# Supplementary information

# Integrated cascade catalysis of AuPtCu nanozymes and glycolysis inhibition for synergistic breast cancer therapy via metabolism regulation

Kanglong Wang[a], Cheng Ni[a], Junhao Huang[a], Yunfei Huang[a], Xueyan Cao[a], Jing Ma[b]*, Xiangyang Shi[a]*, and Rui Guo[a]*

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Shanghai Engineering Research Center of Nano-Biomaterials and Regenerative Medicine, College of Biological Science and Medical Engineering, Donghua University, Shanghai 201620, China

[b] Section of echocardiography, Department of Cardiology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

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* Corresponding author. E-mail: majing0709@163.com (J. Ma), xshi@dhu.edu.cn (X.Y. Shi) and ruiguo@dhu.edu.cn (R. Guo)

## Materials and methods

**Materials.** Generation 5 (G5) PAMAM dendrimers were purchased from Dendritech (Midland, MI). 4-(Bromomethyl) phenylboronic acid (PBA) was purchased from Bide Pharmatech Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd (Shanghai, China). Acetic anhydride ($Ac_2O$), triethylamine (TEA), from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Copper (II) Chloride Dihydrate was purchased from J&K Chemical (Beijing, China). Potassium hexachloroplatinate was acquired from Shanghai Titan Scientific Co., Ltd (Shanghai, China). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), sodium borohydride ($NaBH_4$) and hydrogen peroxide ($H_2O_2$, 30%) were provided by Sigma-Aldrich (St. Louis, MO). 2-Deoxy-D-glucose (2-DG) was obtained from Macklin Co., Ltd. (Shanghai, China). Cy5.5 with N-hydroxysuccinimide (NHS) group (Cy5.5-NHS) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. Regenerated cellulose dialysis membranes, 3,5-dinitrosalicylic acid (DNS) reagent and phosphate buffered saline (PBS) were from Shanghai Yuanye Biotechnology Corporation (Shanghai, China). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Murine 4T1 breast cancer cells were provided by the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). Penicillin and streptomycin were sourced from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Cell Counting Kit-8 (CCK-8) was purchased from Sea Biotech Co., Ltd (Shanghai, China). 4', 6-Diamidino-2-phenylindole (DAPI) was from BestBio Biotechnology Co., Ltd. (Shanghai, China). Reactive oxygen species (ROS) assay kit, 2, 7-dichlorofluorescin diacetate (DCFH-DA), JC-1 Mitochondrial Membrane Potential Assay Kit, annexin V-FITC/PI apoptosis detection kit, adenosine triphosphate (ATP) assay kit and bicinchoninic acid (BCA) assay kit from Beyotime Biotechnology (Shanghai, China). Glutathione (GSH) assay kit was provided by Nanjing Jiancheng Biotechnology Research Institute Co., Ltd (Nanjing, China). The lipid peroxidation (LPO) sensor of C11-BODIPY581/591 was from GlpBio Technology (Montclair, CA). Lactate assay kit

was from Abbkine Scientific Co., Ltd. (Wuhan, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm. All chemicals reagents are commercially available and can be used without further purification unless otherwise stated.

**Synthesis of G5.NHAc-P.** G5.$NH_2$-PBA was synthesized by dropwise addition of a solution of PBA (24.78 mg, 10 mL DMSO) to a solution of G5 (50 mg, 10 mL DMSO), followed by stirring at 70 °C for 24 h. Subsequently, the mixture solution was dialyzed against water (9 times, 2 L) using a dialysis membrane with an MWCO of 3500 Da for 3 days, and then lyophilized to yield the G5.$NH_2$-PBA.

Next, the G5.$NH_2$-PBA (20 mg, in 5 mL water) was mixed with TEA (6.63 μL) with stirring for 30 min. Subsequently, $Ac_2O$ (3.32 μL) was added to the mixture, and stirring was continued for 24 h to acetylate a portion of the remaining terminal amine groups.[1] Then the mixture solution was dialyzed against water (9 times, 2 L) using a dialysis membrane with an MWCO of 3500 Da for 3 days and lyophilized to obtain the G5.NHAc-P product, which was stored at -20 °C for subsequent use.

**Characterization Methods.** $^1$H NMR spectroscopy was performed using a Bruker AV 400 NMR spectrometer (Karlsruhe, Germany) with $D_2O$ as the solvent. UV-vis spectra were collected using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Waltham, MA). Samples were dispersed in water before measurements. The morphology and size of the samples were investigated by a JEOL 2100F transmission electron microscope (TEM, Tokyo, Japan) at a 200-kV accelerating voltage. Samples were by dropping onto a carbon-coated copper grid and air-dried before measurement. The Zeta potential and dynamic light scattering (DLS) were tested using a Malvern Zetasizer Nano ZS system (Worcestershire, UK) equipped with a standard 633 nm laser. The concentration of Au, Pt and Cu in the synthesized samples was analyzed using a Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). XPS spectra were measured by X-ray photoelectron spectroscopy (ESCALAB 250Xi). Dissolved oxygen measurement was recorded by JPBJ-605F.

***In Vitro* Release Profile of 2-DG and Metal Ions.** The *in vitro* response release kinetics of 2-DG and Au, Pt, Cu from G5.NHAc-PG@APC was investigated in various buffer solutions of different pH (7.4, 6.5, and 5.5) with or without $H_2O_2$ (1 mM). G5.NHAc-PG@APC (5 mg) nanoplatforms were dispersed in 1 mL of PBS buffer solution with corresponding pH values and then seal the dispersed solution in a dialysis bag with MWCO of 3500Da. Then immerse the dialysis bag in 9 mL of PBS buffer solution corresponding to pH (with or without 1 mM $H_2O_2$) and placed in a shaker at 37 °C for 48 hours. Within a specified time interval (0.5, 1, 2, 4, 8, 12, 24, 48 h), 1 mL sample solution was taken out from the buffer solution and replenish an equivalent amount of fresh buffer solution.

The concentration of 2-DG was detected by DNS method. Briefly, first prepare 2-DG standard solutions with different concentration gradients and react them with DNS reagents at 100 °C water bath for 5 min. Dilute with water and measure the absorbance at 535 nm by UV-vis spectroscopy, and obtain a standard curve. The absorbance of 2-DG in the sample solution was obtained with the same method, and then calculate the corresponding concentration. Meanwhile, the concentration of Au, Pt and Cu in the solutions at different time points was determined by ICP-OES.

**Blood Compatibility Test.** All animal experiments were performed by the guidelines from the Institutional Committee for Animal Care and Use of Donghua university and also with the regulations of the National Ministry of Health. Hemolysis assay was performed to assess the hemocompatibility of the G5.NHAc-PG@APC. Briefly, 2 mL whole blood was taken from the BALB/c mice and transferred into an anticoagulant tube. After centrifugation (3000 rpm, 5 min) and washing sediment with PBS 5 times, the red blood cells (RBCs) were collected and then diluted 10 times with PBS. 100 μL of the above diluted RBCs were added to 7 centrifuge tubes containing 900 μL of G5.NHAc-PG@APC solution ([G5] = 2, 5, 10, 20, and 50 μM), ultrapure water (positive control) or PBS (negative control) and mixed evenly. After incubation at 37 °C for 2 h, the all samples were centrifuged at 10000 rpm for 5 min and the UV absorbance of each tube at 540 nm was measured. The hemolysis rate is calculated according to the following formula:

$$\text{Hemolysis rate (\%)} = (D_t - D_{nc}) / (D_{pc} - D_{nc}) \times 100\ \% \quad (1)$$

$D_t$ is the absorbance value value of the test sample at 540 nm, while $D_{pc}$ and $D_{nc}$ are the absorption value of the positive control and the negative control at 540 mm, respectively.

