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In situ quantitation of intracellular microRNA in whole cell cycle with a functionalized carbon nanosphere probe[†]

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Experimental

Materials and reagents. D-(+)-Glucose (analytical purity, Sinopharm Chemical Reagent Co. Ltd, China). A549, HepG2 and HaCaT cells, pH 7.4 PBS, 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 late endosomal/lysosomal tracker were obtained from Invitrogen Corporation (USA). Folic acid (FA) and polyethylenimine (PEI, 25 K) were obtained from Sigma-Aldrich Inc. (USA). DNA hybridization buffer (HB) was phosphate-buffered saline (137 mM NaCl, 2.5 mM Mg²⁺, 10 mM Na₂HPO₄, and 2.0 mM KH₂PO₄, 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 (0.08 mg mL⁻¹) at 37 °C in a humidified atmosphere containing 5 % CO₂, while Opti-MEM was used during the transfection process. 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.

Oligonucleotides	Oligonucleotide sequences
miRNA-18a	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'
anti-miRNA (inhibitor probe)	5'-CUAUCUGCACUAGAUGCAC CUUA-3'
sense mimic sequence	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'
antisense mimic sequence	5'-AUCUGCACUAGAUGCACCUUAU U-3'
Cy5-ssDNA	5'-Cy5-CTATCTGCACTAGATGCACCTTA-3'
single-base mismatched strand	5'-UAAGGUGCAUCGAGUGCAG AUAG-3'
three-base mismatched strand	5'-UAAGGAGCAUCUCGUGCAU AUAG-3'
non-complementary RNA	5'-CAAGUCACUAGUGGUUCCGUU-3'
background calibration strand	5'-Cy5-ATCAGTCTGATAAGCTA-3'

Table S1. Oligonucleotides employed in this work

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). The emission intensity of Cy5 was collected from 650 to 720 nm with an excitation wavelength of 633 nm. Scanning electron microscopic (SEM) image was obtained on a Hitachi S-4800 scanning electron microscope (Japan) at an acceleration voltage of 5.0 kV. Dynamic light scattering (DLS) was observed on a 90 Plus/ BI-MAS equipment (Brook haven, USA). Fourier transform-infrared (FT-IR) spectra were measured on a Nicolet 400 FT-IR spectrometer (Madison, WI). 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-vis spectrophotometer (Nanodrop-2000C, Nanodrop, USA). 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).

Synthesis of CNSs. The CNSs was prepared from glucose under hydrothermal condition with some modifications.^{S1} 3.2 g glucose was dissolved in 70 mL water, which was placed in a 100 mL Teflon-sealed autoclave and maintained at 180 °C for 3.5 h. The product was isolated by centrifugation, cleaned by three cycles of centrifugation/washing/redispersion in water and then in alcohol, and oven-dried at 80 °C for more than 4 h.

Preparation of functionalized CNS probes. 1.2 mL 1.0 mg mL⁻¹ CNSs was dispersed in 8.3 mL 0.1 M pH 7.4 PBS and sonicated for 5 min to obtain a homogeneous dispersion. Subsequently, 0.5 mL 20 μM Cy5-ssDNA was added to the dispersion and stirred 3 h at room temperature. After the mixture was centrifuged at 15000 rpm for 20 min, the supernatant was collected to characterize the amount of Cy5-ssDNA loaded on CNS by FL detection with a standard curve method. The average quantity of Cy5-ssDNA loaded on 1.0 mg L⁻¹ CNS was 7.68 nM. The resulting CNS probe was dispersed in 10 mL pH 7.4 PBS containing 6 μM FA and stirred for 1 h at room temperature to obtain the f-CNS probe. After the mixture was washed with 0.1 M pH 7.4 PBS and centrifuged at 15000 rpm for 5 min for three times, the resulting f-CNS probe was redispersed in 0.5 mL 0.1 M pH 7.4 PBS and stored at 4 °C. FA functionalized CNSs (FA-CNSs) were prepared by mixing the dispersion of CNSs and FA solution with a similar process. The concentration of f-CNS probe or FA-CNSs was expressed as the amount of CNSs. The CNS probe using background calibration (BC-CNS probe) was prepared with a similar process as f-CNS probe using background calibration strand instead of Cy5-ssDNA.

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 FA-CNSs (30 to 360 mg L⁻¹), 2.0% Lp2000 or 0.5 mM PEI 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 ($A_{test}/A_{control}$) ×100.

Apoptosis assay. The apoptosis experiments were carried out with Annexin VFITC/PI double staining. Briefly, 1.5×10^5 HepG2 cells were seeded for 12 h in a 6-well plate containing 1.5 mL fresh Opti-MEM in each well. These cells were then incubated with 1.5 mL Opti-MEM or Opti-MEM containing 120 mg L⁻¹ FA-CNSs or 2.0% Lp2000 for 3 h. The resulting cells were collected, stained with 5.0 µL Annexin V-FITC and 5.0 µL PI for 10 min, and analyzed by flow cytometry.

