Electronic Supplementary Material (ESI) for Nanoscale.

Electronic Supplementary Information

Enhanced Delivery of Theranostic Liposomes through NO-Mediated Tumor Microenvironment Remodeling

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Experimental Section

Materials.1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cholesterol lipids, L-Arginine (L-Arg), oleylamine, oleic acid, 1-octadecene (ODE), poly(acrylic acid) (MW~1800), N,N'-dicyclohexylcarbodiimide (DCC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich. DSPE-PEG-NHS (MW: 2000) lipids was purchased from Xi'an Ruixi. $PbCl_2$, CdO, and sulfur powder were purchased from Alfa. Phosphate-buffered saline (PBS) was purchased from Hyclone. mPEG-amine (MW~5K) was purchased from Laysan Bio. The cyclo (RGDyK) was purchased from China Peptides. Other reagents were of analytical grade. Ultrapure water was used in all experiments.

Synthesis of 5K-PEGylation of PbS/CdS QDs. OPA-modified PbS/CdS QDs (2.5 mg) synthesized as previously reported were dissolved in 1mL pH 8.5 MES buffer[1]. 10 mg of mPEG-amine (MW~5K) was dissolved 100 μL MES, and gradually added to QDs dispersion with stirring. 5 mg of EDC was dissolved in 100 μL MES, and quickly added to the above dispersion with vibrating. The mixture was stirred at room temperature overnight. The PEGylated QDs were purified by 100 kDa filter, and washed three times with 1x PBS to remove excess reactants. The purified product was dissolved in 1x PBS and stored at 4 °C.

Preparation of Arg/QDs@Lip liposomes. The liposomes were prepared by the thin-layer evaporation and R-NHS/NH_2-R cross-linking method[2,3]. DPPC, cholesterol, and DSPE-PEG-NH_2 (MW~2000) were dissolved in chloroform at a molar ratio of 60 : 25 : 15. The chloroform solvents were removed using rotary evaporator until a thin lipid film was formed, which was further dried for 3 h under vacuum at room temperature. Arg and QDs with a weight ratio of 1 : 1 were mixed and dissolved in PBS buffer. Then the aqueous phase mixture and lipid film with a weight ratio of 1 : 4 were mixed for ultrasonic hydration. And the mixture was heated in a water bath at 65 °C and freezing-thawing for 5 cycles using liquid N_2. The formed liposome dispersion was extruded 20 times through a polycarbonate membrane of 200 nm pores to produce small vesicles. The liposomes were dialyzed for 24 h at room temperature in a dialysis bag (MWCO 1000 Da) to remove unencapsulated Arg.

Synthesis of Arg/QDs@Lip-RGD liposomes. Equimolar amounts cRGD with DSPE-PEG-NHS were weighed and dissolved in PBS. Then it was added to liposome solution prepared above and reacted overnight in the dark with shaking for 12 h. The formed modified-liposome dispersion was extruded 20 times through a polycarbonate membrane of 200 nm pores to produce small vesicles. The cRGD-

modified liposomes were dialyzed for 24 h at room temperature in a dialysis bag (MWCO 1000 Da) to remove unreacted cRGD. This solution was stored at 4 °C for further use.

Characterizations. Transmission electron microscopy (TEM) images were obtained on a JEM-1400Plus electron microscope (operating at 120 kV acceleration voltage). Atomic force microscopy (AFM) experiments were performed in air or fluidic mode using a FastScan atomic force microscope (Bruker, USA) at room temperature. Dynamic light scattering and zeta potential data were recorded on a Malvern Nano-ZS ZEN3600 zetasizer. Photoluminescence spectra was measured by a FLS1000 fluorescence spectrometer (Edinburgh Instruments) provided with a xenon lamp exciter and a near infrared photomultiplier detector (PM1700). The fluorescence *in vivo* images of the whole body were captured with an NIR-II small animal imaging system (NIR vana, Princeton Instruments, USA). Wide-field imaging experiment parameters: 1 x magnification objective, 808 nm laser, 980 nm and 1500 nm long-pass filters, laser power: 25 mW cm^{-2}, exposure time: 200 ms; High resolution imaging experiment parameters: 10 x magnification objective, 808 nm laser, 980 nm and 1500 nm long-pass filters, laser power: 25 mW cm^{-2}, exposure time: 200 ms.