**POD-like Activity Assay.** The POD-like activity of the G5.NHAc-PG@APC was investigated using TMB as the substrate in the presence of $H_2O_2$ at room temperature. G5.NHAc-PG@APC (20 μg $mL^{-1}$) dissolved in PBS buffer solution (pH 5.5) containing TMB (0.25 mg $mL^{-1}$) and $H_2O_2$ (10 mM) with a final reaction volume of 2 mL. After a 5 min, The POD like activity performance was demonstrated by detecting the absorption spectrum of colorimetric reactions. Subsequently, the enzymatic reaction kinetics of the POD-like activity of the G5.NHAc-PG@APC were investigated. Briefly, the carried out in PBS (final volume of 3 mL, pH 5.5) at room temperature, using the G5.NHAc-PG@APC (20 μg $mL^{-1}$) as the catalyst, TMB (0.25 mg $mL^{-1}$) as the chromogenic agent, and varying concentrations of $H_2O_2$ (1, 2, 5, 10, 20, 40 mM) as the substrate. The absorbance at 652 nm was monitored every 20 s after the initiation of the reaction. Finally, the maximum velocity ($V_{max}$) and the Michaelis-Menten constant ($K_m$) of the enzymatic reaction were determined using the following formula:

$$A = \varepsilon bc \#(2)$$

$$v_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \#(3)$$

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \#(4)$$

$v_0$ refers to the initial reaction velocity, $\varepsilon$ is the molar absorption coefficient of oxTMB (where ε = 39000 $L \cdot M^{-1}\ cm^{-1}$), $b$ = 1 cm, $V_{max}$ signifies the maximum reaction velocity, $[S]$is the concentration of $H_2O_2$ substrate, and $K_m$ is the Michaelis-Menten constant.

The POD-like activities of the G5@AuPt, G5@PtCu, and G5@AuPtCu nanozyme materials were determined using the same method.

**CAT-like Activity Assay.** The CAT-like activity and enzymatic kinetics of the G5.NHAc-PG@APC nanoplatforms were investigated at room temperature using $H_2O_2$ as the substrate, with the generated oxygen content recorded by a JPBJ-605F dissolved oxygen meter. Different concentrations of $H_2O_2$ (1, 2, 5, 10, 20, 40 mM) were rapidly injected into a PBS solution (final volume of 10 mL, pH 6.5) containing the G5.NHAc-PG@APC (10 μg $mL^{-1}$). Oxygen generation was recorded every 30 s, and the maximum velocity ($V_{max}$) and Michaelis-Menten constant ($K_m$) were derived from the Michaelis-Menten curve. The influence of pH on the CAT-like activity of the G5.NHAc-PG@APC was evaluated in PBS buffers at pH values of 5.5, 6.5, and 7.4. An $H_2O_2$ solution (final concentration of 10 mM) was rapidly injected into PBS buffers of varying pH (final volume of 10 mL) containing the G5.NHAc-PG@APC (10 μg $mL^{-1}$). The oxygen production was subsequently recorded every 30 s for 10 min.

**SOD-like Activity Assay.** The SOD-like activity of the G5.NHAc-PG@APC nanozyme was assessed using a Superoxide Dismutase (SOD) Activity Assay Kit (WST-8 method), following the manufacturer's protocol. Different concentrations of the G5.NHAc-PG@APC were mixed with the substrate and incubated. The absorbance of WST-8 at 450 nm was subsequently measured. The corresponding inhibition rate was then calculated to evaluate the SOD-like activity of the nanozyme.

**Cell Culture.** 4T1 and L929 cells were cultured in RPMI 1640 and DMEM media, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37 °C in an incubator (Waltham, MA) with 5% $CO_2$. The culture medium was replaced every other day, and subculture was performed when the cells reached approximately 80% confluency.

**Cell Viability.** The cytotoxicity of various treatments against 4T1 cells were evaluated using the CCK-8 assay. Briefly, 4T1 cells in the exponential growth phase were seeded into 96-well plates at a density of $1\times10^4$ cells per well and cultured overnight. After removing the culture medium, the cells were washed three times with PBS. Subsequently, fresh medium containing 10% PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC and G5.NHAc-PG@APC ([G5] = 0, 1, 2, 4, 8, 16, or 32 μM; [2-DG] = 0, 7.1, 14.2, 28.4, 56.8, 113.6, or 227.2 μM) were added, and the cells were incubated for 24 h. Then, the medium in

each well was replaced with 100 μL of serum-free medium containing 10% CCK-8 reagent, followed by further incubation for 1 h. The absorbance at 450 nm was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA) to determine cell viability. Cells treated with PBS were used as the control group, and their corresponding cell viability was set as 100%.

***In Vitro* Cellular Uptake.** The cellular uptake of G5.NHAc-PG@APC by 4T1 cells at different time points was investigated using confocal laser scanning microscopy (CLSM). Initially, the G5 dendrimer within G5.NHAc-PG@APC was labeled with Cy5.5 according to an established protocol.[2] 4T1 cells were seeded into confocal dishes at a density of $1\times10^5$ cells per dish and cultured overnight in 1 mL of complete medium. Subsequently, at designated time points, the original medium was replaced with 1 mL of serum-free medium containing G5.NHAc-PG-Cy5.5@APC. The cells were then incubated with the material for respective durations of 2, 4, 6, and 8 h. Following incubation, the cells were washed three times with PBS, fixed with 2.5% glutaraldehyde for 15 min, and then stained for nuclei with DAPI at 37 °C for 15 min. After additional washes with PBS, the samples were imaged using a CLSM.

Furthermore, the intracellular levels of Pt in 4T1 cells after different treatment durations were determined by ICP-OES. 4T1 cells were seeded into 6-well plates at a density of $2\times10^5$ cells per well in 2 mL of complete medium and cultured for 12 h. Subsequently, the medium was replaced with 1 mL of serum-free medium containing G5.NHAc-PG@APC, the cells from different groups were then incubated for varying periods (2, 4, 6, and 8 h). After incubation, the cells were washed three times with PBS, trypsinized, collected, and counted. The harvested cells were digested in aqua regia for 12 h, and the Au, Pt, and Cu contents were subsequently determined using ICP-OES. Additionally, the intracellular content of Pt in L929 cells after different treatment times was investigated using the same methodology.

To further elucidate the cellular uptake mechanism of G5.NHAc-PG@APC, 4T1 cells were pretreated with various endocytosis inhibitors, including chlorpromazine (inhibitor of clathrin-dependent endocytosis, 20 μM), amiloride (inhibitor of macropinocytosis, 100 μM), and genistein (inhibitor of caveolae-dependent endocytosis, 700 μM), as well as free PBA (400 μM), for 2 h at 37 °C. Subsequently,

the culture medium was removed, and the cells were incubated with G5.NHAc-PG@APC -containing medium for an additional 6 h. Finally, the cells were collected, digested with aqua regia, and the intracellular Pt content was quantified using ICP-OES.

**Intracellular ROS Detection.** Intracellular ROS levels in 4T1 cells were detected using the fluorescent probe DCFH-DA, analyzed by both CLSM and flow cytometry. 4T1 cells were seeded into confocal dishes at a density of $1\times10^5$ cells per dish and cultured overnight in 1 mL of complete medium. The old medium was then replaced with fresh complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, followed by incubation for 6 h. After treatment, the medium was removed and the cells were washed three times with PBS. Then, 1 mL of serum-free medium containing DCFH-DA (10 μM) was added under light-protected conditions and incubated for 15 minutes. Following another three washes with PBS, the cells were fixed with 2.5% glutaraldehyde for 15 min and subsequently stained with DAPI for nuclei visualization at 37 °C for 10 min. After a final wash with PBS, intracellular ROS levels were observed and imaged using CLSM.

Similarly, 4T1 cells were seeded in 6-well plates at a density of $1\times10^5$ cells per well and cultured overnight. After treatment with various materials for 6 h, the medium was aspirated and the cells were washed three times with PBS. Subsequently, under light-protected conditions, 1 mL of serum-free medium containing DCFH-DA (10 μM) was then added to treat the cells for 15 min. Following incubation, the cells were washed with PBS, trypsinized, and collected by centrifugation. Finally, the cells were resuspended in PBS and quantitatively analyzed for intracellular ROS levels using flow cytometry.

