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Supporting Information

Localized NIR-II photo-immunotherapy through the synergy of photothermal ablation and *in situ* generated interleukin-12 cytokine for efficiently eliminating primary and abscopal tumors

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Materials and methods

Materials

Tetraethyl orthosilicate (TEOS, 96%), 2-bromoisobutyryl bromide (BIBB,＞98%) and 2-(dimethylamino) ethyl methacrylate (DMAEMA,＞98.5%) were purchased from TCI (Shanghai) Development Co., Ltd. (Shanghai, China). *N,N,N',N'',N''*-pentamethyl diethylenetriamine (PMEDTA, 99%), copper (I) bromide (CuBr, 98%) and branched polyethylenimine (PEI, $M_w \approx 25$ kDa) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Hexadecyltrimethylammonium chloride (CTAC, 97%) was obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). Anhydrous tetrahydrofuran (THF, 99.5%) and sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$, 99%) was purchased from Shanghai Titan Scientific Co., Ltd. (Adamas-beta, Shanghai, China). 3-mercaptopropyltriethoxysilane (MPTES, 95%) was obtained from J&K Scientific Ltd. (Beijing, China). Sodium hydroxide (NaOH), hydrochloric acid (HCl), triethanolamine (TEA), triethylamine (TETN), sodium chloride (NaCl), copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), ethanol, methanol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DMAEMA was used after inhibitor removal through a ready-to-use disposable inhibitor-removal column (Sigma-Aldrich). Deionized water with the resistivity of 18.2 MΩ cm was obtained from a Milli-Q Gradient System (Millipore, Bedford, MA, USA), and it was used for all experiments.

Synthesis of CuS-SiO_2-PD (CSP) nanoparticle

Thiol-functionalized mesoporous SiO_2: 2g of CTAC (6.25 mmol) and 18 μL of TEA were dissolved in 20 mL of water at 80℃ under intensive stirring for 1 h, and further, a mixture of 1.5 mL of TEOS and 150 μL of MPTES was added dropwise. After another 1 h of reaction, the resulting nanoparticle thiol-functionalized mesoporous SiO_2 were collected by centrifugation, washed with ethanol thrice, and then extracted for 3 h with 1wt% NaCl methanol solution at room temperature to further remove the surfactant template CTAC.

SiO_2-Br: The immobilization of the ATRP initiator on silica nanoparticles was carried out *via* the reaction of -OH groups on silica nanoparticles with BIBB. Typically, 0.1 g of SiO_2 obtained from the above steps was dispersed in 5 mL of anhydrous THF, and 0.5 mL of BIBB together with 0.5 ml of TETN was subsequently added at 0 ℃. The reaction was maintained at 0 ℃ for 3 h and then kept at room temperature overnight. The resultant SiO_2-Br were separated by centrifugation, washed with

the mixture of methanol and water (1/1, v/v) for several times, and dried under vacuum.

CuS-SiO_2: The obtained SiO_2-Br was dispersed in 20 mL of water (adjusting the pH to 7-8 with 0.1M NaOH), followed by the addition of 200 μL of $CuCl_2 \cdot 2H_2O$ (0.1 M). After 6 h of stirring, the Cu^{2+}-loaded SiO_2 nanoparticles were centrifuged and then re-dispersed in 20 mL of water (adjusting the pH to 3 with 0.1M HCl), followed by the addition of 400 μL of $Na_2S \cdot 9H_2O$ solution (0.1 M). The mixture solution was stirred at 90 ℃ for 15 min and turned dark green. Finally, the product was centrifuged with water thrice to obtain CuS-SiO_2.

CuS-SiO_2-PD (CSP): Employing typical atom transfer radical polymerization method, 20 mg of initiator-modified CuS-SiO_2, 0.5 mL of DMAEMA monomer, and 30 μL of PMEDTA were dissolved in 5 mL of a mixture of methanol and water (2/3, v/v). Subsequently, 10 mg of CuBr (0.07 mmol) was added into the mixture under oxygen free condition. The mixture was maintained at 30 ℃ for 30 min, and then purified by dialyzing against water using a dialysis membrane (MWCO 3500), and the final product CuS-SiO_2-PD (CSP) was collected by lyophilization.