Detecting NO in PBS solution. The classical Griess reagent (Beyotime, China) was used to detect NO in PBS solution. Equivalent of Arg both in the above prepared liposomes (Arg/QDs@Lip-RGD) and free state were separately diluted into PBS solution containing H_2O_2 so that the final Arg concentration was 5 mg/mL and H_2O_2 concentration was 5 mM, 10 mM, 20 mM, respectively. Then the solutions were incubated statically protected from light. Centrifuged (3000 rpm) with ultrafiltration tube (MWCO 3000 Da) at different time points (5, 10, 15, 20, 25 h) and take a small amount of solution (50 μL) at the bottom for NO detection. Added room temperature Griess reagent I, 50 μL, Griess reagent II, 50 μL, respectively. The absorbance was measured by the UV Visible spectrophotometer method (A540 nm). The same method was used to detect NO generation in 20 mM H_2O_2 solutions with different pH (7.4, 6.8, 5.6).

Cell viability assay. Cell viability was determined by CCK-8 assay. 4T1 cells were seeded into 96-well plates (8000 cells per well) and cultured with RPMI-1640 for 24 h, then the cells were incubated with different concentrations of QDs@Lip-RGD, Arg/QDs@Lip-RGD ranging from 0 to 5 mg/mL in 0.1 mL of RPMI-1640 for 24 h. Then the wells were washed 3 times with PBS, a CCK-8 working solution was used to replace the culture medium and cultured for another 15 min. Finally, a microplate reader was

used to measure the absorbance at 450 nm of each well. Cell viability was calculated by comparing with the absorbance of the cells in control group and expressed as the percentage histogram.

***In Vitro* Anti-tumor PTT.** 4T1 cells were cultured in a 35 mm^2 culture dish and co-incubating with QDs@Lip-RGD and Arg/QDs@Lip-RGD liposomes (3 mg/mL) for 30 minutes. After being irradiated with 808 nm laser at the power density of 1.5 W/cm^2 for 10 minutes, the live and dead cells were co-stained with calcein-AM (Green, live cells) and propidium iodide (Red, dead cells)for 20 min and finally observed with 20x lens under the inverted fluorescence microscope.

Detecting NO by DAF-FM DA in 4T1 cells. The NO specific probe, DAF-FM DA, was used to characterize the NO release in both tumor cells and tissues. DAF-FM DA can react with NO to form a highly fluorescent benzotriazole (Ex 495 nm, Em 515 nm). The 4T1 cells (50,000 per well) were seeded in the 4-well tissue culture dish covered with coverslip glass. After 24 h culture, the cells were incubated with fresh medium containing DAF-FM DA (5 μM) for 20 min. Then, the cells were incubated with fresh medium containing Arg@Lip-RGD, Arg/QDs@Lip-RGD at concentration of 5 mg/mL for 24 h. After paraformaldehyde fixation and hoechest staining, the fluorescence images reflecting NO release were obtained using fluorescence microscope.

Animal Handing. Female BLAB/c mice aged 6 weeks were obtained from School and Hospital of Stomatology, Wuhan University. After mice were depilated, 5×10^5 4T1 cells were injected into the right back subcutaneously. The tumor size was measured by vernier calipers every two day. When the volume of the tumor was about ~100 mm^3, it represented the tumor model was successfully established. The tumor volume was calculated according to the following formula: $V = 0.5 \times length \times width^2$.

