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Supporting information

for

Ruthenium Nitrosyl Functionalized Graphene Quantum Dots as an Efficient Nanoplatform for NIR-light-controlled and Mitochondria-targeted Delivery of Nitric Oxide Combined with Photothermal Therapy

Min Guo,[a] Hui-Jing Xiang,[a] Yi Wang,[b] Qian-Ling Zhang,[b] Lu An,[c] Shi-Ping Yang,[c] Yinchu Ma,[d] Yucai Wang,[d] and Jin-Gang Liu[a]**

[a] Key Laboratory for Advanced Materials of MOE, School of Chemistry & Molecular Engineering, East China University of Science and Technology, Shanghai, 200237, P. R. China, E-mail: liujingang@ecust.edu.cn

[b]Shenzhen Key Laboratory of Functional Polymer, College of Chemistry and Environmental Engineering, Shenzhen University, Shenzhen, 518060, P. R. China, E-mail: zhql@szu.edu.cn

[c] Key Laboratory of Resource Chemistry of MOE & Shanghai Key Laboratory of Rare Earth Functional Materials, Shanghai Normal University, Shanghai, 200234, P. R. China.

[d] School of Life Sciences and Medical Center, University of Science & Technology of China, Hefei, 230027, P. R. China

Chemicals and methods:

All reagents were commercially available and used without further purification unless otherwise noted. $[(tpy^{COOH})Ru(OPDA)(Cl)](PF_6)_3$,[S1] $[(tpy^{COOH})Ru(OPDA)(NO)](PF_6)_3$,[S1] $[Ph_3PC_3H_6COOH]Br$ (TPP^{COOH}),[S2] and N-GQDs [S3] were prepared according to previously reported methods.

Transmission electron microscopy (TEM) was performed on a JEOL JEM-2011 transmission electron microscope operating at 100 kV. AFM images were obtained with a Micronano new SPM atomic force microscope (MicroNano D5-A). XPS data were collected on a Thermo Escalab 250

XPS instrument with a monochromatic Al Kα X-ray source ($h\nu$=1486.6 eV). All binding energies were referenced to the C1s peak (284.6 eV) arising from adventitious carbon. FTIR spectra were recorded on a Shimadzu Fourier transform infrared spectrometer (IRPrestige-21). UV-vis absorption spectra were recorded on a Shimadzu UV-visible spectrophotometer (UV-2600). Fluorescence spectra were acquired on a Horiba Fluoromax-4 fluorescence spectrophotometer.

The intensity of 808 nm NIR light irradiation on the sample cell was measured using a CEL-NP2000 intensity meter. The amount of NO released was measured using a NO-sensitive electrode (World Precision Instrument, ISO-NO meter, equipped with a TBR1025 free radical analyzer for measuring nitric oxide from 0.3 nM to 100 μM.). The amperometric data collected from the electrode was then converted into the corresponding nitric oxide concentration. Fluorescence images were acquired by using a Leica TCS SP5 II inverted microscope with a Leica DMI 6000B confocal scanning system. Flow cytometry (FCM) was performed on a Beckman Coulter flow cytometer (Quanta SC, USA).

Synthesis of amine-modified N-GQDs (NH_2-N-GODs)

N-GQDs were dissolved in 2.0 mL of DI water, and activated by an EDC·HCl /NHS solution for 1 h. Then, 1.0 mL of ethylenediamine was added to react for 24 h at room temperature. Following that, the solution was dialyzed in dialysis tube (molecular weight cutoff 1000 Da) against DI water for 48 h. The solid NH_2-N-GODs were collected by freeze-drying.

Synthesis of {N-GQDs@Ru-NO@TPP} (nanoplatform 1)

$[(tpy^{COOH})Ru(OPDA)(NO)](PF_6)_3$ (152.0 mg, 0.16 mmol) and TPP^{COOH} (9.0 mg, 0.02 mmol) were dissolved in 2.0 mL DMF, and activated by an EDC·HCl/NHS solution for 1 h. Then, NH_2-N-GQDs (100 mg) were added to react for 24 h at room temperature. Following that, the solution was dialyzed in dialysis tube (molecular weight cutoff 1000 Da) against DI water for 48 h. Nanoplatform **1** was finally obtained by freeze drying.

