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Supporting Information

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Detection of β-Amyloid by Sialic Acid Coated Bovine Serum Albumin Magnetic Nanoparticles in a Mouse Model of Alzheimer's Disease

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Materials

Iron (III) acetylacetonate [$Fe(acac)_3$], oleyl amine, 1,2-hexadecanediol, oleic acid (OA), benzyl ether, BSA, ethyl-(3,3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), 2,2′-(ethylenedioxy)bis(ethylamine), tetramethylammonium hydroxide were purchased from Sigma-Aldrich and hydrogen peroxide (30%) was purchased from CCI. Amberlite IR 120 hydrogen form (Amberlite H+) was purchased from Fluka. Buffered 10% formalin solution was purchased from Azer Scientific. Potassium ferrocyanide trihydrate ($K_4Fe(CN)_6$. $3H_2O$) was purchased from Mallinckrodt. 3,3′,5,5′-tetramethylbenzidine (TMB) and 1,1,1,3,3,3-hexafluoro-2-propanol 99.9% were purchased from Acros Organics. Thioflavin T (ThT), UltraPure Grade was purchased from AnaSpec. Aβ(1-42) was purchased from GL Biochem. (Shanghai) Ltd. bEnd.3 endothelial cells were purchased from American Type Culture Collection (ATCC). Phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), sodium pyruvate (100 mM), glutamine, penicillin−streptomycin (Pen Strep) mixture were purchased from Gibco. The anti-Aβ 1-16 (6E10) mAb, SIG-39320 was purchased from Covance, and goat anti-mouse HRP-conjugated secondary antibody was purchased from BioRad. Tween 20 was purchased from BioRad. CellTiter 96 Aqueous One solution containing MTS was purchased from Promega. Ultrathin-carbon type A, 400 mesh copper grids for TEM were purchased from Ted Pella, Inc. Ultrafiltration membranes and centrifugal filters were purchased from Millipore, while dialysis tubings were obtained from BioDesign Inc. bEnd.3 cells were cultured in DMEM. All cell culture media was supplemented with 10% inactivated FBS, 1% Pen-Strep mixture, and L-glutamine (2 mM).

Synthesis of Sia-BSA$_x$-NP

The magnetic NPs were synthesized by conjugating the sialic acid moieties with the Fe_3O_4 magnetite magnetic NPs. The superparamagnetic nanocrystals were prepared via the thermal

decomposition method.[1] Iron (III) acetylacetonate (2 mmol) were mixed with 1,2-hexadecanediol (10 mmol), oleic acid (6 mmol), and oleyl amine (6 mmol) in benzyl ether (40 mL), then heated for two hours at 200ºC following 1 hour reflux at 300ºC under argon flow. The oleic acid coated magnetic NPs were washed in ethanol (50 mL) three times then collected by centrifuge. The obtained NPs were dispersed in hexanes in the presence of oleic acid (50 μL) and oleylamine (50 μL) and centrifuged to precipitate large particles. Then, hexanes were removed by rotavap and NPs (10 mg) were dispersed in chloroform (5 mL) for ligand exchange with TMAOH 3% in water (10 mL). The reaction mixture was stirred for 8 hours and NPs migrated from the organic layer to the aqueous phase. The resulted water dispersed NPs were added to a BSA solution in water (10 mg in 15 mL water) and the reaction was stirred overnight to obtain BSA coated NPs. It is noteworthy that the stirring should be done in a medium speed and in room temperature to prevent protein denaturing, usually observed as bubble formation. NP-BSA were washed using ultrafiltration (molecular weight cutoff MWCO: 100,000) to remove unbound BSA.

The cationization of NP-BSA was performed by treating the NPs (10 mg) with 2,2′-(ethylenedioxy)bis(ethylamine) in the presence of EDCI (100 mg) to yield positively charged NP-BSA_x. The extent of cationization was greatly dependent on the pH of the reaction where pH = 6 was found to be optimal. Conjugation of sialic acid was achieved by EDCI (0.375 mmol) coupling of sialic acid **2** (0.125 mmol) to NP-BSA_x (50 mg) dispersed in 15 ml pH=8.5 carbonate buffer and the reaction was stirred for 12 hours at room temperature. To hydrolyze methyl ester in the sialic acid conjugated, the NPs were treated with sodium hydroxide (0.01 M) and pH was carefully brought to 9. After 20 minute stirring, the pH value was dropped to 7.5 by adding hydrochloric acid solution (0.01 M). Finally, NP-BSA_x-Sia were washed by ultrafiltration (MWCO 100,000)

and dispersed in PBS buffer. For transcytosis and confocal microscopy experiments, NP-BSA_x-Sia were labeled with the fluorophore FITC to yield NP-BSA_x-Sia-FITC.

