Supplementary Information

A Facile Approach for the Delivery of Inorganic Nanoparticles into Brain by Passing through the Blood-Brain Barrier (BBB) **

Yeong Shin Yim[a], Jin-sil Choi[b], Gun Tae Kim[a], Chul Hoon Kim[a], Tae-Hyun Shin[b], Dong Goo Kim[a,*], and Jinwoo Cheon[b,*]

[a]Department of Pharmacology, Yonsei University College of Medicine, 250 Sungsan-ro, Seodaemun-gu, Seoul 120-752, [b]Department of Chemistry, Yonsei University, 50 Yonsei-Ro, Seodaemoon-gu, Seoul 120-749, Korea

Serum albumin (SA) coated magnetic nanoparticles (MNPs)

MNPs were prepared following previously reported methods. $MnCl_2$ (0.082 g) and iron (III) acetylacetonate ($Fe(acac)_3$) (1.765 g) were mixed in octyl ether (15 ml) in the presence of surfactants (oleic acid and oleylamine). The reaction solution was heated at 300 °C for 1 hr and the reaction mixture was cooled down to a room temperature. Then, MNPs were centrifuged and precipitated after the ethanol addition. The organic ligands were removed via treatment of 1 M trimethylammonium hydroxide solution and serum alubumin (SA) was introduced onto the MNP's surface. Then, the SA on the MNPs were further cross-linked with 2,2-(ethylenedioxy)bis(ethylamine), which resulted in the enhancement of the stability of surface coating. SA-MNPs (2 mg) were dispersed in 1 ml of 0.01 M phosphate buffered saline (PBS), pH 7.2. 2,2-(-(ethylenedioxy)bis(ethylamine), 9.6 mg (50 μmol) of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 1.2 mg (5 μmol) of N-hydroxysulfosuccinimide were added to the solution. The reaction was carried out for 2 hrs at a room temperature. Cross-linked SA-MNPs were purified with desalting column (GE Healthcare, USA).

SA-MNPs were labeled with a fluorescent dye (fluorescein, FITC) to visualize the presence of SA-MNPs in the tissue and cells. The prepared SA-MNPs were labeled with N-hydroxysulfosuccinimide fluorescein isothiocyanate (NHS-FITC, Pierce, USA), and extra FITC was removed with a desalting column.

There was no difference between the hydrodynamic size of the SA-coated nanoparticles before and after the cross-linking process (*ca*. 30 nm; Fig. S1a). The mixture of SA and MNPs had a negative (-25 mV) charge, which was similar to that of native albumin (zeta potential: *ca*. -23 mV) as shown in Fig. S1b. After the cross-linking process, the surface charge of SA-MNPs was changed into weakly negative (-4 mV), because the glutamic acid and aspartic acid residues of albumin on the nanoparticles were interlinked to each other or linked to small molecules with amine end, (Fig. 1a in the main text). The serum albumin which was amine-functionalized under the same reaction condition without nanoparticles also possessed almost a neutral charge (-2 mV) like SA-MNPs.

In the electrophoresis gel, nanoparticles moved slightly toward the positive side because their negative surface charge was relatively weak (Fig. S1 c(i)). When they were stained with Coomassie blue, which binds to proteins with arginine and histidine groups, the blue band appeared at the same location as the nanoparticles (Fig. S1 c(ii)). Moreover, nanoparticles labeled with *N*-

hydroxysuccinimide-fluorescein (NHS-FITC) on their albumin coating showed no fluorescence signals except at the same site as the nanoparticles (Fig. S1 c(iii)).

