Electronic Supplementary Information

pH-Responsive hyaluronic acid-cloaked polycation/gold nanohybrids for tumor-targeted synergistic photothermal/gene therapy

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Experimental

Materials: Chloroauric acid ($HAuCl_4$) and ascorbic acid (AA) were obtained from Sinopharm Group Co. Ltd (China). Silver nitrate (99.8%), sodium borohydride (98%), cetyltrimethylammonium bromide (CTAB, 99%), ethyl 2-bromoisobutyrate (98%), copper (I) bromide (CuBr, 98%), glycidyl methacrylate (GMA, 98%), branched polyethylenimine (PEI, Mw~25,000Da), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fluorescein diacetate (FDA), and propidium iodinate (PI) were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Sodium periodate ($NaIO_4$, 98%), ethylenediamine (ED, 98%), *N*-hydroxysuccinimide (NHS, 98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC-HCl, 98%), and α-Lipoic acid (LA, 99%) were obtained from Energy Chemical Co., Ltd (Shanghai, China). GMA were used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma Aldrich). 4T1 and HEK293 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The plasmid pRL-CMV (encoding Renilla luciferase) from Promega Co. (Cergy Pontoise, France), plasmid pEGFP-N1 (encoding enhanced green fluorescent protein (EGFP)) from BD Biosciences (San Jose, USA), and 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 used as received and were of analytical grade. Radioimmunoprecipitation (RIPA) buffer and Annexin V-FITC/PI Apoptosis Kit were purchased from Beijing Solarbio Science & Technology Co., Ltd. China.

Synthesis of gold nanorods (Au NRs): The synthetic procedures of Au NRs were based on the previous report.[1] In brief, gold seeds were prepared by chemical reduction of $HAuCl_4$ with $NaBH_4$. 2.5 mL of 0.2 M CTAB was mixed with 74 μL of

24 mM $HAuCl_4$ and 1 mL of water. Then, 0.5 mL of 10 mM ice-cold $NaBH_4$ solution was added to the mixture and the solution was stirred vigorously for 2 min. The Au seeds were placed in a water bath at 27 °C for 1 h before use. For the growth solution, 25 mL of 0.2 M CTAB, 1.65 mL of 4 mM $AgNO_3$, 1.55 mL of 24 mM $HAuCl_4$ and 20 mL of H_2O were mixed to produce a yellowish solution. Then, 620 μL of 0.0788 M AA was added and the solution turned colorless. Finally, 500 μL of gold seeds was injected and the growth solution was kept constant at 27 °C for 24 h. The resulting gold nanorods were collected by centrifugation and re-dispersed in deionized water.

Synthesis of PGED-LA: For the synthesis of PGMA, 44.2 μL of ethyl 2-bromoisobutyrate, 3 mL of GMA, and 93.8 mg of 2,2'-bipyridine were dissolved completely into DMSO. Then, CuBr (43 mg) was added to the mixture, and the flask was sealed under a nitrogen atmosphere. The reaction proceeded for 60 min. For the preparation of PGED, 2.0 mL of ED and 0.45 g of PGMA were first added into the flask containing 5 mL of DMSO. The resultant reaction mixture was stirred at 80 °C for 60 min under a nitrogen atmosphere. The final mixture was dialyzed against deionized water using a 3.5 kDa molecular weight dialysis membrane at room temperature for 24 h.

For the preparation of PGED-LA, PGED (200 mg), NHS (23 mg, 0.198 mM), and EDAC (31 mg, 0.198 mM) were dissolved in 5 mL of deionized water and LA (34 mg, 0.165 mM) was dissolved in 1 mL of ethyl alcohol. Then the two kinds of solution were mixed and stated at 30 °C for 24 h under the protection of nitrogen. The final mixture was dialyzed against deionized water using a 3.5 kDa molecular weight dialysis membrane at room temperature for 24 h. The final products were freeze dried.

Synthesis of Au-PGED (AP): The synthetic details on the preparation of AP were reported in our earlier work.[2] In brief, 5 mg of PGED-LA was dissolved in 5 mL of

deionized water. 2 mg of the as-prepared Au NRs purified by centrifugation was then added to 5 mL of the PGED-LA solution, resulting in the concentration of 0.4 mg mL^{-1} for Au NRs. The mixture was kept at 40 °C for 48 h. The final products of AP were obtained by centrifugation at 12000 rpm for 20 min.

Synthesis of HA-CHO: The aldehyde groups-bearing hyaluronic acid (HA-CHO) was obtained by the reaction of hyaluronic acid and sodium periodate, following the previous report.[3] Briefly, 1 g of hyaluronic acid was dissolved in 100 mL of deionized water, and then 0.532 g of sodium periodate was added. The mixed solution was stirred at room temperature for 24 h. Subsequently, 0.6 mL of ethylene glycol was added. The product was dissolved in water, dialyzed against deionized water, and obtained by lyophilization. The oxidation degree of the HA-CHO was determined by the titration analysis of hydroxylamine hydrochloride.[4]

Preparation of AP/pDNA-HA Complexes: For the preparation of AP/pDNA-HA complexes, AP/pDNA complexes were first prepared with a fixed N/P ratio of 15. In brief, 2.5 μg of pDNA and 48.4 μg of AP were vortexed and kept constant for 30 min. Then different amounts of HA-CHO (5.7, 11.4, 17.1 or 22.8 μg) were added to the mixture respectively. The mixture was vortexed and kept constant for 30 min and then AP/pDNA-HA complexes were prepared. The weight ratio of HA/PGED in AP/pDNA-HA was 25%, 50%, 75% and 100%, respectively.