Characterization and physicochemical properties of NP-BSA_x-Sia

The prepared NPs were imaged under transmission electron microscope (TEM) (JEM-2200FM) operating at 200 kV using Gatan multiscan CCD camera with Digital Micrograph imaging software. Following each step of NP synthesis, the size and charge of the NPs were measured by dynamic light scattering (DLS) using a Zetasizer Nano zs apparatus (Malvern, U.K.). Thermal Gravimetric Analysis (TGA) was performed to quantify the total amount of organic content of NPs. Sialic acid was cleaved under mild acidic condition and thiobarbituric acid assay was performed to determine the amount of sugar on the surface of NPs.

To test the colloidal stability of the NPs, a solution of NP-BSA_x-Sia in PBS was kept at 4 °C and the hydrodynamic sizes and surface charge of NPs were continuously monitored by DLS over three weeks. As shown in **Figure S1d**, there were little changes of either hydrodynamic sizes or surface charges suggesting the high colloidal stability of the NPs.

To determine the magnetic relaxivitiy of the NP, serial dilutions of the NP were prepared and T_2* weighted MRI was performed. ($1/T_2$*) value was plotted against Fe concentration, and r_2* was the slope of the best linear fit.

Procedures for quantifying the amount of sialic acid on NP-BSA_x-Sia

To quantify the amount of sialic acid on NP-BSA_x-Sia, NP-BSA_x-Sia (2.9 mg mL^{-1}) were incubated in an aqueous H_2SO_4 (0.1 N) solution with 0.2% sodium dodecylsulfate (SDS) at 85°C for 1 hour. They were further incubated at 37°C for another 0.5 hour followed by periodic acid (50 μL) treatment (25mM H_5IO_6 in 125 mM HCl) for 0.5 hour at 37 °C. To inactivate the excess amount of periodic acid, 40 μL of sodium meta-arsenite $NaAsO_2$ (2%) in HCl (0.5M) was added.

When the yellow-brownish color of liberated iodine disappeared, 200 µL of thiobarbituric acid (0.1M pH = 9, pH was adjusted with NaOH (0.1M)) was added. The reaction tubes were placed in boiling water for 10 minutes then transferred into an ice bath for 2 minutes and kept at 37ºC for another 2 minutes. A butanol/HCl solution (500 µL, n-butanol containing 5.7% HCl (10.5M)) was used to extract the colored complex. Two phases were separated by centrifuge and the upper phase was transferred into a 96 well plate to determine the absorbance at 550nm using a microplate reader.

The number of sialic acid for each NP-BSA_x-Sia was calculated as follows: based on TGA, 26% of NP-BSA_x-Sia weight was Fe_3O_4 and TEM showed these nanoparticles are spherical and they are on average 5 nm in diameter. Since the lattice volume for magnetite is 592 $Å^3$, on average, each NP-BSA_x-Sia core contains 110.5 lattices based on the volume. From the crystal structure, each lattice contains 8 Fe_3O_4 molecules. Thus, the average number of Fe_3O_4 molecules in each NP-BSA_x-Sia core is 884. Sialic acid accounted for 7% of NP-BSAx-Sia weight then the average number of sialic acid (MW:465 for sialic acid-linker) for each NP-BSA_x-Sia is calculated to be 118.

Procedure for quantifying the amount of 2,2'-(ethylenedioxy)bis(ethylamine) on cationized BSA

2,2'-(Ethylenedioxy)bis(ethylamine) (50.8 mg, 0.34 mmol) was dissolved in PBS (5 ml) and pH value of the solution was adjusted to 6 by dropwise addition of diluted HCl. BSA (10 mg) was dissolved in PBS (10 ml) and added to the diamine solution together with EDCI (131.3 mg, 0.68 mmol). The reaction was allowed to continue overnight. The resulting cationized BSA was purified using extensive steps of dialysis (MWCO 3.5 kDa) in water and PBS. MALDI MS analysis (**Figure S1a**) was used to determine molecular weight of BSA after cationization. The

MS difference before and after diamine functionalization indicated that on average 33 molecules of 2,2'-(ethylenedioxy)bis(ethylamine) have been attached to each molecule of BSA.

