Supporting Information for:

Sequential Drug Release and Enhanced Photothermal and Photoacoustic Effect of Hybrid Reduced Graphene Oxide-Loaded Ultrasmall Gold Nanorod Vesicles for Cancer Therapy

Jibin Song,[†,‡] Xiangyu Yang,[‡] Orit Jacobson,[‡] Lisen Lin,[‡] Peng Huang,[‡] Gang Niu,[‡] Qingjie Ma,[†] Xiaoyuan Chen[‡]**

[†]China-Japan Union Hospital of Jilin University, Changchun, Jilin, 130033, China

[‡]Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health, Bethesda, Maryland 20892, United States

Experimental Section

Materials and Equipments

Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$) was from Alfa Aesar. 2-hydroxyethyl

disulphide, methoxy-poly(ethylene glycol)-thiol (MPEG-SH) with a molecular weight of 5 kDa, cetyltrimethylammonium bromide (CTAB, 98%), ascorbic acid (99%), sodium borohydride (96%), sodium citrate(99%), silver nitrate (99%), polyvinyl alcohol (PVA, MW 9,000~10,000), and hydrazine hydrate (50-60%) were purchased from Sigma-Aldrich. Radio metal [^{64}Cu] was produced by the PET Department, NIH Clinical Center. Calcein AM and Hoechst 3342 were purchased from Life technology. All solvents unless specified were obtained from Sigma-Aldrich and used as received.

Scanning electron microscopy images were obtained on a Hitachi SU-70 Schottky field emission gun Scanning Electron Microscope (FE-SEM). Transmission Electron Microscopy (TEM) was conducted on a Jeol JEM 2010 electron microscope at an acceleration voltage of 300 kV. UV-vis absorption spectra were recorded by using a Shimadzu UV-2501 spectrophotometer. Gel permeation chromatography (GPC) was measured on a Shimadzu HPLC system using chloroform as the eluent, and the molecular weight was calibrated with polystyrene standards. ^{1}H NMR spectra were obtained on a Bruker AV300 scanner using $CDCl_3$ as the solvent. Fluorescence images of cancer cells were collected using a Photometrics CoolSNAP-cf cooled CCD camera. Thermogravimetric analysis (TGA) was performed on a Perkin-Elmer Diamond TG/DTA. Samples were placed in platinum sample pans and heated under a nitrogen atmosphere at a rate of 10 °C/min to 100 °C and held for 40 min to completely remove residual solvent. Samples were then heated to 700 °C at a rate of 10 °C/min.

Synthesis of PEGylated Reduced Graphene Oxide (rGO-PEG)

To prepare reduced graphene oxide (rGO), GO was first prepared by the modified hammer's method as we previously reported. 10 mL GO aqueous solution at a concentration of 2 mg/mL

was sonicated for 1 h, followed by adding NaOH (0.1 g/mL) to neutralized the solution. The GO was purified by centrifugation for several times and dispersed in deionized water. 1 mL polyethylene glycol-amine (PEG-NH_2) (5 mg/mL) was added into 5 mL GO solution (0.4 mg/mL), and the mixed solution was sonicated for 10 min. An aqueous solution of N-(3-dimethylaminopropyl-N'-ethylcarbodiimide) hydrochloride (EDC) (2 mg/mL) was then slowly added into the solution. The mixed solution was stirred at room temperature for 10 h. PEGylated GO was obtained by centrifugation for three times. To obtain PEGylated rGO (rGO-PEG), 5 mL PEGylated GO (0.5 mg/mL) was further reduced by hydrazine solution (65 wt% in deionized water, 0.015 mL) under stirring and heating at 85 ^{o}C for 1 h. The rGO-PEG was centrifuged and washed with deionized water.

Preparation of DOX laoded rGO-DOX-PEG

1 mL aqueous solution of DOX (2 mg/mL) was slowly added into 1 mL of rGO-PEG (0.5 mg/mL) aqueous solution (pH=9), and the mixture was stirred for 12 h in dark. Free Dox was removed by centrifugation. The loading centet of DOX on the surface of rGO-PEG was caculated by measuring it's UV-vis spectrometry at 485 nm. The obtained DOX loaded rGO-PEG was stored at 4 ^{o}C for further use.

Synthesis of Thiolated PLGA (PLGA-SH)

To synthesize PLGA-SH, 0.067 g of 2-hydroxyethyl disulfide, 3 g of LA and 2.5 g of GA were added into a glass flask with nitrogen for 40 min. Afterwards, 16 μL of tin(II) 2-ethylhexanoate (SnOct, ~95%) was injected and flushed with nitrogen for another 20 min. The polymerization reaction was processed at 125 ^{o}C for 35 h. Then, 15 mL chloroform was added into the flask to

dissolve the as-prepared product. To purify the product (PLGA-S-S-PLGA), the solution was precipitated into cold hexane for five times and dried under vacuum. To further obtain PLGA-SH, 1.0 g of the PLGA-S-S-PLGA was dissolved in 15.0 mL of chloroform and then 150 μL of tributyl phosphine was added as the reduction catalyst to reduce the disulfide bond. After reaction for 30 min, PLGA-SH was obtained by precipitated into cold hexane and dried under vacuum at 40 ^{o}C. The yield is 82% and molecular weight of the PLGA-SH is Mn=72 000 based on GPC result.

