Electronic Supplementary Material (ESI) for Chemical Science.
This journal is © The Royal Society of Chemistry 2023

Supporting for:

Quantitative detection of microRNA-21 in vivo by in-situ assembled photoacoustic and SERS nanoprobes

Liting Zheng,[‡a] Qingqing Li,[‡a] Ying Wu,[b] Lichao Su,[a] Wei Du,[a] Jibin Song,[b] Lanlan Chen*[a] and Huanghao Yang*[a]

[a] *Fujian Science & Technology Innovation Laboratory for Optoelectronic Information of China; MOE key laboratory for analytical science of food safety and biology, College of Chemistry, Fuzhou University, Fuzhou 350108, China.*

[b] *State key Laboratory of Chemical Resource Engineering, College of Chemistry, Beijing University of Chemical Technology, Beijing 10010, China.*

Email: hhyang@fzu.edu.cn; llchen@fzu.edu.cn

Experimental materials and reagents: Thiolated methoxy polyethylene glycol (mPEG-SH), N, N-dimethylformamide (DMF, ≥99.5 %), Cetyltrimethylammonium bromide (CTAB, 99%), sodium borohydride ($NaBH_4$, 96%), Sodium hydroxide (96%), poly(lysine), Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), L-ascorbic acid, sodium citrate (99%), poly (vinyl pyrrolidone) (PVP, MW = 40,000), Silver nitrate ($AgNO_3$), 2-Naphthalenethiol (2-NAT) was purchased from Sigma-Aldrich. Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) was purchased from Sangon biotechnology Co., Ltd. CCK-8 kit was obtained from Dojindo Molecular Technologies, Int. 2-proponal, 4-Mercaptobenzonitrile (4-MBN), poly (acrylic acid) (PAA, MW=5,000) were purchased from J&K Scientific and n-butanol were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Lyso-Tracker Green were purchased from the Beyotime Biotechnology Co., Ltd. (Shanghai, China). All DNA oligonucleotides (listed in Table S1) were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). All the reagents were analytical grade. Ultrapure and deionized water acquired from a Millipore water purification system (18.2 MΩ cm^{-1} resistivity, Millipore).

Apparatus: Transmission Electron Microscopy (TEM) images were taken with a Hitachi HT7700 and a 100 kV accelerating voltage (Japan). The UV-vis-NIR absorption spectra were measured by using a UV-Visible/NIR Spectrophotometer UH4150 (HITACHI, Japan). Zeta-potential was measured and particle size was counted using a Dynamic Light Scattering (DLS) machine from Malvern Instruments Ltd. (England). Confocal laser scanning microscopy (CLSM) studies was performed by a Nikon A1 confocal fluorescence microscope (Nikon, Japan). Dark-field spectroscopic experiments was performed by an inverted optical microscope (Eclipse Ti2-E, Nikon, Japan) assembled with a dark-field condenser (0.8 < NA < 0.95) and a 40x objective lens (NA = 0.7). Raman spectra were recorded in vitro through Renishaw inVia™ confocal Raman microscopy (Renishaw, England). Raman mapping were performed by Horiba Xplora Plus (Horiba scientific, Japan) and Renishaw InVia Raman microscope (Renishaw, Hoffman Estates, IL). Photoacoustic (PA) imaging was obtained by applying the Vevo LAZR-X 3100 PA imaging system (Visual-Sonics Co. Ltd, Toronto, Canada).

Synthesis of Au@(PEG/NAT) Nanoparticles: In the first step, we synthesized 16 nm Au nanoparticles using the previously reported sodium citrate reduction method.[1] And then, in order to prepared Au@(PEG/NAT) nanoparticles. we first placed mPEG-SH onto the surface of the above prepared 16 nm Au nanoparticles, and stirred vigorously for 6 h to obtain Au@PEG nanoparticles. Thereafter, 900 μL, 10 mg mL^{-1} of NAT solution in DMF was added to the 10 mL DMF solution with 2 mL Au@PEG solution and stirred for 6 h. The Au@(PEG/NAT) was obtained by centrifugation at 12000 g for 15 min, and finally stored in H_2O.

Synthesis of AuNNP with NAT in the Nanogap: We synthesized AuNNP by using the previously reported Ag etching method.[2]

The prepared 8 mL of Au@(PEG/NAT) solution was added into a 50 mL, 1% PVP and 5 mL, 0.1 M CTAB mixed solution. Then the 0.01 M, 1.5 mL $AgNO_3$ and 0.1 M,1.25 mL AA solutions were added, following add the 0.1 M, 2.5 mL sodium hydroxide solution. After 15 minutes, the Au@(PEG/NAT)@Ag nanoparticles formed and cellected by centrifugation at 15000 g, 20 min. Subsequently, the above prepared Au@(PEG/NAT)@Ag solution in PVP was added to a solution with 100 mL PVP and 0.1 M, 10 mL CTAB, and then added a 0.4 mg mL^{-1}, 5 mL chloroauric acid solution. After stirring for 15 min. 0.1 M, 5 mL AA solution was then added, and the product was collected by centrifugation at 12000 g, 12 min. Finally, the product was stored in 2 mL of H_2O for further use.

Synthesis of AuNP/MBN Nanoparticles: The 10 mg mL^{-1}, 20 μL MBN was added to the above synthesized 16 nm AuNP solution under stirring. After reaction for 11 h, the products MBN coated AuNP (AuNP/MBN) were purified by centrifugation at 10000 g for 12 min and further redispersed in 20 mL of H_2O.

Oligonucleotide Functionalization of AuNP/MBN or AuNNP/NAT: DNA functionalization of AuNP/MBN according to the previously reported INDEBT method.[3] First, 10 μL, 10 mM of TCEP was added to 10 μL, 100 μM thiolated DNA to activate for 1 h at room temperature. And then, thiolated DNA was added to the above 16 nm AuNP solution in the presence of 10 μL, 0.1 M NaCl. After that, 900 μL of n-butanol was immediately added to the above solution with a quick vortex-mixing. Following, 200 μL of 0.5×TBE (Tris, 44.5 mM; EDTA, 1 mM; boric acid, 44.5 mM; pH 8.0) buffer was added to the above solution followed by another quick vortex-mixing and a brief centrifugation for several seconds at 2000 g to facilitate a liquid phase separation. Then, DNA-functionalized AuNP/MBN were obtained as a sublayer. Then, DNA functionalization of AuNNP/NAT followed a similar process, except that sodium chloride was not added.

The as-obtained AuNP/MBN/DNA_1 and AuNNP/NAT/DNA_2 were centrifugation at 10000 g for 12 min and 8000 g for 10 min respectively, then redispersed in PB buffer. This was repeated three times to remove excessive DNA. The final precipitates of DNA-capped AuNP/MBN and AuNNP/NAT (termed as AuNP probe and AuNNP probe) were dispersed in PB buffer (10 mM) and stored at 4 °C for further use.