Procedure for preparation of Aβ fibrils and oligomers

Synthetic Aβ (1-42) peptide (1 mg) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (3 mL) and sonicated for 15 min, then lyophilized for 72 h. The thin peptide film was dissolved in 0.22 μm filtered solution of 10 mM NaOH solution (0.25 mL). After 10 minute sonication, the solution was carefully neutralized with diluted HCl solution to pH= 6. The neutralized solution was diluted with PBS buffer to the desired final volume of 1.5 mL (the stock Aβ solution concentration is 147.7 μM). For experiments requiring fibril formation, the previous solution was incubated at 37°C for 48 h while for Aβ oligomers preparation the solution was incubated in 22°C for 24h. To verify formation of Aβ fibrils and oligomers, a native-PAGE gel electrophoresis was run and stained by Coomassie blue to visualize Aβ peptides (**Figure S3a**).

ELISA Assay of NP-BSA$_x$-Sia Binding with Aβ Oligomers

To evaluate the binding of NP-BSA$_x$-Sia with Aβ oligomers, an ELISA assay was run. To a well in a Nunc MaxiSorp 96-well ELISA plate was added a solutino of NP-BSA$_x$-Sia (0.5 mg/mL, 50 μL), which was incubated at 37 °C for 12 hours. After washing the wells with PBST (3 × 300 μL), Aβ oligomer solutions at various concentrations were added to the wells (50 μL, 0.0021, 0.0042, 0.0085, 0.017, 0.034, 0.068, 0.136, 0.2734, 0.547 and 1.09 μM respectively) and incubated at 22 °C for 12 h. The plate was washed with PBST (3 × 300 μL) and the anti-Aβ(1-16) IgG (6E10) monoclonal antibody, SIG-39320 (50 μL per well, 0.137nM, 1:24 000 in 1% (w/v) BSA containing PBS) was added, which in turn was incubated at 22 °C for 1 h. After washing with PBST (3 × 300 μL), the solution was discarded and the wells were washed three times with PBST (300 μL) and incubated for 1 h with the goat anti-mouse HRP-conjugated secondary antibody (50 μL, 1.7 nM,

1:18 000 in 1% (w/v) BSA in PBS). The solution was discarded, and the wells were washed three times with PBST (300 μL). To a freshly prepared 3,3′,5,5′-tetramethylbenzidine (TMB) solution (5 mg of TMB was dissolved in 2 mL of DMSO and then diluted to 20 mL with citrate phosphate buffer), H_2O_2 (20 μL) was added. This mixture (150 μL) was immediately added to the ELISA wells. Blue color was allowed to develop for 10-20 min. The reaction was quenched (yellow color) by addition of 0.5 M H_2SO_4 (50 μL/well) and the absorbance was measured at 450 nm on an SpectraMax M3 microplate reader. Binding of Aβ oligomers with NP-BSA$_x$-Sia is shown in **Figure S3**.

NP-BSA$_x$-Sia-FITC uptake by bEnd.3 endothelial cells using flow cytometry and laser confocal imaging

bEnd.3 cells (1 x 10^5 cells/well, 3 mL) were allowed to attach in a 6-well plate overnight at 37 °C and 5% CO_2. The cells were washed twice with PBS, and NP-BSA$_x$-Sia-FITC (83 μg mL^{-1}, 3 mL) in serum-free DMEM was added. The plate was incubated for 1, 3 and 7 hours at 37 °C under 5% CO_2. The cells were then washed with PBS (3x) and trypsinized with 0.25% trypsin-EDTA (1 mL). Trypsin was neutralized with serum-containing DMEM, and the cells were collected by centrifugation (2500 rpm; 4 °C). The cells were resuspended in DMEM (300 μL) and transferred to FACS tubes. The cells were stored on ice till the time of FACS analysis. The endocytosis of NP-BSA$_x$-Sia-FITC vs NP-BSA$_x$-FITC was also studied by repeating the same procedure but with 3 hour NP incubation.

To study endocytosis of NP-BSA$_x$-Sia into brain endothelial cells, confocal microscopy was performed. bEnd.3 cells (3 x 10^5 cells per well, 2 mL) were cultured in a 4- well chambered plate at 37 °C and 5% CO_2 for 24 hours. The culture medium was removed and the cells were washed with PBS (3x). NP-BSA$_x$-Sia-FITC in serum-free DMEM was added. The cells were

incubated with the NPs for 5 hours. Lysotracker red (1 mM, 50 µL per well) was added 1 hour before completion of incubation. The supernatant was removed. The cells were washed trice with PBS, and fixed with 10% formalin (0.5 mL per well) for 15 min. Formalin was removed and the cells were washed trice with PBS. DAPI (300 nM, 300 µL per well) was added, and the cells were incubated for 4–5 min. The supernatant was removed, and the cells were washed with PBS and water. The plate was covered by an aluminum foil and stored at 4 °C until imaging time. Confocal microscopy was performed on an Olympus FluoView 1000 CLSM.

