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Supporting information

Multifunctional hybrids with versatile types of nanoparticles via self-assembly for complementary tumor therapy

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Experimental

Materials

Chloroauric acid ($HAuCl_4$) and ascorbic acid (AA) were obtained from Sinopharm Group Co. Ltd. Silver nitrate ($AgNO_3$, 99.8%), sodium borohydride ($NaBH_4$, 98%), cetyltrimethylammonium bromide (CTAB, 99%), ethyl bromoisobutyrate (98%), glycidyl methacrylate (GMA, 97%), anhydrous *N,N*-dimethylformamide (DMF, 99.8%), dimethyl sulfoxide (DMSO, 99.8%), copper(I) bromide (CuBr, 98%), branched polyethylenimine (PEI, M_w ~25,000 Da), ethanolamine (EA, 99.5%), ethylenediamine (ED, 99.5%), β-cyclodextrin (β-CD, 99%), 1-adamantanecarboxylic acid (Ad-COOH, 98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 98%), α-lipoic acid (LA, 99%), *N,N′*-Dicyclohexylcarbodiimide (DCC, 99%), 4-dimethylaminopyridine (DMAP, 98%), *N*-hydroxysuccinimide (NHS, 98%), *N,N,N′,N″,N″*-pentamethyl diethylenetriamine (PMDETA), iron(III) acetylacetonate (99%), benzyl ether (98%), oleyl amine (C18: 80%-90%), and diethylene glycol (DEG, 99%) were purchased from Energy Chemical Co., Shanghai, China. Poly(acrylic acid) (PAA, $M_n \approx 2{,}000$) was purchased from Aladdin Chemical Co., Ltd., Shanghai, China. GMA was used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma-Aldrich) and other chemicals were used as received without further purification. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), penicillin and streptomycin were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Hep G2 and C6 cell lines were purchased from China Infrastructure of Cell Line Resources, Beijing, China. Plasmid pRL (encoding Renilla luciferase) from Promega Co. (Cergy Pontoise, France), plasmid pEGFP (encoding enhanced green fluorescent protein (EGFP)) from BD Biosciences (San Jose, USA) and constructed plasmid p53 (encoding P53 protein) were amplified in *Escherichia coli* and purified according to the supplier's protocol (Qiagen GmbH, Hilden, Germany). All other chemicals were of analytical grade and used without purification.

Synthesis of PGED-CD and PGED-CD-LA

Poly glycidyl methacrylate (PGMA) was synthesized as described in our previous work.[S1] Briefly, 0.42 mmol of ethyl bromoisobutyrate, 3.6 mL of GMA, and 0.84 mmol of PMDETA were dissolved completely into DMSO. Then, CuBr (60 mg, 0.42 mmol) was added to the mixture, and the flask

was sealed under nitrogen atmosphere. The reaction proceeded for 40 min. For the preparation of PGED, 4.0 mL of ED and 0.5 g of PGMA were first added into flask containing 5 mL of DMSO. The resultant reaction mixture was stirred at 80 ^{o}C for 40 min under nitrogen atmosphere. The final mixtures were precipitated with excess diethyl ether prior to being re-dissolved in deionized (DI) water and dialyzed against deionized water using a 3.5 kDa molecular weight dialysis membrane at room temperature for 24 h.

PGED-CD were prepared by combining multiple epichlorohydrin-modified CDs (CD-ECH) with PGED. To prepare CD-ECH, β-CD (1.5 g) was firstly dissolved in the mixture of 5 mL of DMSO and 5 mL of isopropyl alcohol. And 12.5 mL of 1 mol L^{-1} NaOH aqueous solution was then added into the solution, followed by the addition of 2 mL of epichlorohydrin. The reaction was kept with stirring at room temperature in a nitrogen atmosphere for 48 h. The pH value of the reaction solution was adjusted to about 7.0 with concentrated hydrochloric acid, and excess acetone was used to precipitate the reaction mixture. The raw CD-ECH was dissolved in a small amount of deionized (DI) water and dialyzed against DI water (4×5 L) with dialysis membrane (MWCO, 1000Da) at room temperature for 4 h. Finally, CD-ECH was collected by lyophilization.

For the preparation of PGED-CD, CD-ECH (0.5 g) and PGED (100 mg) were dissolved in 5 mL of H_2O. The resulting mixture was kept with continuous stirring at 80 °C for 72 h in a nitrogen atmosphere. PGED-CD was obtained by lyophilization after dialyzing against DI water (4 × 5 L) with dialysis membrane (MWCO, 7000Da) at room temperature for 48 h.

To prepare PGED-CD-LA, PGED-CD (200 mg), NHS (23 mg, 0.198 mmol) and EDAC (31 mg) were dissolved in 5 mL of deionized water and LA (34 mg, 0.165mmol) was dissolved in 1 mL of ethyl alcohol. Then the two kinds of solution were mixed and stated at 30 ^{o}C for 24 h under the protection of nitrogen. The final mixture were precipitated with excess diethyl ether prior to being dissolved in DI water and dialyzed against DI water using a 3.5 kDa molecular weight dialysis membrane at room temperature for 24 h. The final products were freeze dried.

Synthesis of SiO_2-Ad

The synthesis of chiral silica nanorods (NRs) with diameter ~50 nm and length ~200 nm was constructed by employing F127 and CTAB as a binary template.[S2] In a 250 mL flask, 0.2 g of F127

and 0.4 g of CTAB were dissolved in 100 mL DI water and then 1.2 mL of ammonia and 1.2 mL of TEOS were introduced in the mixture, respectively. The silica solid products were collected by centrifuge, washed with water for three times, and dried at 60 °C overnight. The surfactant templates were removed by calcination in air at 550 °C for 6 h.

The silanization process of SiO_2–NH_2 was performed by adopting similar procedures to those reported earlier.[S3] Briefly, 0.3 mL of APTES was added to the mixture of 18 mL of ethanol and 2 ml of DI water, and the solution was stirred for 30 min. Then, 0.2 g of chiral silica NRs was added, and the resultant reaction solution was stirred for 6 h at room temperature. Next, 0.16 mL of TEA was introduced, and the reaction solution was stirred for another 12 h before separation. The SiO_2–NH_2 were centrifuged at 11,000 rpm, washed with ethanol and cured at 130 °C to strengthen the silane coating by formation of a polysiloxane network.