**Intracellular GSH Detection.** To evaluate the intracellular glutathione (GSH) levels in 4T1 cells following treatment with different materials, a GSH assay kit was used according to the manufacturer's instructions. Briefly, 4T1 cells were seeded in 6-well plates at a density of $2\times10^5$ cells per well and cultured overnight. The cells were then treated with complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively, for 6 h. After incubation, the cells were

trypsinized, collected by centrifugation, and lysed via rapid freeze-thaw cycles. The intracellular GSH content was subsequently detection following the protocol provided with the assay kit.

**Intracellular LPO Detection.** The level of lipid peroxidation (LPO) in 4T1 cells was detected using the fluorescent probe C11-BODIPY581/591 and CLSM. Briefly, 4T1 cells were seeded at a density of $1\times10^5$ cells per dish in confocal dishes and incubated overnight in 1 mL of complete medium. The old medium was then replaced with fresh complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, followed by another 6 hours of incubation. After removal of the medium, the cells were washed three times with PBS. Subsequently, 1 mL of serum-free medium containing C11-BODIPY581/591 (10 μM) was added under light-protected conditions and incubated for 20 min. Following another three washes with PBS, the cells were fixed with 2.5% glutaraldehyde for 15 min and then subjected to nuclear staining with DAPI at 37 °C for 10 min. After a final wash with PBS, the intracellular LPO levels were visualized and analyzed using CLSM.

Similarly, after treating cells with the respective materials for 6 h, the culture medium was aspirated, and the cells were washed three times with PBS. Following trypsinization, the cells were collected by centrifugation and subsequently resuspended in 1 mL of serum-free medium containing C11-BODIPY581/591 (10 μM) under light-protected conditions. The cells were then incubated at 37 °C for 20 min. After incubation, the cells were centrifuged, washed twice with PBS, and finally resuspended in PBS for quantitative analysis of intracellular LPO levels using flow cytometry.

**Intracellular Dissolved Oxygen Detection.** To examine the alterations in intracellular $O_2$ levels in 4T1 cells after treatment with different materials, the $O_2$ probe $[Ru(dpp)_3]^{2+}Cl_2$ was employed and detected using CLSM. 4T1 cells were seeded in confocal dishes at a density of $1\times10^5$ cells per dish and cultured overnight. The medium was then removed, and the cells were treated with serum-free medium containing the $[Ru(dpp)_3]^{2+}Cl_2$ (5 μM) probe for 2 h. Subsequently, the medium was replaced with complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC,

respectively, followed by another 6 h of incubation. After treatment, the cells were washed three times with PBS and then observed under CLSM for fluorescence.

**Cell Migration Assay.** A wound healing assay was performed to evaluate the migration ability of 4T1 cells. The cells were seeded in 6-well plates at a density of $2\times10^5$ cells per well and cultured overnight. When the cells reached approximately 80% confluence, a straight wound was created in each well using pipette tip. After washing with PBS, the cells were further cultured in fresh medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively. Images of the wounds were captured using a phase-contrast microscope at 0 h and 24 h after scratching.

To further investigate the effect of G5.NHAc-PG@APC on cell migration, 4T1 cells were resuspended in serum-free medium containing the respective materials and seeded into the upper chambers of a Transwell plate (24-well, 8.0 μm pore polycarbonate membrane, NEST Biotechnology, Wuxi, China) at a density of $5\times10^4$ cells per well. The lower chambers were filled with complete medium containing 20% FBS as a chemoattractant. The plate was incubated at 37 °C in a 5% $CO_2$ atmosphere for 36 h. Subsequently, the medium was removed, and the cells were fixed with 500 μL of 2.5% glutaraldehyde for 30 min. After three washes with PBS, both chambers were stained with 1% crystal violet (200 μL in the upper chamber and 500 μL in the lower chamber) for 10 min at room temperature in the dark. Non-migrated cells on the upper surface of the membrane were gently removed using a moistened cotton swab after washing with PBS. The migrated cells on the lower surface of the membrane were then observed and photographed under an inverted phase-contrast microscope. For quantitative analysis, the crystal violet was eluted with 300 μL of 3% acetic acid, and the absorbance at 595 nm was measured using a microplate reader.

**Mitochondrial Integrity Assay.** To investigate changes in mitochondrial membrane potential (MMP) in 4T1 cells following treatment with different materials, JC-1 fluorescent probe was used as a dye and detected by CLSM. 4T1 cells were seeded at a density of $1\times10^5$ cells per dish in confocal dishes and incubated overnight. The old medium was then replaced with complete medium containing PBS,

G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively, followed by further incubation for 6 h. After treatment, the cells were washed three times with PBS, incubated with 1 mL of freshly prepared JC-1 staining working solution in the dark for 20 min, and finally washed with serum-free medium before observation under the confocal microscope.

Similarly, after treating cells with the various materials for 6 h, the culture medium was aspirated, and the cells were washed three times with PBS. Following trypsinization, the cells were collected by centrifugation and subsequently resuspended in 1 mL of JC-1 staining working solution under light-protected conditions. The cells were then incubated at 37 °C for 20 min. After incubation, the cells were centrifuged, washed twice with PBS, and finally resuspended in PBS for quantitative analysis of intracellular mitochondrial membrane potential using flow cytometry.

**Intracellular ATP Detection.** To evaluate the intracellular ATP levels in 4T1 cells after treatment with different materials, an Enhanced ATP Assay Kit was employed. Briefly, 4T1 cells were seeded in 6-well plates at a density of $2\times10^5$ cells per well and cultured overnight. The cells were then treated for 12 h with complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively. After incubation, the cells were lysed according to the manufacturer's instructions, and the intracellular ATP content was detected. Subsequently, the protein concentration in the cell lysates was quantified using a BCA Protein Assay Kit, and the relative ATP content was calculated based on the protein concentration.

**Detection of Extracellular Lactate Levels.** The extracellular lactate levels of 4T1 cell after different treatments were detected using a lactate assay kit. In brief, 4T1 cells were seeded in 6-well plates at a density of $2\times10^5$ cells per well and cultured overnight. The cells were then treated for 6 h with complete medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively. Subsequently, the extracellular lactate levels were measured according to the manufacturer's instructions for the lactate assay kit.

**Cell Apoptosis Assay.** To investigate the apoptosis of 4T1 cells after treatment with different materials, an apoptosis detection kit was employed. 4T1 cells in the logarithmic growth phase were seeded in 6-well plates at a density of $2\times10^5$ cells per well and cultured for 24 h. The old medium was then discarded and replaced with 2 mL of fresh medium containing PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively, followed by another 24 h of incubation. Subsequently, the cells were washed with PBS, trypsinized, and collected by centrifugation. After resuspension, 5 μL of Annexin V-FITC and 5 μL of PI were added, and the cells were incubated in the dark for 15 min before analysis by flow cytometry.

**Animal Models.** To evaluate the antitumor efficacy of nanozymes *in vivo*, a subcutaneous 4T1 tumor model was established in female BALB/c mice (4-6 weeks, Shanghai JieSiJie Laboratory Animal Center, Shanghai, China). 4T1 cells in the logarithmic growth phase were harvested and resuspended in PBS buffer at a concentration of $1.5\times10^7$ cells/mL. Subsequently, 100 μL of the cell suspension was subcutaneously injected into the right hind flank of each mouse to initiate tumor formation. The *in vivo* antitumor experiment was initiated when the tumor volume reached approximately 100 $mm^3$.

***In Vivo* Biodistribution.** To investigate the *in vivo* distribution of the synthesized nanozymes, 100 μL of G5.NHAc-PG@APC (25 mg/kg, [G5] =18.2 mg/kg) was administered to mice via tail vein injection. The mice were euthanized at 1, 3, 6, 12, 24, and 48 hours post-injection, and major organs along with tumors were collected. The harvested tissues were weighed, cut into small pieces, and digested in aqua regia for 4 days. The content of Au, Pt, and Cu in the digested samples was subsequently quantified using ICP-OES.

**Pharmacokinetic Study.** For pharmacokinetic analysis, blood samples were collected from the retro-orbital plexus of mice at 0.1, 0.5, 1, 3, 6, 12, and 24 h following tail vein injection of G5.NHAc-PG@APC. The blood samples were digested with aqua regia, and the Au content was determined by ICP-OES. The blood concentration-time profile was generated by fitting the data to a first-order exponential decay (ExpDec1) model.

