Electronic Supplementary Information (ESI) for Chemical Communications

Simultaneous sensing of intracellular microRNAs with a multi-functionalized carbon nitride nanosheet probe†

Xianjiu Liao, Quanbo Wang and Huangxian Ju*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P.R. China

Experimental

Materials and reagents. Graphene oxide (GO) was obtained from XFNano Materials Tech Co., Ltd. (Nanjing, China). HepG2, A549 and HaCaT cells, 3-(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT), Annexin V-FITC, propidium iodide (PI) and pH 7.4 binding buffer were from KeyGen Biotech. Co. Ltd (Nanjing, China). DNase I endonuclease was from Thermo Fisher Scientific Inc. (USA), and ssDNA binding protein (SSB) was from Promega Corporation (USA). Lipofectamine 2000 (Lp2000), serum-free medium (Opti-MEM), Hoechst 333342 as nuclear tracker and LysoTracker Green DND-26 as endosomal/lysosomal tracker were obtained from Invitrogen Corporation (USA). Folate (FA) and 2-mercaptoethanol were obtained from Sigma-Aldrich Inc. (USA). DNA hybridization buffer (HB) was Tris-HCl buffer (20 mM, containing 100 mM NaCl, 5.0 mM KCl, and 5.0 mM $MgCl_2$, pH 7.4). HepG2 and HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum, penicillin (80 U mL^{-1}), and streptomycin (80 μg mL^{-1}) at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2, while Opti-MEM was used for cell transfection. Carbon nanosphere (CNS, 110 nm) was prepared according to the literature.[S1]

Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all runs. All RNA and DNA sequences (Table S1) were purchased from Shanghai GenePharma Co., Ltd. and Sangon Biological Engineering Technology Co., Ltd. (Shanghai, China), respectively.

Table S1. Oligonucleotides employed in this work

Oligonucleotides	**Oligonucleotide sequences**
Cy3-ssDNA (probe 1,P1)	5'-Cy3-AACGGAACCACTAGTGACTTG-3'
miRNA-224 (target 1,T1)	5'-CAAGUCACUAGUGGUUCCGUU-3'
single-base mismatched T1 (smT1)	5'-CAAGUCACU*C*GUGGUUCCGUU-3'
three-base mismatched T1 (tmT1)	5'-CAAGUC*G*CUAG*A*GGUU*A*CGUU-3'
inhibitor-224	5'-AACGGAACCACUAGUGACUUG-3'
background calibration T1	5'-Cy3-AAAAAAAAAAAAAAAA-3'
Cy5-ssDNA (P2)	5'-Cy5-CTATCTGCACTAGATGCACCTTA-3'
miRNA-18a (T2)	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'
single-base mismatched T2 (smT2)	5'-UAAGGUGCAUC*G*AGUGCAGAUAG-3'
three-base mismatched T2 (tmT2)	5'-UAAGG*A*GCAUCU*C*GUGCA*U*AUAG-3'
inhibitor-18a	5'-CUAUCUGCACUAGAUGCACCUUA-3'
background calibration T2	5'-Cy5-AAAAAAAAAAAAAAAA-3'
non-complementary RNA	5'-UGGAGUGUGACAAUGGUGUUUG-3'
NH_2-Poly A	5'-NH_2-AAAAAA-3'
Cy5-Poly A	5'-Cy5-AAAAAA-3'

Mismatched bases are highlighted in italic type.

Apparatus. Laser scanning confocal microscopic (LSCM) images were gained on a TCS SP5 laser scanning confocal microscope (Leica, Germany). Transmission electron micrograph (TEM) was obtained using a JEM-2100 TEM instrument (JEOL, Japan). Zeta potential analysis was performed on a Zetasizer (Nano-Z, Malvern, UK). Flow cytometric (FCM) analysis was

performed on a Coulter FC-500 flow cytometer (Beckman-Coulter, USA). UV-vis absorption spectra were obtained with a UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Japan). Fluorescence (FL) spectra were recorded on a RF-5301PC spectrofluorometer (Shimadzu, Japan). MTT assay was performed on a microplate reader (680, Bio-Rad, USA). Cell number was determined using a Petroff-Hausser cell counter (USA). Dynamic light scattering (DLS) was observed on a 90 Plus/ BI-MAS equipment (Brook haven, USA). X-ray diffraction (XRD) pattern was recorded on a D8 Advance X-ray diffractometer (Bruker, Germany) using Cu Kα radiation (λ = 1.5418 Å). X-ray photoelectron spectroscopic (XPS) measurement was performed using an ESCALAB 250 spectrometer (Thermo-VG Scientific, USA) with an ultrahigh vacuum generator.

Synthesis of CNNS. Carbon nitride nanosheet **(**CNNS) was prepared from bulk graphitic-phase carbon nitride (g-C_3N_4) according to the literature.[S2] Firstly, bulk g-C_3N_4 was prepared by polymorization of melamine molecules at high temperature. Then CNNS was prepared by sonicating the bulk g-C_3N_4 in water. The sonicated mixture was first centrifuged at 5,000 rpm to remove aggregated bulk g-C_3N_4 particles and obtain the CNNS suspension, which was centrifuged at 15,000 rpm to obtain CNNS. The CNNS was redispersed in ultrapure water to a concentration of 1.5 mg mL^{-1}.

