Electronic Supplementary Information

Supramolecular nanovesicles for synergistic glucose starvation and hypoxia-activated gene therapy of cancer

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Materials and Methods

Materials and characterization

Tetrachloroauric acid ($HAuCl_4 \cdot 4H_2O$), L-ascorbic acid, sodium borohydride ($NaBH_4$), ethanol, 25% glutaraldehyde, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), amantadine, glucose oxidase (GOx), tetrahydrofuran (THF), β-D-Glucose, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) were purchased from Aladin and used as received. Sodium citrate was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The transfection agent Polyjet^{TM} was purchased from Beyotime (China). Hypoxia probe Cy5-HIF-1α was purchased in Bioss (China). COOH-PLA-PEI was purchased from Xi'an Ruixi (China). β-CD-NH_2 was purchased from Zhiyuan (China).

Milli-Q Integral system (Merck) was used to supply Milli-Q water in this work. The morphology of NPs was observed by transmission electron microscopy (TEM, JEOL 2100F, Japan). The zeta potential of nanomaterials was measured on a Zetasizer (Malvern) at 200KV. NMR spectra were obtained using a Bruker Ultra Shield 600 PLUS NMR spectrometer. Fluorescence images were acquired by confocal laser scanning microscopy (CLSM, Leica TCS SP8, German) and inverted fluorescence microscope (Olympus IX73). Fourier transform infrared (FT-IR) spectra and UV-vis-NIR absorbance spectra were measured on IFS-66V/S and Shimadzu UV-1800 spectrophotometers. MTT assays were measured using a microplate reader (Infinite F200 Pro, TECAN). Intracellular uptake and cell apoptosis were both quantified by a flow cytometer (Beckman coulter). Biodistribution was obtained by in vivo imaging system (IVIS, Lumina XR III).

Preparation of Au NPs

Au NPs were prepared by following method: $HAuCl_4 3H_2O$ solution (0.51 mL, 50 mmol) was dispersed in 100 mL ultrapure water, and the mix solution was boiled with vigorous stirring. Then a sodium citrate solution (0.7 mL, 1 wt%) was dropped into the boiling solution. After 10 min reaction, the Au nanoparticle solution was prepared.

Preparation of Au@Fc NPs

The SH-Fc was dissolved in DMSO and added into Au NPs aqueous solution, followed by 1 h sonication and 12h stirring. The Au@Fc NPs were centrifuged and washed with the solution of DMSO : water = 1:5 to remove the excessive SH-Fc.

Preparation of β-CD-PLA-PEI

β-CD-PLA-PEI was synthesized by the reaction of COOH-PLA_{2w} -PEI_{2k} (M.W.=22000, 1 equiv.) with excess NH_2-β-CD (5 equiv.). EDC (5 equiv.) and NHS (5 equiv.) in 10 mL DMSO and the mixture was stirred at 70 ºC temperature for 24 h. The solution was dialyzed with dialysis cassette (MWCO, 3500 Da) against water overnight to remove the unreacted β-CD and lyophilized to obtain β-CD-PLA-PEI. The obtained β-CD-PLA-PEI was confirmed by ^{1}H NMR.

Preparation of Au@PLA-PEI NPs

20 mg β-CD-PLA-PEI was dissolved in 100uL DMSO respectively and dropwise added into 1 mL Au@Fc aqueous solution. The mixture was stirred for 1 h and centrifuged to obtain the Au@PLA-PEI NPs.

Preparation of Au NVs

Au@PLA-PEI NPs were dispersed in 200 μL THF and dropwise added into 1 mL plasmid and GOx aqueous solution followed by sonication at room temperature for microemulsion. The plasmid loaded vesicles were obtained after 15 min sonication followed by 24 h stirring to evaporate the THF. The Au NVs were centrifuged to remove the excessive plasmid and GOx. The obtained P/GAu NVs were dispersed in 200 μL water.
The GAu NVs without plasmid were prepared via the same method by dispersing Au@PLA-PEI NPs in THF and dropwise added into water for microemulsion.

