Electronic Supplementary Information

Nitrogen-doped graphene quantum dots coupled with photosensitizers for one-/two-photon activated photodynamic therapy based on a FRET mechanism

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MATERIALS AND METHODS

Materials.

All reagents and solvents used were obtained from commercially available sources and used without further purification. Nitrogen-doped graphene quantum dots (N-GQD) was synthesized and purified according to our previous literature. Rose Bengal (RB), 4,7,10-trioxa-1,13-tridecanediamine, 9,10-anthracenediylbis(methylene) dimalonic acid (ABDA), propidium iodide (PI), Hoechst 33342, Alexa Fluor 488 and 3-(4,5-dimethylthiazolyl-2)-2,5-dipheny tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from J&K Chemicals (Shanghai, China). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 Medium and phosphate buffered saline (PBS) were purchased from HyClone/Thermosher (Beijing, China). Dimethyl sulfoxide (DMSO) was purchased from Beijing Chemical Works (Beijing, China). The breast cancer cell line MCF-7 was purchased from the Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Science (Beijing, China). The ultrapure water was obtained by a Milli-Q A10 purification system (Millipore) with 18.2 MΩ cm^{-1}.

Apparatus and characterization

Experiments of transmission electron microscopy (TEM) were performed on an F20 S-TWIN (Tecnai G2, FEI company). Confocal laser scanning microscope (CLSM) images were obtained from an Olympus FV1000 equipped with 405, 488, 559, 635 nm one-photon laser and MaiTai Deepsee two-photon laser. UV-vis spectra were obtained on a Shimadzu 2600 UV-vis spectrometer. FT-IR spectra were recorded in the range 1000-4000 cm^{-1} on a TENSOR-27 spectrometer of Bruker using CaF_2 pellets. Spectra of time-resolved fluorescence were obtained by the Imaging Spectrometer (Horiba Jobini, HR320) with a Nano LED source (excitation at 455 nm).

Preparation of N-GQD-RB.

To synthesize N-GQD-RB, 9.6 mg of EDC was added to the N-GQD solution (0.6 mg mL^{-1}, 150 μL) and stirred evenly. Then, 1.44 mg of NHS was added and stirred for 30 minutes for carboxyl activation. Finally, 200 μL of 4,7,10-trioxa-1,13-tridecanediamine was added and stirred for 18 hours. The procedure was shielded from light. The product was purified by dialysis with 500 MW dialysis tube. Afterwards, 9.6 mg of EDC was added to the RB solution (1 mg mL^{-1}, 300 μL) and then 1.44 mg of NHS was added and stirred for 30 minutes. Then the above solution was added and stirred for

another 18 hours. The final solution was purified using dialysis with 2000 MW dialysis tube. The concentration of N-GQD-RB was confirmed by the RB standard curve.

FRET analysis of N-GQD-RB.

FRET was confirmed by a photo-bleaching experiment. For the bleaching acceptor experiment, the following settings were used: 10 μL of N-GQD-RB suspension was placed onto a slide glass and dried in vacuum for CLSM analysis. Acceptors (RB) were bleached using a 559 nm laser at 95% intensity for 240 seconds, then samples were imaged by the CLSM, with a 405 nm laser at 5% intensity and 559 nm laser at 5% intensity. Time-resolved fluorescence spectra were also used to confirm the energy transfer from donor N-GQD to acceptor RB. The suspensions of N-GQD and N-GQD-RB were dispersed uniformly and measured at the maximum emission wavelength of N-GQD (525 nm) to confirm the energy transfer from the donor to the acceptor. Equally, pure RB and N-GQD-RB were measured at the maximum emission wavelength of RB (580 nm). N-GQD, RB and N-GQD-RB were excited by a one-photon laser (405 nm 5% intensity) and a two-photon laser (800 nm, 5% intensity) to obtain the fluorescence spectra by confocal laser scan microscope through the spectrum scanning mode, respectively.

Detection of singlet oxygen.

ABDA was chosen as the chemical probe to detect singlet oxygen. In this method, 1.5 mL N-GQD-RB (0.01 mg mL^{-1}) water dispersions were mixed homogeneously with 25 μL ABDA (10 mM, in DMSO) and irradiated by a xenon lamp (100 W) with a 480 nm and a 530 nm cut-off filter, respectively. The UV-vis spectra of mixture were measured from 300 nm to 450 nm for a period of irradiation time. The control experiments were carried out with ABDA by the same irradiation.