Preparation of functionalized CNNS probes. The FA-Poly A was firstly prepared by adding 3.2 mg EDC and 4.8 mg NHS in 7.91 mL pH 6.0 MES containing 6 μM FA. After the mixture was stirred at room temperature for 5 min, 10 μL 2-mercaptoethanol was added to quench the excess EDC. Then, 80 μL 300 μM NH_2-Poly A was added to the mixture and stirred at room temperature for 4 h.[S3] The concentration of the obtained FA-Poly A was 3 μM, expressed as the amount of NH_2-Poly A.

The multiple dye-ssDNA and folate functionalized CNNS probe (mf-CNNS probe) were prepared as follows: 400 μL 1.5 mg mL^{-1} CNNS was diluted with 2.52 mL 20 mM pH 7.4 Tris-HCl buffer and sonicated for 5 min to obtain a homogeneous dispersion. Subsequently, 15 μL 10 μM Cy5-ssDNA and 15 μL 10 μM Cy3-ssDNA were added to the dispersion and stirred for 5 min at room temperature, then 50 μL 3 μM fresh FA-Poly A was added to the mixture and stirred another 5 min. After the mixture was centrifuged at 15000 rpm for 20 min, the resulting mf-CNNS probe was redispersed in 400 μL or 15 μL 20 mM pH 7.4 Tris-HCl buffer and stored at 4 ^{o}C, the concentration of mf-CNNS probe was 1.5 mg mL^{-1} or 40 mg mL^{-1}, expressed as the amount of CNNS. The supernatant was collected to characterize the amount of dye-ssDNA loaded on CNNS by FL detection with a standard curve method. The average quantity of both Cy5-ssDNA and Cy3-ssDNA loaded on 1.0 μg mL^{-1} CNNS was 0.23 nM.

The single dye-ssDNA and folate functionalized CNNS probe (f-CNNS probe) was prepared with a similar process as mf-CNNS probe using 200 μL 1.5 mg mL^{-1} CNNS, 15 μL 10 μM Cy3-ssDNA (f_1-CNNS probe) or Cy5-ssDNA (f_2-CNNS probe). The background calibration probes were also prepared using corresponding background calibration T1 or T2 instead of dye-ssDNA. The folate functionalized CNNS (f-CNNS) was prepared by mixing the dispersion of CNNS and folate solution, and the m-CNNS probe was prepared by mixing the CNNS dispersion and Cy5-ssDNA and Cy3-ssDNA solutions with a similar process. The f-CNS and f-GO were also prepared by mixing folate solution with the dispersion of CNS or GO.

MTT assay. 1.0×10^4 HepG2 cells were seeded in a 96-well plate containing 100 μL Opti-MEM in each well for 12 h. These cells were incubated with 100 μL Opti-MEM as control and 100 μL Opti-MEM containing f-CNNS (50 to 800 μg mL^{-1}), 200 μg mL^{-1} f-CNS or 200 μg mL^{-1} f-GO for 3 h. 50 μL MTT (1 mg mL^{-1}) was then added to each well. After incubation for 4 h, the media

were removed, and 150 μL dimethylsulfoxide was added to solubilize the formed formazan dye. After 15 min, the absorbance of each well was measured at 490 nm. The relative cell viability (%) was calculated by $(A_{test}/A_{control}) \times 100$.

Gel electrophoresis experiments. The gel electrophoresis was performed as follows: 14 μL sample was added in the mixture of 3 μL loading buffer and 3 μL GelRed. The mixture was injected into polyacrylamide hydrogel in tris-borate-EDTA (TBE) buffer. Electrophoresis was carried out at 90 V in TBE buffer for 1 h. The resulting board was observed under UV irradiation.

Cell-specific transfection. 1.0×10^4 HepG2 cells were cultivated for 12 h in 35-mm confocal dish. Then the cells were treated with 500 μL Opti-MEM containing 200 μg mL^{-1} f-CNNS or CNNS for different times. As control, A549 and HaCaT cells were treated with 500 μL Opti-MEM containing 200 μg mL^{-1} f-CNNS for different times.

Confocal microscopy was also used to optimize the incubation time of mf-CNNS probe. With a similar process, the HepG2 cells were treated with 200 μg mL^{-1} mf-CNNS probe for different times. The amount of mf-CNNS probe was optimized by incubating HepG2 cells with different amounts of mf-CNNS probe for 3 h.

Subcellular localization of mf-CNNS probe. The subcellular localization of mf-CNNS probe was observed with confocal microscopy by incubating HepG2 cells with 200 μg mL^{-1} mf-CNNS probe for 3 h and then 1.0 μM Hoechst 333342 for 20 min, or 200 μg mL^{-1} mf-CNNS probe or f-CNNS for 3 h and then 1.0 μM LysoTracker Green DND-26 for 20 min. Hoechst 333342 was excited at 405 nm to collect the emission from 410 to 480 nm, while LysoTracker DND-26 was excited at 488 nm, and the emission was collected from 500 to 580 nm.