Exocytosis of the NP-BSA$_x$-Sia-FITC from bEnd.3 endothelial cells

bEnd.3 cells ($3x10^5$ cells/well) were cultured in a 35 mm cell culture plate overnight at 37 °C and 5% CO_2. The supernatant was removed and the cells were washed with PBS trice. NP-BSA$_x$-Sia-FITC (0.1 mg mL^{-1}, 2 mL) in serum free DMEM media was added and the cells were incubated for 5 hours at 37 °C and 5% CO_2 after which the supernatant was removed and the cells were washed with PBS six times to ensure the complete removal of NPs from the surface of the cells. Fresh serum-free DMEM (2 mL) was added to the plate, which was incubated at 37 °C and 5% CO_2. The supernatant (100 µL) was transferred at specific time points to a black, clear bottom 96-well plate, and fluorescence was measured on a plate reader (excitation wavelength 488 nm; emission wavelength 520 nm). The drawn samples were returned to the plate to maintain a constant volume through the experiment.

Cytotoxicity assay of NP-BSA$_x$-Sia

bEnd.3 cells were plated into 96-well plates at a density of $4 x10^4$ cells per well in 10% DMEM cell culture media for 24 hours at 37 °C and 5% CO_2. The culture medium was replaced with non-serum solution of different concentrations of NPs (0.0625, 0.125, 0.25, 0.5, 1 mg mL^{-1}/well). After 4 hours incubation at 37 °C, the medium was replaced with MTS solution (20 µL in

200 μL) in culture medium and incubated for 1 hour at 37 °C. The developed brown color in the wells was an indication of live cells. The absorption of the plate was measured at 490 nm in an iMark microplate reader (BioRad). Wells without cells (blanks) were subtracted as background from each sample.

***Ex-vivo* brain Aβ/NP binding experiments (MRI)**

The brains of C57BL/6 mice were harvested and fixed in buffered 10% formalin solution for 24 hours. After washing with deionized water, brains were incubated with 27 μM Aβ (4 mL) for 12 hours at 4 °C. The Aβ treated brains were incubated with NP-BSA_x-Sia and NP-BSA_x (0.6 mg mL^{-1}, 4 mL) for 12 hours at 37 °C after washing with deionized water. After incubation, the brains were washed thoroughly and placed in a 24-well plate in PBS. For the sialic acid competition experiment, 0.1 M free sialic acid was added with NP-BSA_x-Sia (4 mL). Two control brains were treated in a similar manner (concentrations, temperatures and duration), however one was first incubated with Aβ only then PBS after water washing, and the other was incubated with PBS and then with NP-BSA_x-Sia after water washing. The brains were imaged with the following parameters (T_2* weighted sequence): wrist coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, echo times = 9.93 ms, time of repetition = 20.4 ms, receiver bandwidth = ±7.8 kHz, field of view = 6 cm, slice thickness = 0.5 mm, number of slices = 40, acquisition matrix = 256 × 256, and number of excitation = 3.

Prussian blue staining of mouse brains

The brains used in the *ex vivo* Aβ/NP-Sia MRI binding experiment were stained directly after imaging. First, brains were soaked in potassium ferrocyanide trihydrate ($K_4Fe(CN)_6$. $3H_2O$) solution 10% for 10 minutes. Then they were immersed in a 1:1 mixture of ($K_4Fe(CN)_6$. $3H_2O$)

10% and HCl 20% for another 10 minutes. The tissues were washed with water four times. A blue color was observed on the areas bearing iron oxide NPs.

Histological studies

Following soaking in sucrose solution, mouse brains were covered with optimum cutting temperature (OCT) formulation and frozen on a dry ice-methanol bath. Brains were sectioned at 10 μm using cryostat and tissues were placed on glass slides for further staining. To localize iron oxide NPs, Prussian blue staining was used and tissues were examined under bright field microscope. Thio-S staining was applied on prepared brain tissue samples to locate Aβ plaques and samples were viewed under a confocal microscope (Nikon, A1 CLSM).

