Science Advances
AAAS

advances.sciencemag.org/cgi/content/full/5/10/eaaw6264/DC1

Supplementary Materials for

Gold-DNA nanosunflowers for efficient gene silencing with controllable transformation

Shuaidong Huo*, Ningqiang Gong*, Ying Jiang, Fei Chen, Hongbo Guo, Yaling Gan, Zhisen Wang, Andreas Herrmann, Xing-Jie Liang*

*Corresponding author. Email: huosd@nanoctr.cn (S.H.); liangxj@nanoctr.cn (X.-J.L.)

Published 2 October 2019, *Sci. Adv.* **5**, eaaw6264 (2019)
DOI: 10.1126/sciadv.aaw6264

This PDF file includes:

Fig. S1. Characterization of the as-synthesized NPs.
Fig. S2. Cytotoxicity evaluation of MCF-7 cells with NIR irradiation at different times.
Fig. S3. Stability test of self-assembled nanostructures.
Fig. S4. Cellular uptake and intracellular distribution of NPs.
Fig. S5. *C-myc* mRNA level determined in cytoplasm and nucleus separately by real-time PCR.
Fig. S6. Safety evaluation of the nanosunflower structure.
Fig. S7. Penetration behavior study of fluorescein isothiocyanate–labeled nanosunflowers in multicellular spheroid model.
Fig. S8. Quantitative biodistribution of average Au content in tissues including the heart, liver, spleen, lung, kidney, and tumor after different treatments.
Table S1. Hydrodynamic size distribution of Au-POY2T NPs and nanosunflowers before and after NIR irradiation for 3, 10, 12.5, or 15 min, respectively.
Table S2. Histopathological scoring results of the H&E staining images.

Chemicals. Chloroauric acid ($HAuCl_4$), sodium borohydride, tris(2-carboxyethyl) phosphine hydrochloride ($C_9H_{15}O_6P\cdot HCl$, TCEP·HCl, >98%), and fluorescein isothiocyanate isomer I (≥90%, FITC, $C_{21}H_{11}NO_5S$) were purchased from Sigma-Aldrich. N-(2-Mercaptopropionyl) glycine ($C_5H_9NO_3S$, Tiopronin) was supplied by Dalian Meilun Biological Technology Co. Ltd., China. Amino-modified thiol-polyethylene glycol (SH-PEG-NH_2, MW=2000, 99%) was supplied by Beijing SeaskyBio Technology Company. All oligonucleotide sequences (SH-POY2T: SH-C6-AAAAAATGGGTGGGTGGTTT GTTTTTGGG and CA: SH-C6-CCCAAA AACAAACCAC) were synthesized and provided by Beijing Sunbiotech Company. A stock standard solution of Au (1000 μg/mL) was acquired from the National Analysis Center for Iron and Steel, China. Hydrochloric acid and Nitric acid (MOS grade) were obtained from Beijing Chemical Reagents Institute, China. All chemicals were used without further purification. *Aqua regia* (**highly corrosive and must be used with extreme caution!**) was prepared by mixing HCl and HNO_3 with the volume ratio of 3:1, all glassware was cleaned with freshly prepared *aqua regia*. Milli-Q water (18.2 MΩ, Millipore System Inc.) was used throughout the experiments.

Characterization of as-prepared nanoparticles and nanostructures. The morphology of the prepared gold nanoparticles and self-assembled structures were examined using a Tecnai G^2 20 S-TWIN Transmission Electron Microscope (TEM, Philips, Netherlands) with 200 kV accelerating voltage and an electron microscope (HT7700, Hitachi, Japan) with 80 kV accelerating voltage. The surface topography of self-assembled nanostructures was observed using a scanning electron microscope (SEM, Hitachi S4800, Japan). Optical absorption spectra were measured with a Lambda 950 UV/vis/NIR spectrophotometer (25 °C, Perkin-Elmer, USA). Hydrodynamic diameter of the prepared Au NPs and self-assembled structure were determined by a Nano ZS Zetasizer (25 °C, Malvern, England). The concentration of gold element was determined *via* an Optima 5300DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Perkin-Elmer, USA), or an ELAN DRC-e Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Perkin-Elmer, USA).

