Supporting Information

A cascade nanoplatform for the regulation of tumor microenvironment and combined cancer therapy

Xiaochun Hu[a, b, 1], Wenrong Zhao[a, 1], Ruihao Li[a], Keke Chai [a], Fangjian Shang[c], Shuo Shi[a,*] and Chunyan Dong[a*]

[a] Department of Comprehensive Cancer Therapy, Shanghai East Hospital, School of Medicine, Shanghai Key Laboratory of Chemical Assessment and Sustainability, School of Chemical Science and Engineering, Tongji University, Shanghai 200092, P. R. China

[b] School of Medicine, Shanghai University, Shanghai 200444, China

[c] College of Aeronautical Engineering, Binzhou University, Binzhou 256603, China

[1] These authors contributed equally to this work.

* Corresponding authors.

E-mail addresses: shishuo@tongji.edu.cn (S. Shi), cy_dong@tongji.edu.cn (C. Dong).

Experimental section

Materials

Dopamine hydrochloride was purchased from sigma. Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$) and DTC were purchased from Aladdin. Unless otherwise noted, all chemicals were used without further purification. Fetal bovine serium (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), penicillin streptomycin (PS), Cell counting Kit-8 (CCK-8) and trypsin were ordered from KeyGEN BioTECH. Anti-Calreticulin Antibody was purchased from ProMab Biotechnologies (California, USA). The following antibodies were bought from DAKEWE: Zombie NIR™ Fixable Viability Kit(REF. NO. 423105, Biolegend), FITC anti-mouse CD45(REF. NO. 103108, Biolegend), TruStain fcX™ anti-mouse CD16/32(REF. NO. 101319, Biolegend), PerCP/Cyanine5.5 anti-mouse CD8a Antibody(REF. NO. 100734, Biolegend), Alexa Fluor® 647 anti-human/mouse Granzyme B Antibody(REF. NO. 515406, Biolegend), Brilliant Violet 421™ anti-mouse F4/80(REF. NO.123131, Biolegend), Brilliant Violet 605™ anti-mouse/human CD11b Antibody (REF. NO. 101257, Biolegend), PE anti-mouse CD206(REF. NO. 141706, Biolegend), PE/Cyanine7 anti-mouse CD86(REF. NO. 105013, Biolegend). Mouse Tumor Dissociation Kit was purchased from Miltenyi Biotec (REF. NO. 130-096-730, Miltenyi Biotec). Female BALB/c nude mice were purchased from Slac Experimental Animal Centre (Shanghai, China). All the animal experiments were approved by the Ethics Committee of Tongji University Animal Experiment Center (approval number was TJBB00720101).

Characterizations

SEM was carried out on a Hitachi S-4800 scanning electron microscope. TEM images were obtained with a TEOL JEM-2100 transmission electron microscope. Zeta-potential measurements were conducted on a Malvern Zetasizer Nano ZS instrument. Confocal fluorescence imaging was performed on a laser confocal scanning microscope (Leica TCS SP5). 808 nm NIR lasers (Changchun Laser) were used to carry out the PTT study. The camera (DALI TECHNOLOGY) was

utilized to monitor the photothermal conversion. The whole-body fluorescent imaging was employed by a small animal imaging system (Aniview 100, Biolight Biotechnology, Guangzhou).

Synthesis of DQ

The DQ was synthesised according to the reported reference [1]. Briefly, 4-(Bromomethyl)benzeneboronic acid pinacol ester (0.50 g, 1.68 mmol) in tetrahydrofuran (10 mL) was added into sodium diethyldithiocarbamate (DTC, 3.80 g, 16.85 mmol) in methanol (10 mL) under stirring at room temperature. After an overnight reaction, the solution was concentrated and redissolved in ethyl acetate, and washed with water and brine, and removed water by anhydrous sodium sulfate. And then, the filtrate was concentrated. The final product was purified by silica gel column chromatography using ethyl acetate/hexane (1:10) as an eluent to afford target compound as white solid. ESI-MS (*m/z*): $[M + H]^+$ calcd. for $C_{18}H_{29}BNO_2S_2$ 366.17, found 366.1724. ^{1}H NMR (600 MHz, DMSO) δ 7.62 (d, 2H), 7.39 (d, 2H), 4.54 (s, 2H), 3.96 (q, 2H), 3.72 (q, 2H), 1.28 (s, 12H), 1.19 (dt, 6H). ^{13}C NMR (151 MHz, DMSO) δ 193.75 (s), 140.62 (s), 135.03 (s), 129.11 (s), 84.10 (s), 49.70 (s), 47.00 (s), 41.08 (s), 25.12 (s), 12.88 (s), 11.82 (s).

Synthesis of M-Cu/DQ

The MPDA NPs were prepared referring to the previously reported method [2, 3]. 20 mg $CuCl_2$ was added into MPDA dispersion (water, 2 mg/mL, 10 mL) under stirring at room temperature for 12 h. The M-Cu was collected by centrifugation and washed with water for several times. After that, 20 mg M-Cu was added into DQ solution (DMF, 2.5 mg/mL, 4 mL) under stirring at room temperature for 24 h. And then, the M-Cu/DQ was collected by centrifugation and washed with DMF to remove the unloaded DQ.

Synthesis of M-Cu/DQ-GOx- FA/PEG (MCDGF)

M-Cu/DQ (10 mg), GOx (10 mg) were stirred in Tris-HCl (5mL, 10 mM, pH=8.5) overnight. After that, MCDG was separated from the dispersion by centrifugation and washed with water several times. MCDG (10 mg), NH_2-PEG-FA (10 mg) were stirred in Tris-HCl (5mL, 10 mM, pH=8.5) overnight. After that, MCDGF was separated from the dispersion by centrifugation and washed with water several times.

