Supporting Information

Targeted Brain Tumor Imaging by Using Discrete Biopolymer-Coated Nanodiamonds across the Blood Brain Barrier

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KEYWORDS

Nanodiamond • blood brain barrier • bioimaging • glioblastoma • targeting

1. Experimental Section

Materials bEnd.3, C8-D1A and U-87 MG were purchased from American Type Culture Collection (ATCC). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), retinoic acid, streptavidin and urea were purchased from J&K Scientific. Fluorescein (FAM) maleimide, 6-isomer and cyanine 7 (Cy7) maleimide were purchased from Lumiprobe. Amicon® ultra 15 mL centrifugal filters (30K and 100K). Falcon permeable support for 24-well with 1 μm transparent PET membrane was purchased from Corning Life Science. Monocystalline Diamond (ND) powder with MSY 0-0.05micro was purchased from Microdiamant. Adenosine 3',5'-cyclic monophosphate (cAMP), bovine serum albumin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), disodium hydrogen phosphate, ethylenediamine dihydrochloride, fluorescein isothiocyanate–dextran (average molecular weight: 10,000 Da), glucose, hydrochloric acid, hydrocortisone, insulin-transferrin-sodium selenite media supplement, nitric acid, perchloric acid, phosphodiesterase inhibitor, poly-L-lysine, sodium acetate, sodium chloride, sodium hydroxide, sodium phosphate monobasic dihydrate, sulfuric acid, tris(2-carboxyethyl)phosphine (TCEP) and trifluoroacetic acid were purchased from Sigma Aldrich. Ham's F-12 medium, fetal bovine serum (FBS), fibronectin human protein, L-glutamine, Dulbecco's Modified Eagle Medium (DMEM), penicillin streptomycin solution, phosphate buffered saline (PBS) and trypsin containing 0.25% EDTA were purchased from Invitrogen. Glutaldehyde, formaldehyde, osmium tetraoxide, Spurrs's resin were purchased from Electron Microscopy Sciences. Biotinylated polyethylene glycol succinimdyl valerate (MW: 5000) was purchased from Laysan Bio Products. Biotinylated RGD peptide was purchased from 1st BASE.

Preparation of carboxyl-NDs 50 mg of pristine nanodiamonds (NDs) were acidified by refluxing with a 1:1:1 (v/v) mixture of sulphuric acid, nitric acid and perchloric acid at 110 ^{o}C

for 24 h. The acidified NDs were washed with deionized water three times after the oxidation, and then refluxed with 0.1 M of NaOH at 110 ^{o}C for 24 h. In the final step, the alkaline-treated NDs were heated with 0.1 M of HCl at 110 ^{o}C under reflux for 24 h. The carboxyl-NDs were washed by water three times and the final product obtained was dissolved in 5 mL of water. The final concentration of carboxyl-NDs obtained was 10 mg/mL.

Chemical synthesis of modified BSA 150 mg of BSA was added into 1.2 mM of ethylenediamine dihydrochloride solution with pH 4.75 (50 mL), followed by 800 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and stirred for 15 min. 4 M of sodium acetate buffer with pH 4.75 (2 mL) was added to terminate the reaction. The cationized BSA (cBSA) solution was then concentrated and purified with autoclaved water five times using protein ultra-centrifugal filters 30K. 50 mg of cBSA was dissolved in degassed 50 mM phosphate buffer with pH 8 (50 mL), then 100 mg of NHS-PEG-Biotin was added and stirred for 2 h at room temperature. cBSA-PEG was washed by autoclaved water five times with the use of protein ultra-centrifugal filters. 50 mg of cBSA-PEG was added to 50 mM phosphate buffer containing 5 M of urea and 2 mM of EDTA (50 mL) followed by 1 mg of TCEP and stirred for 4 h. Next, FAM-maleimide/Cy-7 maleimide was added to the reaction mixture and stirred overnight at room temperature. After reaction, dye labelled protein was washed with water and concentrated to ~10 mg/mL by ultra-centrifugal filters.

