Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information for:

In Vivo Multi-Photon Luminescence Imaging of Cerebral Vasculature and Blood-Brain Barrier Integrity Using Gold Nanoparticles

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Experimental Section

Preparation of PEGylated Gold Nanoparticles: To prepare citrate-coated gold nanoparticles with diameters of 10, 20, and 40 nm, sodium citrate (Sigma Aldrich, S4641, Sodium citrate tribasic dehydrate) was used as a reducing agent as described in the previously established method.[1, 2] Briefly, 1 ml of 0.1 % (w/w) $HAuCl_4$ (Sigma Aldrich, 520918, Gold(lll)chloride trihydrate) was added to 99 ml of deionized (DI) water. 2.1ml (10 nm), 1.7 ml (20 nm), or 900 µl (40 nm) of 1 % (w/w) sodium citrate (in DI water) was added to the $HAuCl_4$ solution after boiling, respectively. Then, the mixture solution was kept heated and stirred vigorously for 5 min. To prepare citrate-coated gold nanoparticles with a diameter of 5 nm, sodium citrate and sodium borohydride (FLUKA, 71321) were used as reducing agents as described in the previously established method.[2] Briefly, 1 ml of 1 % (w/w) $HAuCl_4$ was added to 90 ml of DI water, and the $HAuCl_4$ solution was sonicated in an ultrasonic bath (Bransonic, 3510E-DTH) for 1 min. Then, 2 ml of 38.8 mM sodium citrate (in DI water) was added to the $HAuCl_4$ solution and the mixture solution was sonicated for 1 min. Finally, 1 ml of ice-cold 0.075 % (w/w) sodium borohydride in 38.8 mM sodium citrate solution was added to it, and the final mixture solution was sonicated for 5 min. To coat gold nanoparticles with polyethylene glycol (PEG) molecules, 1 ml of 16.05 mg/mL mPEG-sulfhydryl (Sunbio, P1SH-5, MW = 5000) (in DI water) was added to 600 ml of citrated-coated gold nanoparticles, and the mixture solution was vigorously stirred overnight. PEGylated gold nanoparticles were washed and concentrated with an amicon ultra centrifugal filter (Millipore, UFC810024, Amicon Ultra-4 PL 100K) by centrifugation at 4,000 R. The prepared PEGylated gold nanoparticles (PEG-GNP) were

stored in PBS at 4°C before use. The physical properties of PEG-GNPs were characterized via dynamic light scattering (DLS) and transmission electron microscopy (TEM). For TEM imaging, an aliquot of PEG-GNPs dispersed in water was dropped onto the Formvar/carbon grid (Ted Pella, Inc.), which was then gently wiped off after approximately 1 min and air-dried. TEM images were obtained using a high-resolution FE-TEM (JEOL, JEM-2100F). The hydrodynamic size and polydispersity index value were obtained using a Zetasizer ZS90 dynamic light scattering machine (Malvern Instruments). The absorbance spectra of PEG-GNPs were obtained using the SpectraMax Plus384 absorbance microplate reader (Molecular Devices).

In vitro multi-photon luminescence: 30 µl taken from each of the 100µgAu/ml PEG-GNPs samples was placed into the microscopy chamber well (Ibidi, 81826, µ-Slide 18 well-flat, ibiTreat, tissue culture treated, sterile) and covered with a cover glass to use a water immersion lens (Carl Zeiss**,** 420967-9900-000, Objective W N-Achroplan 40x/0.75 M27). The PEG-GNP samples were then irradiated with femtosecond pulsed lasers operating in the NIR wavelengths ranging from 720 nm to 900 nm with 80 MHz repetition ratio using multi-photon laser scanning microscopy (Carl Zeiss, LSM510). The multi-photon luminescence of PEG-GNPs was collected using band-pass filters in the wavelength range of 570 nm to 640 nm, in which the multi-photon luminescence emission of PEG-GNPs was mainly observed regardless of their sizes. The images were then analyzed with ImageJ software to measure the mean luminescence intensity of samples at each excitation wavelength. The multi-photon luminescence intensity of PEG-GNPs was obtained by subtracting the measured luminescence intensity of DI water from the measured

luminescence intensity of PEG-GNPs at the same excitation wavelength. The mean multi-photon luminescence intensity was then normalized to the laser power at the excited wavelength. To study the nanoparticle concentration-dependent luminescence, PEG-GNP samples with diameters of 5 and 40 nm at different Au concentrations (0 μgAu/ml, 10 μgAu/ml, 50 μgAu/ml, and 100 μgAu/ml) were irradiated at the excitation wavelength of 800 nm, and the multi-photon luminescence intensity of each sample was measured.