Characterization

The morphology and elemental mapping of the products were characterized using a high resolution transmission electron microscope (TEM; FEI Company, TALOS F2000) operated at 200-kV accelerating voltage. For TEM experiments, the nanoparticle suspension was dropped onto the carbon-coated copper grid and dried naturally. The particle size of the samples was measured using a Zetasizer Nano ZS (Malvern Instruments) with a detection angle of 173° and using a 3-mW He-Ne laser, which was operated at the wavelength of 633 nm. Measurement of zeta potential was performed on a Nano ZS system by using the M3-PALS technology. The FT-IR spectra were analyzed on a Fourier transform infrared spectrometer (Perkin-Elmer, Spectrum-2000) with the spectral region of 400-4000 cm^{-1}, where the KBr was mixed with samples and compressed to a plate. The absorbance of the products was measured by a UV-Vis-NIR spectrophotometer (Agilent, Cary 5000). The amounts of modified components on SiO_2 were analyzed with thermal gravimetric analyzer (TGA, NETZSCH, TG 209 F3). The TGA measurement was carried out in the air from RT to 800 ℃, with a heating rate of 20 ℃ min^{-1}.

The CSP solution (Cu concentrations: 0-50 μg mL^{-1}) in centrifuge tube was irradiated by a 1064 nm NIR-II laser (0.5-1 W cm^{-2}) for 800 s to evaluate the photothermal performance. The temperature

of the solutions was monitored using a thermocouple microprobe (Φ = 0.5 mm) (Xiamen Baidewo Technology Co., STPC-510 P) submerged in the solution. To evaluate the photo-stability of CSP, the CSP solution (Cu concentration: 10 μg·mL^{-1}) was irradiated by a 1064 nm laser at 0.8 W cm^{-2} for 800 s (laser on), followed by naturally cooling to room temperature (laser off). The laser on and laser off cycles were repeated for four times.

The CSP stock solution was prepared at a nitrogen concentration of 10 mM (3.8 mg mL^{-1}) in sterilized water and then stored at 4 ℃ for further usage. The CSP to *pDNA* ratios were expressed as molar ratios of nitrogen (N) in PDMAEMA to phosphate (P) in *pDNA* (denoted as N/P ratios). The CSP/pDNA complexes at various N/P ratios were obtained by mixing different amounts of CSP with constant amounts of DNA, where volumes of CSP and *pDNA* solutions were equal. The mixture was then incubated for 30 min at RT to obtain the final CSP/pDNA complexes.

The gene condensation ability of CSP was evaluated by agarose gel electrophoresis. The CSP/*pDNA* complexes at various N/P ratios were obtained by mixing serious amounts of CSP from 1.7 μg (N/P = 0.5) to 85 μg (N/P = 25) with 0.3 μg *EGFP*. Each CSP/*EGFP* complex (20 μL) was mixed with 6 × DNA loading buffer (4 μL) and analyzed using 1% agarose gel containing 10000× GelStain. The gel was run in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) with a voltage of 120 V for 25 min in a Sub Cell system (Bio-Rad Lab, Hercules, CA). The DNA bands were finally visualized using a UV transilluminator and photographed using a Gel Doc XR imaging system (Bio-Rad, Lab, Hercules).

In vitro experiments

Cell culture: The murine melanoma cell line B16F10 was maintained as a monolayer culture in the RPMI-1640 medium, while the mouse embryonic fibroblast cell line NIH 3T3 was cultured in Dulbecco's Modified Eagle Medium (DMEM), both supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA) at a humid atmosphere (37 ℃, 5% CO_2).

***In vitro* cytotoxicity assay**: The CCK-8 viability assay was performed to evaluate the cytotoxicity of the CSP in NIH 3T3 cell line using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded into a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h. The culture medium were then replaced with 100 μL of fresh medium containing serial concentration CSP

(1.2-5.8 μg, equal to the CSP concentration at various N/P ratios from 5 to 25), and then the cells were incubated for another 24 or 48 h. Thereafter, 10 μL of CCK-8 stock solution in PBS (5 mg mL^{-1}) and 90 μL of culture medium were added into each well. After 2 h, the absorbance at the wavelength of 450 nm of each well was measured using a Spectra Max M5 microplate reader. The cell viability (%) relative to control cells was calculated from $([A]_{test} - [A]_{blank})/([A]_{control} - [A]_{blank}) \times 100\%$, where $[A]_{test}$ and $[A]_{control}$ are the absorbance values of the cells with and without the nanoparticle treatment, respectively. $[A]_{blank}$ is the absorbance of the CCK-8 reagent itself at 450 nm. For each sample, the final absorbance value was the average of those measured from five wells in parallel.

Determination of cellular internalization of CSP*@pDNA*: To evaluate the intracellular localization of the CSP/*pDNA* complexes in B16F10 cell line, the plasmid pCDH-CMV-EF1α-EGFP (*EGFP*) was labeled with YOYO-3 fluorescent dye (YOYO™-3 Iodide, Invitrogen, Eugene, OR, USA) according to the manufacturer's protocol. B16F10 cells were seeded into 20 mm confocal laser dishes at the density of 5×10^4 cells in 1 mL of growth medium/dish. The CSP/YOYO-3-labeled *EGFP* complexes (N/P = 15) were obtained as mentioned above. The complexes containing 2 μg of *EGFP* and 34.5 μg of CSP were applied to each dish. After 5 h of incubation with the nanocomplexes, the cells were washed with PBS, labeled with Hoechst and then imaged using a confocal laser scanning microscope (CLSM; Zeiss LSM780).