***In Vivo* Antitumor Activity Assay.** Tumor-bearing mice were randomly divided into four groups (n = 5). On days 1, 4, 7, and 10, the mice received intravenous injections via the tail vein of PBS, G5.NHAc-P, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively. Tumor volume and body weight were measured every two days. Tumor volume (V) was calculated using the formula $V = (W^2 \times L) / 2$, where W and L represent the width and length of the tumor, respectively. The relative tumor volume (RTV) was determined by $RTV = V / V_0$, where $V_0$ denotes the tumor volume on the first day of measurement.

**Histological and Biosafety Examinations.** At the end of the treatment, all mice were euthanized, tumor tissues and major organs (heart, liver, spleen, lungs, and kidneys) were collected were collected and fixed in 4% paraformaldehyde, followed by paraffin embedding and sectioning. The tumor sections were then subjected to hematoxylin and eosin (H&E) staining, Ki67 immunohistochemical staining, TUNEL and HIF-1α immunofluorescence staining to further evaluate the antitumor efficacy in vivo, according to the literature.[3,4] Additionally, major organs were stained with H&E and histological changes were observed to assess the in vivo biosafety of the treatment, according to the standard protocols.[5]

Additionally, healthy mice were intravenously injected via the tail vein with PBS, G5.NHAc-P, G5.NHAc-P@APC, or G5.NHAc-PG@APC, respectively. After being fed for 7 days, blood samples were collected from the orbital sinus of each group and blood routine were analyzed using an automated blood cell counter (BC-2800 Vet Analyzers, Mindray, Shenzhen, China), including red blood cell (RBC), platelet (PLT), white blood cell (WBC), and hemoglobin (HGB). Then the blood samples were centrifugated at 3000 rpm for 15 min at 4°C to obtain the serum, and the serum biochemistry markers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), and uric acid (UA) were analyzed by Servicebio Technology Co., Ltd. (Wuhan, China).

**Table S1.** Au, Pt and Cu yield in different samples revealed by ICP-OES.

| Samples | Au yield (%) | Pt yield (%) | Cu yield (%) | G5: Au: Pt: Cu (n/n) |
|---|---|---|---|---|
| G5.NHAc-P@APC | 91.7 | 80.5 | 52.3 | 1: 4.99: 12.02: 2.62 |
| G5.NHAc-PG@APC | 90.3 | 79.2 | 50.2 | 1: 4.91: 11.83: 2.51 |

**Table S2.** The hydrodynamic size, polydispersity index, and zeta potential of different samples.

| Samples | Size (nm) | PDI | Zeta Potential (mV) |
|---|---|---|---|
| G5.$NH_2$-P | 185.0 ± 14.3 | 0.49 ± 0.07 | 40.7 ± 6.0 |
| G5.NHAc-P | 129.8 ± 0.9 | 0.21± 0.01 | 15.4 ± 1.2 |
| G5.NHAc-P@APC | 104.2 ± 5.6 | 0.46 ± 0.19 | 9.9 ± 0.9 |
| G5.NHAc-PG@APC | 123.2 ± 0.3 | 0.15 ± 0.02 | 8.9 ± 1.1 |

**Table S3.** Au, Pt and Cu content in different samples revealed by ICP-OES.

| Samples | Au (μg/mL) | Pt (μg/mL) | Cu(μg/mL) | G5: Au: Pt: Cu (n/n) |
|---|---|---|---|---|
| G5@AuPtCu | 36.66 | 95.56 | 4.87 | 1: 3.87: 10.19: 1.60 |
| G5@AuPt | 36.08 | 95.92 | - | 1: 3.81: 10.23: 0 |
| G5@PtCu | - | 95.23 | 5.23 | 1: 0: 10.16: 1.71 |

**Table S4.** The hydrodynamic size, polydispersity index, and zeta potential of different samples.

| Samples | Size (nm) | PDI | Zeta Potential (mV) |
|---|---|---|---|
| G5@AuPtCu | 193.1 ± 14.8 | 0.47 ± 0.02 | 37.3 ± 4.9 |
| G5@AuPt | 180.5 ± 8.9 | 0.54 ± 0.14 | 33.4 ± 3.8 |
| G5@PtCu | 203.3 ± 26.3 | 0.56 ± 0.11 | 32.7 ± 2.1 |