Confocal microscopic analysis of intracellular miRNAs. 1.0×10^4 HepG2 cells were cultivated in 35-mm confocal dish for 12 h and treated with 500 μL Opti-MEM, or 500 μL Opti-MEM containing 50 nM inhibitor-18a and inhibitor-224, 50 nM inhibitor-224, or 50 nM inhibitor-18a for 48 h at 37 ^{o}C. These HepG2 cells and 1.0×10^4 HeLa cells treated with 500 μL Opti-MEM were then transfected with 200 μg mL^{-1} mf-CNNS probe or background calibration probe at 37 ^{o}C for 3 h, respectively. After washing with PBS, these cells were used to perform confocal imaging analysis for multiple miRNAs detection. The signal from background calibration probe was used to eliminate background by adjusting the "offset" button of LSCM. The FL intensity was digitized by Leica Application Suite Advanced FL (LAS-AF) software.

Flow cytometric analysis of intracellular miRNAs. 1.0×10^5 HepG2 or HeLa cells were cultivated in 60 mm culture dish for 12 h and treated with 5mL Opti-MEM containing different miRNA inhibitors for 48 h, and then with fresh 5mL Opti-MEM containing 100 μg mL^{-1} f_1-CNNS probe or f_2-CNNS probe for 3 h to perform the flow cytometric analysis. The background calibration probe transfected cells were also collected for background calibration.

Characterization of CNNS and mf-CNNS probe

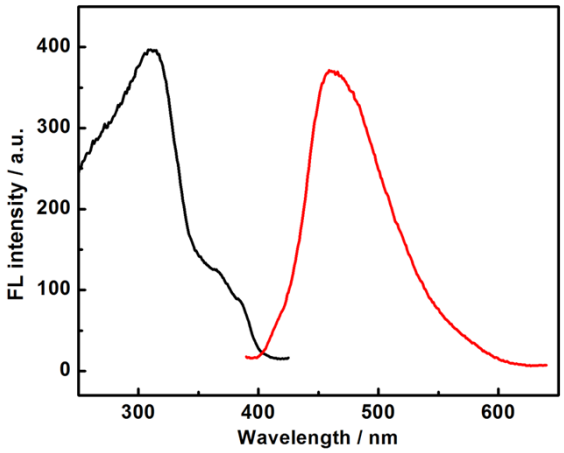

Fig. S1 FL excitation and emission spectra of 150 μg mL^{-1} CNNS.

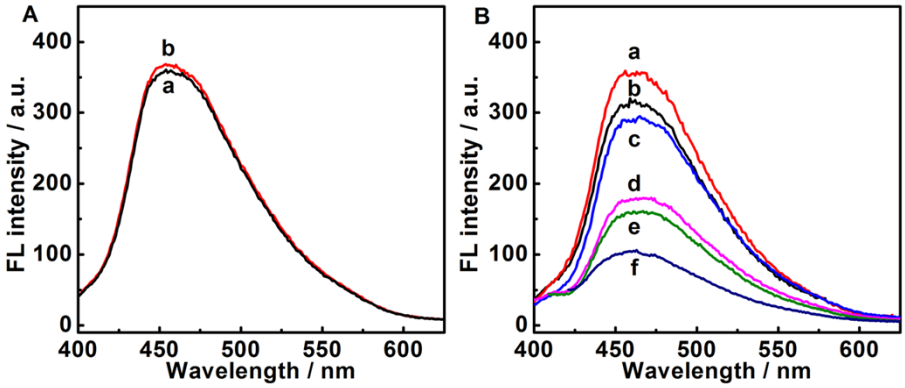

Fig. S2 FL spectra of (A) 150 μg mL^{-1} CNNS (a) and mf-CNNS probe (b), and (B) 150 μg mL^{-1} CNNS with excitation wavelength of (a) 310, (b) 290, (c) 330, (d) 350, (e) 370 and (f) 390 nm.

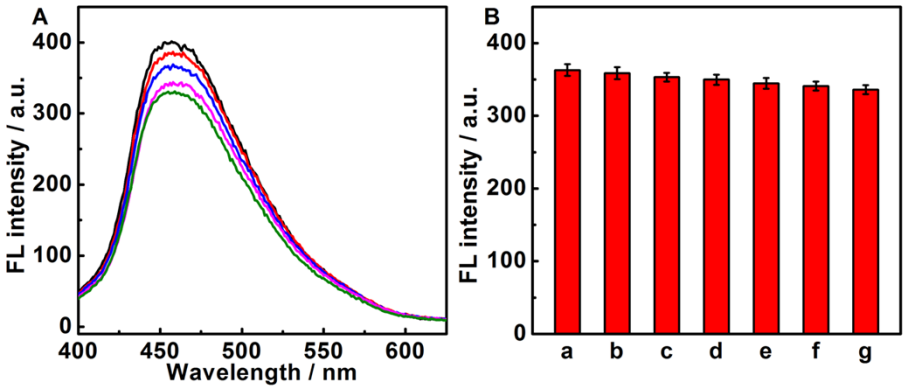

Fig. S3 (A) FL spectra of 150 μg mL^{-1} CNNS at 15, 25, 35, 45 and 55 ^{o}C (from top to bottom). (B) FL intensity of 150 μg mL^{-1} CNNS in 20 mM pH 7.4 Tris-HCl buffer containing 100, 200, 400, 600, 800, 1000 and 1500 mM NaCl (from a to g).