The control sample of {N-GQDs@Ru-Cl@TPP} was prepared in a similar way by substitute $[(tpy^{COOH})Ru(OPDA)\ (NO)](PF_6)_3$ with $[(tpy^{COOH})Ru(OPDA)(Cl)](PF_6)_3$, and {N-GQDs@Ru-NO} was obtained similar to that of {N-GQDs@Ru-NO@TPP} in the absence of TPP^{COOH}.

Light triggered NO release

Nanoplatform **1** (1.0 mg/mL) was suspended in saline solution (150 mM) in a quartz cuvette with a gentle stirring using a magnetic stirring bar. NO release was initiated by irradiation with an 808 nm

NIR laser. The amount of NO released was analyzed using a NO-sensitive electrode. The tip of the electrode was placed outside the light path. The electrode was accurately calibrated by mixing standard solutions of $NaNO_2$ with H_2SO_4 (0.1 M) and KI (0.1 M), according to the protocol indicated in the manufacturer manual. The amperometric data collected from the electrode was then converted into the corresponding NO concentration.

NO quantum yield measurement

An 808 nm NIR laser was used for NO quantum yields measurement. Light intensity was determined before each photolysis experiments by an actinometry meter (measured intensity of ~5 mW). The solution of nanoplatform was placed in a 1.0 cm-path-length quartz cuvette, 1.0cm away from the light source. The solution was prepared to ensure sufficient absorbance (>90 %) at the irradiation wavelength and agitated periodically during the photolysis experiment. NO quantum yields (Φ) were calculated based on NO concentrations, obtained by NO meter measurement. The calculated values were plotted versus time. These plots were linear, with a negative slope, for the first 20-25% of the reaction. The extrapolated quantum yield at t = 0 (y intercept) was taken as Φ_{NO} for the photolabilization of NO from the nanoplatform solution.

The *in vitro* photothermal effect

Nanoplatform **1** (200, 400, 600, 800, 1000 μg/mL) and DI water (1.0 mL) was irradiated with light irradiation (808 nm, 1.0 W/cm^2, 10 min), and the solution temperature was recorded using a thermal probe.

Cell culture

Human cervical carcinoma cells (HeLa cells) were obtained from Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Science (CAS, China). HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo,USA) at 37 °C under 5% CO_2 atmosphere, supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) and 1% (v/v) penicillin/streptomycin (Thermo, USA).

MTT assay

All the cells were seeded on a 96-well plate with a density of 5×10^4 cells per well and incubated in a humidified 5% CO_2 atmosphere for 24 h. The cell culture medium were removed and washed with PBS. Following that, different concentrations of the nanoplatform **1** (0, 25, 50, 100, 150, 200 μg/mL) suspended in cell culture medium were added and incubated further for a period of 12 or 24 h at

37 °C in a humidified 5% CO_2 atmosphere. MTT (100 μL, 500 μg/mL) solution was added to each well. After 4 h of incubation at 37 °C, the cell culture medium was removed and the formazan crystals were lysed with 150 μL of DMSO. The absorbance was then measured at 490 nm using a microplate reader (Multiskan MK3, USA).

808 nm NIR irradiation experiments: After incubation of the cells with different concentrations of the nanoplatform for 4 h, light irradiation was applied (808 nm, 600 mW/cm^2, 10 min), and the cells were incubated for another 1 h. Subsequently, the same procedures, as described above, were performed to obtain the final absorbance measurement at 490 nm using a microplate reader.

Confocal laser scanning microscopy

Fluorescence imaging was performed with a Leica DMI 6000B confocal scanning system. A 405 nm laser was used as the excitation source and the corresponding emissions were collected in the wavelength range of 420–490 nm. HeLa cells were seeded on a plastic-bottomedμ-dish of diameter 35 mm, with a density of 104 cells and maintained at 37 °C in 5% CO_2 atmosphere for 24 h. The cells were then treated with nanoplatform **1** (50 μg/mL) for 2 h. After incubation, the cells were washed twice with PBS and subjected to confocal fluorescence microscopy analysis.

Real-time intracellular NO detection experiments: After incubating the cells with nanoplatform **1** solution (50 μg/mL) in the cell culture medium for 4 h, the cells were washed twice with PBS, treated with DAF-FM-DA (5.0 μM), and then incubated for 30 min. After that, the cells were washed twice with PBS and imaged with serum-free medium in the absence or presence of light irradiation (808 nm, 200 mW/cm^2, 2 min). Excitation was carried out with lasers at λ = 405 nm or 488 nm, and emissions were recorded in the wavelength range of 420–490 nm or 500–550 nm, respectively.

