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Supplementary data

Multifunctional nanocarriers based on graphitic-C_3N_4 quantum dots for tumor-targeted, traceable and pH-responsive drug delivery

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1. Experimental details

1.1 Chemicals and materials

Urea, concentrated nitric acid, and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co. (China). RGD, 2-(N-morpholino) ethanesulfonic acid (MES), glutaraldehyde (50% in water), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 4`,6-diamidino-2-phenylindole (DAPI) and diamine-terminated oligomeric poly (ethylene glycol) were purchased from Sigma-Aldrich (USA). DOX, dialysis bags (MWCO 3500Da), penicillin-streptomycin, 4% paraformaldehyde, and Roswell Park Memorial Institute medium (RPMI) were purchased from Solarbio Science & Technology Co. (China). Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher Scientific Corporation Co (China). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang biotechnology Co. (China). Trypsin-EDTA solution (0.25%) was obtained from Life Technologies (USA). MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was purchased from Nanjing Infogate biochemical technology Co. (China). BCA Assay Kit was purchased from Enjing biotechnology (China). All chemicals were used as received without any further purification.

1.2 Preparation of g-CNQDs-PEG-RGD

The g-CNQDs were synthesized as previously described[1]. First, bulk g-C_3N_4 was synthesized by a standard pyrolysis method, using urea as the precursor, which was heated at 500 ℃ for 3 h with a ramp rate of 5℃ min^{-1} in a crucible. Then, bulk g-C_3N_4 powder (1g) was refluxed in HNO_3 (6 M) for 24 h. This solution was heated at 120℃ to evaporate water and HNO_3, and a white solid was collected. The resulting powder was subsequently washed with deionized water for several times until a neutral solution was obtained. The white product was dried at 40℃ and the sample was marked as g-C_3N_4-HNO_3. Finally, g-C_3N_4-HNO_3 (100 mg) was dispersed in deionized water (30 mL) and ultrasonicated for 30 min. The as-obtained aqueous suspension was transferred into a poly (tetrafluoroethylene) (Teflon)-lined autoclave (50 mL) and heated at 180℃ for 12 h. After cooling to room

temperature, the solution was filtered with a microporous membrane (0.22 μm) and centrifuged at about 5000 rpm to yield a clear g-CNQDs solution. PEG was covalently conjugated to carboxyl-functionalized g-CNQDs through its -NH_2 group by using the cross-linking reagent EDC and NHS. In a typical procedure, g-CNQDs (50 mg) was dispersed into MES buffer (pH 6.0, 20 mM) with sonication for 5 min and then activated by using EDC (2 mg mL^{-1}) and NHS (2 mg mL^{-1}) for 30 min. Seventy-one microliters of PBS buffer (100 mM, pH 7.4) was then added to the mixture, followed by the addition of 15 mg PEG at room temperature with continuous stirring for 24 h. The resulting PEG-modified g-CNQDs (g-CNQDs-PEG) were dialyzed to ensure all free unreacted PEG, EDC, and NHS were removed from g-CNQDs-PEG. The target peptide RGD was covalently bound onto g-CNQDs-PEG by using the cross-linking reagent glutaraldehyde. Briefly, 20 mg of g-CNQDs-PEG was dispersed in 5 mL PBS buffer and 5 mL of 2.5% glutaraldehyde solution was added and stirred for 12 h. Subsequently, excess glutaraldehyde was removed from the precursor by dialysis. The glutaraldehyde-modified g-CNQDs-PEG was dispersed into 4 mL water with sonication and 1 mL RGD (5 mg mL^{-1}) in PBS was added. The components were allowed to react at room temperature for 24 h. The resultant g-CNQDs-PEG-RGD complexes were dialyzed with water and lyophilized for further use.

1.3 Characterization

The morphology of g-CNQDs and g-CNQDs-PEG-RGD was characterized by high-resolution transmission electron microscopy (HRTEM, TecnaiG2 F20, USA). FT-IR spectra were obtained on an Avatar 370 spectrometer (Thermo Fisher Scientific, USA) in a KBr pellet, scanning from 4000 to 400 cm^{-1} at room temperature. The Z-potential and hydrodynamic sizes of g-CNQDs, g-CNQDs-PEG and g-CNQDs-PEG-RGD were tested on a Malvern Zetasizer NanoZS90 (Malvern, United Kingdom). UV-vis absorption spectra and photoluminescence spectra of the samples were acquired on a U-3900 and an F-4600 spectrophotometer (Hitachi, Japan), respectively.