Protein solution in concentration of 0.5 mg/mL was mixed with sinapinic acid matrix (sinapinic acid was saturated in 1:1 of water and ACN containing 0.3 % of trifluoroacetic acid) in a 1:1 ratio. The protein was crystalized on a metallic plate, and the mass was determined by ABI 4800 Plus MALDI TOF/TOF Analyzer. 1 mg of dried protein was mixed with 49 mg of KBr solid and ground into fine powder. Samples containing KBr pellets were analyzed by Fourier-transform infrared spectrometer (Perkin Elmer Spectrum 100). The signal from a pure KBr pellet was subtracted as background.

Synthesis and characterization of fluorophore-labeled RGD-dcBSA-PEG-NDs carboxyl-NDs were firstly diluted by water to 0.1 mg/mL, then they were slowly added drop-by-drop to 40 mg of cBSA-PEG-Dye; the mixture was then stirred overnight. After reaction, excess water was removed by amicon ultra-centrifugal filters 100K; Fluorophore-labeled PEG-dcBSA-NDs were washed with water to remove the unbound protein and finally dissolved in 1 X PBS for storage. Fluorophore-labeled dcBSA-PEG-NDs were added into 2 mg/mL of streptavidin and incubated for 4 h. Excess streptavidin was removed by centrifugation, and the NDs were washed with water three times. The NDs were re-suspended in 1 X PBS. Streptavidin conjugated NDs (SA-dcBSA-PEG-NDs) were added to 1 mg/mL of biotinylated RGD peptide and incubated overnight. Excess peptide was removed by centrifugation, and the NDs were finally re-suspended in 1 X PBS.

100 μL of purified FAM-labeled dcBSA-PEG-NDs and carboxyl-NDs (10 mg/mL) were dried by a speed vacuum concentrator. The mass of dried NDs was determined by a five decimal place analytical balance; the mass of protein coated on ND surface was calculated by the difference in mass between FAM-labeled dcBSA-PEG-NDs and carboxyl-NDs.

The size and zeta potential characterizations of NDs were determined by Malvem Zetasizer Nano ZS. 50 μg of NDs was suspended in 1 mL of solvent, water, 1X PBS, 1 M of NaCl, 10 mM HEPES containing 0.1 % of BSA and DMEM containing 10 % of FBS respectively. The refractive index of carbon (2.42) was chosen as a reference to calculate the size distribution of NDs in terms of intensity.

Cell Cultivation bEnd.3, C8-D1A and U-87 MG were cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % of FBS and 1 % of penicillin with streptomycin. Cells were incubated at 37 ^{o}C in a humidified 5% carbon dioxide atmosphere. When the confluency

reached above 90 %, cells were sub-cultured into one third of population to maintain healthy growth.

***In vitro* Blood-Brain Barrier (BBB) Model Setup** The setup of in vitro BBB followed the procedures as in previous researches.[1,2] The abluminal sides of cell insert was coated with poly-L-lysine by addition of 5 μg/cm^2 of solution and dried. 5 μg/cm^2 of fibronectin was then added to the luminal side and dried. 12,000 C8D1A cells were seeded on the abluminal side of insert and incubated for 2 days. 20,000 bEnd.3 cells were cultured on the luminal side for 5 days. The culture medium was changed to enhanced medium (DMEM:Ham's F-12 (1:1) supplemented with 2 mM L-glutamine, 550 nM hydrocortisone, 312.4 μM cAMP, 17.5 μM phosphodiesterase inhibitor, 1 μM retinoic acid, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite and 1% penicillin with streptomycin) 2 days after bEnd.3 was seeded. The experiment was performed on day 5.

MTT Assay 20,000 cells were seeded on a 96-well plate and incubated overnight. FAM-labeled RGD-dcBSA-PEG-NDs were added to the cells in different concentrations (25, 50, 100, 500 and 1000 μg/mL) and incubated for 24 h. Cells were then incubated with fresh medium containing 0.5 mg/mL MTT for 2 h at 37 °C for the cytotoxicity assay. After incubation, the medium was removed and the solution of DMSO and ethanol (1:1) was added. The absorbance at 570 nm was measured using a Biotek Powerwave xs Microplate Reader.