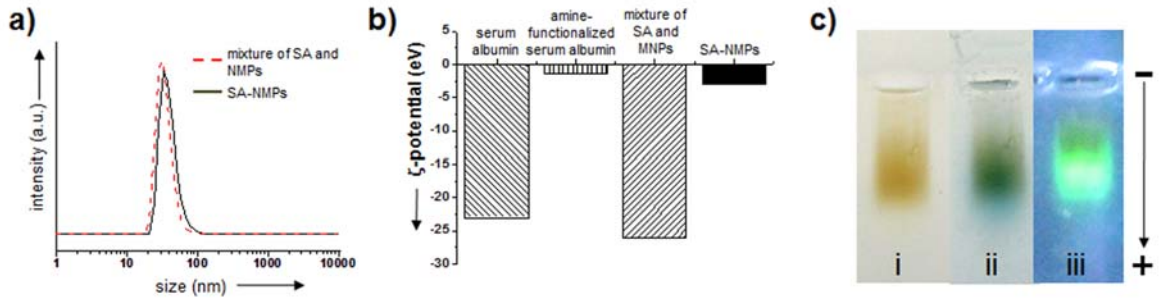

Fig. S1 a) Hydrodynamic size of the mixture of SA and MNPs and SA-MNPs. b) Comparison of ξ-potential of SA, amine-functionalized SA, mixture of SA and MNPs, and SA-MNPs. c) The movement of nanoparticles in the electrophoresis of agarose gel (100V, for 20 min). The SA-MNPs were labeled with FITC. When the agarose gel was stained with the protein-staining Coomassie blue at the same location as the brown band (i), the protein was stained blue (ii). In addition, under UV light irradiation, the green band (iii) was at the same position of SA-MNPs.

Preparation of primary cultured endothelial cells

Primary endothelial cells were cultured from the brain of 8-to-12-week-old BALB/c mice. After mice were sacrificed and each of their brains was dissected, only the gray matter was chopped into 2-3 mm pieces and centrifuged at 600 g and 4 ^{o}C for 5 min. Then, the enzymic dissociation technique was applied collagenase/dispase solution (Sigma, USA), DNase I (Sigma, USA) TLCK (tosyl-lysine-chloromethylketone, Sigma, USA) for 1 hr at 37 ^{o}C in order to degrade extracellular matrix. The suspension was triturated with a pasteur pipette, centrifuged for 5 min at 600 g, and resuspended with 25% BSA. This was centrifuged again at 1000 g for 10 min in order to separate capillary fragments from myelin, neurons, astrocytes, and other single cell contaminants. After pouring off the top layer, the capillary pellet was resuspended in a second collagenase/dispase solution, incubated for 3 hrs at 37 °C, and washed afterwards. Then, in the culture dish prepared be beforehand by coating it with collagen (Sigma, USA) and fibronectin (Invitrogen, USA), cells were seeded in a complete culture media (DMEM, GIBCO, USA; 20% FBS, GIBCO, USA; 50x amino acid, Sigma, USA; 100x vitamins, Sigma, USA; 50 μg/ml gentamycin, Sigma, USA).

Preparation of a primary cultured hippocampal neuron

Primary hippocampal neurons were prepared from 18-day-old embryonic Sprague-Dawley rats. Briefly, the dissected hippocampus was incubated for 12 min at 37 °C in dissection solution (HBSS, GIBCO, USA; HEPES and Pen/Strep, Invitrogen) containing 0.05% trypsin. The medium was decanted and cells were washed three times with 5 ml of dissection solution. The cells were suspended with a Pasteur pipette in a chopping solution (dissection solution with DNase (Sigma, USA)), and centrifuged at 1000 rpm for 5 min. The chopping solution was removed and the cells were suspended in plating medium (Neurobasal medium, GIBCO, USA; B-27, Invitrogen, USA; L-glutamine, Sigma, USA). Then, they were spread on cover-slips in 12-well plates. Ten to fourteen days after the growth, the primary cultured neurons were utilized.

Preparation of primary cultured hippocampal astrocyte

Primary hippocampal astrocytes were prepared from 1 day-old BALB/c mice. The dissected hippocampus was suspended with a Pasteur pipette in dissection solution (MEM, GIBCO, USA; Glucose, Sigma, USA; Sodium bicarbonate, Sigma, USA), and centrifuged at 1000 rpm for 5 min. The dissection solution was removed and with the cells suspended in plating medium (dissection solution within 10% Horse serum, GIBCO, USA and 10% Bovine serum, GIBCO, USA), they were spread onto 10 cm plates. Ten to fourteen days after growth, the primary cultured astrocyte was incubated with Ara-C for 24 hr. Then, the cells were placed in growth medium (dissection solution within 10% Bovine serum, GIBCO, USA).