In vivo multi-photon luminescence imaging: Eight-week-old male ICR mice were purchased from Koatech (Gyeonggi-do, Republic of Korea). Mice were anesthetized with intraperitoneal (i.p.) injection of urethane (1.5–2.0 mg/g body weight) dissolved in saline. A craniotomy (2.0 mm by 2.0 mm) was made using a high speed dental drill over the somatosensory cortex. A custom-made head frame was attached to the skull with dental cement, and the dura was carefully removed with fine forceps. For *in vivo* multi-photon luminescence imaging of mouse cerebral vasculature, 200 μl of PEG-GNPs (30 mgAu/kg body weight) was injected via tail vein using a 30 gauge needle and polyethylen-10 tubing, and immediately, cerebral vasculature within the 150 μm deep cortex was imaged with 10 μm depth increments at the excitation wavelength of 800 nm (0.15 W for all images). To determine the imaging depth, cerebral vasculature within the 300-μm-deep cortex was imaged with 1.16 μm depth increments at the excitation wavelength of 800 nm. The laser intensity was gradually increased from 0.15 W at the surface to 0.21 W at the depth of 300 μm. The slice images were further processed for maximum intensity projection. The multi-photon luminescence ratio of vessel to extravascular tissue was calculated using ImageJ software (http://rsbweb.nih.gov/ij/index.html). The multi-photon luminescence signals

within blood vessels with diameters larger than 20 μm and the multi-photon luminescence signals in the extravascular regions were used for this calculation (n = 3). To measure blood residence times of PEG-GNP luminescence, 50 μl of blood was extracted from the retro-orbital sinus using heparinized capillary tubes (Fisher Scientific, Leicestershire, UK) at different times (3, 60, 120, and 240 min or 720 and 1440 min) after intravenous injection of PEG-GNPs (30 mgAu/kg body weight). The extracted blood was collected in anticoagulant-coated tubes (Fisher Scientific, Leicestershire, UK; 05-407-34) and then transferred to the microscopy chamber well to measure the multi-photon luminescence in blood (n = 3).

To image cerebral vasculature in the photothrombosis-induced mouse model, a craniotomy (2.0 mm by 2.0 mm) was prepared as described previously. For a pre-injection image, cerebral vasculature was imaged before intravenous injection of PEG-GNPs. Then, 200 μl of PEG-GNPs (30 mgAu/kg body weight) was injected via tail vein using a 30 gauge needle and polyethylene 10 tubing, and immediately following, cerebral vasculature within the 150 μm deep cortex were imaged with 10 μm depth increments at the excitation wavelength of 800 nm (0.15 W). To induce photothrombosis selectively in the cortical region, 100 μl of 0.67% (w/w) rose bengal (photosensitizer) was injected via tail vein, and the photoactivation was performed by exposing a cortical area in the middle of the craniotomy to the 535 nm green light (HBO 100-W arc lamp, 535+_25nm; ~3mW) for 5 min. Then, the photothrombosis-induced region was serially imaged at 0.5, 1 and 2 h after photoactivation (n = 3). The images were analyzed to quantify the luminescence of PEG-GNPs in the extravascular tissue near the cerebral microvasculature after photothrombosis.