1.4 DOX loading and release behaviors

DOX was loaded by adding 5 mL of DOX (1 mg mL^{-1}) into 5 mL g-CNQDs-PEG-RGD PBS solution (5 mg mL^{-1}) at different pH values (7.4 and 9.0) and stirred for 24 h, followed by dialysis with PBS buffer to remove the unbound DOX. The DOX loading amount was determined by fluorescence spectroscopy at 590 nm with an excitation wavelength of 490 nm. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated with the following equation, where DOX_t is the total amount of DOX; DOX_f is the free amount of DOX in dialysate.

$$DLC(\%)=\frac{DOX_t\text{-}DOX_f}{\text{mass of drug carrier}}\times 100\%$$

$$DLE(\%)=\frac{DOX_t\text{-}DOX_f}{DOX_t}\times 100\%$$

The g-CNQDs-PEG-RGD-DOX complex (5 mL, 2 mg mL^{-1}) prepared in pH=9.0 was loaded in a dialysis bag (MWCO 3500 Da) and dialyzed in PBS buffer (50 mL) at various pH values (5.0, 6.0, and 7.4) for various times at 37°C. At each time point, 3 mL of the outer dialysate was extracted for analysis and replaced with an equal volume of fresh PBS buffer. The amount of released DOX was analyzed by fluorescence spectroscopy at 590 nm.

1.5 Cell experiment

1.5.1 Cell culture

The HepG2 and HeLa cells were cultured in RPMI and DMEM medium，respectively. Both mediums supplemented with 1% antibiotics (penicillin and streptomycin), and 10% fetal bovine serum. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

1.5.2 Confocal microscopic imaging

HepG2 cells were incubated with free DOX or g-CNQDs-PEG-RGD-DOX (containing 2.5 μg mL^{-1} DOX) at 37℃ for different times. After being washed several times with PBS, the cells were observed with a confocal laser scanning microscope (CLSM, Nikon, Japan). The g-CNQDs-PEG-RGD was excited by a 405 nm laser and signals were collected in the range

of 525±50 nm, and DOX was excited by a 488 nm laser and signals were collected in the range of 595±50 nm. For DAPI staining experiments, cells were immersed in DAPI for 10 min before imaging by CLSM. DAPI was excited by a 405 nm laser and signals were collected in the range of 450±50nm. Z-stack confocal microscopic images of HepG2 cells were taken from the top to the bottom of the cells.

1.5.3 Flow cytometry analysis

HepG2 and HeLa cells were cultured with DOX or g-CNQDs-PEG-RGD-DOX in a 6-well plate for different DOX concentrations. The cells were then washed with PBS three times and harvested. The cells were resuspended in PBS buffer (0.5 mL) for flow cytometric measurement using FACSCalibur (BD, USA).

1.6 MTT assay

HepG2 and HeLa cells were used to study the toxicity of g-CNQDs, g-CNQDs-PEG, g-CNQDs-PEG-RGD, g-CNQDs-PEG-RGD-DOX, and free DOX via the MTT assay. In detail, the cells were seeded into 96-well plates at a density of 5×10^3 cells in each well for 24 h. Subsequently, g-CNQDs, g-CNQDs-PEG, g-CNQDs-PEG-RGD, g-CNQDs-PEG-RGD-DOX and free DOX in PBS with varying concentrations were added into the culture medium and the cells were cultured for an additional 24 h. The wells containing cells grown in culture medium with PBS treatment served as the controls. For the MTT assay, MTT solution (20 μL, 5mg mL^{-1} in PBS) was added to each well and incubated for 4 h at 37℃ to form formazan. After removing the culture medium, DMSO (150 μL) was used to dissolve the formazan, and the optical density (OD) was measured using a microplate reader (Infinite M200 Pro, Tecon, Switzerland) at 490 nm. Cell viability was calculated using the following equation.