Characterization: Transmission electron microscopy (TEM), thermal gravimetric analysis (TGA), particle size and zeta potential measurements were used to characterize the obtained products. For TEM measurements, the samples was droped onto a formvar-covered copper grid, followed by drying naturally. TEM was performed using a Tecnai G2 analytical electron microscope (FEI Company, Hillsboro, OR) operating at 200 kV. A Tarsus TG 209 F3 thermogravimetric analyzer

(Netzsch, Germany) was used to determine the amount of grafted polymers in AP. The hydrodynamic diameters and zeta potentials of the particles were provided by a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). The zeta potential changes of AP/pDNA-HA in buffers at pH 5.0 and 7.4 were measured at different time points. Agarose gel electrophoresis was used to test the pDNA condensation ability of AP, AP/HA' and AP-HA.[5] The stability of pDNA after AP/pDNA-HA irradiated with NIR light laser (1 W/cm^2, 5 min) was evaluated by agarose gel electrophoresis. The electrophoresis test was carried out in TAE running buffer in a Sub-Cell system (Bio-Rad Lab, Hercules, CA). DNA bands were visualized and photographed by a UV transilluminator and BioDco-It imaging system (UVP Inc., Upland, CA).

In Vitro Gene Transfection Assay: Cells were seeded into 24-well plates at a density of 7×10^4 cells/well in 500 μL of medium and cultured for 24 h, and then the medium was replaced with 500 μL of fresh medium containing AP/pDNA or AP/pDNA-HA at different N/P ratios (the concentration of AP was from 12.9 to 77.4 μg/mL). For the preparation of AP/pDNA-HA, HA-CHO (HA/PGED weight ratio of 50%, from 1.5 to 9.1 μg) was added to the AP/pDNA mixture. After 4 h, each well was replaced with 500 μL of the fresh media containing 10% fetal bovine serum (FBS) and cultured for another 20 h, resulting in a total transfection time of 24 h. The cultured cells were washed twice with PBS, and 70 μL of cell lysate (Promega Co., Cergy Pontoise, France) was added. Luciferase gene expression was quantified using a luminometer (Berthold Lumat LB 9507) and a commercial kit (Promega Co.). Bicinchoninic acid assay (Biorad Lab) was carried out to analyze the protein concentration in the cells. Results were expressed as relative light units (RLUs)/mg of cell protein lysate (RLU/mg protein). To observe the intuitive expression, AP/pDNA or AP/pDNA-HA

mediated gene transfection was also assessed by employing plasmid pEGFP-N1 (BD Biosciences, San Jose, CA) as another reporter gene in 4T1 cells. The transfected cells were imaged using a Leica DMIL fluorescence microscope and the percentage of the EGFP-positive cells was determined from flow cytometry (FCM, Beckman Coulter, USA).

In Vitro Cytotoxicity: To evaluate the cytotoxicity of AP/pDNA and AP/pDNA-HA in 4T1 and HEK293 cell lines, pDNA complexes with various N/P ratios were prepared by mixing different amounts of AP nanohybrids with 0.33 μg of pDNA. The HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% FBS, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 ºC, under 5% CO_2 and 95% relative humidity atmosphere. For MTT assay, the cells were seeded in 96-well plates at a density of 10^4 cells per well. After the cells were cultured for 24 h, 100 μL of fresh medium containing serial dilutions of AP/pDNA or AP/pDNA-HA complexes was added and the cells were incubated for another 24 h. As the N/P ratio was from 5 to 30, the concentration of AP was from 21.5 to 129.0 μg/mL. For the preparation of AP/pDNA-HA, HA-CHO (HA/PGED weight ratio of 50%, 0.5 to 3.0 μg) was added to the AP/pDNA mixture. Thereafter, 10 μL of sterile-filtered MTT stock solution in PBS (5 mg/mL) was added to each well to produce a final MTT concentration of 0.5 mg/mL. After that, the unreacted dye was removed by aspiration and 10 μL of MTT (5 mg/mL in PBS) was added to each well and cultured for 4 h. Finally, 100 μL of DMSO was added to dissolve the produced formazan crystals. Cells cultured in a medium without complexes were used as control. The absorbance at a wavelength of 570 nm was measured by a Bio-Rad model 680 microplate reader. Cell viability was calculated from $[A]_{test}/[A]_{control} \times 100\%$, where

$[A]_{test}$ is the absorbance of the complexes and $[A]_{control}$ is the absorbance of the control.