Cytotoxicity.

Breast cancer cells (MCF-7) were used for the cell experiments. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium containing 10% FBS and 100 U mL^{-1} penicillin-streptomycin in 5% CO_2 and a humidified atmosphere of 95% at 37 °C. Exponential growing cells were placed in 96-wells culture plates with the density of 1×10^4 cells/well and proliferated until spreading to more than 80% of the bottom of the culture well. For photo toxicity, different volumes (0, 10, 20, 30, 40, 50, 60 μL) of test drugs solutions were added to the cells. They were co-cultured for about 4 hours before irradiation. Afterwards, the cells were irradiated by a xenon lamp (100 W) with a 480 nm cut-off filter for 10 minutes. Then, the cells were continued to

culture with drugs. After 20 hours, the cells were washed by PBS to remove drugs. 100 μL of RPMI 1640/FBS and 20 μL of MTT (5 mg mL^{-1}) were added to each well. Finally, after 4 hours' incubation, 150 μL of DMSO was added to each well to replace the culture medium and MTT and measured by the microplate reader with the absorbance at 490 nm. Dark toxicity was detected using the above protocol but without irradiation.

Two-photon induced toxicity of N-GQD-RB to MCF-7 Cells.

MCF-7 cells were incubated in a 35 mm confocal dish and incubated until spreading to more than 50% of the bottom in 5% CO_2 at 37 °C. Then, cells were cultured with N-GQD-RB (0.005 mg mL^{-1}, 100 μL), N-GQD (0.005 mg mL^{-1}, 100 μL) and RB (0.005 mg mL^{-1}, 100 μL), respectively. Then, after 4 hours' incubation in the dark, the cells were washed twice to remove drugs without uptake by the cells. Before irradiation, 1 mL of fresh culture medium with 10% FBS and 10 μL of PI (1 mg mL^{-1}, in PBS) was added in the dish for staining the dead cells. Then, MCF-7 cells were irradiated by a two-photon laser (800 nm) at 5% intensity for 20 minutes. Afterwards, the cells were cultured for 12 hours in the darkness. Then, the images were obtained with 559 nm laser at 5% intensity for detecting dead cells. The control experiment was tested according to the above-mentioned protocol.

Experiments *in vivo* for blood vessel bioimaging and closure.

The protocol of animal experiments was approved by the Institutional Ethical Committee of Animal Experimentation of National Center for Nanoscience and Technology, and all experiments on animals were performed in accordance with governmental and international guidelines. Female white mice (NIH line; ~20 g; 4 weeks of age; Beijing Vital River Laboratory Animal Technology Co., Ltd) were housed in cages and fed with standard mouse chow and water. According to requirements for Biosafety and Animal Ethics, all efforts were made to minimize the number of animals used and their suffering.

For in vivo ear blood vessel imaging and closure experiments of mice, the mice were firstly anaesthetized by intraperitoneal injection of 10% chloral hydrate. After a few minutes, the mice were injected with 200 μL N-GQD-RB (0.01 mg mL^{-1}) via tail vein. Afterwards, the ear hair was cleaned and prepared for two-photon imaging. An inverted confocal microscope was used for bioimaging of the ear blood vessel via two-photon excitation.

For two-photon ear blood vessel imaging, a section of the blood was irradiated as a series of 512

× 512 pixel images, each 10 μm apart through the depth, and total scan depth was 300 μm. An objective lens (20×, NA=0.75) was used to focus the two-photon laser to irradiate the ear blood vessel. The laser power used for two-photon imaging was measured to be 5 mW at the focal plane. For ear blood vessel closure, a section of the vessel was irradiated as a series of 512 × 512 pixel images, each 1 μm apart through the depth, and total scan depth is 40 μm. After 14 minutes continues scan with an 800 nm two-photon laser at 20% intensity, the two-photon images of before and after were obtained. An oil-immersion objective lens (60×, NA=1.35) was used to focus the two-photon laser to irradiate the ear blood vessel. The laser power used for two-photon imaging was measured to be about 0.5 mW at the focal plane (with Newport Power Meter, Model 1918-C).