Co-localization experiments: the cells were incubated with nanoplatform **1** solution or {N-GQDs@Ru-NO} (50 μg/mL) in the cell culture medium for 8 h, the cells were washed twice with PBS, then treated with Mito-Tracker Green (5.0 μM), and followed by 30 min incubation. After the incubation period, the cells were washed twice with PBS and imaged with serum-free medium. Excitation was carried out with lasers at λ = 405 nm or 488 nm, and emissions were recorded in the wavelength range of 420–490 nm or 500–550 nm, respectively.

Flow Cytometry (FCM)

HeLa cells were incubated with the nanoplatform **1** solution (50 μg/mL) in cell culture medium for 2 h at 37 °C. All the cells were washed twice with PBS and harvested by trypsinization, followed by

centrifugation at 1,500 rpm for 6 min. The precipitate thus obtained was re-suspended in PBS and analyzed using a flow cytometer.

Animal model

The mice (NOD/SCID, female) were purchased from Beijing HFK Bioscience Company, Ltd. Animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee. The HeLa cells were implanted subcutaneously in the right flank of the mice.

***In vivo* phototherapy**

When the tumors had grown to a mean volume of approximately 80 mm^3, the mice were randomly separated into four groups and intratumorally injected with PBS, nanoplatform **1**, and N-GODs in physiological saline, respectively. The first group of mice received PBS with laser irradiation as a control group; the second group was injected with N-GQDs (4.0 mg/kg) and with laser irradiation; the third group was injected with nanoplatform **1** (4.0 mg/kg) without laser irradiation; and the fourth group was injected with nanoplatform **1** (4.0 mg/kg) and with laser irradiation. One hour later, the mice were intraperitoneally injected with sodium pentobarbital, and were anesthetized. The hair on the tumor surface was removed with a depilatory cream, and the tumor site was irradiated by an 808 nm laser (1.0 W/cm^2, 10 min). The mice in all groups received only once injection of the nanoplatform and the laser irradiation groups were treated with light irradiation once as well. The temperature of the tumor site was measured using an infrared camera. The tumor sizes and the mouse weight were monitored every three days and normalized to their initial values. Tumor size was measured using a caliper, and tumor volume was calculated using the following equation: $V = (\pi/6) \times LW^2$, where L is the long diameter and W represents the short diameter.[S4]

After phototherapy, the tumors from all groups were taken by surgery, and photos were taken. The tumors from each group were then marinated in formalin, and followed by TUNEL staining, which was performed with a TUNEL apoptosis detection kit (FITC-labeled) and DAPI, respectively, according to the manufacturer's instruction.

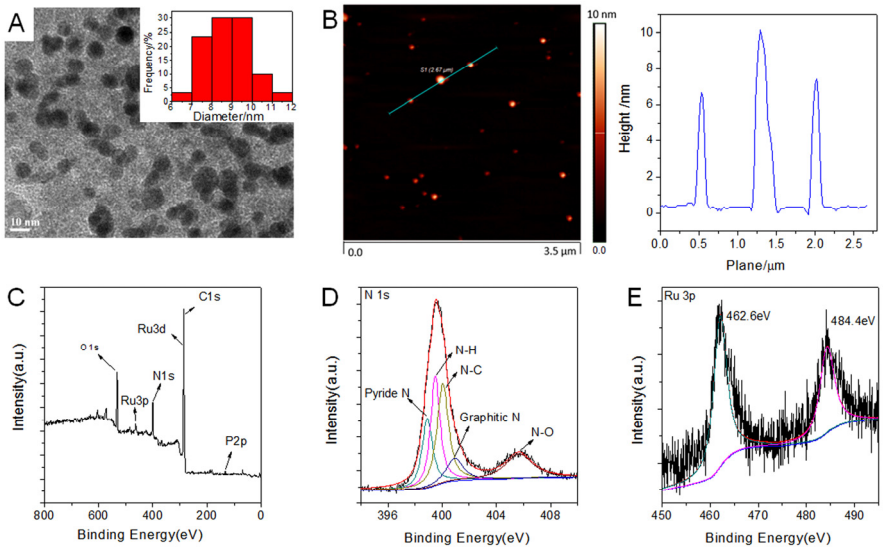

Figure S1 (A) TEM image of nanoplatform **1**. The inset shows the corresponding statistical size distribution. (B) AFM image of nanoplatform **1** (C) XPS survey spectrum of nanoplatform **1**. (D) N1s and (E) Ru 3p core level spectra of nanoplatform **1**.