Synthesis of Ultrasmall Gold Nanorod

A newt seedless method was employed to synthesize small gold nanorod. Briefly, 5 mL of cetyltrimethylammonium bromide (CTAB) solution (0.2 M) in deionized water was stirred at 30 ^{o}C for 5 min until the solution became clear, followed by adding 5.0 mL gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) (1.0 mM). After stirred for 5 min, 300 μL of $AgNO_3$ (4.0 mM) and 12.0 μL of HCl (37%) were sequentially added into the mixed solution. Afterwards, a solution of ascorbic acid (75 μL, 85.8 mM) was rapidly added to the solution, followed by injected 7.5 μL of $NaBH_4$ (0.01 M). After reaction for 6 h, the CTAB modified small gold nanorod (AuNR@CTAB) was purified by several cycles of centrifugation and washed with deionized water.

Calculation of the Ratio of PEG and PLGA Grafts on the AuNR Surface

^{1}H-NMR measurement (**Figure S2**) shows that the resonance of -CH_2-CH_2-O- (3.65 ppm) of PEG and that of -CO-CH_2-O- group (1.54 ppm) of PLGA has a ratio of 2:1, which leads to a molar ratio of 1:1 for ethylene glycol (EG) and (LGA) monomer. With the molecular weights of

PEG (M_n=5 KDa) and PLGA (M_n=7.2 kDa), the ratio of PEG and PLGA grafts can be calculated using Equation S1, where M_{nLGA} is the molecular weight of LGA monomer. Because of the ratio of LA to GA is 1:1, thus the molecular weight of LGA is: $Mn_{LGA} = 0.5\ Mn_{LA} + Mn_{GA}$. M_{nEG} is the molecular weight of EG monomer. The PEG to PLGA ratio is thus 1:1 (PEG:PLGA).

$$Ratio\ (PEG{:}\,PLGA) = Ratio(EG{:}\,LGA)\left(\frac{Mn_{PLGA}/Mn_{LGA}}{Mn_{PEG}/Mn_{EG}}\right) \qquad (Equation\ S1)$$

Calculation of PEG/PLGA Graft Density from TGA Data.

Given the size of a gold atom (0.0125 nm^3), the number of gold atom ($N_{Au\ atom}$) in a gold nanorod (~9 × 2 nm) can be calculated using Equation S2, where r is the radius and L is the length of the gold nanorod. There are 11,966 gold atoms per small nanorod and therefore the molar mass ($M_{Au\ nanorod}$) of the gold nanorods is 197 $N_{Au\ atom.}$ Combining the molar mass of the gold nanorod, the ratio of PEG and PLGA and the weight fraction obtained in TGA analysis, the average number of polymer grafts can be calculated by Equation S3, where W_{polymer} is the weight fraction (31.5%) of the organic part, $W_{\text{Au nanorod}}$ is the weight fraction of gold nanorod and $M_{PEG+PLGA}$ is the sum of the molar mass of 1 PEG and 1 PLGA grafts. Therefore there are 24 grafts per small gold nanorod, which include 12 PEG chains and 12 PLGA chains, and the graft density is ~0.385 chains/nm^2.

$$N_{Au\ atom} = \frac{V_{Au\ nanorod}}{V_{Au\ atom}} = \left(\frac{\pi r^2 L}{V_{Au\ atom}}\right) \qquad (Equation\ S2)$$

$$N_{grafts\ per\ nanorod} = \left(\frac{2W_{polymer}/Mn_{PEG+PLGA}}{W_{Au\ nanorod}/M_{Au\ nanorod}}\right) \qquad (Equation\ S3)$$

Preparation of rGO Loaded Ultrasmall Gold Nanorod Vesicles

rGO loaded plasmonic AuNR@PEG/PLGA vesicle was prepared using double emulsion water-in-oil-in-water ($W_1/O/W_2$) method. Briefly, 0.4 mg rGO-PEG was dissolved into 0.12 mL of D.I. water (internal aqueous phase, W_1) and then added to 1 mL of chloroform (oil phase, O) containing 5 mg AuNR@PEG/PLGA. The primary W_1/O emulsion was prepared by pulsed sonication (100 watts and 22.5 kHz, MISONZX ultrasonic liquid processors, XL-2000 series) for 1 min. In this procedure, an aqueous solution (W_1) of the rGO-PEG was emulsified into the chloroform solution containing amphiphilic AuNR@PEG/PLGA. It was immediately poured into 5 ml of 2% (w/v) PVA aqueous solution (re-emulsification solution, W_2) and further homogenized for 1 min at 6500 rpm to produce a $W_1/O/W_2$ double emulsion. The emulsion was further stirred at room temperature for 12 h to evaporate the oil phase. By removing the chloroform solvent, the microspheres were formed and rGO was loaded into the cavity of the vesicle. After the chloroform was completely evaporated and the solution became clear, the resulting rGO loaded vesicles were purified by centrifugation (3500g, 5 min) for three times to remove the free rGO-PEG. The obtained rGO-AuNRVe was dispersed in D.I. water for further use.

