Supporting Information

Identifying glioblastoma margin using dual-targeted organic nanoparticles for efficient in vivo fluorescence image-guided photothermal therapy

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Materials. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (DSPE-PEG_{2000}), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(folate(polyethylene glycol)-2000) (DSPE-PEG_{2000}-Folate) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-maleimide-(polyethyleneglycol)-2000) (DSPE-PEG_{2000}-Mal) were ordered from Avanti Polar Lipids. cRGD peptide was a commercial product customized by GenicBio, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA solution, phosphate buffered saline (PBS), paraformaldehyde (PFA) and tetrahydrofuran (THF) and fluorescein diacetate (FDA) were purchased from Sigma-Aldrich and used as received. Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium alpha (MEM α), penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Thermo Scientific and used as received. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Bedford, USA).

Preparation of TNSP NPs. TPETPAFN and SP were synthesized according to literature.[1-2] TNSP NPs were synthesized through a modified nanoprecipitation method. For preparation of NPs without surface targeting ligand, 1 mg of DSPE-PEG_{2000}, 0.125 mg of TPETPAFN and 0.125 mg

of SP were dissolved in 1 mL of THF solution to form a clear solution. For preparation of NPs with different surface targeting ligands, 1 mg mixture of DSPE-PEG_{2000}-Folate and DSPE-PEG_{2000}-Mal with different mass ratios (100:0, 75:25, 50:50, 25:75, 0:100) were dissolved in 1 mL of THF solution containing TPETPAFN (0.125 mg) and SP (0.125 mg). The mixed solutions were rapidly injected into 10 mL of MilliQ water under sonication by a microtip probe sonicator (XL2000, Misonix Incorporated, NY) at 12 W output for 60 s. THF was evaporated by stirring for 12 h at room temperature. Syringe filters with a cut-off of 200 nm were applied to filter off the NP suspensions. Thiol-functionalized cRGD dissolved in DMSO with a molar ratio of 2:1 to DSPE-PEG-Mal was added into the NP suspensions. Following an overnight reaction at room temperature, excess cRGD and DMSO were removed by dialyzing against MilliQ water with 12000-14000 kDa membrane for 3 days.

Preparation of TPETPAFN NPs and SP NPs. TPETPAFN NPs were synthesized through a modified nanoprecipitation method. 1 mg of DSPE-PEG_{2000}, 0.25 mg of TPETPAFN or SP were dissolved in 1 mL of THF solution to form a clear solution. The mixed solutions were rapidly injected into 10 mL of MilliQ water under sonication by a microtip probe sonicator (XL2000, Misonix Incorporated, NY) at 12 W output for 60 s. THF was evaporated by stirring for 12 h at room temperature. Syringe filters with a cut-off of 200 nm were applied to filter off the NP suspensions.

Characterization. The UV-vis absorption spectrum was measured using a Shimadzu UV-1700 spectrophotometer. The fluorescence spectrum was measured using a fluorometer (LS-55, Perkin Elmer, USA). The average particle sizes were determined by laser light scattering with a particle size analyzer (90 Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90° at room temperature. The zeta potential was measured by a zeta potential analyzer (ZetaPlus, Brookhaven

Instruments Corporation) at room temperature. The morphologies of the NPs were studied by field emission transmission electron microscopy (FE-TEM) (JEM-2010F, JEOL, Japan). Thermogravimetric analysis was performed on thermal analyser (DTG-60AH, Shimadzu) with a heating rate of 15 °C min^{-1} under N_2 atmosphere. Elemental analysis was performed on elemental analyser (vario MICRO cube, Elementar).

***In vitro* cell imaging.** The GBM cell line, U87MG, and mouse embryo fibroblast cell line, NIH/3T3, were cultured in DMEM medium with penicillin-streptomycin (10,000 U/mL) and 10% FBS using T25 flasks. The cells were sub-cultured into 8-well chambers to achieve 80% confluence and incubated with the NPs with different surface functionalization for 4 h. The cells were then washed twice with 1× PBS buffer and fixed in 4% paraformaldehyde. The fixed cells were imaged by confocal microscope (Leica SP8, Germany).

The neuronal glia mixture cells were derived adult rat hippocampus neural stem cells according to previous literatures, which was then clonally expanded.[3-4] They differentiate into neurons, astrocytes and oligodendrocytes. Neural stem cells were maintained in a proliferative media containing EGF and FGF growth factors as described previous.[3] Neuronal glia mixed culture was obtained from differentiation of neural stem cells, plated in a 4-well chamber with medium containing; DMEM : F12, B27 (Gibco) supplemented, 20 ng/mL of brain-derived neurotropic factor (BDNF; R&D Systems), and 1.0 µmol/L all-trans retinoic acid (Sigma-Aldrich). The cells were incubated with the FA-cRGD-TNSP NPs for 4 h, washed twice with 1× PBS buffer and fixed in 4% paraformaldehyde. The fixed cells were imaged by confocal microscope (Leica SP8, Germany).