Quantification of plasmid concentration

The plasmid concentration in Au NVs was quantified by measuring the P content by ICP-MS according to our previous work. Firstly, the plasmid loaded Au NVs were washed by water and dispersed in 100 μL water. Then, 100 μL aqua regia was added to decompose Au and destroy DNA structure. The solution was diluted by 1% HNO_3 and the P content was measured by ICP-MS. The plasmid concentration was calculated according to a standard curve.

Quantification of GOx concentration

The GOx concentration in Au NVs was quantified by measuring the H_2O_2 generation by 3,3',5,5'-Tetramethylbenzidine (TMB). Firstly, excessive adamantanamine was added into the GAu NVs solution to induce GOx burst release and the GOx solution was obtained by ultrafiltration (1000 Da). TMB solution was added into the GOx solution and incubated for 4h at room temperature, 50 μL of 2 M H_2SO_4 as a stopping solution was added. Then, the UV absorption spectrum was obtained and the value at 448 nm was recorded and normalized as a standard curve($Y = 0.031*X - 0.062$).

Release profile of Au NVs

PTX was used as a model payload in Au NVs and GAu NVs for release profile studies by using a dialysis method. 15 mL phosphate-buffered saline (PBS) solution was used as the release medium to study the PTX release profile under different condiation: 1) 5 mM H_2O_2, 1 mg/ mL glucose and 1U GOx; 2) 1 mM H_2O_2, 1 mg/ mL glucose and 1U GOx; 3) 0 mM H_2O_2, 1 mg/ mL glucose and 1U GOx; 4) 5 mM H_2O_2, 0 mg/ mL glucose and 1 U GOx(O_2 elimination); 5)1 mM H_2O_2, 0 mg/ mL glucose and 1U GOx(O_2 elimination); 6) 0 mM H_2O_2, 0 mg/ mL glucose and 1U GOx (O_2 elimination); 4) 5 mM H_2O_2, 0 mg/ mL glucose and 0 U GOx; 5) 1 mM H_2O_2, 0 mg/ mL glucose and 0 U GOx; 6) 0 mM H_2O_2, 0 mg/ mL glucose and 0 U GOx; 7) SH-Au NVs in 5 mM H_2O_2, 1 mg/ mL glucose and 1U GOx. 100 μL dialysate was suck out and replenished at 0 min, 10 min, 15min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 20 h, 24 h, 40 h, 48 h. The PTX release was quantified by HPLC. The chromatographic conditions were as follows: the column used was an XDB C18 (4.6 × 250 mm, 5 mm), and the mobile phase consisted of acetonitrile and water (60/40, v/v).

Cell Culture and in Vitro Cytotoxicity Assays.

B16 cell line (melanoma cell line) was cultured in 25 cm^2 flasks using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin & streptomycin (PS) solution at 37 ℃ with 5% CO_2.

Controlled EGFP expression by *RTP801::EGFP* loaded Au NVs

B16 cells were incubated by *RTP801::EGFP* plasmid loaded Au NVs for 24 h under hypoxia and normoxia responsively. A parallel-group incubated with CMV::EGFP loaded Au NVs was conducted for positive comparison and a parallel group incubated with empty Au NVs without plasmid loading was used as the negative control. The internalized fluorescence images were acquired by a fluorescence microscope (IX73) on the cells after washing with PBS.

Controlled p53 expression by *RTP801::p53* loaded Au NVs

B16 cells were incubated by *RTP801::p53* plasmid loaded PAu NVs and P/GAu NVs for 24 h under hypoxia and normoxia responsively. A parallel-group incubated with polyjet^{TM} transfected *RTP801::p53* plasmid was conducted for positive comparison. The AF647 conjugated p53 antibody was used for fluorescence detection via flow cytometry.

Biosafety evaluation

B16 cells were incubated with Au NVs (50, 100, 150, 200, 250 μg Au/mL) for 24 h before cell viability evaluation by MTT assays.