***In Vitro* Cytotoxicity of Hybrid rGO-AuNRVe**

A standard Cell Counting Kit-8 (CCK-8) was utilized to analyze the cytotoxicity of rGO-AuNRVe following a general protocol. Briefly, U87MG cells were seeded in a 96-well plate with the concentration of 5×10^4 cells/well. After incubation at 37 °C for 24 h, rGO-AuNRVe with a final concentration of 0.3, 0.6, 1.2 or 2.4 nM of gold nanorod were incubated with cells for 2, 4, 8, 16 and 24 h, respectively, after which 10 μl of CCK-8 solution was added to each well of the

96-well plate and incubated for another 4 h. The amount of an orange formazan dye, produced by the reduction of WST-8 (active gradient in CCK-8) by dehydrogenases in live cells, is directly proportional to the quantity of live cells in the well. Therefore, by measuring the absorbance of each well at 450 nm using a microplate reader, cell viability could be determined with the calculation of the ratio of absorbance of experimental well to that of the cell in control well. All experiments were triplicated and results were averaged.

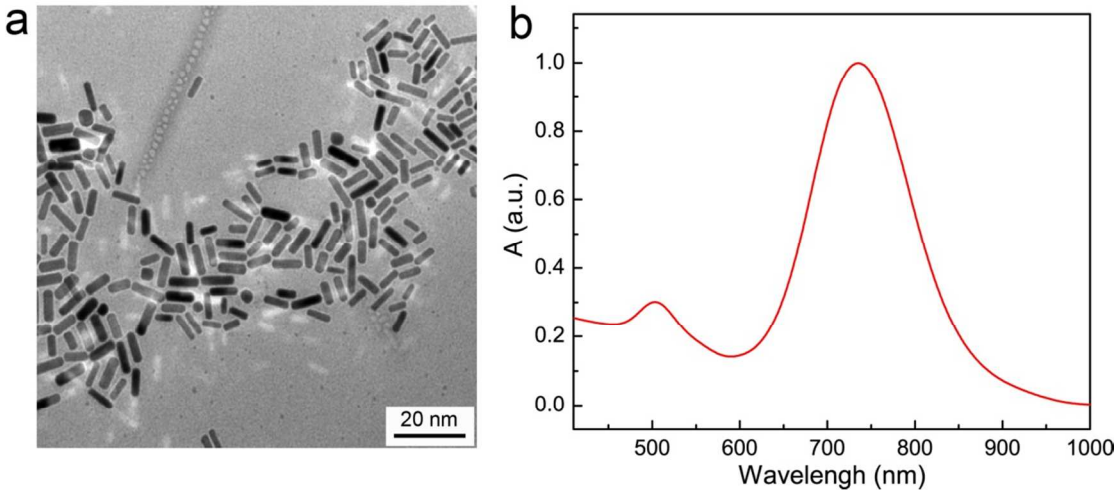

Figure S1. TEM image (A) and UV-vis spectra (b) of small gold nanorod (dimension: ~9 × 2 nm) coated with CTAB.

