Supplementary Information

Orderly Nucleic Acid Aggregates by Electrostatic Self-assembly in Single Cells for miRNA Detection and Visualizing

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S1. Reagents and Apparatus

Reagents: HeLa and MCF-7 cells were purchased from KeyGEN biotechnology Company (Nanjing, China). Human hepatocellular liver carcinoma cell line HepG2 was bought from Shanghai Bioleaf Biotechnology Company (Shanghai, China), and human normal hepatocytes L-02 and K562 cells were from Silver Amethyst Biotech. Co. Ltd. (Beijing, China). MirVana miRNA isolation kit and Fetal bovine serum were purchased from Life Technologies (Carlsbad, California). Tris-HCl, NaCl, $MgCl_2$, EDTA, 3-aminopropyltriethoxysilane (APTES), triethanolamine ($TEAH_3$), and tetraethyl-orthosilicate (TEOS) were purchased by Aladdin. All the water used in the work was RNase-free. Hybridization buffer (pH 7.4) contained 10 mM Tris-HCl, 50 mM NaCl, 1mM EDTA, and 10 mM $MgCl_2$. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), and dimethyl sulphoxide (DMSO) were bought from Sigma Chemical Company. Cetyl-trimethylammonium tosylate (CTATos) was purchased from Merck. Unless otherwise mentioned, ultrapure water was used throughout the experiments. All other reagents employed in this work were analytical

grade and used without further purification. All oligonucletides used in the present study were purchased by Sangon Biotech Co., Ltd. (Shanghai China). MicroRNAs sequences were synthesized and purified by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The sequences were as follows:

N1: 5'-ATC AGA CTG ATG TTG A CAA AGT T CAA CAT CAG TCT GAT AAG CTA-3'

N2: 5'-ACT TTG TCA ACA TCA GT-(ROX)-C TGA T TAG CTT A TCA GAC T-(BHQ)-GA TGT TGA-3'

miR-21: 5'-UAG CUU AUC AGA CUG AUG UUG A-3'

let-7a: 5'-UGA GGU AGU AGG UUG UAU AGU U-3'

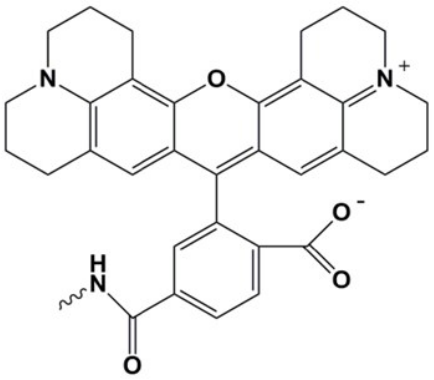
let-7b: 5'-UGA GGU AGU AGG UUG UGU GGU U-3'

let-7c: 5'-UGA GGU AGU AGG UUG UAU GGU U-3'

let-7d: 5'-**A**GA GGU AGU AGG UUG **C**AU AGU U-3'

Apparatus: Fluorescence imaging was performed by a Leica TCS SP8 inverted confocal microscope (Leica, Germany). The cellular images were acquired using a 100 × objective. Solid laser (561 nm) was used as excitation source for Rox-labeled probe, and a 562-700 nm bandpass filter was used for fluorescence detection. Transmission electron microscopy (TEM) was measured on a JEOL JEM-2100 instrument. All fluorescence measurements were carried out on a F4600 fluorometer (Hitachi, Japan).

The structure of Rox



S2. Cell Culture and miRNAs Preparation

MCF-7, Hela, HepG2 and L-02 cells were respectively cultured in RPMI 1640 (Hyclone, penicillin 100 U/mL, streptomycin 100 μg/mL) adding 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere including 5% CO_2, in terms of the instructions of the American Type Culture Collection. Hela cells were selected as a representative for analyzing the intracellular miR-21 level. Hela cells were gathered and centrifuged at 3000 rpm for 7 min in a culture medium, washed three times with phosphate buffered solution (pH 7.4), and then spun down at 3000 rpm for 7 min. The cell pellets were suspended in 700 μL of lysis solution. Total RNA was extracted from Hela cells using the mirVana miRNA Isolation Kit on the basis of the manufacturer's procedures. The sample of miR-21 in these cells was diluted, afterwards, analysed for the subsequent miRNA experiments.