The luminescence (in the region within 30 μm from the outer wall of vessels) from the vessels larger than 20 μm, which are presumably vein and artery, was excluded to primarily analyze the PEG-GNPs leaked from the cerebral microvasculature. To verify that the green light irradiation alone does not damage the vessels in which PEG-GNPs are circulating, the same experiments were performed without rose bengal injection (n = 3). Additionally, to verify that the multi-photon luminescence of rose bengal is negligible in the images after 30 min post-photoactivation, blood residence time of rose bengal was measured based on their multi-photon luminescence signal. A craniotomy (2.0 mm by 2.0 mm) was prepared as described previously. 100 μl of 0.67% (w/w) rose bengal was injected via tail vein, and the multi-photon luminescence of cerebral vasculature was imaged every 2 min up to 20 min and then imaged at 30, 60, 90, and 120 min post-injection (n = 3). All procedures were approved by the Institutional Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology, and performed in compliance with the relevant laws and institutional guidelines. Student's *t* test was used for statistical analysis of the results.

Evans blue extravasation: The craniotomy (2.0 mm by 2.0 mm) was prepared as described previously. The mice were injected intravenously with 50 μl of Evans blue (4% w/v) immediately after induction of photothrombotic stroke and were killed 2 h later. Brain was rapidly removed and fixed in 4% paraformaldehyde for 24 h, after which photograph of the whole brain was taken. Then coronal sections of the brain were cut to a thickness of 30 μm using a cryotome. Brain sections were mounted and coverrslipped using fluorescent mounting medium. Evans blue fluorescence from the brain sections was imaged with an

automated microscope (ImagXpress Micro; objective, 10x). Images were processed by MetaXpress software and stitched together to generate an entire brain section.

Histological examination: Mice were transcardially perfused with PBS, followed by 4% paraformaldehyde (in PBS). After the perfusion, the brain was post-fixed with 4% paraformaldehyde at 4 ^{o}C for 30 min, and then incubated in 30% sucrose overnight to prevent ice crystal formation. Coronal sections of the brain were cut on a cryotome (thickness 20 μm), and collected serially on positively-charged glass slides. To identify the ischemic region induced by photothrombosis, cresyl violet staining was performed by applying 0.2% cresyl violet solution to the brain sections for 5 min. The sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped with Canada balsam. The cresyl violet-stained sections were then imaged with a light microscope equipped with a CCD camera. To confirm the GNP extravasation in the photothrombotic region, the brain sections were collected onto positively-charged glass slides and immediately cover-slipped with aqueous mounting medium. Multi-photon luminescence images of the brain sections were then obtained at the excitation wavelength of 800 nm using multi-photon laser scanning microscopy. The image acquisition was done with the same parameter as used for the *in vivo* imaging.

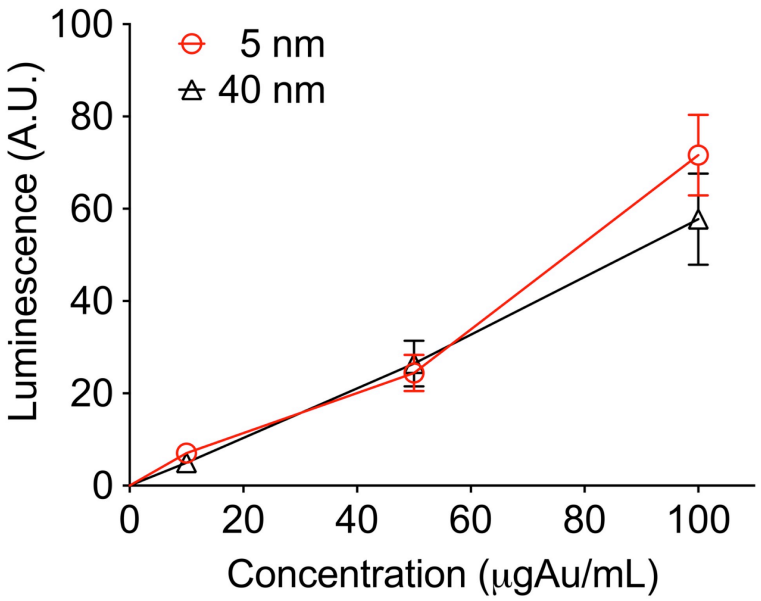

Fig. S1 Concentration-dependent multi-photon luminescence of PEGylated gold nanoparticles when exposed to the excitation wavelength of 800 nm.

