Supporting information

Materials

Catalase (CAT), glucose oxidase (GOx), *N*-acryloxysuccinimide (NAS), hydrogen peroxidase (H_2O_2), protoporphyrin IX (PpIX), (4-penten-1-yl)triphenylphosphonium bromide (PTPB), acrylamide (AAm), *N, N'* -methylene bisacrylamide (BIS), fluorescein isothiocyanate, rhodamine B isothiocyanate and ammonium persulfate (APS) were commercially obtained from Sigma-Aldrich. Horseradish peroxidase (HRP), tetramethylethylenediamine (TEMED), dimethyl sulfoxide (DMSO) and fluorescamine were purchased from Tokyo Chemical Industry. Sodium carbonate (Na_2CO_3), o-dianisidine (ODS) and sodium bicarbonate ($NaHCO_3$) were purchased from Alfa Aesar. MTT assay reagent (YEASEN), DAPI reagent (Beyotime), MitoTracker Red CMXRos (YEASEN), DCFH-DA analysis kit (Beyotime), JC-1 analysis kit (Beyotime), cleaved caspase-3 and cytochrome c antibody (Abcam), Cy3 conjugated secondary antibody (Beyotime), H&E staining kit (YEASEN).

Preparation of acryloxylated CAT

The preparation of acryloxylated CAT was operated according to former work.[1] 60 mg of CAT was firstly dissolved in 9.0 mL of sodium carbonate buffer solution (20 mM, pH = 9.3), then 28 mg of NAS dissolved in 1 mL of DMSO was added into the above solution. Dialysis against phosphate buffer solution was carried out to remove unreacted NAS and DMSO after stirring at room temperature for 2.5 h. The number of acryloyl groups that are modified onto CAT surface was estimated by assessing the number of unreacted amino groups by the fluorescamine assay. Around 19 of the 28 amino groups on CAT were modified by acryloyl groups.

Preparation of acryloylated GOX

The operation procedure for preparing acryloxyated GOX was the same as that for preparing acryloyated CAT, except for CAT replaced with GOX. Around 9 of the 15

amino groups were modified by acryloyl groups.

Preparation of mitochondria targeted GOX-CAT-PpIX (tGCP) nanogel

Acryloylated GOX and CAT (GOX/CAT, 1:1, mol/mol) were firstly dissolved in phosphate buffer saline (PBS) solution (pH=7.0, 50mM). After AAm (AAm/protein, 20000:1, mol/mol) was added into the above mixture, mitochondrial targeting PTPB, BIS and PpIX (BIS/PTPB/PpIX/protein, 700:117:17:1, mol/mol) were dissolved in DMSO and added slowly into the above mixture. TEMED (TEMED/APS, 2:1, w/w) and APS (APS/protein, 500:1, mol/mol) were added into the above mixture solution under nitrogen atmosphere to initiate the polymerization. The mixture was kept and stirred at room temperature for 2 h, The prepared nanogel solution was firstly dialyzed against PBS for purifying. The porphyrin content in the solution was estimated by calibration curve measured by UV−vis.

Preparation of mitochondria targeted GOX-PpIX (tGP) nanogel

Preparing tGP nanogel was the same as preparing tGCP nanogel except that CAT was removed.

Preparation of GOX-CAT-PpIX (GCP) nanogel

Preparing GCP nanogel was the same as preparing tGCP nanogel except that mitochondrial targeting PTPB was removed.

The comparation for the activity of CAT before and after polymerization: H_2O_2 was added into PBS solution (pH=7.0, 50 mM) at the final concentration of 1.0 mM, then CAT-PpIX nanogel or native CAT was added at the final catalase concentration of 0.85 μg/mL, the rate of catalytic reaction was immediately monitored by the absorbance change at 240 nm.

The comparation for the activity of GOX before and after polymerization: Native GOX or GOX-PpIX nanogel and HRP were firstly added into PBS solution (pH=7.0,

50 mM) with the final concentration of 2 μg/mL and 20 μg/mL, respectively. Then ODS dissolved in ethanol was added at the final concentration of 0.5 mM, glucose was finally added into above mixture at the final concentration of 2.5 mM to initiate the reaction. The rate of reaction was immediately monitored by the absorbance change at 460 nm.