Agarose Gel Electrophoresis. The 3% agarose gel (containing Roti dye) was prepared and used to determine the DNA exchange efficiency of the 2 nm POY2T NPs. The electrophoresis was performed as follows: 20 μL nanoparticle and glycerin (1/4) mixture was loaded onto an agarose gel. Electrophoresis was carried out at a voltage of 130 V for 30 min in TBE (1×) running buffer. Finally, the results were pictured and analyzed by a VILBER E-Box gel imaging system.

ICP-MS sample preparation and ICP-MS measurements. For the quantitative determination of Au content in the cellular uptake, nuclei localization and tissue biodistribution study, the lysed cell, extracted cell nuclei or isolated tissues were digested with 0.5 mL fresh *aqua regia* (**highly corrosive and must be used with extreme caution!**) for 10 min. Then each digested sample was diluted to 10 mL with de-ionized water. A series of gold standard solutions (20, 10, 5, 2, 1, 0.5, 0.2, and 0 ppb) were prepared at the same time. Each gold standard solution also contained 5% *aqua regia*. The gold standard solutions and prepared sample solutions were measured on a Perkin-Elmer ICP mass spectrometer. Each experiment was done in triplicate, and each replicate was measured 5 times by ICP-MS. After the amount determination of gold atoms in incubated cells, following equations were used to convert the number of gold atoms to a number of gold nanoparticles. For a sphere of diameter D, the number of atoms (U) fitting into each volume of gold nanoparticles was determined. In this calculation, *a* refers to the edge of a unit cell, which has a value of 4.0786 Å on the edge. M is the measured number of gold atoms from ICP-MS.

$$U = \frac{2}{3}\pi\left(\frac{D}{a}\right)^3 \qquad N = \frac{M}{U}$$

Following equations were used to do the calculation in the study:

Uptake number of NPs by each cell= Number of NPs taken up by whole cells / Number of cells

Cell uptake percentage (%) = (Number of NPs taken up by cells / Number of NPs taken up by normalized cell group) × 100 %

Number of NPs in each cell nucleus= Total number of NPs localized in cell nucleus / Number of cells

Intranuclear NP percentage (%) = (Number of NPs localized cell nucleus / Number of NPs localized in normalized cell group) × 100 %

Inhibition percentage of cell uptake (%) = (Number of NP uptake in the presence of inhibitors / Number of NP uptake in the absence of inhibitors) × 100 %

RT-PCR. C-myc gene expression was quantitatively determined by reverse transcriptase polymerase chain reaction (RT-PCR). MCF-7 cells were treated with 2 nm Au- POY2T NPs, self-assembled nanoflowers or POY2T with an equivalent dose of 1 μM (POY2T). After that, mRNA was extracted (together or separately extracted from cytoplasm and nucleus) and first-strand cDNA was synthesized using a

commercialized kit (cat. number, RR047A, Takara, Japan). GAPDH was used as a reference gene. Primer sequences were as follows:

c-myc fwd, 5'-TGAGGAGACACCGCCCAC-3';
c-myc rev, 5'-CAACATCGATTTCTCTCATCTTC-3';
GAPDH fwd, 5'-GACTTCAACAGCAACTCCCAC-3';
GAPDH rev, 5'-TCCACCACCCTGTTGCTGTA- 3'.

Western blot. MCF-7 cells were cultured in 6-well plates at a density of 10^6 cells per well. After 80% confluences, 2 nm Au-POY2T NPs, self-assembled nanoflowers or POY2T with an equivalent dose of 1 μM (POY2T) were added to the wells. In self-assembled nanostructures treated groups, the cells were irradiated with 808 nm NIR laser (1 W/cm^2) for 10 min after pre-incubated with the nanostructures for 1, 3, 6 or 12 h. After each irradiation, the cells were continued for incubation until 24 h. Finally, cells were lysed and total proteins were extracted and stored in -80 °C. 40 μg of total protein was separated by 10% SDS-PAGE gel, then transferred to a NC membrane, incubated with primary c-myc antibody (D3N8F, cat. number, 13987, cell signaling technology, USA), and finally imaged using a BIO-RAD GelDoc XR system (BIO-RAD, USA).