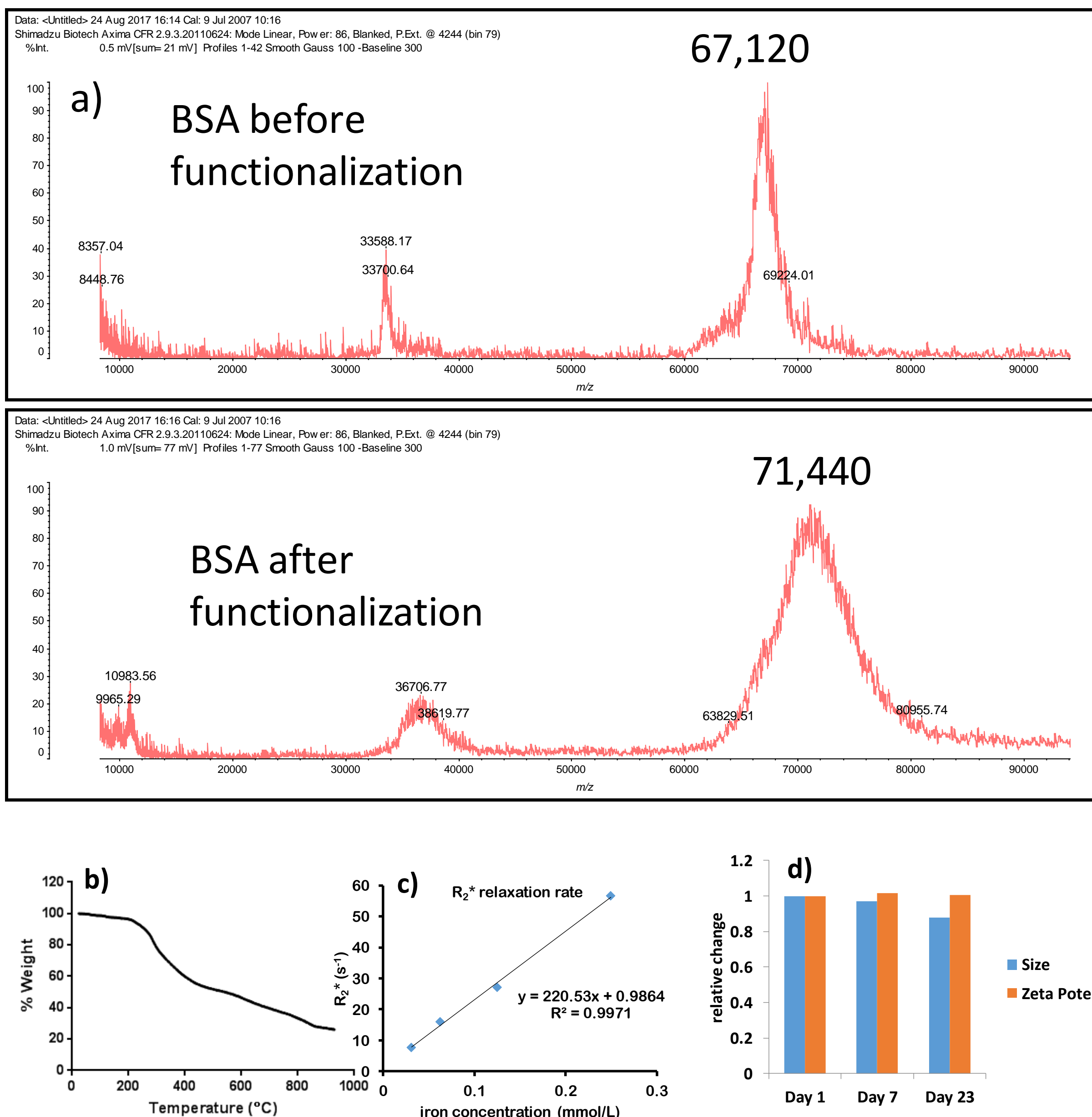


Figure S1. MALDI-MS of BSA (a) before and (b) after reaction with 2,2'-(ethylenedioxy)bis(ethylamine) **1**. Functionalization with diamine **1** caused the average molecular weight of BSA to increase from 67,120 to 71,440. This mass change corresponds to on average the addition of 33 molecules of **1** to each BSA following amide formation. (b) Thermogravimetric analysis (TGA) of NP-BSA_x-Sia; (c) R_2* relaxivity measurement of NP-BSA_x-Sia; and (d) There were little changes in size and zeta potential for NP-BSA_x-Sia in PBS over 23 days indicating the colloidal stability of the NPs..