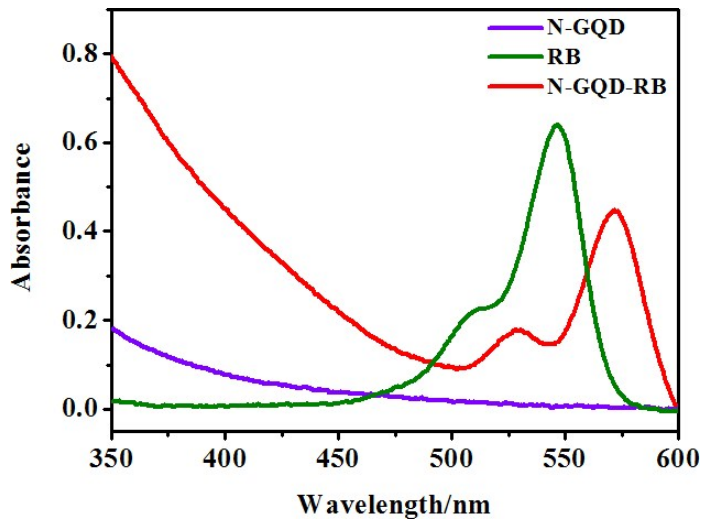

Figure S1 UV/Vis absorption spectra of N-GQD, RB and N-GQD-RB.

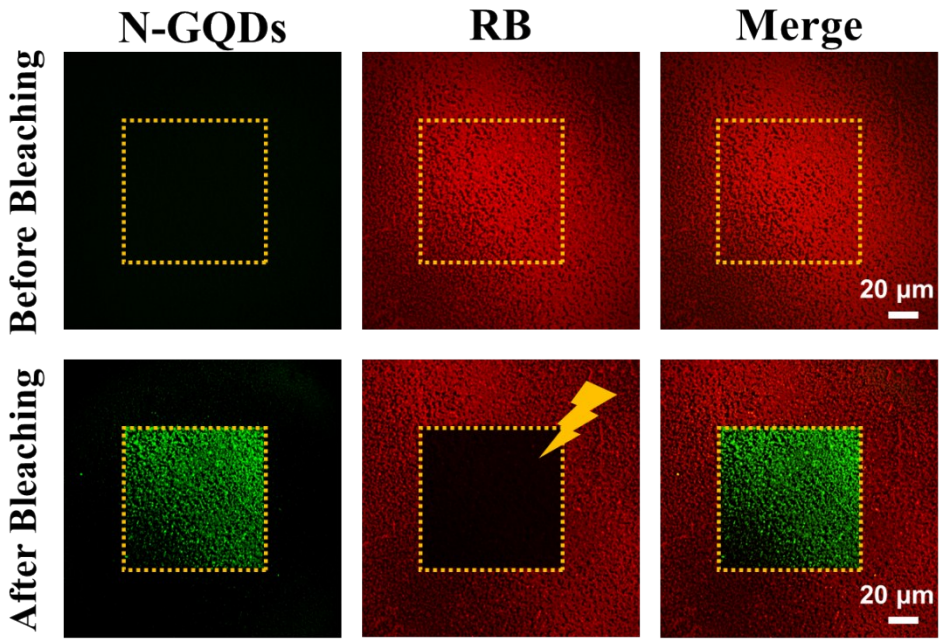

Figure S2 CLSM images of N-GQD–RB before and after local region bleaching.

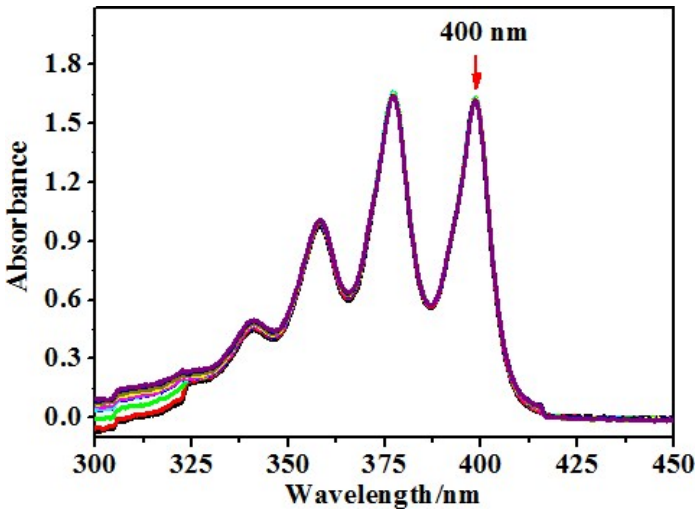

Figure S3 Absorption spectra of ABDA under irradiation with 480 nm irradiation in the absence of N-GQD-RB.