Animal model with GBM. All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the National University of Singapore. The

animals were housed at an animal care facility with constant temperature and humidity with free access to food and water. Male NCr nude mice weighing 25 - 30 g and 8-10 weeks of age (InVivos Pte Ltd, Singapore) were used in this study and 12 mice were divided into 4 groups (a control group and 3 experimental groups), including three animals in each of the experimental groups and the control group. The mice were anesthetized by intraperitoneal injection of ketamine (10 mg/mL) and xylazine (1 mg/mL) and mounted on a stereotaxic frame to reduce motion artifacts during the experiment, and the scalp was excised to expose the bregma landmark. The body temperature was measured using a rectal probe and was maintained at 37 ± 0.5°C using a self-regulating thermal plate (TCAT-2 Temperature Controller, Physitemp Instruments, Inc., Clifton, NJ, USA). U87MG maintained in MEM α culture medium with penicillin-streptomycin (10,000 U/mL) and 10% FBS using T75 flasks for implantation into the experimental animals. One million U87MG cells suspended in 10 μL of MEM α were injected via a Hamilton microliter syringe at a rate of 0.1μL/min into the right primary motor cortical region of the mice (M1 region; ML: +2 mm, AP: 1.5 mm and DV: 2 mm.[5-6] The experimental animals in different groups were sacrificed at the respective time points (*i.e.*, days 3, 5, and 7 post tumour implantation) for histological assessments. The mice were sacrificed following perfusion with 0.1 M PBS and 4% PFA. Paraffin-embedded brain tissue sections (7 μm) were stained with hematoxylin and eosin (H&E) and imaged by light microscope (Leica, Germany).

***Ex vivo* and *in vivo* brain imaging.** A group of tumour-implanted mice received intravenous injections of 150 μL of TNSP NPs (0.5 mg/mL based on TPETPAFN mass concentration) with different surface functionalization via tail vein injection at different time points of 3, 5 and 7 days post tumour implantation. The control group received saline injection. At 4 h following NP injection, the mice were sacrificed following perfusion with 0.1 M PBS and the brain tissue was

carefully extracted. Frozen brains were then sectioned into 14 μm slices. The brain slices were further stained with DAPI and immunostained with primary anti-EGFR antibody and secondary antibody conjugated with Alexa Fluor® 488 to identify the tumour lesion. The brain slices were imaged using a confocal microscope (Zeiss LSM800, Germany). Another group of tumour-implanted mice were injected 150 μL of FA-cRGD-TNSP NPs through intravenous injection (0.5 mg/mL based on TPETPAFN mass concentration) at 7 days post tumour implantation. In vivo fluorescence imaging was conducted on IVIS Spectrum In Vivo Imaging System (PerkinElmer).

Photothermal conversion efficiency calculation. The photothermal conversion efficiency of TNSP NPs were calculated based on literatures[7-8] and Figure S11, which was 59% at 808 nm.

***In vitro* and *in vivo* photothermal therapy.** U87MG cells were sub-cultured onto 8-well chambers; neuronal glia mixed culture was plated onto 4-well chamber. After incubation with the FA-cRGD-TNSP NPs for 4 h, one group of cells were irradiated with 808 nm continuous-wave (CW) laser for 5 min at a power of 0.8 W while the other group of cells was kept in a dark environment. The cells were stained by FDA and imaged by confocal microscope (Leica SP8, Germany). Furthermore, U87MG cells and neuronal glia mixed culture were plated onto 96-well plates and incubated with the FA-cRGD-TNSP NPs for 4 h. The cells were washed twice by 1× PBS buffer. Selected wells were irradiated by 808 nm CW laser for 5 min at a power of 0.8 W. The cells were further incubated for 24 h and 100 μL of MTT solution (0.5 mg/mL) was added into each well. After 3 h incubation, the MTT solution was removed and DMSO (100 mL) was added into each well. The absorbance of MTT at 570 nm was studied with a microplate reader (Genios Tecan). Cells without any treatment were kept in darkness and served as the control.

PTT efficacy was also studied *in vivo* by administration of the FA-cRGD-TNSP NPs (150 µl, 0.5 mg/mL based on SP mass concentration) to the mice via tail vein injection at 5 days post tumour implantation. At 4 h after NP injection, the right frontal hemisphere was irradiated with 808 nm CW laser for 5 min with a power of 0.8 W. The control groups of mice with tumour implantation were untreated or treated with NPs and did not receive any laser irradiation. At 14 days post tumour implantation, the mice were perfused with, 0.1M PBS and sacrificed. Brains were sectioned into 7 µm slices for H&E staining. Stained brain slides were imaged by light microscopy (Leica, Germany).

***In vivo* cortical temperature assessment following PTT**. The photothermal capability of the FA-cRGD-TNSP NPs was assessed *in vivo* using a microprobe thermometer (Ultra fine flexible microprobe IT-24P, Physitemp Instruments, Inc., Clifton, New Jersey, USA) inserted in the brains of tumour-bearing mice. The animals were anesthetized, scalp excised and two burr holes were drilled carefully in the bilateral primary motor cortical regions of the mice (M1 region; ML: +2 mm, AP: 1.5 mm and DV: 2 mm) to facilitate placement of 2 microprobes. We then connected the ultrafine microprobe to a data acquisition system (Thermes USB, Physitemp Instruments, Inc., Clifton, New Jersey, USA) to continuously monitor the cortical temperature. At day 5 post tumour implantation, NPs or saline were injected in 2 groups of tumour-bearing mice. 4 hours post NP administration, the baseline temperature was recorded bilaterally for 5 mins, and the tumour region (right M1 cortex) was subject to PTT by irradiation with 808 nm CW laser for 5 min at a power of 0.8 W. The cortical temperature was recorded continuously to the nearest 0.01°C, every second throughout the treatment and for 5 min posttreatment. The core temperature was monitored and maintained at 37±0.5°C by means of a self-regulating thermal blanket.