Preparation of fluorescence-labeled GOX-CAT (FGC) nanogel

GOX modified with fluorescein isothiocyanate (FITC-GOX): DMSO solution of fluorescein isothiocyanate was added into 10 mg/mL of GOX aqueous solution (pH=8.2). After stirring for 12 h at room temperature, dialysis against PBS solution was used for purifying.

CAT modified with rhodamine B isothiocyanate (RhB-CAT): DMSO solution of rhodamine B isothiocyanate was added into 10 mg/mL of CAT solution (pH=8.2). After stirring for 12 h at room temperature, dialysis against PBS solution was used for purifying.

The ratio of fluorescence molecule to protein on RhB-CAT and FITC-GOX were determined with the extinction coefficients of 108000 $M^{-1}cm^{-1}$ for RhB at 555 nm, 324000 M^{-1} cm^{-1} for CAT at 405nm, 81000 $M^{-1}cm^{-1}$ for FITC at 495 nm, 44100 M^{-1} cm^{-1} for GOX at 280 nm by UV-Vis spectra.

The method for synthesizing fluorescence-labeled GOX-CAT nanogel using FITC-GOX and HRP-CAT was similar to that for synthesizing tGCP nanogel except that mitochondrial targeting PTPB and PpIX was removed.

Cytotoxicity analysis

MTT assay was used for cytotoxicity analysis. Mouse breast cancer 4T1 cells were inoculated in a 96-well plate according to the density of 10,000 cells/well. After cultured in a cell incubator for 24h, different samples were treated under different conditions according to the concentration gradient. After continuous culture for 24h, MTT assay reagent was added into the 96-well plate for further incubation for 4 hours.

Finally, the medium was removed, DMSO was added to dissolve the product, and the multifunctional enzyme plate analyzer was used to detect and characterize the product at 490 nm.

Hemolysis Test

2ml rat blood was placed in 2ml EP tube that was rinsed with 1% heparin sodium, and then centrifuged at 3500 rpm and 4℃ for 5min to collect red blood cells. Next, red blood cells were suspended twice with normal saline and centrifuged under the same conditions. After that, 1ml red blood cells were diluted to 10ml with normal saline. 200uL diluted red blood cell solution was added to the normal saline prepared with different concentrations of composited enzyme nanogels, and distilled water was used as the positive control. After incubation at 37℃ for 3h, centrifugation was performed at 4℃ at 5000 rpm for 5min. Finally, photos were taken and the supernatant was absorbed to determine the absorption value at 580nm. Hemolysis (%) = $[(ABS - ABS_0) / (ABS_{100} - ABS_0)] \times 100\%$. ABS_0 represents the absorption value of the negative control (normal saline), ABS_{100} represents the absorption value of the positive control (distilled water), and ABS represents the absorption value of different sample groups. Each experiment was repeated three times.

Flow cytometry analysis

To further characterize the cytotoxicity of our biocatalytic reactor, we performed flow cytometry analysis. The cells (4T1, 20,000 cells/well) were grown in a 24-well plate for 24 hours, and then treated with different prepared samples for 24 hours. At the end of the incubation, the cells were trypsinized and collected in a 2 mL sterile EP tube. The flow cytometer is used to analyze the samples. For each sample, data are collected from 10,000 single-cell events to record forward and side scatter information, in triplicate. The results were analyzed and plotted using FlowJo X software.

Cell mitochondrial targeting analysis

10,000 4T1 cells were added to each of the 48 holes of the pre-laid glass slide, and

cultured in a cell incubator for 24 hours. Then, the matched samples were added, and the matched red mitochondrial fluorescent probe was added to each well at different time points and incubated for 40 min. Finally, the glass slide is mounted with a mounting agent containing DAPI. After drying and fixation, the cell uptake behavior was observed and photographed under a Zeiss LSM5 confocal microscope.

Evaluation of ROS production in living cells

50,000 4T1 cells were seeded in a special confocal culture dish and incubated for 24 hours. Different sample groups containing the same amount of porphyrin were added respectively, and the incubation continued for 12 hours. Next, the cells were washed with PBS and incubated with DCFH-DA (5 uM) for 40 minutes, and then treated in the dark or light for 5 minutes (600 nm laser, 100 mW/cm^2). Finally, a confocal microscope is used for observation and photographing records.

