Electronic Supplementary Information

Targeted dual-mode imaging and phototherapy of tumors using ICG-loaded multifunctional MWCNTs as a versatile platform

Yong Hu,‡[a] Ruizhi Wang,‡[b] Yiwei Zhou,‡[a] Nuo Yu,[c] Zhigang Chen,[c] Dongmei Gao,*[d] Xiangyang Shi*[a] and Mingwu Shen*[a]

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, P. R. China

[b] Department of Radiology, Huadong Hospital, Fudan University, Shanghai 200040, P. R. China

[c] College of Materials Science and Engineering, Donghua University, Shanghai 201620, P. R. China

[d] Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, P. R. China

---

* To whom correspondence should be addressed. E-mail addresses: gdm_325@163.com (D. Gao), xshi@dhu.edu.cn (X. Shi) and mwshen@dhu.edu.cn (M. Shen)

‡ These authors contributed equally to this work.

Part of experimental section

Materials

Multiwalled carbon nanotubes (MWCNTs) (diameter = 30-70 nm, length = 100 nm-2 μm) were synthesized and characterized as described in previous reports.[1, 2] Dual-functional polyethylene glycol (PEG) with one end of the amine group and the other end of the carboxyl group (NH_2-PEG-COOH, Mw = 2000) was purchased from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). Folic acid (FA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC), indocyanine green (ICG), and 1,3-Diphenylisobenzofuran (DPBF) were supplied by J&K Chemical Ltd. (Shanghai, China). polyethyleneimine (PEI) (Mw = 25000), succinic anhydride (SAH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and all the other chemicals and solvents were supplied by Aldrich (St. Louis, MO). All of the chemicals were used without further purification. HeLa cells (a human cervical cell line) were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were acquired from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cell counting kit-8 (CCK-8) was obtained from 7Sea Pharmatech Co., Ltd. (Shanghai, China). Live & dead viability/cytotoxicity assay kit (component A: calcein acetyl methoxy methyl ester (AM); component B: propidium iodide (PI)) was from Keygen Biotech Co., Ltd. (Nanjing, China). Water with a resistivity exceeding 18.2 MΩ.cm consumed in all experiments was passed through a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA). Regenerated cellulose dialysis membranes with a molecular weight cutoff (MWCO) of 1000, 14000 or 50000 were acquired from Fisher (Pittsburgh, PA).

Formation of MWCNT-PEI

The acid-treated MWCNTs (80 mg) were activated by EDC (80 mg) and NHS (48 mg) in DMSO (80 mL) under vigorous magnetic stirring for 3 h to activate the carboxyl groups on the surface of MWCNTs. Then, branched PEI with abundant amines was reacted with MWCNTs. Typically, a PEI solution (80 mg, in 20 mL of DMSO) was dropwise added into the solution of MWCNTs with carboxyl groups activated under magnetic stirring. After 3 days, the reaction mixture was dialyzed against phosphate buffered saline (PBS, 3 times, 2 L) and water (6 times, 2 L) for 3 days using a dialysis membrane (MWCO = 50000), followed by lyophilization to obtain the MWCNT-PEI product.

Characterization techniques

Fourier transform infrared (FTIR) spectra were collected using a Nicolet Nexus 670 FTIR spectrophotometer (Nicolet-Thermo, Waltham, MA). Samples were dried and mixed with ground KBr crystals and pressed as pellets before measurements. Transmission electron microscopy (TEM) images were taken on a JEOL 2010F electron microscope (Tokyo, Japan) at an operation voltage of 200 kV. TEM sample was prepared by depositing an aqueous suspension of MWCNT-PEI.SAH-FITC-PEG-FA (5 μL, 0.1 mg/mL) onto a carbon-coated copper grid and air dried at room temperature. Zeta potential measurements were carried out using a Malvern Zetasizer (Nano Series-Zen 3600, Worcestershire, UK). A minimum of 10 measurements were acquired for each sample. Thermal gravimetric analysis (TGA) was performed using a TG 209 F1 thermal gravimetric analyzer (NETZSCH Instruments Co., Ltd., Selb/Bavaria, Germany). The samples were heated from ambient temperature to 900 ^{o}C under N_2 atmosphere at a rate of 10 ^{o}C/min. UV-vis spectroscopy of the specimens was conducted using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA).