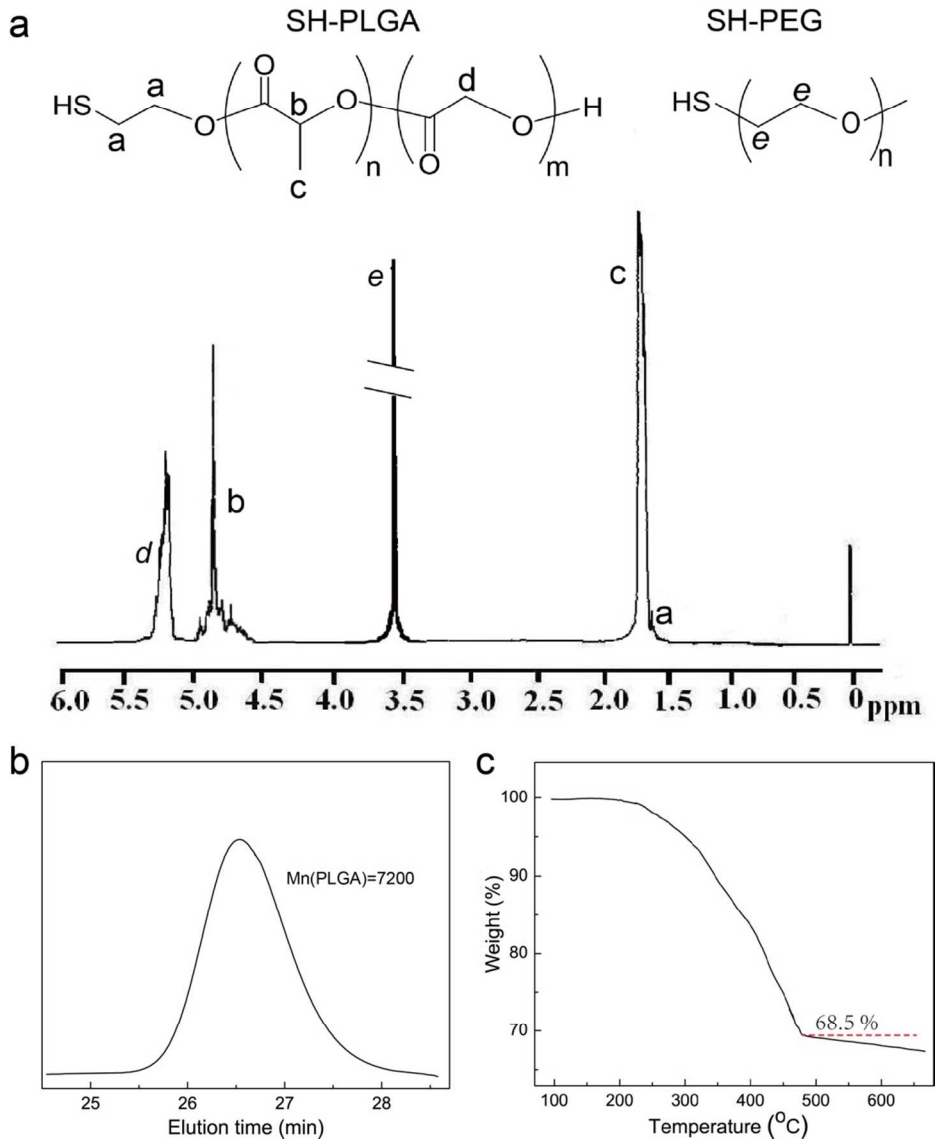

Figure S2. (a) ^{1}H NMR (300 MHz, δ, ppm, $CDCl_3$) of SH-PEG and SH-PLGA: 3.66 (-CH_2-CH_2-O-), 1.58 (SH-CH_2CH_2-), 1.71 (-CH_3), 4.78 (-CO-C*H*-O), 5.26 (-CO-CH_2-O-). The ratio of LA to GA is 1:1 calculated from the ratio of b:d is 1:2. (b) GPC traces of the SH-PLGA (Mn = 7.2 kDa, PDI= 1.27). (c) TGA analysis of the gold nanorods grafted with mixed polymer brushes of poly(ethylene glycol) and copolymer of poly(lactic-*co*-glycolic acid) (the weight fraction of the polymer brushes is 31.5%).

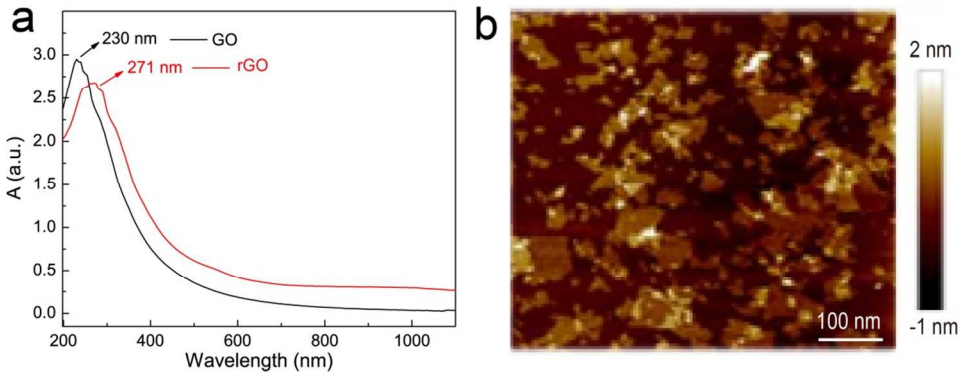

Figure S3. (a) UV-vis spectra of GO and rGO in aqueous solution. (b) AFM images of rGO.

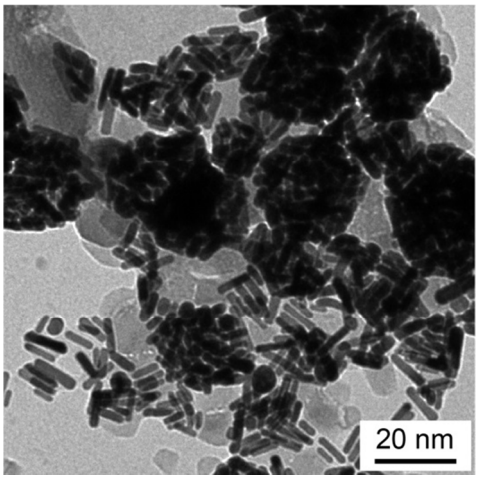

Figure S4 TEM image of the dis-assembed pH-responsive rGO loaded AuNR@PEG/PMMAVP vesicles.