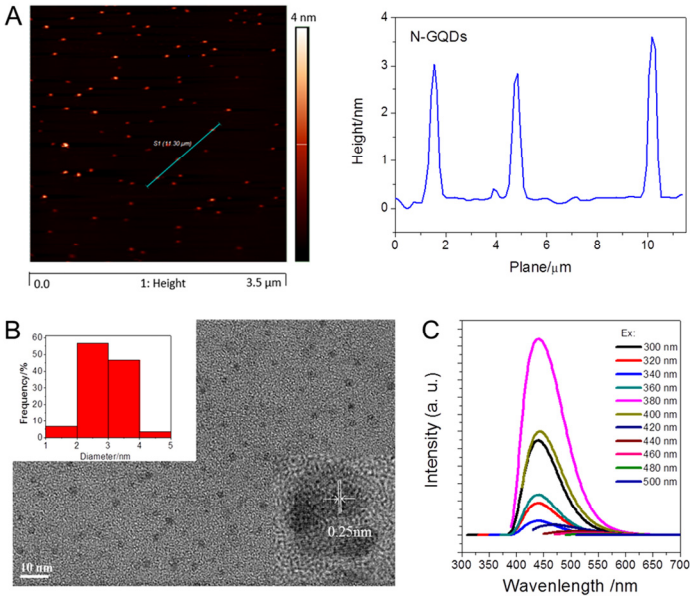

Figure S2 (A) AFM and (B) TEM image of N-GODs. The inset shows the corresponding statistical size distribution. (C) Fluorescence spectra of N-GQDs in water as obtained by varying the excitation wavelength from 300 to 500 nm.

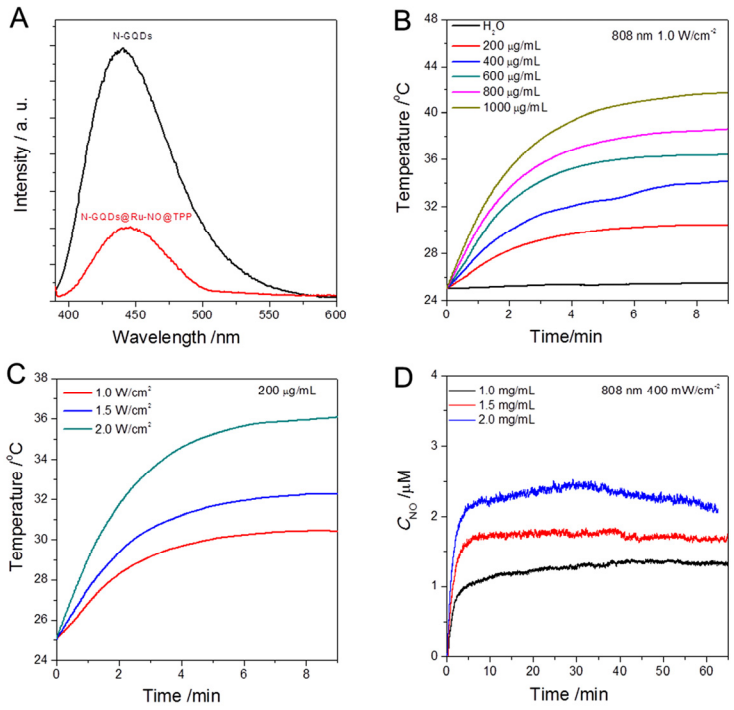

Figure S3 (A) Fluorescence spectra of N-GQDs (black line) and nanoplatform **1** (red line) in water. Ex: 380 nm. (B) Photothermal effect of nanoplatform **1** with different concentrations (0, 200, 400, 600, 800, and 1000 μg/mL) in water irradiated by 808 nm NIR light with powder density of 1.0 W/cm^2. (C) Photothermal effect of nanoplatform **1** (200 μg/mL) in water irradiated by 808 nm NIR light with powder density of 1.0, 1.5, and 2.0 W/cm^2, respectively. (D) NIR light-induced NO release from nanoplatform **1** with concentration of 1.0, 1.5, 2.0 mg/mL, respectively, in anaerobic saline solution by constant 808 nm light illumination with powder density of 400 mW/cm^2.