***In vitro* internalization of SA-MNPs into primary cultured cells**

Cells (5×10^5) were incubated with 100 μg/ml of the FITC-labeled SA-MNPs at 37 °C for 1 h. For the confocal microscopic (LSM700, Carl Zeiss, USA) observation, the cells including endothelial cells, astrocytes, and neurons were stained by incubation with phalloidine, anti-GFAP, and anti-NeuN (Chemicon, USA), respectively. In the case of astrocytes and neurons, an additional secondary antibody (RITC-conjugated anti-mouse, Zymed, USA) treatment process was required. After washing with PBS, they were then stained with the nuclear marker DAPI (Invitrogen, USA) for 5 min. Finally, a glass cover-slip was placed on the slide for mounting in an anti-fade reagent (ProLong Gold anti-fade reagent, Invitrogen, USA). Fluorescence of the sections was observed by confocal microscope (Carl Zeiss, USA) with the LSM 510 program.

Animals and housing

The BALB/c mice were supplied by the division of laboratory animal medicine, Yonsei University College of Medicine. All animal experiments were approved by the committee for the care and use of laboratory animals at Yonsei University (Project license number: #00062). Mice were cared for in accordance with the guide for animal experiments as well as with the NIH guide for the care and use of laboratory animals. All animals were raised in a specific pathogen free (SPF) environment, with maintained temperature (22 ± 1°C), controlled humidity (55%), and a 12:12 hour light/dark cycle (with light starting at 7:00 am). They were divided by sex, weighed, and culled on postnatal day (PND) 2.

***In vivo* internalization of SA-MNPs into the brain**

SA-MNPs were injected in the tail vein of mice with a 20 mg/kg dose. At 2 weeks post-injection, the animals were sacrificed and their blood was perfused through the heart puncture with saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Each brain was removed quickly and fixed with sucrose for 24 hrs. Post-fixing, the brains were cut in coronal sections with a cryotome to 20 μm thick. Sections were blocked with 3% bovine serum albumin (BSA, Sigma, Germany) in PBS for 1 hr and then stained by incubation with anti-NeuN (Chemicon, USA) overnight. After washing with PBS, the sections were incubated with a secondary antibody (RITC-conjugated anti-mouse, Zymed, USA) for 1 hr. Then they were stained with the nuclear marker DAPI (Invitrogen, USA) for 5 min. Finally, a glass cover-slip was placed on the slide for mounting in an anti-fade reagent (ProLong Gold anti-fade reagent, Invitrogen, USA). Fluorescence of the sections was observed by CLSM (Carl Zeiss, USA) with the LSM 510 program. The mouse brain was also imaged with 3T MRI (Achieva, Philips Medical Systems, Best, Netherlands). The MR image was obtained by

T2-weighted fast spin echo sequence at 3T (repetition time (TR) = 4000 ms, echo time (TE) = 100 ms, matrix = 256 × 256, field of view (FOV) = 10 × 10 cm, slice thickness = 1 mm, number of acquisitions = 2).

The biodistribution of SA-MNPs

For the biodistribution study, SA-MNPs were labeled with the radioisotope ^{125}I ($t_{1/2}$ = 60.14 days). SA-MNPs and ^{125}I were mixed in the presence of IODO-BEADs (Pierce, USA) and incubated for 30 min. Then, the unreacted ^{125}I was removed by using a 100 kDa filter (Amicon, USA). The radioactivity of SA-MNPs was measured with a gamma counter (Perkin Elmer, France) and they were injected in the tail vein of mice at a dose of 20 mg/kg. Mice were kept separately in metabolic cages with free access to food and water after the administration. At week 2 after the injection, mice were anesthetized with 1 ml/kg chloropent (pentobarbital 1 g; chloral hydrate 42.5 g; magnesium sulfate 21.3 g; ethyl alcohol 100 ml in 1 L solution). The heart was punctured and the blood was collected in a tube containing heparin. A whole-body perfusion was performed by forced injection with cold phosphate buffered saline (PBS) through the heart puncture. After the perfusion, liver, lung, heart, and brain were excised, subsequently. Tissues were weighed and homogenized in a glass homogenizer with a homogenization buffer (0.32 M sucrose, 100 mM HEPES, pH 7.4). Lysates of homogenized tissue were centrifuged and the supernatants were resuspended in lysis buffer (1% Triton X-100, 100 mM NaCl, 0.1% SDS, and 0.5% sodium deoxycholate). Solutions were incubated for 30 min in a 4°C rotator and then centrifuged. Finally, the radioactivity of the supernatant from each organ was measured by the gamma counter (Perkin Elmer, France).