H_2O_2-sensitivity of DQ tested by ^{1}H NMR and UV-vis

DQ (10 mg) was dissolved in DMSO-d_6/D_2O (450 μL/50 μL) in NMR tube and 60 μL of H_2O_2 (30 wt%) was added. The tube was tested for ^{1}H NMR at determined points. For control group, H_2O_2 was replaced with H_2O. The mixture solution was prepared in a quartz cuvette containing 1.94 mL 1×SBF buffer, 0.02 mL DQ (5 mg/mL in DMSO), 0.02 mL $CuCl_2$ (20 mg/mL in water) and 0.02 mL H_2O_2 (50 mM in water). The UV-vis spectrum of this mixture was collected from 400 to 600 nm at determined points. There was free of H_2O_2 for control group.

Photothermal conversion experiments

The photothermal conversion capability of MCDGF was explored by monitoring the temperature variety of the nanoplatform dispersion (25-200 μg/mL) under 808 nm laser irradiation (0.25-1.3 W/cm^2). The deionized water was

used as the control group under the same condition. The temperature of the dispersions was measured by infrared camera every 30 s.

The photothermal conversion efficiency (η) is calculated according to the reported method[4] as below, in which m is 1 g, c is 4.2 J/g:

$$\eta = m \times c \times (T_{max} - T_{surr})/(I \times (1 - 10^{-A}) \times \tau_s)$$

$$\tau_s = -t/ln\theta$$

$$\theta = (T - T_{surr})/(T_{max} - T_{surr})$$

Detection of gluconic acid and H_2O_2

Gluconic acid was a product of glucose oxidation reaction, which was detected by a reported method [5]. MCDGF dispersion (10, 50, 100 μg/mL) containing 4.5 mg/mL of glucose was kept at 37 °C for 3 h, and 3.2 mL of the supernatant was obtained by centrifugation. And then, 1 mL aqueous solution (5 mM EDTA and 0.15 mM trimethylamine) and 25 μL of 3 M hydroxylamine was added into above supernatant. After incubating at 37 °C for 0.5 h, 0.5 mL aqueous solution (1 M HCl, 0.1 M $FeCl_3$ and 0.25 M CCl_3COOH) was added. The absorbance of mixture solution was recorded by the UV-vis spectrophotometer. As another product of glucose oxidation reaction, H_2O_2 was detected by hydrogen peroxide assay (Beyotime).

In vitro DQ and Cu^{2+} release

To study the DQ and Cu^{2+} release properties from MCDGF, 2 mL dispersion (MCDGF or DCDF) (0.5 mg/mL, containing 0.1%w/v Tween 80) was added into a centrifuge tube. The tube was put into a shaker and vibrated at 200 rpm at 37 ℃. At determined time intervals, the above dispersion was imposed to laser irradiation (808 nm, 1.5 W cm^{-2}) for 5 min. The supernatant was obtained and used for ICP analysis. The pellet was resuspended and put back into the shaker for subsequent measurement. For the control group, the dispersion would not be imposed to 808 nm laser irradiation.

Cytotoxicity assay

4T1 murine breast cancer cell line (ATCC® CRL-2539TM) was purchased from Chinese Academy of Sciences Cell Library and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37 °C in a humidified atmosphere containing 5% CO_2.

5×10^4 4T1 cells were plated in triplicate in 96-well plates, and after 24-h incubation, the cells were treated with DQ + 100 μM H_2O_2, DQ + 20 μM Cu(II) + 100 μM H_2O_2 respectively at different concentrations for 24 h. Cell viability was measured using the CCK8 assay. 100 μL of DMEM (without FBS) containing 10% CCK8 was added into each well and incubated for 4 h at 37 °C. The light absorbance at 450 nm was measured and the cell viability was calculated according to the following formula: Cell viability = (mean absorbance of test wells - mean absorbance of medium control wells) / (mean absorbance of untreated wells - mean absorbance of medium control wells) × 100%. The viability of 4T1 cells treated with nanoparticles (M-PEG, M-PEG + PTT, M-GOx-PEG, M-Cu/DQ-PEG,

MCDG+PTT, MCDGF+PTT) were measured following the method mentioned above. The PTT experiments were performed with 1.5 W/cm^2 808 nm laser irradiation for 10 min.

Live/dead cell staining assay.

The live/dead staining assay of 4T1 cells were distinguished by using the AM/PI staining kit. Cells were incubated with different treatments of 20 μg/mL in 6-well plates (2×10^5 cells/well) for 4 h. After that, cells were stained with AM/PI according to the protocol. The laser confocal microscope was employed to evaluate their live/dead status.

Cellular uptake of Ce6 labelled MCDGF

Confocal laser scanning microscopy (CLSM) (Leica Microsystems) and CytoFLEX LX flow cytometry (Beckman Coulter Life Sciences) were used to examine the accumulation of MCDG-Ce6 and FA-modified NPs in breast cancer cells. 4T1 cells were seeded in confocal dishes with 10×10^4 cells per well. After incubating for 24 h, the cells were treated with Ce6 labeled MCDG or MCDGF (20 μg/mL), respectively. The culture medium was removed after incubating for 2 h or 4 h. Then the cells were washed thrice with PBS and fixed with 4 % paraformaldehyde. The samples were examined by confocal microscope to evaluate the cellular uptake of nanoparticles. For the flow cytometry experiment, 4T1 cells were incubated in 6-well plates with 20×10^4 cells per well for 24 h. MCDG-Ce6 or MCDGF-Ce6 (20 μg/mL) were added into each well. After incubating for extra 2 h, 4 h and 6 h, the cells were harvested and washed thrice with PBS and then assessed by CytoFLEX LX flow cytometry (Beckman Coulter Life Sciences).