$$\text{Cell viability (\%)}=\frac{OD_{sample}}{OD_{control}}\times100\%$$

2. The stability of g-CNQDs and g-CNQDs-PEG

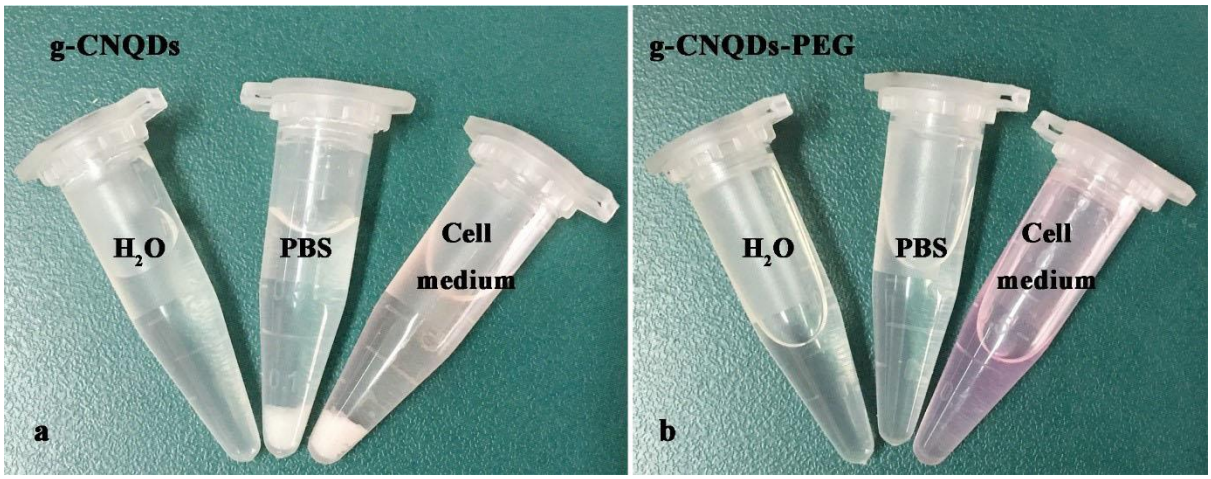

Fig. S1 The stability of (a) g-CNQDs in water, PBS, and cell medium after 48 h; and the stability of (b) g-CNQDs-PEG in water, PBS, and cell medium after 40 days.

3. Zeta potential of the g-CNQDs, g-CNQDs-PEG, and g-CNQDs-PEG-RGD

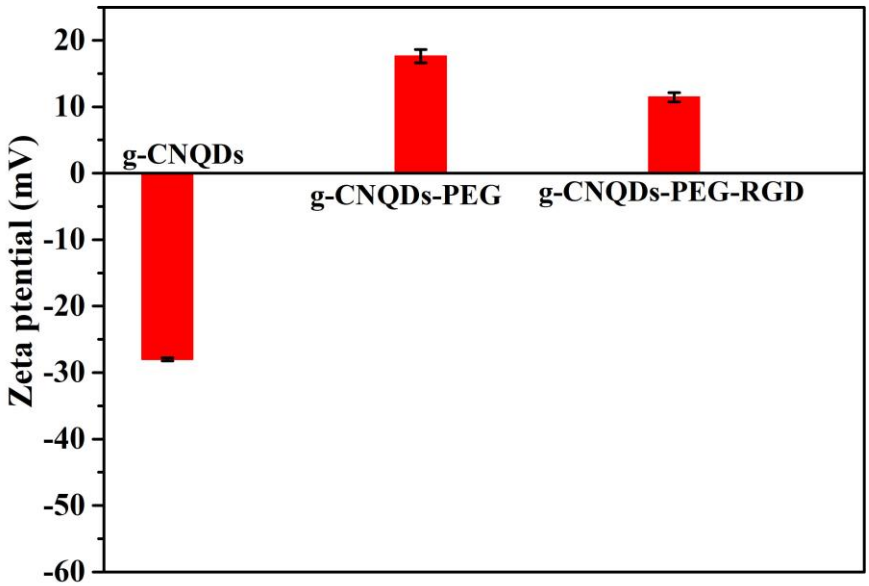

Fig. S2 Zeta potential of the g-CNQDs, g-CNQDs-PEG, and g-CNQDs-PEG-RGD.