Mitochondrial apoptosis pathway analysis

The 4T1 cells were seeded in a special confocal culture dish and cultured overnight. Then the cells were washed with PBS and incubated with fresh medium containing composite enzyme nanogel (8 μg/mL) for 12 hours. The cells were washed several times with PBS to remove non-internalized nanomaterials, and irradiated with a 660 nm NIR laser (100 mW/cm^2) for 10 minutes. After 24 hours of incubation, the cells were fixed with paraformaldehyde (4%), permeabilized with 0.1% Triton X-100 for 20 min, and washed several times with PBS. Then the cells were blocked with BSA (5%) for 1 hour, stained with cleaved caspase-3 or cytochrome c antibody at room temperature for 1 hour, and counter-stained with Cy3 conjugated secondary antibody for 1 hour. The nucleus was stained with DAPI. Finally, the cells were washed 3 times with PBS and imaged with CLSM.

Anti-tumor effect *in vivo*

BalB/C mice (with a weight of about 20g) inoculated with 4T1 tumor on their backs were randomly divided into 6 groups (5 mice in each group). When the tumor volume

reached 100 mm^3, mice were injected subcutaneously with PBS, GCP (1 mg/mL, 100 μL), tGP (1 mg/mL, 100 μL), tGCP (1 mg/mL, 100 μL). All nanogel groups follow the same amount of GOX and photosensitizer. After 24 hours, the mice were irradiated with laser (660nm, 1 W/cm^2) or without any treatment. The body weight and tumor size of the mice were recorded every two days. The tumor size was calculated according to the following formula: Volume = tumor width squared × length / 2. After 14 days, tumors and major organs were taken out from the sacrificed mice for histological analysis.

Histological analysis

The isolated tumors or major organs (heart, liver, spleen, lung, kidney) are routinely processed before frozen sectioning. The Leica microtome is used to slice tissues with a thickness of 6 μm, and then hematoxylin and eosin are used for tissue staining analysis