Cellular Internalization: To quantify the cellular internalization ratio of AP/pDNA and AP/pDNA-HA complexes at the N/P ratio of 15, 4T1 cells were seeded into 6-well plates at a density of 8×10^5 cells per well in 3 mL of RPMI medium and cultured for 24 h. Then the medium in each well was replaced with 3 mL of fresh medium containing AP/pDNA or AP/pDNA-HA complexes (6 μg of pDNA labeled by the fluorescent dye YOYO-1[6]) at the optimal N/P ratio of 15 for 4 h (the concentration of AP was 38.7 μg/mL). For the preparation of AP/pDNA-HA, HA-CHO (HA/PGED weight ratio of 50%, 27.3 μg) was added to the AP/pDNA mixture. Then the cells were removed from the culture medium, washed three times with PBS, trypsinized, centrifuged and re-dispersed in 1 mL of PBS. Flow cytometry (MoFlo XDP, Beckman) was used to analyze the fluorescence intensity. Furthermore, the cells were labeled with 200 μL of 4'6'-diamidino-2-phenylindole[7] (DAPI, 0.15 mg/mL in PBS) for 10 min, and imaged on a Leica DMI3000B fluorescence microscope. To obtain the real-time images, CLSM was used for cell imaging treated with AP/pDNA or AP/pDNA-HA complexes. 4T1 cells were seeded at a density of 7×10^4 cell/culture dish in 2 mL of RPMI and incubated for 24 h. Then the AP/pDNA or AP/pDNA-HA (labeled with YOYO-1 for 2 h) were added and incubated for 0.5, 4, and 6 h, respectively (the concentration of AP was 38.7 μg/mL). For the preparation of AP/pDNA-HA, HA-CHO (18.2 μg) was added to the AP/pDNA mixture. Before observation, the cells were washed by PBS for three times. Finally, the nuclei were stained with DAPI for 10 min. The cells were visualized with a 100 × oil immersion lens.

Apoptosis Assay: 4T1 cells were seeded in a 6-well plate at a density of 5×10^5 cells per well and treated with AP/p53 and AP/p53-HA at the N/P ratio of 15 for 48 h. Thereafter, the cells were harvested *via* trypsin digestion and then washed with cold

PBS twice. The apoptosis percentage was measured by using an Annexin V-FITC/PI Apoptosis Kit and flow cytometry (FCM, Beckman Coulter, USA).

Western Blot: The P53 protein and *β*-actin (the loading control) expression levels were determined by anti-P53 antibody (Abcam) and a monoclonal anti-*β*-actin (Abcam), respectively. After 48 h transfection, both proteins from 4T1 cells were extracted with RIPA buffer. 20 μL of protein extracts were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane.

Complementary PTT/GT in Vitro: The combined PTT/GT *in vitro* was evaluated by MTT assay and FDA-PI double staining assay. The 4T1 cells were seeded in 96-well plates at a density of 10^4 cells per well and incubated for 24 h. For the GT group, 100 μL of AP/p53-HA complex (AP: 3.9 μg, HA-CHO: 0.9 μg) was added to the well and incubated for 4 h. Then 100 μL of fresh medium was added to replace the medium and incubated for another 44 h. For the PTT group, 100 μL of AP-HA complexes (AP: 3.9 μg, HA-CHO: 0.9 μg) was added to the well and incubated for 4 h. Then 100 μL of fresh medium was added to replace the medium and the cells were irradiated by an 808 nm laser (1 W cm^{-2}) for 5 min, and followed the same procedure. To assess the antitumor effect of combined GT/PTT *in vitro*, 4T1 cells were incubated with AP/p53-HA (AP: 3.9 μg, HA-CHO: 0.9 μg) complexes for 4 h. Then 100 μL of fresh medium was added to replace the medium and the cells were irradiated by an 808 nm laser (1 W cm^{-2}) for 5 min, and followed the same procedure. The FDA-PI double staining assay was also applied to evaluate the complementary effect of PTT/GT *in vitro.* The cells were seeded into 24-well plates at a density of 7×10^4 cells/well in 500 μL of medium. After the treatment similar to the MTT assay, the cells were incubated with FDA and PI in a dark room for 10 min and imaged using a Leica

fluorescence microscope.

Hemolysis Assay: 1 mL of rat blood was suspended in 10 mL of PBS. The mixture was centrifuged at 3000 rpm for 15 min to separate red blood cells (RBCs). The RBCs were then washed with PBS until the supernatant was clear. Then, the RBCs were resuspended in PBS at the volume concentration of 4%. 100 μL of AP/p53 (N/P =15) and AP/p53-HA (N/P =15) solutions in PBS with concentrations of 3.6 mg/mL were added in 100 μL of RBC suspension in PBS (4%). The final volume concentration of RBCs was 2%. RBCs treated with water and PBS were used as the positive and negative controls, respectively. The resultant RBC suspension were cultured at 37 ºC for 3 h and then centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 545 nm. The hemolysis ratio was calculated using the following equation:

Hemolysis ratio (%) = (ODtest - ODneg)/(ODpos - ODneg) ×100%

where ODtest, ODneg, and ODpos are the OD values of samples, the negative control, and the positive control, respectively.