Calculation of brain tumour volume. Brain tumour volume was quantified histologically by tracing the tumour margin and the integrated tumour volume was calculated using the ImageJ software (NIH Image). The tumour volume was calculated by adding the tumour regions of all sections and multiplying by the slice thickness.

***In vivo* toxicity study**. Male NCr nude mice weighing 25 - 30 g and 8-10 weeks of age (InVivos Pte Ltd, Singapore) were used to assess toxicity of the FA-cRGD-TNSP NPs. Mice were randomly assigned to 2 groups and each group contained 3 mice. On day 0, the NPs (4 mL/Kg with a NP concentration of 0.5 mg/mL based on SP mass) were intravenously injected into each mouse in one group and mice in the other group were untreated. The normal organs of mice including heart, liver, spleen, lung and kidney were subsequently isolated for histology observations. Briefly, organs on day 30 were fixed in 10% neutral buffered formalin, which were then processed routinely into paraffin, sliced at thickness of 5 μm, and stained with hematoxylin and eosin (H&E). The H&E-stained slices were imaged by optical microscopy (Leica, Germany) and assessed by 3 independent pathologists. Moreover, *ex vivo* fluorescence imaging of important organs from mice sacrificed at different time (4 h, 24 h, 2 days and 10 days) post injection of FA-cRGD-TNSP NPs was conducted on IVIS Spectrum In Vivo Imaging System (PerkinElmer). *Ex vivo* fluorescence imaging of brain from mice sacrificed at different time (4 h, 1 day, 3 days and 7 days) post injection of FA-cRGD-TNSP NPs and PTT treatment was conducted on IVIS Spectrum In Vivo Imaging System (PerkinElmer).

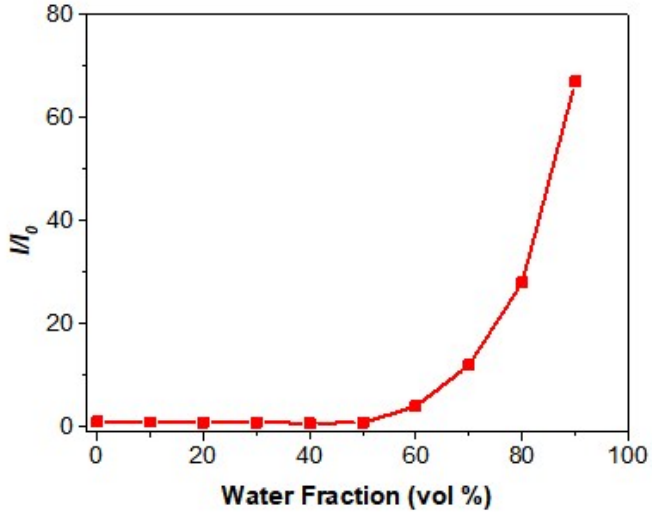

Figure S1. Fluorescence intensity evolution of TPETPAFN in water/THF mixture with different water concentration, where I_0 is the fluorescence intensity in pure THF and I is that in different water/THF mixtures, respectively.

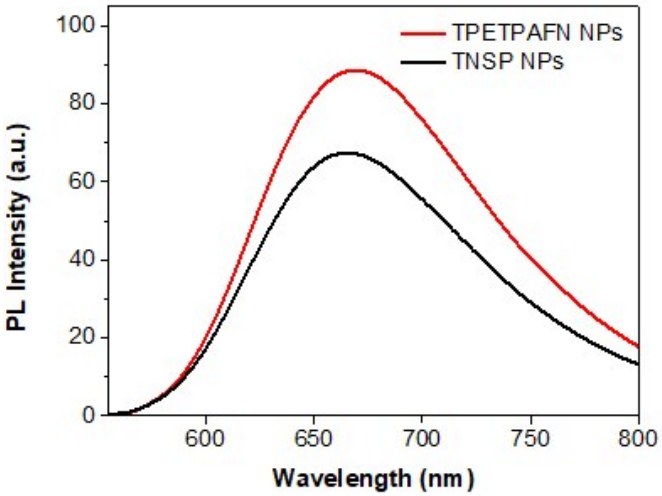

Figure S2. PL spectra of TPETPAFN NPs and TNSP NPs with same concentration of TPETPAFN (0.01 mg/mL) in aqueous media.