In vitro detection of miR-21: To study the responsiveness of miR-21 detection in vitro, AuNNP probe/AuNP probe were incubated with various concentrations of miR-21 in PB buffer (10 mM). The AuNNP probe/AuNP probe was incubated at 37°C for 5 h to allow specific hybridization to occur. The assembly process was recorded by TEM, and DFM. Then, the optical absorption, PA signals and SERS signals of AuNNP probe/AuNP probe incubated with different concentrations miR-21 were measured. The probes were excited with laser irradiation at 785 nm and the SERS spectra over the range 1300 to 2300 cm^{-1} were collected. The SERS spectra was achieved after excitation for 1 s (785 nm excitation, 250 mW, 50× objective lens).

Specificity detection of miR-21: To illustrate the specificity of AuNNP probe/AuNP probe, the target miR-21, miR-144, miR-155, miR-429, let-7a, and mismatch-1 were incubated with the AuNNP probe/AuNP probe in PB buffer for 5 h at 37 °C.

Cell lines and mice: MCF-7 cells (human mammary cancer cell line), HeLa cells (human epithelial cancer cell line) and L02 cells (human hepatocyte cell line cells) were cultured in Roswell Park Memorial Institute Medium (RPMI, HyClone) containing 10% (v/v) fetal bovine serum (FBS, Gibco) and 100 IU mL^{-1} penicillin-streptomycin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

BALB/c nude mice (female, 6-8 weeks) were purchased from Shanghai SLAC Co., Ltd (Shanghai, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Fujian Medical University.

Cytotoxicity assay: In brief, MCF-7 cells or LO2 cells were cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a 5% CO2 incubator. MCF-7 cells or LO2 cells were then seeded in 96-well plates for 24 h, then different concentrations of AuNNP probe/AuNP probe were added to the 96-well plates. After 24 hours of incubation, each well was washed 2-3 times with PBS, followed by incubating with 10 μL of CCK-8 solution for 2 h at 37 °C and the absorbance at 450 nm was recorded.

QRT-PCR: Total RNAs was extracted from MCF-7 cells, Hela cells, LO2 cells, MCF-7 tumor tissue, Hela tumor tissue and muscle tissue, respectively, using Trizol reagent according to the manufacturer's instructions. We performed the reverse transcription (RT) reaction with SweScript RT I First Strand cDNASynthesis Kit (Servicebio) to prepared the cDNA samples. qRT-PCR analysis of miRNA was tested by using 2×SYBR Green qPCR Master Mix (Servicebio), according to the indicated protocol on a CFX Connect™ RealTime PCR Detection System (Bio-Rad, CA, USA). The relative expression of miR-21 was evaluated in triplicate by using the $2^{-\Delta\Delta Ct}$ method. The primers were described in Table S1.

Lysosome localization test: MCF-7 cells (1×10^5) were placed in the confocal dish and incubated for 24 h. Then, AMCA-labeled AuNNP probe and Cy-5-labeled AuNP probe were added to the dish and further incubated for different times. After incubation, the cells were washed with PBS to remove the free AuNNP probe/AuNP probe. Subsequently, the Lyso-Tracker

was incubated with the cells for 30 min to make lysosomes labeled with green fluorescence. After that, fluorescence images of the probes were acquired by CLSM.

DFM, bio-TEM and intracellular Raman mapping: To verify the responsiveness of AuNNP probe/AuNP probe to intracellular miR-21, AuNNP probe/AuNP probe (2 mg mL^{-1}) were incubated with MCF-7 cells or LO2 cells over different durations by using bio-TEM and DFM imaged. Briefly, for DFM imaging, the cells (approximately 10,000) were first seeded on cell climbing films pretreated with poly(lysine), then cultured in an atmosphere containing 5% CO_2 at 37°C for 24 h. The cells were then incubated with 2 mg mL^{-1} AuNNP probe/AuNP probe for 8, 12, 18, or 24 h. The cells were immobilized using 4% paraformaldehyde after rinsed with PBS three times. Dark-field images were collected using DFM using a color charge-coupled device.

To image thin sections with TEM, MCF-7 cells or LO2 cells were incubated with AuNNP probe/AuNP probe (2 mg mL^{-1}) for either 8, 12, 18, or 24 h at 37°C, then scraped by a cell scraper and collected by centrifugation. The cells were then fixed, embedded in paraffin, solidified, heated and then finally transferred onto copper grids for bio-TEM imaging.

To detect intracellular Raman signals and mapping, approximately 30,000 MCF-7, Hela or L02 cells were seeded onto quartz plates then incubated overnight at 37 °C in an atmosphere containing 5% CO_2. 2 mg mL^{-1} of AuNNP probe/AuNP probe were added to the cell samples, and cultured for another 4, 8, 12, 18, or 24 h. The cells were immobilized using 4% paraformaldehyde after rinsed with PBS three times. SERS spectra of AuNNP probe/AuNP probe treated cells were then recorded by using a Horiba Xplora Plus Raman confocal microscope (Horiba Scientific, Japan) with excitation at 785 nm. The SERS spectra was achieved after excitation for 1 s (785 nm excitation, 250 mW, 50× objective lens). Single cell SERS mapping was achieved after excitation for 2 s (785 nm excitation, 2 μm step size, 25 mW, 50× objective lens). The Raman images were measured for 20 μm × 20 μm area (440 pixels, 2 s/per pixel).

In vivo quantitative detection based on PA and ratiometric SERS: For in vivo experiments, a subcutaneous mammary cancer tumor model was established in healthy BALB/c nude mice (6-8 weeks old). MCF-7 cells (3×10^6) were injected subcutaneously and after the tumors had grown to approximately 80-100 mm^3. For each tumor model, the mice were randomly divided into 2 groups (injecteion of AuNNP probe/AuNP probe or PBS, n = 3 for each group).

To test in vivo PA imaging, AuNNP probe/AuNP probe in PB buffer (50 μL, 2 mg mL^{-1}) or PBS alone were injected locally into the tumor of the MCF-7 tumor-bearing mice. The AuNNP probe/AuNP probe or PBS was injected in symmetrical positions across the tumor. Following injection of AuNNP probe/AuNP probe or PBS, PA signals were collected at various time intervals, including 0.5, 4, 8, 12, 24, and 36 h. The acquired PA images were analyzed by VevoLAB software.