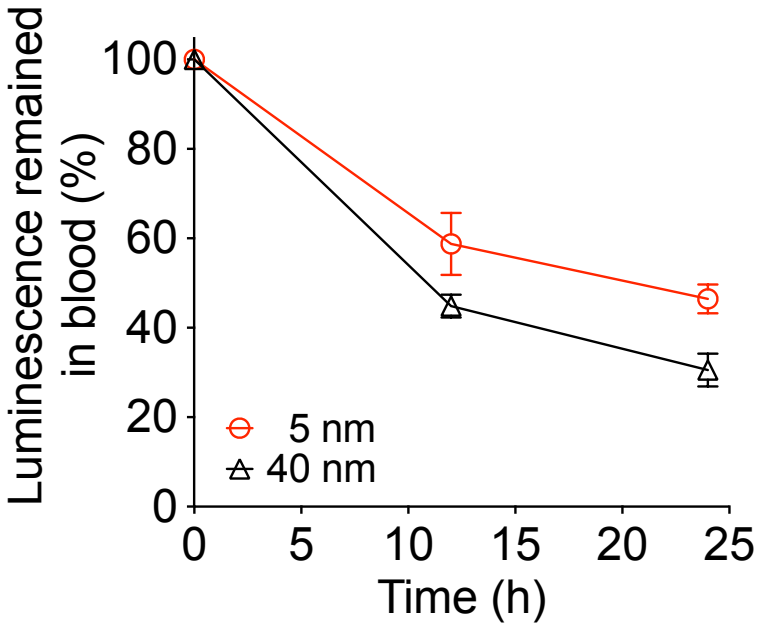

Fig. S2 Percentage of multi-photon luminescence remained in the blood vessels as a function of time after intravenous injection of 5 nm or 40 nm PEG-GNPs. The images were obtained at 12 h and 24 h after intravenous injection of the PEG-GNPs (n = 3, mean with s.d.).

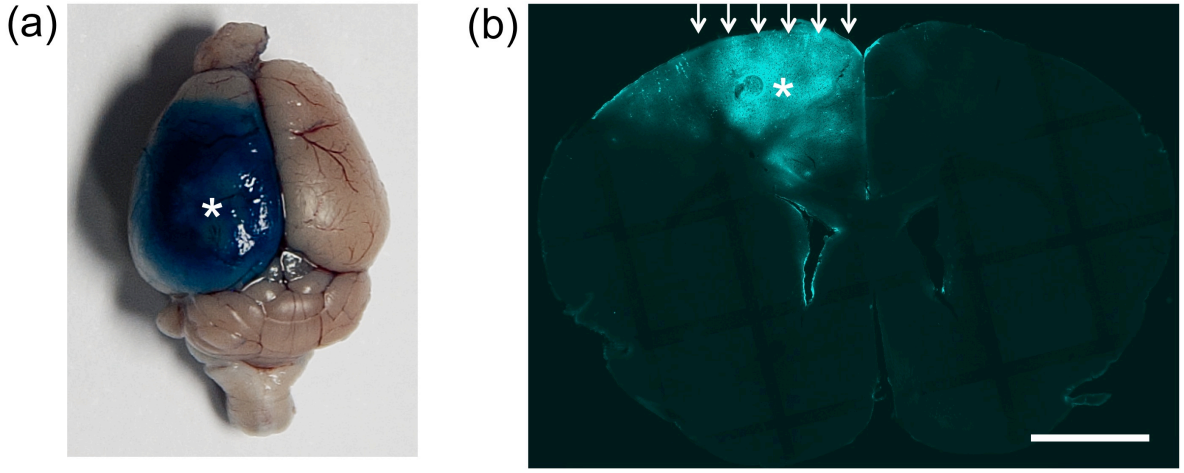

Fig. S3 Evans blue extravasation 2 h after induction of photothrombotic stroke. (a) Photograph of whole brain and (b) fluorescence image of coronal section 2 h after Evans blue injection immediately following photothrombosis induction. Arrows indicate green light irradiation; asterisks indicate the photothrombotic region induced by irradiation. Scale bar is 2 mm.

