Glyconanoparticle Aided Detection of β-Amyloid by Magnetic Resonance Imaging and Attenuation of β-Amyloid Induced Cytotoxicity

Supporting Information

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Materials and Instrumentation

Unless otherwise indicated, all starting materials, reagents and solvents were obtained from commercial suppliers and used as supplied without further purifications. Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from Honeywell Riedel-de Haen, Iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), dextran (9−11 kDa), epichlorohydrin, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), ethyl-(3,3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), and sodium chloride were purchased from Sigma-Aldrich. Ammonium hydroxide (NH_4OH, 28−30%) and hydrogen peroxide (30%) were purchased from CCI. Amberlite IR 120 hydrogen form (Amberlite H^+) was purchased from Fluka. 1,1,1,3,3,3-Hexafluoro-2-propanol 99.9% was purchased from Acros. Buffered 10% formalin solution was purchased from Azer Scientific. Potassium ferrocyanide $K_4Fe(CN)_6$ trihydrate was purchased from Mallinckrodt. 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Acros Organics. Thioflavin T (ThT), UltraPure Grade was purchased from AnaSpec. Beta amyloid (1-42) was purchased from GL Biochem. (Shanghai) Ltd. (No. 52487). SH-SY5Y 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, the Aβ 1-16 (6E10) monoclonal antibody, 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 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega. Ultrathin-carbon type A, 400 mesh copper grids for TEM were purchased form Ted Pella, Inc. Ultrafiltration membranes and centrifugal filters were purchased from Millipore, while dialysis tubings were obtained from

BioDesign Inc. SH-SY5Y cells were cultured in DMEM. All cell culture media was supplemented with 10% inactivated FBS, 1% Pen-Strep mixture, glutamine (2 mM), and sodium pyruvate (1 mM). Dynamic light scattering (DLS) and zeta potential measurements were performed on a Zetasizer Nano zs apparatus (Malvern, U.K.). Transmission electron microscopy (TEM) images were collected on a JEM-2200FS operating at 200 kV using Gatan multiscan CCD camera with Digital Micrograph imaging software. Thermogravimetric analysis (TGA) was carried on a Thermal Advantage (TA-Instruments-Waters LLC) TGA-Q500 series and the samples were burned under nitrogen. Fluorescence emission spectra were recorded on a HITACHI F-4500 Fluorescence. Native-PAGE gel analysis was performed via ImageJ 1.42q (NIH). Spectrometer provided with a continuous (CW) high power Xe lamp as the excitation source. FACS experiments were conducted on a BD Vantage SE flow cytometer. HRMAS NMR experiments were carried out on a Varian Inova-500 NMR spectrometer equipped with a 4 mm gHXNanoprobe.

Experimental procedures

Synthesis of SPION

To a solution of dextran (10 g) in 0.22 µm filter filtered deionized water (23 mL), an aqueous solution of (0.7g, 2.6 mmol) $FeCl_3.6H_2O$ (1 mL) was added, and the mixture was cooled in an ice bath (ice/water). To improve the magnetic properties of the iron oxide nanoparticles, the mixture was stirred under Argon gas for 2 hours to eliminate oxygen from the reaction flask. A freshly prepared 0.22 µm filtered aqueous solution containing $FeCl_2.4H_2O$ (0.28 g, 1.4 mmol) in water (1 mL) was added to the cooled solution. Chilled 30% NH_4OH (1 mL) was added