For in vivo SERS imaging, AuNNP probe/AuNP probe in PB buffer (50 μL, 2 mg mL^{-1}) were injected locally into the tumor or hindlimb muscles of the MCF-7 tumor-bearing mice. The AuNNP probe/AuNP probe was injected in symmetrical positions across the tumor or hindlimb muscles. The SERS spectra at 8, 12, and 18 h and mapping images were collected after NIR laser excitation using a Renishaw InVia confocal Raman microscope (Hoffman Estates, IL, USA) (785 nm excitation, 25 mW, 1 μm step size, 50× objective lens, 5 s exposure time).

Histological examination: The AuNNP probe/AuNP probe (2 mg mL^{-1}, 50 μL) was injected via intratumor injection of the mice. At 48 h post-injection, the mice were sacrificed and the organs (heart, liver, spleen, lung and kidney) were taken for H&E staining test.

Statistical analysis: All data were presented as mean ± s.d. (standard deviation). Comparison of the data was conducted with a t-test. Differences were regarded as statistically significant (* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

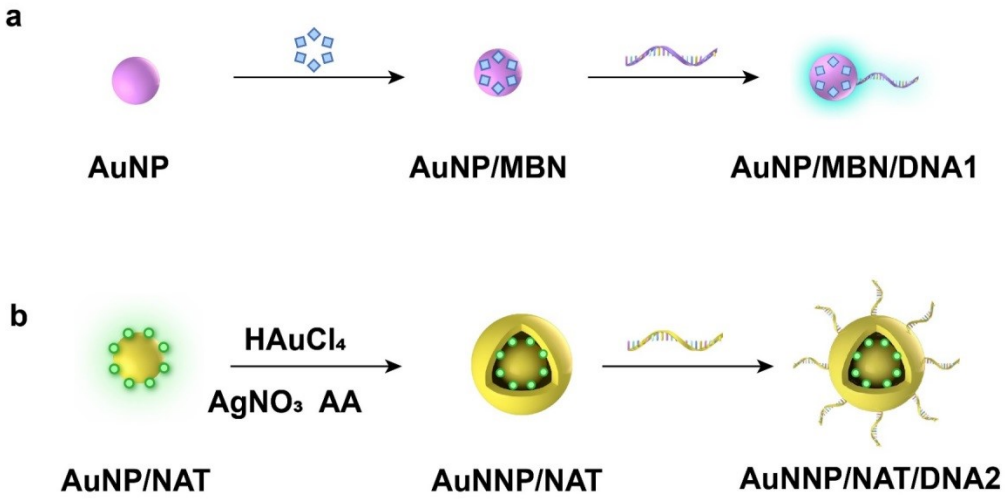

Fig. S1 Schematic diagram showing the fabrication process of AuNP probe (a) and AuNNP probe (b).

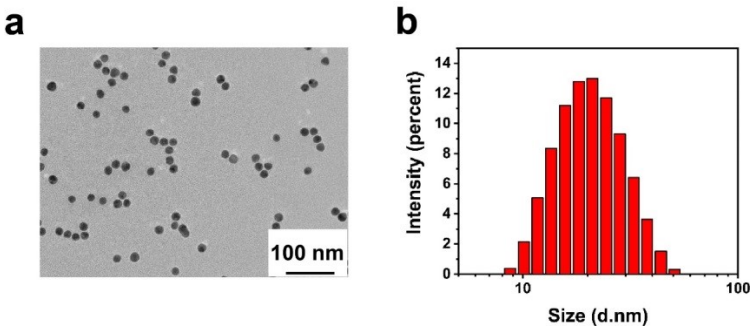

Fig. S2 TEM image (a) and size distribution (b) of AuNP.

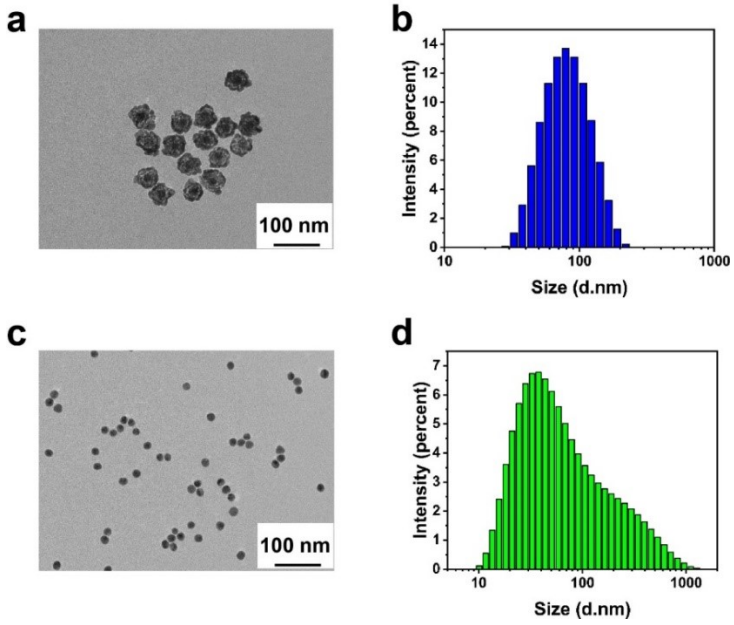

Fig. S3 TEM image (a) and size distribution (b) of AuNNP/NAT, TEM image (c) and size distribution (d) of AuNP/MBN.

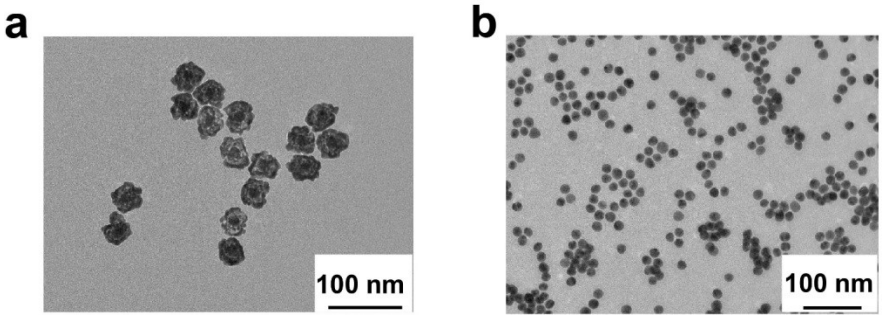

Fig. S4 TEM images of AuNNP probes (a) and AuNP probes (b).