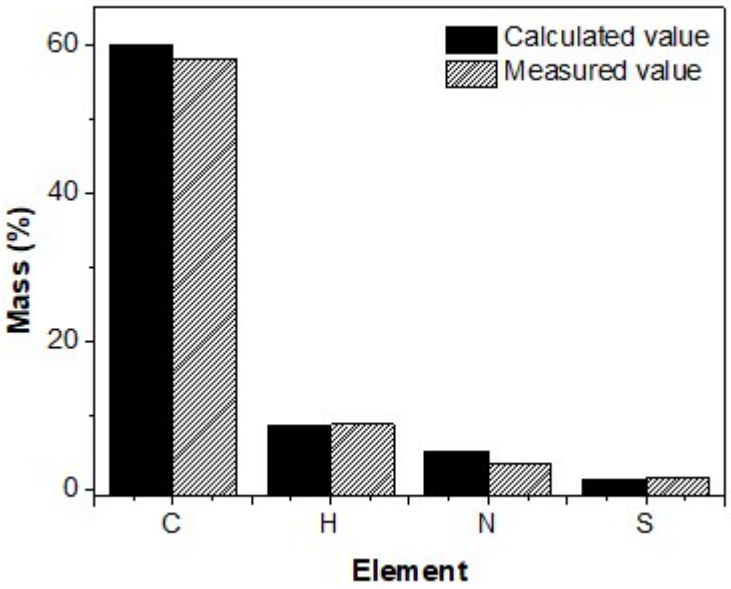

Figure S3. Elemental analysis of TNSP NPs.

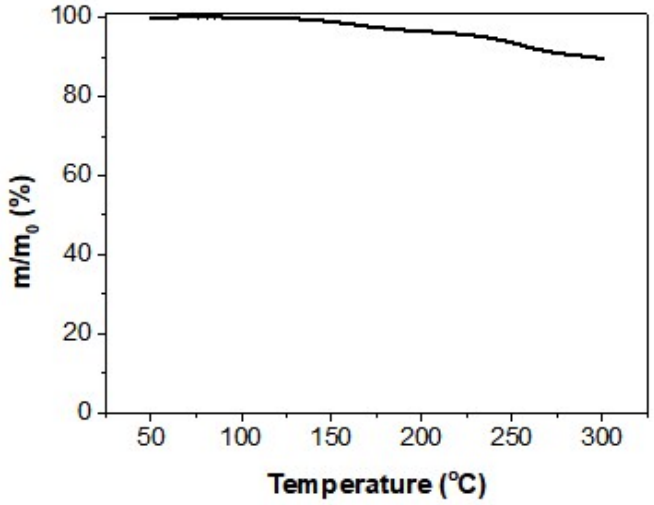

Figure S4. Thermogravimetric analysis of TNSP NPs.

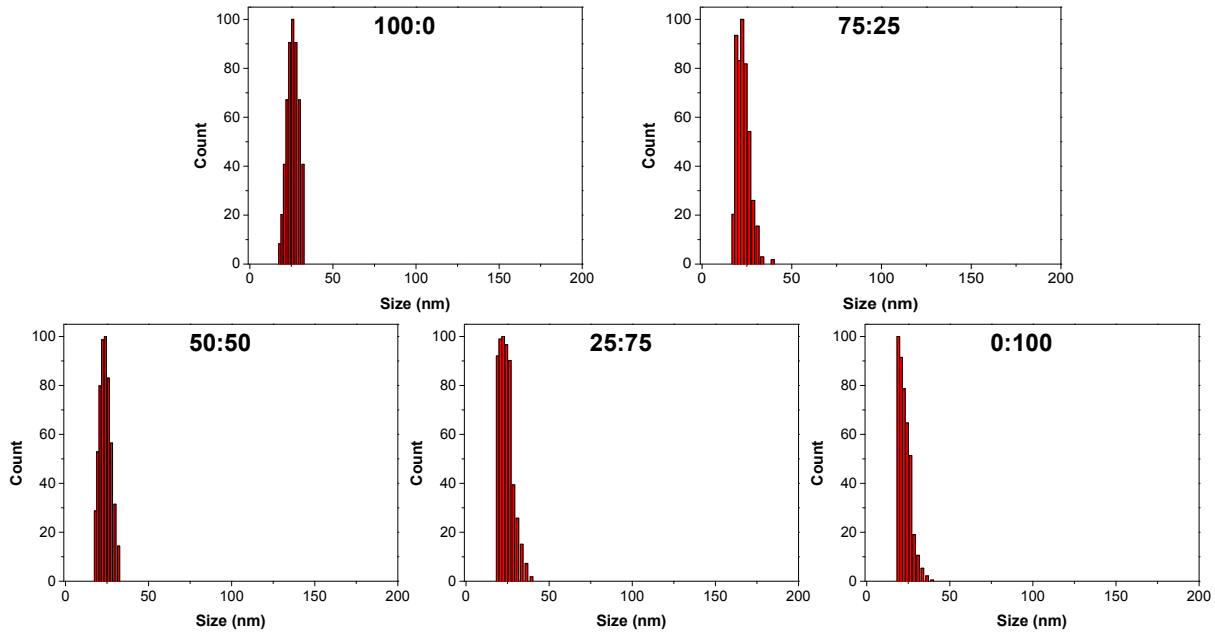

Figure S5. LLS size distributions of TNSP NPs with different ratio of FA: cRGD.