Ad-COOH was then added to react with the amino groups of SiO_2-NH_2 in the presence of EDAC and NHS. Ad-COOH (0.52 g, 2.9 mmol), EDAC (0.60 g, 3.2 mmol), and NHS (0.31 g, 2.0 mmol) were dissolved in 5 mL of DMSO, and then 0.8 mL of TEA was added. The mixture was stirred at 37 °C for 2 h and mixed with 20 mg of SiO_2-NH_2. Then 0.3 mL of TEA was added, and the reaction mixture was stirred for 48 h at 37 °C. The resultant SiO_2-Ad was washed exhaustively with DI water, prior to lyophilization.

Synthesis of Fe_3O_4-PGED-CD

Monodisperse hydrophobic Fe_3O_4 nanocrystals were prepared and transferred to water through ligand exchange. Hydrophobic Fe_3O_4 nanocrystals were synthesized in a three-neck flask equipped with condenser, thermometer and heating mantle. Iron(III) acetylacetonate (706 mg, 2 mmol) was dissolved in 20 mL of benzyl ether/oleyl amine mixture (1:1, V/V), then oxygen was removed by repeated vacuum-argon cycles. The mixture was conducted at 110 °C for 1 h, and the temperature of mixture was then up to 300 °C for 2 h. Finally, the solution was cooled down to room temperature. Excess ethanol was added to the solution to precipitate the sample, which was then collected by centrifugation (8,000 rpm for 20 min). The resulting black powder was redispersed into toluene (20 mg/mL) for the following ligand exchange procedure. A DEG solution (10 mL) containing PAA (0.5 g) was heated to 110 °C with vigorous stirring under N_2 flow. A toluene solution of Fe_3O_4

nanocrystals (1.0 mL) was injected to the hot solution which became turbid immediately. The system was heated to 240 °C and kept at this temperature for 1 h until the solution became clear. After the solution was cooled down to room temperature, excess dilute hydrochloric aqueous solution was added, and a brown-black magnetic powder was obtained by centrifuging. The powder was washed three times with pure water.

To prepare Fe_3O_4-PGED-CD, PGED-CD (20 mg), NHS (3 mg), EDAC (3 mg) and PAA-modified Fe_3O_4 nanoparticles (5 mg) was dissolved in 5 mL of deionized water. The mixture was kept at 40 ^oC for 48 h. The final products of Fe_3O_4-PGED-CD were obtained by centrifugation at 5,000 rpm for 20 min and freeze-dried.

Synthesis of Au-PGED-CD

Au NRs were synthesized based on the classic seed-mediated growth method according to previous literature.[S4] In brief, Au seeds were prepared by chemical reduction of $HAuCl_4$ with $NaBH_4$. First, the ice-cold $NaBH_4$ solution was added to the CTAB and $HAuCl_4$ mixed aqueous solution and the solution was stirred vigorously for 2 min. Then, the Au seeds were placed in a water bath at 27 ^oC for 1 h before use. For growth solution, CTAB, $AgNO_3$ and $HAuCl_4$ were melted into the aqueous solution to give a yellowish solution. Then, AA was added and the solution turned colorless. Finally, the growth solution containing 500 μL of Au seeds was kept constant at 27 °C for 24 h. Au NRs were collected by centrifugation and had a longitudinal surface plasma resonance band at 790 nm in water.

To prepare Au-PGED-CD, 10 mg of PGED-CD-LA was dissolved in 5 mL of deionized water. The as-prepared Au NRs purified by centrifugation were then added to 5 mL of the PGED-LA solution. The mixture was kept at 40 ^oC for 48 h. The final products of Au-PGED were obtained by centrifugation at 12,000 rpm for 20 min and freeze-dried.

Self-assembly of SiO_2@Fe_3O_4-PGED (SFP) and SiO_2@Au-PGED (SAP). To prepare SFP, SiO_2-Ad (10 mg) in DMSO (0.5 mL) was added dropwise into the Fe_3O_4-PGED-CD solution. The mixture was gently stirred at room temperature for 24 h. The resultant nanohybirds (SFP) was centrifuged and washed with deionized water three times, prior to lyophilization. SAP was prepared by using similar procedures.

Characterization

The molecular structures of PGMA, PGED, CD-ECH, PGED-CD and PGED-CD-LA were characterized by nuclear magnetic resonance (NMR) spectroscopy (Bruker ARX, 400 MHz), and the molecular weight of PGMA was determined by gel-permeation chromatography (GPC, Waters). The SFP and SAP nanohybrids were characterized by transmission electron microscopy (TEM, FEI Tecnai G2, 200 kV, Hillsboro, OR, USA), atomic force microscopy (AFM, Bruker Dimension Icon, Santa Barbara, CA, USA), fluorescence spectrophotometer (Hitachi F-7000, Tokyo, Japan), UV-vis spectroscopy (UV-3600, SHIMADZU, Kyoto, Japan), confocal laser scanning microscopy (CLSM, TCS SP8, Leica, Mannheim, Germany), and particle size and zeta-potential measurements (Zetasizer Nano ZS90, Malvern, Worcestershire, UK). For TEM measurements, the suspension of nanoparticles was dropped on Formvar-covered copper grids and then dried naturally. TGA was performed using a TG 209 F3 Tarsus thermogravimetric analyzer (Netzsch, Selb, Germany). The TGA measurements were carried out in air from room temperature to 1,000 °C at a heating rate of 10 °C min^{-1}. Agarose gel electrophoresis was performed using a Sub-Cell system (110 V, Bio-Rad Laboratories, Hercules, CA, USA) to examine the ability of the nanohybrids to bind pDNA, according to the previously published procedure.[S5] DNA bands were visualized and photographed using a UV transilluminator and a BioDco-It imaging system (UVP Inc., Upland, CA, USA).

Cytotoxicity assay

The cytotoxicity of the nanohybrids was evaluated using MTT assay in Hep G2 and C6 cell lines following the procedure described in our previous work.[S6] The cells were cultured with 100 μL of fresh Dulbecco's modifed eagle medium (DMEM) containing PEI/pDNA, PGED/pDNA, SFP/pDNA, or SAP/pDNA complexes at different W/W ratios and kept for 4 h. Then, the complexes were removed completely and the cells were cultured in fresh normal medium for additional 20 h. The final absorbance of the produced formazan in DMSO at a wavelength of 570 nm was measured using a Bio-Rad Model 680 Microplate Reader (UK). The cell viability (%) was defined according to the formula $[A]_{test}/[A]_{control} \times 100\%$, where $[A]_{test}$ and $[A]_{control}$ represent the absorbance values of the wells with polycations and controls (without complexes), respectively.