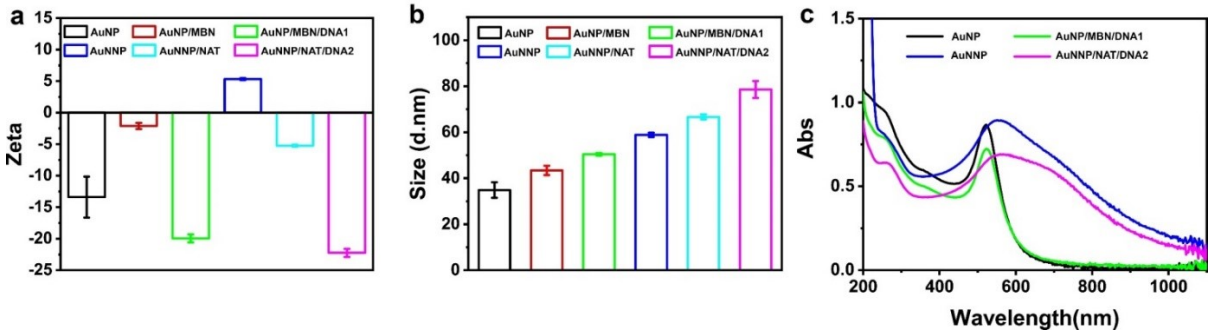

Fig. S5 Zeta potentials (a) and sizes (b) of AuNP, MBN modified AuNP, DNA_1 modified AuNP/MBN, AuNNP, NAT modified AuNNP, and DNA_2 modified AuNNP/NAT. (c) UV-vis spectra of AuNP, DNA_1 modified AuNP/MBN, AuNNP, and DNA_2 modified AuNNP/NAT.

Fig. S6

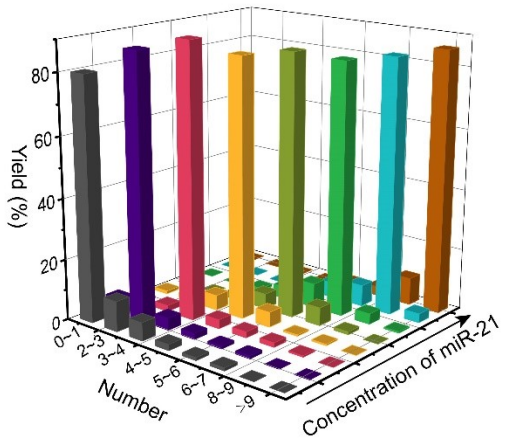

Fig. S6 Statistical analysis of TEM images with respect to different assembly products.

Fig. S7

$S_{AuNNP}=4\pi R^2$ (R=51 ± 2 nm)

$S_{AuNP}=4\pi r^2$ (r=16 ± 1 nm)

$N=S_{AuNNP}/S_{AuNP}=R^2/r^2=11 \pm 2$

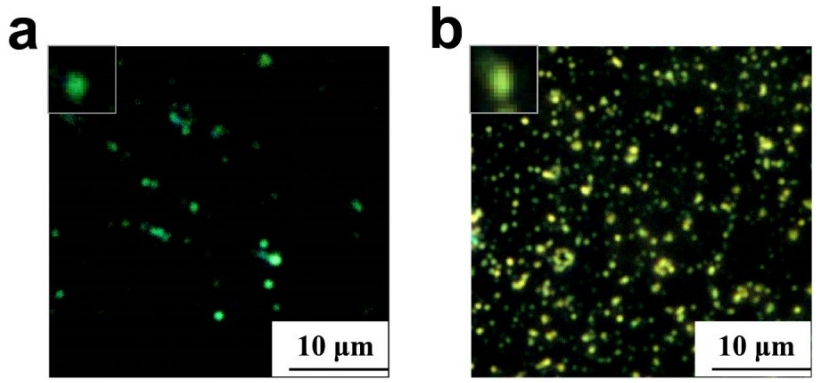

Fig. S8 DFM images of AuNP probes (a) and AuNNP probes (b).

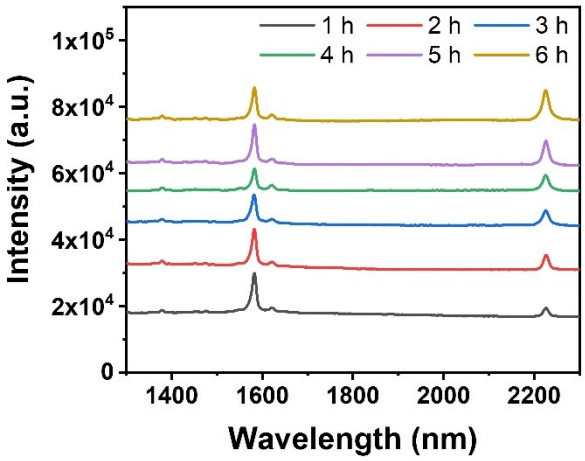

Fig. S9 SERS spectra of AuNNP probe/AuNP probe at different incubation time (from 1 to 6 h).

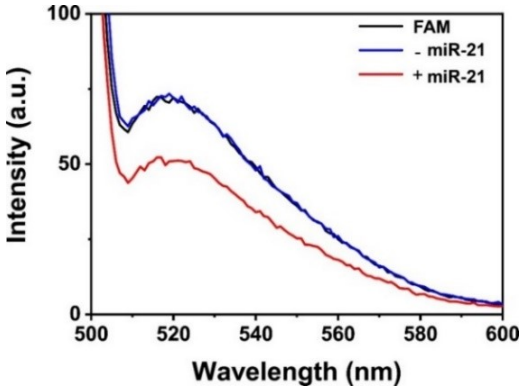

Fig. S10 The fluorescence intensity of DNA_1-FAM, in the present of miR-21 or in the absence of miR-21 incubated with DNA_1-FAM and DNA_2-BHQ1.

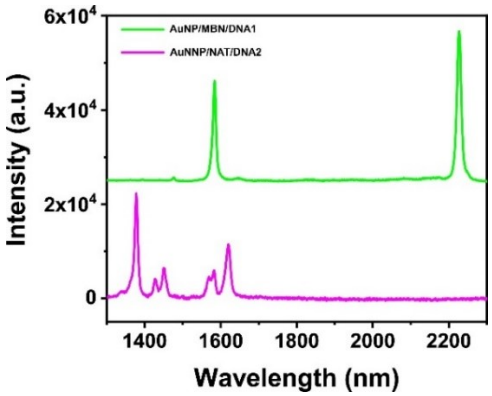

Fig. S11 The SERS spectra of AuNNP probe and AuNP probe.