Detecting NO by DAF-FM DA in tumor tissues. To characterize NO release in tumor tissues, the mice with orthotopic 4T1 tumors (~100 mm^3) were intravenously administered with 200 μL of Arg@Lip-RGD, Arg/QDs@Lip-RGD at a Arg concentration of 2.5 mg/mL. After 4 h, DAF-FM DA (100 μL, 5 μM) was intratumorally injected to the mice. After 30 min, the tumor tissues were harvested and embedded in OCT and frozen sliced for observation by scanner (Pannoramic DESK, P-MIDI, P250). PBS and QDs@Lip-RGD were set as control in all tests.

Analyzing the expression of MMP-9. Western blot was used for MMP examination. Twenty-four hours after i.v. injection of the liposomes, the tumors were excised and immerged in RIPA buffer containing PMSF (Servicebio, China) and Phos-STOP Phosphatase Inhibitor Cocktail (Roche, Switzerland) and homogenized at 2000 g for 5 min. The total proteins were obtained through centrifugation and 20 μg proteins were processed for Western blot assay using specific rabbit Actin and anti-MMP-9 antibodies (Servicebio, China).

Collagen I Assay. Twenty-four hours after liposomes injection, the tumor tissues were harvested, fixed in 4% paraformaldehyde solution, and embedded in OCT. Then the tumor was realize antigen retrieval in EDTA antigen retrieval buffer (pH 8.0) for staining with anti-Collagen I rabbit pAb and Cy3. Fluorescent images (emitted at 590 nm) were obtained using scanner (Pannoramic DESK, P-MIDI, P250).

***In vivo* fluorescence imaging in the NIR-IIb window.** When the tumor of mice grew up to ~100 mm^3, 200 μL liposomes with a QDs concentration at 2.5 mg/mL were injected into the tail vein of mice. The fluorescence *in vivo* images of the whole body were taken by NIR-II small animal imaging system (NIR vana, Princeton Instruments, USA). The exposure time was 200 ms; the power density of 808 nm laser was 25 mW/cm^2. Then the high-resolution fluorescence imaging of the tumor site in mice was taken with a high-magnification lens (10 × objective). Exposure time :200 ms; 808 nm laser; 980 nm and 1500 nm long-pass filters; the power density: 25 mW/cm^2. Four hours after liposomes injection, the tumor tissues were taken out and washed in PBS solution. Then, the tumor was frozen sliced in equal thickness and imaged in the NIR-IIb window. Exposure time :200 ms; 808 nm laser; 980 nm and 1500 nm long-pass filters; the power density: 25 mW/cm^2.

***In vivo* Anti-tumor PTT and immunohistochemical study.** When the tumor volume of mice reached around 100 mm^3, the mice were randomly divided into four groups and treated as followed: Arg/QDs@Lip-RGD / QDs@Lip-RGD / QDs@Lip and only PBS injection with the PTT treatment (200 μL liposomes with a QDs concentration at 2.5 mg/mL). After tail vein injection, the mice were irradiated with 808 nm laser at a power density of 1.0 W/cm^2 for 45 s. The temperature of the tumor surface was recorded by NIR thermal imaging camera, and the thermal images was taken at 0 s and 45 s, and maintained the final temperature for 20 min. After different treatments, the weights of the mice and the volume of tumors were recorded carefully every other day. On the 7th day, the control and Arg/QDs@Lip-RGD groups were carried out biochemical analyses of serum including aspartate

aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin, total bilirubin (TB),alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine (CRE) to verify the biosafety of liposomes. On the 14th day, all the mice were sacrificed. The tumor tissues and normal tissues from each animal were fixed in 4% paraformaldehyde solution, and then processed routinely into paraffin section for hematoxylin and eosin (H&E) assay. Tumor tissue sections were also stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