Detection of immunogenic cell death

4T1 cells were incubated for 24 h, and then treated with PBS or M-PEG + PTT or Cu/DQ or M-Cu/DQ-PEG or MCDG+PTT or MCDGF+PTT, respectively. PTT was performed by irradiation for 10 min with 808nm NIR laser after 12 h of incubation. After 24 h incubation, the cells were collected and washed three times by PBS, then resuspended in PBS. Anti-Calreticulin (CRT) Antibody was added to the cell suspension and stained for 20 minutes in the dark. The immunogenic cell death of 4T1 cells (the ratio of CRT^+) was assessed by CytoFLEX LX flow cytometry (Beckman Coulter Life Sciences).

In vivo anti-tumor therapy

5×10^5 4T1 cells were injected into the right breast fat pad of BALB/c mice (6-8 weeks old) to establish murine triple-negative breast cancer model. The tumor size was calculated as follows: tumor size (mm^3) = ½ (length × width^2). Tumor size was monitored every 2 days by measuring tumor diameters. Mice were randomly grouped for treatments when tumor volume reached 100 mm^3.

The mice were randomly divided into 8 groups (5 mice per group) administered intravenously alone or combined with PTT for 14 days as follows: PBS (PBS 100μL daily), M-PEG (20 mg kg^{-1} M-PEG once every 2 days), Cu/DQ (1.3 mg kg^{-1} Cu/DQ daily), M-GOx-PEG (20 mg kg^{-1} M-GOx-PEG daily), M-Cu/DQ-PEG (20 mg kg^{-1} M-Cu/DQ-PEG daily), M-PEG + PTT (20 mg kg^{-1} M-PEG once every 2 days + PTT), MCDG+PTT (20 mg kg^{-1} MCDGF once

every 2 days + PTT) and MCDGF+PTT (20 mg kg^{-1} MCDGF once every 2 days + PTT). PTT was performed by laser irradiation (1.5 W/cm^2, 808 nm, 10 min) on every following day after each intravenous administration. The body weight and tumor size of each mouse were monitored every two days. Tumor size was recorded in two perpendicular dimensions (a=length and b=width) with a vernier caliper, and the tumor volume (mm^3) was calculated according to the formula ($a \times b^2$)/2. On day 14, the mice were sacrificed and tumors were excised and weighed. The major organs (heart, liver, spleen, lung, and kidney) were collected and then stained by hematoxylin and eosin (H&E) to detect histopathological lesions.

In vivo photothermal effect

The *in vivo* photothermal effect of MCDG and MCDGF was evaluated on 4T1 tumor bearing mice when tumor size reached 300 mm^3. The mice were randomly divided into three groups and administrated by intravenous injection of PBS, MCDG and MCDGF. The laser irradiation (1.5 W/cm^2, 808 nm, 10 min) was given to each mouse at 24 hours after injection. The changes in tumor temperature over time were monitored and imaged using an infrared camera.

The bio-distribution of MCDG and MCDGF NPs

The AniView100 2.0 Multi-mode Live Animal Imaging System was employed to examine the whole-body fluorescent imaging of tumor-bearing mice after intravenous injection of ICG-labelled NPs. Mice injected with PBS, MCDG or MCDG NPs were observed at 2 h, 6 h, 12 h and 24 h. Finally, all mice were sacrificed to excise the tumors and main organs (heart, lung, liver, spleen and kidney) for further observation *in vitro*.

Blood routine examination, serum biochemical and cytokine assays

Blood samples were collected by eyeball enucleation from anesthetized mice in each group. 0.2 ml of blood was transferred to a tube containing EDTA for routine hematological assay while 1.3 ml of blood was centrifuged at 3240 rpm for 15 minutes to obtain serum for blood biochemical test and cytokine assay. The detection of TNF-α and IFN-γ in mouse serum was quantified by mouse precoated ELISA kit (mlBio).

Toxicity assessment of major organs

After anesthesia, the major organs of mice including heart, liver, spleen, lungs and kidneys were harvested and kept in 4% paraformaldehyde before embedded in paraffin. Then, hematoxylin and eosin (HE) staining of paraffin sections was used to analyse organ damage.

Preparation of single-cell suspension from tumors

The tumors were cut into small pieces of 2-4 mm and transferred into gentle MACS C Tubes containing the enzyme mix (prepared from Tumor Dissociation Kit, mouse). Tightly close C Tubes and attach it upside down onto the sleeve of the gentle MACS Dissociator (Miltenyi Biotec, 130-093-235) then dissociate the tumors into single-cell suspensions.

Lyse red blood cells in tumor single-cell suspension

Dilute 10× Red Blood Cell (RBC) Lysis Buffer to 1× working concentration with DI water and resuspend the single tumor cells in 3 ml 1× RBC Lysis Buffer for 5 minutes. Then stop cell lysis by adding 10ml Cell Staining Buffer to the tube. The cells were collected by centrifugation and washed in PBS twice.

Fluorescence-activated cell sorting (FACS) analysis of TAMs and CTLs

Resuspend 1×10^6 cells in 1:1000 diluted 100 μL Zombie NIR™ solution and incubate in the dark for 15-30 minutes at room temperature. After washing one time and resuspending in Cell Staining Buffer at 1×10^7 cells ml^{-1}, Fc receptors of samples were blocked with anti-mouse CD16/32 antibody for 10 minutes on ice. Then add CD45 (0.5μL/100μL), CD11b (1.25 μL/100μL), F4/80 (5 μL/100μL), CD86 (5 μL/100μL), CD8 (5 μL/100μL) antibodies into cell suspensions and incubate on ice for 20 min in the dark. Wash twice with Cell Staining Buffer by centrifugation at 350g for 5 minutes before fixing cells in 0.5 ml/tube Fixation Buffer in the dark for 20 min at room temperature. Next, wash cells twice with Cell Staining Buffer and resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350g for 10 minutes. Repeat the previous step twice before resuspending permeabilized cells in residual Intracellular Staining Perm Wash Buffer, and then add CD206 and granzyme B antibodies (5μL/100μL respectively). After incubating for 20 min in the dark at room temperature, wash the samples in Intracellular Staining Perm Wash Buffer twice and resuspend in Cell Staining Buffer for flow cytometry analysis using the BD FACS AriaIII sorter (BD Biosciences).