4. TG curves of the g-CNQDs and g-CNQDs-PEG

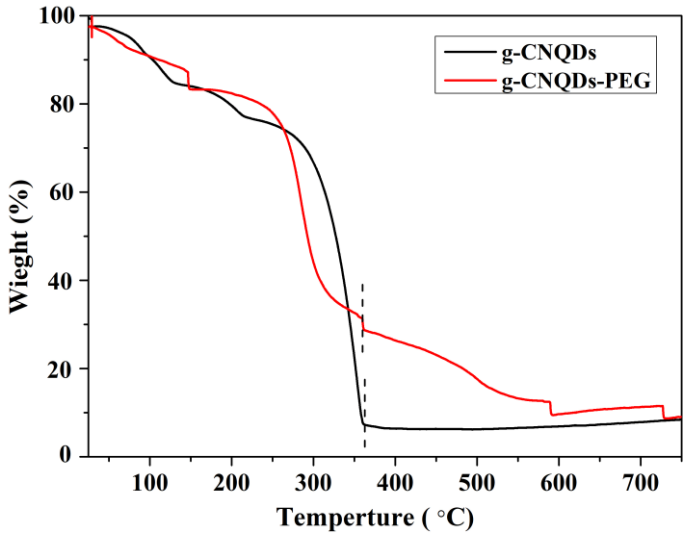

Fig. S3 TG curves of the g-CNQDs and g-CNQDs-PEG.

5. BCA assay

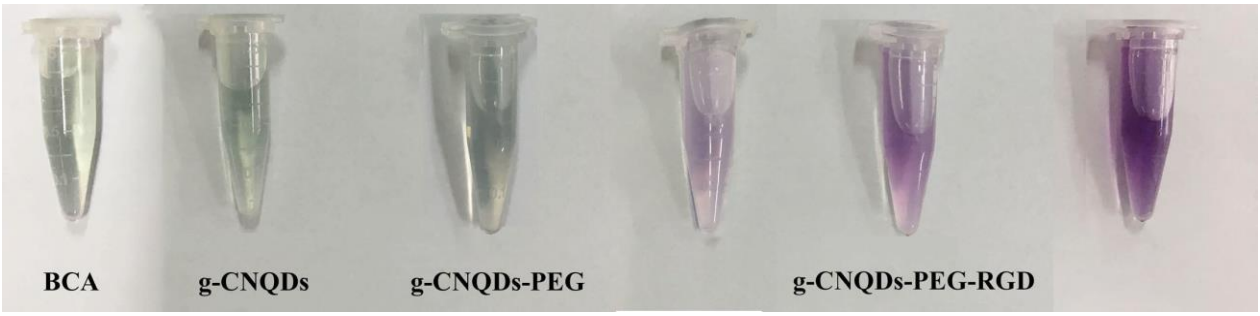

Fig. S4 Photos of BCA solution treated with g-CNQDs, g-CNQDs-PEG, and g-CNQDs-PEG-RGD with different amounts of anchored RGD (increase from left to right).

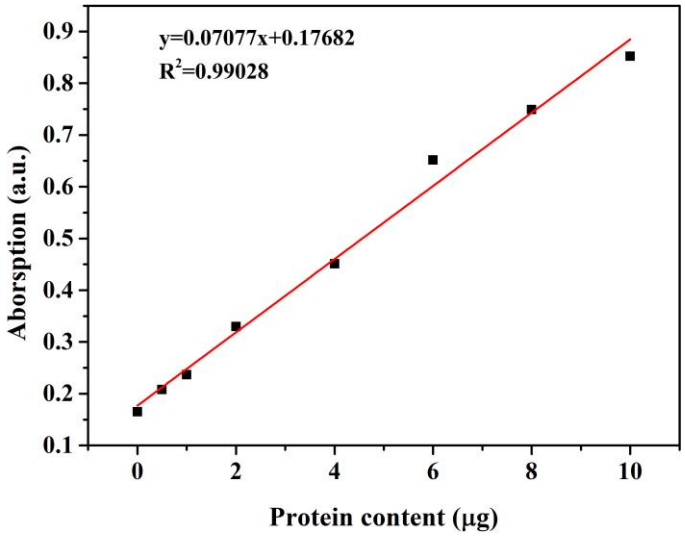

Fig. S5 Standard curve for quantitative protein detection by BCA.