Hemolytic and cytotoxicity assays

Fresh human blood stabilized with heparin was kindly provided by Shanghai Xinhua Hospital (Shanghai, China) with the approval of the Shanghai Xinhua Hospital Ethical Committee. The human red blood cells (HRBCs) were obtained according to the previous reported protocol.[3] After that, 0.1 mL of 10-fold diluted HRBC suspension was gently mixed with 0.9 mL of PBS containing MWCNT-PEI.SAH-FITC-PEG-FA at different concentrations (100, 200, and 400 μg/mL, respectively). The mixtures were maintained still for 2 h and centrifuged (10000 rpm) for 1 min at room temperature. Then, the photographs of the samples were taken while a PerkinElmer Lambda 25 UV-vis spectrophotometer was used to measure the absorbance of the hemoglobin. PBS and water were separately used as negative and positive control, respectively. The hemolysis percentages of different samples were calculated according to a previously reported method.[4]

For cell culture, HeLa cells were routinely cultured and passaged in a 25-cm^2 flask with regular DMEM (without FA) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37 ^oC within a humidified environment of 5% CO_2. Cells cultured in DMEM without FA express high-level FA-receptor (FAR) (denoted as HeLa-HFAR cells),[5, 6] while cells grown in the DMEM containing 2.5-μM free FA for 24 h or longer express low-level FAR (denoted as HeLa-LFAR). Without specific statement, the term of "HeLa cells" is always deemed to be "HeLa-HFAR cells".

CCK-8 cell viability assay was employed to evaluate the *in vitro* cytotoxicity of the MWCNT-PEI.SAH-FITC-PEG-FA under standard manufacturer's instructions. In brief, HeLa cells were seeded into a 96-well plate at a density of 1×10^4 cells per well. After cultivation with 200 μL of DMEM at 37 °C and 5% CO_2 for 12 h, the adherent cells were incubated with 200 μL of fresh medium containing PBS (control) and MWCNT-PEI.SAH-FITC-PEG-FA at varied concentrations (10, 25, 50, 100, and 200 μg/mL, respectively). After an additional 24 h incubation, 20 μL of CCK-8 was added into each well, and the cells were then incubated for another 4 h at 37 ^oC and 5% CO_2.

After that, the absorbance in each well was monitored at 450 nm using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo scientific, Hudson, NH). Cell viability was calculated according to equation (S1):

$$\text{Cell viability} = \frac{A_1 - B}{A_0 - B} \times 100\% \quad \text{(S1)}$$

where A_0 and A_1 represent the absorbance of CCK-8 in DMEM containing HeLa cells after treatment with PBS and multifunctional FA-targeted MWCNTs, respectively. B represents the absorbance of CCK-8 in DMEM. Average value and standard deviation (SD) of 5 parallels for each sample were reported.

Live/dead staining was performed using live & dead viability/cytotoxicity assay kit to further evaluate the cytotoxicity of the MWCNT-PEI.SAH-FITC-PEG-FA. Typically, HeLa cells after treated with the MWCNT-PEI.SAH-FITC-PEG-FA for 24 h were washed 2 times with PBS and immersed in serum-free DMEM containing calcein-AM (2 μM) and PI (8 μM) for 15 min. After replacing the medium with PBS, a Zeiss inverted fluorescence microscope (Axio Vert. A1, Jena, Germany) was used to capture the images of cells.

***In vitro* specific cellular uptake assay**

Flow cytometry was performed to quantify the specific uptake of the MWCNT-PEI.SAH-FITC-PEG-FA by HeLa-HFAR cells. Both HeLa-HFAR and HeLa-LFAR cells were seeded in 12-well cell culture plates at a density of 2×10^5 cells/well. After incubation in 1 mL of DMEM overnight at 37 ^oC and 5% CO_2 to bring the cells to confluence, the medium was carefully aspirated and replenished with 1 mL of fresh medium containing PBS (control) and MWCNT-PEI.SAH-FITC-PEG-FA under different concentrations (10 and 25 μg/mL, respectively). After incubation for an additional 4 h, the cells were successively rinsed with PBS for 5 times, harvested by trypsinization

and centrifugation, and resuspended in 1 mL of PBS. Then, a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was employed to analyze the fluorescence signal intensity of cells. The FL1-fluorescence of 10000 cells was measured and the measurement was repeated for 3 times.