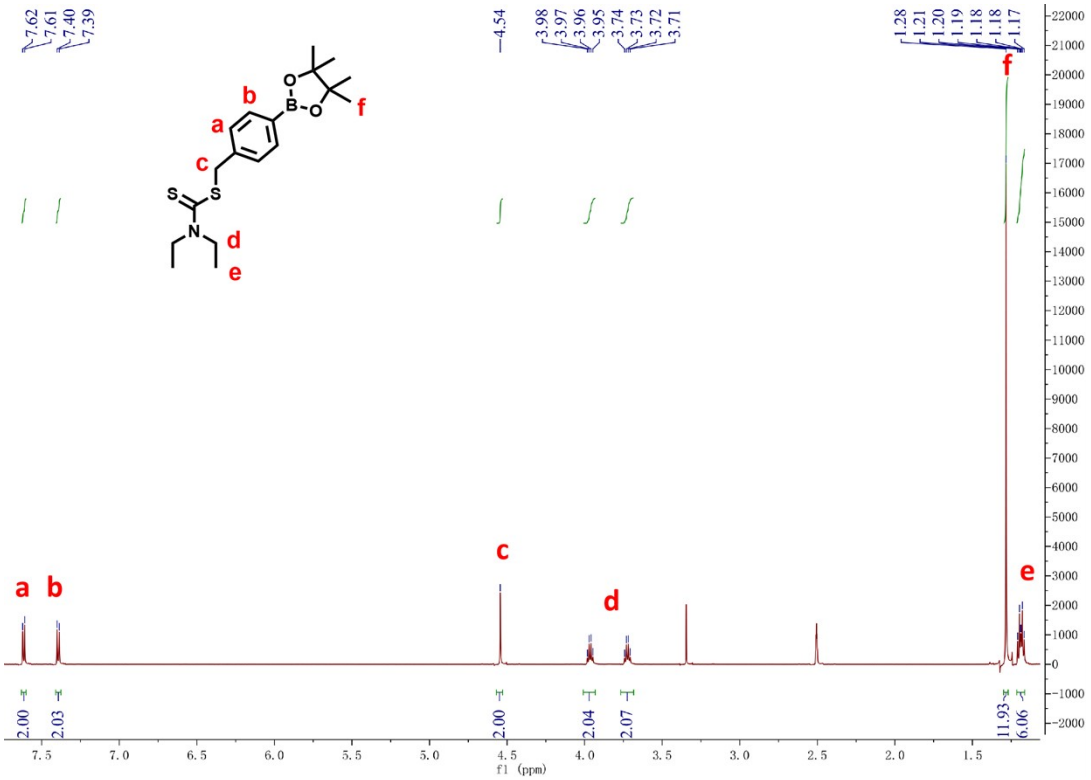

Figure S1. ^{1}H NMR spectrum of DQ in dimethyl sulfoxide-d_6.

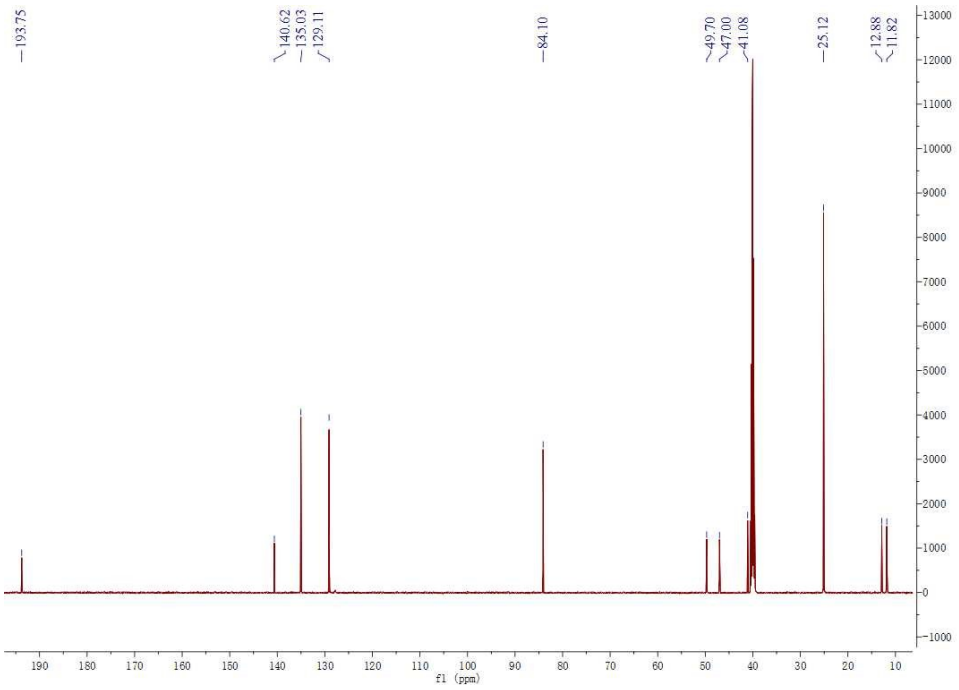

Figure S2. ^{13}C NMR spectrum of DQ in dimethyl sulfoxide-d_6.