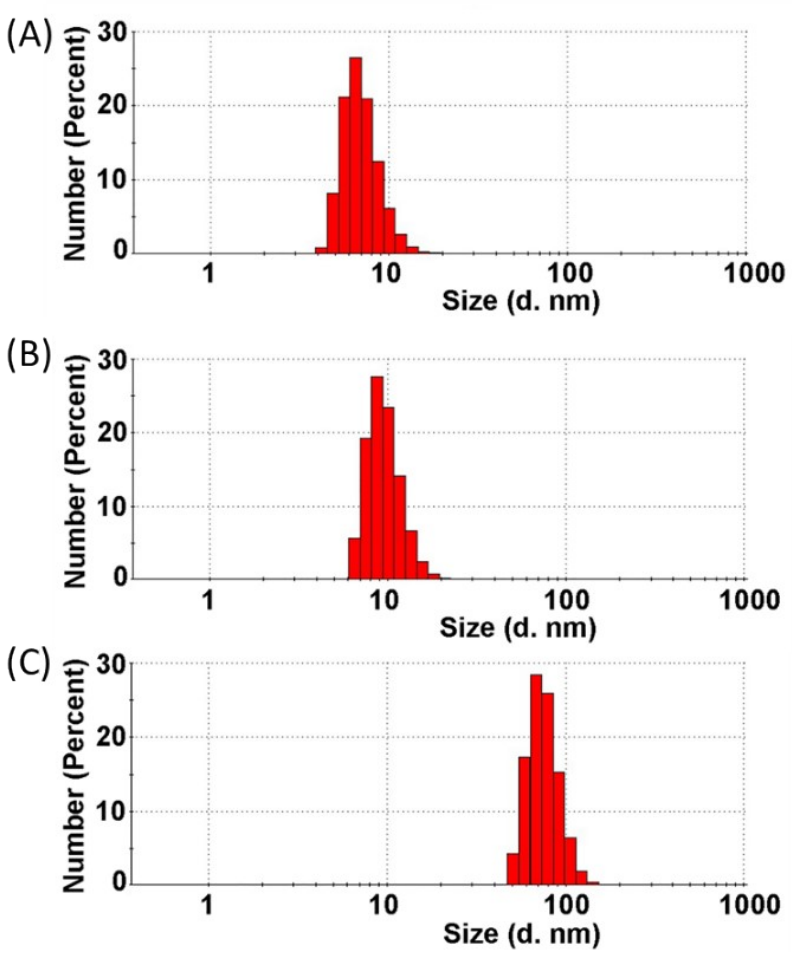


Figure S1. The DLS analysis of (A) GOX, (B) CAT, and (C) freshly prepared tGCP nanogel.

Figure S2. The macroscopic images of the solutions from left to right: 10 mM H_2O_2, tGCP nanogel, tGCP nanogel and 10 mM H_2O_2.

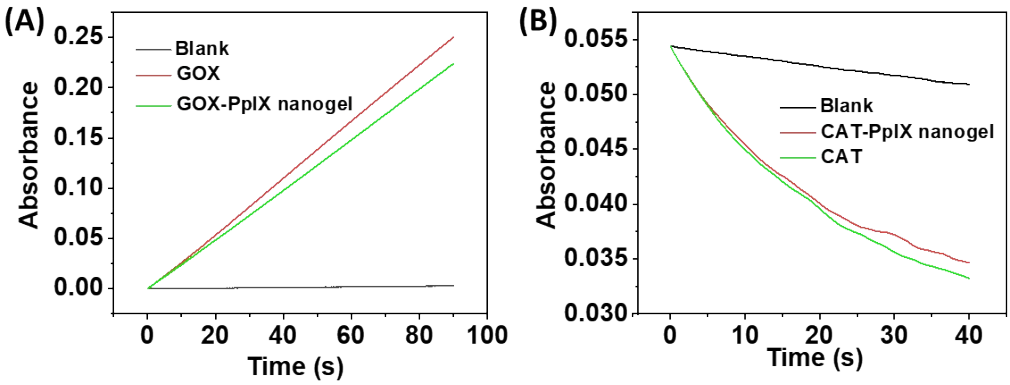


Figure S3. (A) The comparation of the GOX activity for the native GOX and GOX-PpIX nanogel. (B) The comparation of the CAT activity for the native CAT and CAT-PpIX nanogel. The spectra indicated the catalytic activity of GOX-PpIX kept 89.3% of native GOX activity, CAT-PpIX nanogel kept 96.9% of the native CAT activity.