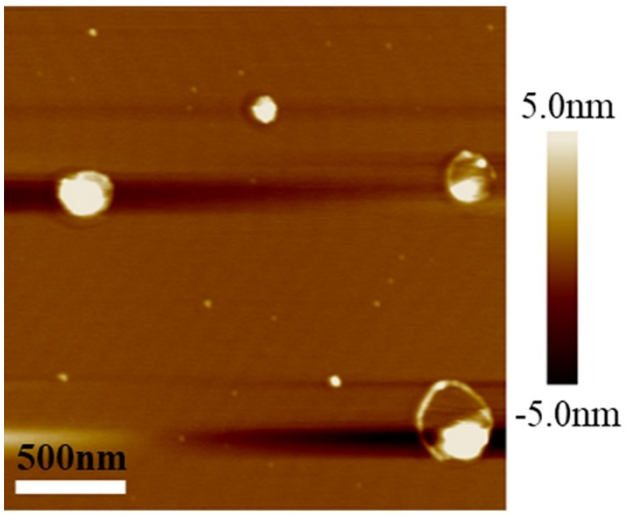

Figure S1. AFM image of Arg/QDs@Lip-RGD.

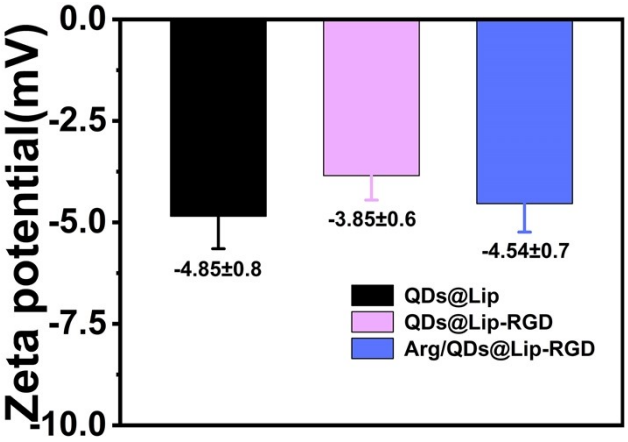

Figure S2. Zeta potential of QDs@Lip, QDs@Lip-RGD and Arg/QDs@Lip-RGD.

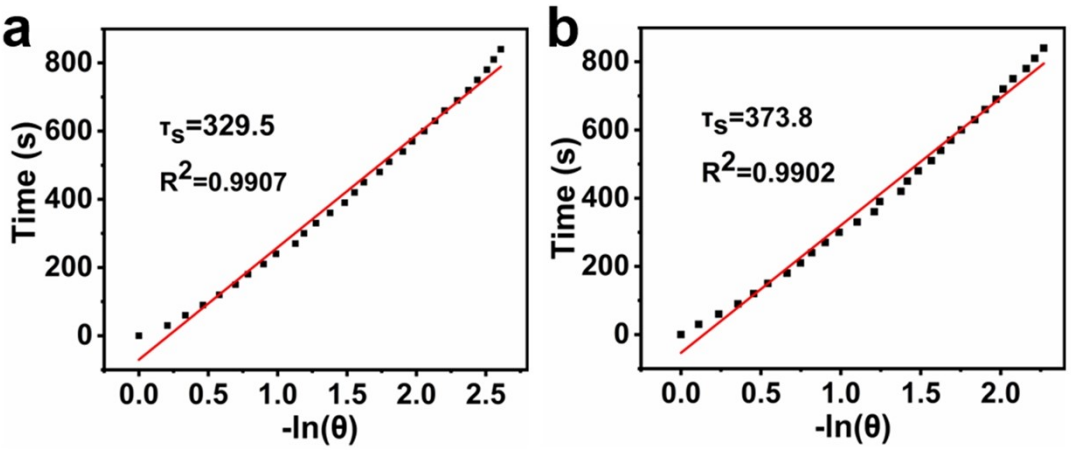

Figure S3. Linear time data versus −ln θ obtained from the cooling period. a) Arg/QDs@Lip-RGD; b) Free QDs. Calculation of photothermal conversion efficiency was referred to the previous report.