FITC labeling of Au-POY2T NPs. The FITC-labeled nanoparticle was synthesized using a two-step method. Firstly, FITC solution was mixed with SH-PEG2000-NH_2 at a mole ratio of 10:1, the reaction was lasted for 24 h under magnetic stirring at room temperature in dark. After that, the mixture was dialyzed (molecule cut off: 3500) against water for 48 h and the mixture was lyophilized to obtain SH-PEG2000-FITC. Then, SH-PEG2000-FITC (1/10 amount of POY2T sequence) was mixed with Au-POY2T in PBS and the reaction was lasted for another 24 h. After that, unmodified SH-PEG2000-FITC was removed by dialysis (molecule cut off: 8000-14000) to obtain FITC-labeled Au-POY2T NPs.

Multicellular spheroids (MCSs) study. Firstly, 96-well plate was covered with 1.5 w/v% hot agarose solution (50 μL/well). And after cooling to room temperature, the plate was washed three times with culture medium. Then MCF-7 cells (3000 cells/well) were added and cultured for 12 days to grow into spheroids.

Bio-TEM observation. Bio-TEM was utilized to determine the subcellular distribution of the nanosunflowers in treated MCF-7 cells. MCF-7 cells were seeded in a 35 mm culture dishes (10^5 cells /well). The cells were then incubated with nanosunflower for 12 h. After that, cells were irritated with 808 nm NIR laser, and

incubated several hours before fixing. The cells were washed three times with PBS, fixed using paraformaldehyde and osmium tetraoxide, and then dehydrated with ethanol. After that, the cells were embedded in Spurr resin, sectioned (70 nm in thickness) and observed under TEM system.

Hemolysis assay. Red blood cells (RBCs) were obtained by centrifuging freshly collected mouse blood at 10,000 g for 10 min. Then, RBCs were washed with saline until the supernatant was colorless. The obtained RBCs were then re-suspended in 6 mL saline and divided into 12 tubes (500 µL each), followed 500 µL of nanoparticles was added to each tube to obtain a final concentration of 1 nM, 10 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM, 20 µM (equivalent in POY2T sequence). At the same time, 500 uL of RBCs suspension were mixed with 500 µL saline or 500 µL distilled water as the negative and positive controls, respectively. Finally, the samples were mixed and kept at room temperature for 2 h, and then centrifuged at 10,000 g for 10 min. For the measurement, 100 µL supernatant of each sample was collected and the absorbance (Abs) at 570 nm was recorded. The percent hemolysis was calculated using the following equation: %Hemolysis = (sample absorbance - negative control absorbance)/ (positive control absorbance - negative control absorbance) ×100.

Determination of necrotic areas. Necrotic level in organs and tumors were estimated using a semi-quantitative scoring system based on the H&E-stained slides: 0, no necrotic cells; 1, solitary necrotic cells; 2, less than 50% the cells are necrotic cells; 3, more than 50% of the cells are necrotic cells.