In Vivo PAI: The PA signals were acquired on the multispectral optical tomography system (MSOT in Vision 256-TF, iThera Medical, Germany) under excitation wavelengths (680–850 nm). Reconstructed PA images were then acquired using the software supplied by the manufacturer. The energy was 100 mJ/cm^2 and the spatial resolutions was 150 μm. For *in vivo* PA imaging, female BALB/c nude mice (6 weeks old, weight 16-18 g) were purchased from Beijing Vital River Laboratory Animal Technology 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. 100 μL of AP (290.3 μg) and AP-HA (AP: 290.3 μg, HA-CHO: 68.2 μg) were i.v. injected into 4T1-tumor bearing

mice. The PA signals in mice were logged at 0, 2, 4, 6, 8, 10, 12 and 24 h post-injection and the PA images were recorded accordingly.

Complementary PTT/GT in Vivo: For tumor therapy, the tumor bearing BALB/c nude mice were randomly divided into four groups with four mice in each group, which were treated by intravenously injection of 100 μL of PBS (control group), AP/p53-HA (GT group), AP-HA+NIR (PTT group), and AP/p53-HA+NIR (PTT/GT group), respectively. For PTT and PTT/GT groups, the 15 μg of p53 for per mouse was utilized. The treatments were administrated once every other day for ten days. The tumors in the PTT and PTT/GT groups were irradiated with an 808 nm laser (1 W/cm^2, 5 min) only once at 10 h after the first intravenous injection. A caliper was used to measure the size of tumors after treatment every two days. The volume was calculated by the formula: tumor length × (tumor width)^2/2. Relative volume V/V_0 (V_0 as the initial tumor volume before treatment) was used to evaluate the tumor growth. After the treatment, all the mice were euthanized and the tumors were weighed, imaged, and dissected, prior to H&E and immunohistochemical analysis, which were performed according to the procedures reported previously.[8]

Statistical Analysis: All experiments were repeated at least three times. Data were presented as means ± standard deviation. Statistical significance was evaluated by using Student *t*-test when only two groups were compared. *$p < 0.05$ was considered to be statistical significance. **$p < 0.01$ and ***$p < 0.001$ were considered to be extreme significance.

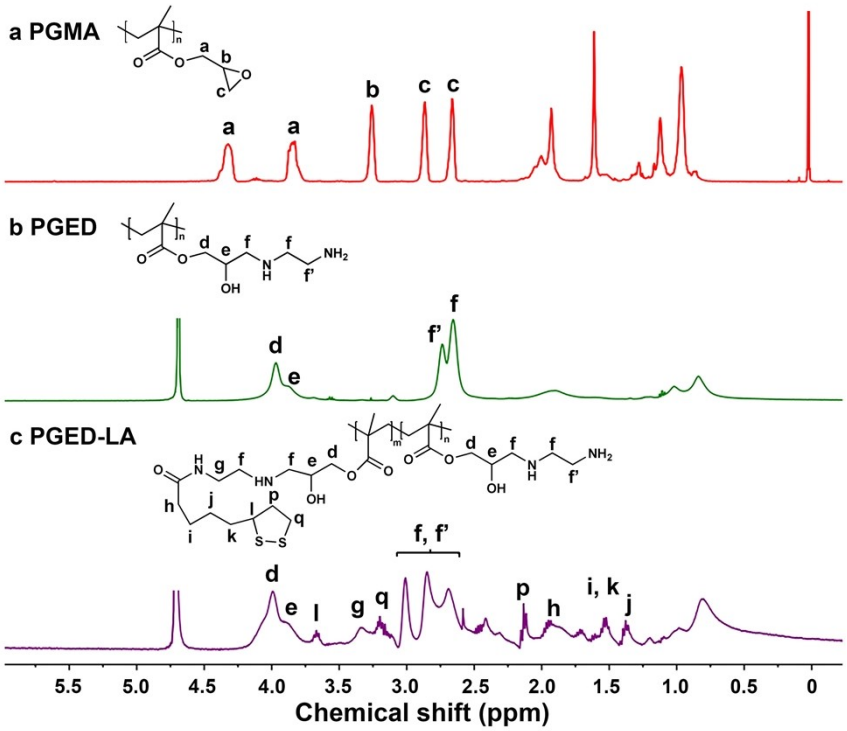

Fig. S1 ^{1}H NMR spectra of (a) PGMA in $CDCl_3$, (b) PGED in D_2O and (c) PGED-LA in D_2O.