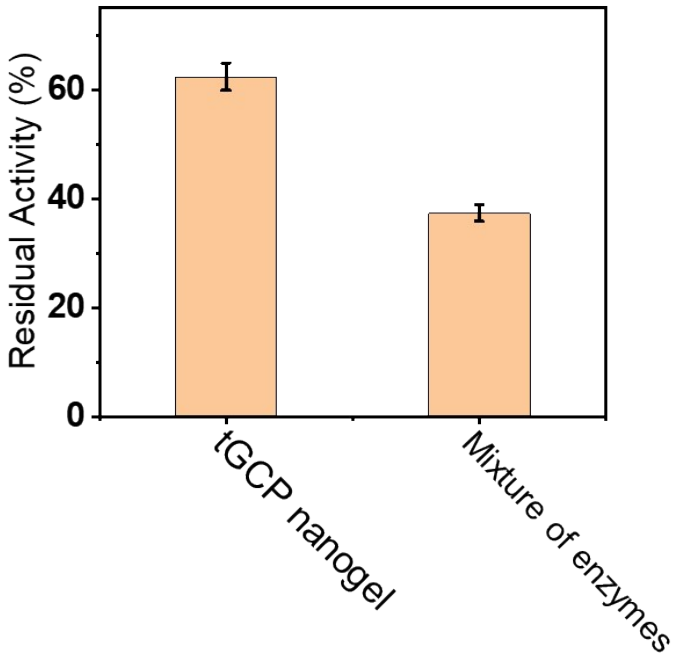
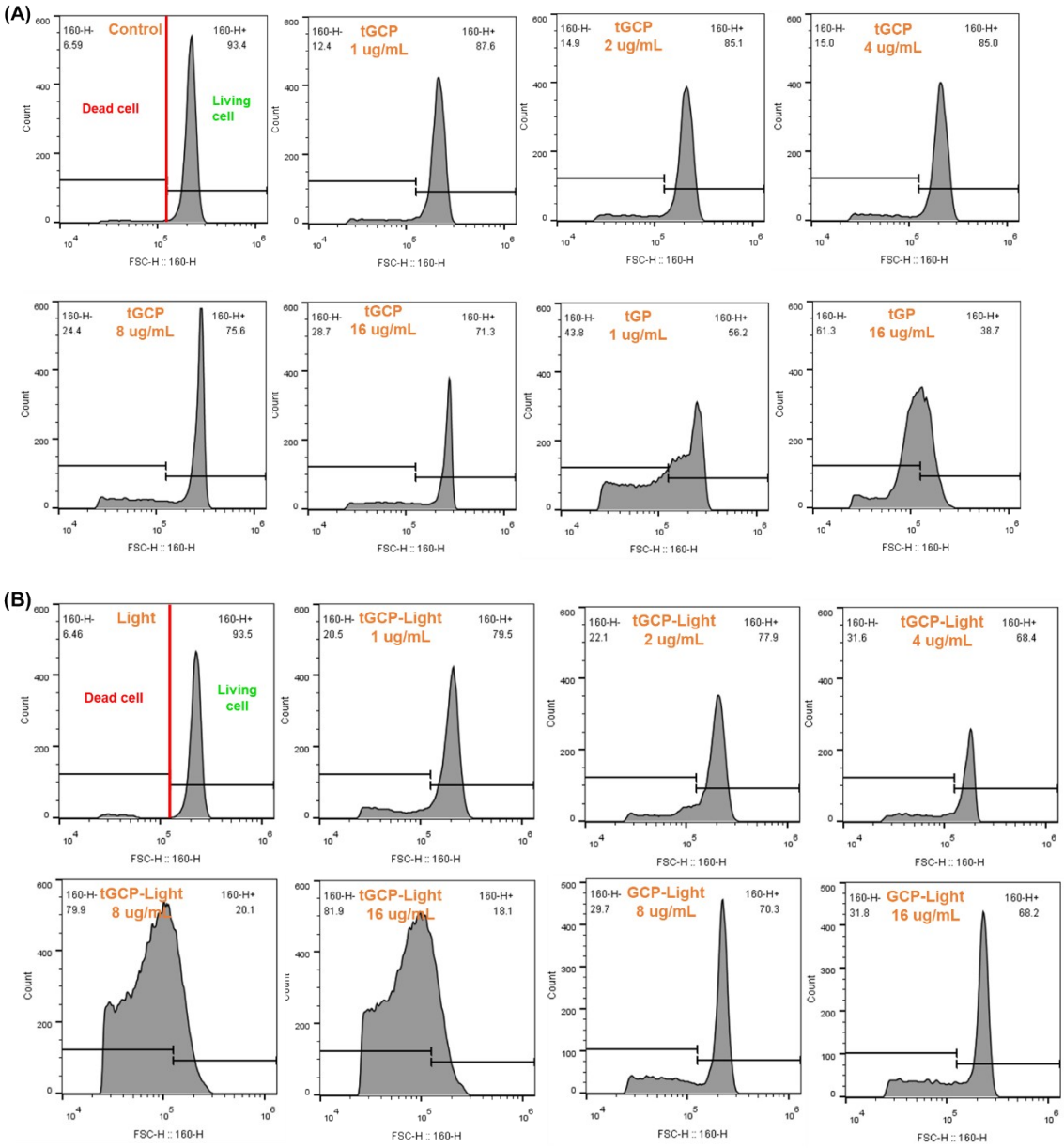


Figure S4. Thermal stability of the mixture of native GOX and CAT, and tGCP nanogel by monitoring the oxygen consumption rate after incubated at 50 ℃ for 30 minutes. The cascaded catalytic activity between native GOX and CAT can maintain around 37.3 % of its original activity, and the cascaded catalytic activity between GOX and CAT within tGCP nanogel can maintain 62.3% of its original activity, indicating when the double enzymes system polymerized into nanogel can improve the cascade system thermal stability.


