Supporting Information

Promoting Transcellular Traversal of Blood-Brain Barrier by Simultaneously Improving Cellular Uptake and Accelerating Lysosomal Escape

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List of Materials:

Materials	Supplier names	CAS numbers or catalog numbers
$Cu(NO_3)_2 \cdot 3H_2O$	Aladdin	CAS: 10031-43-3
mercaptoacetic acid	Aladdin	CAS: 68-11-1
succinic anhydride (SA)	Aladdin	CAS: 108-30-5
$Na_2S \cdot 9H_2O$	Macklin	CAS: 1313-84-4
NaOH	Macklin	CAS: 1310-73-2
chitosan quaternary ammonium salt	Macklin	Cat: 850126
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)	Macklin	CAS: 25952-53-8
N-Hydroxysuccinimide (NHS)	Macklin	CAS: 6066-82-6
citraconic anhydride (CA)	Macklin	CAS: 616-02-4
Sulfo-Cyanine 5 carboxylic acid (Cy5-COOH)	Xi'an KaiXin	Cat: K-R-3036
COOH-PEG_{1000}-iRGD	Xi'an ruixi	Cat: HT20230411004
D-Luciferin potassium salt	Beyotime	Cat: ST196
3,3′-dioctadecyloxacarbocyanine perchlorate (DiO)	Beyotime	Cat: C1038
hoechst 33342	Beyotime	Cat: C1028
membrane protein extraction kit	Beyotime	Cat: ST506
phenylmethyl sulfonyl fluoride (PMSF)	Beyotime	Cat: ST507
Dulbecco's modified Eagle's medium (DMEM)	Gibco	Cat: C11995500CP
RPMI 1640 medium	Gibco	Cat:C11875500BT
fetal bovine serum (FBS)	Yeasen	Cat: 40130ES76
penicillin-streptomycin solution (PS)	Yeasen	Cat: WP9405010
PBS (pH 7.4, 10 mM)	Gibco	Cat: C10010500BT

- All chemical reagents were analytical or higher grade, and used without further purification.
- All aqueous solutions were prepared with ultrapure water.
- CAS stands for CAS number. Cat stands for catalog numbers.

Cell Culture and Animals

4T1 cells (mouse breast cancer cells) were obtained from Hunan Fenghui Biotechnology Co.,

Ltd. U87MG cells (human glioblastoma cells), hCMEC/D3 (immortalized human brain microvascular endothelial cells), RAW 264.7 cells (mouse monocyte macrophage cells) and Luciferase-expressing human glioblastoma tumor (U87MG-Luc) were obtained from Procell Life Science &Technology Co., Ltd. (Hubei, China). 4T1 cells were incubated in RPMI 1640 medium supplied with 10 % FBS and 1 % PS. The hCMEC/D3 cells were incubated in endothelial cell culture basal medium supplied with 5 % FBS, 1 % endothelial cell culture supplement and 1 % PS. U87MG cells were incubated in MEM medium containing with 10 % FBS, 1 % antibiotics, and 1 % Non-Essential Amino Acids (NEAA) and 1mM Sodium Pyruvate (SP). U87MG-Luc cells were grown in DMEM medium with 10 % FBS, 1 % antibiotics, and 1 % NEAA, 1% SP and 1 % penicillin and 1ug/ml puromycin. All the cells were incubated at 37 °C in a humidified incubator with 5 % CO_2.

Female BALB/c nude mice and female BALB/c mice (6-7-week-old) were purchased from Wuhan Experimental Animal Center. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and the procedures were approved by the Wuhan University of China Animal Care and Use Committee (SYXK 2015-0027, Wuhan, China).

Synthesis of CuS NPs

The CuS NPs were synthesized according to previous reports with some modification.[1] The aqueous solution of $Cu(NO_3)_2 \cdot 3H_2O$ (50 mg, 150 mL) and 100 μL of mercaptoacetic acid were mixed and the pH of the mixture was adjusted to 9.0 with NaOH. After the mixture was bubbled with nitrogen for 30 min, $Na_2S \cdot 9H_2O$ aqueous solution (170 mg, 150 mL) was added to the mixture solution under stirring at room temperature. After 48 hours, the black-green solution was subjected to centrifugal ultrafiltration (molecular weight cut-off: 10 kDa, 6000 rpm for 5 min) to remove excess agents. Subsequently, a black powder (CuS NPs, 27.3 mg) was obtained by freeze-drying.

Synthesis of CS

The CuS aqueous solution (2 mg/mL, 0.5 mL) was added to the chitosan aqueous solution (2 mg/mL, 0.5 mL), followed by addition of EDC (1 mg) and NHS (1.5 mg) to the resulting mixture. The mixture was then incubated in the dark and stirred for 4 hours at room temperature. Upon centrifugation of the mixture (10000 rpm, 10 min), the supernatant was removed to obtain CS.

Synthesis of iRGD-CS

The Cy5-COOH aqueous solution (1 mg/mL, 0.5 mL), EDC aqueous solution (1 mg/mL, 1 mL) and NHS aqueous solution (1.5 mg/mL, 1 mL) were added to CS aqueous solution (1 mg/mL,

1 mL) and reacted for 2 hours. Subsequently, COOH-PEG_{1000}-iRGD (4 mg) was added to the above mixture and further reacted for 2 hours. The resulting mixture was centrifuged at 13300 rpm for 10 min at 4 °C, and the collected precipitate was dispersed with 1 mL of deionized water to get the solution of iRGD-CS.