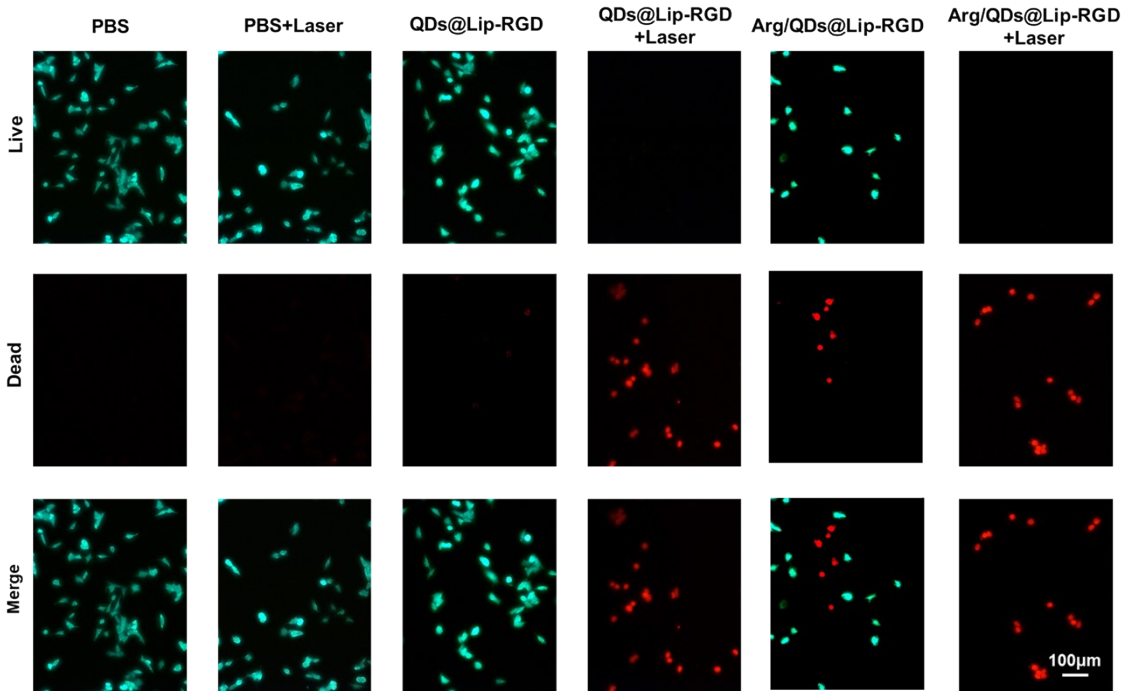

Figure S4. Microscopy images of calcein-AM (Green, live cells) and propidium iodide (Red, dead cells) co-stained 4T1cells after different treatment.

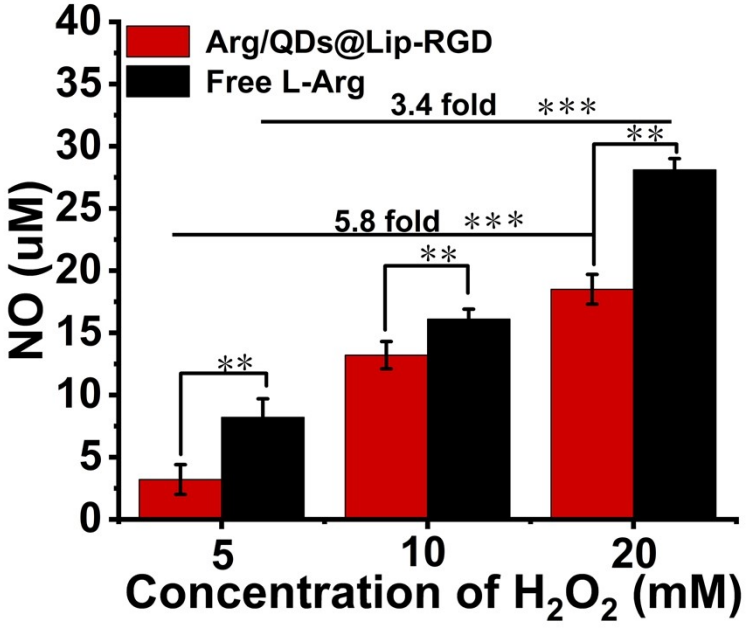

Figure S5. H_2O_2 concentration-dependent NO generation.