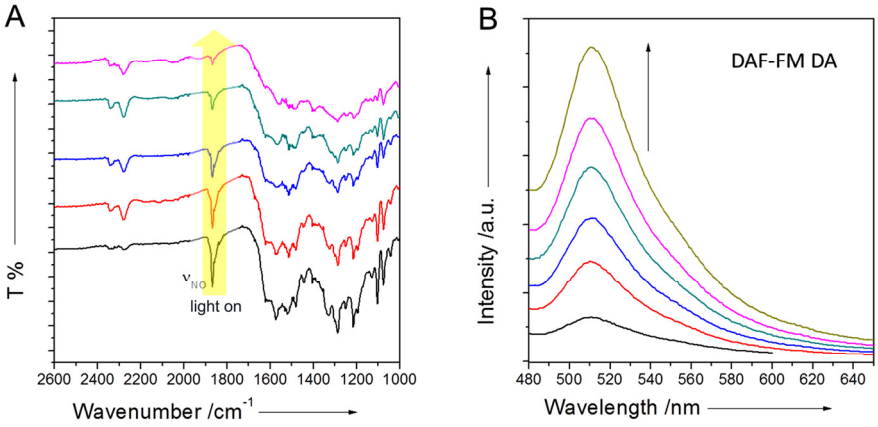

Figure S4 (A) FT-IR spectra of nanoplatform **1** upon 808 nm light irradiation. (B) Fluorescence spectra of DAF-FM DA, a NO probe, in water after 808 nm light irradiation of the nanoplatform **1** that were deposited on the bottom of the cuvette. Spectra were taken every 2 min.

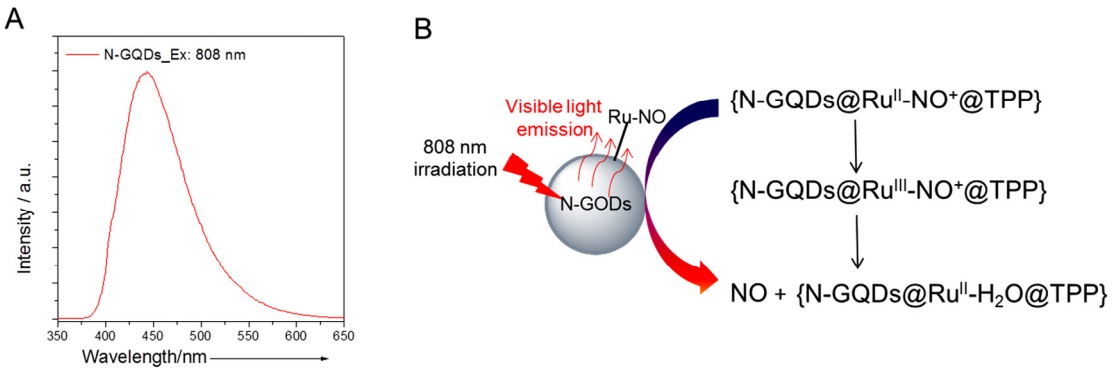

Figure S5 (A) Fluorescence spectra of N-GQDs in water with excitation at wavelength of 808 nm. (B) Plausible photochemical pathways for NO release from nanoplatform **1** under 808 nm laser irradiation.

Carbon dots (CDs) have been reported to display up-converted photoluminescence (Kang, Z. et al. *Dalton Trans.* 2012, *41*, 9526; Li Q. et al. *J. Mater. Chem.* 2012, *22*, 15522; Huang, S.-M. et al. *J. Mater. Chem.* 2012, *22*, 16714.). Here, the N-GQDs were also found to have the unique up-converted property which can convert NIR light into short wavelengths light (Fig. S5A). Consequently, we proposed a plausible mechanism for NO release from nanoplatform 1. NIR light (808 nm) excitation of the carrier of N-GQDs results in visible light emission (Fig. S5A) that is absorbed by the attached Ru-NO, triggering the release of NO (Fig. S5B). Ruthenium nitrosyls derived from polypyridine ligand are photolabile, and release of NO upon their exposure to light excitation is often accompanied by the replacement of NO with solvent molecule, e. g. H_2O, in the coordination sphere of the ruthenium ion (P. K. Mascharak, *Coord. Chem. Rev.* 2008, **252**, 2093; *Acc. Chem. Res.* 2008, **41**, 190.).