Molecular Structures of PGMA, PGED and PGED-LA. Fig. S1 shows the representative chemical structures of PGMA, PGED, and PGED-LA. For PGMA (Fig. S1a), the peaks at δ = 3.8 and 4.3 ppm correspond to the methylene protons adjacent to the oxygen moieties of the ester linkages (a, O=C-O-$\underline{CH_2}$). The signals at δ = 3.2 ppm and peaks at δ = 2.6 and 2.8 ppm can be assigned to the methylidyne protons (b, CH_2-$\underline{CH}$(O)-CH_2) and methylene protons of the epoxide ring (c, CH_2-CH(O)-$\underline{CH_2}$), respectively. The ratio of peak area of a, b, and c is about 2:1:2, indicating that the epoxy groups of PGMA remained intact throughout ATRP process. After the ring-opening reaction of PGMA with ED, the NMR spectra of PGED were shown in Fig. S1b. The peaks at δ = 3.8 and 4.3 ppm (a) shifted to a position (d) at δ = 3.95 ppm. The scope of 3.9-3.5 ppm was attributed to the methylidyne (e, $\underline{CH}$-OH) protons adjacent to the hydroxyl groups. The peaks located at the scope of 3.0-2.5 belonged to the methylene protons (f, $\underline{CH_2}$-NH_2 or f', $\underline{CH_2}$-NH) adjacent to primary or secondary

amine groups. The area ratio of peaks f and f' and peaks d and e is about 2.0, indicating that all the epoxy groups participated in the ring-opening reaction. PGED-LA was generated via amidation reaction, as shown in Fig. S1c. The peaks at 1.96 ppm (h, NH-C(O)-$\underline{CH_2}$) belonged to the carbonyl moiety of amide groups. The signal δ = 3.66 ppm was associated with the methylene protons adjacent to the disulfide groups (l, S-S-$\underline{CH_2}$). The peak at 2.13 was related to the methylene protons adjacent to the methylidyne groups in the ring of lipoyl groups (p, S-S-CH-$\underline{CH_2}$). NMR analysis confirmed the successful conjugation of LA with PGED.

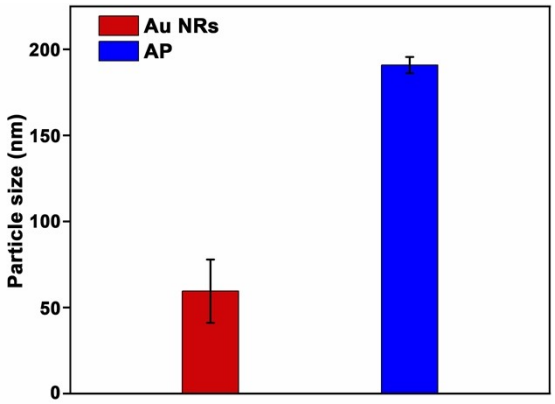

Fig. S2 Particle size of Au NRs and AP nanohybrids determined by dynamic light scattering.

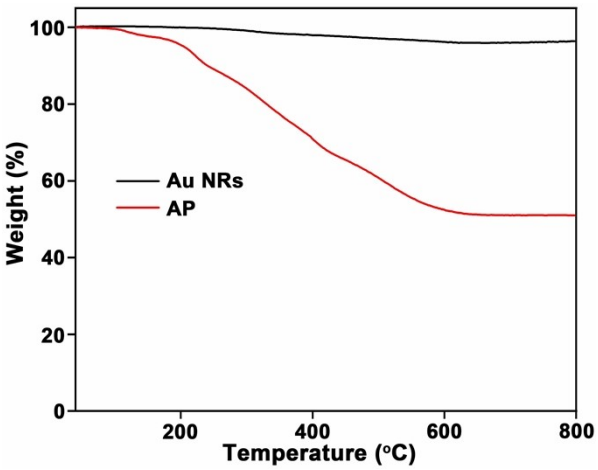

Fig. S3 Thermogravimetric analysis of Au NRs and AP nanohybrids.

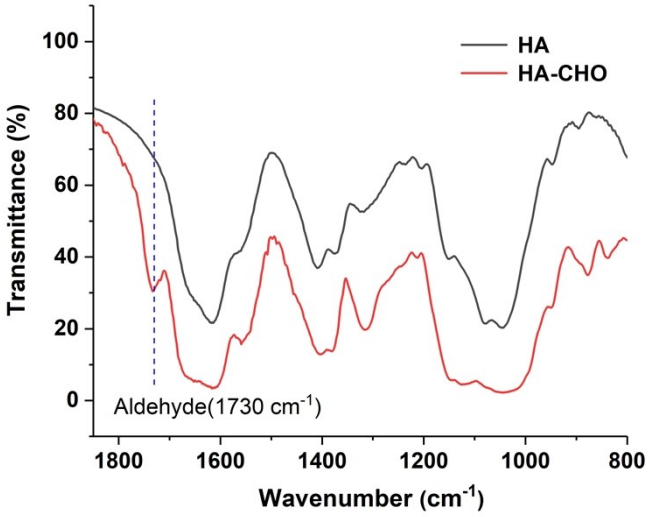

Fig. S4 FTIR spectra of HA and HA-CHO.