Cell synchronization and flow cytometric analysis. Cells were synchronized at G1-S boundary by a double thymidine block.^{S2} In brief, 1.0×10^5 HepG2 cells were firstly seeded in a 6-well plate containing 1.5 mL fresh Opti-MEM in each well for 16 h. The exponentially growing HepG2 cells were sequentially treated with 1.5 mL Opti-MEM containing 2.5 mM thymidine for 18 h, 1.5 mL fresh Opti-MEM for 9 h, 1.5 mL Opti-MEM containing 2.5 mM thymidine for another 18 h, and re-fed with 1.5 mL fresh Opti-MEM for 4 h, 8 h and 24 h to collect the cells at S-, G2/M- and G1-phases, respectively.

The cells synchronized at different phases were fixed in 500 μ L 70% ice-cold ethanol and incubated at 4 °C for at least 2 h. After the cells were washed with PBS, centrifuged and

resuspended in 100 μL RNase A at 37 °C for 30 min, 400 μL PI was added in the suspension at 4 °C for another 30 min to perform cell-cycle analysis with flow cytometry.

Cell-specific transfection. 6.8×10^4 S-phase cells were firstly gained with the double-block method in 35-mm confocal dish. These cells were treated with 500 µL Opti-MEM containing 120 mg L⁻¹ f-CNS probe or CNS probe for for different times. For the competitive test, prior to transfection with f-CNS probe, the cells were incubated with 500 µL Opti-MEM containing saturated FA for 1 h to saturate the FR sites on the cell surface. As control, A549 and HaCaT cells were treated with 500 µL Opti-MEM containing 120 mg L⁻¹ f-CNS probe for different times.

Subcellular localization. The subcellular localization of f-CNS probe was observed with CLSM by incubating HepG2 cells with 1.0 μ M mixture of LysoTracker Green DND-26 and Hoechst 333342 for 20 min, or 120 mg L⁻¹ f-CNS probe for 20 min, 1 h and 2 h, respectively, and then 1.0 μ M LysoTracker Green DND-26 or 1.0 μ M Hoechst 333342 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.

Detection of intracellular miRNA. The amount of intracellular miRNA was measured with standard curve extrapolation using miRNA-18a as a model analyte. 6.8×10^4 HepG2 cells were cultivated in 35-mm confocal dish. Before the first block during the synchronization process, the cells were firstly incubated with 500 µL Opti-MEM containing miRNA-18a mimic of 0, 0.5, 1.0, 2.0, 3.0, 5.0, and 7.0 µg mL⁻¹ for 6 h. The average amount of miRNA-18a mimic delivered into each cell was obtained by detecting the miRNA-18a mimic left in the supernatant with UV absorbance at 260 nm and then calculating with (M_{before} - M_{after})/transfected cell number. Here M_{before} and M_{after} are the amount of miRNA-18a in the media before and after incubation. After

the double-block procedure to obtain the S-, G2/M- and G1-phase cells in 35-mm confocal dish, these cells were treated with fresh 500 μ L Opti-MEM containing 120 mg L⁻¹ f-CNS probe or BC-CNS probe for another 3 h, and then washed with PBS to perform confocal imaging analysis. The signal from the latter was used to eliminate background by adjusting the "offset" button of LSCM. The f-CNS probe transfected cells were also collected and resuspended in pH 7.4 binding buffer for flow cytometric analysis. The FL intensity was digitized by Leica Application Suite Advanced FL (LAS-AF) software. At each miRNA-18a mimic concentration the mean FL intensity was obtained from 5 transfected cells.

To obtain the real samples, the cells were firstly treated with 500 nM anti-miRNA (inhibitor) for 48 h to perform the inhibition experiment. The intracellular miRNA change was then monitored by confocal imaging after the cells were synchronized and treated with fresh 500 μ L Opti-MEM containing 120 mg L⁻¹ f-CNS probe for 3 h.





Fig. S1 DLS characterization of (A) CNSs and (B) f-CNS probe dispersed in ultrapure water with stirring for 5 min.



Fig. S2 (A) Characterization of f-CNS probe. SEM of (A) CNSs and (B) f-CNS probe, (C) FT-IR spectra of CNSs (a) and FA-CNSs (b), and (D) zeta potentials of CNSs (a), CNS probe (b) and f-CNS probe (c).

The FT-IR spectrum of CNSs (Fig. S2C) showed three bands centered at about 3300, 1720 and 1620 cm⁻¹ and one band in the range of 1050-1350 cm⁻¹ (curve a), similar to the previous report. ^{S1} The band at 3300 cm⁻¹ was attributed to the broad coupling of O–H vibrations, and the bands at 1720 and 1620 cm⁻¹ resulted from the C=O and C=C vibrations, respectively. The wide band in the range of 1050-1350 cm⁻¹ could be attributed to the C–OH stretching and O–H bending vibrations. These bands supported the aromatization of glucose and indicated the existence of –OH and –COOH groups. After FA was adsorbed on CNS surface through the π - π interaction between CNSs and the aromatic rings of FA, the bands for C–OH or O–H vibrations were weakened (curve b), suggesting the decreasing amount of free –OH group due to the formation of hydrogen bonding between –OH or –COOH groups of CNSs and –OH, –COOH or –NH₂ groups of the FA. In vitro fluorescence quenching/recovery and Gel electrophoresis for miRNA sensing



Fig. S3 (A) FL intensity of 1.0 μ M Cy5-ssDNA after incubation with 0, 20, 60, 100, 120, 140, 160 and 200 mg L⁻¹ CNSs for 3 h (a), and then 6.0 μ M miRNA-18a at 37 °C for 1 h (b) in HB with emission/excitation wavelength of 660/640 nm. (B) Electrophoresis image of 5.0 μ M Cy5-ssDNA (a), supernatant obtained after mixing 5.0 μ M miRNA-18a and 5.0 μ M f-CNS probe at 37 °C for 1 h (b), and 5.0 μ M miRNA-18a (c).