Transfection assay: Transfection assay was first performed by employing plasmid pCDH-CMV-EF1α-EGFP as the reporter gene (*EGFP*) in B16F10 cells. Briefly, the cells were seeded into 24-well plates at the density of 5×10^4 cells per well in 500 μL of medium and incubated for another 24 h. Then, 500 μL of fresh medium without serum was added to replace the medium. CSP/*EGFP* complexes (20 μL) at various N/P ratios containing 5.7-28.7 μg of CSP and 1.0 μg of *EGFP* were obtained using a method similar to that as described above. The obtained nanocomplexes were subsequently added into the transfection medium and then further incubated for 5 h. Afterwards, the cells were further cultured in fresh medium for another 43 h, thus resulting in total transfection time of 48 h. The transfected cells were imaged using a fluorescence microscope (Zeiss Axio Vert.A1), and the mean fluorescence intensities of EGFP were determined by flow cytometry (FCM; BD FACSVerse).

Furthermore, the secretion levels of IL-12 protein was investigated by ELISA assay. The plasmid

pCDNA3.1-CMV-IL12 (*IL-12*) was employed to form the CSP@*IL-12* nanocomplexes. B16F10 cells were seeded into six-well plates at the density of 1×10^5 cells per well in 1.5 mL of medium, and then, the plate was incubated for 24 h. The cells then were replaced with fresh medium without serum, followed by incubation with CSP@*IL-12* nanocomplexes (at the N/P ratio of 15, containing 51.7 μg of CSP and 3 μg of *IL-12* for 5 h. After another 43 h of incubation, the cell culture supernatant was collected for ELISA assay.

***In vitro* photothermal therapy in the NIR-II window**: The CCK-8 viability assay was performed to quantitatively evaluate the photothermal cell toxicity of the CSP in B16F10 cell line. B16F10 cells in 96-well plates were incubated with serious amounts of CSP (from 1.7 μg to 11.9 μg in 100 μL of medium, equal to the CSP concentration at various N/P ratios from 5 to 25) for 5 h and then washed with PBS buffer to remove the free nanoparticles. Afterwards, the cells were cultured in fresh medium and exposed to NIR-II laser radiation (1064 nm, 0.8 W cm^{-2}) for 10 min. After laser irradiation, the cells were further incubated for 24 h. The cell survival rate was evaluated using the CCK-8 assay, according to the same procedures as described above.

For qualitative analysis, the *in vitro* photothermal therapy effect of CSP was further proved by live/dead staining and apoptosis assay. For live/dead staining, after 24 h of the light irradiation, the cells were stained with both calcein AM (calcein acetoxymethyl ester) and PI (propidium iodide) for 10 min, using LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Eugene, OR, USA)), and then the cells were observed under a fluorescence microscope (Zeiss Axio Vert.A1). For apoptosis assay, the treated cells were stained with annexin V-FITC/PI solution using annexin V-fluoroisothio cyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Dojindo Laboratories, Kumamoto, Japan), and detected by flow cytometry (FCM; BD FACSVerse) after 24 h of the light irradiation.

The induced immune responses by PTT: To evaluate the immune responses induced by PTT, the ICD markers CRT, HMBG1 and ATP, as well as the DC maturation after PTT were determined by immunofluorescence analysis and flow cytometry, respectively. Briefly, B16F10 cells were seeded into 20 mm confocal laser dishes at the density of 5×10^4 cells in 1 mL of growth medium/dish. After 24 h of incubation, the cells were treated with CSP (34.5 μg) for 5 h and then exposed to 1064 nm laser (0.8 W·cm^{-2}, 10 min) according to above similar procedures, and incubated for another 24 h. For analysis of the CRT expression after PTT, the treated B16F10 cells in confocal laser dishes were

stained with anti-CRT (abcam, ab2908) and Hoechst 33342. Finally, the cells were observed by CLSM (Zeiss LSM780). For quantitative analysis of CRT expressing cells, the cells after treatment were stained with anti-CRT and then detected by flow cytometry (FCM; BD FACSVerse). The intracellular HMGB1 and ATP level of B16F10 cells after CSP mediated PTT were examined using Mouse HMGB1 ELISA Kit (CUSABIO, China) and ATP assay kit (Beyotime Biotechnology Co. Ltd, China) according to vendor's instruction, respectively. For determine the DC maturation after PTT, the supernatant in the dishes was added into the 24-well plates seeded with DC cells (derived from the bone marrow of C57BL/6 mouse after stimulated with GM-CSF, at the cell density of 4×10^5) and then incubated for 48 h. In the control group, fresh medium instead of supernatant was added into the DC seeded plate. Subsequently, the cells were stained with anti-CD11c-APC (eBioscience™, 17-0114-82), anti-CD80-PE (eBioscience™, 12-0801-82) and anti-CD86-PE-Cy7 (eBioscience™, 25-0862-82), and finally detected by flow cytometry (FCM; BD FACSVerse).