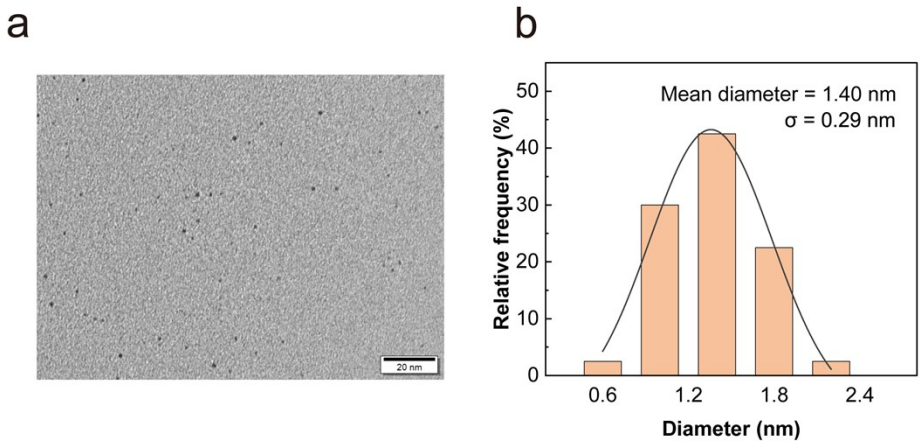


**Figure S1.** TEM micrograph and the corresponding size distribution profile of G5.NHAc-P@APC.

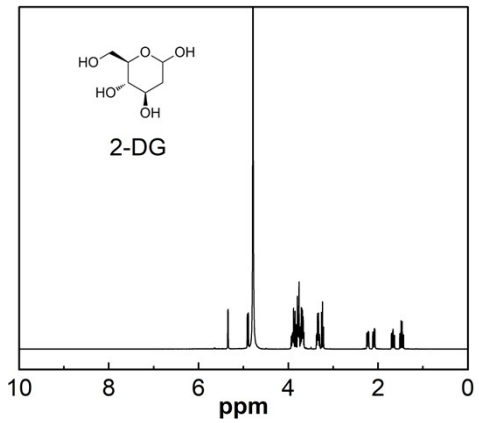


**Figure S2**. $^{1}$H NMR spectra of 2-DG. 2-DG was dissolved in $D_2O$ before measurements.

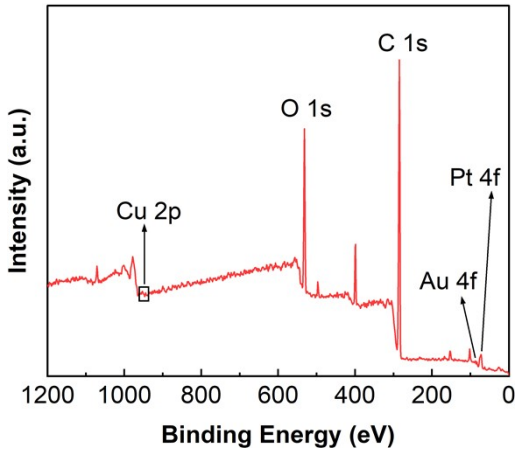


**Figure S3**. High-resolution XPS spectra of G5.NHAc-PG@APC.

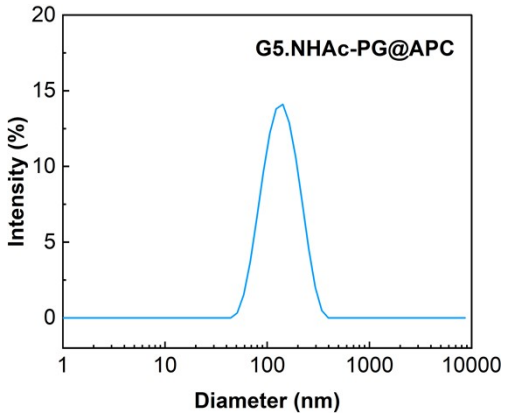


**Figure S4**. Hydrodynamic size distribution of the G5.NHAc-PG@APC.

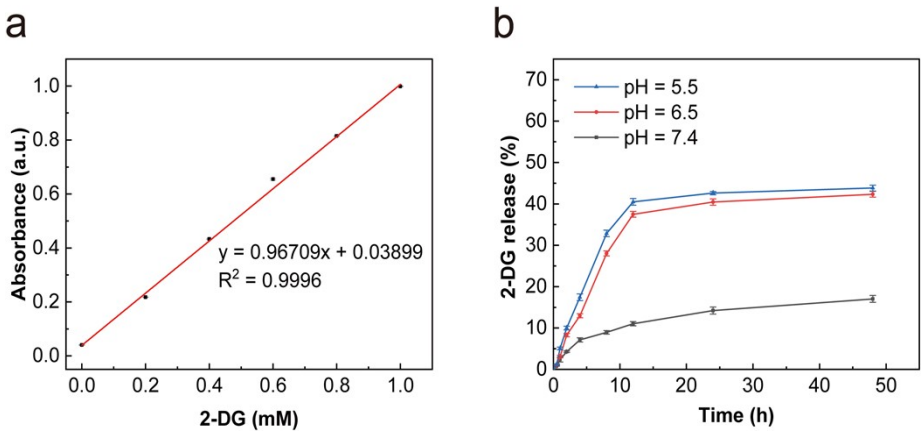


**Figure S5.** (a) The standard curve of 2-DG measured by DNS method. (b) Release behaviors of 2-DG in G5.NHAc-PG@APC under different pH conditions.

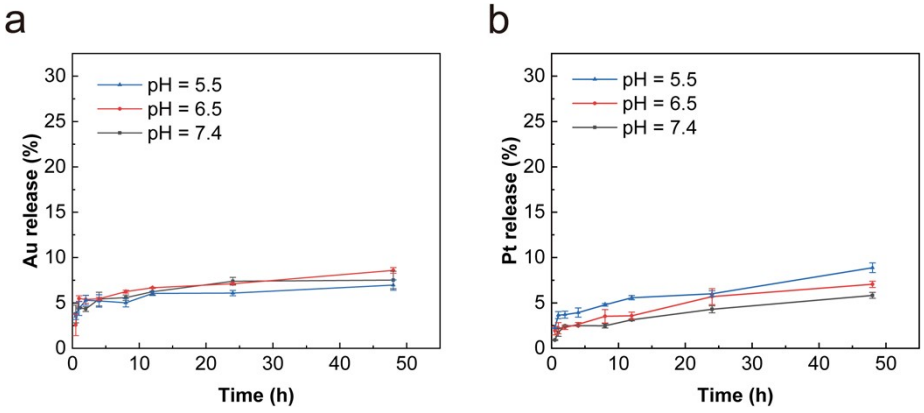


**Figure S6.** Release behaviors of Au (a) and Pt (b) in G5.NHAc-PG@APC under different pH conditions.

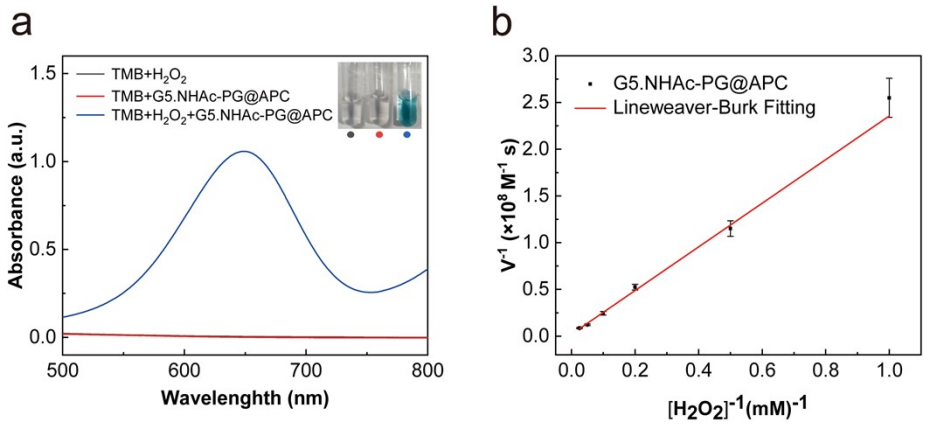


**Figure S7.** (a) Absorbance spectra and photographs of different solutions at pH 5.5. (b) Lineweaver Burk plot of POD-like activity of G5.NHAc-PG@APC nanoplatforms.

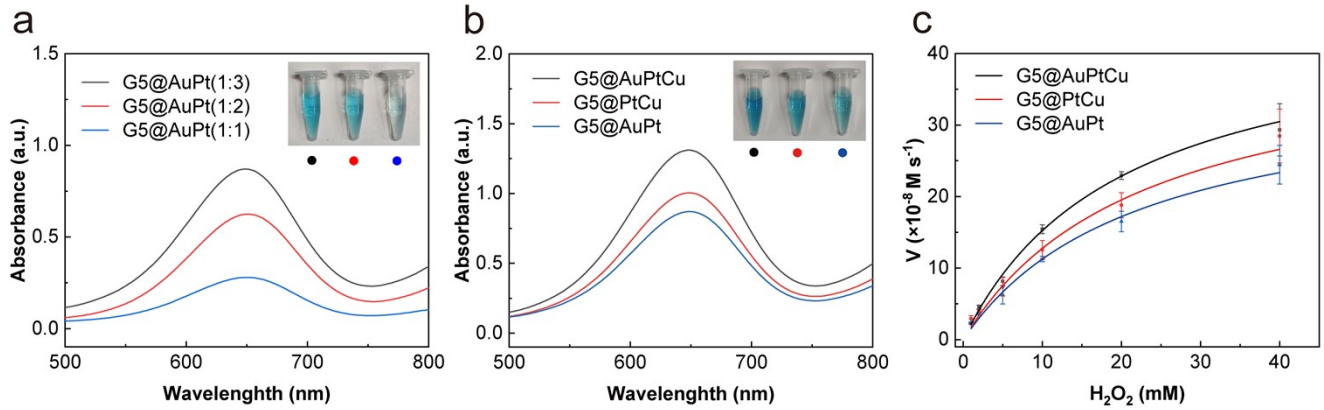


**Figure S8.** (a) POD-like activity of different nanozymes after 5 min reaction with 10 mM $H_2O_2$ and TMB (0.25 mg $mL^{-1}$) at pH 5.5. (b) Michaelis Menten curve of G5@AuPtCu, G5@PtCu and G5@AuPt nanozymes.

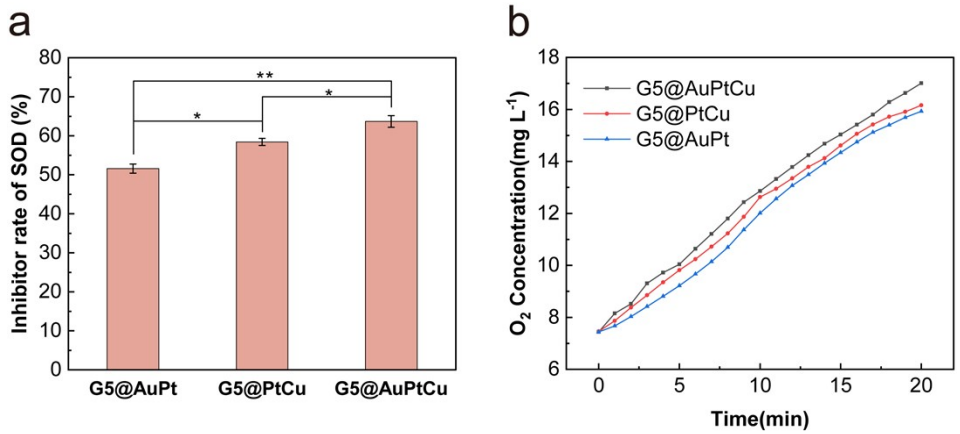

**Figure S9.** (a) SOD-like and (b) CAT-like activities of G5@AuPtCu, G5@PtCu and G5@AuPt nanozymes. *$p < 0.05$, **$p < 0.01$.