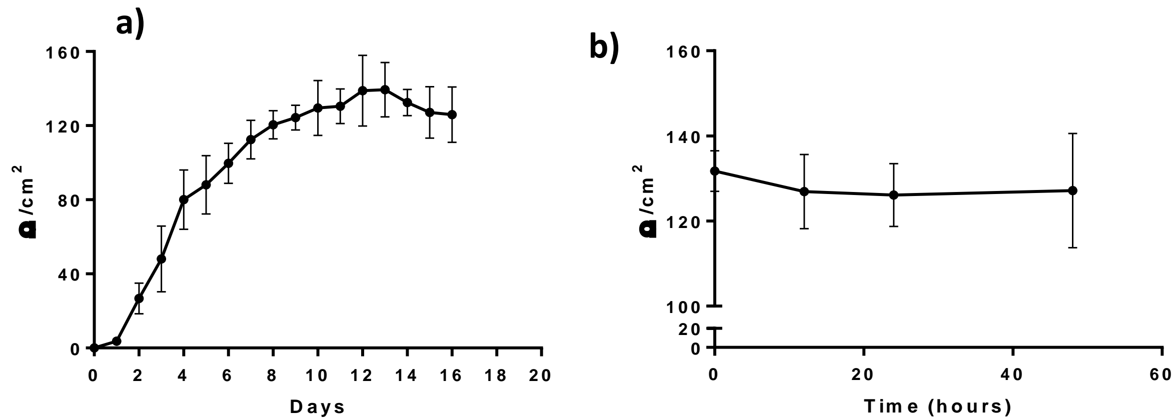
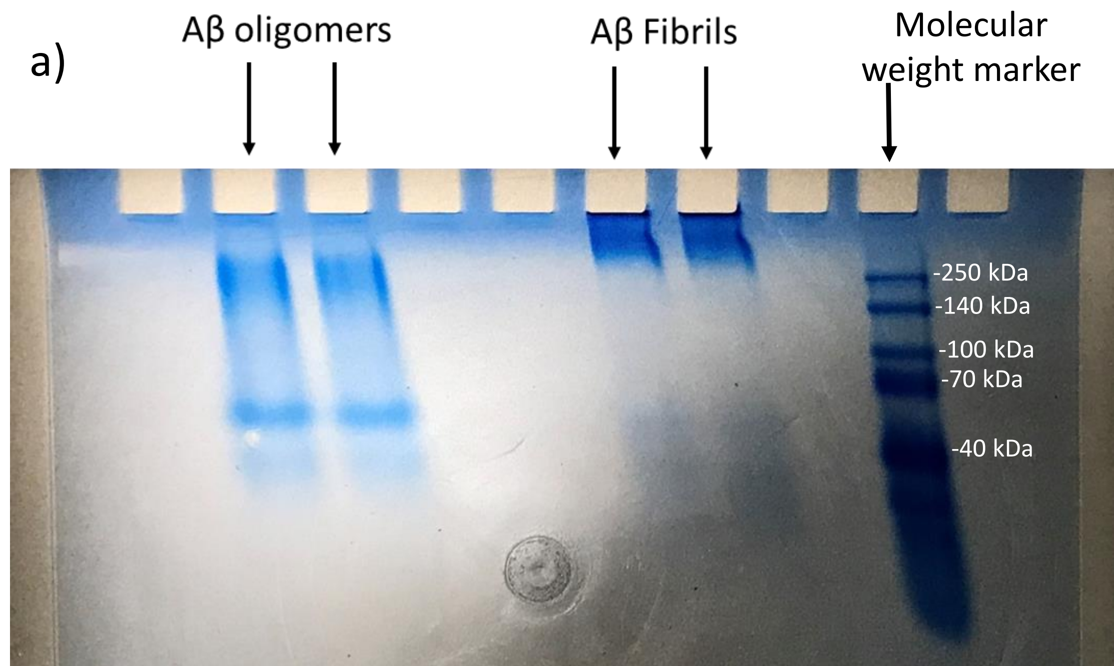


Figure S2. (a) bEnd.3 endothelial cells were grown on a transwell filter. The trans-endothelial electrical resistance (TEER) values reached a plateau after 10 days. The value suggested tight junction between the cells were formed; (b) Incubation of multi-layers of bEnd.3 cells with NP-BSA_x-Sia-FITC did not significantly change the values of the TEER values indicating little impacts on the tight junctions by the NPs.