In vivo experiments

Tumor model: To establish the primary tumor model, 1×10^7 B16F10 cells suspended in 100 μL of PBS were subcutaneously injected into the right flank of each C57BL/6 mouse. For establishment of the distant tumor, 5×10^6 B16F10 cells suspended in 50 μL of PBS were simultaneously injected into the left flank of each mouse. Seven days later, when the primary tumor size reached about 50 mm^3 (the secondary tumor reached 20 mm^3), the tumor bearing mice were randomly divided into different groups.

***In vivo* photothermal imaging**: After the B16F10 tumor-bearing mice receiving local administration (intra-tumoral injection of 172.4 μg of CSP in 30 μL of PBS), the tumor sites were irradiated by 1064 nm laser (0.6 $W \cdot cm^{-2}$) for 5 min. The thermo-graphic images were captured by an IR thermal camera (Ti25 Fluke Co, USA).

***In vivo* synergistic antitumor effect assay:** For *in vivo* synergistic anticancer effect assay, the mice only bearing local B16F10 tumor were randomly divided into six groups. The plasmid pCDNA3.1-CMV-IL12 (*IL-12*) was employed to form the CSP@*IL-12* nanocomplexes. Typically, in the CSP@*IL-12* + laser group, 30 μL of the CSP@*IL-12* solution (N/P = 15) for each mouse was administered as an intra-tumoral injection. After 24 h of injection, the tumor site was irradiated with 1064 nm laser with a power intensity of 0.6 $W \cdot cm^{-2}$ for 5 min (light source: KA64HAMCC-10.00W, BWT Beijing Ltd.). The procedure in other groups is similar as the above, except for injecting

different samples and providing light irradiation. The specific therapeutic method given for each group is listed as below:

(1) Control group: 30 μL of sterilized PBS for each mouse;

(2) Laser group: 30 μL of sterilized PBS for each mouse, followed by 1064 nm (after 24 h of injection) laser irradiation;

(3) CSP group: 30 μL of the CSP solution (172.4 μg of CSP in PBS) for each mouse;

(4) CSP@*IL-12* group: 30 μL of the CSP@*IL-12* solution (172.4 μg of CSP and 10 μg of *IL-12* in PBS) for each mouse;

(5) CSP + Laser group: 30 μL of CSP solution (172.4 μg of CSP in PBS) for each mouse, followed by 1064 nm laser irradiation (after 24 h of injection);

(6) CSP@*IL-12* + Laser group: 30 μL of the CSP@*IL-12* solution (172.4 μg of CSP and 10 μg of *IL-12* in PBS) for each mouse, followed by 1064 nm (after 24 h of injection) laser irradiation;

The therapeutic effects were examined by monitoring tumor volume in each group every day, up to 15 days. The tumor size was measured using a caliper. The tumor volume (V) was calculated using the equation tumor length × (tumor width)^2/2. Relative volume V/V_0 (V_0 as the initial tumor volume before therapy) was used to evaluate the relative tumor growth rate. Notably, for animal ethics and easy statistics concerns, the mice would be sacrificed when the tumor volume exceeded 1500 mm^3. All the tumors were harvested at the end of the studies and stored at -80℃ for the following photographing and weighing. The body weight changes of the treated mice were measured every day to evaluate the side effects. Six mice per group were employed to investigate the survival rate, where a mouse was defined as death when its tumor volume exceeded 1500 mm^3.

To evaluate the histological changes of tumors and protein expression, one tumor-bearing mouse was sacrificed in each group on day 2. The tumors were fixed in formalin after being harvested from mice, and subsequently, these tumors were embedded by paraffin and further sectioned into slices with thickness of 4 mm; then, they were stained with hematoxylin and eosin (H&E) for histopathology evaluation, anti-Ki67 (Servicebio, GB11141) and anti-CRT (abcam, ab2908) for immunohisto-chemical analysis, respectively. IL-12 protein expression was also detected by using IL-12 antibody (abcam, ab203031). The tumor slices were then imaged using a Zeiss microscope (Axio Lab.A1).