Synthesis of CA-iRGD-CS

Citric anhydride (CA) (20 mg) was added to the iRGD-CS aqueous solution (1 mg/mL, 1 mL) and shaken at 37 °C for 12 hours. Then, the mixture was centrifuged (13300 rpm, 10 min) to obtain CA-iRGD-CS. SA-iRGD-CS was synthesized utilizing succinic anhydride (SA) in place of citric anhydride (CA), following the procedure established for the preparation of CA-iRGD-CS.

Preparation of Cell Membrane Coated Nanocarriers

The cell membranes were obtained from 4T1 cells according to previous reports.[2] 4T1 cells were collected and washed with PBS three times, and then were dispersed in membrane protein extraction buffer solutions. After cooling in an ice bath for 15 min, the solution underwent the freezing-thawing cycle three times. Then, the mixture was centrifuged at 4000 rpm for 10 min at 4 °C to collect the supernatant. And the supernatant was further centrifuged at 13.3×1000 rpm for 30 min at 4 °C to obtain the precipitate (cell membrane). Then the precipitate was resuspended in deionized water and stored at -80 °C.

CA-iRGD-CS aqueous solution (0.5 mL, 2 mg/mL) was mixed with the 4T1 cell membranes aqueous solution (0.2 mL, 5 mg/mL) and subjected to sonication for 10 min, resulting in the preparation of CA-iRGD-CS@M nanocarriers. In order to explore pH-responsive property, SA-iRGD-CS@M was obtained by wrapping the 4T1 cell membrane onto SA-iRGD-CS under the same experimental conditions.

Photothermal Conversion Efficiency

The aqueous solution of CS and iRGD-CS in the quartz cells were illuminated by 1060 nm laser (1.5 W cm^{-2}, 15min). Then the laser was turned off and then the temperature went down to the ambient temperature. The photothermal conversion efficiency (η) was calculated according to Equation (1):[3]

$$\eta = \frac{hA(T_{max} - T_{surr}) - Q_{Dis}}{I(1 - 10^{-A_{1060}})} \quad (1)$$

where *h* represented the heat transfer coefficient, A was the surface area of the container, T_{Max} represented the maximum temperature of the samples at 15 min, T_{Surr} was the ambient

temperature, Q_{Dis} represented the heat dissipation from the light absorbed by the solvent and the quartz sample cell, *I* was the laser power (1.5 W cm^{-2}), and A_{1060} is the absorbance of the sample at 1060 nm. The value of *h*A was derived from Equation (2):

$$\tau_s = \frac{m_D c_D}{hA} \quad (2)$$

where τ_s was the system time constant. m_D and c_D were the mass (1.0 g) and heat capacity (4.2 J g^{-1}) of the water. Q_{Dis} represented the heat dissipation from the light absorbed by the water and the quartz sample cell and was calculated according to Equation (3):

$$Q_{Dis} = \frac{c_D m_D (T_{Max} - T_{surr})}{\tau_{s(water)}} \quad (3)$$

where $T_{Max\ (water)}$ was 30.7 °C, $T_{Surr\ (water)}$ was the ambient temperature (22.7 °C) and $\tau_{s\ (water)}$ was 224.8 s. Q_{Dis} was calculated to be 0.15 W.

Cell Cytotoxicity Assay

The cytotoxicity of nanocarriers against various cells (U87MG and hCMEC/D3 cells) was measured by MTT assay. The cells were seeded in 96-well plates at a density of 10000 cells per well and incubated overnight. Subsequently, different nanocarriers at various CuS concentrations (50, 100, 150, 200, 250, 300, 350 and 400 μg/mL) were added to the cells and cultured for further 24 h. 20 μL of MTT (5 mg/mL) was added to each well and incubated for a further 4 h. Finally, the solution in the well plate was completely removed before adding 150 μL DMSO to each well. The absorbance value of each well at 490 nm was measured on the microplate reader. Cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{A_{sample} - A_0}{A_{control} - A_0} \times 100$$

A_{sample} and $A_{control}$ were absorbance recorded for cell cultures incubated with the nanocarriers and MTT, and for the control cell cultures incubated with MTT, respectively. A_0 was absorbance recorded for only cell cultures without the nanocarriers and MTT.

The photothermal toxicity was also measured by MTT assay. U87MG cells were treated with the different nanocarriers (CS and iRGD-CS) (CuS concentration: 100, 200, 300 μg/ml) for 4 hours, washed with PBS three times and irradiated 1060 laser (1.5 W cm^{-2}) for 10 min. After the cells were further incubated for 24 hours, the photothermal toxicity of nanocarriers were detected by the method described above.

In Vitro Cellular Uptake

U87MG cells, hCMEC/D3 cells or RAW 264.7 cells were seeded onto glass-bottom dishes (1×10^5 cells/dish) and incubated for 24 hours. Then the cells were treated with different nanocarriers for 4 hours and washed with PBS three times. The cell nucleus was stained with Hoechst 33342 (5 μg/mL, 10 min). Then the cells were detected by a confocal laser scanning microscope (CLSM, Carl Zeiss LSM880) upon 633 nm laser excitation and the emission was measured in the range of 650-754 nm.

For flow cytometry analysis, the cells were seeded into six-well plates (2×10^5 cells per well) and incubated until the cells attached. Then different nanocarriers were added to the cells and incubated for 4 hours. The cells were washed with PBS three times and digested with trypsin to obtain the cell suspension. The intracellular fluorescence intensity was detected by flow cytometry (BD Accuri C6).