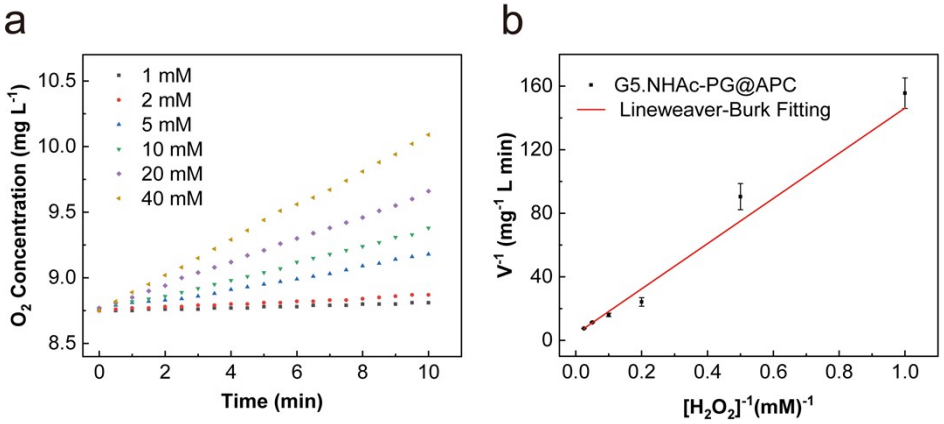


**Figure S10.** (a) $O_2$ generation by CAT-like activity of G5.NHAc-PG@APC (10 μg $mL^{-1}$) under different $H_2O_2$ substrate concentrations at pH 6.5. (b) Lineweaver Burk plot of CAT-like activity of G5.NHAc-PG@APC nanoplatforms.

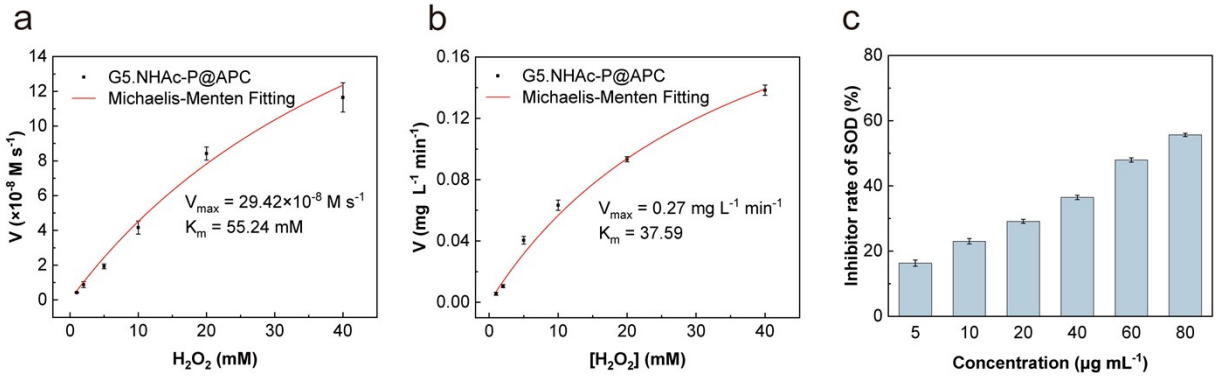


**Figure S11.** (a) POD-like, (b) CAT-like and (c) SOD-like activities of G5.NHAc-P@APC.

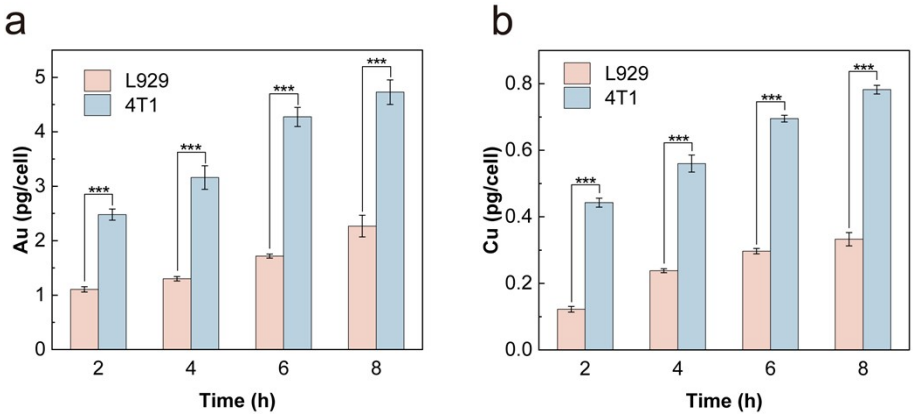

**Figure S12**. Intracellular (a) Au and (b) Cu accumulation in 4T1 and L929 cells after incubation with G5.NHAc-PG@APC at different times.

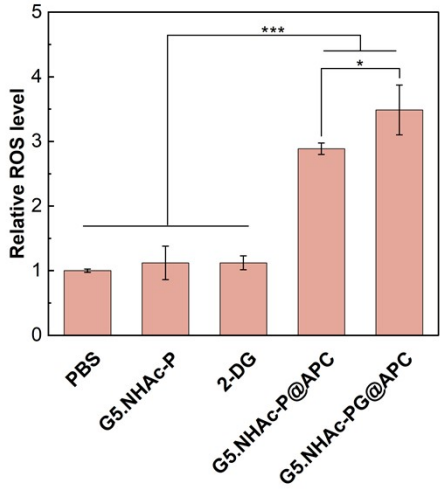


**Figure S13.** Quantitative analysis of ROS levels in flow cytometry results of 4T1 cells after treated with PBS, G5.NHAc-P, 2-DG, G5.NHAc-P@APC, and G5.NHAc-PG@APC for 6 h. *$p < 0.05$, ***$p < 0.001$.

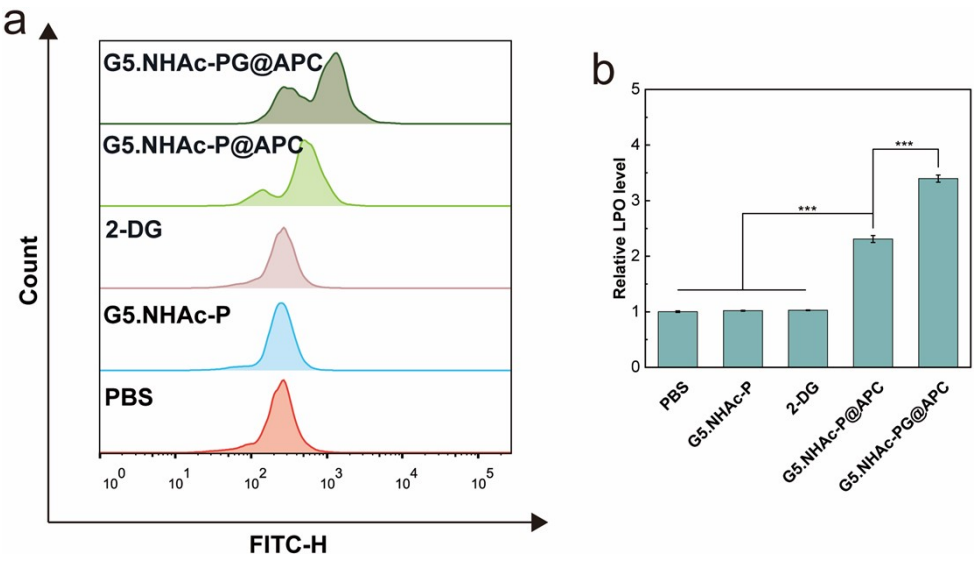


**Figure S14** (a) Flow cytometry analysis and (b) quantification of of intracellular LPO levels in 4T1 cells after various treatments for 6 h. ***$p < 0.001$.