Immune response analysis *in vivo*: Firstly, the CRT expression on day 2 were observed by CLSM (Zeiss LSM780). To further study the immune responses during local administration of PTT and IL-12 cytokine immunotherapy, four tumor-bearing mice were sacrificed in each group on day 4, and the tumor-draining lymph nodes, spleens and tumors were harvested. To examine the DC maturation, the cells isolated from tumor-draining lymph nodes were stained with anti-CD11c-APC (eBioscience™, 17-0114-82), anti-CD80-PE (eBioscience™, 12-0801-82) and anti-CD86-PE-Cy7 (eBioscience™, 25-0862-82), and then detected by flow cytometry (FCM; BD FACSVerse). In addition, the cells harvested from the spleen were stained with anti-CD3-APC (eBioscience™, 17-0032-82), anti-CD4-FITC (eBioscience™, 11-0042-85) and anti-CD8-PE (eBioscience™, 12-0081-82). The level of $CD8^+$ T cells and $CD4^+$ T cells in spleen was finally analyzed by flow cytometry. To evaluate the changes of the tumor microenvironment, one tumor in each group was fixed in formalin and embedded by paraffin and further sectioned into slices; then, they were stained with anti-CD4 (Servicebio, GB13064-2) and anti-CD8 (Servicebio, GB13429) respectively for immunohisto-chemical analysis of T cell infiltration. To analyze the typical cytokines in the tumor sites, the interleukin 12 (IL-12, Boster, EK0422), interferon gamma (IFN-γ, Boster, EK0375), tumor necrosis factor (TNF-α, Boster, EK0527) and Granzyme B (Boster, EK0417) were detected using Mouse ELISA Kit according to vendor's instruction (5mg·mL^{-1} tumor tissues in PBS).

For examination of the therapeutic effects against the distant tumor, the bilateral tumor model was established. The tumor volume and body weight measurement, histopathology evaluation and immunofluorescence analysis of the distant tumors were carried out as indicated above.

Supplementary Figures

Table S1. Characterization of SiO_2, CSP and CSP@*EGFP* measured by DLS.

Sample	Hydrodynamic particle size (nm)	Zeta potential (mV)
SiO_2	118.1±0.5	-21.4±0.8
CSP	156.6±1.2	31.3±0.6
CSP@*EGFP*	143.8±5.0	22.9±0.6

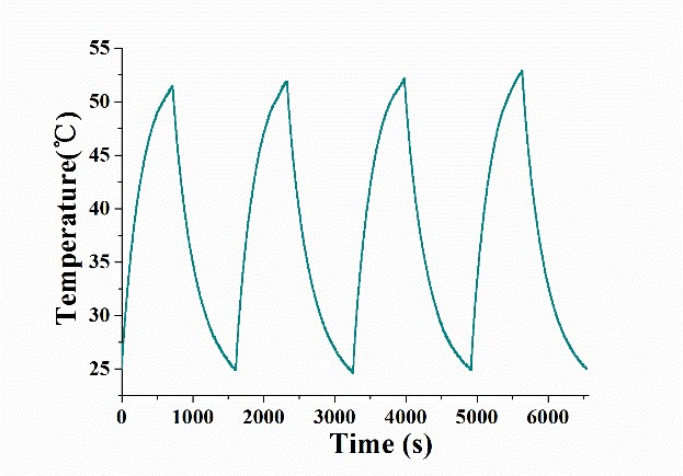

Fig. S1. Temperature elevation curves of CSP suspension at Cu concentration of 10 μg·mL^{-1} over four rounds of on/off cycling (1064 nm laser, 0.8 W·cm^{-2}).

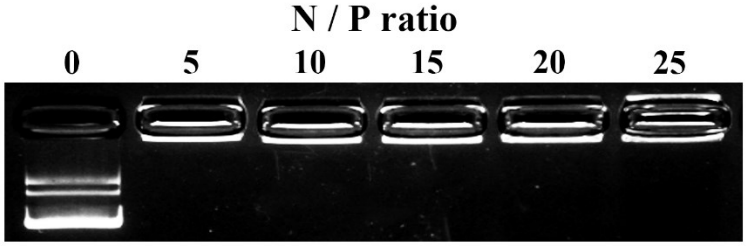

Fig. S2. Electrophoretic analysis of *EGFP* plasmid gene in the complexes of CSP@*EGFP* under various N/P ratios (from 0 to 25).