In Vitro BBB Model Studies

The hCMEC/D3 cells at a density of 5.0×10^5 cells/well were seeded in a 12-well Transwell plate with 0.4 μm of mean pore size membrane, 12 mm diameter of culture plate insert (Labselcet® Transwell® polyester membrane cell culture inserts). The TEER values were monitored by a Millicell-electrical resistance system voltohmmeter during the cell culture process. To study the BBB crossing efficiency of the nanocarriers, the medium containing nanocarriers (500 μL, C_{Cy5}=0.21μg/mL) was added to the apical chamber of BBB model, and the fresh medium (1 mL) was added to the basolateral chamber of BBB model. After the BBB model was set in a 37 °C incubator for 12 hours, the medium in the basolateral chamber were collected to measure the fluorescence intensity by using fluorescence spectrometer.

In order to track the distribution of the coating membranes and the released iRGD-CS NPs during the transcytosis process of CA-iRGD-CS@M across the BBB, U87MG cells (10^4 cells/well) were implanted into the basolateral chamber. The coating membranes of these nanocarriers (SA-iRGD-CS@M and CA-iRGD-CS@M) were all labeled with DiO (3,3′-dioctadecyloxacarbocyanine perchlorate, green fluorescence), while iRGD-CS NPs were modified with Cy5 (red fluorescence). After 12 hours of adding nanocarriers to the apical chamber, U87MG cells in the basolateral chamber were collected for CLSM imaging.

In Vivo Fluorescence Imaging

To construct the U87MG intracranial orthotopic glioblastoma mice model, female BALB/c nude mice (20 ± 2g) were anesthetized with 2.0 % isoflurane and the head of mice was

immobilized in a stereotactic instrument. After the skin overlying the cranium was disinfected and incised, a small hole was created in the skull using a bone drill. Then 5.0×10^5 U87MG-Luc cells were injected into the right striatum (right lateral: 2.0 mm, bregma: 1.0 mm, depth: 2.5 mm) of nude mice by using a mouse adaptor. The growth of intracranial glioblastoma cells was monitored by bioluminescence imaging after the mice were intraperitoneally injected with luciferase substrate D-luciferin potassium (7.5 mg/mL in PBS, 125 μL) at 47 mg/kg for 5 min.

The nanocarriers (CA-iRGD-CS, SA-iRGD-CS@M, CA-CS@M, CA-iRGD-CS@M) were injected into the mice by tail vein, and the fluorescence signal in brain was detected with the in vivo fluorescence imaging system (IVIS Spectrum, PerkinElmer). At 12 hours post-injection, mice were euthanized to collect the main organs (heart, liver, spleen, lung, kidney, and brain) for ex vivo fluorescence imaging.

In Vivo PTT

The mice bearing U87MG-Luc orthotopic glioblastoma were divided into seven groups, including PBS without laser, CA-iRGD-CS@M without laser, PBS + laser, CA-iRGD-CS + laser, SA-iRGD-CS@M + laser, CA-CS@M + laser and CA-iRGD-CS@M + laser. The nanocarriers were injected to mice through tail vein for 12 hours. Then the brains of mice were irradiated with laser (1060 nm, 1.5 W cm^{-2}, 10 min). Bioluminescence imaging was used to monitor the anti-tumor effect and was recorded every three days in 12 days treatment. On day 12, mice were sacrificed to collect the brains for hematoxylin and eosin (H&E) staining.

Biosafety Evaluation

The biosafety of the nanocarriers were evaluated using HE staining and blood test. CA-iRGD-CS, SA-iRGD-CS@M, CA-CS@M and CA-iRGD-CS@M (Concentration of CuS is 15 mg/kg) were intravenously injected into the healthy BALB/c mice (n = 3), respectively. PBS-treated mice were set as control. All mice were euthanized on the 14th day after the injection, and major organs (i.e., heart, liver, spleen, lung, kidney and brain) and the blood samples were collected. The major organs were fixed in 4% paraformaldehyde, paraffin embedded, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and finally imaged by an optical microscope. In addition, the levels of liver function indicators (aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)) and kidney function indicators (creatinine (CREA), blood urea nitrogen (UREA) and creatine kinase (CK)) were detected by a blood biochemistry analyzer.

Statistical Analysis

Statistical significance was performed by using Graphpad Prism. *P<0.05, **P<0.01, ***P<0.00l and ****P<0.0001.

List of abbreviations:

Abbreviation	Description
CA	citraconic anhydride
SA	succinic anhydride
CuS	copper sulfide nanoparticles
Cy5-COOH	sulfo-Cyanine5 carboxylic acid
CS	CuS -loaded chitosan
iRGD-CS	CS modified by Cy5-COOH and iRGD
CA-iRGD-CS	CS modified by Cy5-COOH, iRGD and pH-sensitive citraconic anhydride
CA-iRGD-CS@M	CA-iRGD-CS coated with brain metastatic tumor cell membrane
SA-iRGD-CS	CS modified by Cy5-COOH, iRGD and non-sensitive succinic anhydride
SA-iRGD-CS@M	SA-iRGD-CS coated with brain metastatic tumor cell membrane
CA-CS@M	CS was modified by Cy5-COOH and pH-sensitive citraconic anhydride, then was coated with brain metastatic tumor cell membrane
BBB	blood-brain barrier
CNS	central nervous system
BMECs	brain microvessel endothelial cells
PTT	photothermal therapy
NRP-1	neuropilin-1
AST	aspartate aminotransferase
ALT	alanine aminotransferase
ALP	alkaline phosphatase
CREA	creatinine
UREA	blood urea nitrogen
CK	creatine kinase
DLS	dynamic light scattering

SDS-PAGE	sulfate-polyacrylamide gel electrophoresis
CLSM	confocal laser scanning microscopy
FCM	flow cytometry
MFI	mean fluorescence intensity
TEER	trans endothelial electrical resistance
$-\ln\theta$	negative natural logarithm of the temperature driving force
τ_s	time constant for heat transfer
η	photothermal conversion efficiencies
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
H&E	hematoxylin and eosin