In vitro transfection assay

The gene transfection mediated by SFP/pDNA and SAP/pDNA complexes was first carried out in Hep G2 and C6 cell lines by utilizing plasmid pRL-CMV as a reporter gene. In brief, the cells were seeded in 24-well plates at a density of 5×10^4 cells $well^{-1}$ in 500 μL of DMEM and incubated for 24 h. 20 μL of SFP/pDNA or SAP/pDNA at various W/W ratios prepared by mixing different amount of nanohybrids with 1.0 μg of DNA was added into the transfection medium which contained 10% fetal bovine serum. Luciferase gene expression was investigated using a luminometer (Berthold Lumat LB 9507) and quantifed using a commercial kit (Promega Co.). Bicinchoninic acid assay (Bio-Rad Lab) was carried out to analyze the protein concentration in the cells. Results were expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU per mg protein). The plasmid pEGFP-N1 gene was utilized as the reporter gene in Hep G2 and C6 cell lines at the optimal W/W ratio to observe the intuitive gene transfection. The transfected cells were imaged with a Leica DMI3000B fluorescence microscope. The percentage of the EGFP-positive cells was determined by flow cytometry (MoFlo XDP, Beckman, CA, USA).

Determination of cellular internalization

The cellular uptake was determined by flow cytometry and imaged using fluorescence microscopy. In Hep G2 or C6 cell lines, cells were seeded into 6-well plates at the density of 8×10^5 cells per well and incubated for 24 h. After that, the medium was replaced with 3 mL of DMEM with 10% FBS without antibiotics. pDNA (pRL-CMV) was labeled by fluorescent dye YOYO-1 according to the literature. Then, the cells were incubated with fresh media containing SFP/pDNA or SAP/pDNA (6 μg of labeled pDNA) for 4 h, respectively. The cells were trypsinized, centrifuged, re-suspended in PBS, and then analyzed by flow cytometry (BD LSR II, BD, USA). The cells were washed with PBS three times and stained with DAPI at 150 ng mL^{-1} in PBS for 10 min. The fluorescence images were acquired on a Leica DMI3000B microscope.

Photothermal effects of SFP and SAP in vitro and in vivo

To record the temperature variation of SFP/pDNA or SAP/pDNA samples with different concentrations, the solutions in a quartz cuvette were irradiated under an 808 nm laser (Daheng New Epoch Technology, Inc., Beijing, China) at a power density of 2 W cm^{-2} for 300 s. Temperatures at each time point were recorded by an IR thermal camera (FLIR Systems Inc., Ohio, USA). For in

vitro photothermal experiment, C6 cells were first seeded into 24-well plates with 100 μL of fresh DMEM (5×10^4 cells per well) and incubated for 24 h. Then, the medium was replaced with 500 μL of fresh medium containing SFP or SAP (300 μg mL^{-1}) and the cells were incubated for 30 min and irradiated by an 808 nm laser with an output power density of 2 W cm^{-2} for 5 min. Thereafter, the cells were incubated with FDA and PI in a dark room for 10 min, prior to being imaged using a Leica fluorescence microscope. Meanwhile, C6 cells without SFP or SAP under irradiation were stained and imaged as a control.

For the in vivo NIR thermal imaging experiment, female Balb/c nude mice (6 weeks old, weight 18–20 g) were purchased from Beijing HFK Bioscience Co., LTD. (Beijing, China). Animal studies were approved by Ethical Committee of Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College and performed under legal protocols. Tumor-bearing mice were prepared by subcutaneously injection of 100 μL of 1×10^6 C6 single-cell suspension in PBS into the back of the nude mice. The tumor-bearing mice were taken for in vivo experiments after 10 d when the tumors reached a volume of 100–200 mm^3. Tumor-bearing nude mice were first anesthetized by isoflurane, and then 120 μL of SFP or SAP solution (5 mg mL^{-1}) was intratumorally injected. After 10 min, the tumor site was exposed to an 808 nm laser with a power density of 2 W cm^{-2} for 5 min. During the process of laser radiation, an infrared camera was used to obtain the whole-body infrared thermal images at different time points. Some mice were euthanized 2 h after the laser irradiation and the tumors were collected for hematoxylin-eosin (HE) staining.

PTT/GT therapy in vivo

The C6 tumor-bearing Balb/c nude mice were randomly divided into seven groups with four mice in each group, which were treated by injection of PBS (control group), SFP/p53 (GT group), SAP/p53(GT group), SFP with NIR laser (PTT group), SAP with NIR laser (PTT group), SFP/p53 with NIR laser (GT/PTT group) and SAP/p53 with NIR laser (GT/PTT group), respectively. Basically, 120 μL of PBS were administrated via intratumoral injection for control group, while the concentration of SFP or SAP was 5 mg mL^{-1} for GT, PTT, and GT/PTT groups. The treatments were performed three times a week for 2 weeks. The tumors in the GT/PTT group were irradiated by an 808 nm laser (2 W cm^{-2}, 5 min) only once after the first injection of SFP/p53 or SAP/p53

solutions during the whole trial. The length and width of the tumors were measured by a caliper after treatment every 2 or 3 d. The tumor volume was calculated as: (tumor length × (tumor width)^2)/2. The relative tumor growth ratio was reflected by the relative volume V/V_0 (V_0 as the initial tumor volume before treatment). All mice from seven groups were euthanized 2 weeks later after the first injection. The tumors were then weighed, imaged, and dissected, prior to HE analysis and immunohistochemical analysis.

HE staining and immunohistochemical analysis

The tumors and the major organs including the heart, liver, spleen, lung, and kidney were harvested from mice, fixed with 4% paraformaldehyde solution, embedded in paraffin, and sectioned into slices with a thickness of 4 mm. Thereafter, HE and immunohistochemical staining (p53 protein expression) were carried out according to standard protocols in the previous work. The morphology of tumor sections was observed using a Leica DM IL LED inverted phase contrast microscope. An antiP53 polyclonal antibody (sc-6243, diluted, 1:300, Santa Cruz, CA, USA) was employed to detect P53 protein expression.