***In vitro* BBB Permeability Test** Solutions in both luminal and abluminal compartments were changed from enhanced medium to 10 mM of HEPES buffer which contained 4.5 % glucose with 0.1 % BSA. NDs with a concentration of 500 μg/mL were added to the luminal compartment and incubated at 37 ^{o}C. At each time point (30, 60, 90, 120, 180, 240, 300, 360, 420 and 480 min), 100 μL of buffer was extracted from abluminal compartment for fluorescence measurement, then 100 μL of fresh buffer was replaced in the abluminal compartment. For permeability of sodium fluorescein, 1 μM of solution was added to the

luminal compartment. Fluorescence intensity of sample solutions were analyzed by HORIBA Jobin Yvon FluorMax-4 spectrofluorometer.

Confocal Laser Fluorescence Imaging 20,000 cells were seeded on a glass-bottom confocal dish and incubated overnight. FAM-labeled RGD-dcBSA-PEG-NDs were added to cells to the final concentration of 500 μg/mL and incubated for 24 h. Medium was removed after incubation and replaced with fresh medium. 20,000 U-87 MG cells were seeded on a 24 well-plate and incubated overnight for *in vitro* BBB permeability study. FAM-labeled RGD-dcBSA-PEG-NDs in concentration of 500 μg/mL were added to luminal compartment of transwell insert and incubated for 24 h. After incubation, insert was removed, and the U-87 MG cells were tryspinized to seed on a glass-bottom confocal dish. Before imaging, the cells were stained with 1.62×10^{-5} M of Hoechst 33342 for 15 min, then washed three times with 1X PBS. Imaging was performed by a Leica SP5 confocal laser scanning microscope. The excitation wavelength of Hoechst dye is 405 nm; emission was collected from 430-470 nm. The excitation wavelength of FAM dye is 488 nm; emission collection range was from 500-550 nm. The excitation wavelength of ND is 633 nm; emission was collected at 650-750 nm.

Fluorescence-activated Cell Sorting (FACS) 200,000 cells were seeded on a 6-well plate and incubated overnight. FAM-labeled RGD-dcBSA-PEG-NDs were added to the cells at a concentration of 500 μg/mL and incubated for 24 h. To compare the uptake efficiency of NDs with and without targeting ligand by U-87 MG, FAM-labeled RGD-dcBSA-PEG-NDs and FAM-labeled dcBSA-PEG-NDs were added to the cells at a concentration of 500 μg/mL and incubated for 1 h. For *in vitro* BBB permeability study, procedures are same as in confocal imaging experiment. After incubation, cells were trypsinized and re-suspended in 1X PBS. Emission was collected by FITC channel using FACSCalibur flow cytometer. 10,000 events were recorded per sample. The geometric mean value was taken for calculation to represent fluorescent intensity.

TEM Imaging 40,000 U-87 MG cells were seeded on a coverslip overnight and placed at the bottom of abluminal chamber in *in vitro* BBB model setup. 500 μg/mL of FAM-labeled RGD-dcBSA-PEG-NDs were added to the luminal compartment of transwell insert. After 24 h of incubation, U-87 MG cells were transferred to a glass vial and fixed in primary fixative solution of 2.5 % glutaraldehyde and 2 % paraformaldehyde for 2 h at room temperature. Then, the cells were fixed in 1 % osmium tetroxides with 1.5 % potassium ferrocyanide solution in the dark for 2 h at room temperature. After each fixation, the cells were rinsed with 1X PBS buffer and then dehydrated by graded ethanol (30 % to 100 %), followed by 100 % acetone. Dehydrated cells were infiltrated by Spurr's resin. The cells were embedded in resin and baked at 70 ^{o}C for 2 days. Ultra-thin sections from the baked samples were collected on copper grids then stained with uranyl acetate and lead citrate solutions. TEM imaging was conducted by transmission electron microscope (Philips Technai 12). The procedures above were repeated in tumor harvested from brains of mice after *ex vivo* imaging.