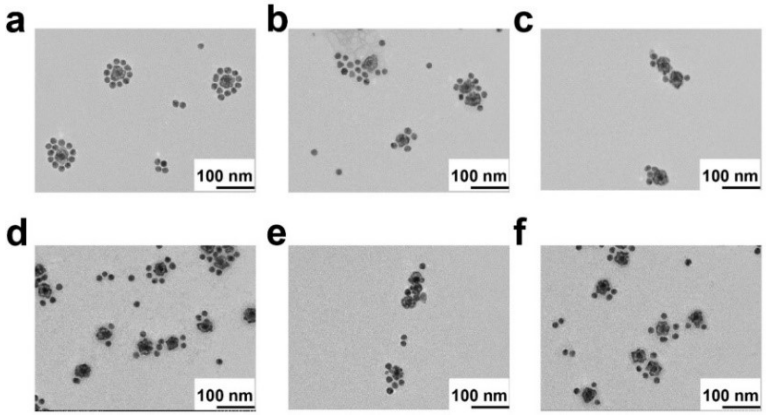

Fig. S12 The TEM images of AuNNP probe/AuNP probe incubation with miR-21 (a), mismatch-1 (b), let-7a (c), miR-144 (d), miR-155 (e) and miR-429 (f) at the concentration of 175 pM.

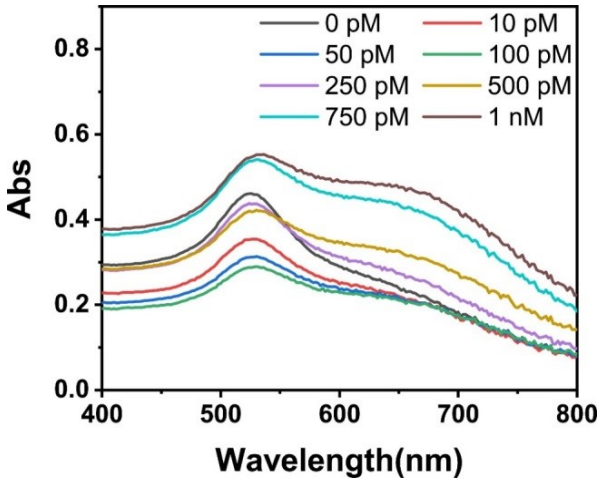

Fig. S13 The UV-vis spectra of different concentrations of miR-21 (from 0 to 1 nM).

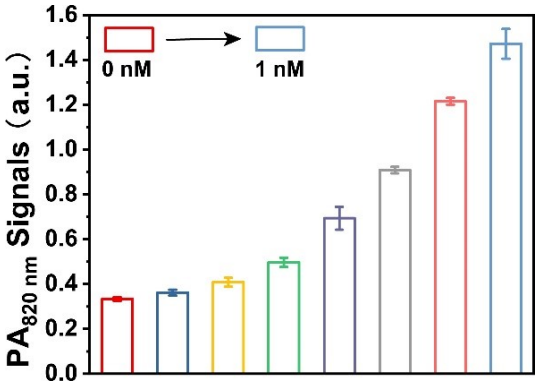

Fig. S14 The PA intensity at 820 nm of different concentrations of miR-21 (from 0 to 1 nM). Error bars represent standard deviation (n = 3).

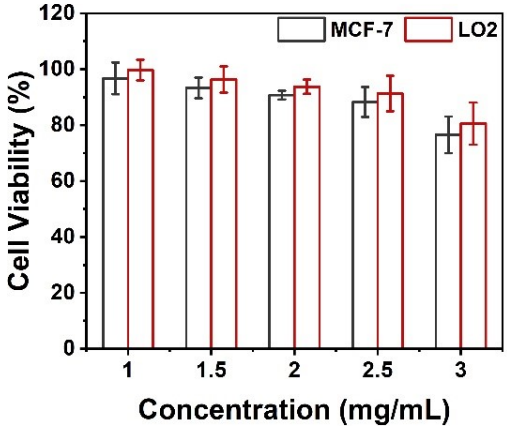

Fig. S15 Cell viability of MCF-7 cells and LO2 cells after incubated with AuNNP probe/AuNP probe. Error bars represent standard deviation (n = 3).

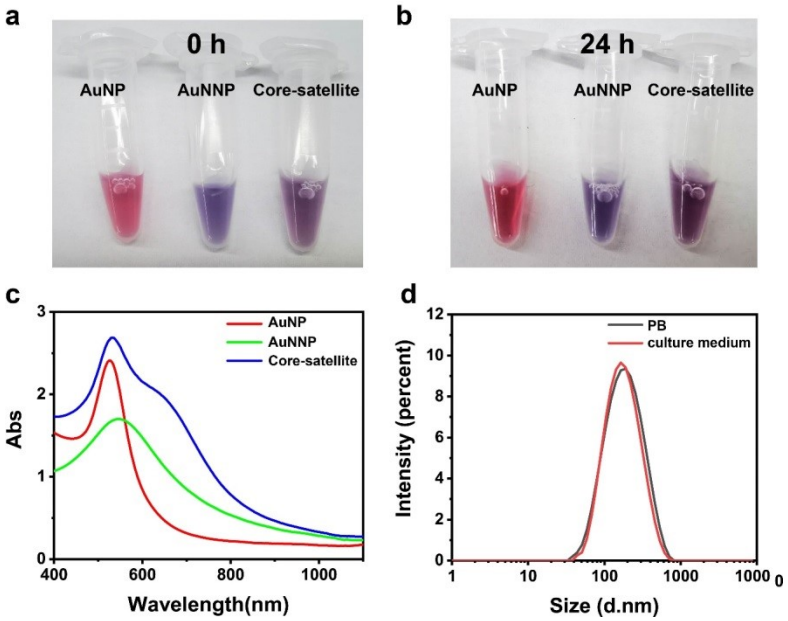

Fig. S16 Pictures of AuNP probe, AuNNP probe, and AuNNP@AuNP core-satellite nanoprobe in 1640 culture medium before (a) and after (b) 24 h incubation. (c) UV-vis spectra of AuNP probe, AuNNP probe, and AuNNP@AuNP core-satellite nanoprobe in 1640 culture medium. (d) DLS of AuNNP@AuNP core-satellite nanoprobe in PB buffer and culture medium.

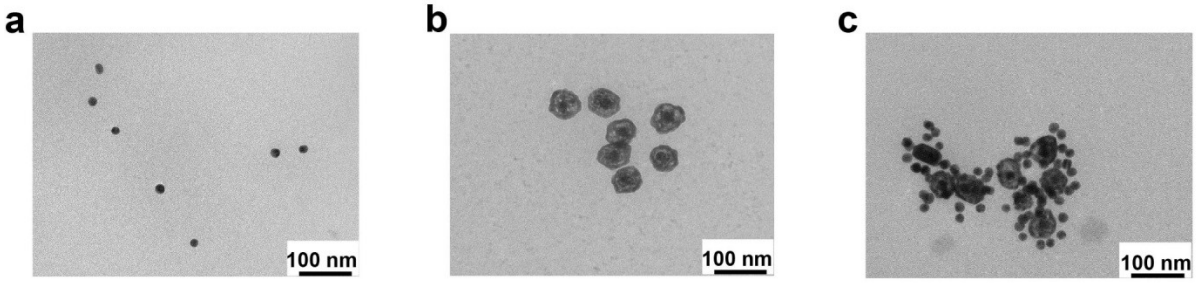

Fig. S17 TEM images of AuNP probe (a), AuNNP probe (b), and AuNNP@AuNP core-satellite nanoprobe (c) in 1640 culture medium.