(A)
160-H- 6.59
Control
160-H+ 93.4
Dead cell
Living cell
160-H- 12.4
tGCP 1 ug/mL
160-H+ 87.6
160-H- 14.9
tGCP 2 ug/mL
160-H+ 85.1
160-H- 15.0
tGCP 4 ug/mL
160-H+ 85.0
160-H- 24.4
tGCP 8 ug/mL
160-H+ 75.6
160-H- 28.7
tGCP 16 ug/mL
160-H+ 71.3
160-H- 43.8
tGP 1 ug/mL
160-H+ 56.2
160-H- 61.3
tGP 16 ug/mL
160-H+ 38.7
Count
FSC-H :: 160-H
(B)
160-H- 6.46
Light
160-H+ 93.5
Dead cell
Living cell
160-H- 20.5
tGCP-Light 1 ug/mL
160-H+ 79.5
160-H- 22.1
tGCP-Light 2 ug/mL
160-H+ 77.9
160-H- 31.6
tGCP-Light 4 ug/mL
160-H+ 68.4
160-H- 79.9
tGCP-Light 8 ug/mL
160-H+ 20.1
160-H- 81.9
tGCP-Light 16 ug/mL
160-H+ 18.1
160-H- 29.7
GCP-Light 8 ug/mL
160-H+ 70.3
160-H- 31.8
GCP-Light 16 ug/mL
160-H+ 68.2
Count
FSC-H :: 160-H