***In vivo* and *Ex vivo* Imaging** NOD-SCID mice with an average weight of 20 g were purchased from the Laboratory Animal Research Unit of the City University of Hong Kong. All procedures were approved by the Animal Research Ethics Sub-Committee of City University of Hong Kong and carried out according to the HKSAR Animal (Control of Experiments) Ordinance. Intracranial glioblastoma-bearing mice were prepared by injecting 150,000 (75,000/μL) U-87 MG cells into the right cerebrum (1.2 mm posterior to bregma, 1.0 mm lateral from bregma; 4.0 mm below the cortical surface) of each animal. Six weeks after injection, T2-weighted MRI images (Bruker BioSpec 3T System) were taken to detect and visualize the brain tumor in each mouse. Mice then received a single intravenous tail vein injection of Cy7-labeled dcBSA-PEG-NDs (n=4), Cy7-labeled RGD-dcBSA-PEG-NDs (n=4); doses were matched at 8.75 mg/kg. Mice injected with 1X PBS served as the control group (n=3). In vivo imaging was then performed 24 hr after injection. After imaging, mice were

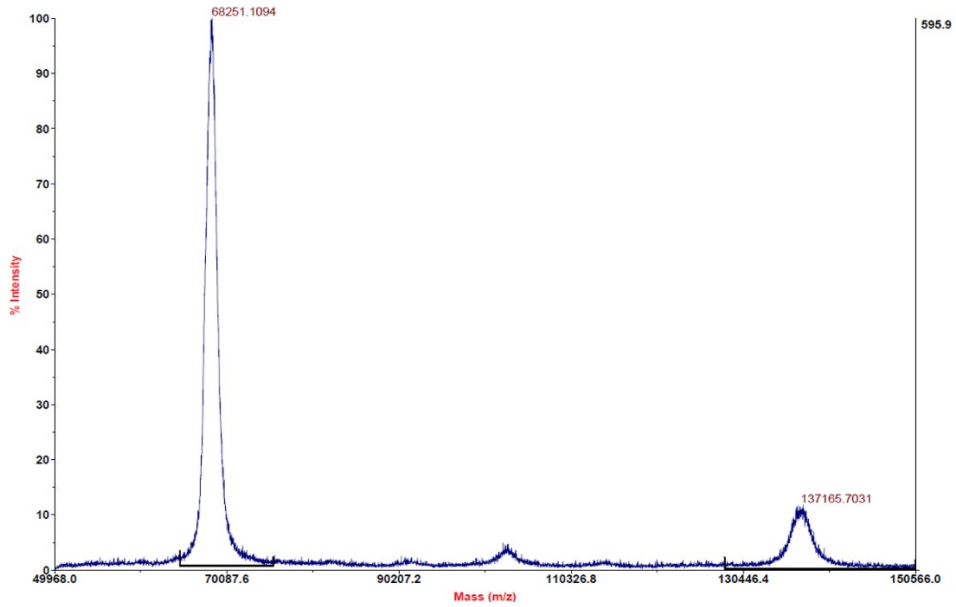
humanely euthanized to harvest their organs for *ex vivo* imaging. The mouse brains were fixed in 10 % formalin and 2.5 % glutaraldehyde, while other tissues were fixed in 10% formalin. All fluorescence imaging were performed using the Bruker In-Vivo Xtreme II imaging system (Bruker, Billerica, MA, USA). The excitation wavelength of Cy 7 is 750 nm and emission was collected at 830nm.

Hematoxylin and Eosin (H&E) Staining The tissues were immersed in 30 % sucrose before sectioning in a cryostat. 20µm thick slices were collected. Tissue slices were stained with Hematoxylin and Eosin (ab245880) according to the manufacturer's instructions. Briefly, rehydrated sections were first stained with Hematoxylin, Mayer's (Lillie's Modification) for 5 min, followed by incubation in Bluing Reagent for 15 s. The slices were then immersed in absolute ethanol, followed by incubation in Eosin Y Solution (Modified Alcohol) for 3 mins. Finally, the slices were rinsed with absolute alcohol and mounted on glass slides and coverslipped for imaging.