In vitro and in vivo PA imaging

For in vitro PA imaging, SAP solutions with various concentrations were loaded in prosthesis supplied by the manufacturer and PA images were acquired using a multispectral optoacoustic tomography imaging system (MSOT invision 256-TF, iThermedical, Germany). The PA intensity was obtained using the software supplied by the manufacturer. For in vivo PA imaging, the tumor-bearing mice were used after 10 d when the tumors reached a volume of 100–200 mm^3. PA images of mice were captured before and 10 min after the intratumoral injection of SAP/p53 solutions under gaseous anesthesia (2% isoflurane, 2 L min^{-1}) using veterinary anesthesia equipment (Mobile 901807, VetEquip, Pleasanton, CA, USA). Tumor-bearing mice for PA imaging were injected with 120 μL of SAP solutions (5 mg mL^{-1}). Laser light was used to illuminate the animal tumor from above, and an ultrasonic detector was used to scan the mouse in its horizontal plane. A series of cross section images were captured along the axial direction using MSOT InVision 256-TF, imaging system (iThermedical, Germany). Reconstructed PA images were then acquired using the software supplied by the manufacturer.

Supporting figures

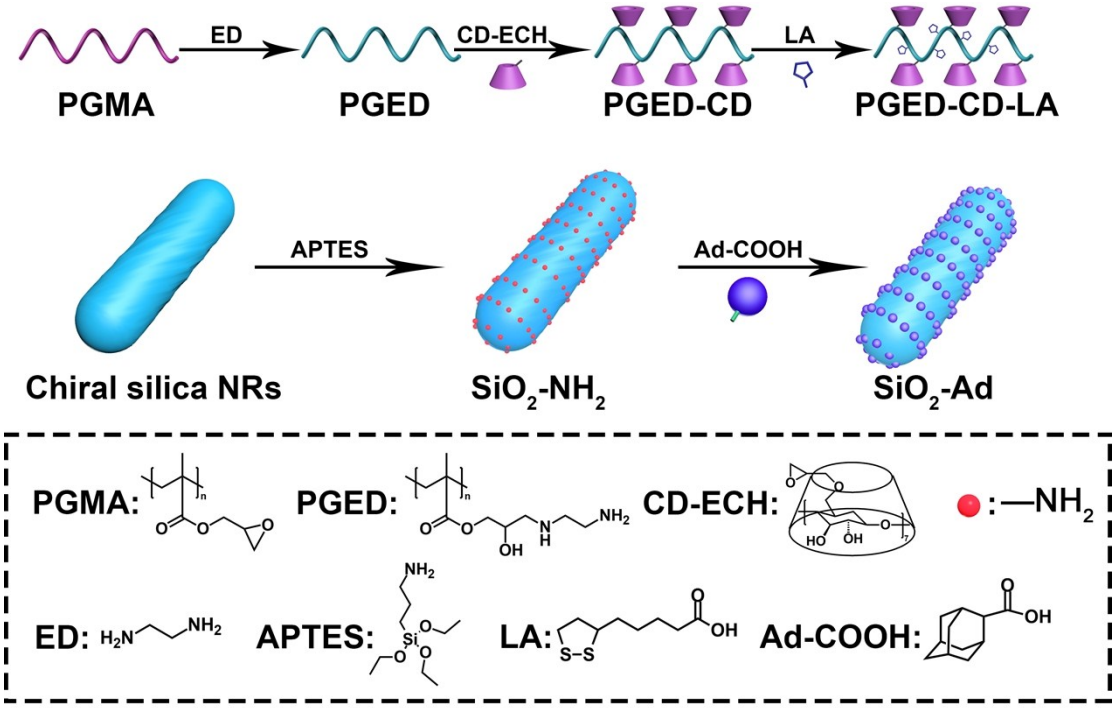

Fig. S1 Schematic illustration of preparation processes of polycations and surface functionalized of chiral silica NRs.

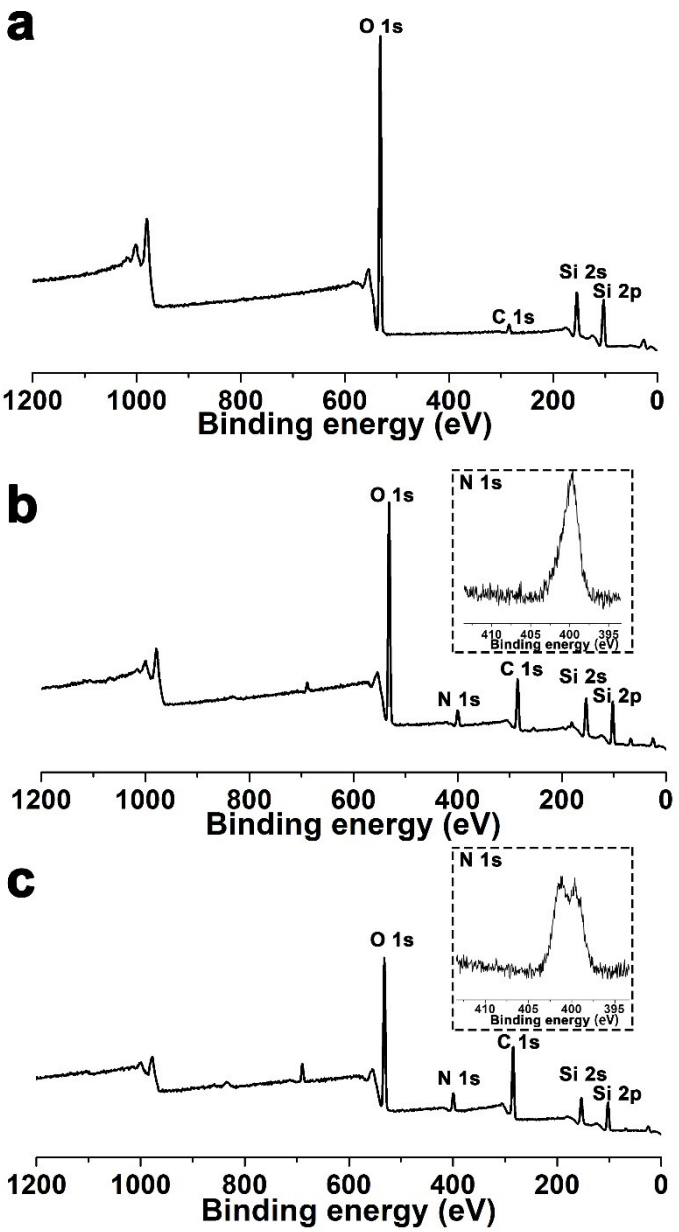

Fig. S2 Typical XPS wide-scan spectra of SiO_2 (a), SiO_2-NH_2 (b) and SiO_2-Ad (c), and N 1s core-level spectra of SiO_2-NH_2 (inset of b) and SiO_2-Ad (inset of c).