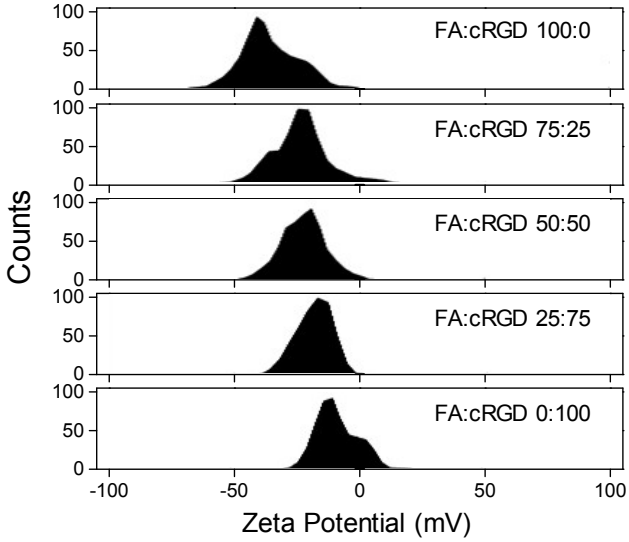

Figure S6. Zeta potentials of TNSP NPs with different surface functionalization.

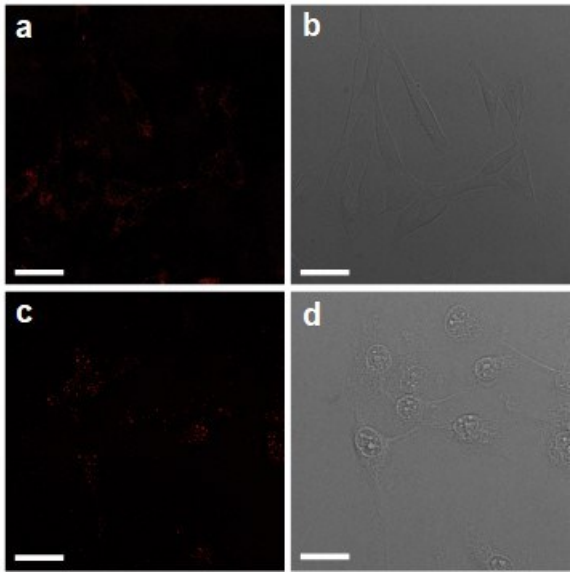

Figure S7. Confocal images of NIH/3T3 normal cells (a, b) and U87MG GBM cells (c, d) after incubation with PEG-TNSP NPs for 4 h at 37 °C. Excitation: 488 nm. Emission: 620 nm - 750 nm. Scale bars: 50 μm.

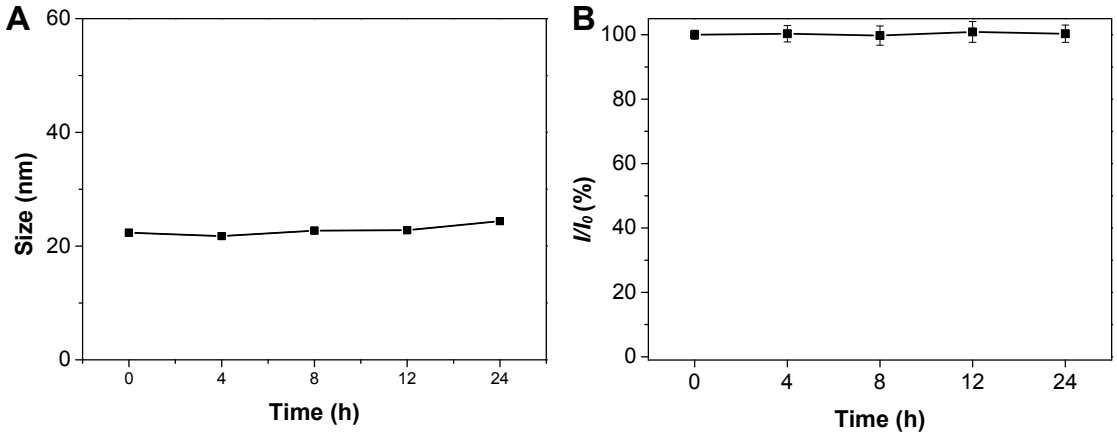

Figure S8. Size change (A) and fluorescence intensity change (B) of TNSP NPs after incubation in 1 × PBS buffer with human serum.