Statistical Analysis: All the results were presented as mean ± standard deviation where applicable. Statistical analysis was conducted by one-way ANOVA with Tukey post hoc test using SPSS Statistics software.

2. Supplementary data

a



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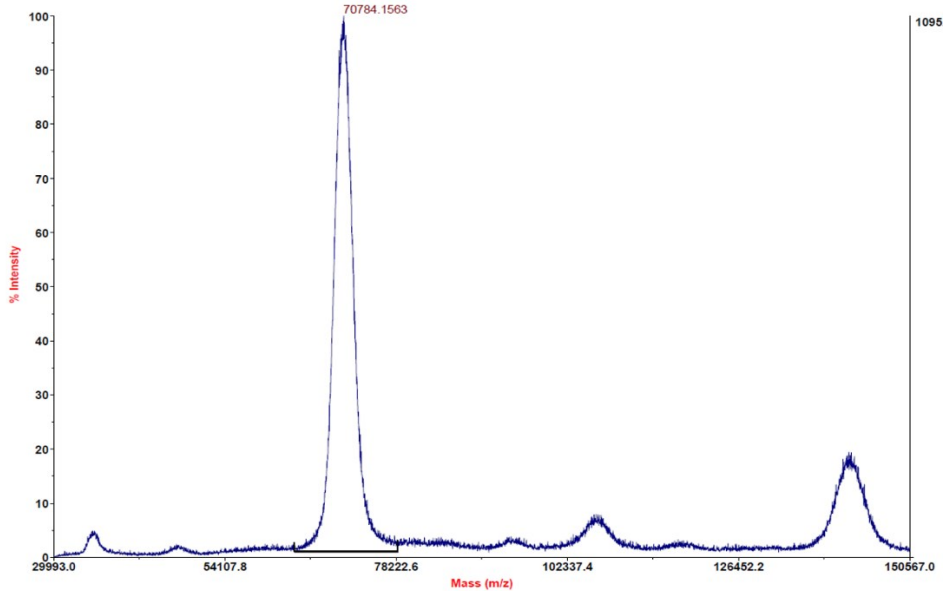