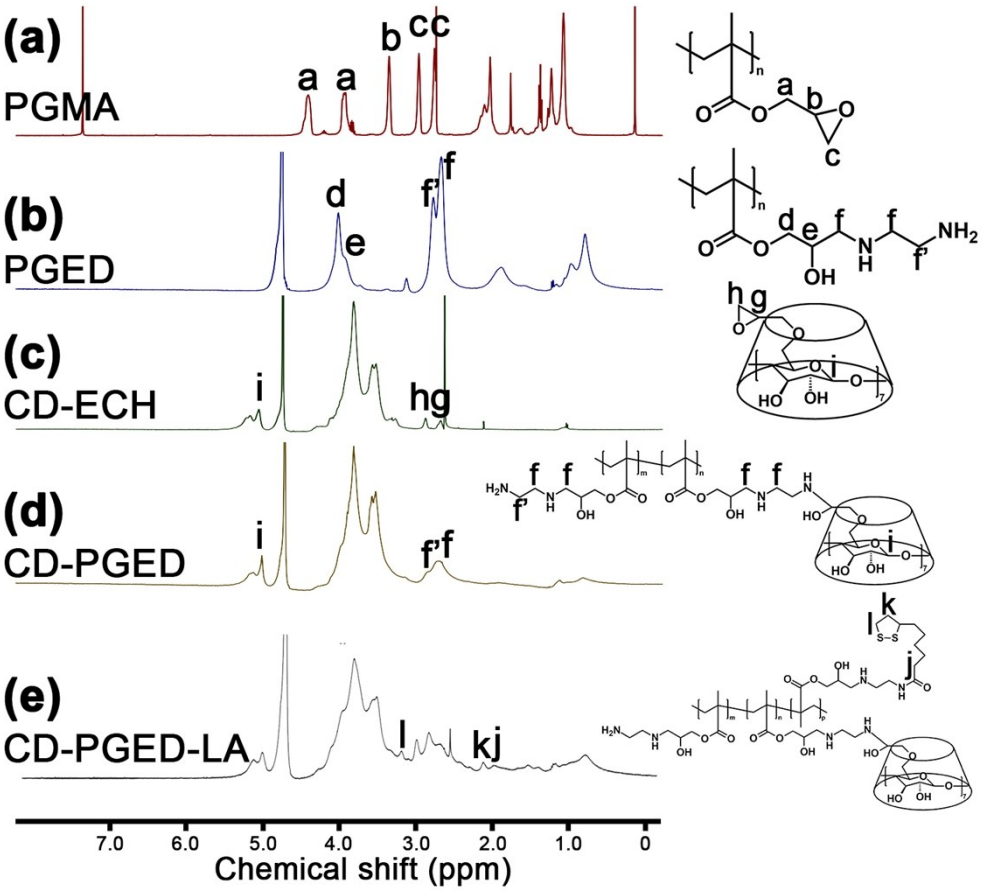

Fig. S3 400 MHz ^{1}H NMR spectra of PGMA (a), PGED (b), ECH-CD (c), PGED-CD (d) and PGEA-CD-LA (e).

The chemical structures of PGMA, PGED, ECH-CD, PGED-CD and PGED-CD-LA were characterized by ^{1}H NMR spectra (Figure S3).

For PGMA (Figure S3a), the characteristic peaks are as follows: δ = 3.8 ppm and 4.3 ppm (a, CH_2-O-C=O), 3.2 ppm (b, CH_2-CH(O)-CH_2), 2.6 ppm and 2.8 ppm (c, CH-CH_2-O). The area ratio of peak a, b and c was 2:1:2, which demonstrated the existence of epoxy groups.

For PGED (Figure S3b), the characteristic peaks are as follows: δ = 3.95 ppm (d, CH_2-O-C=O), 3.9~3.95 ppm (e, CH_2-OH) and 2.5~3.0 ppm (f, CH_2-NH_2 and f ', CH_2-NH). The area ratio of peak f and f' to peak d was 2.0, which was consistent with its chemical structure.

For ECH-CD (Figure S3c), the characteristic peaks are as follows: δ = 2.63 ppm (g, O-CH_2-CH), 2.82 ppm (h, O-CH_2-CH) and 5.0 ppm (i, O-CH-O). The area ratio of i to g and h was 1:4, indicating that one epoxy group was modified to each cyclodextrin molecule.

For PGED-CD (Figure S3d), the characteristic peaks are as follows: δ = 2.5~3.0 ppm (f, CH_2-NH_2 and f ', CH_2-NH) and 5.0 ppm (i, O-CH-O), which proved the cyclodextrin groups have been incorporated with PGED.

For PGED-CD-LA (Figure S3e), the characteristic peaks are as follows: δ = 1.96 ppm (j, NH-C(O) -CH_2), 2.13 ppm (k, S-S-CH-CH_2) and 3.36 (l, S-S-CH_2).