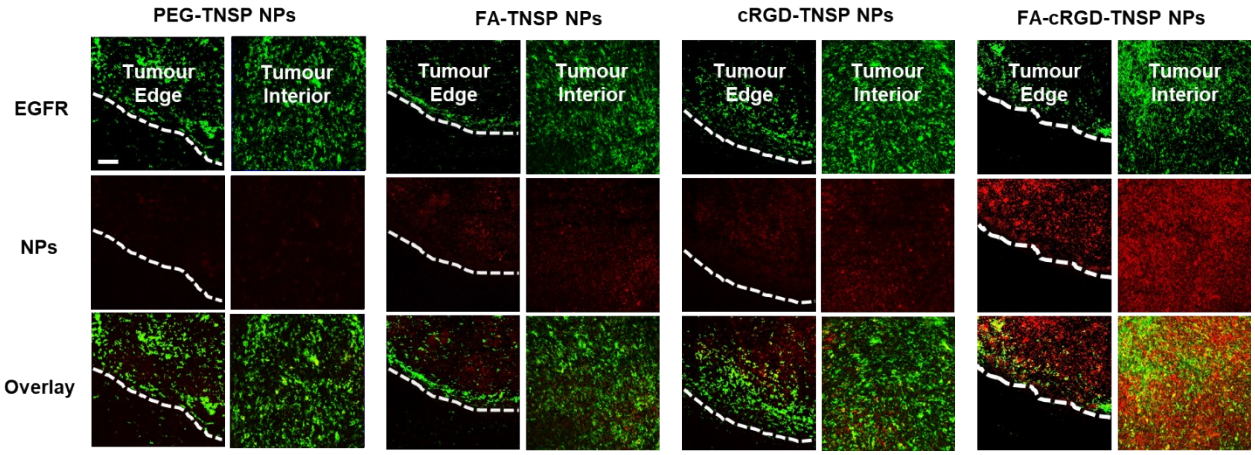

Figure S9. Confocal images of brain slices at 7 days post tumour implantation after administration of TNSP NPs with different surface functionalization for 4 h. The brain slices were immunostained by primary anti-EGFR antibody and secondary antibody conjugated with Alexa Fluor® 488. Scale bar: 150 μm.

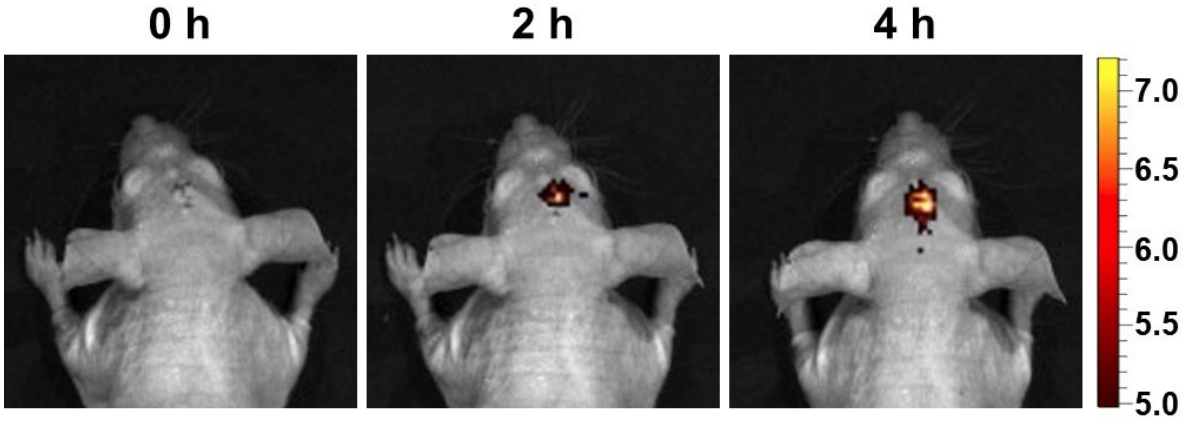

Figure S10. In vivo fluorescence images of mice bearing GBM after FA-cRGD-TNSP NP injection.

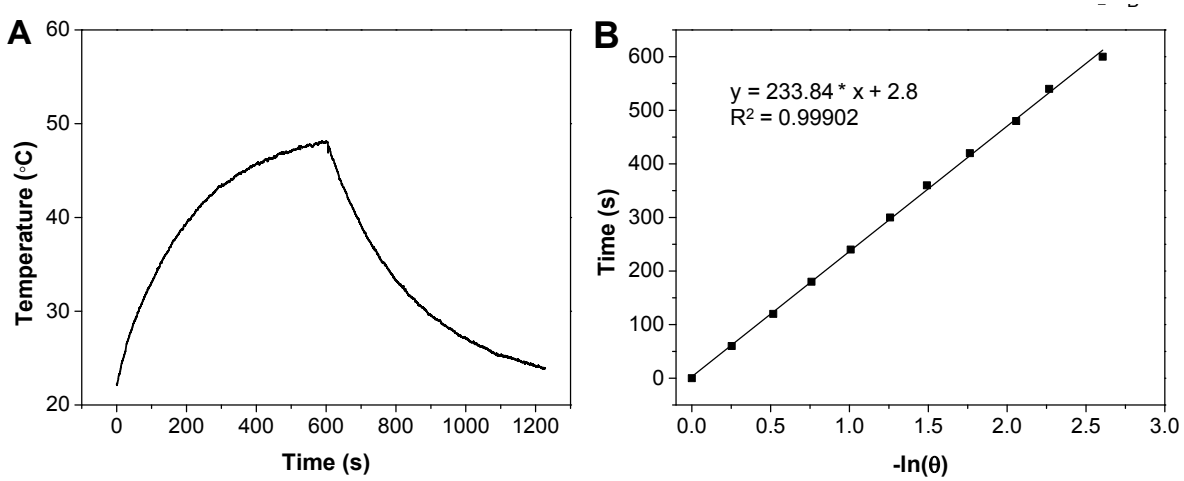

Figure S11. (A)Temperature evolution of TNSP NPs under 808 nm laser irradiation (0.8 W, 10 min) and cooling process after the treatment. (B) Plot of time starting from the cooling stage versus negative natural logarithm of drive force temperature. The slop represents the sample system time constant τ_s.