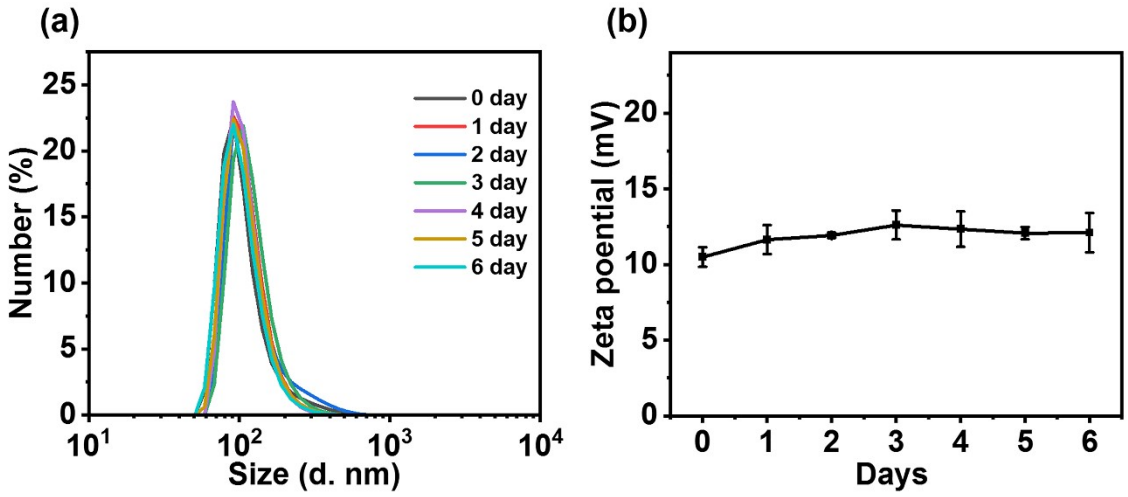

Fig. S1 The changes of the hydrodynamic size distribution (a) and zeta potential (b) of the detached iRGD-CS during the course of 6 days. CA-iRGD-CS@M was incubated in PBS buffer solution (pH 5.0) for 2 hours and centrifuged (4000 rpm, 10 minutes) to collect the precipitate (the detached iRGD-CS). The resulting precipitate (iRGD-CS) was dispersed in 1 ml of water to assess the changes of its hydrodynamic size distribution and zeta potential.

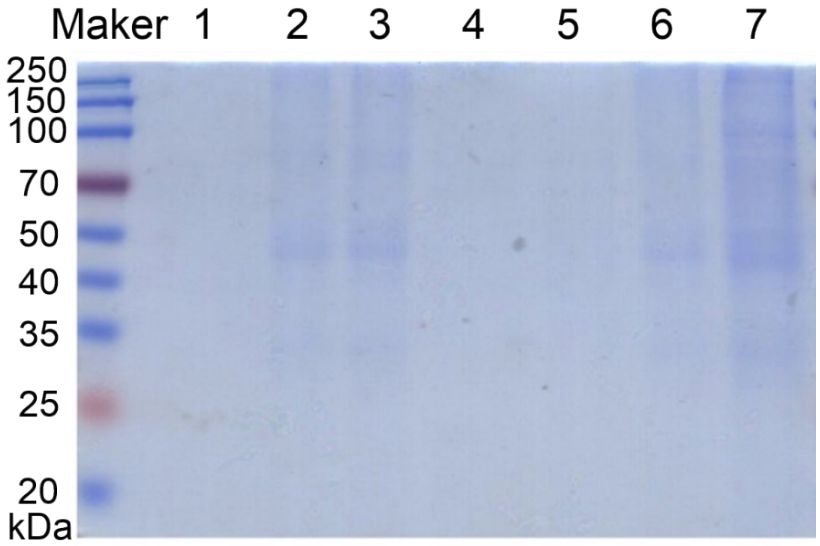

Fig. S2 SDS-PAGE analysis of CA-iRGD-CS@M and SA-iRGD-CS@M. These nanocarriers were incubated in PBS buffer solutions with different pH for 2 hours and centrifuged (4000 rpm, 10 minutes) to collect the supernatants and precipitates. The supernatant (1) and precipitate (2) of SA-iRGD-CS@M in pH 5.0. The supernatant (3) and precipitate (4) of CA-iRGD-CS@M in pH 5.0. The supernatant (5) and precipitate (6) of CA-iRGD-CS@M in pH 7.4. (7): 4T1 cell membrane.

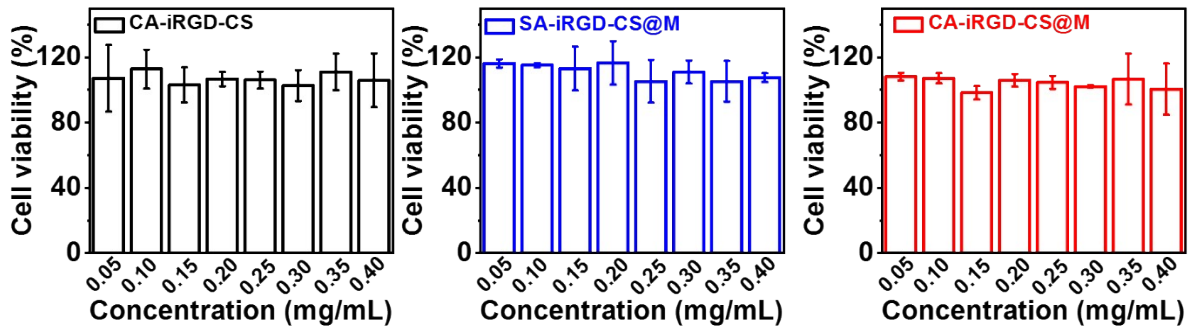

Fig. S3 Cytotoxicity of different nanocarriers against hCMEC/D3 cells after 24 hours incubation. Data are given as the mean ± SD (n = 6).