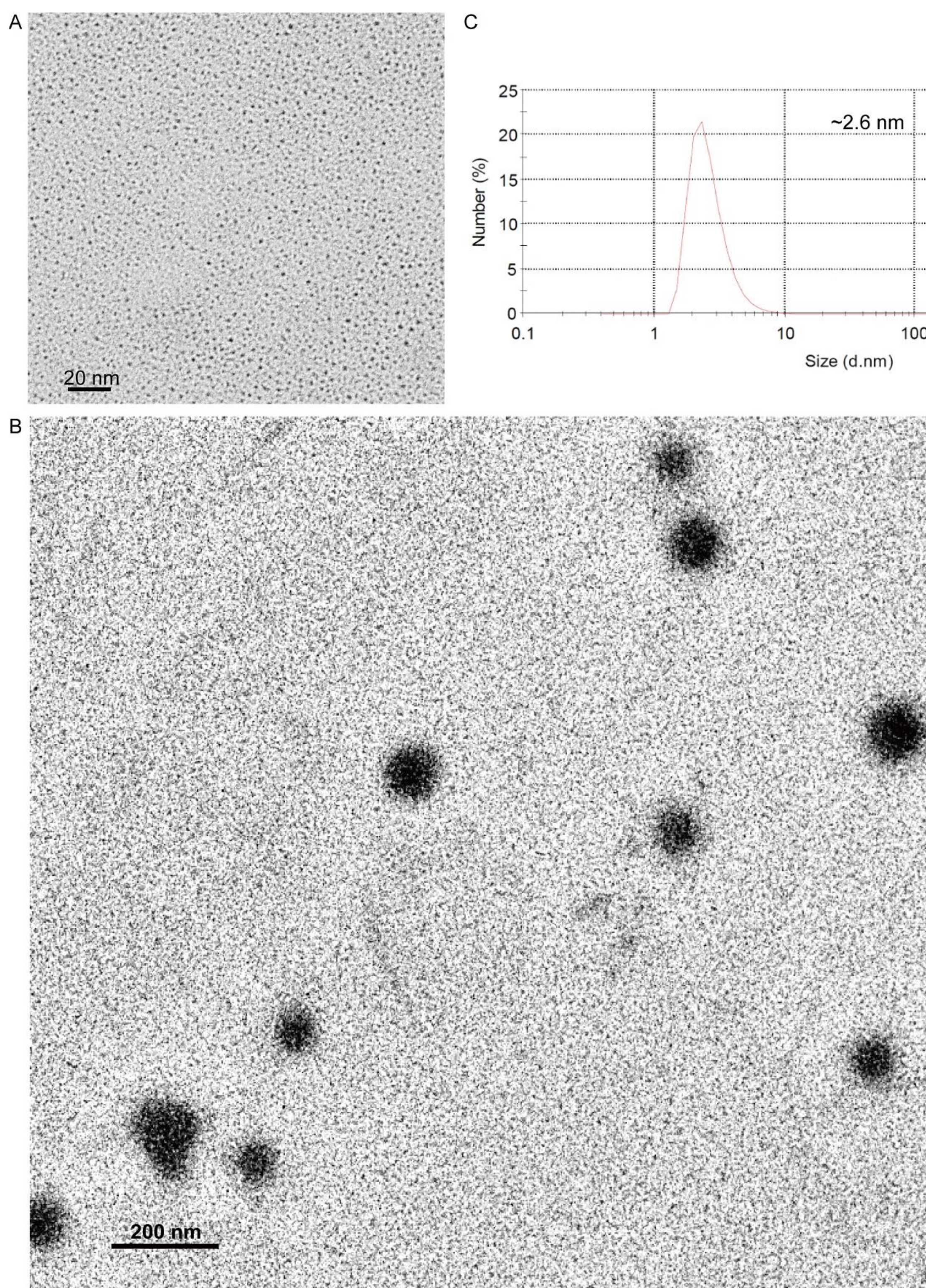

Fig. S1. Characterization of the as-synthesized NPs. (A) Representative TEM image of monodispersed individual 2 nm Au-POY2T NPs. (B) Enlarged representative TEM image of disassembled nanostructures after 10 min NIR irradiation (808 nm). The ultrasmall sized particles (2 nm Au-POY2T NPs) were restored to dispersed state and spread throughout on the grid. (C) Hydrodynamic diameter (DLS) measurement of as-synthesized 2 nm Au-Tiopronin nanoparticles (Au-TIOP NPs).

Table S1. Hydrodynamic size distribution of Au-POY2T NPs and nanosunflowers before and after NIR irradiation for 3, 10, 12.5, or 15 min, respectively.