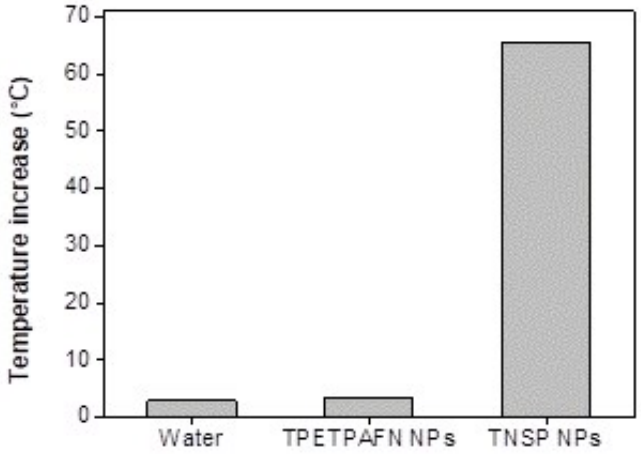

Figure S12. Temperature elevation of water, TPETPAFN NPs, FA-cRGD-TNSP NPs at the concentration of 10 μg mL^{-1} upon 808 nm laser irradiation at a power of 0.8 W for 10 mins.

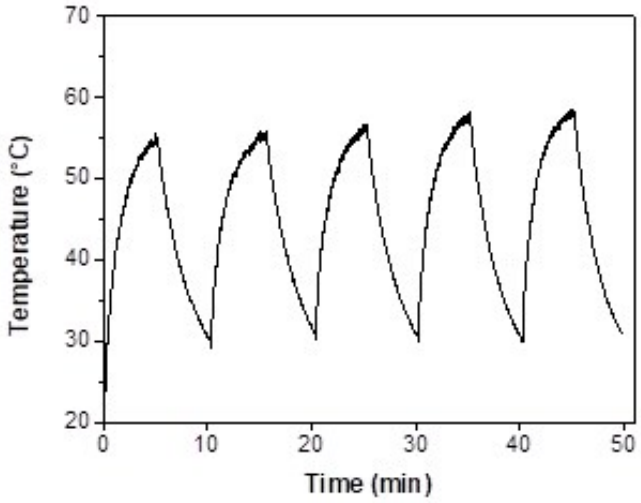

Figure S13. Temperature elevation of TNSP NPs at the concentration of 2.5 μg mL^{-1} over five laser ON/OFF cycles of 808 nm laser irradiation.

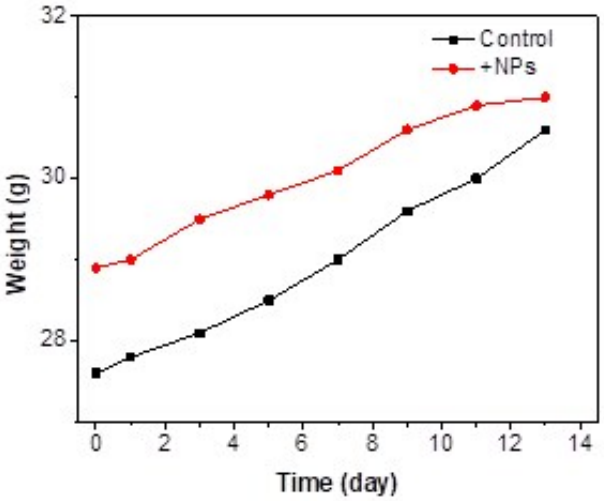

Figure S14. Body weight of mice with or without the administration of FA-cRGD-TNSP NPs.

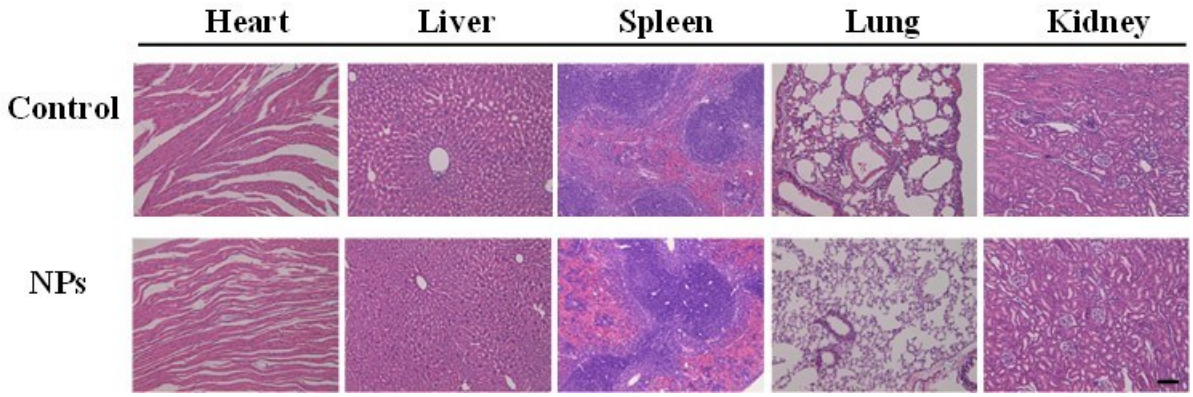

Figure S15. Typical images of H&E-stained heart, liver, spleen, lung and kidney slices from mice on day 30 post injection of FA-cRGD-TNSP NPs. The scale bars in the images are 15 μm.