Cytotoxicity evaluation

B16 cells and A12 cells were incubated with Au NVs, PAu NVs, GAu NVs, P/GAu NVs at plasmid concentration 200, 400, 600, 800,1000 ng/mL, GOx concentration of 44.1, 88.2, 132.3, 176.4 and 220.5 mU/mL and Au concentration of 27.52, 55.04, 82.56, 110.08 and 137.76 μg/mL for 24 h. The cell viability was evaluated by MTT.

Apoptosis rate

Cells after incubation were processed by an Annexin/V-FITC Kit. The fluorescence signal of Annexin V-FITC and PI (positive and negative) obtained by flow cytometer reflected the apoptosis rate.

Biodistribution studies

Cy5 loaded Au NVs (Au concentration of 2 mg/mL) were *i.v.* injected into B16 tumor-bearing C57BL6 mice with a tumor volume of 200 mm^3 and the bio-distribution were observed pre- and 2, 4, 6, 8, 10, 12, 24, 36, and 48 h post-injection by in vivo imaging system (IVIS, Lumina XR III). The mice were sacrificed after in vivo imaging, and the main organs and tumor tissues were collected for ex-vivo imaging.

Tumor inhibition studies

25 B16 tumor-bearing C57BL6 mice were randomly divided into five groups and given the following treatments: 1) PBS, 2) Au NVs, 3) PAu NVs, 4) GAu NVs, 5) P/GAu NVs (with the concentration of Au, RTP801::p53 plasmid and GOx of 2 mg/mL, 14.534 μg/mL, and 3.173 U/mL, respectively for each group). The treatment was repeated at day 0, day 3 and day 6 for total 3 times. The tumor length (a) and width (b) were measured every two days by vernier caliper, and the tumor volume (V) was evaluated by following the equation:

$V = 1/2\ ab^2$.

The body weight of the mice in each group was measured and the survival rate of the mice was recorded every two days in the 14 days treatment. The mice were sacrificed and the tumors in each group were excised and weighed at the 14^{th} day.

The main organs as well as the tumor in each group were sliced and stained according to the hematoxylin and eosin (H&E) and Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL) assay.