The specific uptake of MWCNT-PEI.SAH-FITC-PEG-FA by HeLa-HFAR cells was further examined by confocal microscopy (Carl Zeiss LSM 700, Jena, Germany). Briefly, cover slips were first treated and fixed in a 12-well plate according to our previous report.[7] HeLa-HFAR cells were then seeded into the culture plate at a density of 2×10^5 cells per well with 1 mL of fresh medium and cultured at 37 °C and 5% CO_2 overnight to leave the cells to attach on the cover slips. Then, the medium was replaced with 1 mL of fresh medium containing PBS (control) or the MWCNT-PEI.SAH-FITC-PEG-FA at the concentration of 10 μg/mL. After incubation at 37 °C and 5% CO_2 for 4 h, the cells were rinsed 3 times with PBS, fixed with glutaraldehyde (2.5%) for 15 min at 4 °C, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for 15 min at 37 °C according to a standard procedure. The images of the cells attached on the cover slips were collected by using a 63× oil-immersion objective lens. Meanwhile, HeLa-LFAR cells were also incubated and analyzed under the same conditions.

Statistical analysis

One-way ANOVA analysis of variance was used to assess the significance of the experimental data. Comparisons of two data sets were performed with an unpaired t-test. All data were represented as mean ± standard derivation (SD). A value of 0.05 was used as the threshold of significance, and the data were marked as (*) for $p < 0.05$, (**) for $p< 0.01$, and (***) for $p < 0.001$, respectively.

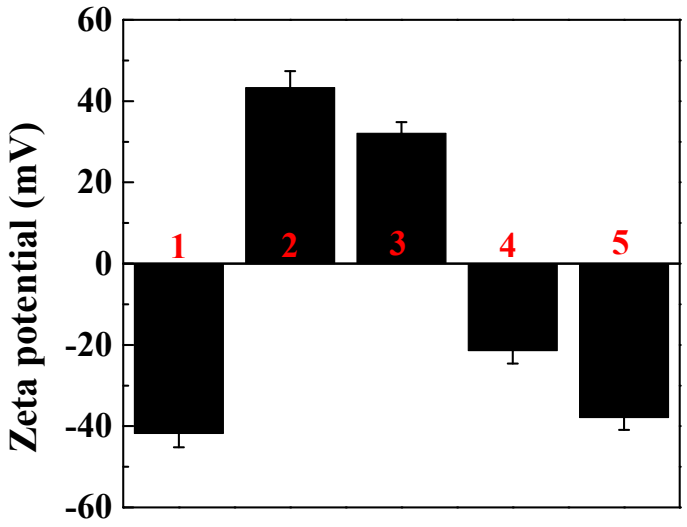

Fig. S1 Zeta potentials of the acid-treated MWCNTs (1), MWCNT-PEI (2), MWCNT-PEI-FITC-PEG-FA (3), MWCNT-PEI.SAH-FITC-PEG-FA (4), and MWCNT-PEI.SAH-FITC-PEG-FA/ICG (5).