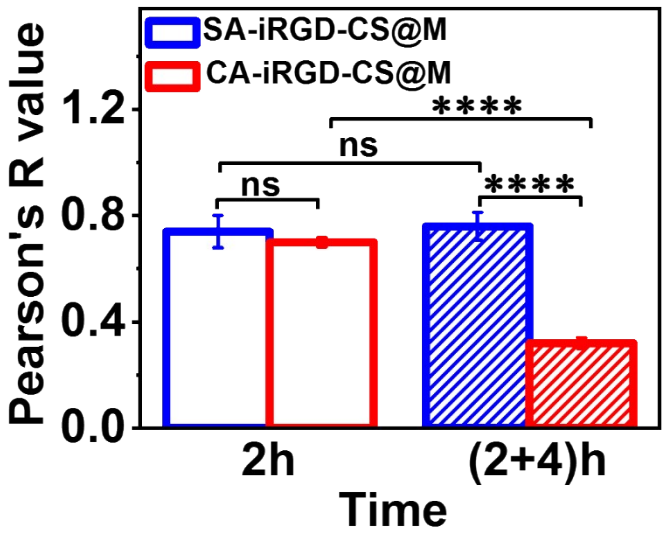

Fig. S4 Pearson's R value of different nanocarriers with lysosomes were calculated by Image J software.

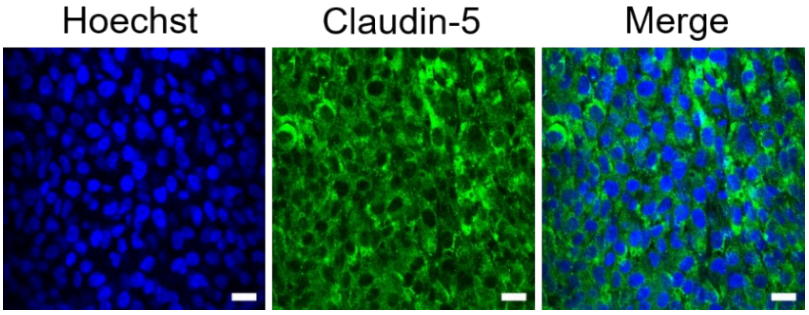

Fig. S5 Immunofluorescence images of claudin-5 (green) in BBB monolayer. Nuclei was stained by Hoechst 33342 (blue). Scale bar: 20 μm.

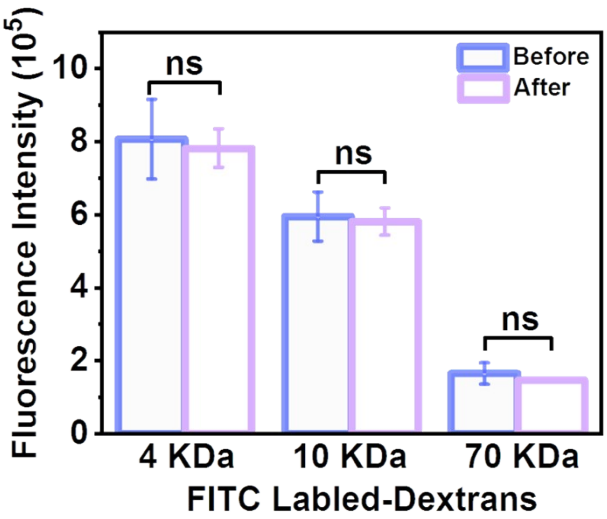

Fig. S6 Fluorescence intensity of FITC-labeled Dextrans with different molecular weights (4 kDa, 10 kDa, 70 kDa) in the basolateral chamber before and after the addition of CA-iRGD-CS@M to the apical chamber for 12 hours.