Sample	Peak 1 Size(nm)/Number(%)	Peak 2 Size(nm)/Number(%)	Peak 3 Size(nm)/Number(%)
Au-POY2T NPs	4.8/100	--	--
NanoSunflowers	208/100	--	--
NanoSunflowers + NIR (3 min)	62.6/49.8	150.2/38.5	6.3/11.7
NanoSunflowers + NIR (10 min)	6.1/73.3	37.9/15.8	91.9/10.9
NanoSunflowers + NIR (12.5 min)	5.8/91.2	24.9/8.8	--
NanoSunflowers + NIR (15 min)	5.5/96.3	20.6/3.7	--

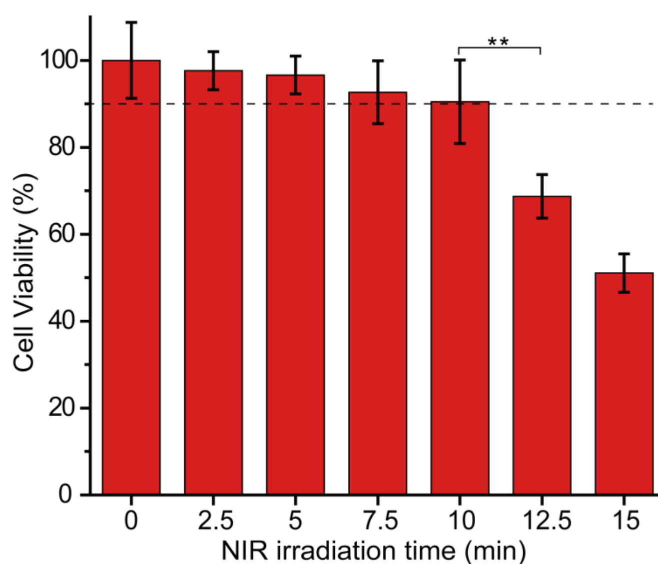

Fig. S2. Cytotoxicity evaluation of MCF-7 cells with NIR irradiation at different time. Mean values ± standard deviation, $N = 3$. Statistical difference was determined by two-tailed Student's t-test, $^{**}P < 0.01$.

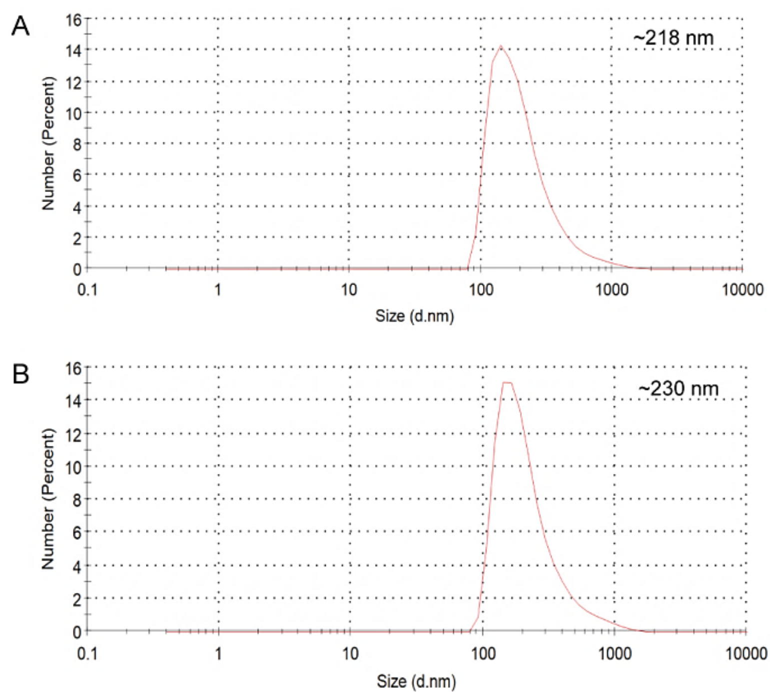

Fig. S3. Stability test of self-assembled nanostructures. (A) Hydrodynamic diameter (DLS) measurement of self-assembled nanostructures dispersed in water after 24 h incubation. (B) Hydrodynamic diameter measurement of self-assembled nanostructures dispersed in 10% FBS (Fetal Bovine Serum)-containing cell culture medium after 24 h incubation.