6. Hydrodynamic diameters of g-CNQDs, g-CNQDs-PEG and g-CNQDs-PEG-RGD

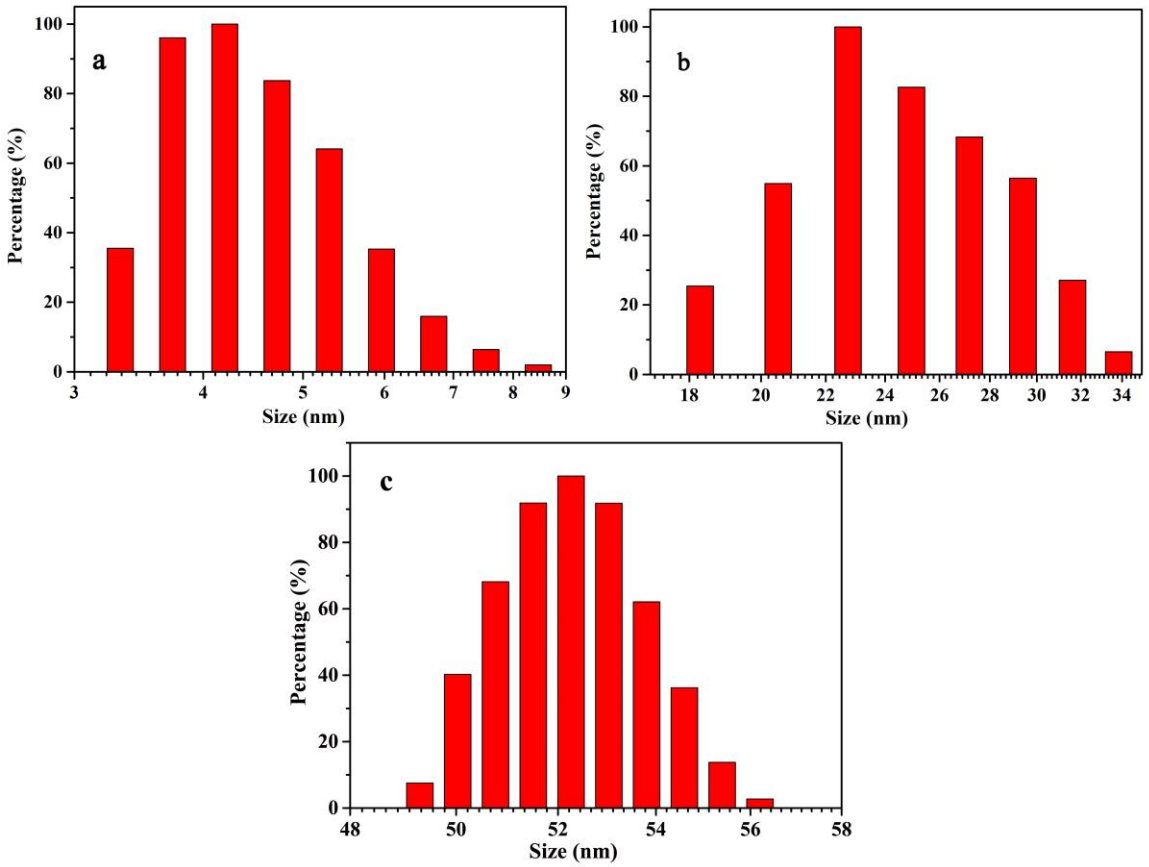

Fig. S6 Hydrodynamic diameters (*D*h) of (a) g-CNQDs, (b) g-CNQDs-PEG, and (c) g-CNQDs-PEG-RGD, as measured by DLS.