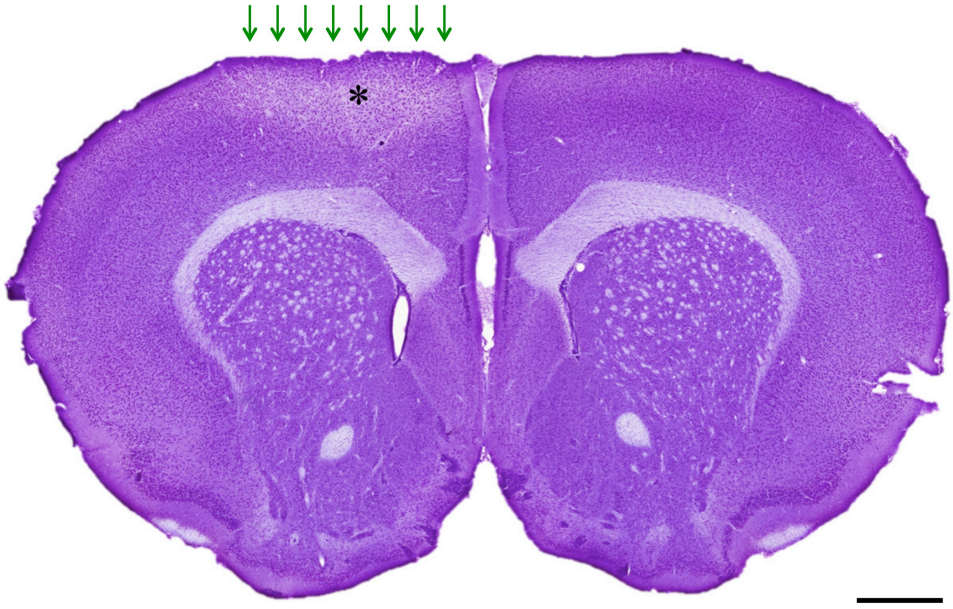

Fig. S4 Cresyl violet staining of a brain section 2 h after induction of photothrombotic stroke. Green arrows indicate green light irradiation; an asterisk indicates the photothrombotic region induced by irradiation. Scale bar is 1 mm.

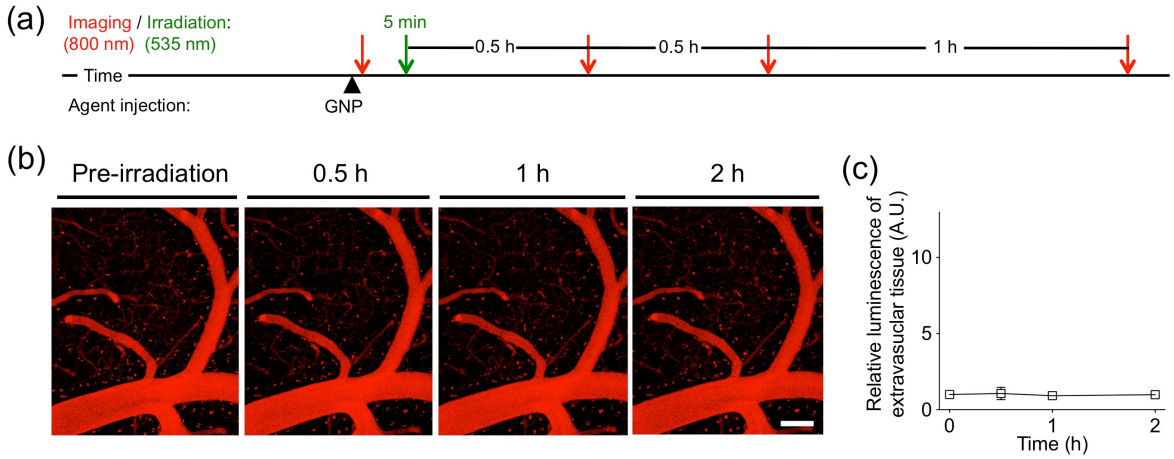

Fig. S5 Effect of green light irradiation on the cerebral vasculature intravenously injected with PEGylated gold nanoparticles (PEG-GNP). (a) Timeline of experiments. An open-skull cranial window was prepared to visualize the cortical vasculature in a live mouse using the multi-photon luminescence of PEG-GNPs. After PEG-GNP injection, the cortical vasculature region was irradiated with green light for 5 min without photosensitizer injection. (b) Time-lapse multi-photon luminescence image of cortical vasculature injected with PEG-GNPs before and after green light irradiation. (c) Relative *in vivo* multi-photon luminescence of PEG-GNPs observed in the extravascular tissues (no significant difference between pre-irradiation and 0.5-2 h post-irradiation, unpaired *t*-test, n = 3; mean with s.d.). Scale bar indicates 100 μm.