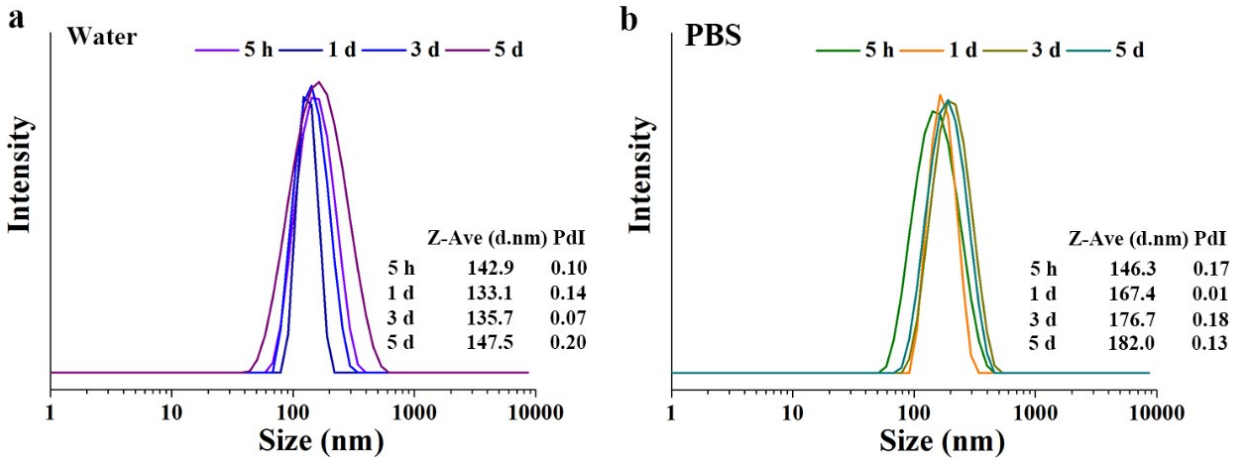

Fig. S3. Size distributions of CSP@*EGFP* complexes (N/P = 15) in (a) water and (b) PBS measured by DLS.

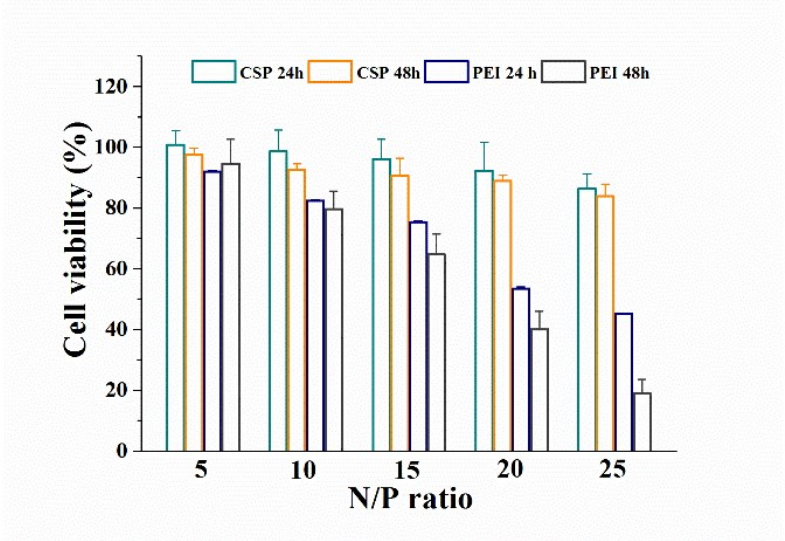

Fig. S4. CCK-8 viability assay of NIH 3T3 cell line treated with CSP at various concentrations (mean ± SD, n = 5).