Results and Discussion

NMR of β-CD-PLA-PEI

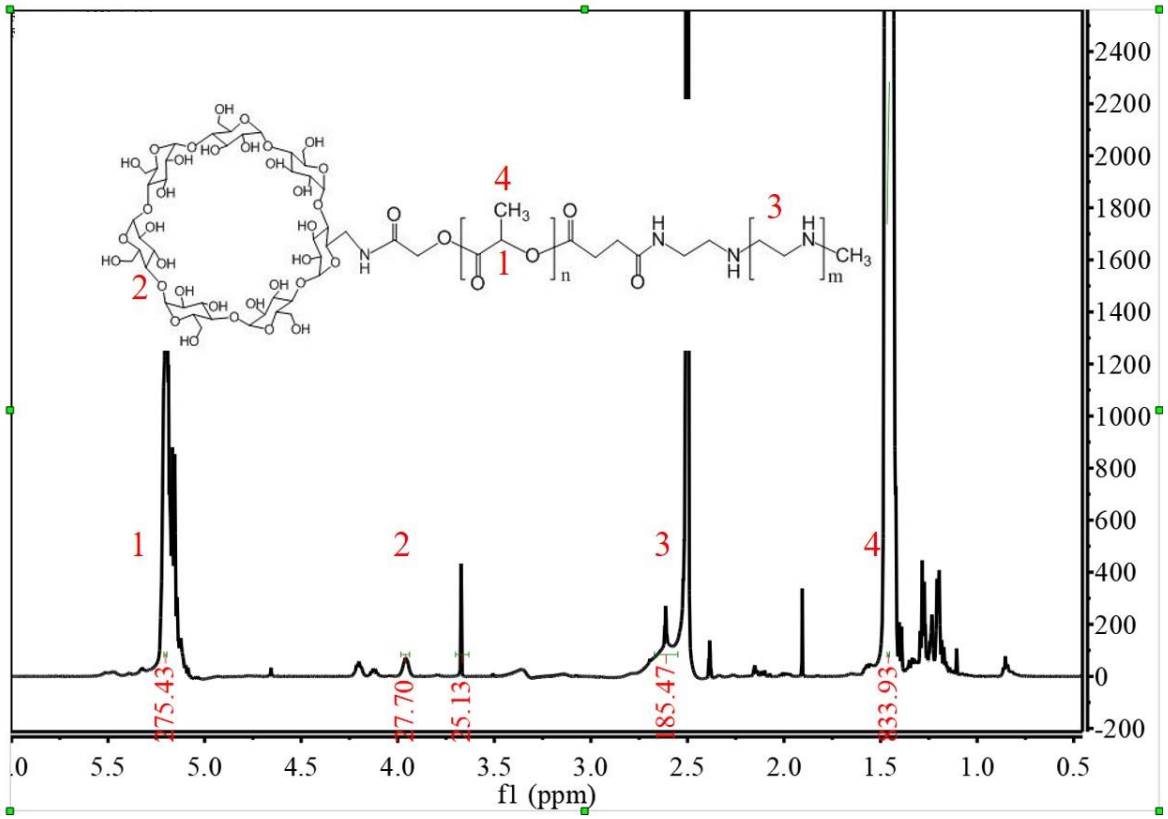

Figure S1. [1]H NMR spectrum of β-CD-PLA-PEI in DMSO-d6.

GOx quantification

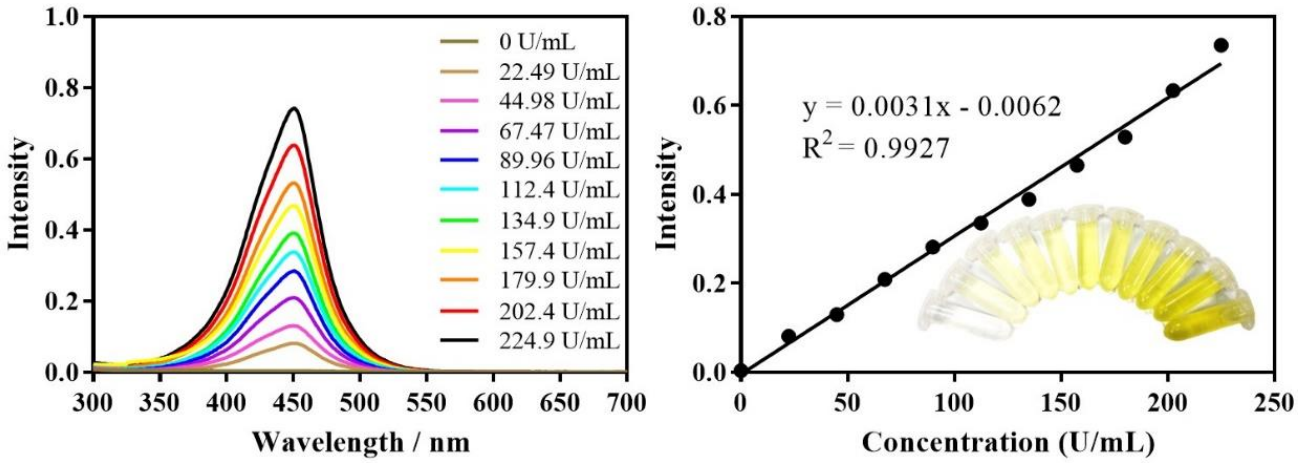

Figure S2. UV absorption spectrum of TMB incubated GOx solution and the standard of the absorption value at 448 nm. Insert is the digital photo of the TMB incubated GOx solution.