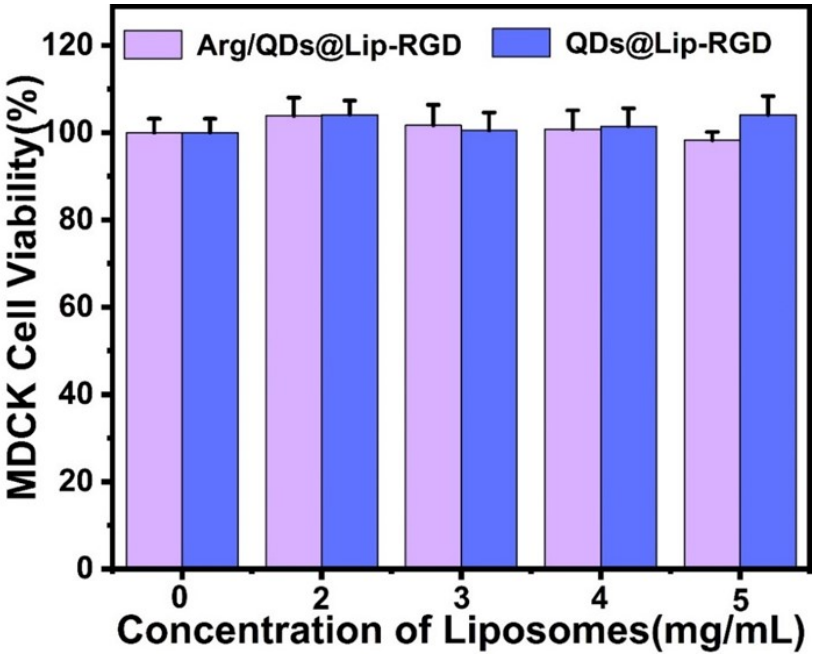

Figure S6. Cell viabilities of DMCK cells incubated with QDs@Lip-RGD and Arg/QDs@Lip-RGD liposomes for 24 hours at various concentrations.

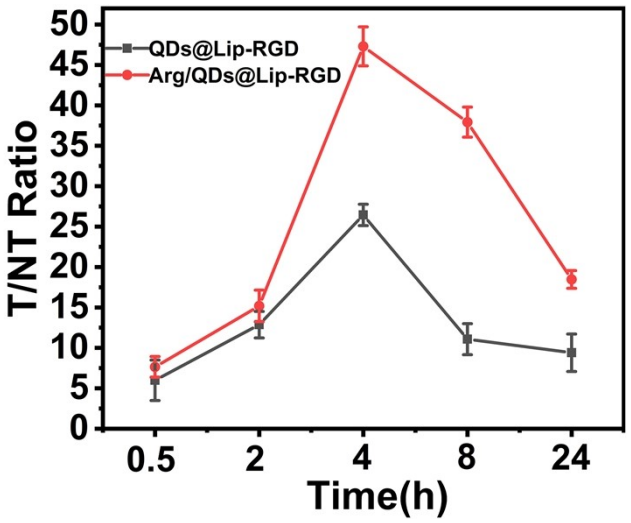

Figure S7. Time course curve of T/NT signal ratios over the course of 24-h p.i.