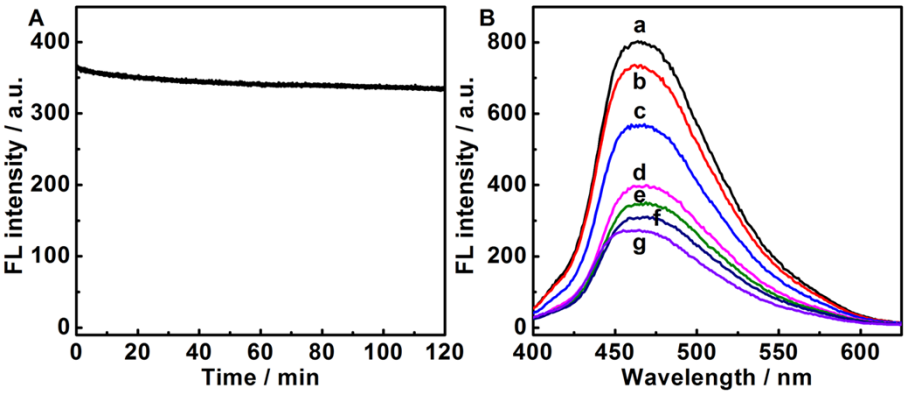

Fig. S4 FL intensity of 150 μg mL^{-1} CNNS at (A) different times, and (B) FL spectra at pH 3, 5, 6, 7, 8, 9 and 11 (from a to g).

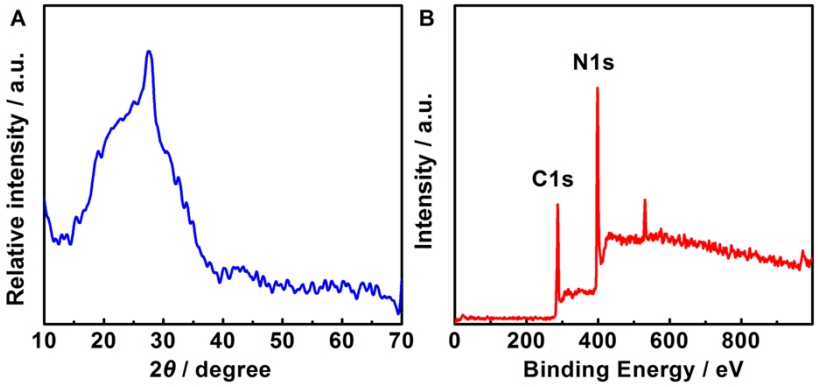

Fig. S5 (A) X-ray diffraction pattern and (B) X-ray photoelectron spectrum of CNNS.

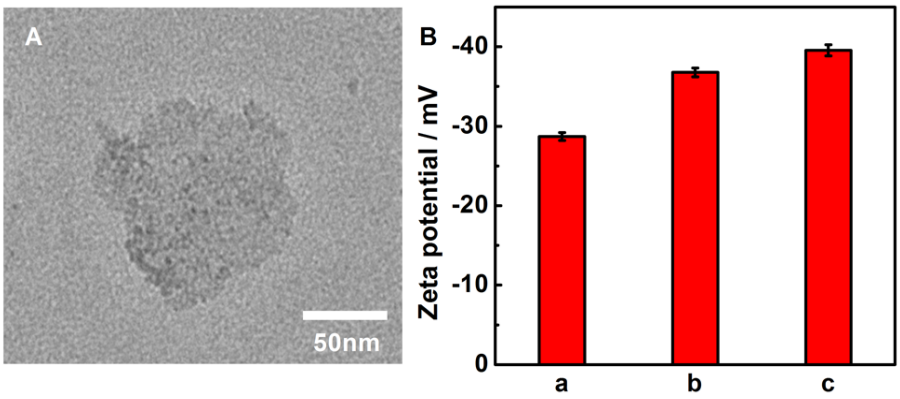

Fig. S6 (A) TEM of CNNS and (B) zeta potentials of 200 μg mL^{-1} CNNS (a), m-CNNS probe (b) and mf-CNNS probe (c).

