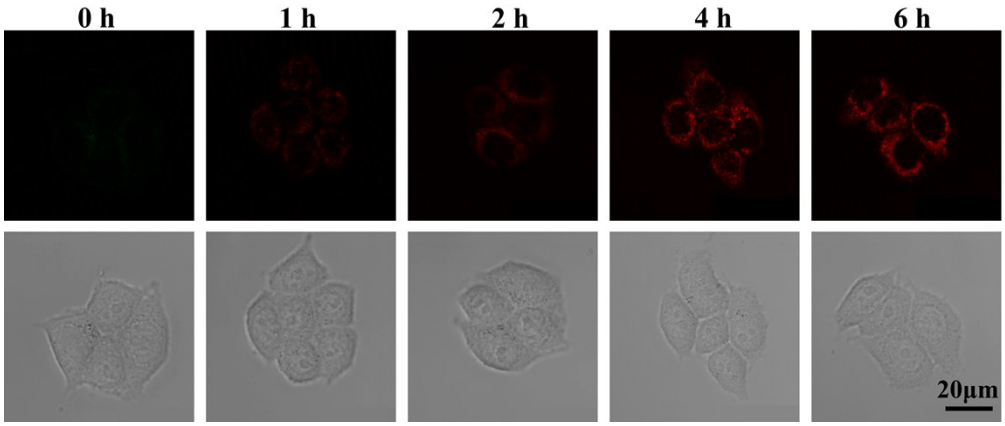

Fig. S8 Fluorescence images of MCF-7 cells after incubated with DNAzyme-MnO_2 nanosystems at variant time.

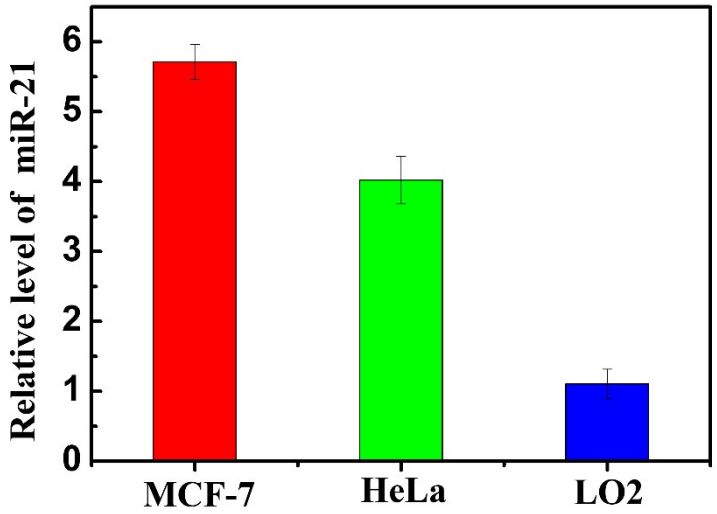

Fig. S9 RT-PCR analysis for the expression of the miR-21 in MCF-7, HeLa and LO2 cells. Error bars represent the standard deviation of three replicates.

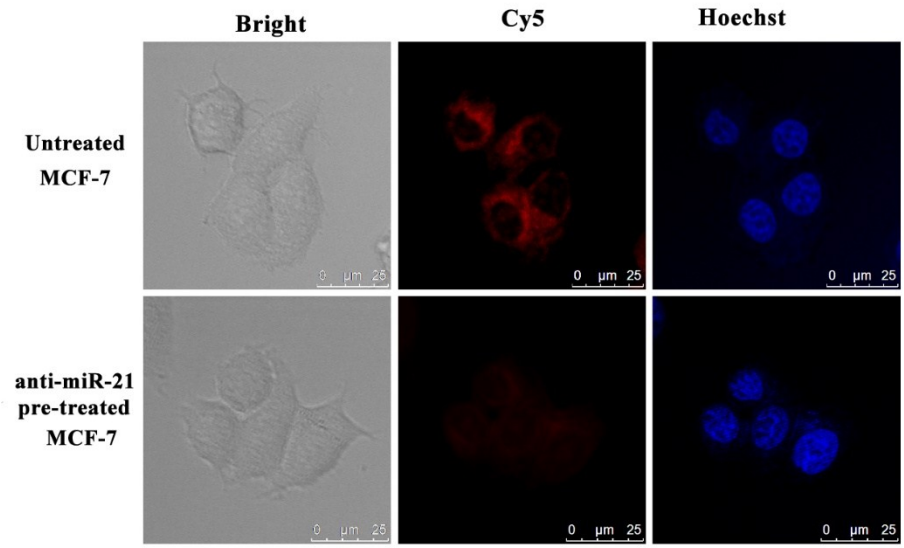

Fig. S10 CLSM images of untreated MCF-7 cells (top) and anti-miR-21 pre-treated MCF-7 cells (bottom) after incubation with DNAzyme-MnO_2 nanosystems for 4 h.

Reference:

1. H. Fan, Z. Zhao, G.Yan, X. Zhang, C.Yang, H. Meng, Z. Chen, H. Liu, W. Tan, *Angew. Chem., Int. Ed.,* 2015, **54**, 4801- 4805.