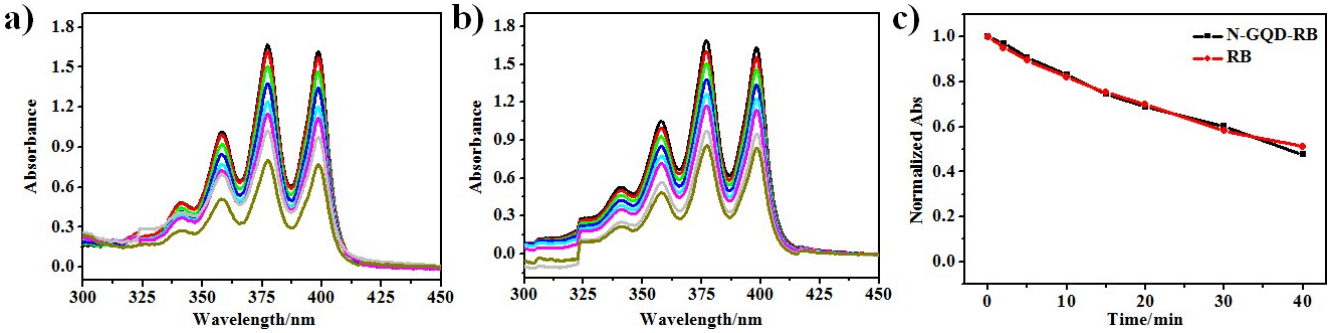

Figure S4 Absorption spectra of ABDA in the presence of a) N-GQD-RB and b) RB under irradiation of 530 nm over different periods of times. c) Normalized absorbance changes of ABDA caused by singlet oxygen oxidation plotted against irradiation time at 400 nm with N-GQD and RB (irradiation at 530 nm).