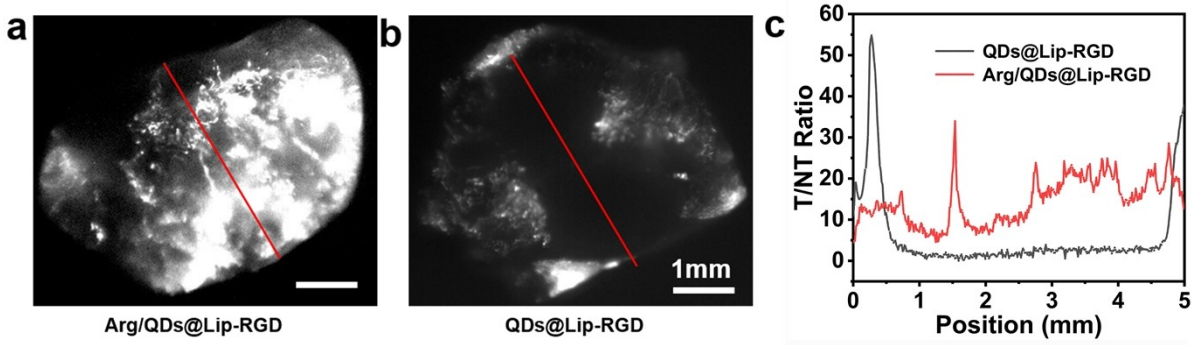

Figure S8. NIR-IIb fluorescence imaging of tumor slices: a) Arg/QDs@Lip-RGD injected group; b) QDs@Lip-RGD injected group; c) Cross-sectional fluorescence intensity profile. Red line: Arg/QDs@Lip-RGD; Black line: QDs@Lip-RGD.

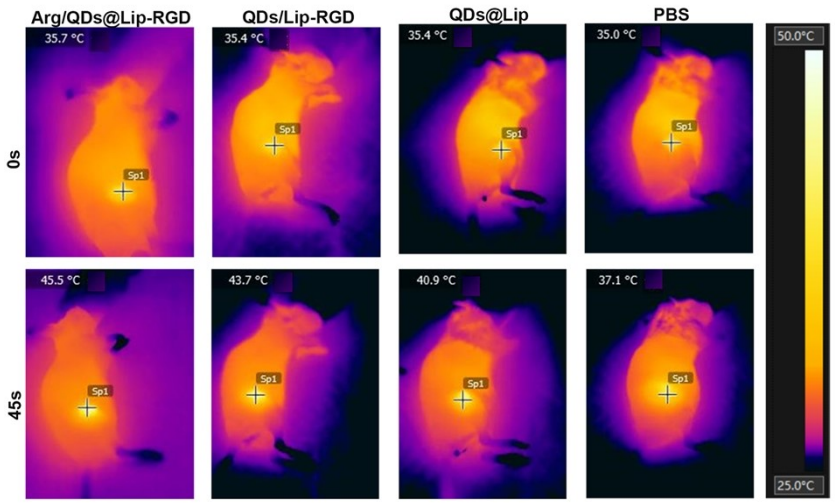

Figure S9. Photothermal images at various time points (0, 45 s) of with different liposomes under the 808 nm laser irradiation.

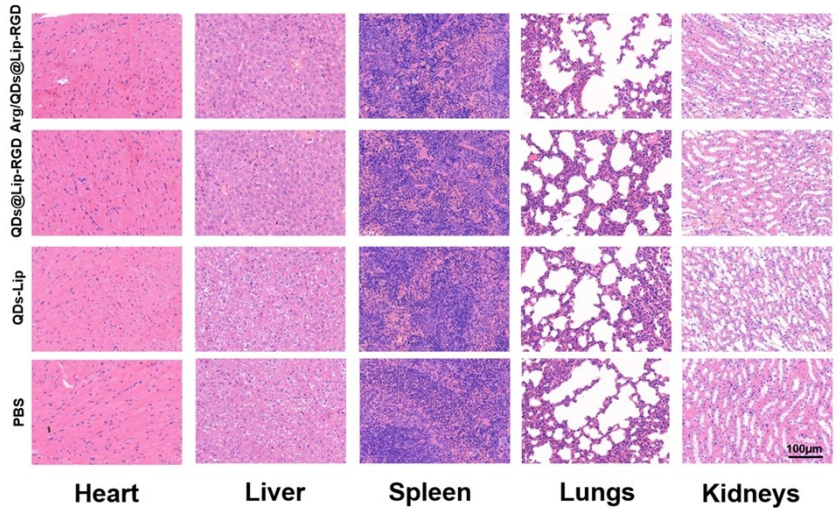

Figure S10. H&E stained images of normal tissues of each group.