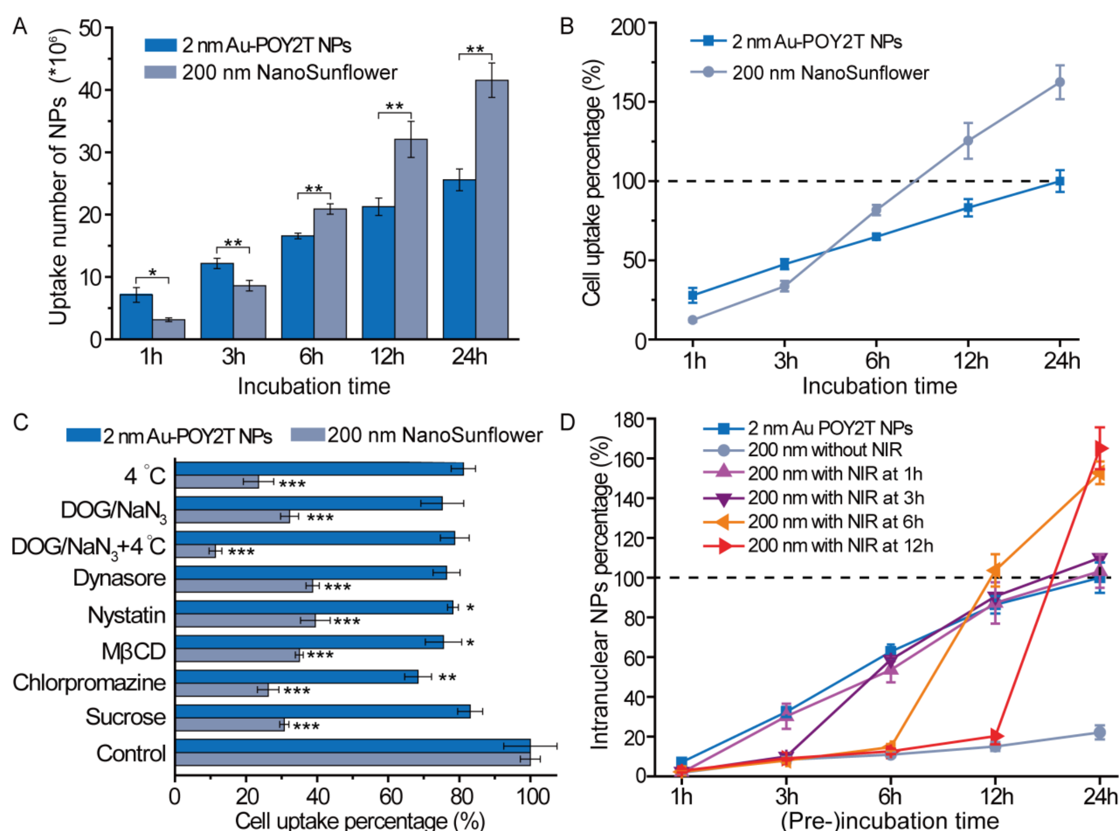

Fig. S4. Cellular uptake and intracellular distribution of NPs. (A) Cellular uptake of 2 nm Au-POY2T NPs and 200 nm nanosunflowers (calculated to the number of Au-POY2T NPs from gold element amount) by MCF-7 cells after different incubation time (1 h, 3 h, 6 h, 12 h, and 24 h). The amount of gold was determined by ICP-MS. Mean values ± standard deviation, $N = 3$. Statistical differences were determined by two-tailed Student's t-test. $*P < 0.05$, $**P < 0.01$. (B) Time-dependent uptake percentage trend of Au-POY2T NPs after treatment of individual Au-POY2T NPs and 200 nm nanosunflowers by MCF-7 cells. The uptake efficiency was normalized to uptake amount of individual 2 nm Au-POY2T NPs after 24 h incubation. Mean values ± standard deviation, N = 3. (C) Cellular uptake pathway study of 2 nm Au-POY2T NPs and 200 nm nanosunflowers by MCF-7 cells using specific endocytosis inhibitors and conditions. Mean values ± standard deviation, $N = 3$. Statistical differences were compared with each control group, determined by two-tailed Student's t-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (D) Percentage trend of Au-POY2T NPs localized in the MCF-7 cell nucleus of individual Au-POY2T NPs, 200 nm nanosunflowers, and 200 nm nanosunflowers with NIR irradiation after different pre-incubation time (1 h, 3 h, 6 h, and 12 h). The percentage was normalized to the amount of 2 nm Au-POY2T NPs treatment after 24 h incubation. Mean values ± standard deviation, $N = 3$.