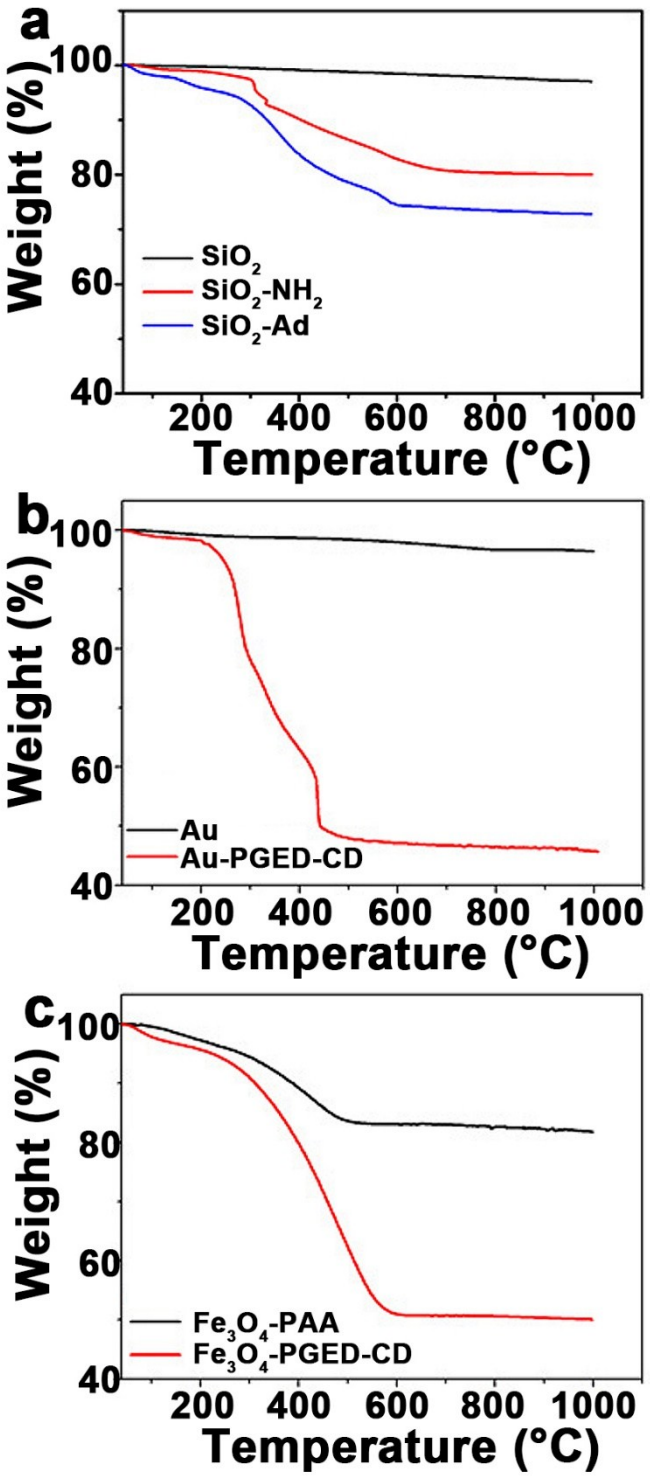

Fig. S4 TG analysis of SiO_2-Ad (a), Au-PGED-CD (b) and Fe_3O_4-PGED-CD (c).

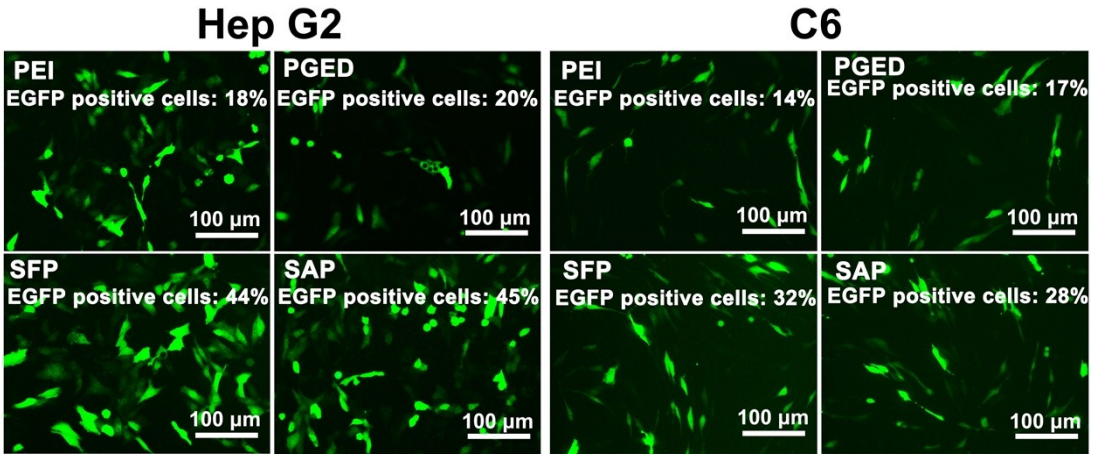

Fig. S5 Representative images of EGFP expression mediated by PEI/pDNA, PGED/pDNA, SFP/pDNA, and SAP/pDNA complexes in Hep G2 and C6 cells at their respective optimal transfection conditions.