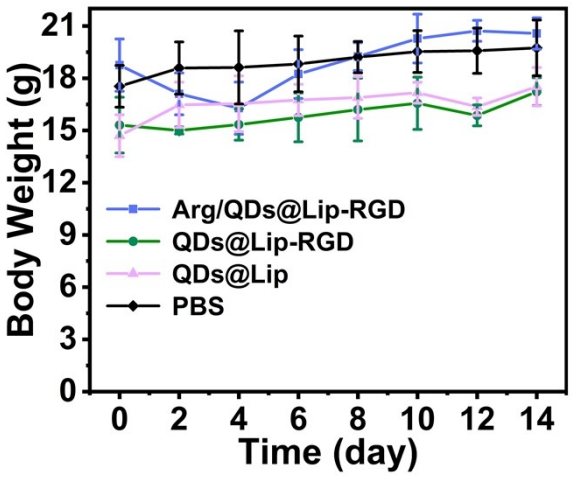

Figure S11. Mouse weight in different groups during 14 days.

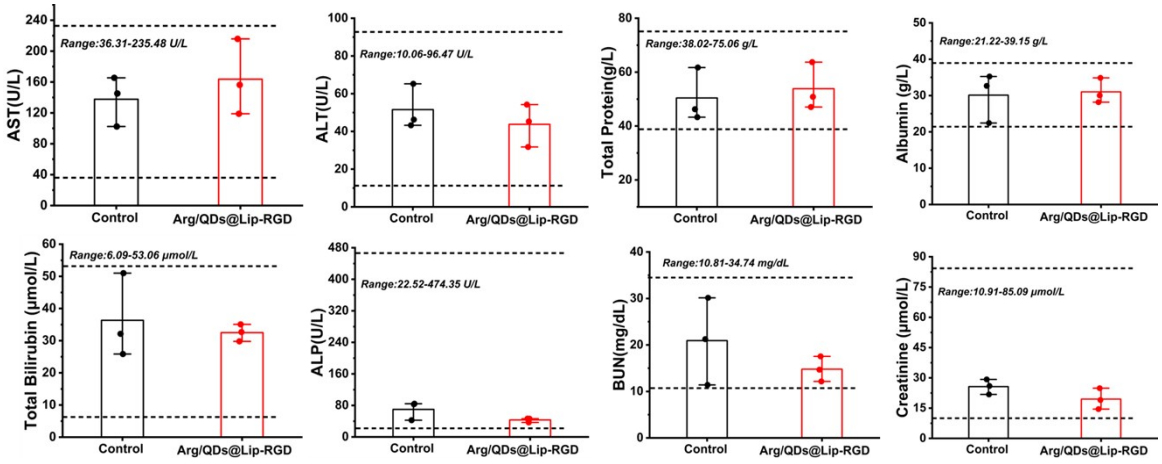

Figure S12. Biochemical analyses of serum including AST, ALT, TP, albumin, TB, ALP, BUN, and CRE.

Table S1. Average tumor volumes in different groups measured at 0- and 14-day.

	Arg/QDs@Lip-RGD	QDs@Lip-RGD	QDs@Lip	PBS
0 day	127 mm^3	130 mm^3	129 mm^3	129 mm^3
14 day	71 mm^3	296 mm^3	473 mm^3	894 mm^3

References

[1]. M. Zhang, J. Yue, R. Cui, Z. Ma, H. Wan, F. Wang, S. Zhu, Y. Zhou, Y. Kuang, Y. Zhong, D. W. Pang and H. Dai, *Proc. Natl. Acad. Sci. U. S. A.,* 2018, **115**, 6590-6595.

[2]. Y. Jia, X. Wang, D. Hu, P. Wang, Q. Liu, X. Zhang, J. Jiang, X. Liu, Z. Sheng, B. Liu and H. Zheng, *ACS Nano,* 2019, **13**, 386−398.

[3]. B. Huang, J. Hu, H. Li, M. Y. Luo, S. Chen, M. Zhang, Z. J. Sun and R. Cui, *ACS Appl. Bio Mater.,* 2020, **3**, 1636−1645.