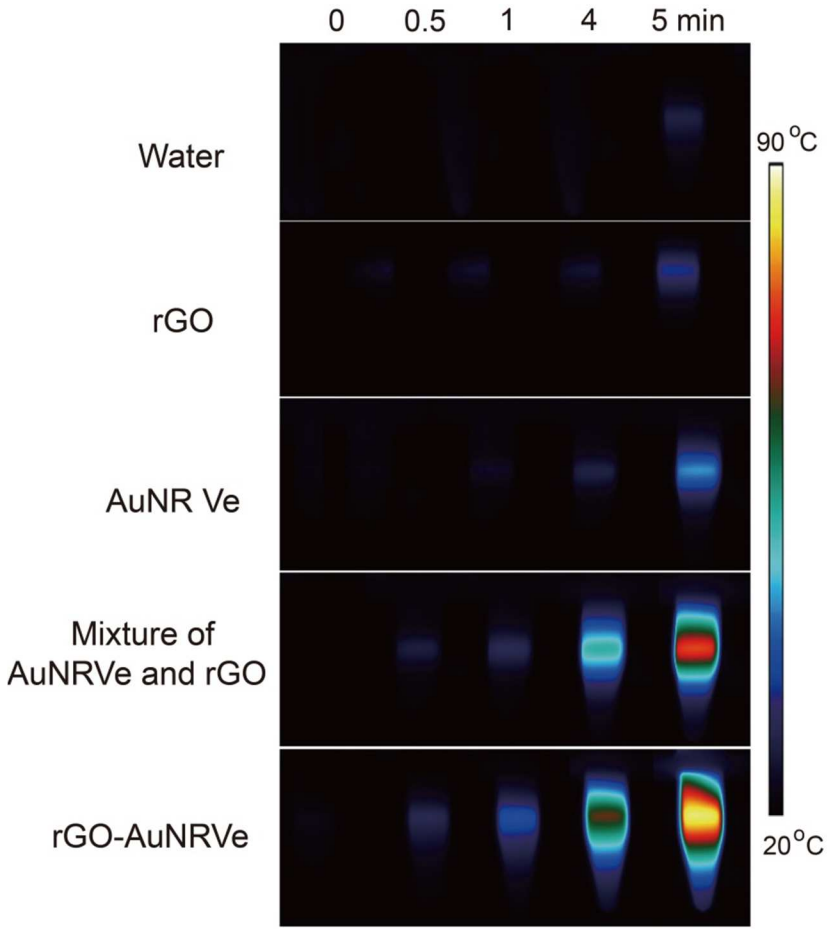

Figure S5. Temperature thermal images of water, rGO, AuNRVe, mixture of rGO and AuNRVe and rGO-AuNRVe irradiated with 808 nm laser at a power density of 0.5 W/cm^2 as a function of time.

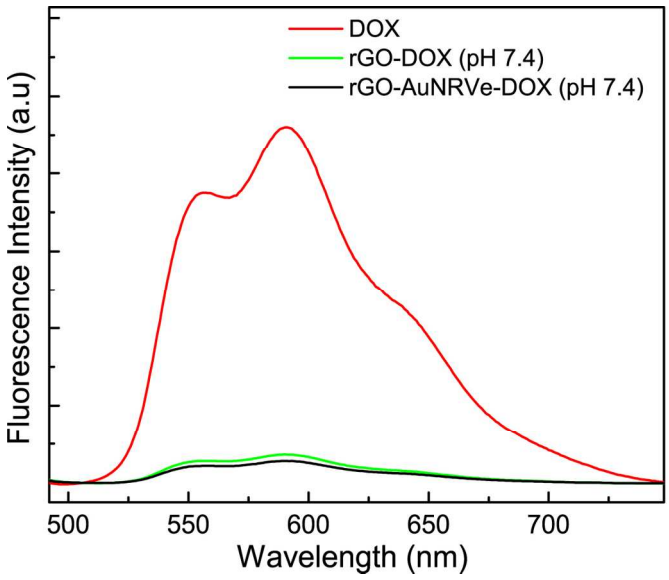

Figure S6. (a) Loading content of rGO-AuNRVe-DOX and AuNRVe-DOX. (b) Fluorescence spectra of free DOX, PEGylated rGO-DOX and rGO-AuNRVe-DOX in pH=7.4 aqueous solution.