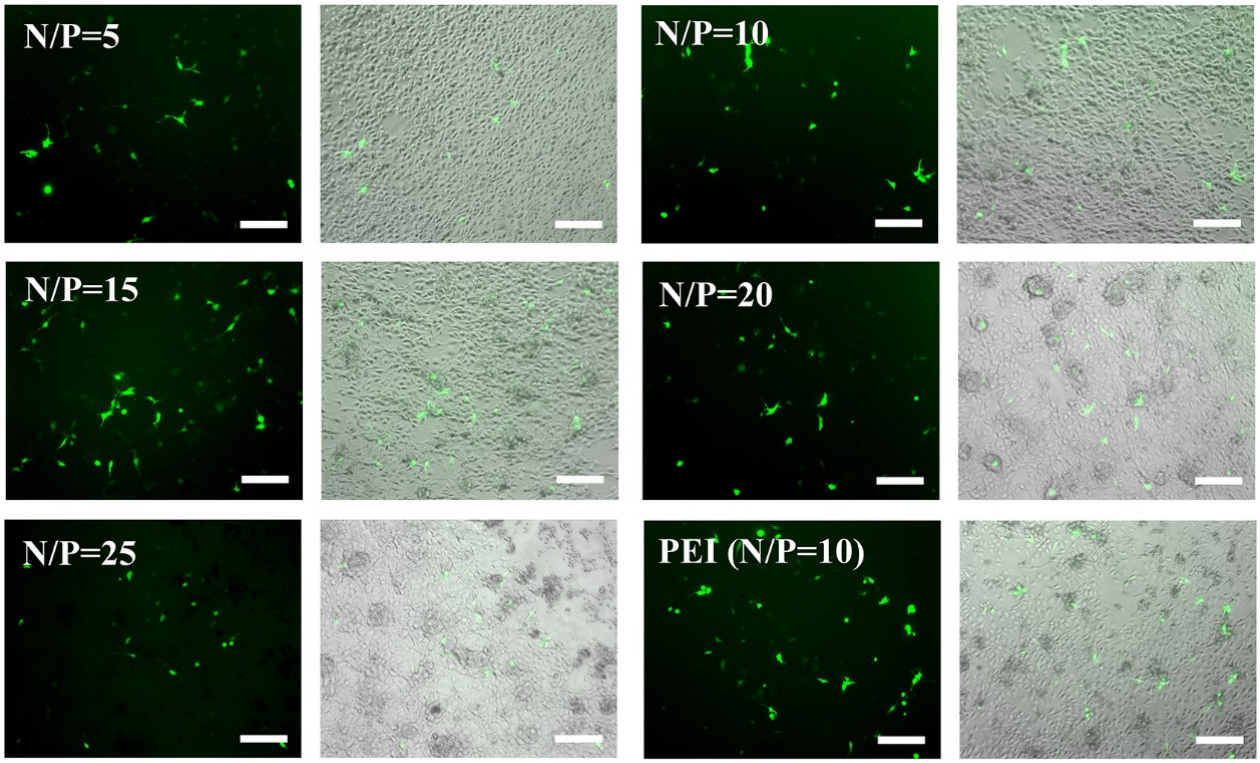

Fig. S5. Fluorescence microscopy images and the corresponding overlay of bright field images of EGFP gene expression mediated by CSP at various N/P ratios, PEI (N/P =10) as comparison. Scale bar: 200 μm.

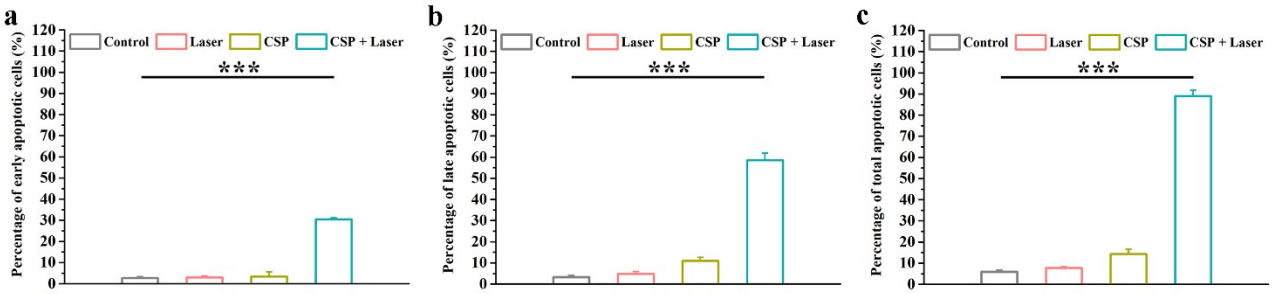

Fig. S6. The percentages of (a) early (Annexin V+/PI−), (b) late (Annexin V+/PI+) and (c) total (early + late) apoptotic cells after indicated procedures (mean ± SD, n = 3). The CSP concentration is 34.5 μg·mL^{-1}, equal to the CSP concentration at the N/P ratio of 15. 1064 nm laser at the power density of 0.8 W·cm^{-2} for 10 min was employed to perform PTT. (*P＜0.05, **P＜0.01, and ***P＜0.001)