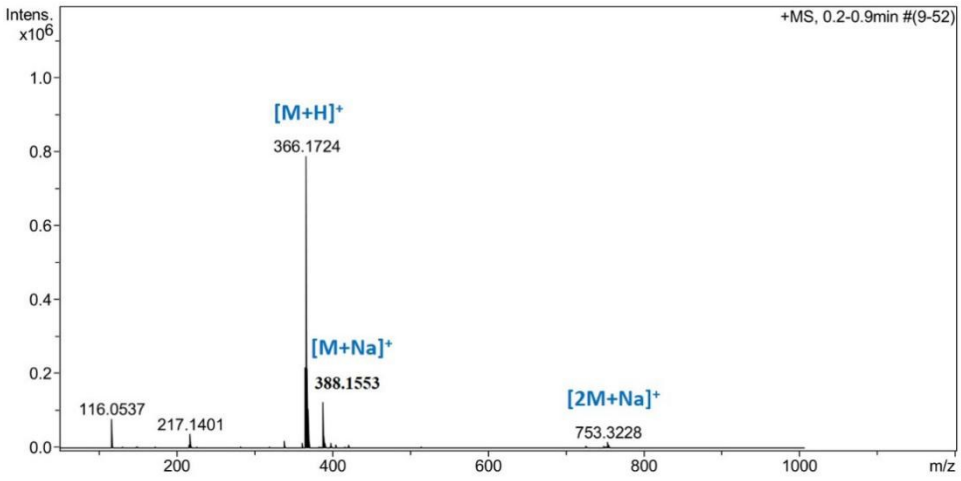

Figure S3. ESI-MS spectrum of DQ.

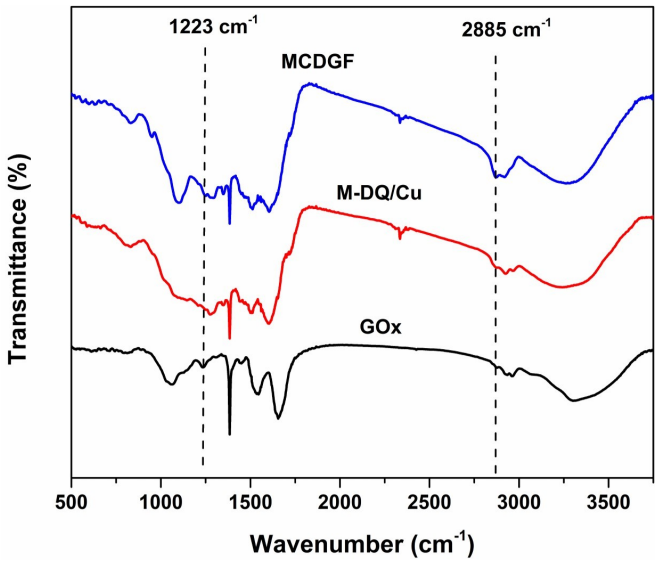

Figure S4. FTIR results of nanoparticles.

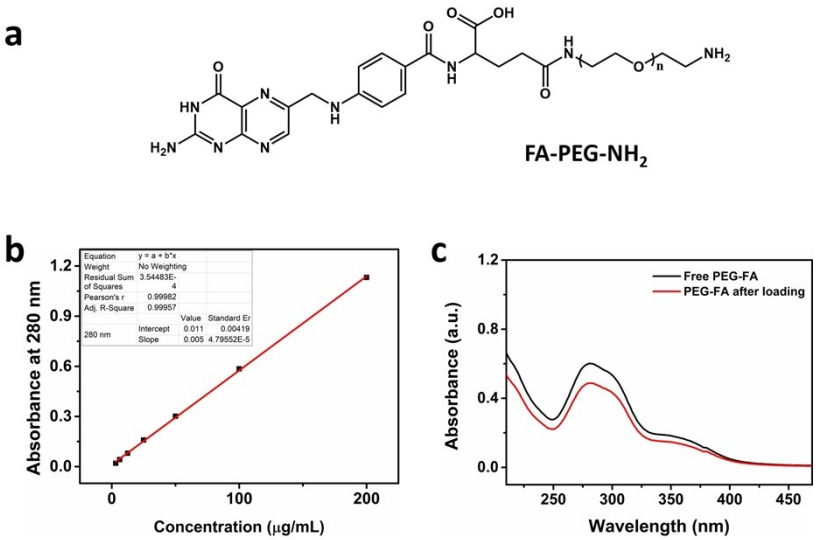

Figure S5. (a) Structure of FA-PEG-NH_2. (b) Standard curve of FA-PEG-NH_2 at 280 nm. (c) The UV/vis absorption spectrum of the supernatant collected after centrifugation and the initial FA-PEG-NH_2 solution.

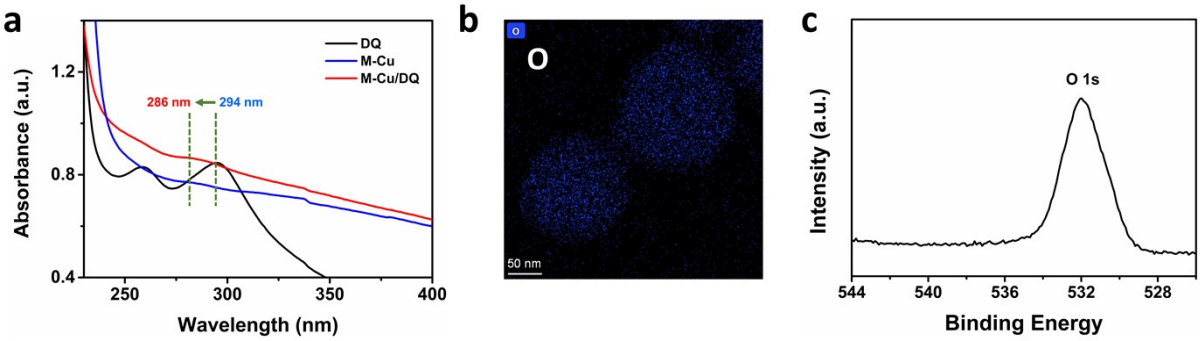

Figure S6. (a) The enlarged spectrogram of DQ, M-Cu and M-Cu/DQ. (b) The enlarged O elemental mapping of MCDGF. (c) XPS high-resolution spectrum of O 1s.