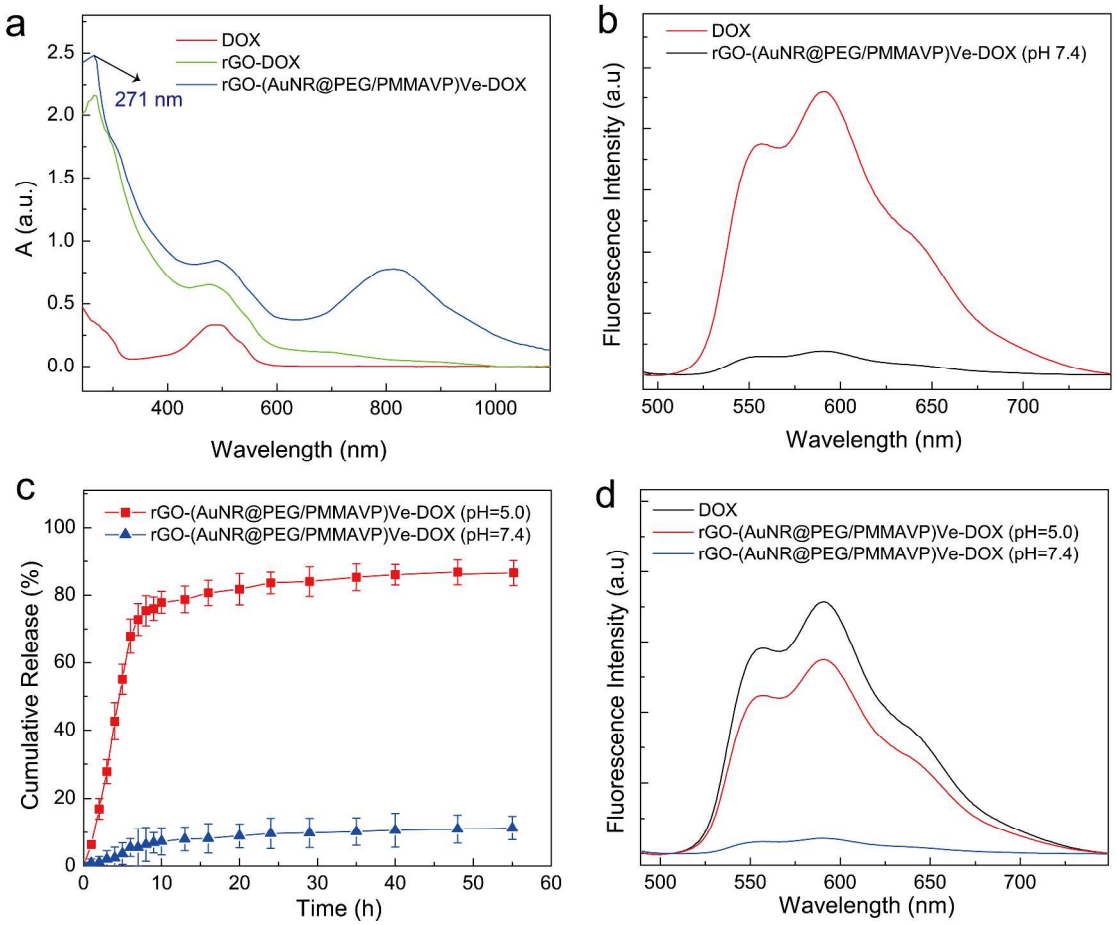

Figure S7. (a) UV-vis spectra of free DOX, PEGylated DOX loaded rGO and DOX loaded small pH-responsive AuNR@PEG/PMMAVP vesicle. (b) Loading content of rGO-(AuNR@PEG/PMMAVP)Ve-DOX and (AuNR@PEG/PMMAVP)Ve-DOX. (c) Stimuli-responsive DOX release profiles of rGO-(AuNR@PEG/PMMAVP)Ve-DOX in pH=5.0 or pH=7.4 aqueous solution. (d) Fluorescence spectra of free DOX and rGO-(AuNR@PEG/PMMAVP)Ve-DOX in pH=5.0 or pH=7.4 aqueous solution.

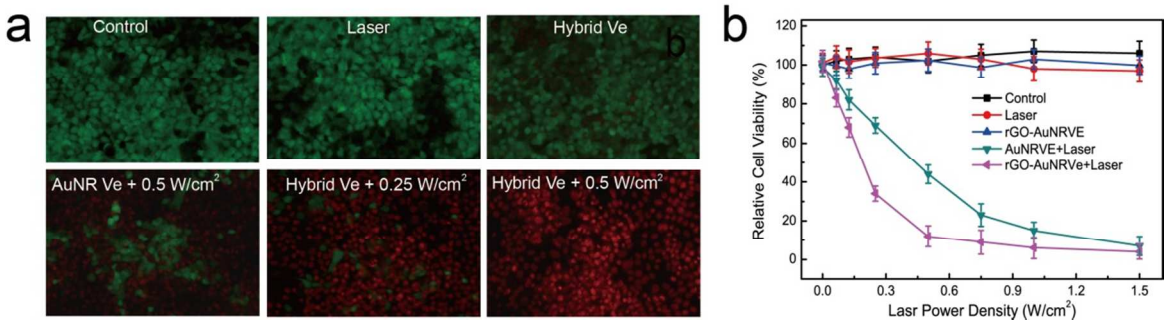

Figure S8. Fluorescence images (a) and cell viability (b) of U87MG cancer cells treated with rGO-AuNRVe or AuNRVe and irradiated with NIR laser (0.25 W/cm^2 or 0.5 W/cm^2) for 3 min. The live and dead cells were stained with calcein-AM (green color: live cell) and propidium iodide (PI) (red color: dead cell) after a total of 24 h incubation.

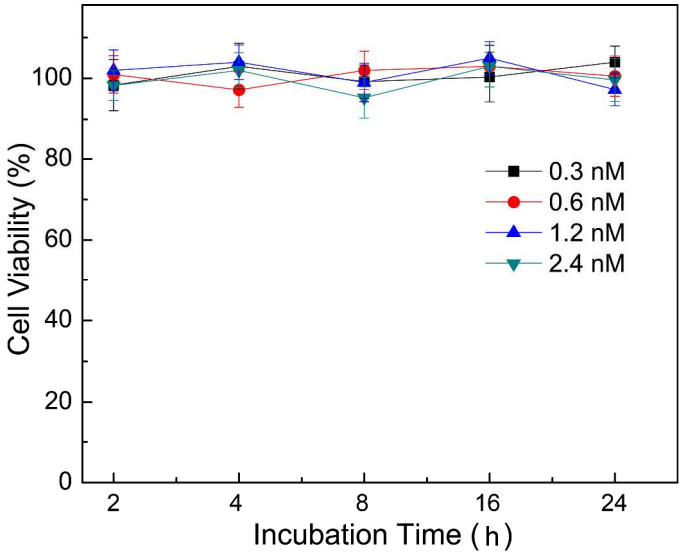

Figure S9. *In vitro* cytotoxicity of the hybrid rGO-AuNR vesicles at different concentrations after an incubation of 2 h, 4 h, 8 h, 16 h and 24 h.