S3. Synthesis and Analysis of PCMSNs

MSNs were prepared with some modifications according to the literature.[S1] 17.35 g $TEAH_3$, 96.0 g CTATos and 5.0 L ultrapure water were stirred at 80 °C for 90 min. Then 729.0 g TEOS was rapidly added into the surfactant solution only after the surfactant was fully dissolved in water including the small organic amine (SOA). This mixture (molar composition, SiO_2 : CTATos : SOA : H_2O =1.0 : 0.06 : 0.026 : 80.0) was stirred at 80 °C for 2 hours. The prepared MSNs were filtered, swashed, and dried in the oven at 100 °C for 20 hours. Then 2 g MSNs were dispersed in 200 ml anhydrous ethanol, then APTES was added and stirred for 6 h at 36 ºC. The synthesized product was filtered and rinsed with ethanol and dried at 60 ºC to get PCMSNs (Fig. S1).

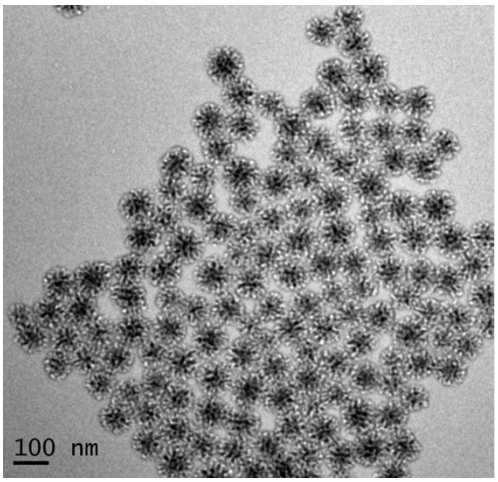


Fig. S1 TEM image of PCMSNs synthesized (about 80 nm)

The N_2 adsorption-desorption isotherms displayed that the narrow Barrett-Joyner-Halenda (BJH) pore-size distributions of PCMSNs and MSNs were different (Fig. S2). Compared with MSNs, the pores of 4 nm diameter were almost completely disappeared in PCMSNs, and the pores of 20 nm diameter were decreased obviously. Moreover, PCMSNs and MSNs have respectively the Burnauer-Emmett-Teller (BET)-surface areas of 237.3 m^2 g^{-1} and 438.7 m^2 g^{-1}. The results demonstrated massive positively charged aminopropyl groups have modified in the pores of PCMSNs.

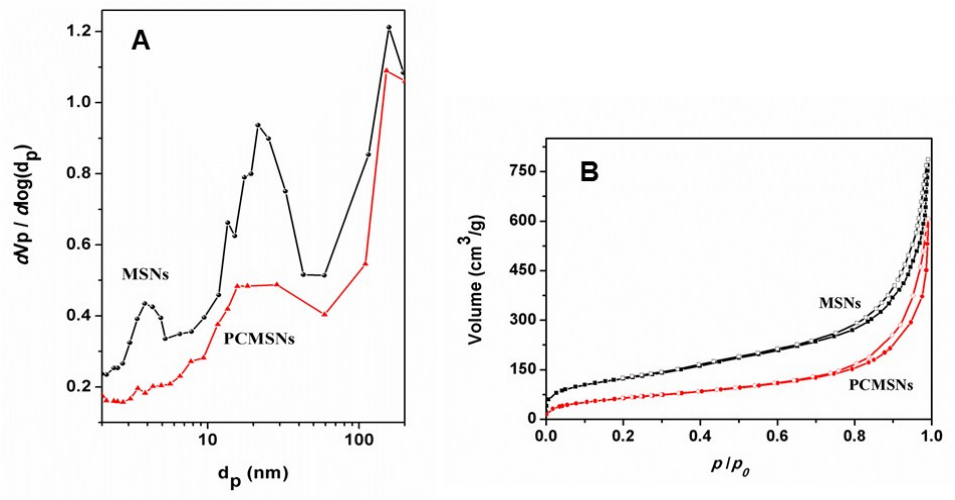


Fig. S2 (A) Pore size distributions of PCMSNs and MSNs; (B) N_2 adsorption-desorption isotherms of PCMSNs and MSNs.

S4. Preparation of the PCMSNs-N1N2 probes

PCMSNs were dispersed in 2 mL hybridization buffer (pH 7.4), and stirred continuously at 37 ^{o}C for 1 h to obtain the PCMSNs solution (10 mg/ml). The hairpin DNA-N1 probe (25ul 1.0 $\times 10^{-5}$ M), the hairpin DNA-N2 probe (25ul 1.0 $\times 10^{-5}$ M), 50 ul of 10 mg/ml PCMSNs and 500 ul hybridization buffer were mixed in a 1.5 mL Eppendorf tube. After the mixture was stirred continuously at 37 °C for 3 h, the excess reagents were moved away by centrifuging at 10000 rpm for 10 min. The sediment was washed and centrifuged repeatedly for two times to get the PCMSNs-N1N2 probes .