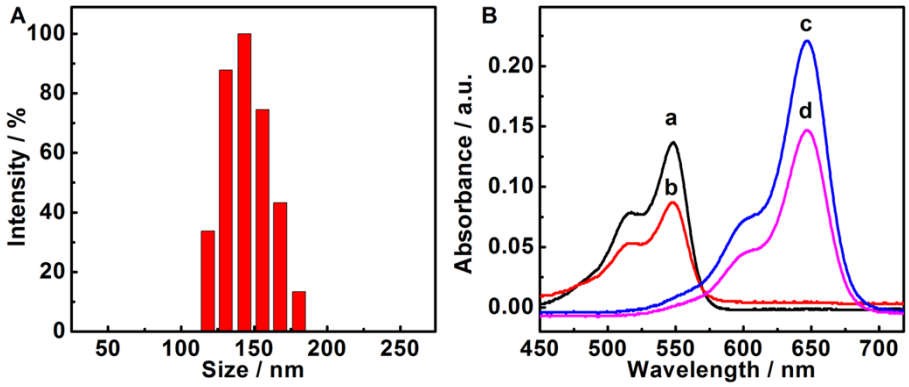

Fig. S7 (A) DLS characterization of 150 μg mL^{-1} CNNS, and (B) UV-visible absorption spectra of 500 nM Cy3-ssDNA in the absence (a) and presence (b) of 1.0 mg mL^{-1} CNNS, and 500 nM Cy5-ssDNA in the absence (c) and presence (d) of 1.0 mg mL^{-1} CNNS. Curves (b) and (d) were measured using 1.0 mg mL^{-1}CNNS as blank.

In vitro fluorescence quenching and recovery for miRNA sensing

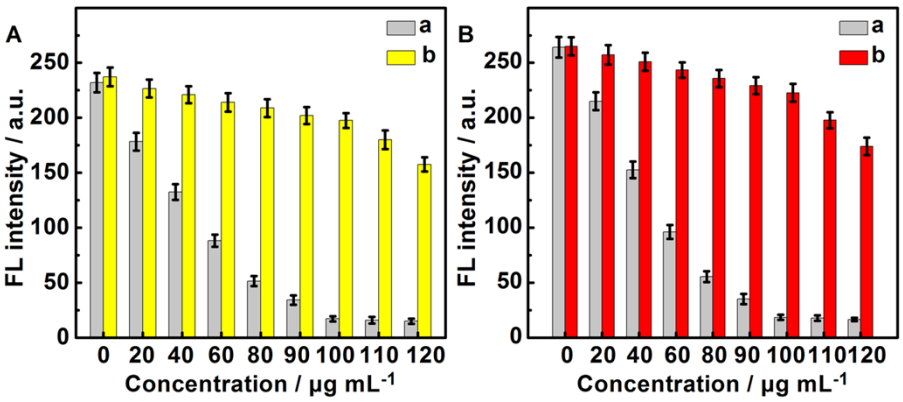

Fig. S8 FL intensity of (A) 50 nM Cy3-ssDNA after incubation with 0, 20, 40, 60, 80, 100, 110 and 120 μg mL^{-1} CNNS for 5 min (a) and then 250 nM miRNA-224 at 37 °C for 1 h (b), and (B) 50 nM Cy5-ssDNA after incubation with 0, 20, 40, 60, 80, 100, 110 and 120 μg mL^{-1} CNNS for 5 min (a) and then 250 nM miRNA-18a at 37 °C for 1 h (b). The emission of Cy3/Cy5 is monitored at 567/667 nm with an excitation wavelength of 543/640 nm. All measurements are performed in HB.

Gel electrophoresis

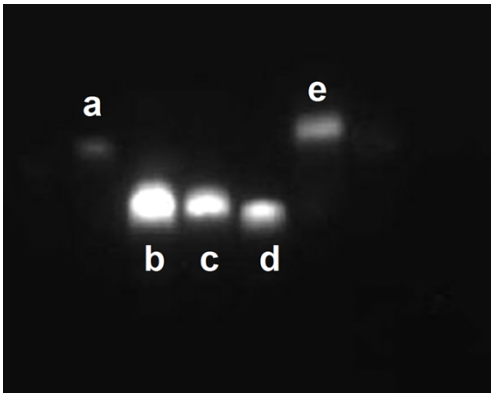

Fig. S9 Electrophoresis image of 5.0 μM miRNA-224 (a), supernatants obtained after mixing 5.0 μM miRNA-224, 5.0 μM miRNA-224 and 20 mg mL^{-1} mf-CNNS probe (b), 5.0 μM miRNA-18a and 10 mg mL^{-1} f_2-CNNS probe (c), and 5.0 μM miRNA-224 and 10 mg mL^{-1} f_1-CNNS probe at 37 °C for 1 h (d), and 5.0 μM Cy3-ssDNA (e).

Specificity of miRNA sensing

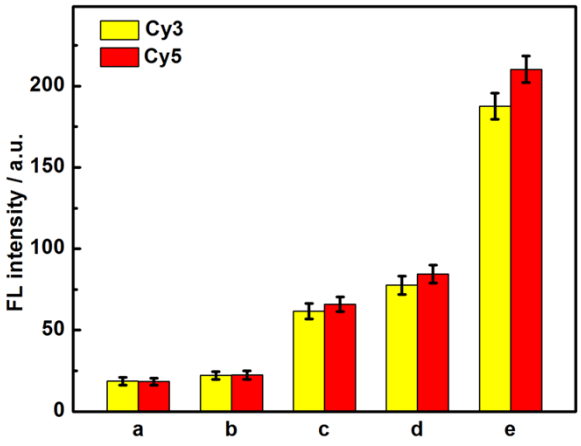

Fig. S10 FL intensity of 200 μg mL^{-1} mf-CNNS probe (a) after incubation with 250 nM non-complementary RNA (b), 250 nM tmT1/tmT2 (c), 250 nM smT1/smT2 (d) and 250 nM T1 /T2 (e) at 37 °C for 1 h. The emission of Cy3/Cy5 is monitored at 567/667 nm with an excitation wavelength of 543/640 nm. All measurements are performed in HB.