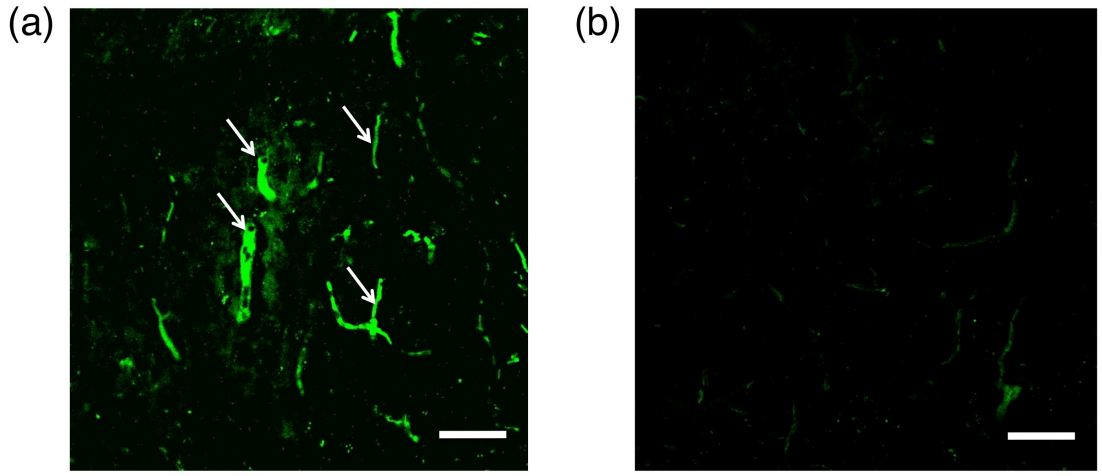

Fig. S6 Multi-photon luminescence images of brain sections from the mouse injected with 5 nm PEG-GNPs at 2 h post-stroke. (a) Photothrombotic region in the left hemisphere. (b) Intact region in the contralateral hemisphere. Arrows indicate blood vessels. Scale bars are 50 μm.

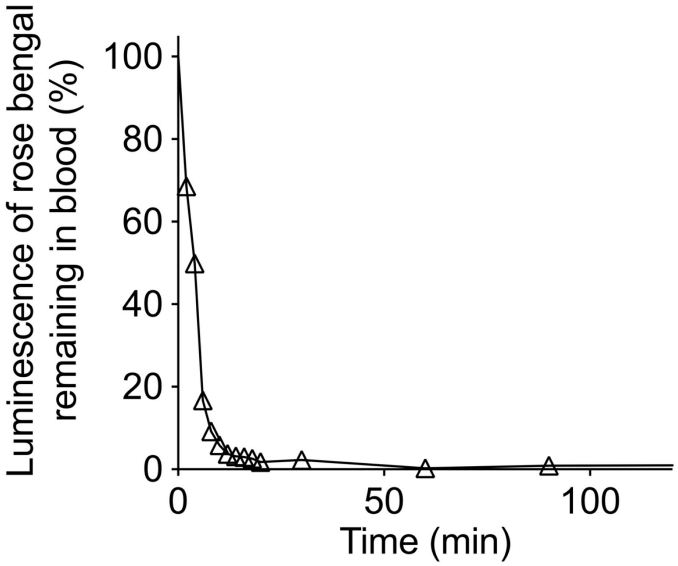

Fig. S7 Blood residence time of intravenously-administered rose bengal. *In vivo* multi-photon luminescence signal of rose bengal observed in the cerebral vasculature was used to measure their blood residence time.

[1] G. Frens, Nature Physical Science 1973, 241, 20.

[2] W. Haiss, N. T. K. Thanh, J. Aveyard, D. G. Fernig, Analytical Chemistry 2007, 79, 4215.