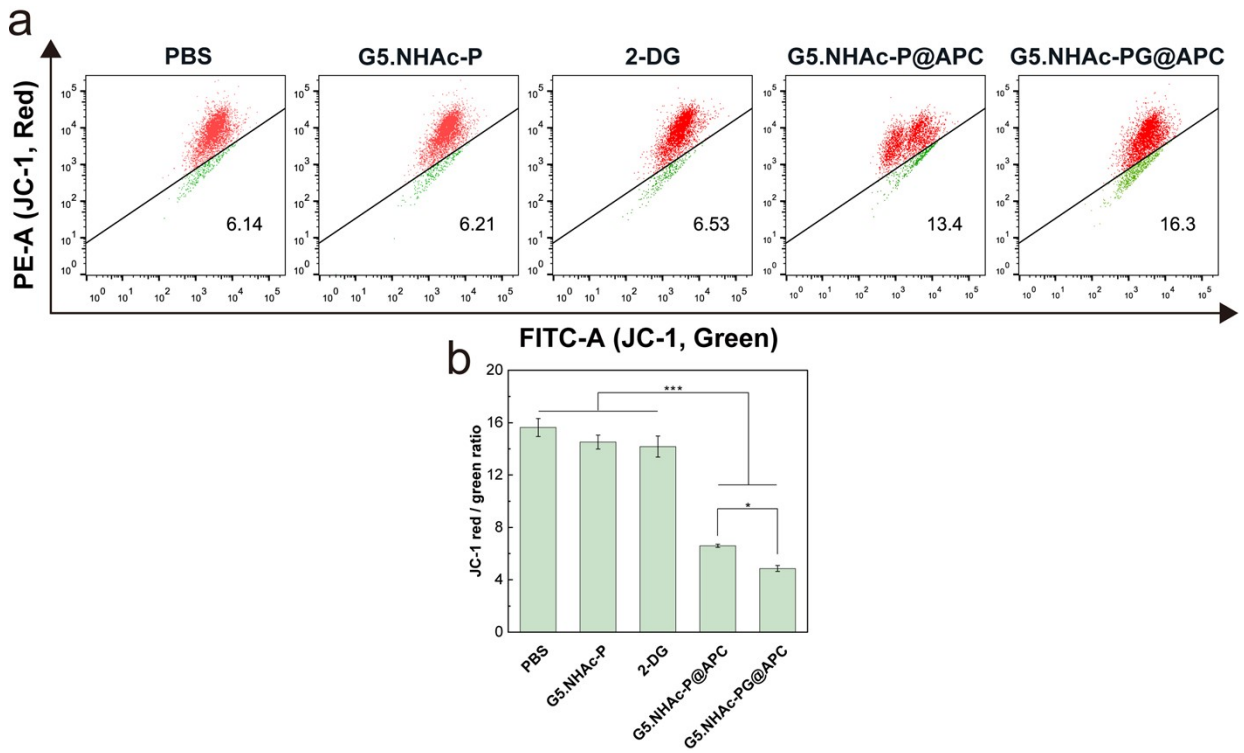


**Figure S15** (a) Flow cytometry analysis and (b) quantification of mitochondrial membrane potential in 4T1 cells after various treatments for 6 h. *$p < 0.05$, ***$p < 0.001$.

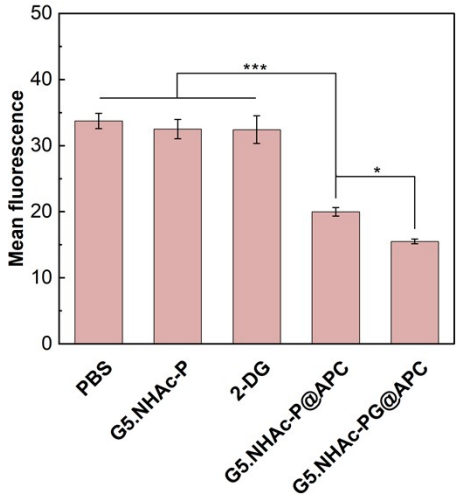


**Figure S16.** Quantitative analysis of mean fluorescence of 4T1 intracellular $[Ru(dpp)_3]^{2+}Cl_2$ $O_2$ probe after different treatments for 6 h. *$p < 0.05$, ***$p < 0.001$.

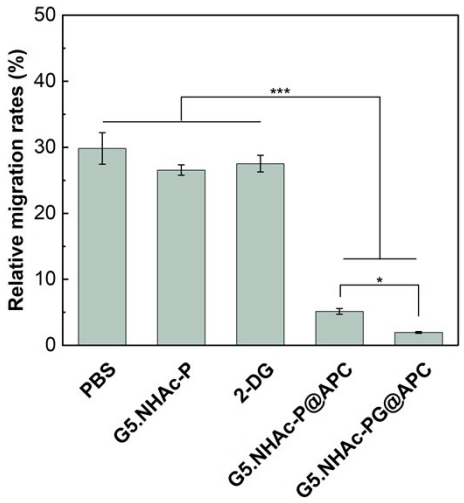


**Figure S17.** Quantification of migration of 4T1 cells after different treatments for 24 h. *$p < 0.05$, ***$p < 0.001$.

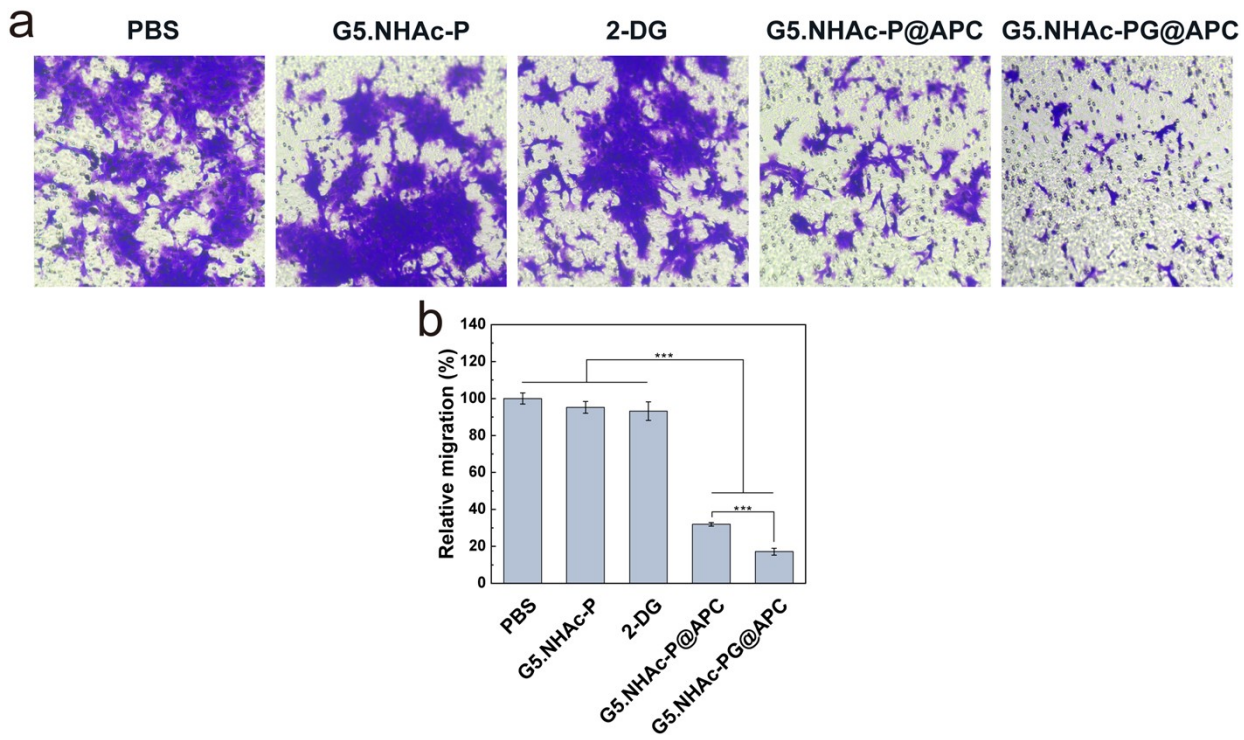


**Figure S18**. (a) Inverted microscopy images and (b) the quantitative analysis of crystal violet detection of 4T1 cells after different treatments. ***$p < 0.001$.