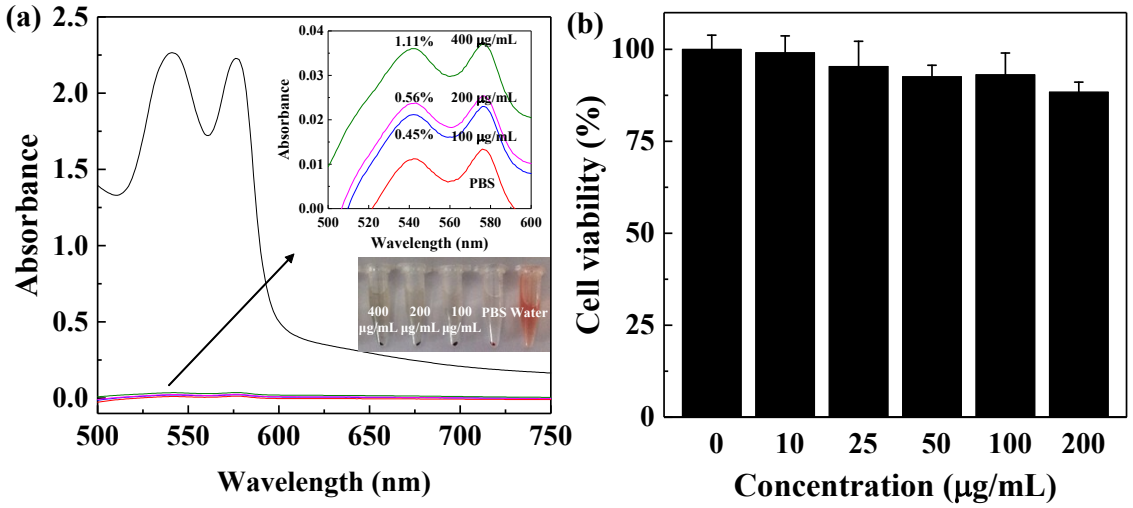

Fig. S2 (a) Hemolytical activity of the FA-targeted MWCNTs at different concentrations (100, 200, and 400 μg/mL, respectively). PBS and water were used as negative and positive controls, respectively. The bottom-right inset shows the photograph of HRBCs exposed to water, PBS, and PBS-containing FA-targeted MWCNTs at different concentrations for 2 h, followed by centrifugation. The upper-right inset shows the enlarged UV-vis spectra. (b) CCK-8 viability assay

of HeLa cells after treatment with the FA-targeted MWCNTs in the concentration range of 10-200 μg/mL for 24 h.

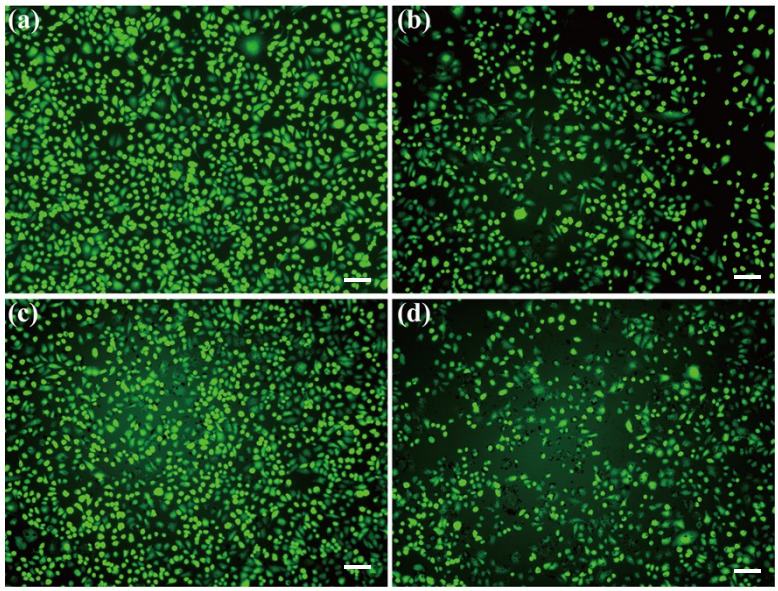

Fig. S3 Live/dead staining images of HeLa cells treated with PBS (a) and FA-targeted MWCNTs at the concentration of 50 (b), 100 (c), and 200 (d) μg/mL, respectively. The scale bar in panels a-d represents 100 μm.