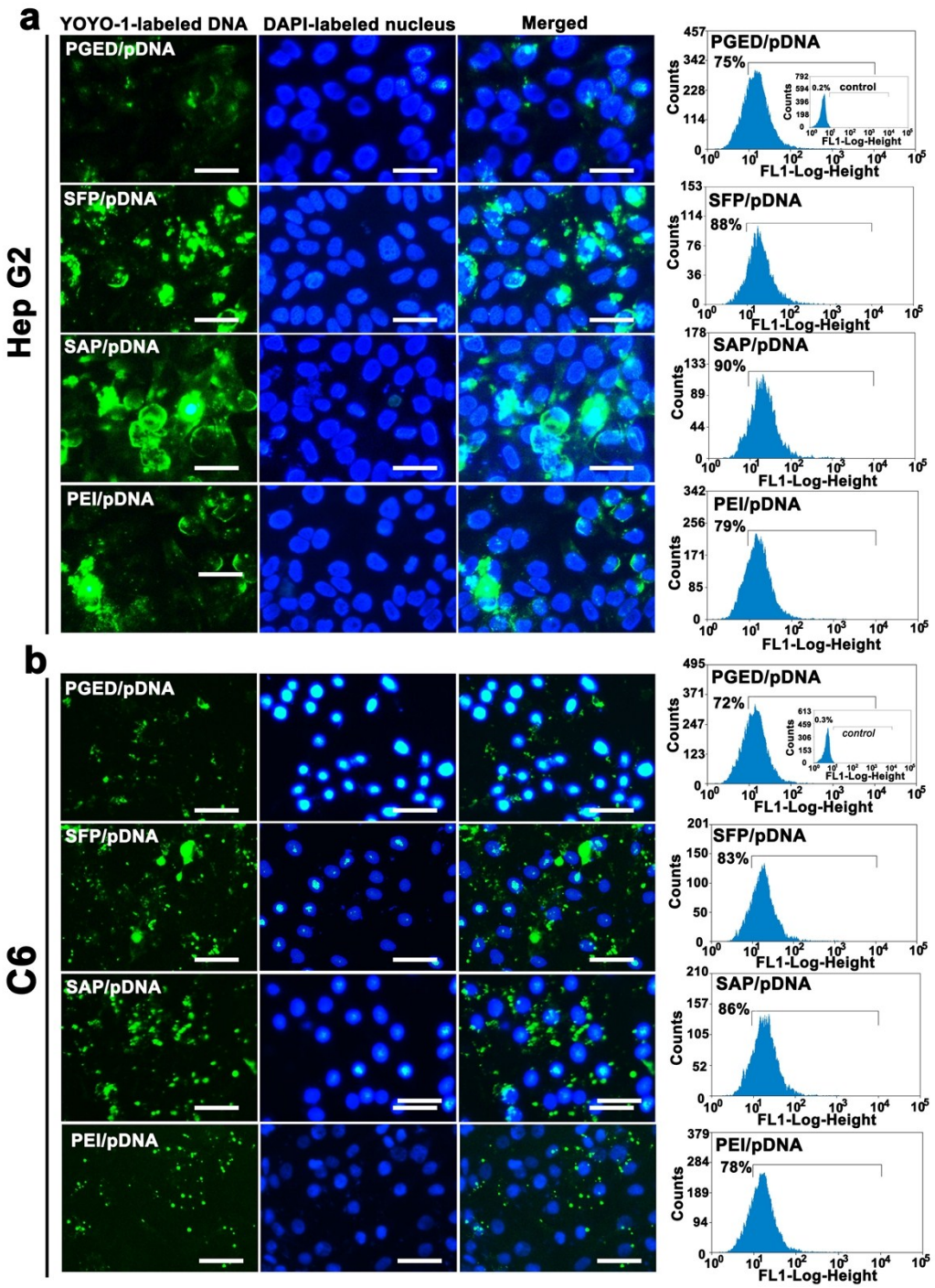

Fig. S6 Representative fluorescent images and flow cytometry analysis of Hep G2 (a) and C6 (b) cells treated with PGED/pDNA at the N/P ratio of 20, SFP/pDNA, SAP/pDNA at the W/W ratio of 30, where the YOYO-1-labeled pDNA was shown in green, and the DAPI-labeled nuclei were shown in blue (scale bar = 20 μm). Hep G2 and C6 cells treated with PEI/pDNA complexes at the optimal transfection condition were taken as controls.

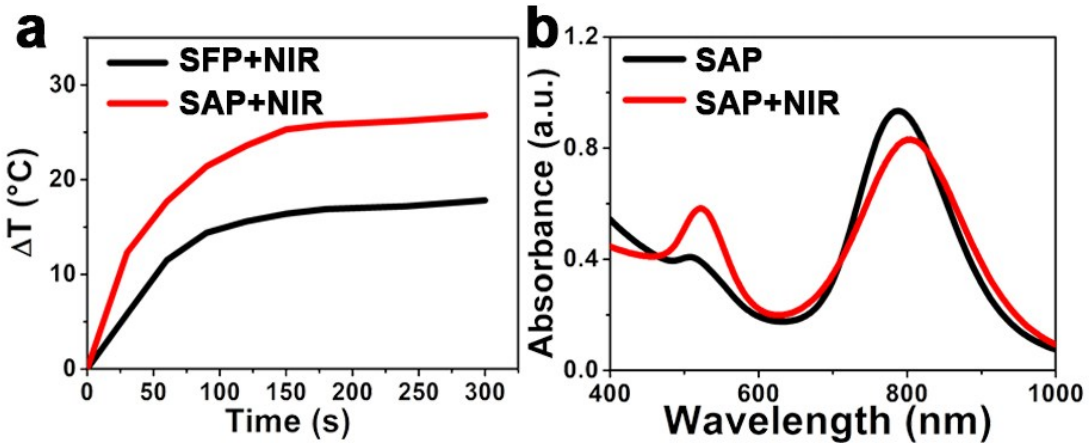

Fig. S7 (a) Temperature elevation of SFP and SAP aqueous solution upon irradiation at 808 nm (2 W cm^{-2}); (b) UV-vis spectra of SAP and SAP+NIR.

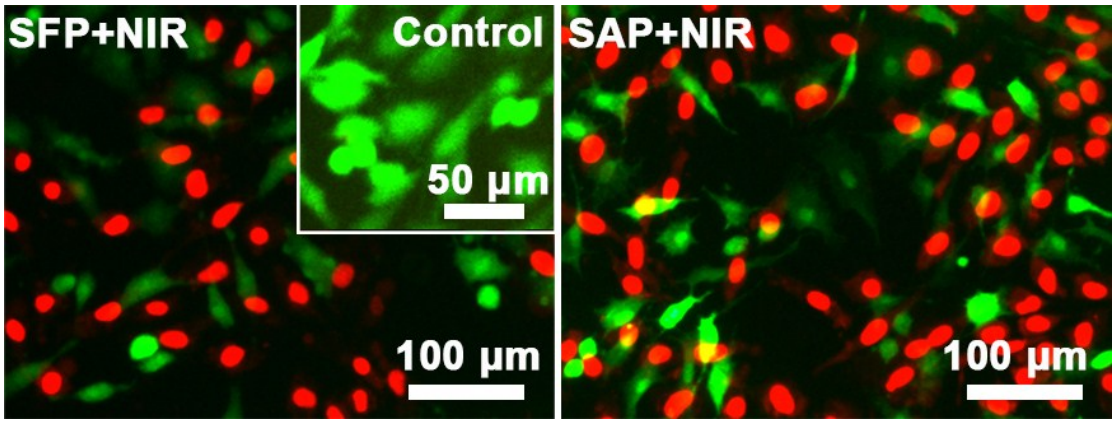

Fig. S8 Representative fluorescence images of FDA/PI stained C6 cells treated with SFP and with SAP after laser irradiation