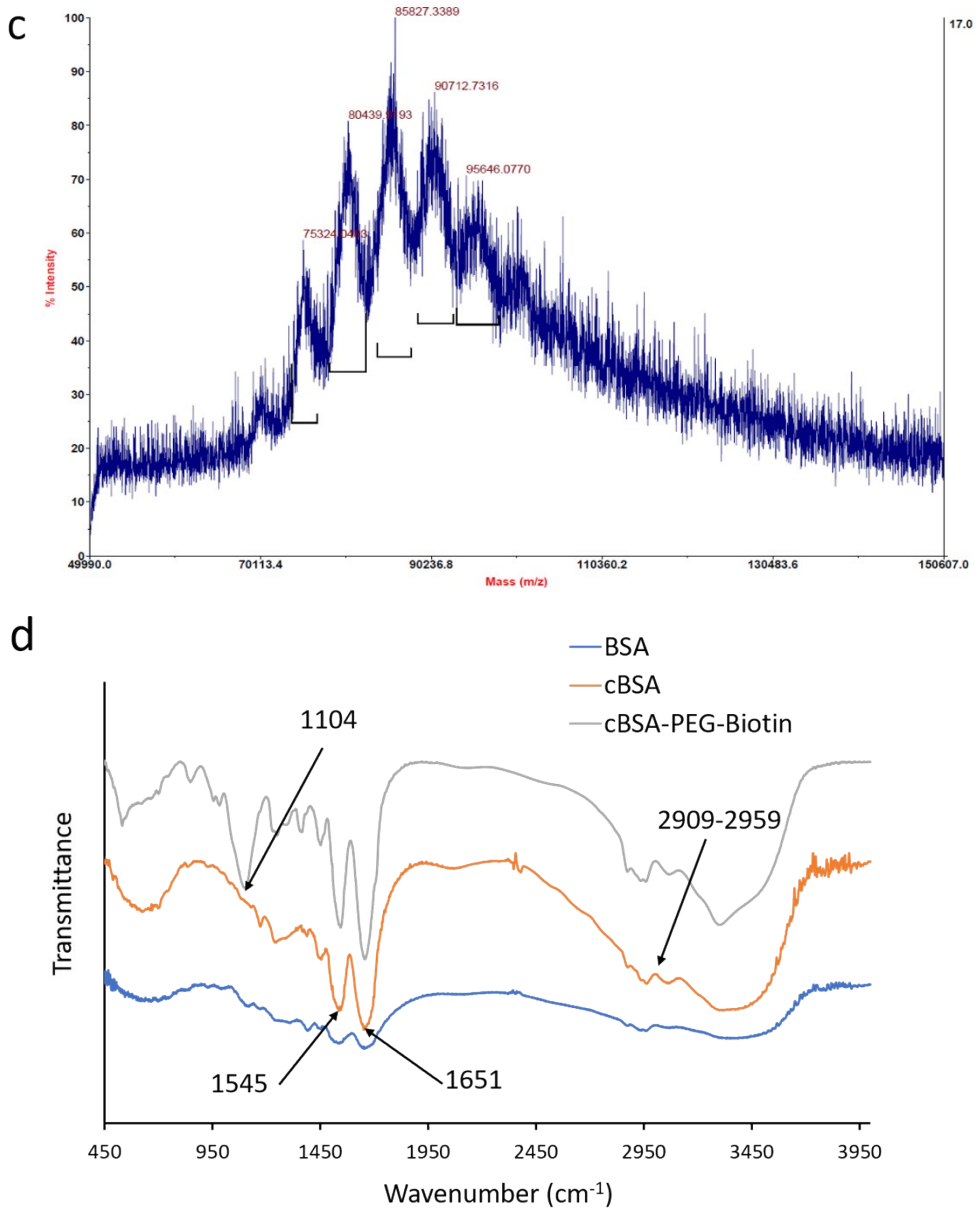

Figure S1. MALDI-TOF spectra of a) raw BSA with m/z peak at 68251.1094 [M^+], b) cBSA with m/z peak at 70784.1563 [M^+] and c) cBSA-PEG with m/z peaks at 75324.0483-95646.0770 [M^+]. (d) Fourier-transform infrared spectra of BSA, cBSA and cBSA-PEG-Biotin.

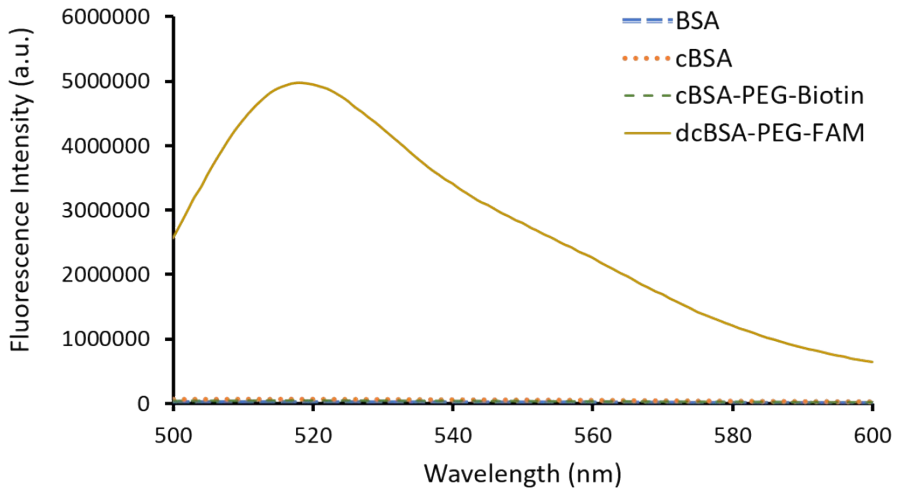

Figure S2. Fluorescence spectra of BSA after different stages of modification.