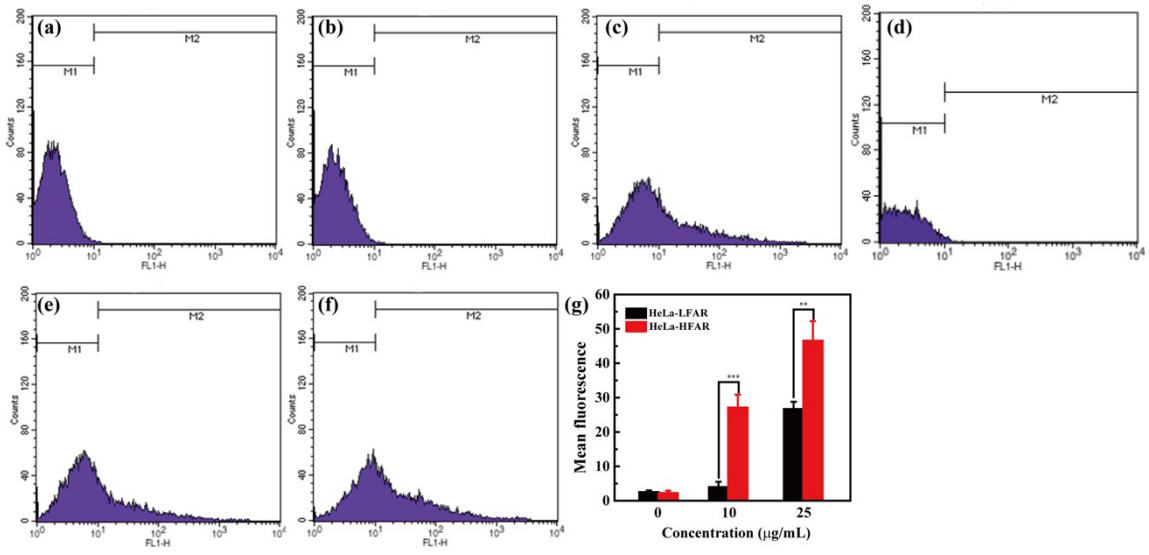

Fig. S4 Flow cytometry analysis of the cellular uptake of FA-targeted MWCNTs at the concentrations of 10 μg/mL (b, e) and 25 μg/mL (c, f) by HeLa-LFAR (b, c) and HeLa-HFAR (e, f)

cells after treatment for 4 h. HeLa-LFAR (a) and HeLa-HFAR (d) cells treated with PBS were used as control. Mean fluorescence of cells (mean ± S.D., n = 3) as a function of the concentration of FA-targeted MWCNTs is shown in (g).