dropwise while stirring rapidly. The black suspension was brought to 70-80 ºC over a 1 hour period, and maintained at this temperature for additional 90 min. After being cooled to room temperature, the suspension was dialyzed (MWCO 14,000) against distilled water (20 L bucket, 4 changes). The excess ammonium hydroxide, ammonium chloride, and dextran were further removed by ultrafiltration (MWCO 100,000). After 4 washes, the suspension was concentrated to ~ 75 mL, collected and centrifuged to remove any large particulates. Cross-linking the dextran coating on the surface of DSPION was achieved by adding an aqueous sodium hydroxide solution (5 M, 20 mL) and epichlorohydrin (10 mL). The mixture was stirred rapidly for 24 hr at room temperature. The excess epichlorohydrin was removed by dialysis against distilled water (MWCO 14,000) in a 20 L bucket (6 changes). The solution was concentrated to ~ 100 mL by ultrafiltration. The introduction of primary amino groups to the surface of the cross-linked dextran coated DSPION was achieved by adding 30% NH_4OH (20 mL) and stirring the mixture at 37 ºC for 36 hours. The colloidal solution was dialyzed (MWCO 14,000) against distilled water (20 L bucket, 4 changes), and then concentrated to 50 mL by ultrafiltration (MWCO 100,000) to yield DSPION-NH_2.

High-Resolution Magic Angle Spinning (HRMAS) NMR

HRMAS NMR experiments were carried out on a Varian Inova- 500 NMR spectrometer equipped with a 4 mm gHXNanoprobe (Variannmr Inc., Palo Alto, CA) available at the University of Tennessee Health Science Center (Memphis, TN). The HR-MAS probe with internal lock is capable of performing either direct or indirect (inverse) detection experiments. Magic angle spinning (MAS) experiments were performed at spinning rates of up to 2.5 kHz using a 40 µL glass rotor. NP-Sia was dissolved in D_2O solvent and was further diluted at different concentrations with D_2O to find out the concentration limit to the NMR signal

broadening. HRMAS ^{1}H-NMR spectra were obtained using 100-600 scans for each experiment. The sample temperature was regulated with an accuracy of ± 0.1 °C.

Thiobarbituric acid Assay (TBA)

NP-Sia was treated with 0.2% sodium dodecylsulfate (SDS) in 0.1 N H_2SO_4 to the final concentration of 2.86 mg/mL at 85°C for 60 minutes to hydrolyze the sialic acid from the nanoparticle. It was then incubated at 37 °C for 30 minutes, and then treated with 50 µL periodic acid (25mM H_5IO_6 in 125mM HCl) for 30 min at 37 °C. The excess periodic acid was inactivated with 40 µL sodium meta-arsenite (2% $NaAsO_2$ in 500mM HCl). When the resulting yellow-brownish color (color of the liberated iodine) disappeared, 200 µL of thiobarbituric acid (0.1M 4,6- dihydroxypyrimidine-2-thiol, pH 9) (pH adjusted with 0.1 mol/L NaOH) was added. The reaction tubes were incubated in a boiling water bath for 10 min. The tubes were transferred to an ice-water bath for 2 min followed by 2-min incubation at 37 °C. (The latter incubation improves the extent and increases the rate of chromophore extraction.) The colored complex was extracted by adding 500 µL butanol/HCl solution (n-butanol containing 5.7% of 10.5M HCl) with vigorous agitation. The two phases were separated by centrifugation for 5 minutes. 250 µL of the upper phase was placed into a 96-well flat-bottomed microtiter plate and the absorbance was determined at 550nm using a microplate spectrophotometer.[1, 2]

Thioflavin-T Assay (ThT)

ThT fluorescence measurements were performed in a clear bottom black 96-well fluorescence plate (COSTAR 3695-96) on a FLUOstar OPTIMA (BMG Labtechnologies). The control solutions were 250 µL of tris buffer, 250 µL of 20 µM Aβ (Aβ (20 µM)), and 250 µL of 20 µM ThT. 50 µL of Aβ (20 µM) solutions were added to 200 µL of different concentrations of (10,

20, and 50 μM) ThT solutions. ThT fluorescence measurements were performed with λ_{ex} = 440 nm (10 nm bandpass) and λ_{em} = 489 nm (10 nm bandpass).