7. PL calibration curve of free DOX

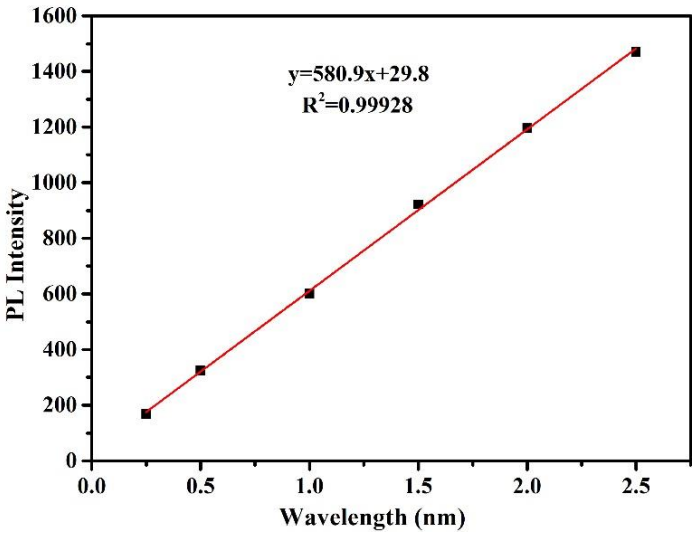

Fig. S7 PL calibration curve of free DOX.

8. DLC and DLE of DOX loaded onto g-CNQDs-PEG-RGD at different pH vaules

Table S1 DLC and DLE of DOX loaded onto g-CNQDs-PEG-RGD at different pH vaules

pH	DLC	DLE
7.4	9.6%	46%
9.0	19.2%	96%

9. PL spectra of g-CNQDs-PEG-RGD in g-CNQDs-PEG-RGD-DOX

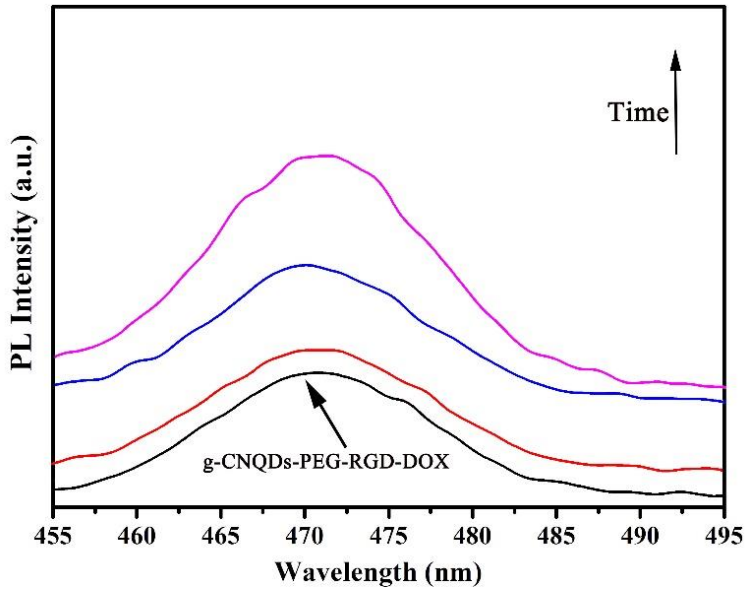

Fig. S8 PL spectra (at 405 nm excitation) of g-CNQDs-PEG-RGD in g-CNQDs-PEG-RGD-DOX with different DOX release times: 0h (black), 2h (red), 8h (blue), and 16h (purple).