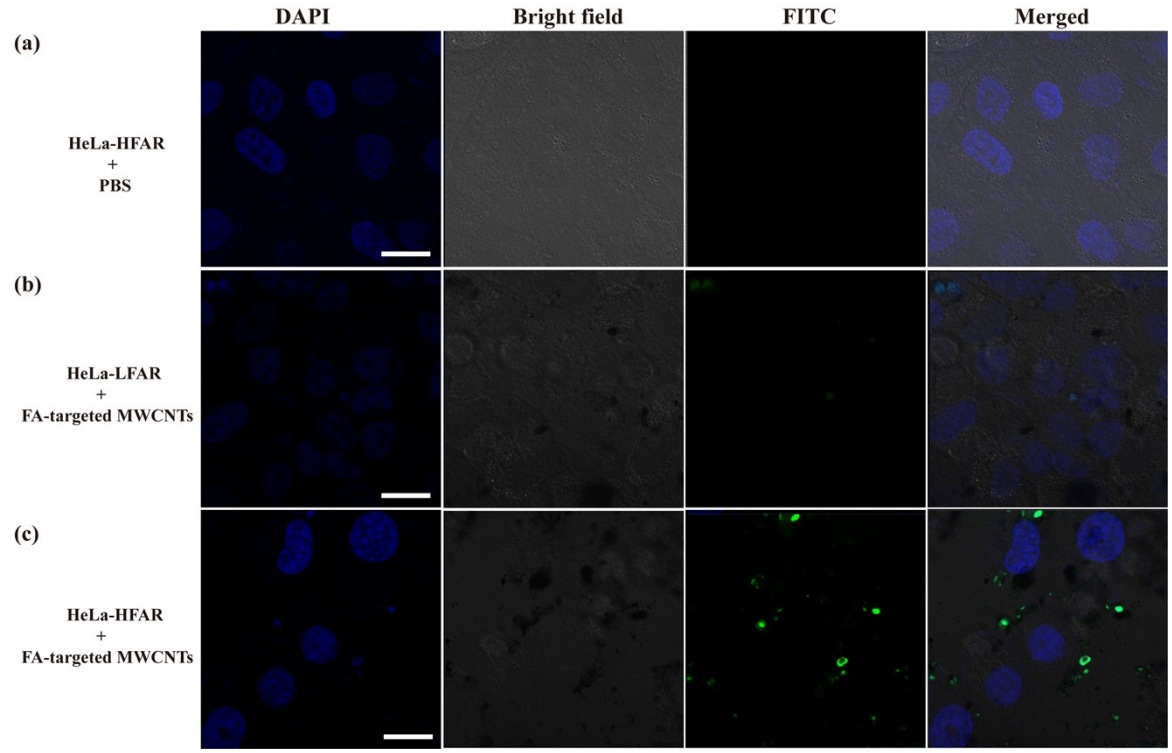

Fig. S5 Confocal microscopic images of the HeLa-HFAR (a, c) and HeLa-LFAR (b) cells after treated with PBS (a) and FA-targeted MWCNTs (b, c) at the concentration of 10 μg/mL for 4 h. The scale bar represents 20 μm.

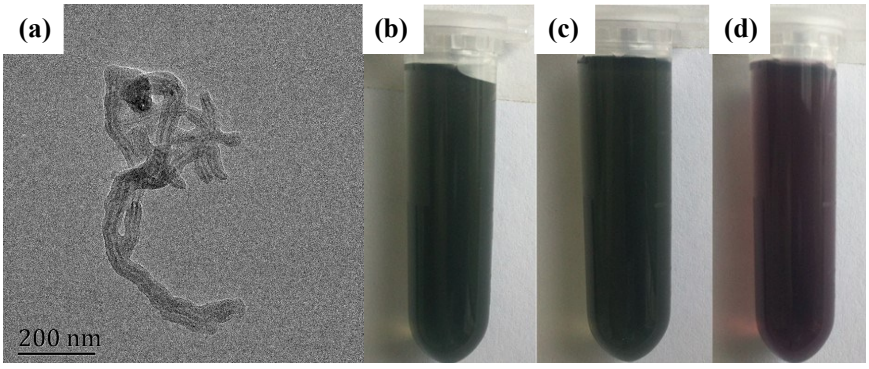

Fig. S6 TEM image of ICG-loaded MWCNTs (a) and photographs of the ICG-loaded MWCNTs

dispersed in water (b), PBS (c), and cell culture medium containing 10% FBS (d), respectively over a period of two months.

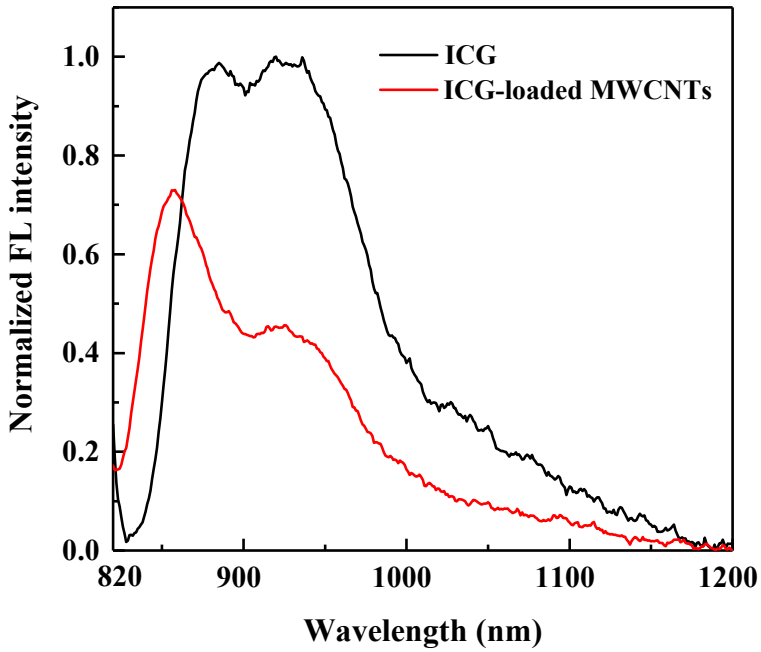

Fig. S7 The normalized FL spectra of aqueous solutions of free ICG and ICG-loaded MWCNTs (λex= 730 nm) according to the highest FL intensity of ICG spectrum, respectively.