Protection properties of CNNS

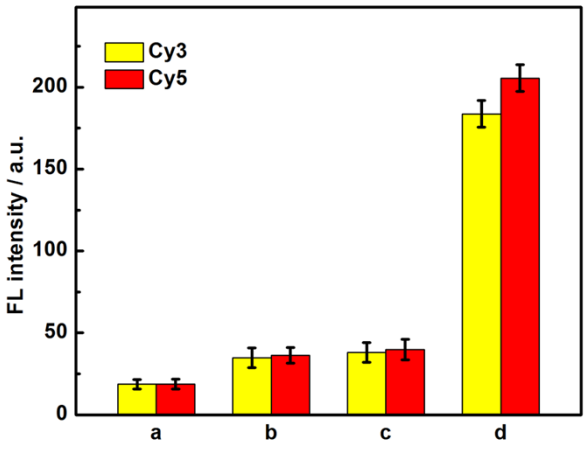

Fig. S11 FL intensity of mf-CNNS probe (a), mf-CNNS probe after incubation with 250 nM SSB (b), 1.0 U DNase I (c) and 250 nM T1 /T2 (d) at 37 °C for 1 h. The emission of Cy3/Cy5 is monitored at 567/667 nm with an excitation wavelength of 543/640 nm. All measurements are performed in HB.

Cytotoxicity of CNNS with MTT assay

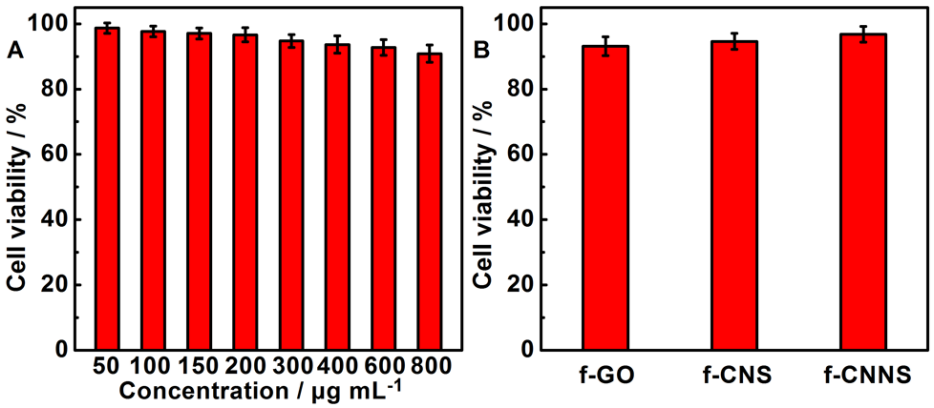

Fig. S12 Viability of HepG2 cells after incubation with (A) 50, 100, 150, 200, 300, 400, 600 or 800 μg mL^{-1} f-CNNS, and (B) 200 μg mL^{-1} f-CNNS, f-CNS or f-GO at 37 °C for 3 h.

Specific delivery of f-CNNS

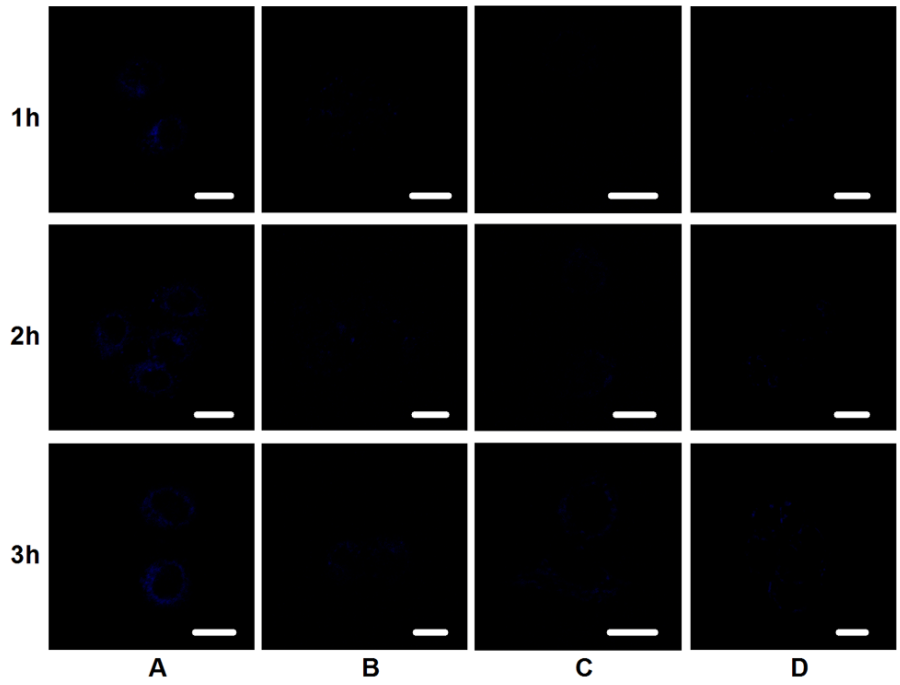

Fig. S13 Confocal FL images of HepG2 cell transfected with 200 μg mL^{-1} (A) f-CNNS and (B) CNNS, and (C) A549 and (D) HaCaT cell transfected with 200 μg mL^{-1} f-CNNS at 37 ^{o}C for 1, 2 and 3 h. Scale bars: 20 μm.