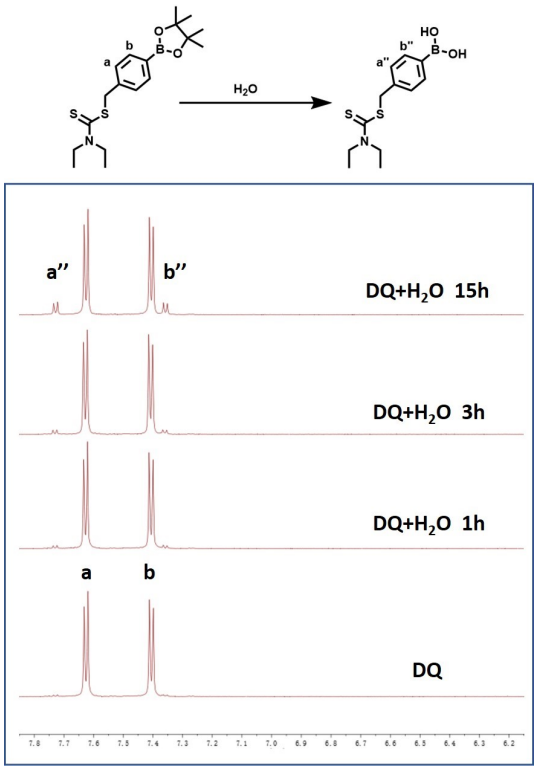

Figure S7. ^{1}H NMR spectra changes of DQ in the absence of H_2O_2.

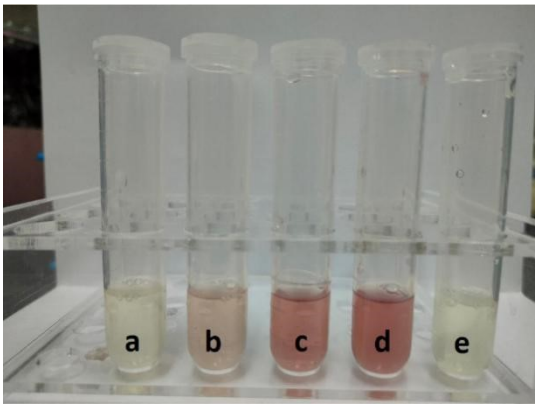

Figure S8. Glucose oxidase ability of NPs, and the pink color indicated the production of gluconic acid. (a) 100 μg/mL MCDF, (b) 10 μg/mL MCDGF, (c) 50 μg/mL MCDGF, (d) 100 μg/mL MCDGF dispersion in the presence of 4.5 mM glucose and (e) 100 μg/mL MCDGF dispersion in water.

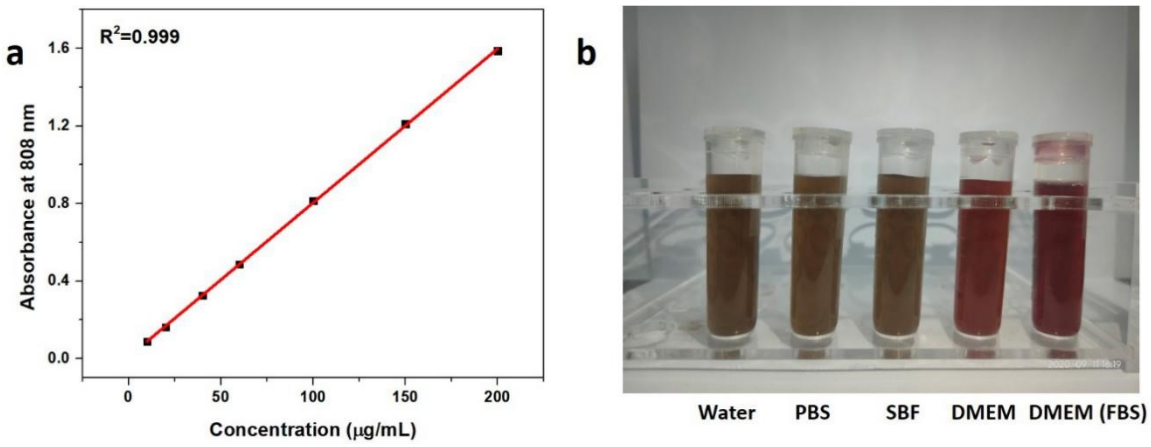

Figure S9. (a) The absorption intensity of NPs at 808 nm. (b) MCDGF in water, PBS, SBF, DMEM and DMEM(FBS) solutions (200 μg/mL).

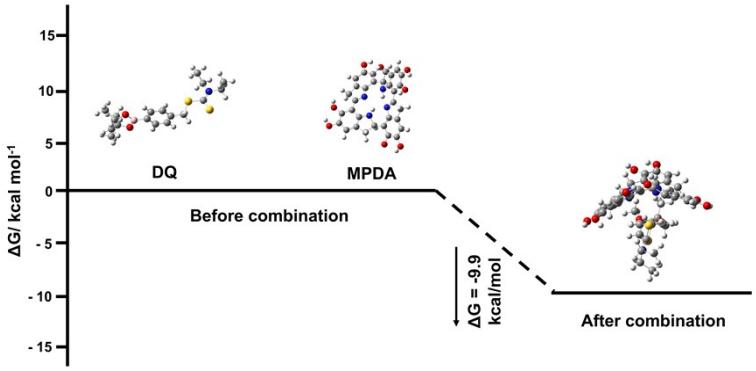

Figure S10. The energy variation of DQ and MPDA before combination and after combination. DFT method: Gaussian 09, b3lyp/6-31 g*, PCM.