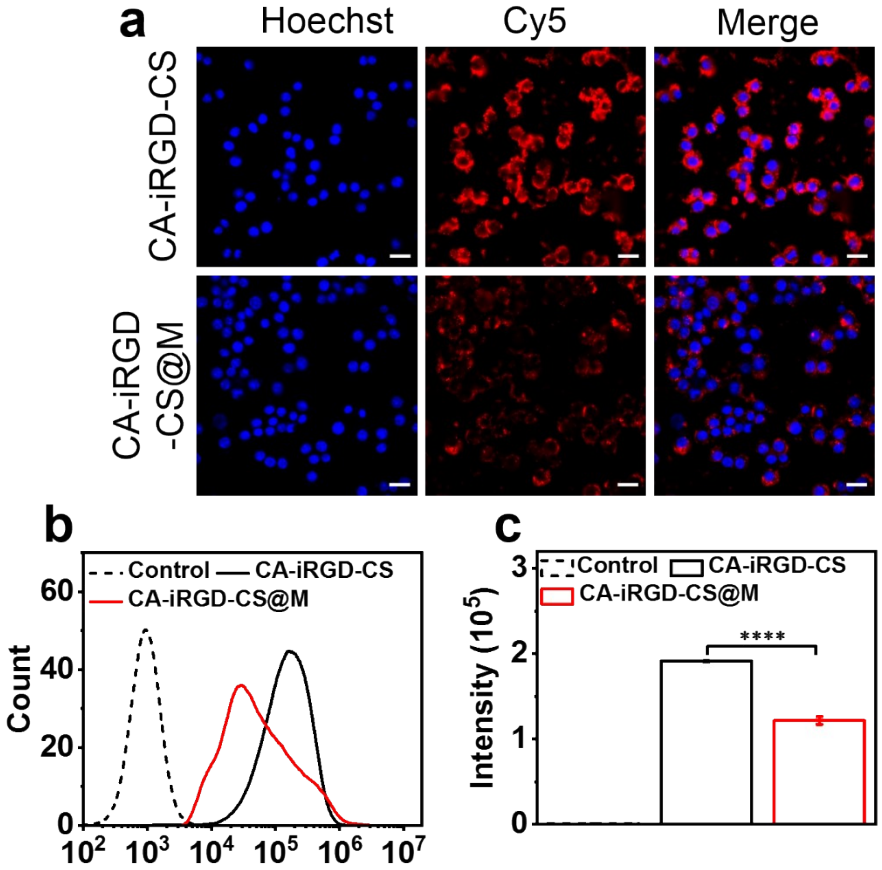

Fig. S7 (a) CLSM images (b) flow cytometry analysis and (c) mean fluorescence intensity (MFI) of RAW264.7 cells treated with CA-iRGD-CS and CA-iRGD-CS@M for 4 hours. Scale bar: 20 μm.

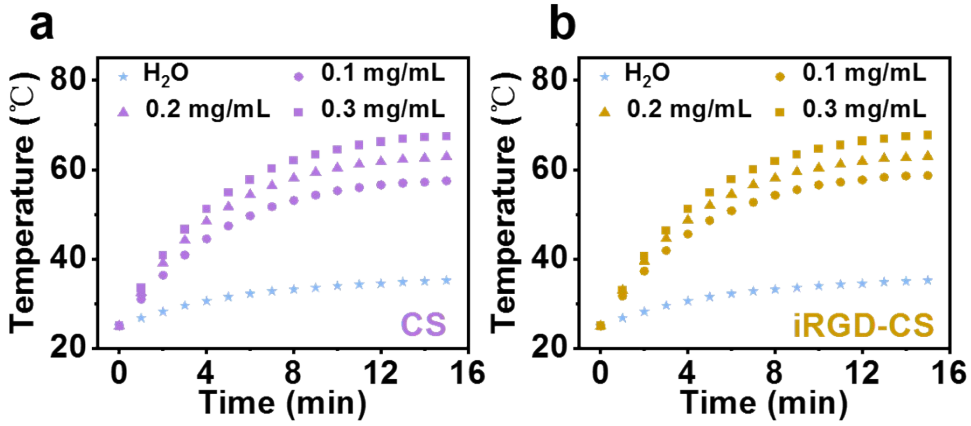

Fig. S8 Photothermal heating curves of CS (a) and iRGD-CS (b) with different concentration upon 1060 nm laser irradiation (1.5 W cm^{-2}).

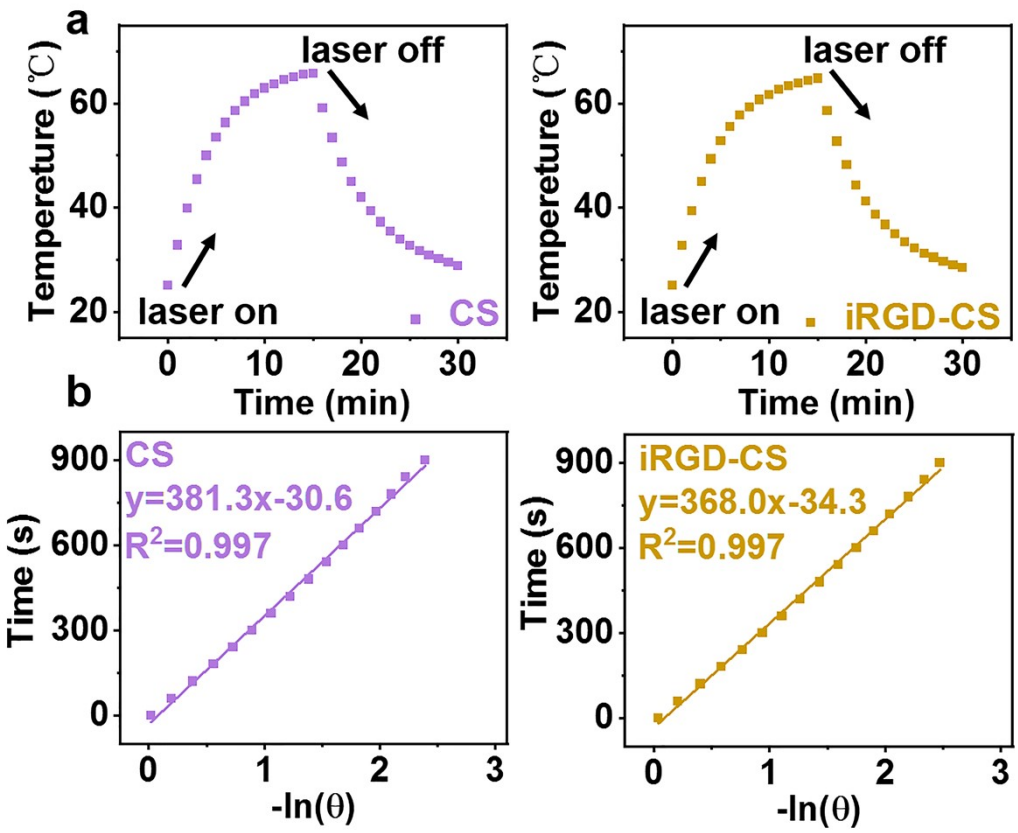

Fig. S9 (a) Photothermal effect of CS and iRGD-CS under irradiation of a 1060 nm laser (1.5 W cm^{-2}), and the laser was turned off after irradiation for 15 min. (b) Plot of cooling time versus negative natural logarithm of the temperature driving force (-lnθ) obtained from the cooling stage.