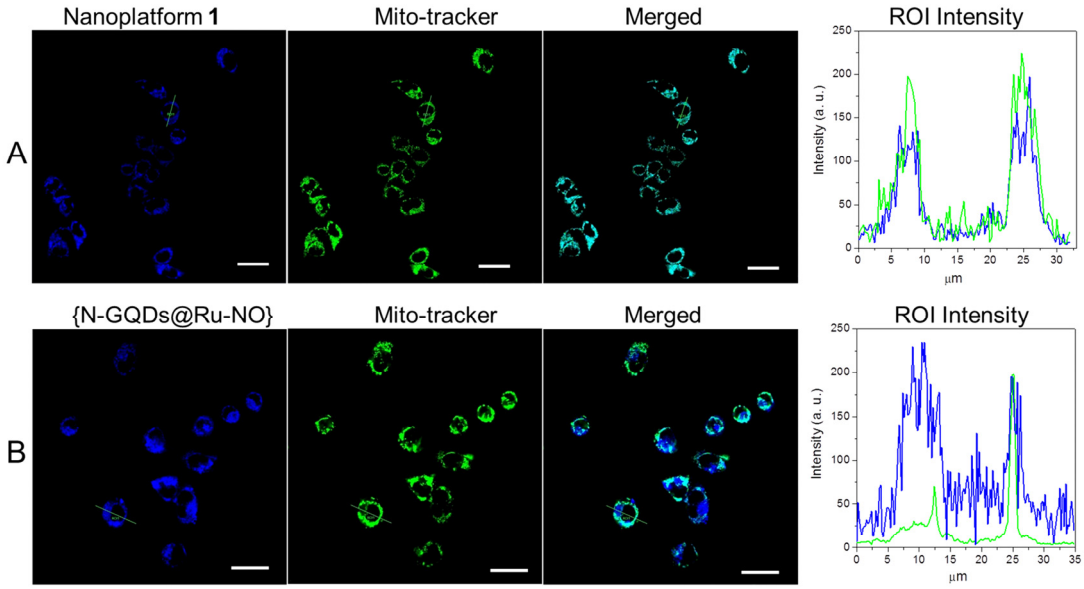

Figure S6 (A) Confocal microscope images of HeLa cells treated with nanoplatform **1** and co-stained with Mito-tracker Green and the intensity profile of ROIs across the cells. (B) Confocal microscope images of HeLa cells treated with the control nanoplatform {N-GQDs@Ru-NO} and co-stained with Mito-tracker Green and the intensity profile of ROIs across the cells. The images were acquired for excitation at 405 nm and 488 nm and recording the corresponding fluorescence in the range of 420–490 nm and 500-550 nm, respectively.

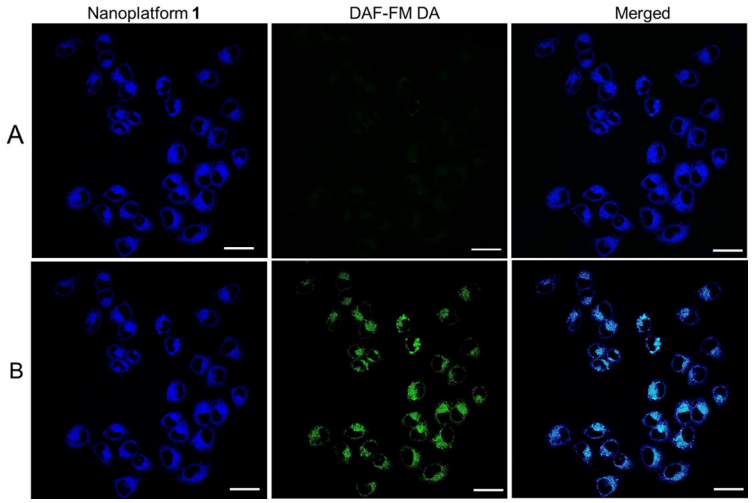

Figure S7 Confocal microscope images of HeLa cells treated with nanoplatform **1** (50 μg/mL) and DAF-FM DA (5.0 μM) before (A) and after (B) 808 nm NIR light irradiation (2.0 min). The blue and green images were obtained for excitation at 405 nm and 488 nm, and recording the corresponding fluorescence in the range of 425–475 nm, and 500–550 nm, respectively. Scale bar: 30 μm.