The amount of SA-MNPs in brain, blood (plasma), lung, heart and liver is measured as 6.0, 0.7, 6.5, 8.6 and 29.6 ng/g, respectively (Fig. S2). Large portion of SA-MNPs exists in liver due to reticuloendothelial system (RES). The amount of SA-MNPs in the brain is comparable to 20% of that in liver. The value is comparable to the previous data of BBB permeable nanoparticles (5~30 %).

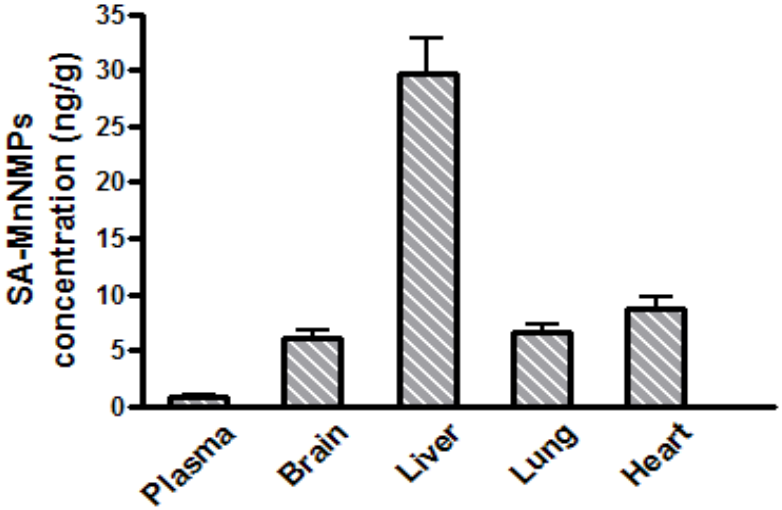

Fig. S2 Biodistribution of ^{125}I labeled SA-MNPs. Nanoparticles were injected in the tail vein at a dose of 20 mg/kg. Mice were sacrificed at week 2 after the injection.

Intact BBB penetration test

To evaluate the intact BBB penetration of SA-MNPs, mice were randomly divided into three

groups; saline-treated, SA-MNPs treated, and hyperthermia groups. In the hyperthermia group, mice were placed in a heated thermal blanket at 41~42°C for 45min. SA-MNPs were injected via the tail vein with a dose of 20 mg/kg. After pre-treatment, 2% EB dye in saline was injected intravenously and allowed to circulate for 30 min. Mice were anesthetized and perfused with PBS through heart puncture. After decapitation, the brain was excised

The toxicity test of SA-MNPs on neurons

The biological safety of nanoparticles is an important issue in its application to living organelles. Therefore, a cellular toxicity by nanoparticles was examined. The cellular toxicity of SA-MNPs was examined in neurons and their viability was measured with MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. SA-MNPs were treated on the primary neuron from 10 to 1000 μg/ml for 24 hrs. As shown in Fig. S3, the live cell was sustained up to 1 mg/ml compared to a non-nanoparticle-treated neuron.

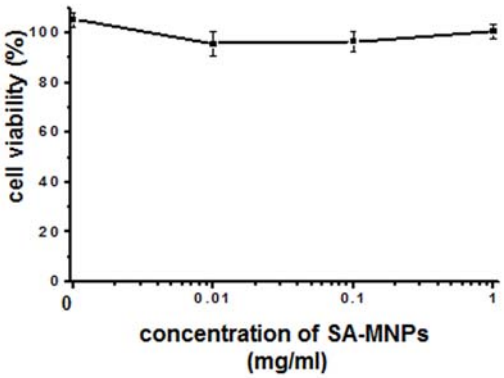

Fig. S3 Toxicity test of SA-MNPs on neuron. Nanoparticles were treated with the concentrations up to 1 mg/ml on neurons for 24 hrs. The viability of cells was measured by using MTT assay.