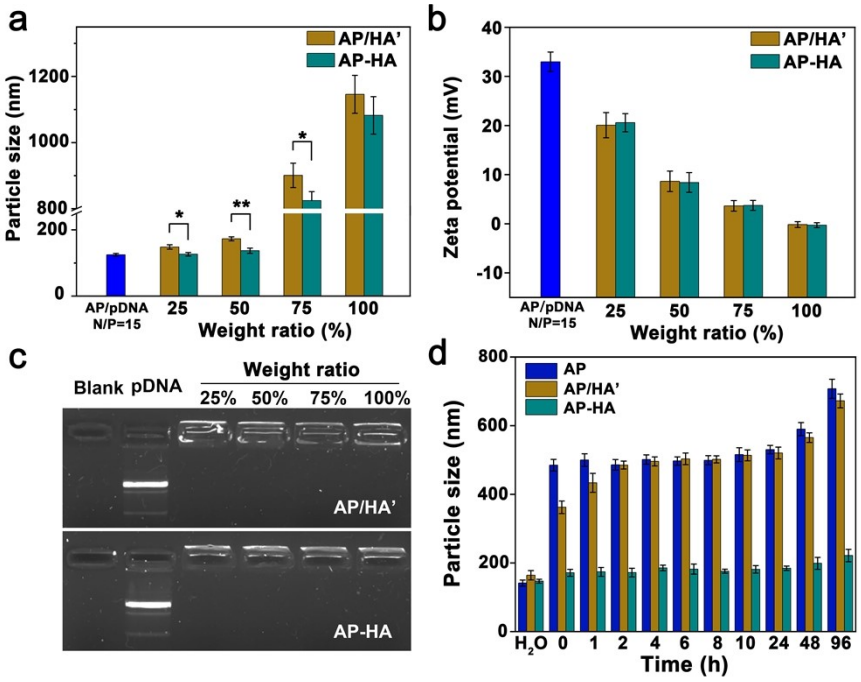

Fig. S5 (a) Particle size and (b) zeta potential of AP/pDNA-HA and AP/pDNA/HA' (N/P =15) at various weight ratios of HA/PGED. (c) Electrophoretic mobility of AP/pDNA/HA' and AP/pDNA-HA at the N/P ratio of 15. (d) Particle size of AP/pDNA, AP/pDNA/HA' and AP/pDNA-HA complexes after incubation in 10% FBS medium for different times.

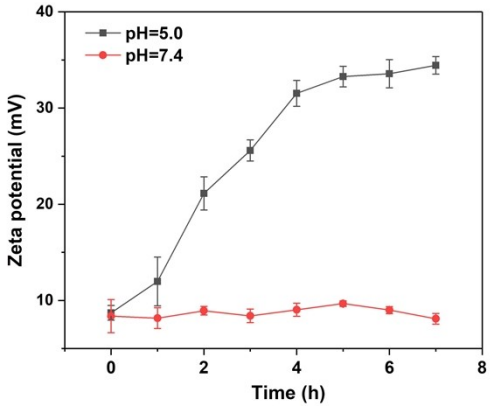

Fig. S6 Zeta potential changes of AP/pDNA-HA (N/P =15) after incubation in buffers at pH 5.0 and pH 7.4 over time.

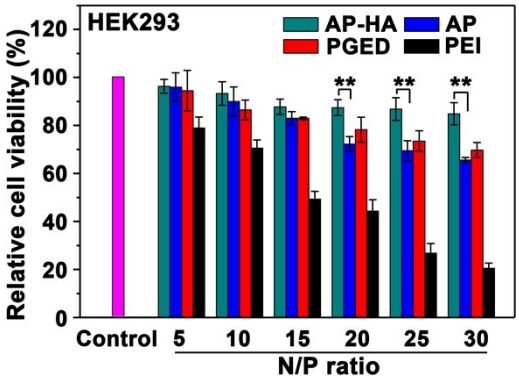

Fig. S7 Cytotoxicity of AP/pDNA and AP/pDNA-HA in HEK293 cell line.

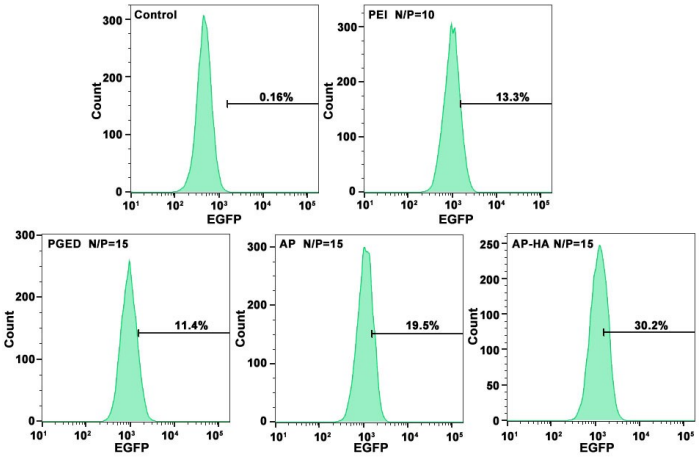

Fig. S8 Flow cytometric analysis of 4T1 cells treated with the complexes of pEGFP-N1 plasmid with PEI, PGED, AP and AP-HA, respectively.