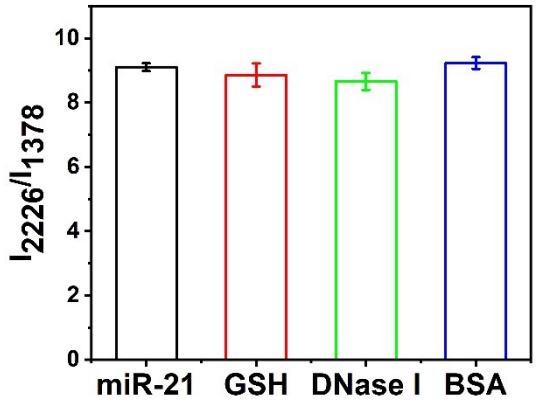

Fig. S18 The ratiometric SERS signals of AuNNP probe/AuNP probe in the presence of miR-21, GSH (1mM), DNase I (10 IU L^{-1}) and 10% BSA in vitro (miR-21 concentration of 175 pM).

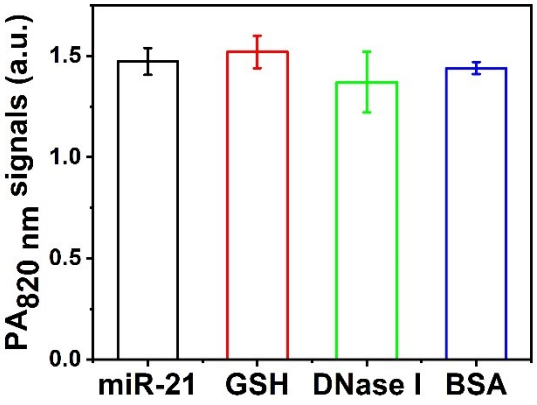

Fig. S19 The average PA intensity at 820 nm of AuNNP probe/AuNP probe in the presence of miR-21, GSH (1mM), DNase I (10 IU L^{-1}) and 10% BSA in vitro (miR-21 concentration of 175 pM).

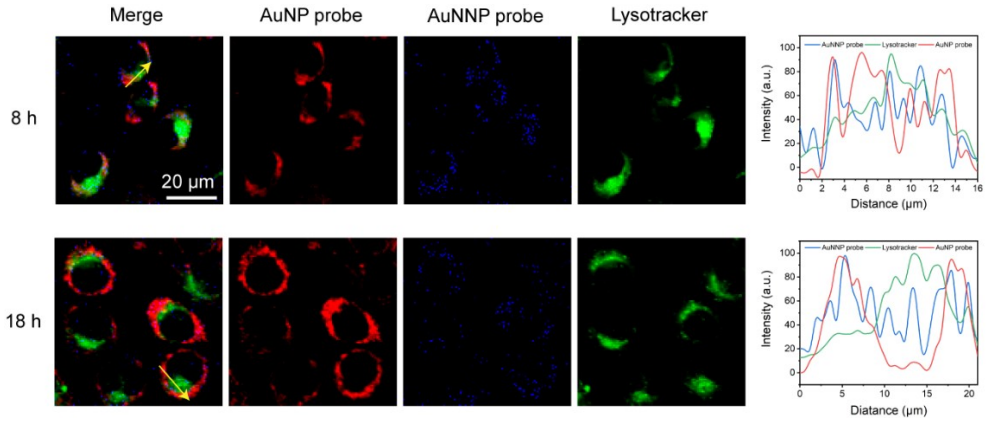

Fig. S20 Confocal laser scanning microscopy images (CLSM) of MCF-7 cells incubated with AuNNP probe/AuNP probe for 8 h and 18 h. The green color comes from the lysosomes stained with lysotracker green, while the red color is from AuNP probe (Cy5 channel) and the blue color is from AuNNP probe (AMCA channel). Right: the linescan profiles of fluorescence intensity variations along the yellow arrow. Scale bar: 20 μm.

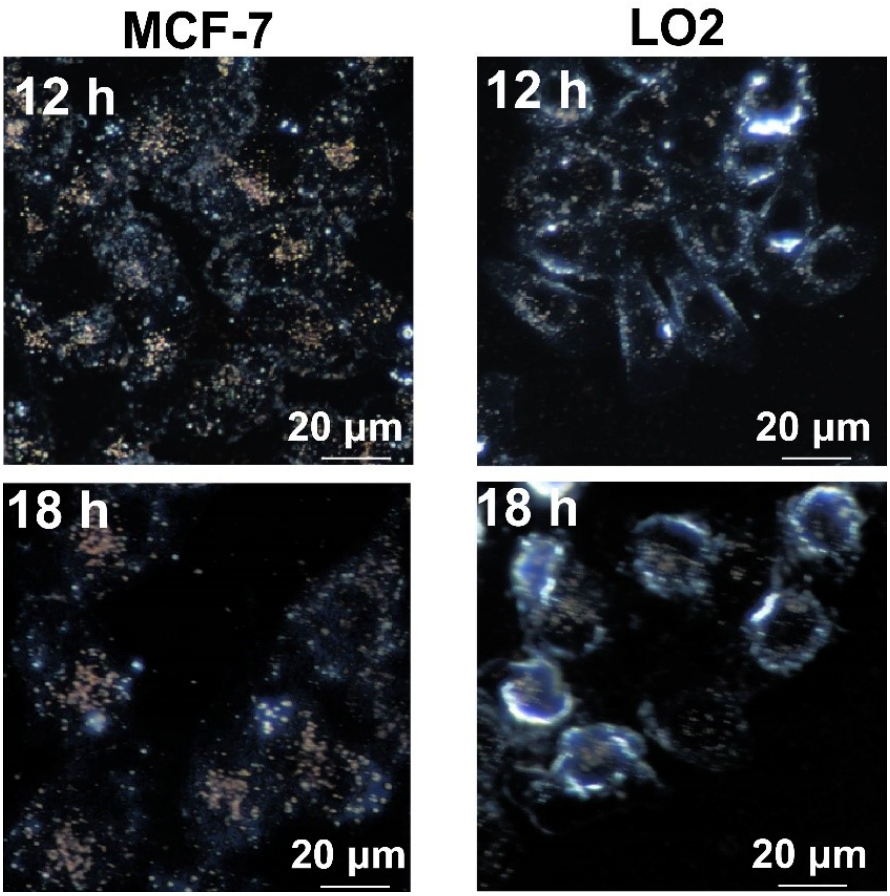

Fig. S21 Enlarged intracellular DFM images of MCF-7 cells and LO2 cells after incubation with AuNNP probe/AuNP probe (2 mg mL^{-1}) for 12 h and 18 h.