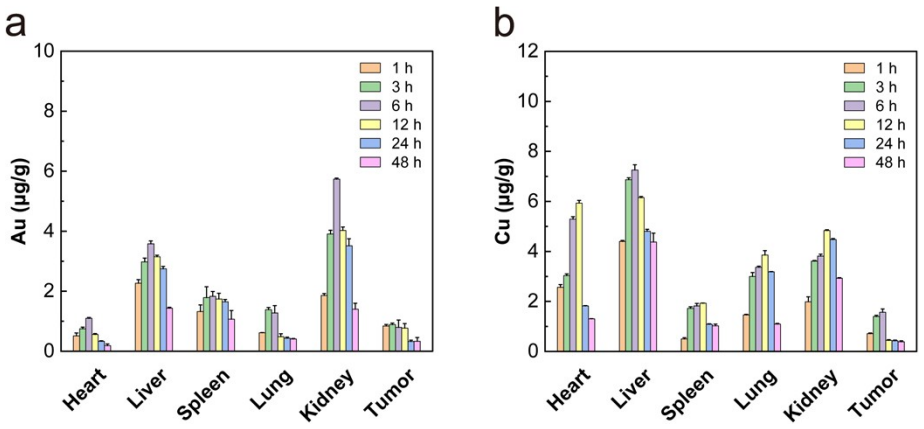


**Figure S19.** The biodistribution of (a) Au and (b) Cu elements in major organs and tumors after G5.NHAc-PG@APC injection.

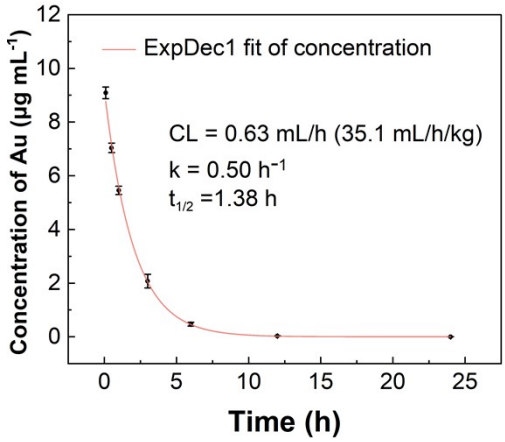


**Figure S20.** Blood drug concentration–time profile following tail vein injection of G5.NHAc-PG@APC.

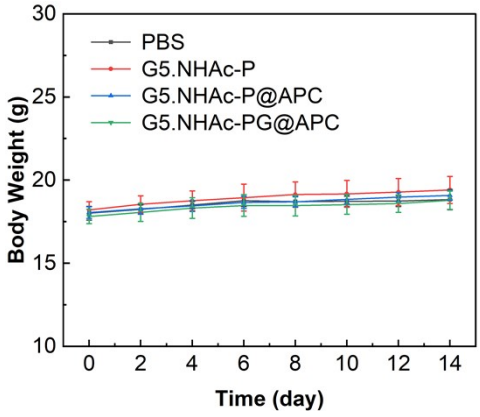


**Figure S21.** Mouse body weight changes of all groups after different treatments.

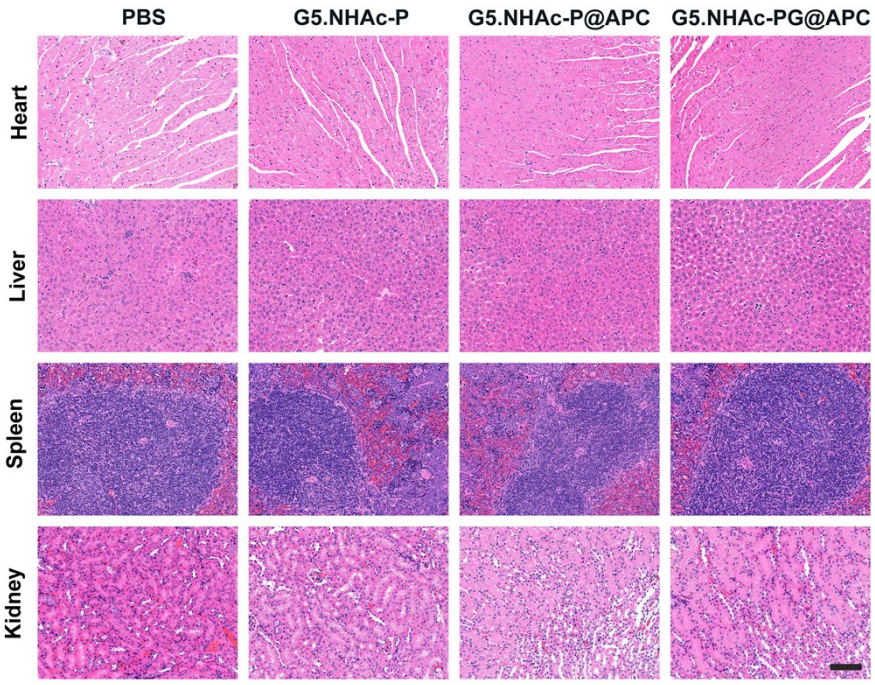


**Figure S22.** H&E staining of sections from major organs of 4T1 tumor-bearing mice after different treatments on day 14. scale bar = 100 μm.

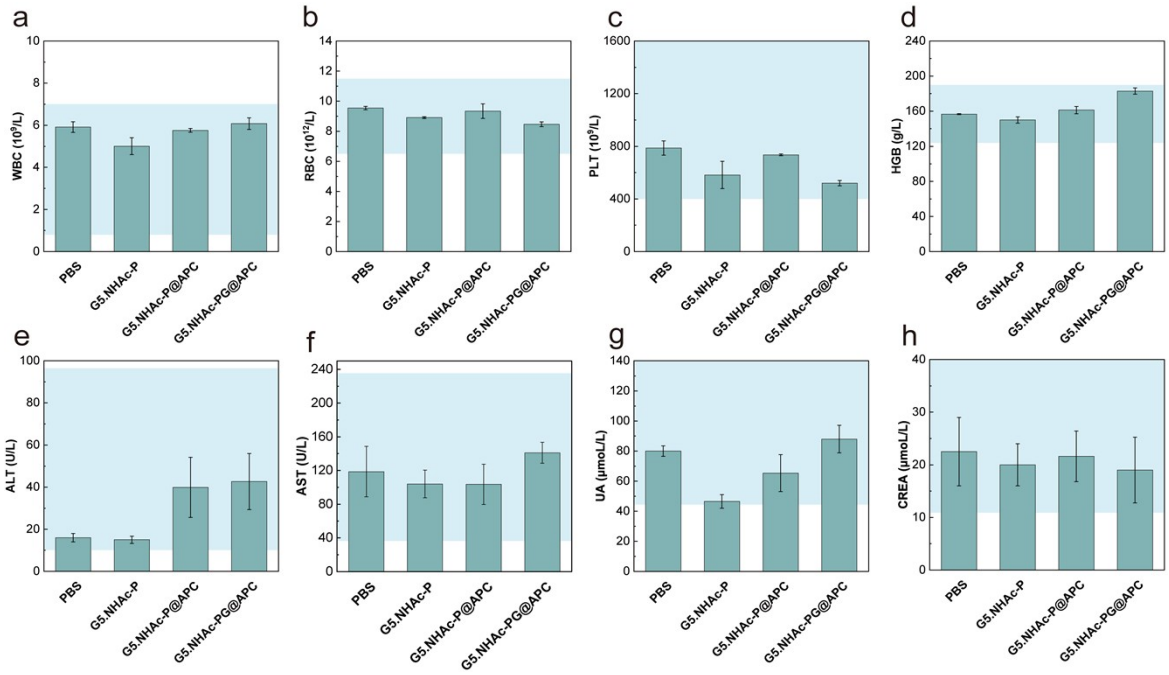


**Figure S23.** Hematological and biochemical analyses in healthy mice 7 days after different treatments of PBS, G5.NHAc-P, G5.NHAc-P@APC, and G5.NHAc-PG@APC (n=3). Panels: WBC, RBC, PLT, HGB, ALT, AST, UA, CREA. Shaded areas denote normal ranges.

## Supplementary References


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