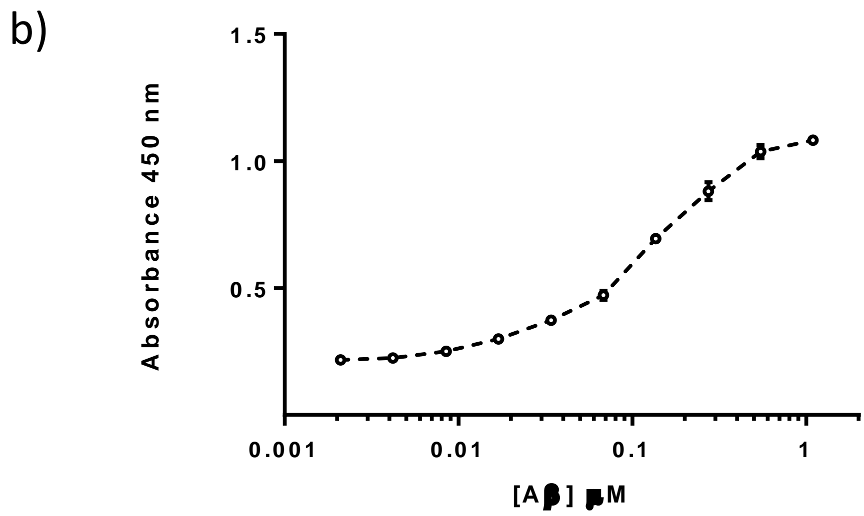


Figure S3. a) Native-PAGE gel electrophoresis following Coomassie blue staining confirmed the preparation of Aβ oligomers and fibrils respectively. b) ELISA showed dose dependent binding of Aβ oligomer by immobilized NP-BSA$_x$-Sia.

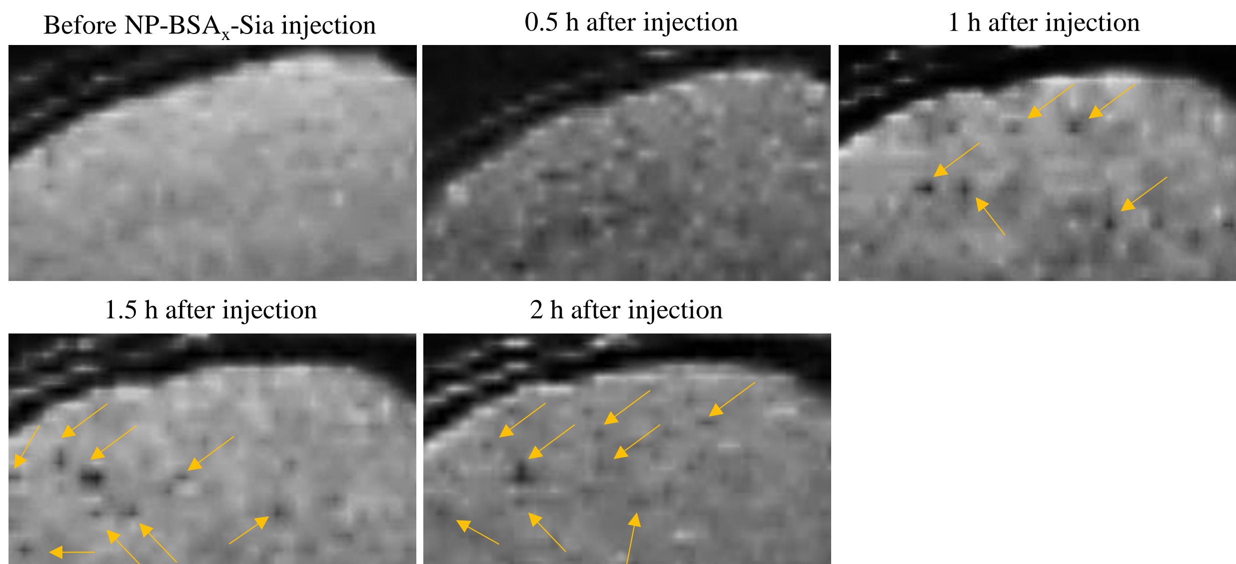


Figure S4. *In vivo* MRI of Tg mouse brain before and after injection of NP-BSA$_x$-Sia showed signal loss in some spots in the brain presumably due to binding of particles with Aβ plaques. The animal was scanned at different time intervals (0.5, 1, 1.5, 2 hours) after injection.