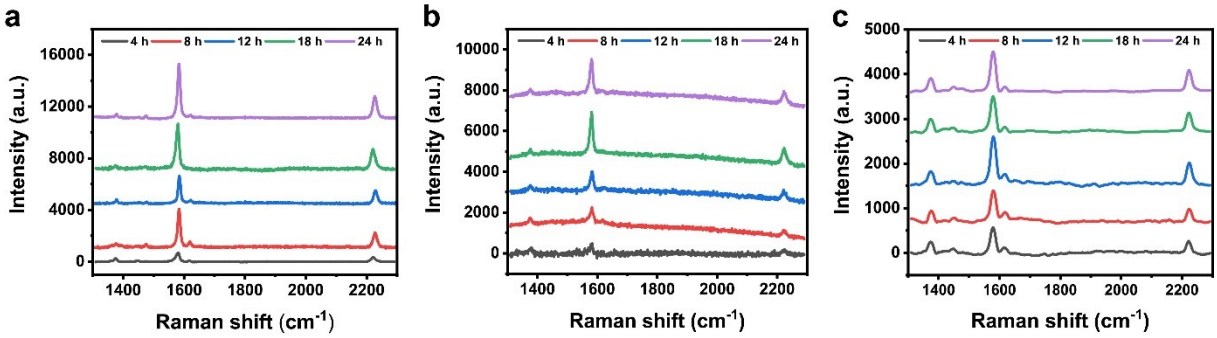

Fig. S22 SERS spectra of MCF-7 cells (a), Hela (b) and LO2 cells (c) after 4 h-24 h of incubation.

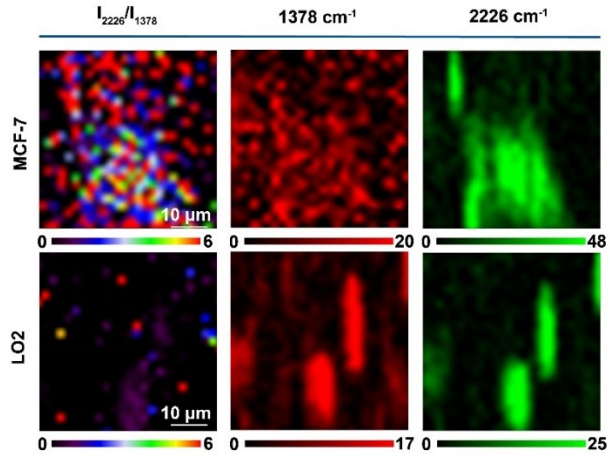

Fig. S23 SERS mapping images of MCF-7 cells and LO2 cells after 4 h of incubation.

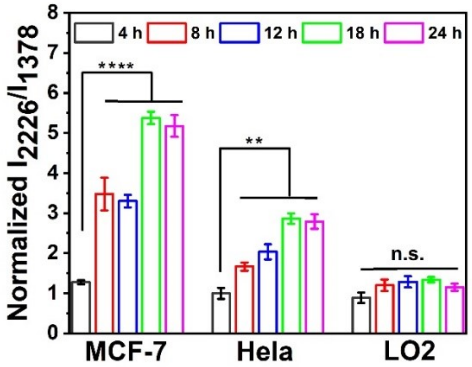

Fig. S24 The ratio intensity of MCF-7 cells, Hela cells and LO2 cells after 4 h-24 h of incubation. Error bars represent standard deviation (n = 3). ****p<0.0001, **p<0.01, n.s.: not significant.

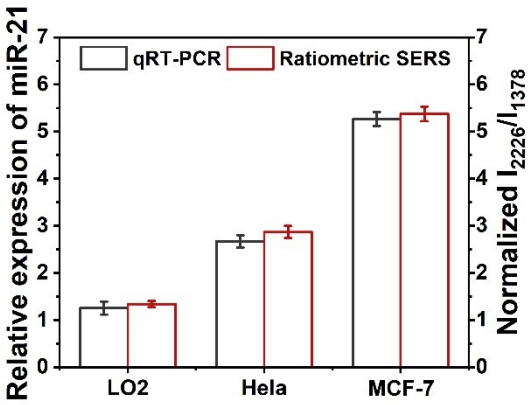

Fig. S25 Histogram of the relative level of miR-21 (qRT-PCR) and the relative SERS signal (normalized I_{2226}/I_{1378}) of LO2 cells, Hela cells and MCF-7 cells at 18 h. The data error bars indicate mean ± SD (n = 3).

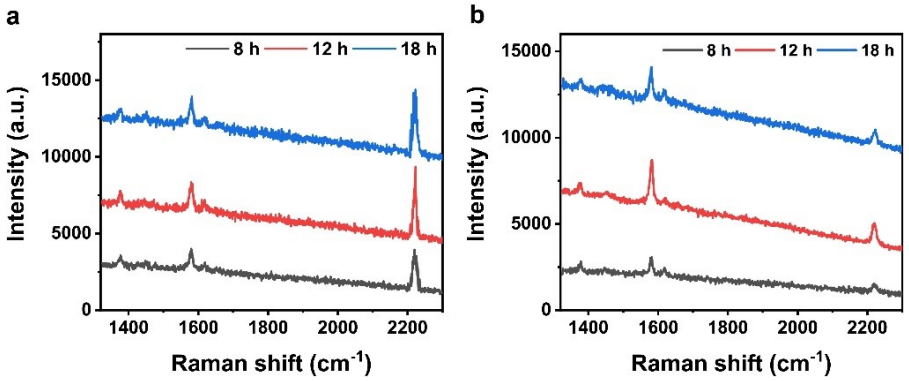

Fig. S26 SERS spectra of AuNNP probe/AuNP probe treated Balb/c nude mice bearing MCF-7 tumor (a) and normal tissue (b).