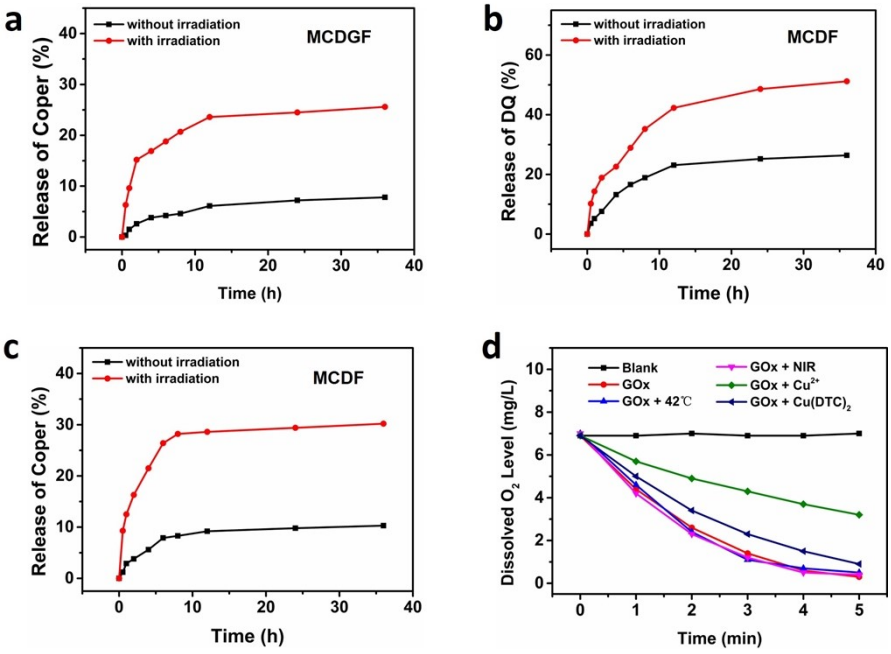

Figure S11. (a) Cu^{2+} release behavior from MCDGF with or without irradiation. (b) DQ and (c) Cu^{2+} release behavior from MCDF with or without irradiation. (d) The consumption of O_2 by GOx under different conditions

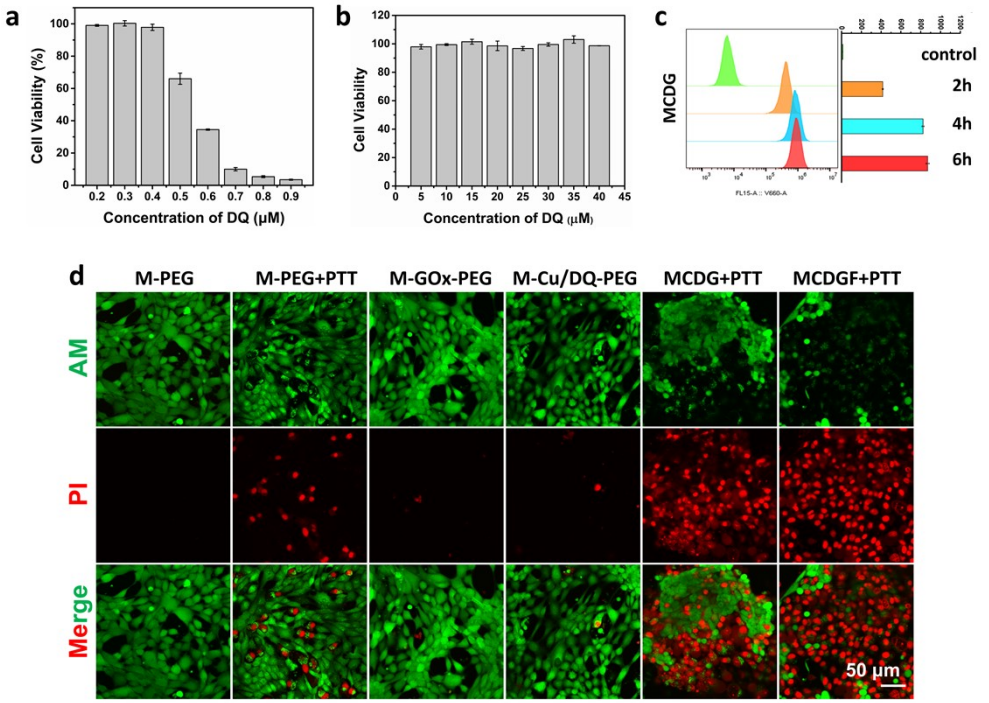

Figure S12. (a) Viability of 4T1 cells treated with DQ+20 μM Cu^{2+}+100 μM H_2O_2. (b) Viability of 4T1 cells treated with different concentration DQ+100 μM H_2O_2. (c) Flow cytometric assay, the corresponding mean fluorescence intensity (MFI) of 4T1 cells incubated with 20 μg/mL Ce6 labeled MCDG for different time periods. (d) Fluorescent images of cytotoxicity in 4T1 cells with different treatments of 20 μg/mL. The live and dead cells are stained with calcein acetyl methyl ester (calcein-AM, green) and propidium iodium (PI, red).

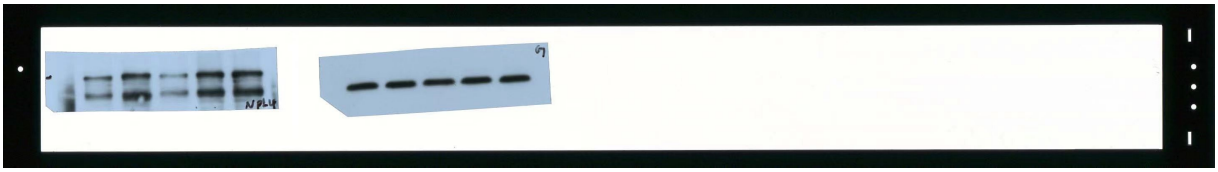

Figure S13. Full raw data for the western blot experiments.