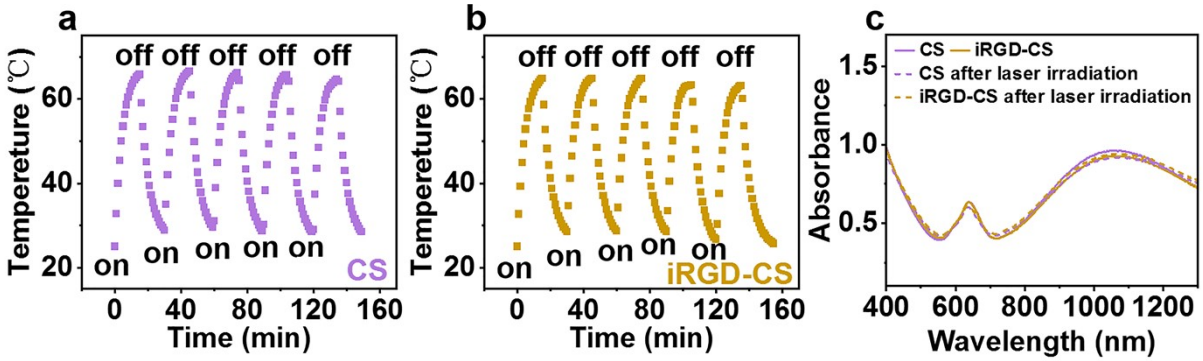

Fig. S10 Temperature variations of CS (a) and iRGD-CS (b) under irradiation at a power density of 1.5 W cm^{-2} for five light on/off cycles (15 min of irradiation for each cycle). (c) Absorption spectra of CS and iRGD-CS before and after 1060 nm laser irradiation (1.5 W cm^{-2}) for five light on/off cycles (15 min of irradiation for each cycle). (C_{CuS}=0.3 mg/mL).

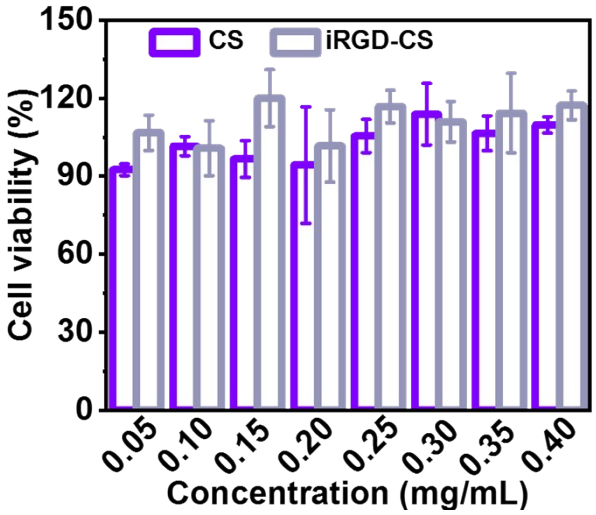

Fig. S11 Cytotoxicity of CS and iRGD-CS against U87MG cells after 24 hours incubation. Data are presented as mean ± SD (n = 6).

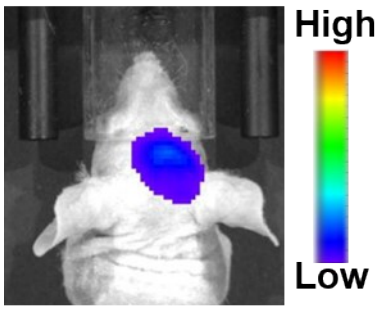

Fig. S12 Bioluminescence intensity in the mice brain after 7 days of tumor cell inoculation.

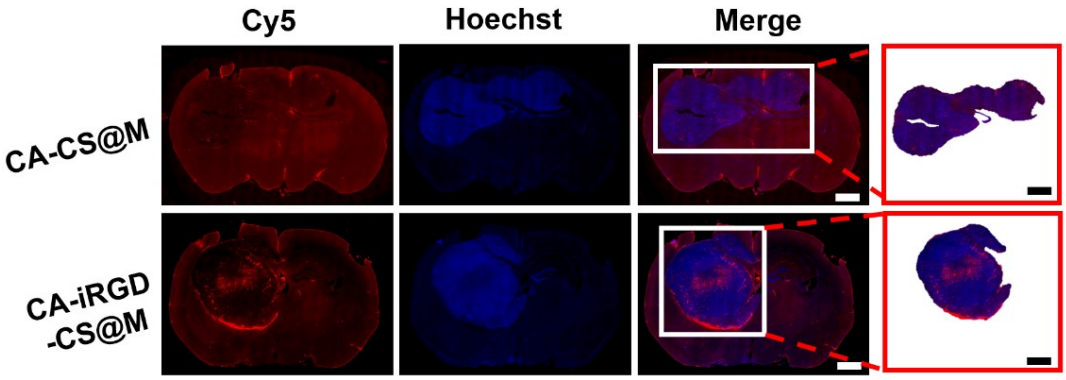

Fig. S13 Fluorescence microscope images of whole brain with tumor. At 12 hours after the injection of Cy5-labeled nanocarriers (CA-CS@M and CA-iRGD-CS@M), the brain tissue with tumor was collected and then sectioned for fluorescence staining. The nucleus was stained with Hoechst 33342 (blue fluorescence). Concentration of CuS is 10 mg kg^{-1}. Scar bar: 1000 μm.