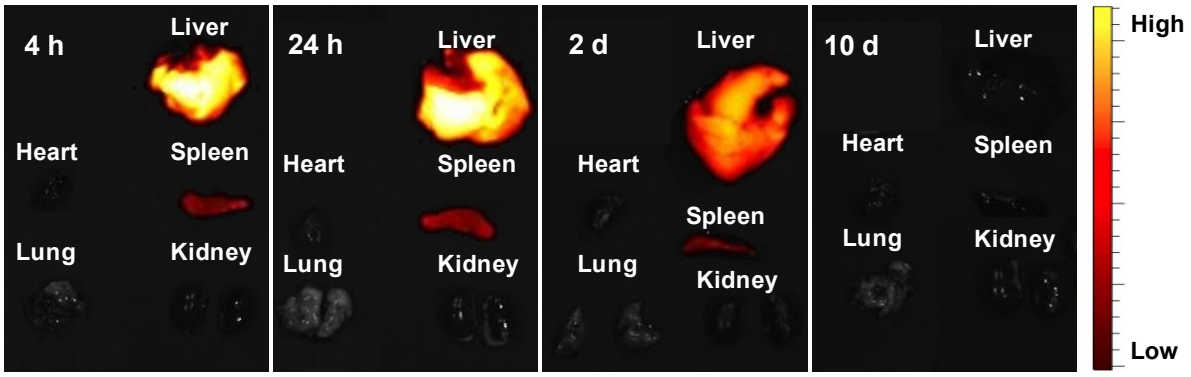

Figure S16. *Ex vivo* fluorescence images of the internal organs from mice sacrificed at different time (4 h, 24 h, 2 days and 10 days) post-injection with FA-cRGD-TNSP NPs.

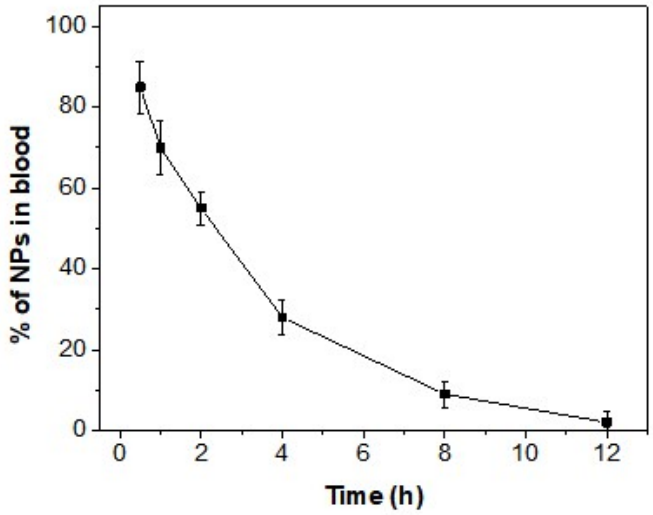

Figure S17. Blood circulation curve of FA-cRGD-TNSP NPs after injection.

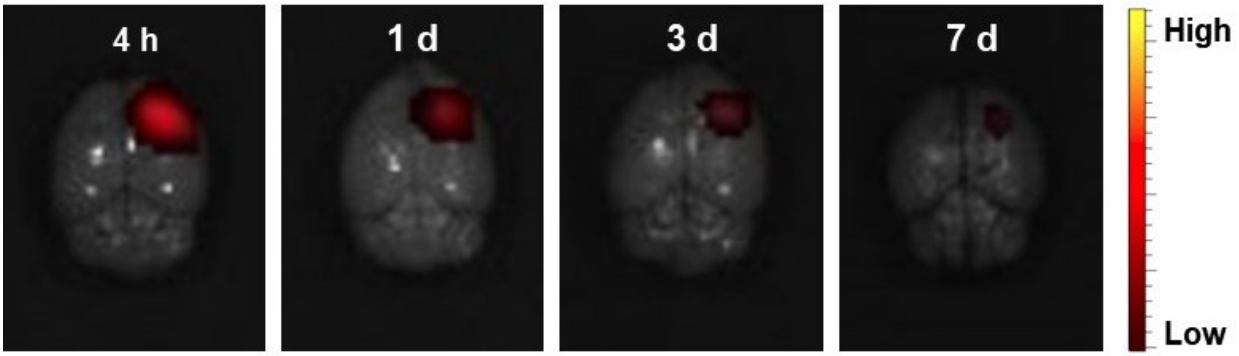

Figure S18. *Ex vivo* fluorescence images of brain from mice sacrificed at different time (4 h, 1 day, 3 days and 7 days) post-injection with FA-cRGD-TNSP NPs.

References

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