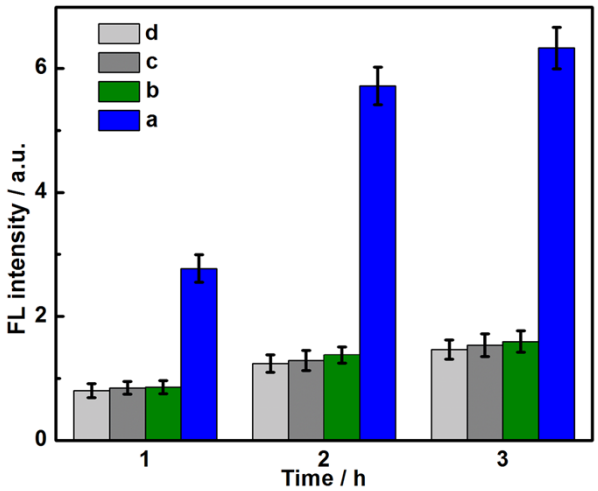

Fig. S14 Confocal FL intensity of HepG2 cell transfected with 200 μg mL^{-1} (a) f-CNNS and (b) CNNS, and (c) A549 and (d) HaCaT cell transfected with 200 μg mL^{-1} f-CNNS at 37 ^{o}C for 1, 2 and 3 h.

Optimization of incubation time and amount of mf-CNNS probe for cell transfection

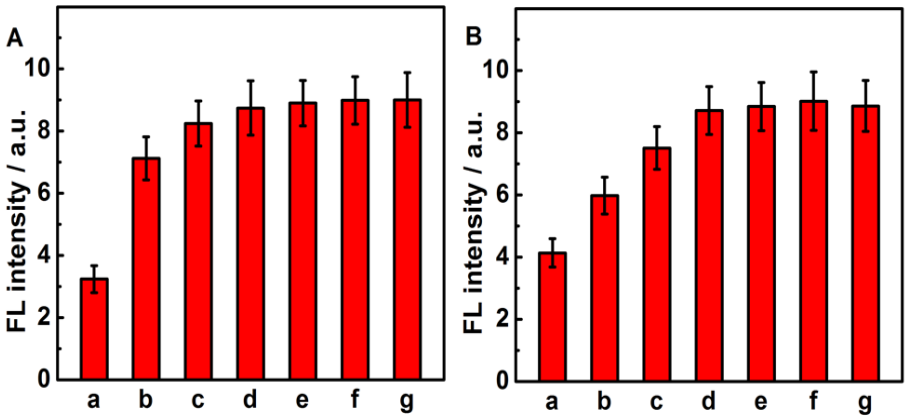

Fig. S15 Confocal Cy5 FL intensity of HepG2 cell after incubation with (A) 200 μg mL^{-1} mf-CNNS probe at 37 °C for 1, 2, 2.5, 3, 3.5, 4 and 5 h (from a to g), (B) 50, 100, 150, 200, 250, 300 and 350 μg mL^{-1} mf-CNNS probe at 37 °C for 3 h (from a to g).

Confocal microscopic analysis of intracellular miRNAs

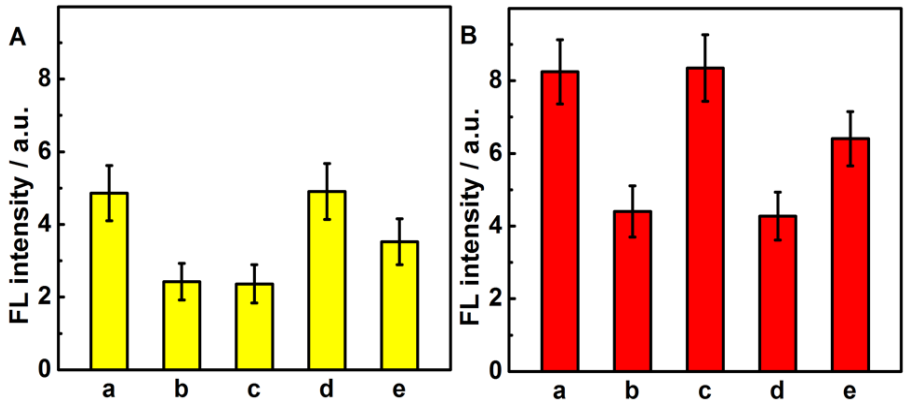

Fig. S16 FL intensity of Cy3 (A) and Cy5 (B) from confocal microscopic analysis of HepG2 cells (a), HepG2 cells treated with 50 nM inhibitor-18a and inhibitor-224 (b), 50 nM inhibitor-224 (c), 50 nM inhibitor-18a (d) for 48 h, and HeLa cells (e), and then transfected with 200 μg mL^{-1} mf-CNNS probe at 37 °C for 3 h.