S5. The Cytotoxicitiy Tests of PCMSNs assembled N1 and N2 probes

The cytotoxicity tests of PCMSNs assembled N1 and N2 probes (PCMSNs-N1N2) were studied with HeLa cells by MTT experiment,[S2, S3] as shown in Fig. S3. Briefly, the cells were incubated with 200 μL culture medium containing 15 μL PCMSNs-N1N2 probes for different times, then were washed three times with 200 μL phosphate buffered solution (pH 7.4), MTT (0.5 mg mL^{-1}, 100 μL) was seeded in the wells and incubated at 37 °C for 4 h. Then 150 μL DMSO was added to each well to dissolve the crystals constituted by the living cells, and the absorbance at 490 nm was tested to acquire the relative cell viability. The test showed that HeLa cells maintained about 90.7% of the cell viability by $(A_{test}/A_{control})\times 100\%$ after incubation with 15 μL probe for 6 h, demonstrating that the excellent biocompatibility for the self-assembly.

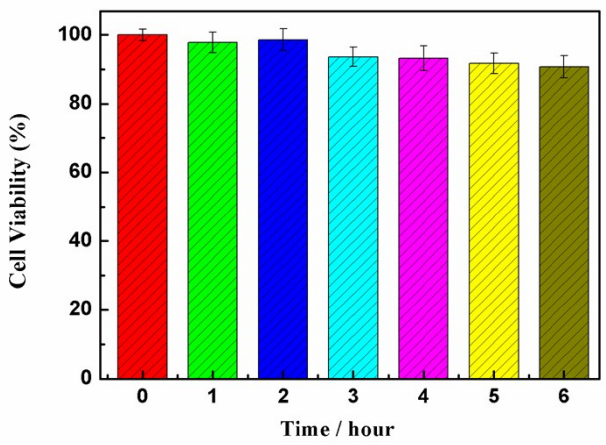


Fig. S3 Viability of HeLa cells (100 μL, 1.0×10^{6} mL^{-1}) after incubation with PCMSNs-N1N2 probes (50 ug/ml)for different times.

S6. Controlled Experiments of Fluorescence Responses and Analysis of the HCR reaction

To evaluate the feasibility of PCMSNs-ONAAs method for miR-21 determination, a series of controlled experiments were performed (Fig. S4). The target miR-21 in a reaction system (curve a) triggered hybridization chain reaction (HCR) to generate ONAAs-PCMSNs, enabling fluorescence intensity being significantly enhanced, which verified the feasibility of this method.

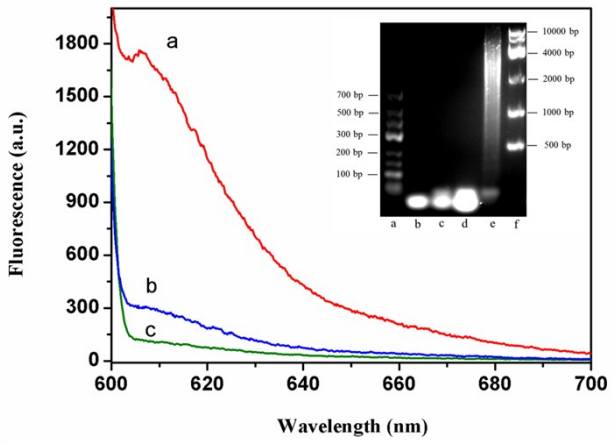


Fig. S4 A series of controlled experiments performed by fluorescence responses: (a) ONAAs-PCMSNs self-assembly by N1 and N2 with target miR-21, (b) PCMSNs carrying the N1 and N2 probes, (c) PCMSNs carrying the N2 probes. **Inset:** the HCR reaction was analyzed by 1.0% agarose gel electrophoresis, (a, f) the marker, (b)N1, (c)N2 ,(d) the mixture of N1 and N2, (e) the HCR product.

S7. Optimization of the experimental conditions

The incubation time, pH and temperature of the reaction solution are three important factors affecting DNA hybridization. For achieving the best sensing performance, a series of control experiments were designed to optimize the incubation time, pH and temperature. Fluorescence intensity enhanced speedily when incubation time increased from 1h to 4h. But after 4h, the fluorescence intensity reduced slightly. So 4h of incubation was considered to be the optimum. Fig. S5 displayed the influence of pH and temperature on the fluorescence signal measured by 1.0×10^{-10} M miR-21. On account of physiological conditions in living cells, pH 7.4 and 37 ℃ were chosen for subsequent experiments.