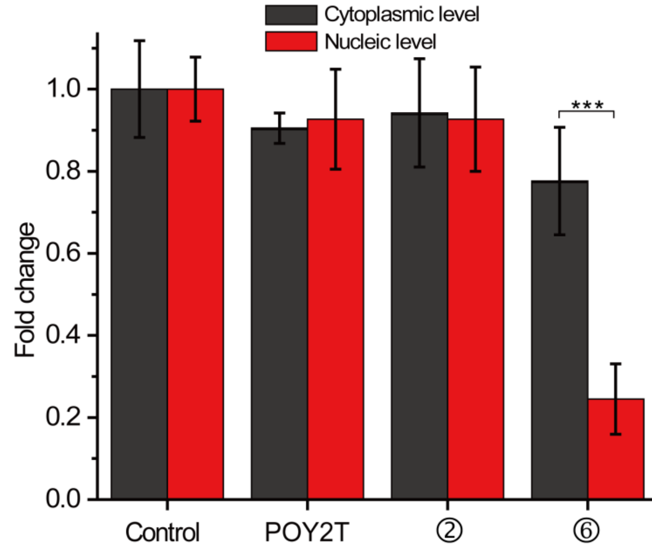

Fig. S5. *C-myc* mRNA level determined in cytoplasm and nucleus separately by real-time PCR. After treatment of nanosunflowers with NIR irradiation at 12 h pre-incubation (group ⑥), compared with control, POY2T sequence only, and nanosunflowers without NIR irradiation (group ②). Mean values ± standard deviation, $N = 3$. Statistical differences were determined by two-tailed Student's t-test. $***P < 0.001$.

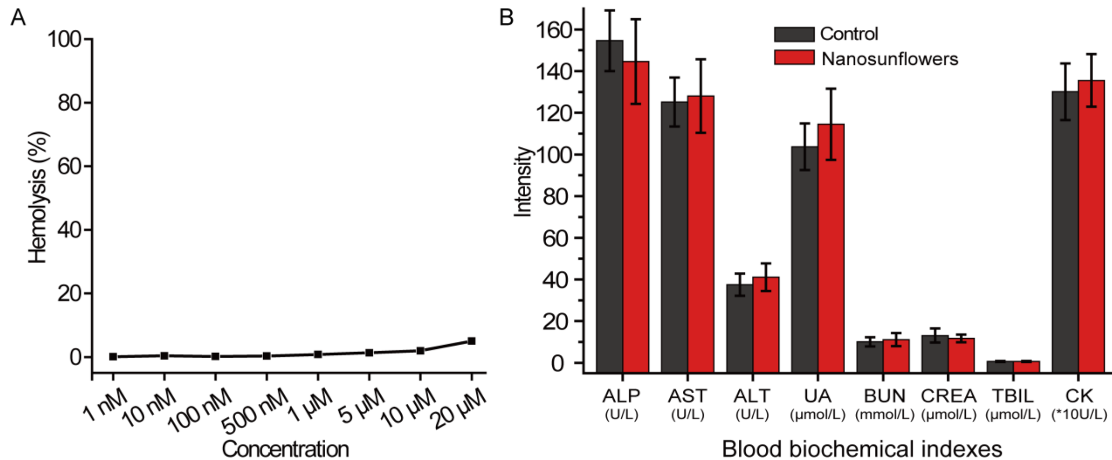

Fig. S6. Safety evaluation of the nanosunflower structures. (A) Hemolytic analysis of mouse red blood cells after treatment with nanosunflowers with different concentrations. Water was used as a positive control and PBS was used as a negative control. (B) Blood biochemistry analysis of mice at 24 h after the administration of nanosunflowers or saline (control). ALP: alkaline phosphatase, AST: aspartate transaminase, ALT: alanine aminotransferase, UA: uric acid, BUN: blood urea nitrogen, CREA: creatinine, TBIL: total bilirubin, CK: creatine kinase. Mean values ± standard deviation, $N = 3$.