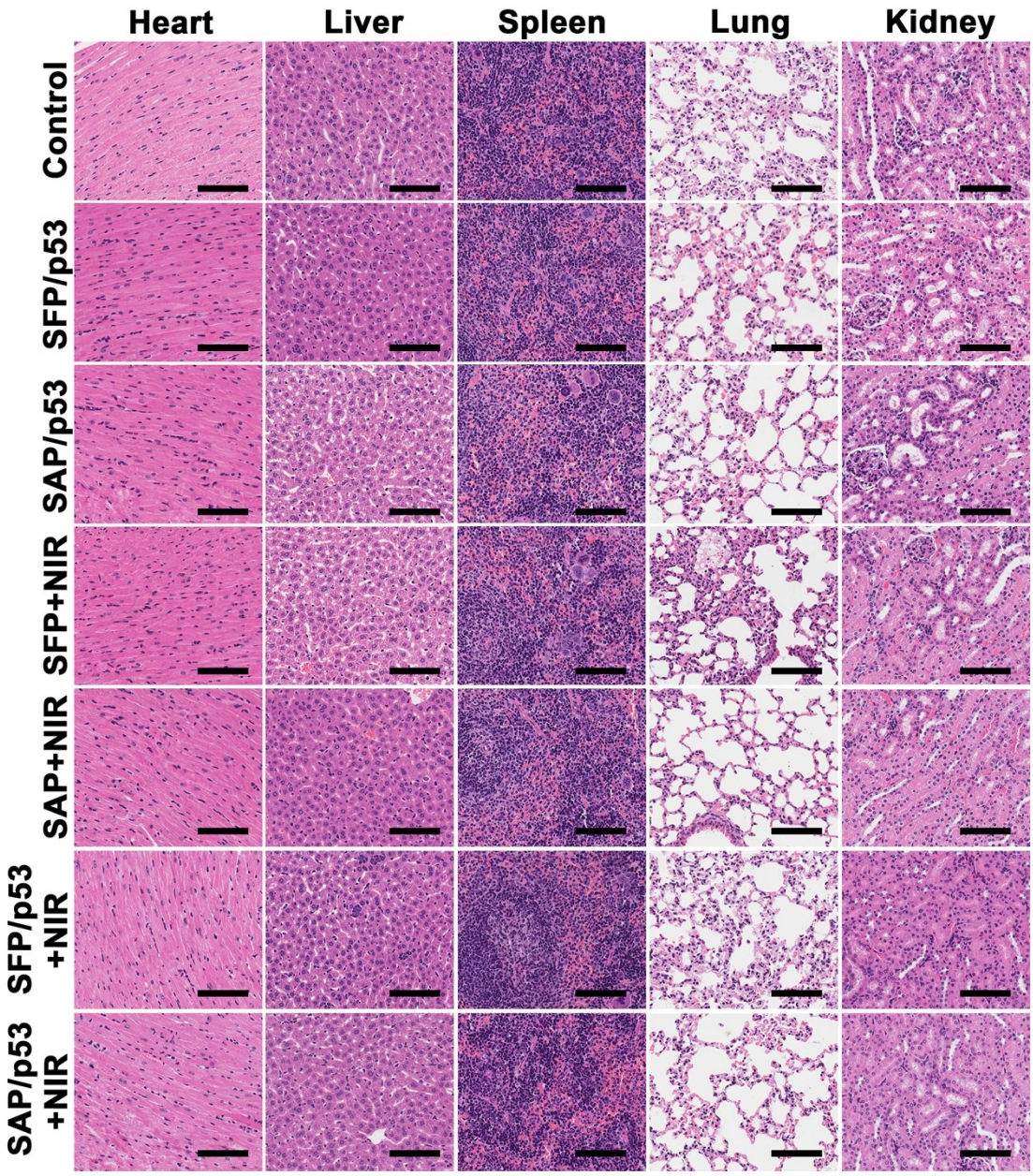

Fig. S9 Histology analysis of major organs (hear, liver, spleen, kidney, and lung) of different groups of mice (scale bar = 100 μm).

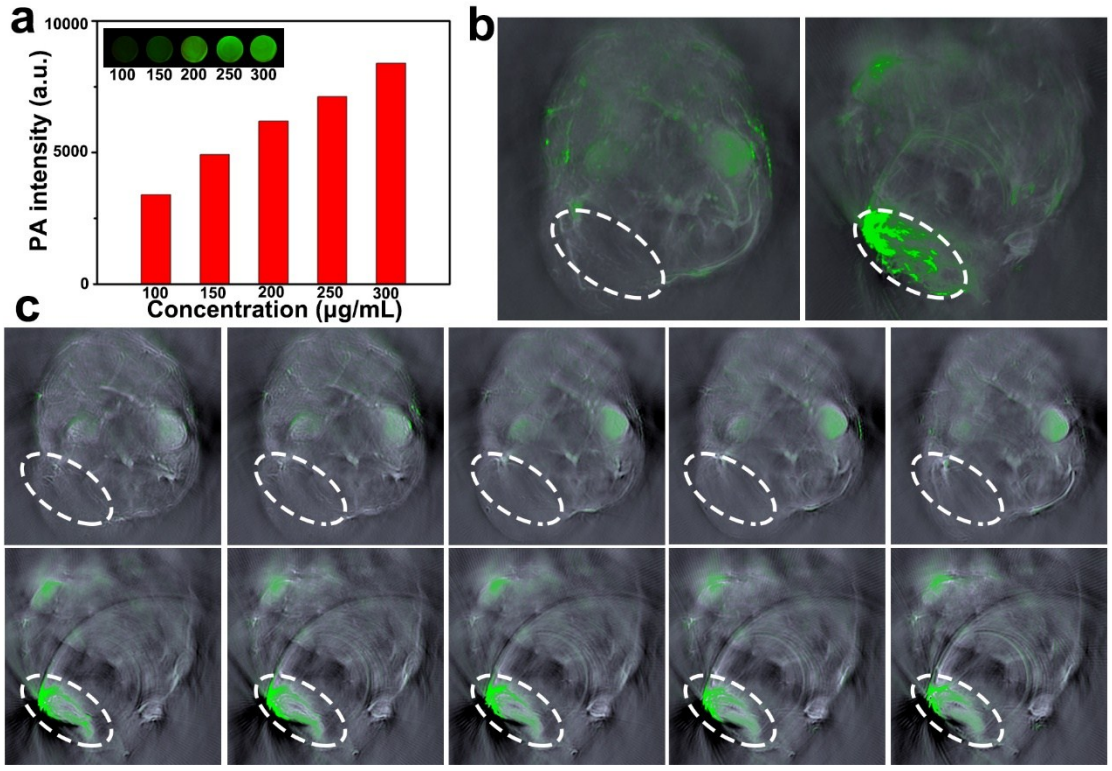

Fig. S10 (a) PA intensity and corresponding PA images of SAP solution in the phantom; (b) PA images of glioma-bearing mice before and after injection with SAP/p53 complexes (5 mg mL^{-1}, 120 μL); (c) PA scans at various axial positions of mice before (the upper line) and after (the lower line) injection with SAP/p53 complexes (5 mg mL^{-1}, 120 μL). Tumor regions are highlighted by the white dashed circles.

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