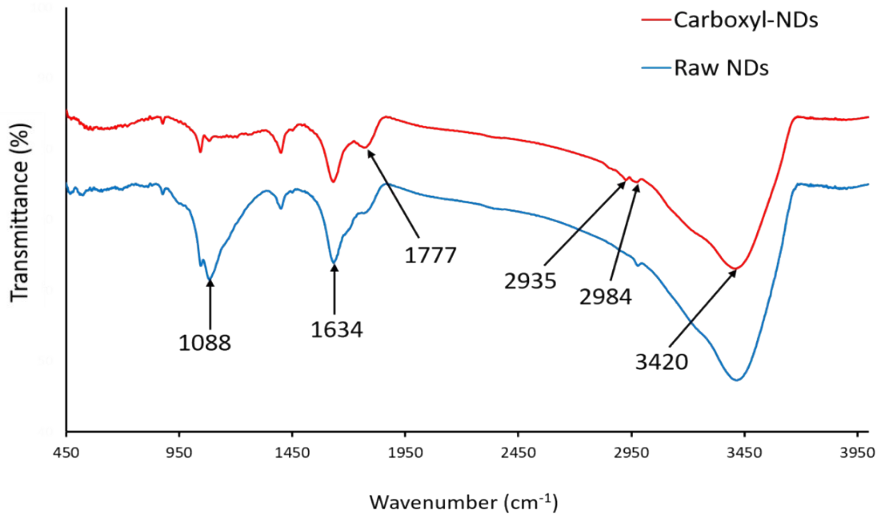

Figure S3. IR spectra of raw NDs and carboxyl-NDs.

Solvent	FAM-labeled dcBSA-PEG-NDs		FAM-labeled RGD-dcBSA-PEG-NDs		FAM-labeled Aggreagated NDs	
	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
Water	62.96 ± 0.61	0.119 ± 0.014	73.28 ± 0.31	0.090 ± 0.012	60.19 ± 0.74	0.162 ± 0.014
1X PBS	66.27 ± 0.48	0.094 ± 0.012	76.57 ± 0.78	0.060 ± 0.015	60.52 ± 1.69	0.135 ± 0.025
1M NaCl	65.63 ±	0.095 ±	78.25 ±	0.083 ±	242.7 ±	0.388 ±

	0.06	0.007	1.65	0.001	19.07	0.088
10mM HEPES with 0.1% BSA	66.09 ± 0.07	0.140 ± 0.003	79.23 ± 2.32	0.148 ± 0.014	724.5 ± 69.8	0.299 ± 0.094
DMEM with 10% FBS	62.41 ± 0.67	0.313 ± 0.001	71.20 ± 0.94	0.283 ± 0.016	1392 ± 436	0.752 ± 0.218

Table S1. Hydrodynamic diameter and PDI values of FAM-labeled dcBSA-PEG-NDs, FAM-labeled RGD-dcBSA-PEG-NDs and FAM labeled aggregated NDs in different media. Data are represented as the mean ± standard deviation (n=3).

Sample	1st trial	2nd trial	3rd trial
FAM-labeled dcBSA-PEG-NDs	63.52 nm	62.31 nm	63.05 nm
FAM-labeled SA-dcBSA-PEG-NDs	71.52 nm	71.62 nm	74.24 nm
FAM-labeled RGD-dcBSA-PEG-NDs	73.45 nm	72.93 nm	73.47 nm

Table S2. Hydrodynamic diameter of different ND samples in water.