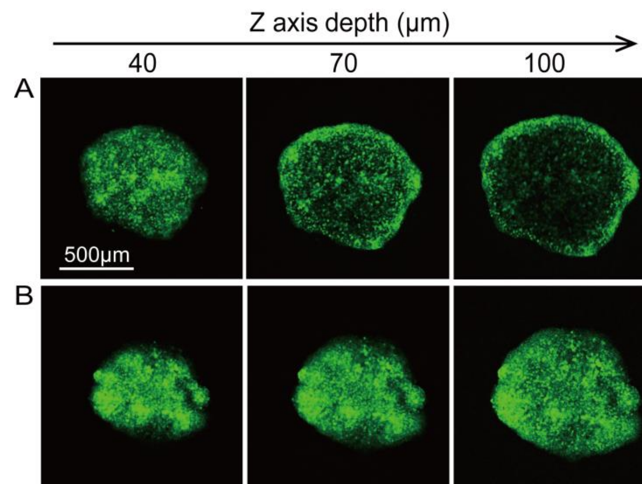

Fig. S7. Penetration behavior study of fluorescein isothiocyanate–labeled nanosunflowers in multicellular spheroids model. Z-stack observation of MCF-7 multicellular spheroid treated with FITC labeled nanosunflowers for 24 h and followed by treatment (A) without or (B) with NIR irradiation.

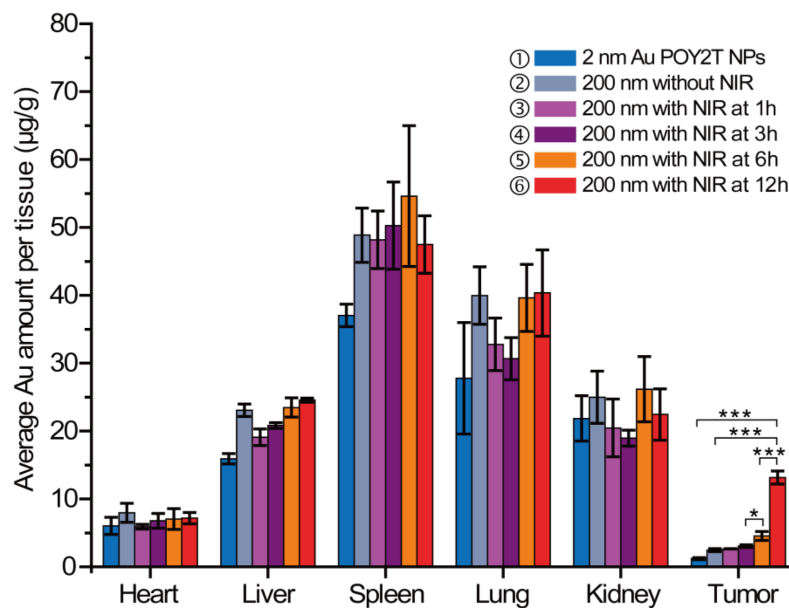

Fig. S8. Quantitative biodistribution of average Au content in tissues including the heart, liver, spleen, lung, kidney, and tumor after different treatments. Group① with 2 nm Au-POY2T NPs, group②, ③, ④, ⑤, ⑥ with 200 nm nanosunflowers and the tumors were irradiated at different time with a NIR laser after each i.v. injection. The mice were killed at day 24 and the amount of gold was determined by ICP-MS. Mean values ± standard deviation, N = 3. Statistical differences were determined by two-tailed Student's t-test, $*P < 0.05$, $***P < 0.001$.

Table S2. Histopathological scoring results of the H&E staining images.

	Control	Light	POY2T	①	②	③	④	⑤	⑥
Heart	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0
Spleen	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0	0
Tumor	0	1	1	1	1	1	1	2	3

Note: (0, no necrotic cells; 1, solitary necrotic cells; 2, less than 50% the cells are necrotic cells; 3, more than 50% of the cells are necrotic cells.)