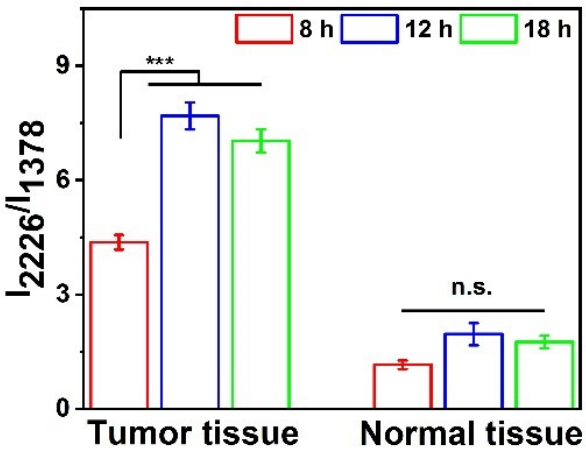

Fig. S27 The I_{2226}/I_{1378} ratio of AuNNP probe/AuNP probe treated Balb/c nude mice bearing MCF-7 tumor and normal tissue. Error bars represent standard deviation (n = 3). ***p<0.001, n.s.: not significant.

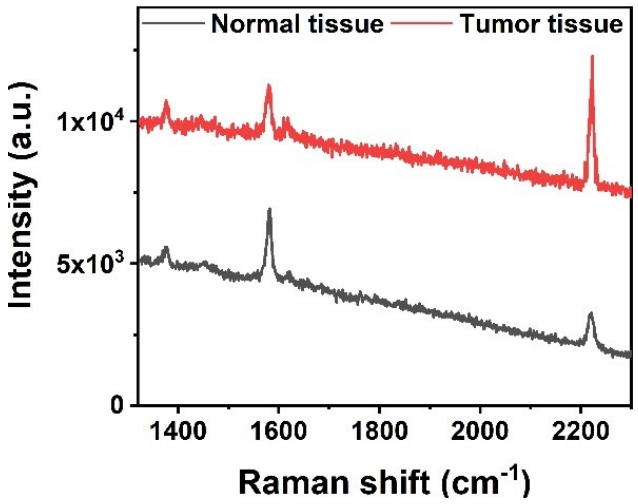

Fig. S28 SERS spectra of AuNNP probe/AuNP probe treated Balb/c nude mice bearing MCF-7 tumor and normal tissue at 12 h post-injection.

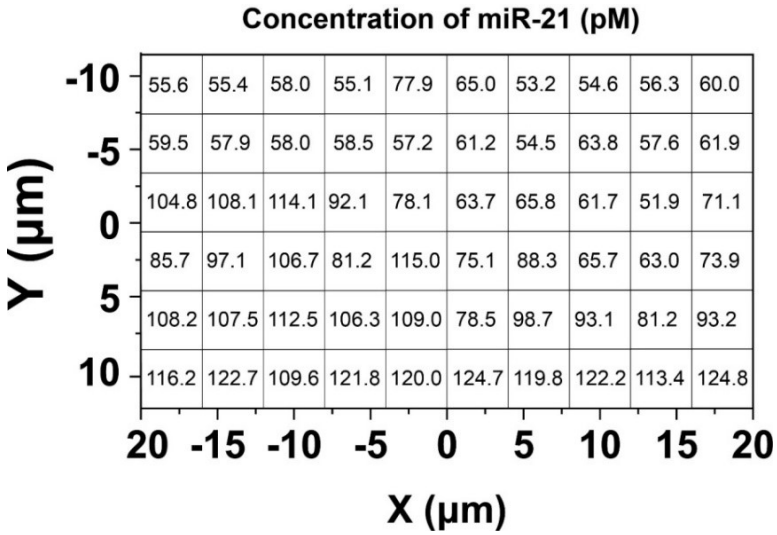

Fig. S29 The average absolute concentration of miR-21 at different tumor sites.

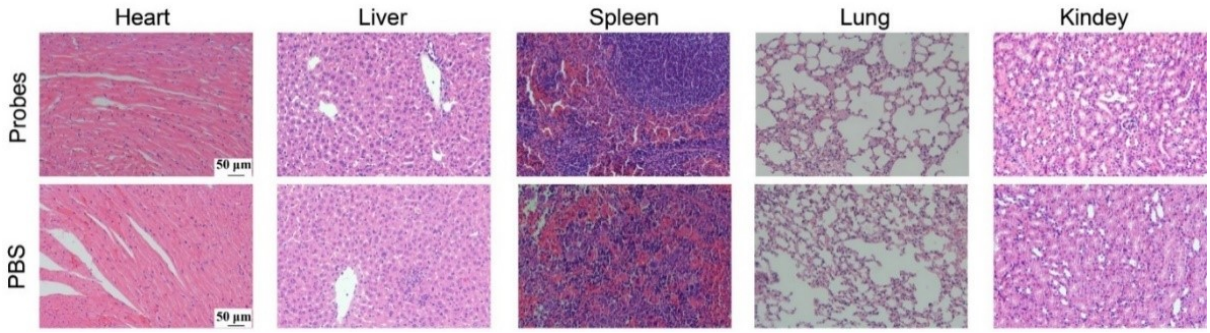

Fig. S30 Representative H&E-stained images of the main organs after AuNNP probe/AuNP probe administered MCF-7 tumor-bearing mice were sacrificed at 48 h post intratumor injection. The PBS administration is chosen as the control group. Scale bars are 50 μm.

Table S1. DNA and miRNA sequences used in this study.

Name	Sequence (5'-3')
miR-21	UAGCUUAUCAGACUGAUGUUGA
DNA1	CTGATAAGCTATTTTTTTTTT-SH
DNA2	SH-TTTTTTTTTTTCAACATCAGT
Cy5-DNA1	Cy5-CTGATAAGCTATTTTTTTTTT-SH
DNA2-AMCA	SH-TTTTTTTTTTTCAACATCAGT-AMCA
mismatch-1	UAACUUAUCAGACUGAUGUUGA
miR-144	UACAGUAUAGAUGAUGUACU
miR-155	UUAAUGCUAAUCGUGAUAGGGGU
miR-429	UAAUACUGUCUGGUAAAACCGU
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
DNA1-FAM	FAM-CTGATAAGCTATTTTTTTTTT
DNA2-BHQ1	TTTTTTTTTTTCAACATCAGT-BHQ1
miR-21 forward	ACACTCCAGCTGGGTAGCTTATCAGACTGA
miR-21 reverse	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC
U6 forward	CTCGCTTCGGCAGCACA
U6 reverse	AACGCTTCACGAATTTGCGT

Reference

1 H. Feng, Q. Fu, W. Du, R. Zhu, X. Ge, C. Wang, Q. Li, L. Su, H. Yang and J. Song, *ACS Nano*, 2021, **15**, 3402-3414.

2 J. Li, Z. Zhu, B. Zhu, Y. Ma, B. Lin, R. Liu, Y. Song, H. Lin, S. Tu and C. Yang, *Anal. Chem.*, 2016, **88**, 7828-7836.

3 Y. Hao, Y. Li, L. Song and Z. Deng, *J. Am. Chem. Soc.*, 2021, **143**, 3065-3069.