Specificity of miRNA sensing



Fig. S4 FL intensity of 120 mg L⁻¹ f-CNS probe (a) after incubation with 5.0 μ M noncomplementary RNA (b), three-base mismatched strand (c), single-base mismatched strand (d) and complementary target (e) at 37 °C for 1 h. The increased FL intensity for noncomplementary, three- and single-base mismatch RNA is only 1.98%, 20.8% and 29.5% of the complementary target, respectively.

Protection properties of CNSs



Fig. S5 (A) FL emission spectra of f-CNS probe (a), f-CNS probe after incubation with 6.0 μ M SSB (b) or 1.0 U DNase I (c) at 37 °C for 1 h, and 1.0 μ M Cy5-ssDNA (d) in HB. Inset: histogram of FL intensities. (B) FL intensity of 120 mg L⁻¹ f-CNS probe in pH 4.0, 5.0, 6.0, 7.0, 7.4 and 8.0 PBS buffer. The emission is monitored at 660 nm with an excitation wavelength of 640 nm.

The f-CNS probe suspension presented low FL intensity, while 1 μ M free Cy5-DNA exhibited a FL intensity of 194.2 (Fig. S5A, curves a and d, ESI[†]). Although the FL intensity of f-CNS probe increased from 18.6 to 32.8 or 39.5 after incubated with 1.0 unit DNase I or 6.0 μ M SSB (Fig. S5A, curves c and b, ESI[†]), the increased value was only 7.3% and 10.7% FL intensity of the free Cy5-DNA, respectively. Thus the CNSs could well resist DNase I cleavage and SSB interaction, demonstrating excellent protection properties of CNSs.

Cytotoxicity of CNSs



Fig. S6 Viability of HepG2 cells after incubation with (A) 30, 60, 120, 180, 240 or 360 mg L⁻¹ FA-CNSs, and (B) 0.5 mM PEI, 2% Lp2000 or 120 mg L⁻¹ FA-CNSs for 3 h. (C) Flow cytometric analysis of cell apoptosis induced by 120 mg L⁻¹ FA-CNSs and 2% Lp2000.





Fig. S7 Evaluation of cell-specific delivery via receptor-mediated endocytosis. (A) Confocal FL images of (a) HepG2 and (b) FR-saturated HepG2 cells transfected with 120 mg L⁻¹ f-CNS probe, (c) HepG2 cells transfected with 120 mg L⁻¹ CNS probe, (d) A549 and (e) HaCaT cells transfected with 120 mg L⁻¹ f-CNS probe at 37 °C for different times. Smart gain: 950 V, scale bars: 20 μ m. (B) FL intensities of HepG2 cells transfected with 120 mg L⁻¹ f-CNS probe at 37 °C for 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 h. (C) Histogram of corresponding mean FL intensities of (A).



Fig. S8 FL intensity of HepG2 cells transfected with 3.0 μ g mL⁻¹ miRNA-18a mimic for 6 h and synchronized at S phase and then incubated with 40, 60, 80, 100, 120, 140, 160 and 180 mg L⁻¹ f-CNS probe (from a to h) at 37 °C for 3 h.

Subcellular localization of f-CNS probe



Fig. S9 Confocal FL images of HepG2 cells transfected with (A) the mixture of 1.0 μ M Hoechst 333342 and LysoTracker Green DND-26 for 20 min, (B) 120 mg L⁻¹ f-CNS probe for 3 h and 1.0 μ M Hoechst 333342 for 20 min, (C) 120 mg L⁻¹ BC-CNS probe for 3 h, and (D-F) 120 mg L⁻¹ f-CNS probe for (D) 20 min, (E) 1 h and (F) 2 h and then 1.0 μ M LysoTracker Green DND-26 for 20 min. Scale bars: 20 μ m.

Cell synchronization and flow cytometric analysis



Fig. S10 (A) Distribution of unsynchronized cells as control, and (B-D) synchronized cells after release for 4 (B), 8 (C) and 24 h (D).

Inhibition experiment for monitoring the change of miRNA expression level s



Fig. S11 Confocal FL images of S-, G2/M- and G1-phase HepG2 cells transfected with 120 mg L⁻¹ f-CNS probe at 37 °C for 3 h (control), and 500 nM LP2000-anti-miRNA at 37 °C for 48 h and then 120 mg L⁻¹ f-CNS probe at 37 °C for 3 h (inhibition). Smart gain: 950 V, scale bars: 20 μ m.

Supporting references

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