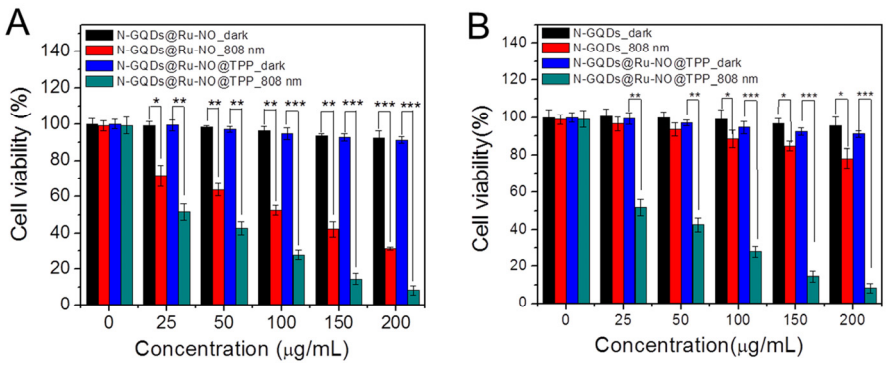

Figure S8 (A) Dark and photo-induced lethality of HeLa cells treated with nanoplatform **1** and {N-GQDs@Ru-NO} with concentrations ranging from 0 to 200 μg/mL for incubation of 4 h, respectively. (B) Dark and photo-induced lethality of HeLa cells treated with nanoplatform **1** and N-GQDs with concentrations ranging from 0 to 200 μg/mL for incubation of 4 h, respectively. Light source: 808 nm NIR light, 600 mW/cm^2; irradiation time: 10 min.