Release profile without O_2

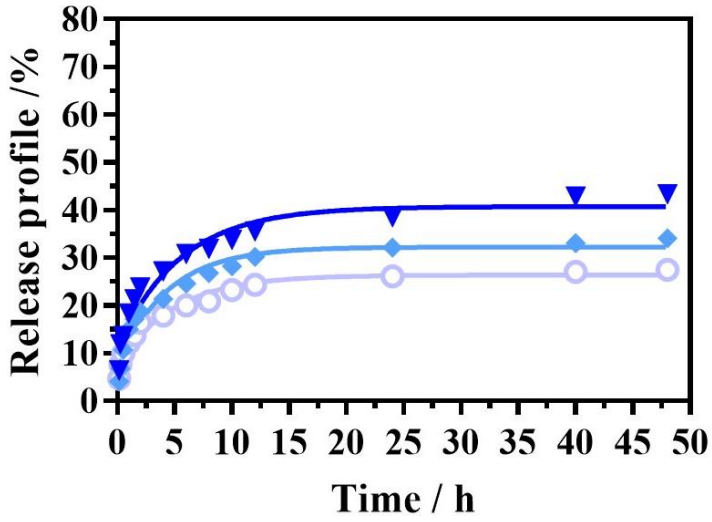

Figure S3. The release profile of PTX without O2. Release profile of PTX in Au NVs with different concentration of H_2O_2, glucose, and GOx.(▼5 mM H_2O_2, 0 mg/ mL glucose and 1 U GOx(O_2 elimination);◆1 mM H_2O_2, 0 mg/ mL glucose and 1U GOx(O_2 elimination); ○0 mM H_2O_2, 0 mg/ mL glucose and 1U GOx(O_2 elimination))

Hypoxia-activated gene expression

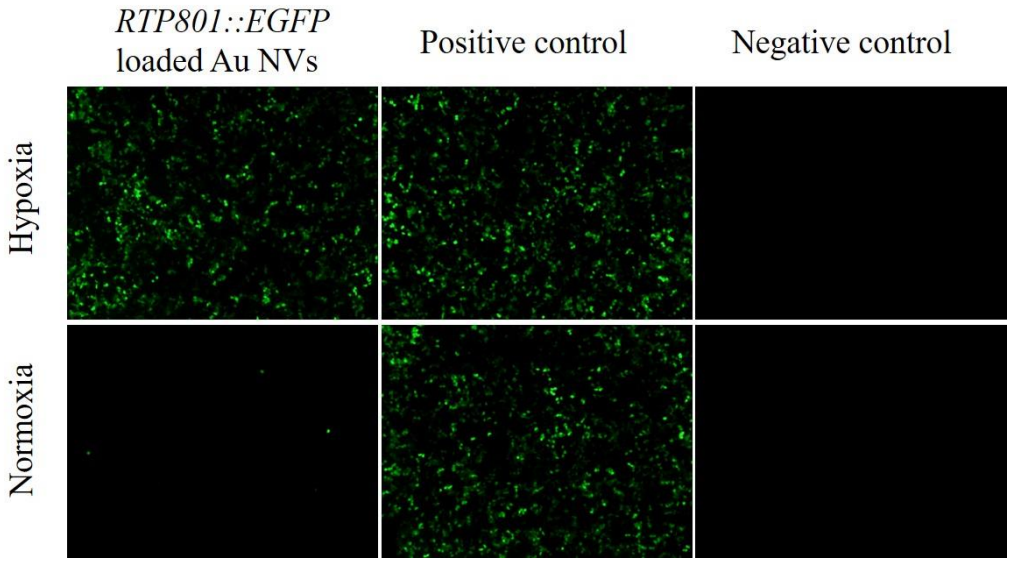

Figure S4. Gene expression in B16 cells after incubation with (left) RTP801::EGFP loaded Au NVs, (middle) CMV::EGFP loaded Au NVs. The untreated B16 cells was used as the negative control. (right) Control.