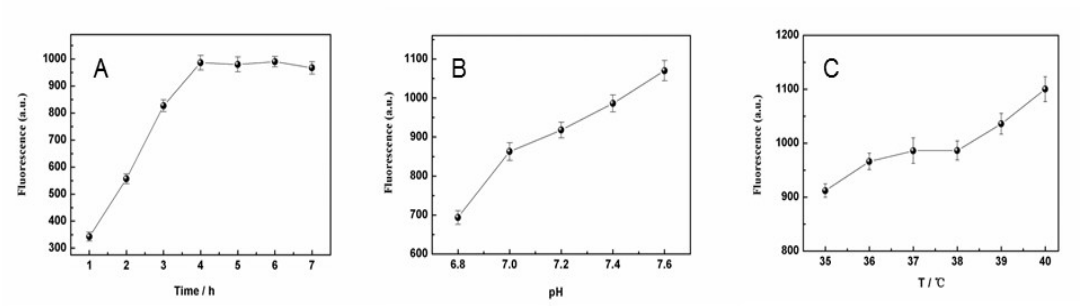
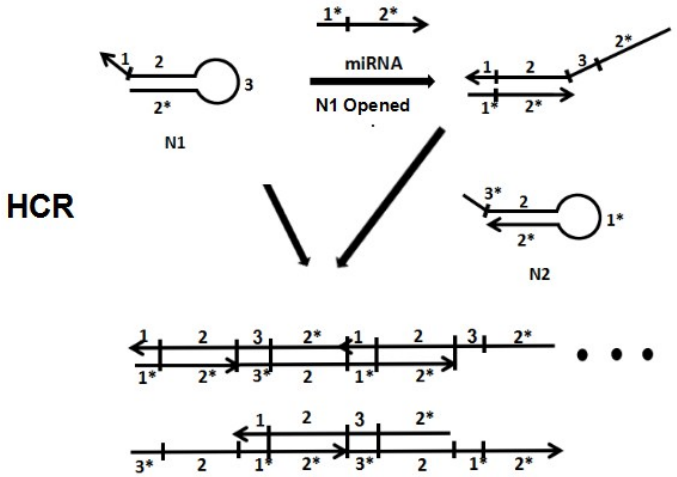


Fig. S5 (A) Effect of the incubation time, (B) pH and (C) temperature, on the fluorescence intensity responding of 1.0 $\times 10^{-10}$ M miR-21 in hybridization buffer.

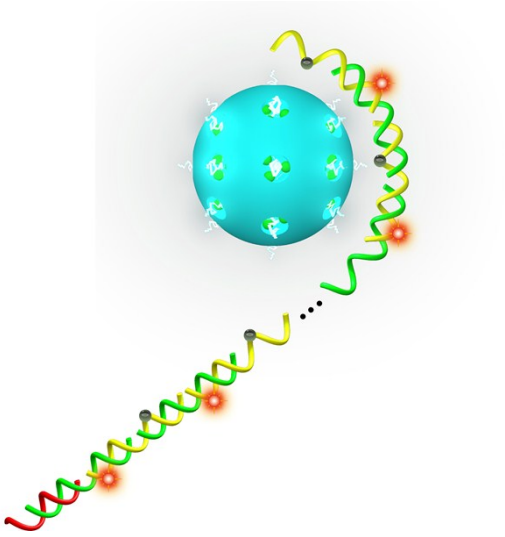
S8. The specific process of HCR hybridization



Scheme S1 Schematic representation of the HCR process[S4-S5].

S9. The Structure of ONAAs-PCMSNs

The ONAAs-PCMSN self-assembly possessed a unique nanostructure with a positively charged interlayer and a PCMSN core, based on a target-initiated hybridization chain reaction.



Scheme S2 The structure of ONAAs-PCMSNs.

S10. Internalization mechanism of PCMSNs-N1N2 probe and evaluation for the fluorescence signals in cytoplasm.