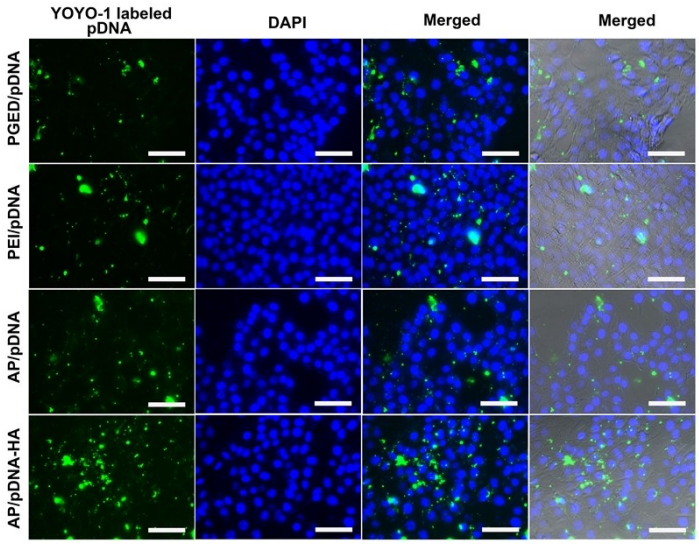

Fig. S9 The typical fluorescence microscopy and merged images of 4T1 cells treated with YOYO-1 labeled PGED/pDNA, PEI/pDNA, AP/pDNA and AP/pDNA-HA for 4 h. Scale bar: 50 μm.

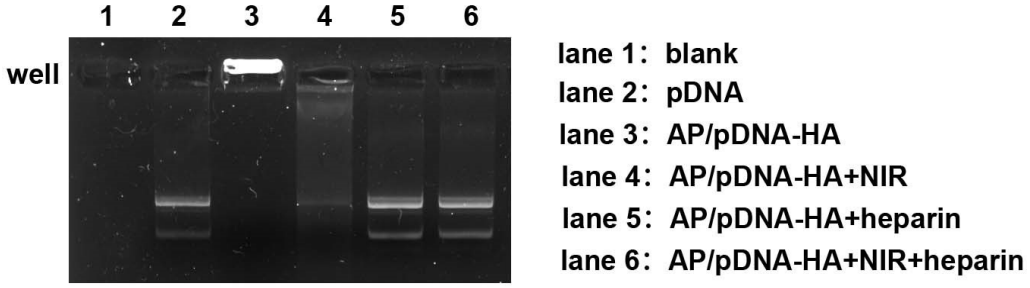

Fig. S10 Agarose gel electrophoresis assay of AP/pDNA-HA (N/P =15) complexes under different conditions with or without NIR irradiation.

Stability of pDNA: From Fig. S10, negative pDNA could be released from the well (lane 2), while pDNA in the AP/pDNA-HA complex was retained in the well (lane 3) due to the electrostatic interaction. After NIR laser irradiation (808 nm, 1 W cm^{-2}, 10 min), successful release of pDNA from AP/pDNA-HA complex was observed. The pDNA could be released from AP/pDNA-HA by heparin treatment (lane 5). After NIR irradiation (1 W cm^{-2}, 10 min) and heparin treatment, pDNA was released from complexes completely (lane 6). Compared with lane 5 and lane 6, it could be speculated that pDNA was not damaged by NIR irradiation.

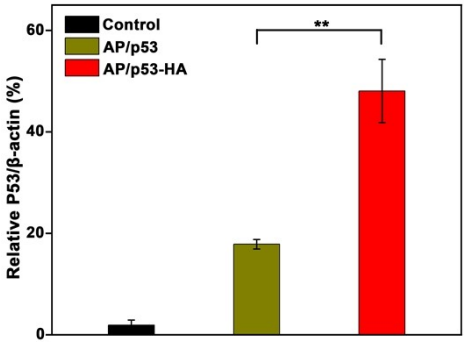

Fig. S11 Quantitative statistics of Western blot of P53 protein expression in 4T1 cells.

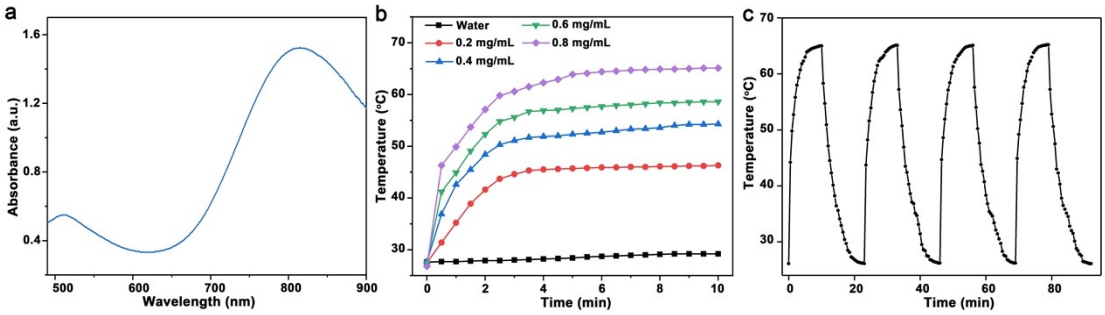

Fig. S12 (a) UV-vis spectra of AP-HA and (b) temperature elevation of AP-HA aqueous solutions upon irradiation with an 808 nm NIR light laser (1 W cm^{-2}, 10 min). (c) Photothermal cycling curve of AP-HA (0.8 mg/mL) upon NIR laser irradiation (1 W cm^{-2}).