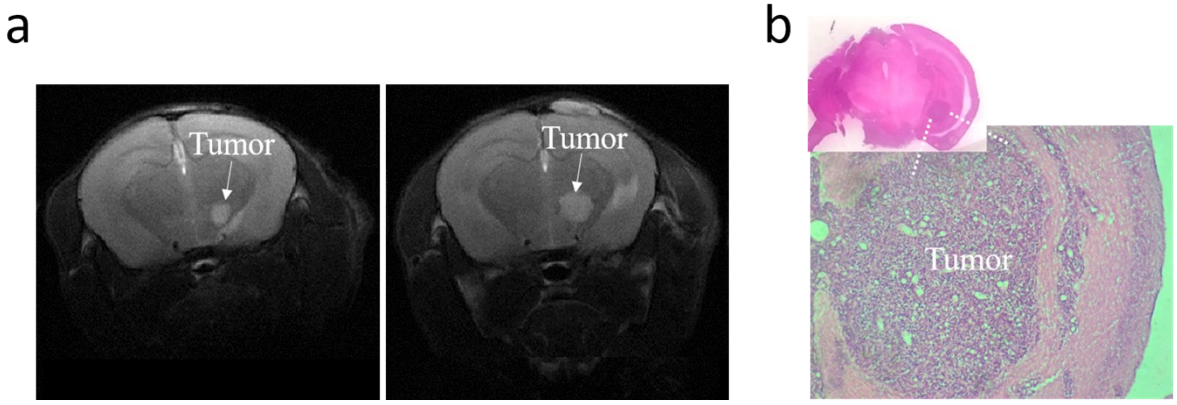

Figure S4. (a) MRI images of glioblastoma-bearing mice six weeks after injection. (b) Images of 20-μm-thick mouse brain slices from Cy7-labeled RGD-PEG-NDs treated mice stained with H&E.

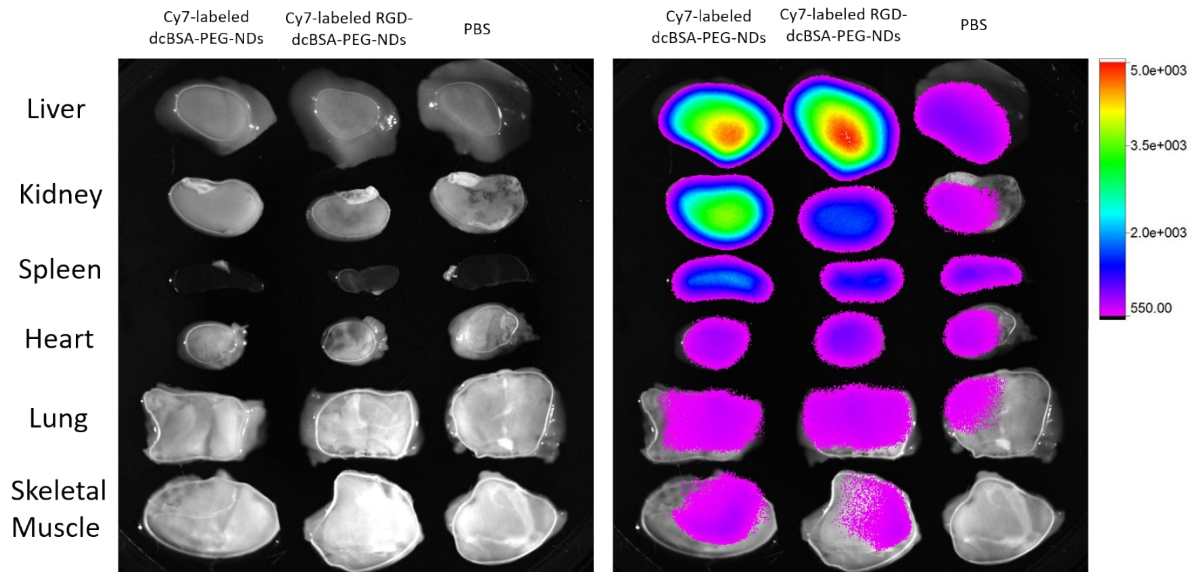

Figure S5. *Ex vivo* fluorescence imaging of different organs in mice injected with Cy7-labeled dcBSA-PEG-NDs, Cy7-labeled RGD-dcBSA-PEG-NDs and PBS. Left is the reflectance image; right is an overlay of the fluorescence and reflectance images.

Reference

1. G. Li, M. J. Simon, L. M. Cancel, Z.-D. Shi, X. Ji, J. M. Tarbell, B. Morrison and B. M. Fu, *Ann. Biomed. Eng.*, 2010, **38**, 2499-2511.
2. D. M. Wuest, A. M. Wing and K. H. Lee, *J. Neurosci. Methods*, 2013, **212**, 211-221.