10. CLSM images of HepG2 cells treated with g-CNQDs-PEG-RGD-DOX and DAPI

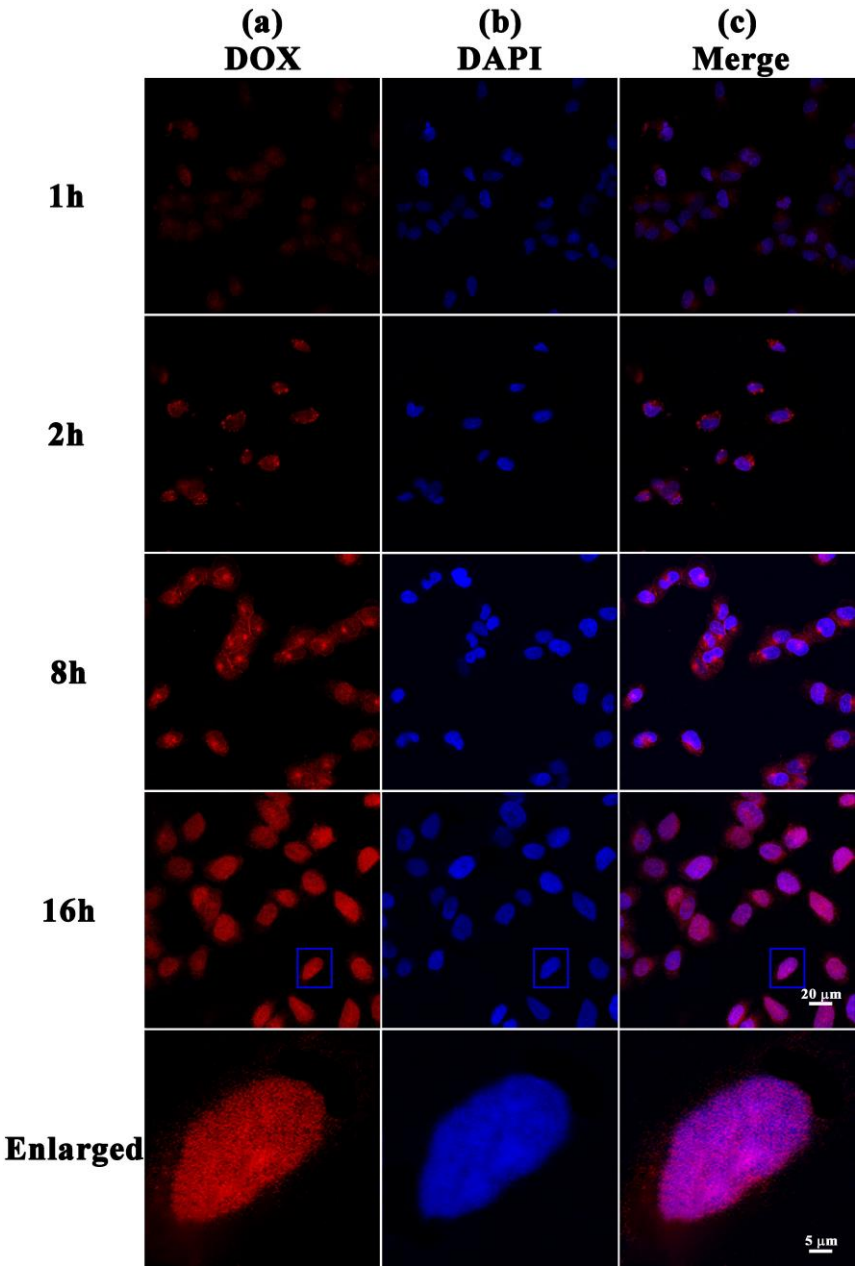

Fig. S9 CLSM images of HepG2 cells treated with g-CNQDs-PEG-RGD-DOX after incubation for 1, 2, 8, and 16 h: (a) DOX, (b) cell nuclei stained with DAPI, and (c) merged images.

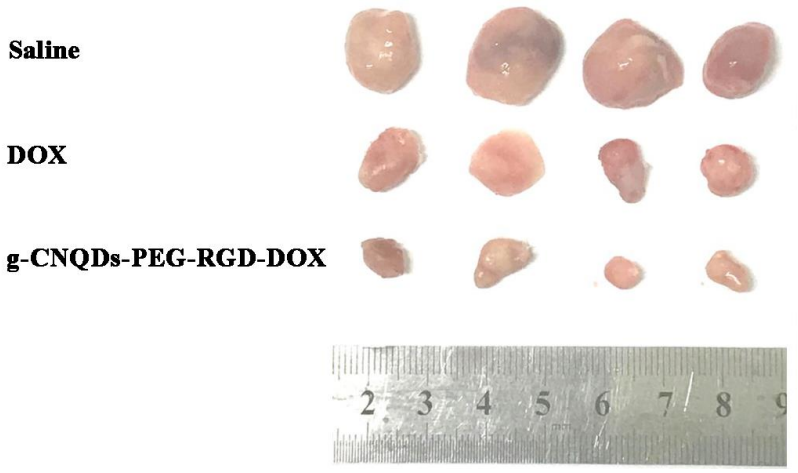

Fig. S10 Photograph of tumors resected from mice after 16-day treatments.

Notes and references

1. J. Dong, Y. L. Zhao, K. Q. Wang, H. Y. Chen, L. Liu, B. L. Sun, M. F. Yang, L. P. Sun, Y. Wang, X. G. Yu, L. F. Dong, *Chemistry Select*, 2018, 3, 1–9.