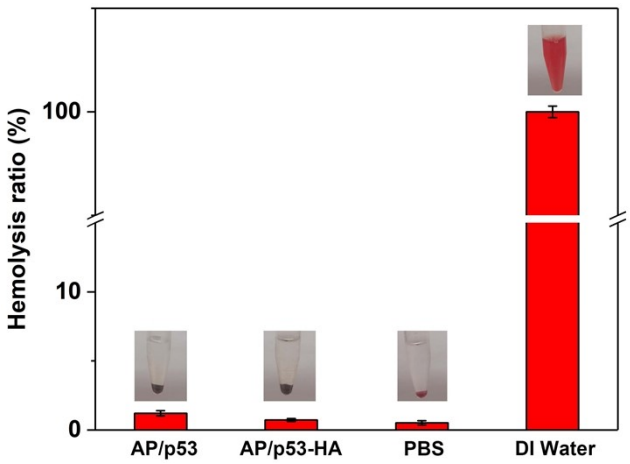

Fig. S13 Hemolysis ratio of RBCs treated with AP/p53 (N/P =15) and AP/p53-HA (N/P =15) at the concentrations of 3.6 mg/mL (mean ± SD, n = 3).

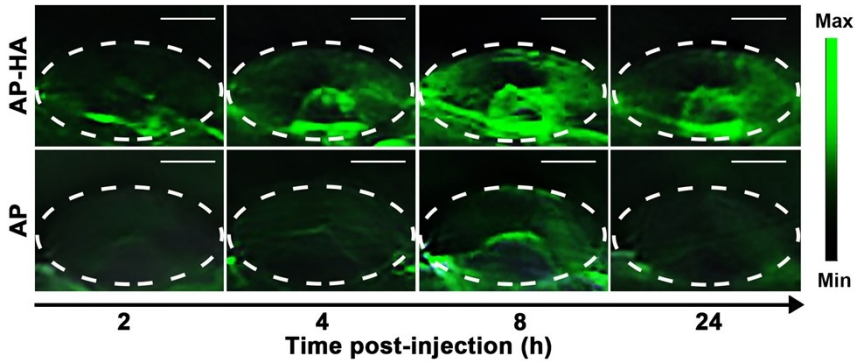

Fig. S14 PA images of the tumor site at different time points after intravenous injection of AP-HA or AP nanohybrids. Scale bar: 2 mm.

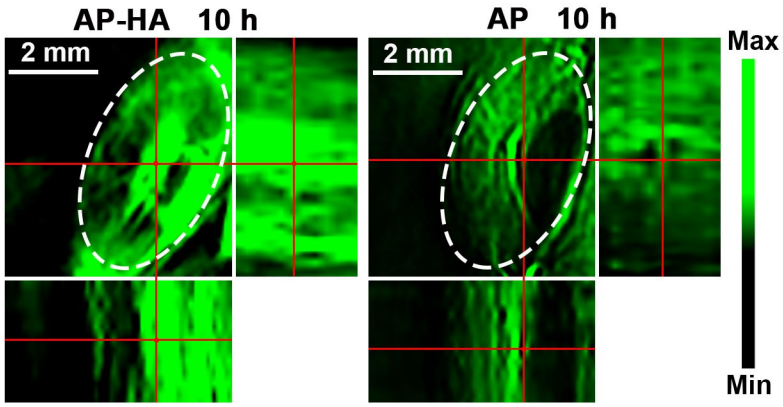

Fig. S15 3D PA images tumors at 10 h post-injection of AP-HA and AP.

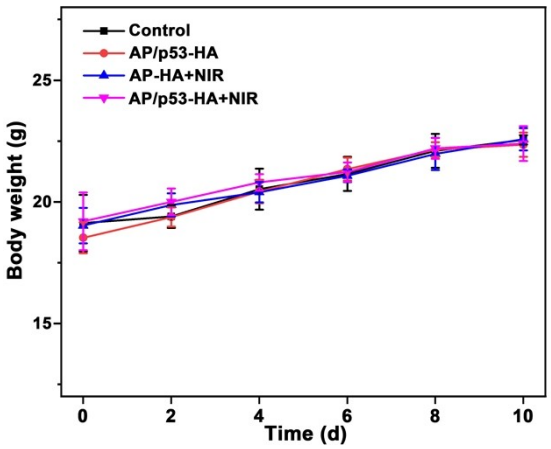

Fig. S16 Body weight evolution of tumor-bearing mice following different administrations: PBS, AP/p53-HA, AP-HA+NIR, and AP/p53−HA+NIR, respectively.

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