According to the references,[S6-S9] PCMSNs can be rapidly taken up by the cells ascribed to the targeted adsorption and endocytosis, which primarily led to the higher level of nanoparticles internalization into cells within 2h. HeLa cells (200ul , 1×10^6 mL^{-1}) were seeded in the wells of 6-well plate for 24 h, and incubated with PCMSNs-N1N2 probe at 4 ^oC and 37 ^oC for 2 h, then were washed three times with phosphate buffered solution (pH 7.4). The internalization and localization of the probe were characterized by inversion fluorescence microscope (Olympus, IX53) in Fig. S6. High temperature (37 ^oC) obviously displayed the internalization of PCMSNs-N1N2 probe in HeLa cells, implying an energy-dependent endocytosis of this PCMSNs-N1N2 probe. It was observed that most of ONAAs-PCMSN signals were localized in cytoplasm nearby the nucleus (37 ^{o} C), as shown in Fig. S6, which were consistent with the endocytosis independent mechanism for cellular uptake of the nanoassembly[S10].

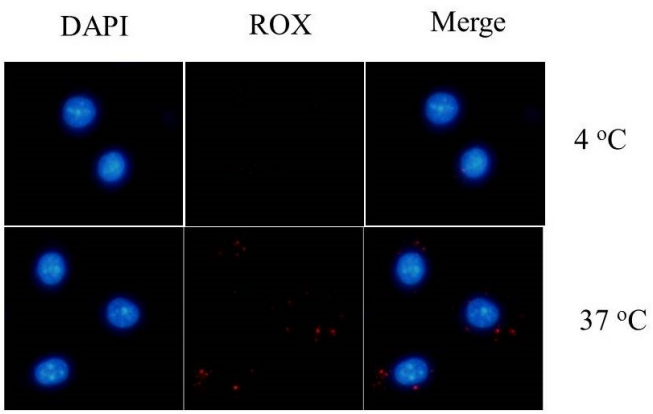


Fig. S6 The fluorescence Images of HeLa cells after incubation with PCMSNs-N1N2 probe (50 ug mL^{-1}) at 4 °C or 37 °C for 2h.

S11. The cells incubation and fluorescence intensity

For the fluorescence intensity and imaging experiments, four types of cells (MCF-7, Hela, HepG2, L-02) were cultured in 6-well slides. The cells were incubated with 600 μL culture medium containing the PCMSNs-N1N2 probes (50 ug/ml) for 4 h, then were washed three times with phosphate buffered solution (pH 7.4). Subsequently, 0.1 mol/L SDS (sodium dodecyl sulfate) solution was added to the mixture to attain an SDS concentration of 20 mmol/L.[S11] After these processed cells were lyzed, the mixture was centrifuged (2000 r/min, 6 min) three times, and each supernatant was gathered. Fluorescence measurements of the reaction products were implemented by a F4600 fluorometer. The fluorescence intensity of the cells treated by the ONAAs-PCMSNs method was evaluated (Fig. S7-A). In Fig. S7-B, upon addition of the PCMSNs-N1N2 probes, the fluorescence intensity of the MCF-7 cells hardly added within initial 30 min. After 60 min, the intensities gradually increased due to the target triggered HCR in living cells. The fluorescence intensity was attained the maximum at 240 min. The fluorescence intensitiy changed hardly after 240 min.

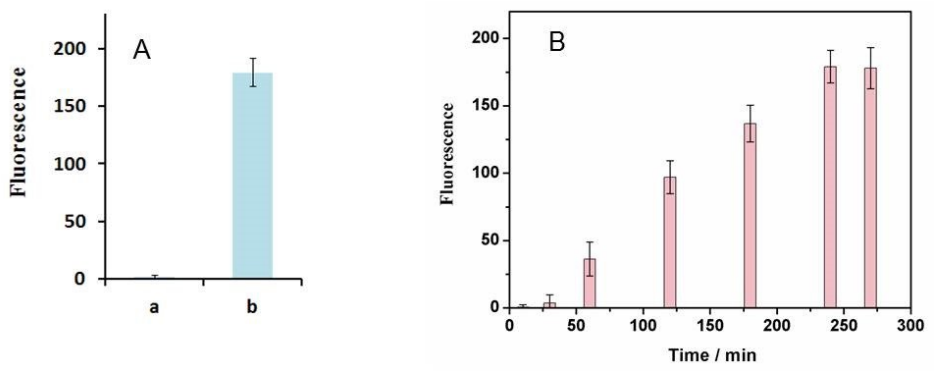


Fig. S7 (A) The fluorescence intensity of the cells treated with non-targeting ONAAs-PCMSNs (a), with ONAAs-PCMSNs (b) in MCF-7 cells; (B) the fluorescence intensities of time course of the MCF-7 cells by the ONAAs-PCMSNs method.

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