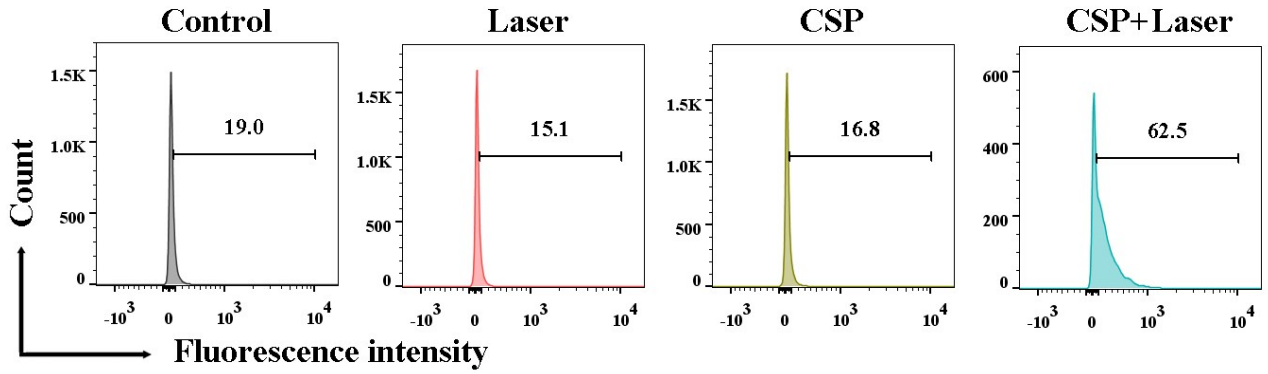

Fig. S7. The flow cytometry analysis of CRT expressing cells after CSP mediated PTT. The CSP concentration is 34.5 μg·mL^{-1}, equal to the CSP concentration at the N/P ratio of 15. 1064 nm laser at the power density of 0.8 W·cm^{-2} for 10 min was employed to perform PTT.

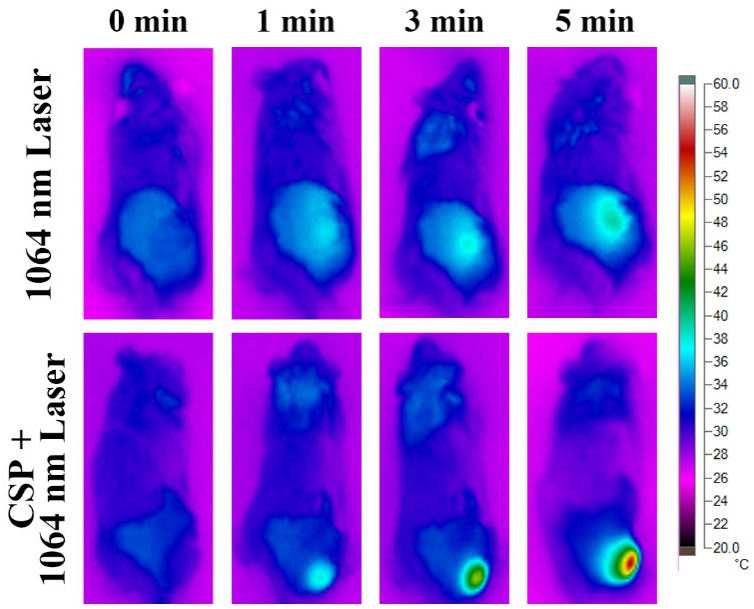

Fig. S8. The IR thermal images of B16F10 bearing mice with the tumor site irradiated by 1064 nm light at power density of 0.6 W·cm^{-2} for 0-5 min, in the presence or absence of intra-tumoral CSP injection (172.4 μg of CSP in 30 μL of PBS).

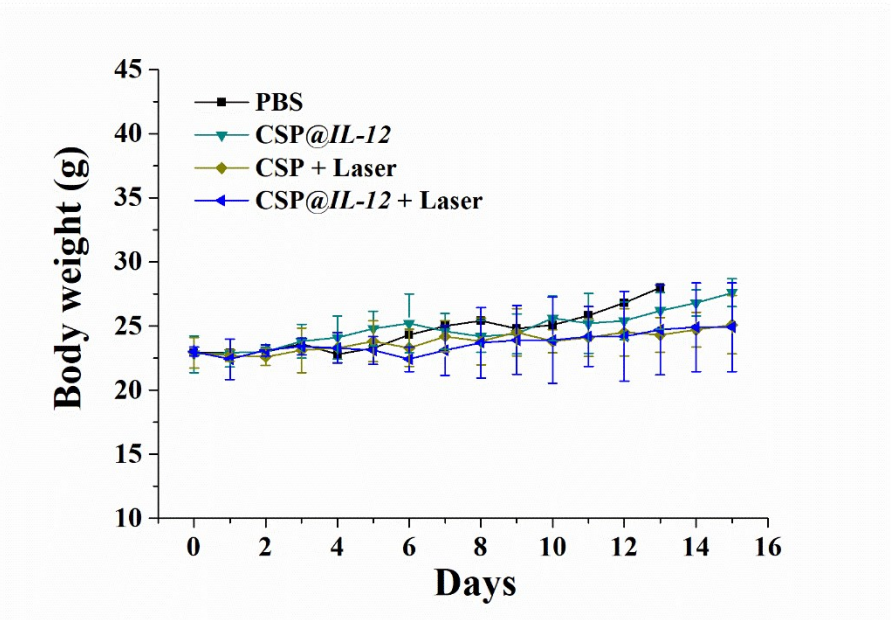

Fig. S9. Mean body weights of the mice in bilateral B16F10 tumor model (n=5).