Cell apoptosis rate of B16 and A12 cells

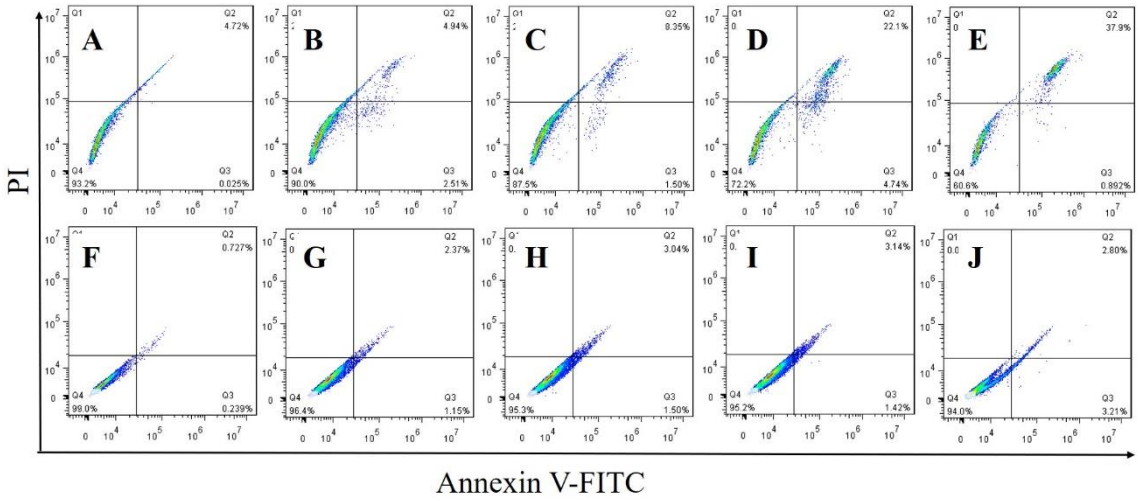

Figure S5. Cell apoptosis rate of B16 and A12 cells after treated by control (A and F), Au NVs (B and G), PAu NVs (C and H), GAu NVs (D and I) and P/GAu NVs (E and J) for 24 h.

Histological analysis of main organs

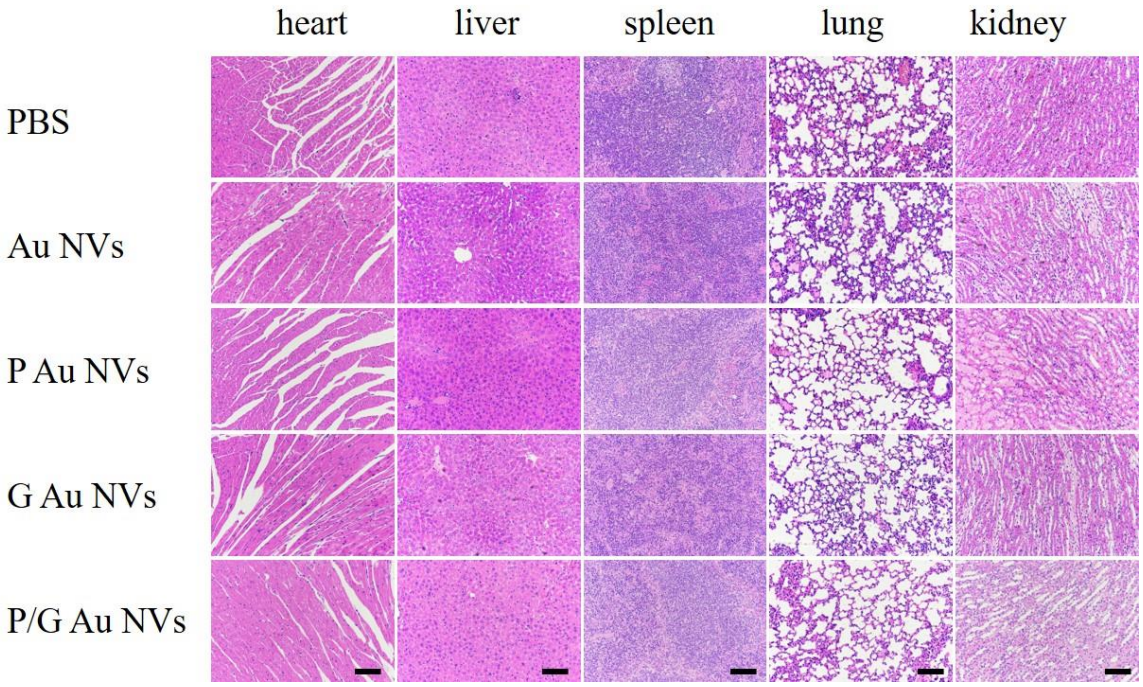

Figure S6. Histological analysis of main organs with H&E staining. Scale bar: 100 μm.