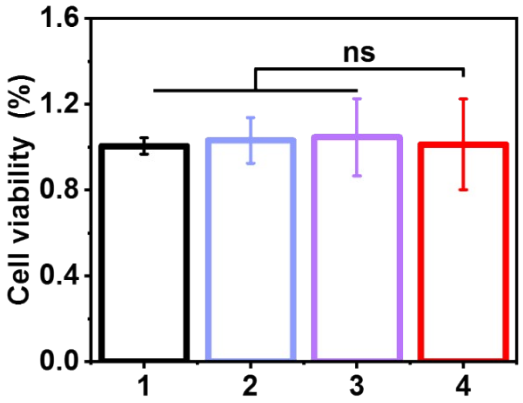

Fig. S14 Viability of hCMEC/d3 cells treated with different conditions. 1: +PBS; 2: +laser; 3: +CA-iRGD-CS@M (0.3 mg/mL); 4: +CA-iRGD-CS@M (0.3 mg/mL) + laser (10 min, 1060 nm, 1.5 W cm^{-2}).

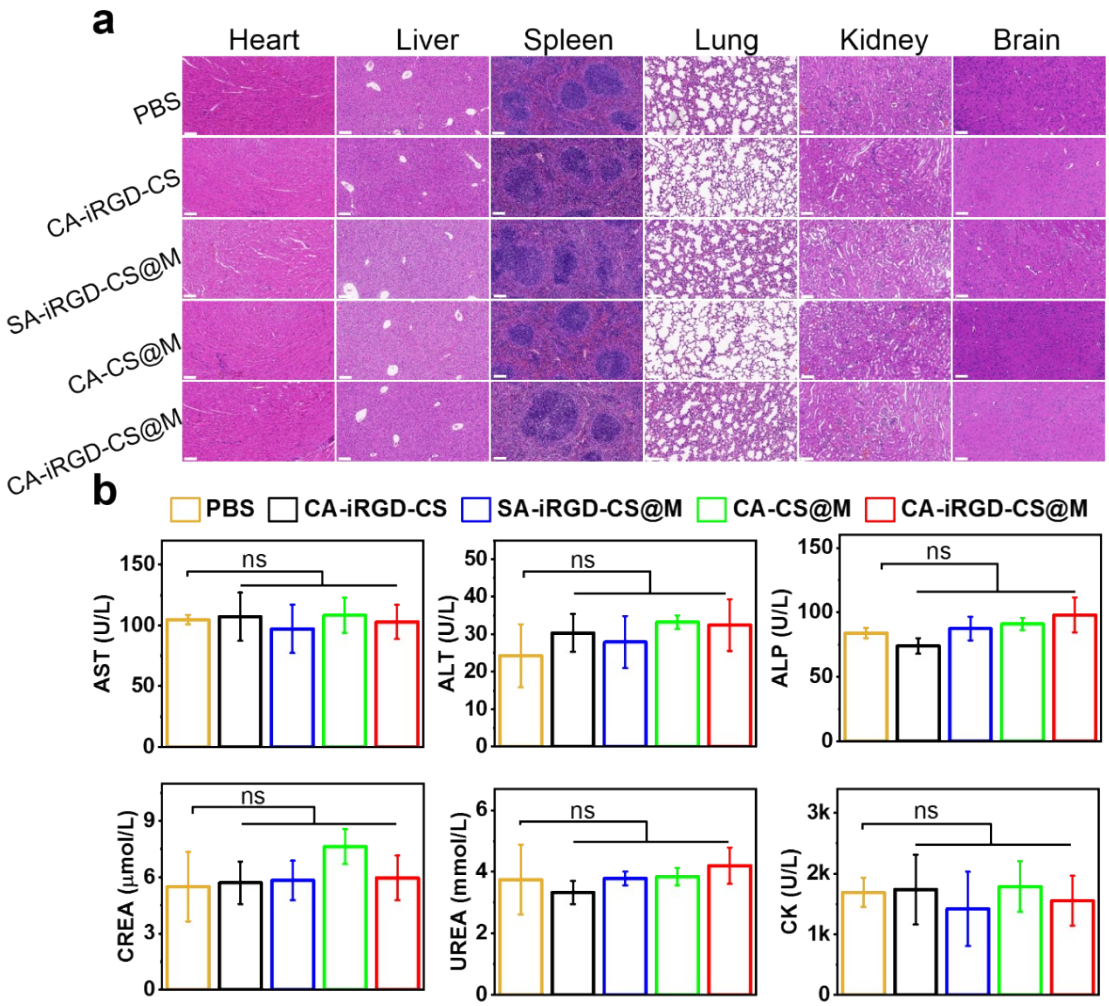

Fig. S15 (a) H&E staining images of major organs (heart, liver, spleen, lung, kidney and brain) of mice after different treatments (PBS, CA-iRGD-CS, SA-iRGD-CS@M, CA-CS@M and CA-iRGD-CS@M). Scar bar: 1000 μm. (b) Blood biochemistry test (AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; CREA: creatinine; UREA: blood urea nitrogen; CK: creatine kinase.) for BALB/c mice with different treatments for 14 days. Data are presented as mean ± standard deviation (SD) (n=3).

Reference

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2. C. Wang, B. Wu, Y. Wu, X. Song, S. Zhang and Z. Liu, *Adv. Funct. Mater.*, 2020, **30**, 1909369.
3. J. Zhang, C. Yang, R. Zhang, R. Chen, Z. Zhang, W. Zhang, S. H. Peng, X. Chen, G. Liu, C. S. Hsu and C. S. Lee, *Adv. Funct. Mater.*, 2017, **27**, 1605094.