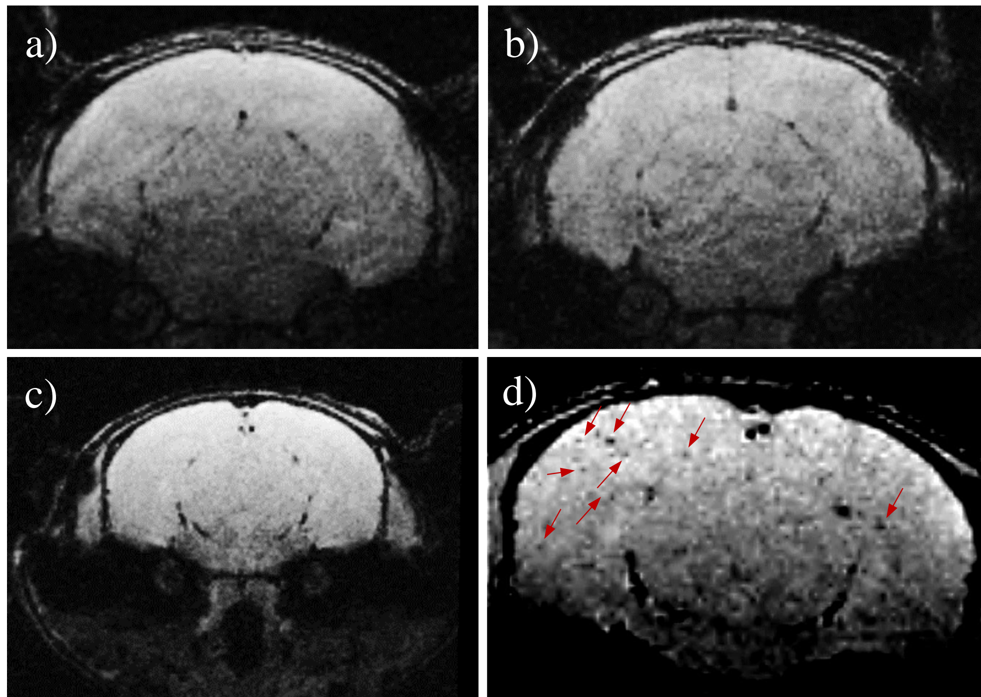


Figure S5. a) *In vivo* MRI of brain of a wild type mouse before and b) 1.5 hour after injection of NP-BSA_x-Sia. (c) and (d) are images of a brain slice of Tg mouse. c) before and d)1.5 hour after injection of nanoparticles respectively. c) and d) are presented here to aid in comparison with the images (a and b) from wild type mice. Regions of interest of signal changes have been highlighted by red arrows.

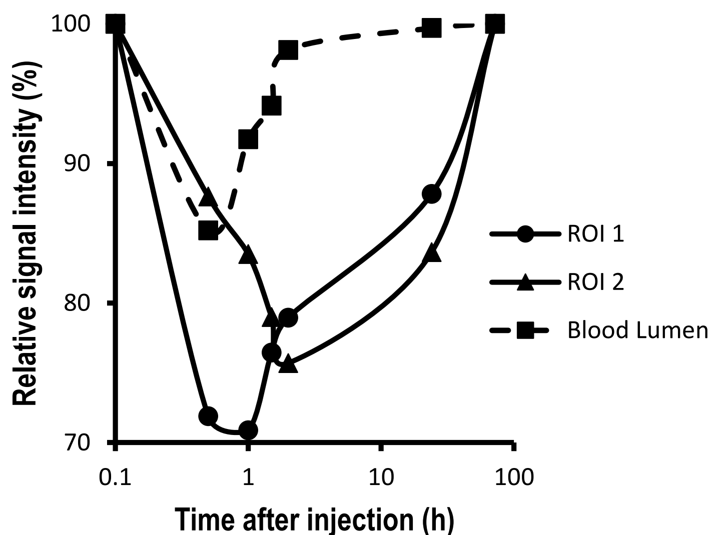


Figure S6. Relative MRI signal intensity changes for two ROIs presumably due to NP binding to Aβ plaques vs lumen of brain blood vessels.

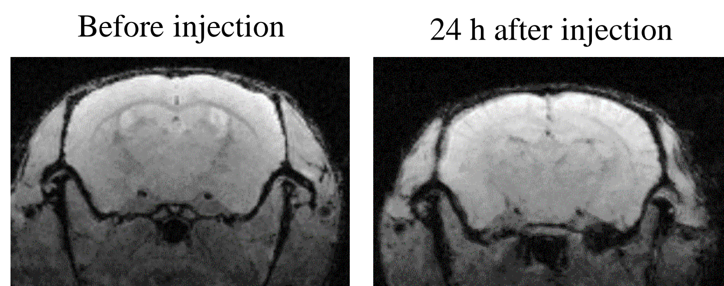


Figure S7. *In vivo* T_2* weighted MRI of Tg mouse brain after injection of Feridex®. Feridex® (8 mg kg^{-1}) was injected to Tg mouse then mouse brain was imaged. In contrast to Tg mouse receiving NP-BSA$_x$-Sia, no significant local changes were observed in MRI suggesting little binding of Feridex to brain tissues.

References:

(1) Xie, J.; Peng, S.; Brower, N.; Pourmand, N.; Wang, S. X.; Sun, S. *Pure Appl. Chem.* **2006**, *78*, 1003.