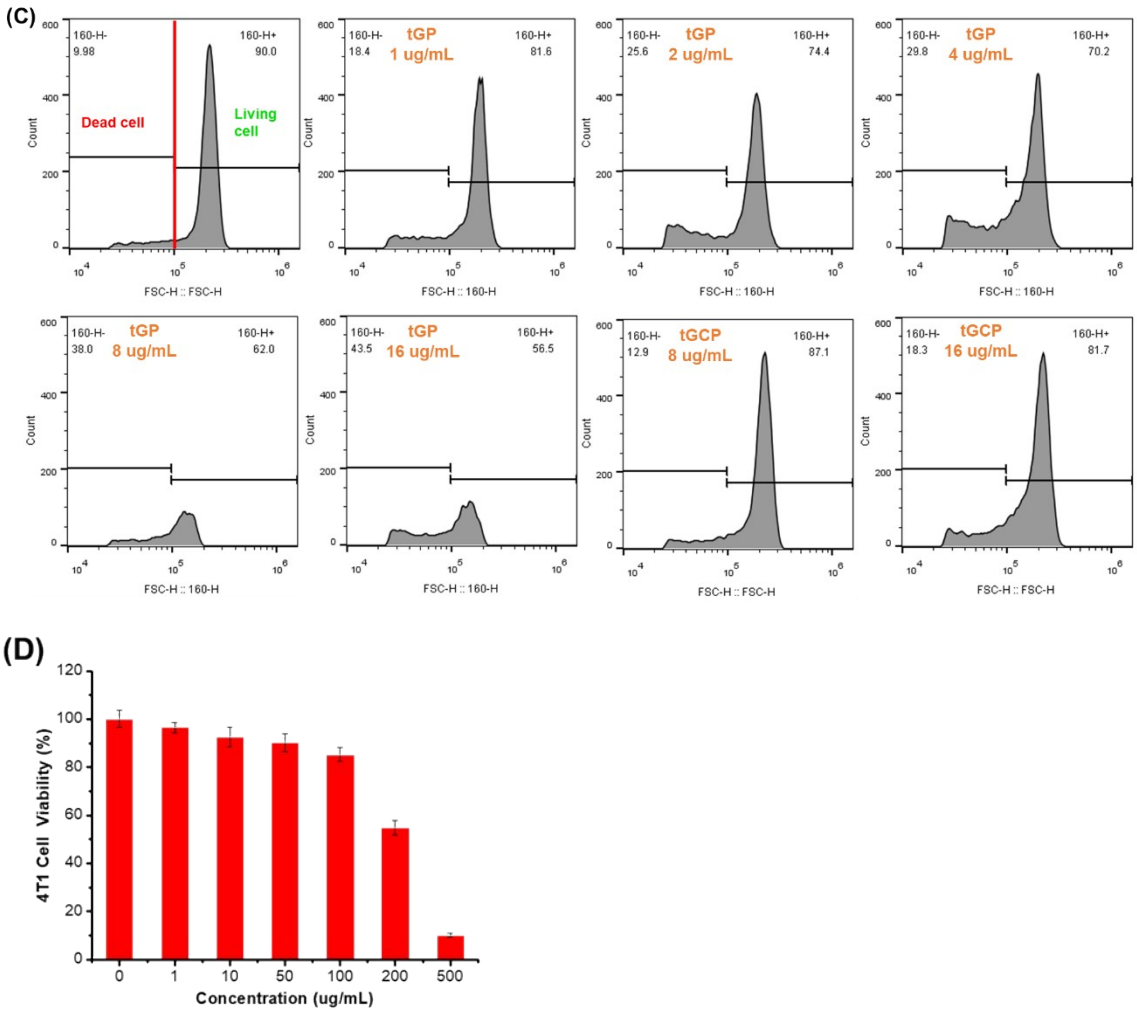


Figure S5. Flow cytometry analysis of cytotoxicity of different compound enzyme nanoparticles. (A) 4T1 cells cytotoxicity of tGCP and tGP without light. (B) 4T1 cells cytotoxicity of GCP and tGCP under light conditions. (C) LO2 cells cytotoxicity of tGP and tGCP. (D) 4T1 cells cytotoxicity of H_2O_2 at different concentrations.

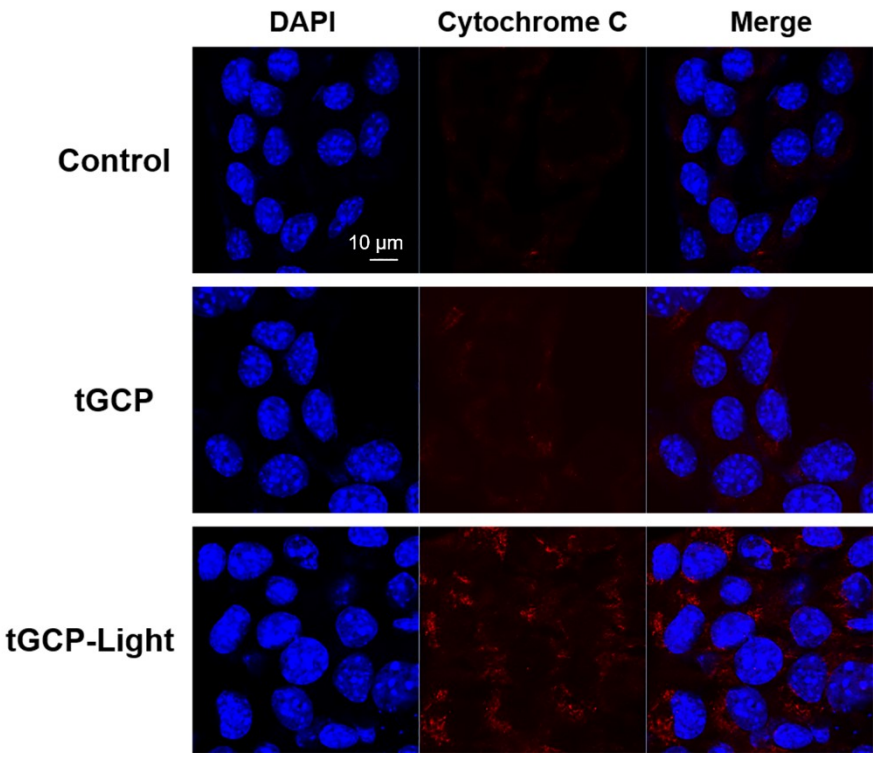


Figure S6. Confocal fluorescence imaging of cytochrome c released from mitochondria in 4T1 cells treated with tGCP with or without light.

Reference

1. M. Qin, Z. Cao, J. Wen, Q. J. Chen, Z. He and Y. Lu, *Advanced Materials*, 2020, **32,** 2004901.