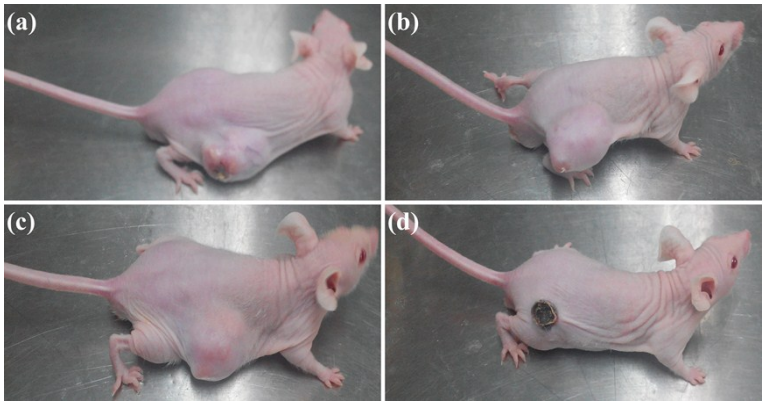

Fig. S8 Representative photographs of the tumor-bearing mice in Control group (a), Laser group (b), ICG-loaded MWCNTs group (c), and ICG-loaded MWCNTs + Laser group (d) at 24 days posttreatment.

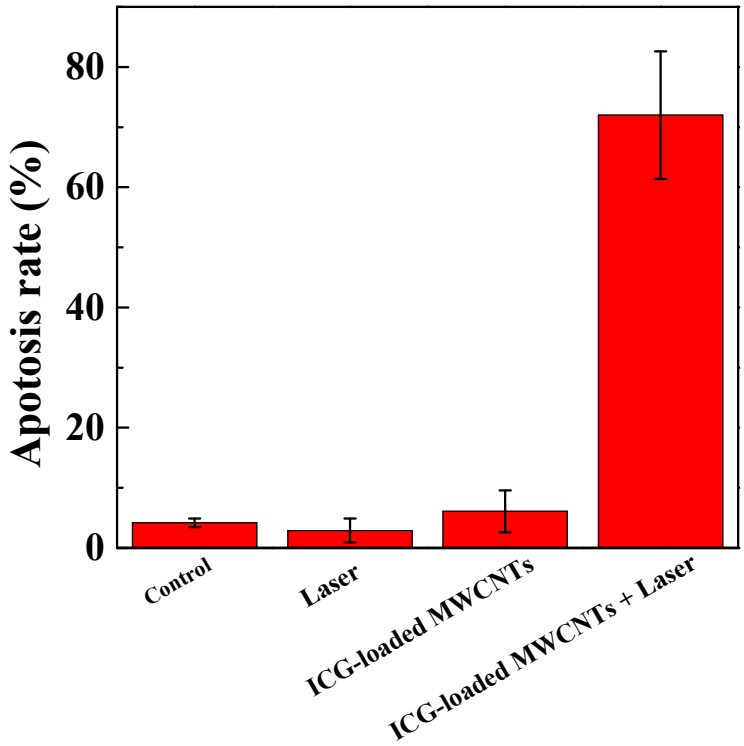

Fig. S9 Apoptosis rate of tumor cells after different treatments by quantification of the TUNEL-positive tumor cells in tumor sections.

References

1. E. J. Petersen, Q. Huang and J. Weber, Walter J, *Environ. Sci. Technol.*, 2008, **42**, 3090-3095.
2. E. J. Petersen, Q. Huang and W. J. Weber Jr, *Environ. Health Perspect.*, 2008, **116**, 496.
3. C. Peng, K. Li, X. Cao, T. Xiao, W. Hou, L. Zheng, R. Guo, M. Shen, G. Zhang and X. Shi, *Nanoscale*, 2012, **4**, 6768-6778.
4. Y. Hu, R. Wang, J. Li, L. Ding, X. Wang, X. Shi and M. Shen, *Part. Part. Syst. Charact.*, 2017, **34**, 1600113.
5. X. Zheng, K. Kelley, H. Elnakat, W. Yan, T. Dorn and M. Ratnam, *Mol. Cell. Biol.*, 2003, **23**, 2202-2212.
6. C. Sun, R. Sze and M. Zhang, *J. Biomed. Mater. Res., Part A*, 2006, **78A**, 550-557.
7. S. Wang, Y. Wu, R. Guo, Y. Huang, S. Wen, M. Shen, J. Wang and X. Shi, *Langmuir*, 2013, **29**, 5030-5036.