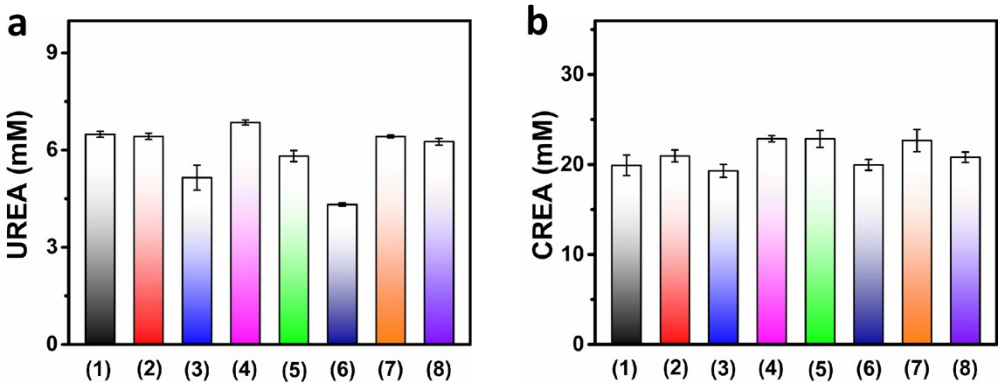

Figure S14. Physiological function assessment of kidney toxicity in different groups. (1) PBS, (2) M-PEG, (3) Cu/DQ, (4) M-GOx-PEG, (5) M-Cu/DQ-PEG, (6) M-PEG+PTT, (7) MCDG +PTT, (8) MCDGF+PTT.

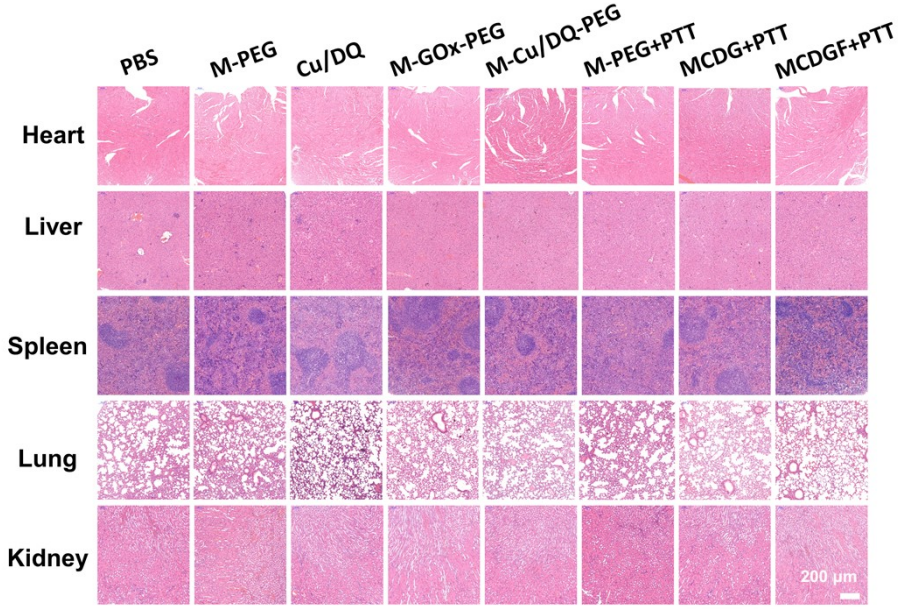

Figure S15. H&E (hematoxylin and eosin) stained images of mice heart, liver, spleen, lung and kidney in different treatment groups (Magnification, ×100).

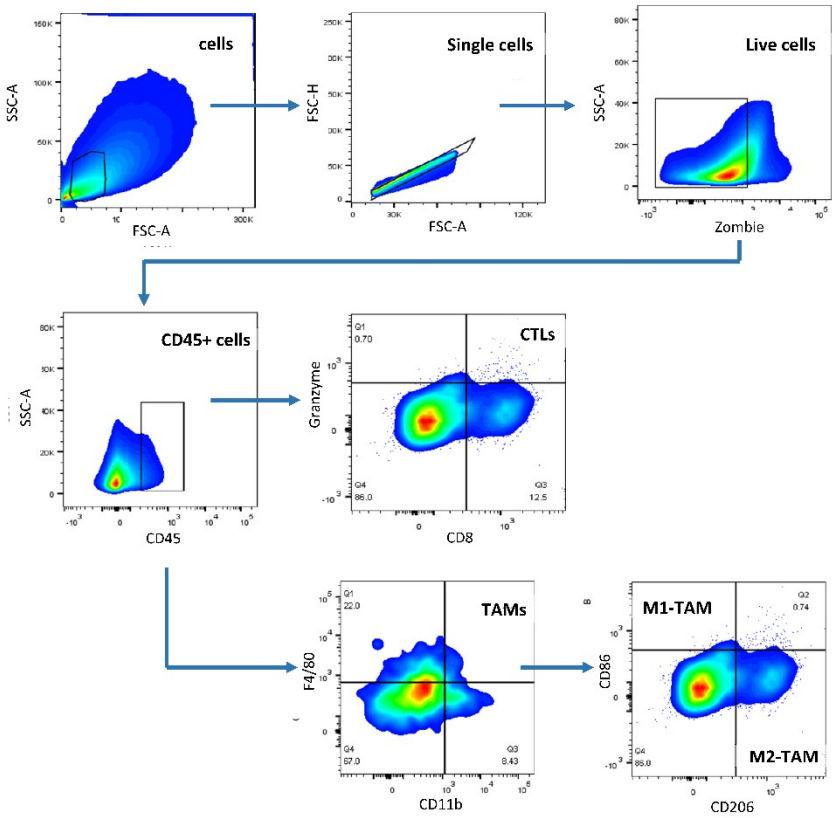

Figure S16. Representative the flow cytometry analysis strategy.

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