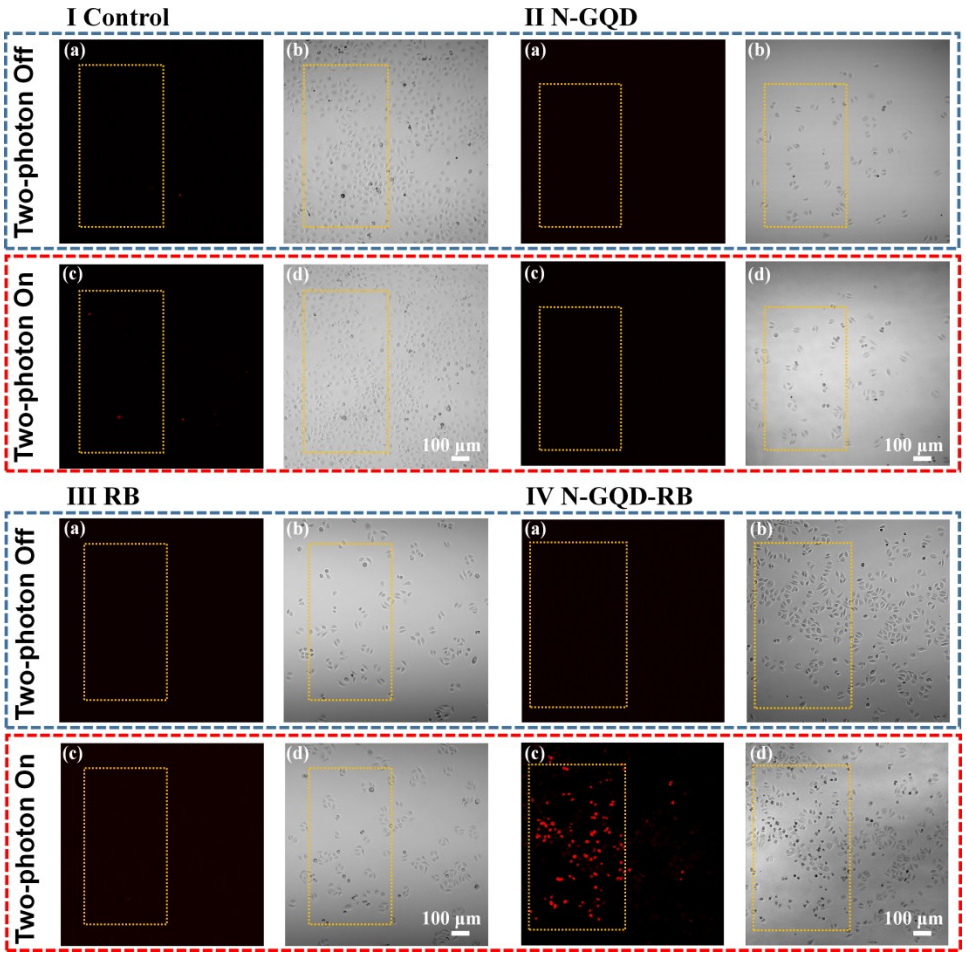

Figure S5 The CLSM images of MCF-7 cells co-cultured with I control; II N-GQD; III RB; and IV N-GQD-RB, and stained with PI. The images were collected before and after irradiation with an 800 nm two-photon laser for 20 minutes and additionally cultured for another 12 hours. (a), (c) images of PI channel (E_m: 590–630 nm); (b), (d) transmission light images.

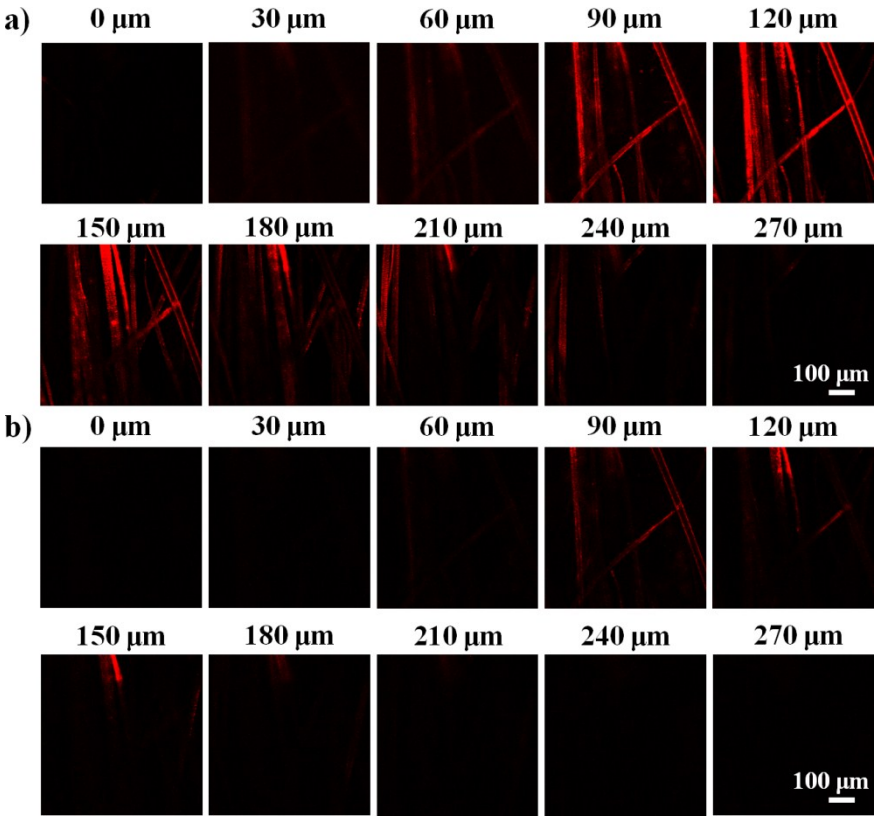

Figure S6 CLSM images of ear blood vessels at different vertical depths, a) with two-photon excitation (Ex:800 nm), and b) with one-photon excitation (Ex:405 nm). Em: 590–630 nm.