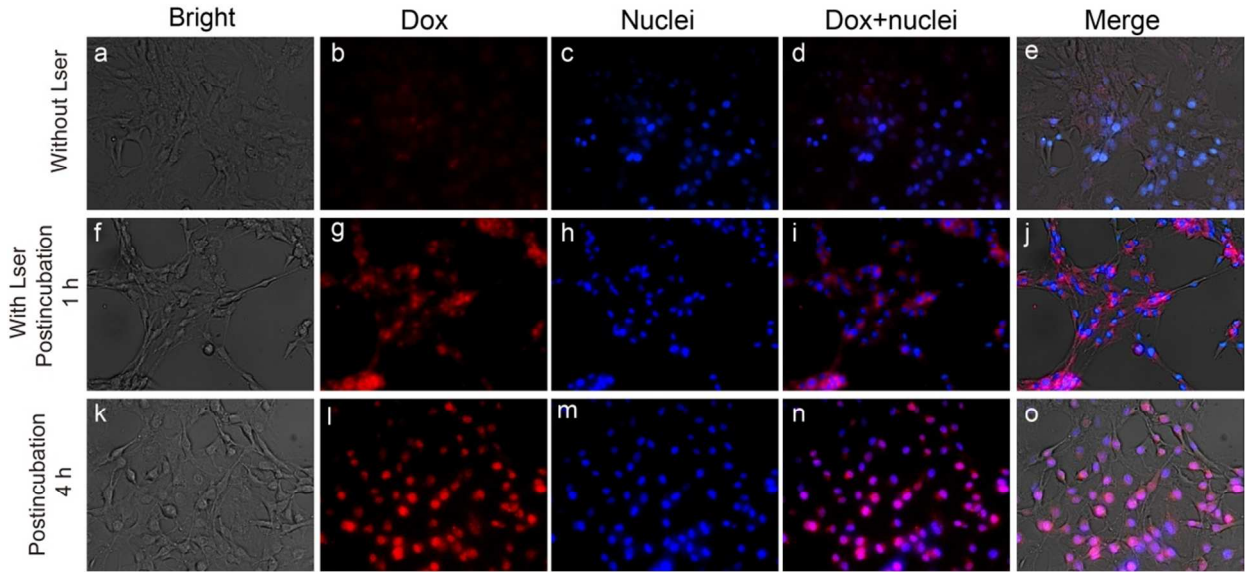

Figure S10. Bright-field, fluorescence and the overlaid images of U87MG cells labeled with DOX loaded small AuNR vesicle after 3 h incubation (a-e), postincubation for 1 h (f-j) and 4 h (k-o) after laser irradiation at 0.25 W/cm^2 for 3 min. DOX has a red fluorescence and cell nuclei were counter-stained with Hoechst 3342 exhibiting blue fluorescence.

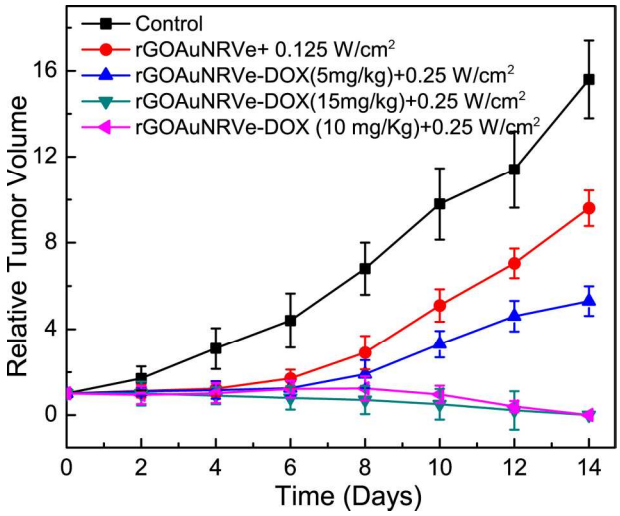

Figure S11. Relative tumor volumes of the tumor-bearing mice after intravenous injection of the samples and exposure to 808 nm laser at different power densities. Tumor volumes were normalized to their initial sizes.