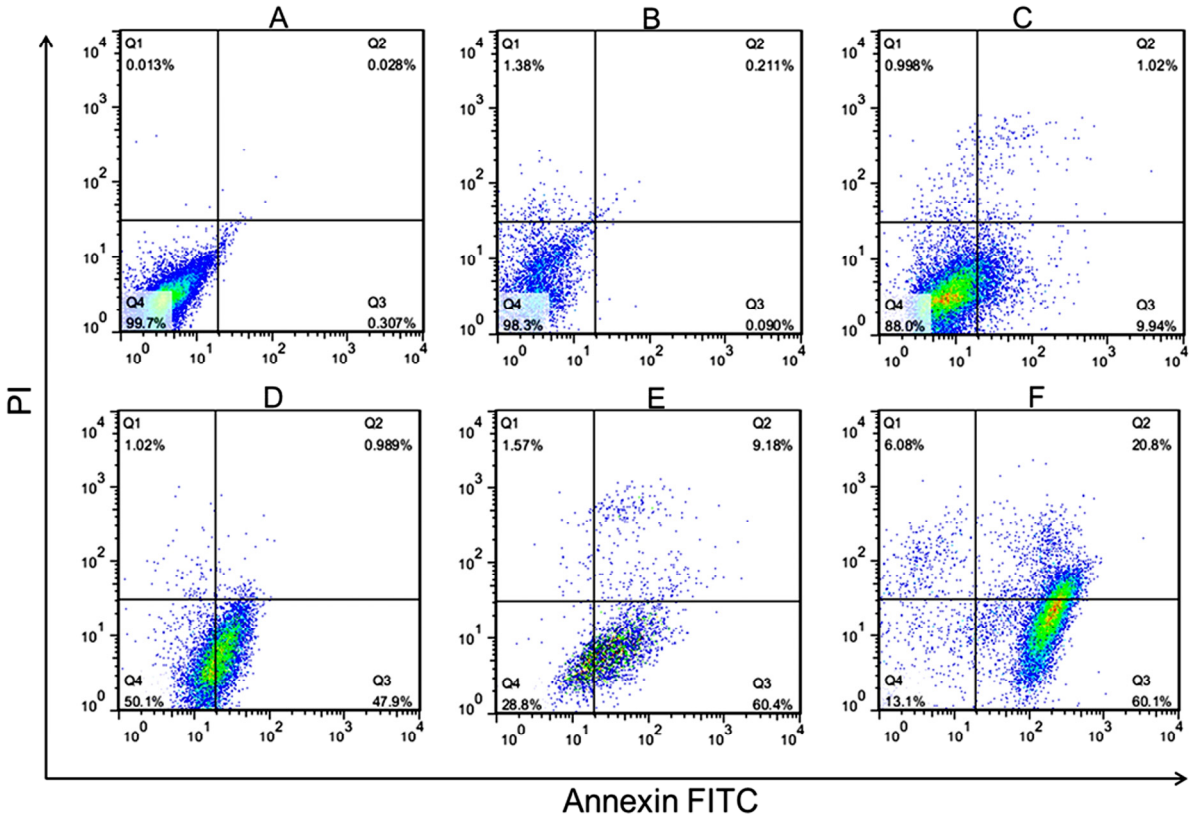

Figure S9 Flow cytometric analysis for early and late apoptotic cells. Control HeLa cells (A) in dark and (B) in the presence of 808 nm NIR laser irradiation (600 mW/cm^2, 10 min). (C) HeLa cells were treated with 200 μg/mL of nanoplatform **1** and incubated for 4 h without NIR light irradiation. From (D-F): HeLa cells were treated with nanoplatform **1** in concentration of (D) 50, (E) 100, and (F) 150 μg/mL, incubated for 4 h, irradiated by an NIR laser (808 nm, 600 mW/cm^2, 10 min), and then followed by incubation of 1 h, respectively.

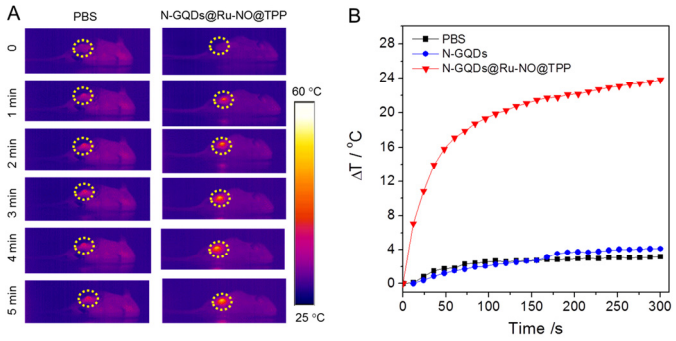

Figure S10 (A) IR thermal images of mice tumor sites intratumorally injected with PBS and nanoplatfrom **1** at 0, 1, 2, 3, 4, and 5 min, respectively, with irradiation of 808 nm laser (1.0 W/cm^2). (B) Temperature changes at the mice tumor sites as a function of irradiation time.

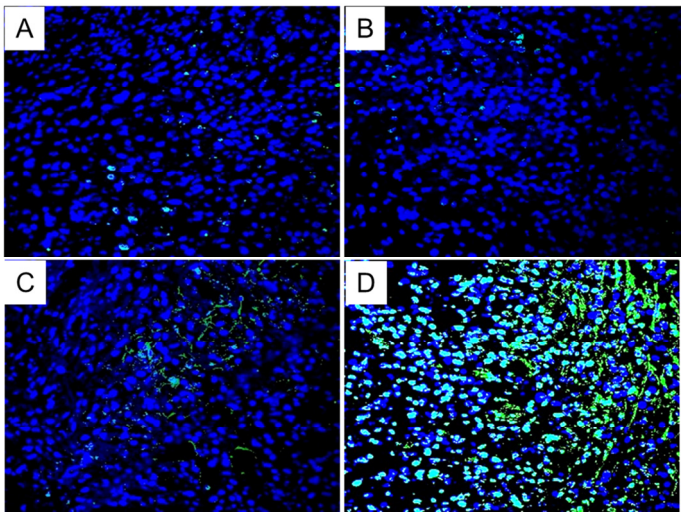

Figure S11 Apoptosis results of tumor tissue dissected from the HeLa tumor-bearing mice with different treatment groups immediately after phototherapy based on TUNEL assay. Blue: DAPI-stained nucleus; green: FITC-labeled apoptosis cells. (A) treated with PBS solution and irradiated by 808 nm laser, 1.0 W/cm^2, 10 min; (B) treated with nanoplatform **1**, without light irradiation; (C) treated with N-GQDs and irradiated by 808 nm laser, 1.0 W/cm^2, 10 min; (D) treated with nanoplatform **1** and irradiated by 808 nm laser, 1.0 W/cm^2, 10 min.

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