Flow cytometric analysis of intracellular miRNAs

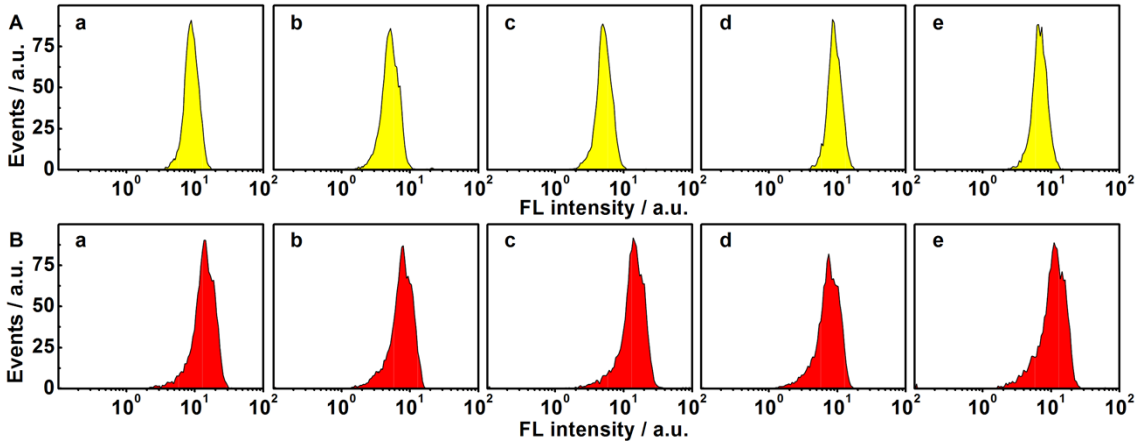

Fig. S17 Histograms of Cy3 (A) and Cy5 (B) from flow cytometric analysis of HepG2 cells (a), HepG2 cells treated with 50 nM inhibitor-18a and inhibitor-224 (b), 50 nM inhibitor-224 (c), 50 nM inhibitor-18a (d) for 48 h, and HeLa cells (e), and then transfected with 100 μg mL^{-1} f_1- and f_2-CNNS probes at 37 ^oC for 3 h, respectively.

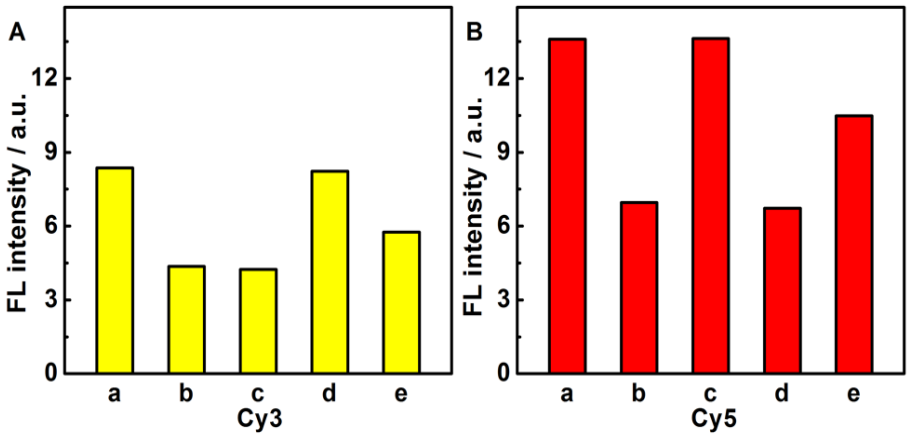

Fig. S18 FL intensity of Cy3 (A) and Cy5 (B) from flow cytometric analysis of HepG2 cells (a), HepG2 cells treated with 50 nM inhibitor-18a and inhibitor-224 (b), 50 nM inhibitor-224 (c), 50 nM inhibitor-18a (d) for 48 h, and HeLa cells (e), and then transfected with 100 μg mL^{-1} f_1- and f_2-CNNS probes at 37 ^oC for 3 h, respectively.

Supporting references

[S1] X. M. Sun and Y. D. Li, *Angew. Chem. Int. Ed.*, 2004, **43**, 597−601.

[S2] X. D. Zhang, X. Xie, H. Wang, J. J. Zhang, B. C. Pan and Y. Xie, *J. Am. Chem. Soc.*, 2013, **135**, 18−21.

[S3] (a) E. Heister, V. Neves, C. Tîlmaciu, K. Lipert, V. S. Beltrán, H. M. Coley, S. R. P. Silva and J. McFadden, *Carbon*, 2009, **47**, 2152−2160; (b) Q. B. Wang, W. Wang, J. P. Lei, N. Xu, F. L. Gao and H. X. Ju, *Anal. Chem.*, 2013, **85**, 12182−12188.