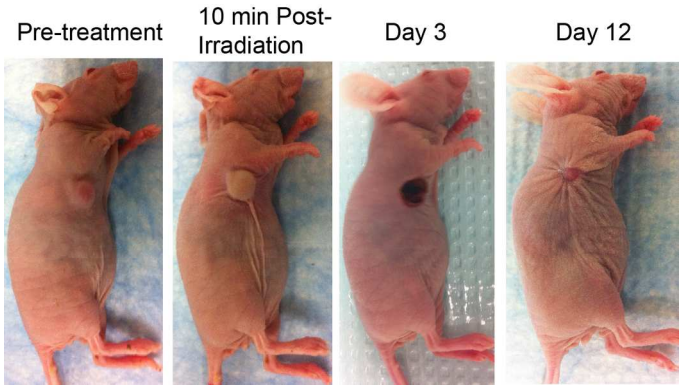

Figure S12. Photographs of U87MG tumor-bearing mice at different times after intravenous injection of the rGO-AuNRVe and exposed to 808 nm laser at a power density of 0.5 W/cm^2.

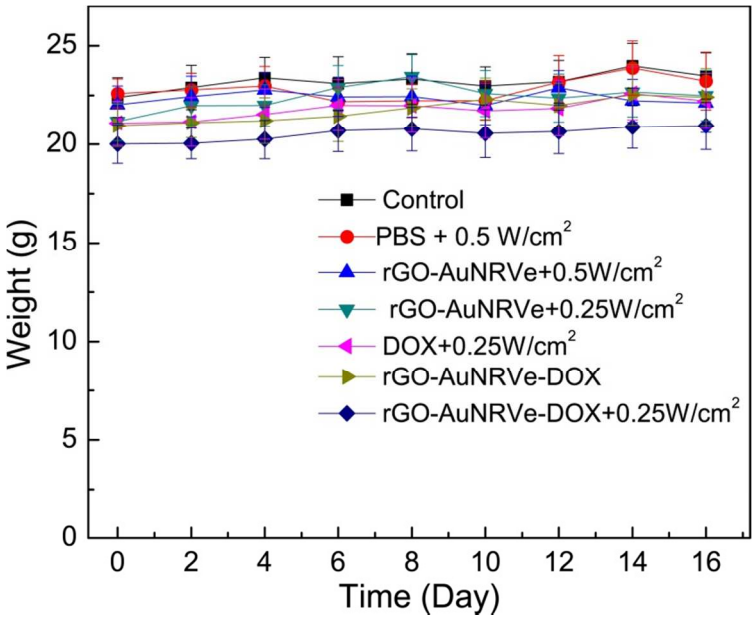

Figure S13. The body weight changes of U87MG tumor bearing mice as a function of time after different treatments.

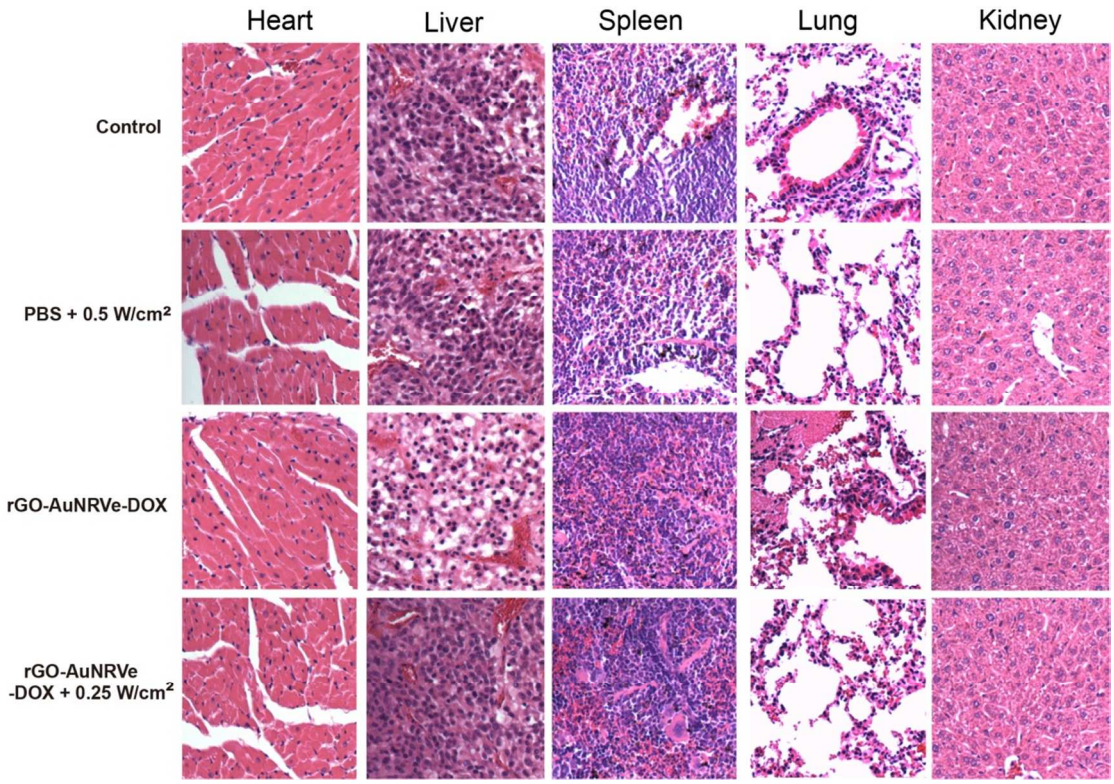

Figure S14. Hematoxylin and eosin staining of different organ sections collected from different groups of mice after laser irradiation of 9 day.