Transmission Electron Microscopy (TEM)

Similar sample preparation protocol to intrinsic tyrosine fluorescence was used for TEM imaging. 10 μL of the samples were deposited on ultrathin-carbon type A, 400 mesh copper grids (Ted Pella, Inc.) and let to evaporate under the hood. Once dry, 1% solution of uranyl acetate was added for 10 seconds and the solution was wicked away with filter paper. The grids were then washed with water, and dried for 15 min at room temperature.

Aβ and NP-Sia binding using Prussian blue staining

Aβ solutions (0.5 mL, 25 and 100 μM) were added to two eppendorf tubes containing solutions of NP-Sia (0.5 mL, 200 μg/mL) respectively. Control solutions were prepared by addition of PBS buffer (0.25 mL) into two eppendorf tubes containing NP-Sia (0.25 mL, 200 μg/mL) or Aβ (0.25 mL, 100 μM). All samples were rotated on a tube rotator overnight at room temperature then incubated for 1 hour at 37 °C. NP-Sia/Aβ mixtures and control solutions (0.5 mL each) were pipetted to 24-well Costar cell culture, flat-bottomed plate and then incubated for 4 hours at 37 °C. After thoroughly washing the wells with PBS (5 x 0.5 mL), the Gomori's modified Prussian blue solution (0.5 mL) was added to each well and incubated for 10-15 minutes. Blue color was developed in wells containing NP-Sia. (Gomori's modified Prussian blue preparation: 2 mL of 10% $K_4Fe[CN]_6$ solution mixed with 2 mL of 20 % v/v HCl solution.)

Native-PAGE Gel Electrophoresis:

Samples preparation was conducted exactly as described for the intrinsic tyrosine fluorescence experimenta1 procedure. 13.5 µL of Aβ in 10 mM NaOH (443 µM) was added to 200 µL of NP-Sia (0.02, 0.2, and 2 mg/mL) in eppendorf® tubes. The tubes were gently mixed and incubated at room temperature for 24 hours. After incubation, 20 µL of the mixture was added to 5 mL of non-SDS sample buffer and was subjected to electrophoresis (200 V) on an 18% native-PAGE gel. The gels were then stained with Coomassie (for Aβ characterization) and silver staining (for Aβ/NP-Sia binding).

Magnetic Resonance Imaging (MRI) Experiments:

All MRI experiments were carried out on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI).

NP-Sia Relaxivity Measurements

Seven different dilutions of the NP-Sia (0.00001, 0.0001, 0.001, 0.0125, 0.05, 0.1 mg/mL) were prepared to a final volume of 5 mL in 15 mL-centrifuge tubes (Corning). The tubes were placed on a polystyrene tube holder. To evaluate the R2* characteristics of the nanoparticles in phantoms, the following parameters were used: Head coil, 2D fast spin echo, flip angle = 90°, eight echo times (TEs) = 8.0 ms, 16.0 ms, 23.9 ms, 31.9 ms, 39.9 ms, 47.9 ms, 55.8 ms and 63.8 ms, time of repetition (TR) = 500 ms, receiver bandwidth (rBW) = ± 31.2 kHz, field of view (FOV) = 16 cm, slice thickness = 3 mm, number of slices = 2, acquisition matrix = 256 × 256 and number of excitation (NEX) = 1. To evaluate the R1 characteristics of the nanoparticles in phantoms, the following parameters were used: Head coil, 2D inversion recovery spin echo, flip angle = 90°, echo time = 15 ms, time of repetition = 2500 ms, inversion time = 50 ms, 100 ms, 400 ms, 700 ms and 1000 ms, receiver bandwidth = ± 15.6 kHz, field of view = 16 cm, slice

thickness = 3 mm, number of slices = 1, acquisition matrix = 256 × 128 and number of excitation = 1. The R1 was estimated based on the spin recovery curve after inversion.

Determination of Detection Limit of Aβ

Eight 15 mL centrifuge tubes of constant concentration of nanoparticles and variable concentration of Aβ were incubated in PBS buffer (total volume of 3 mL/each) for 24 hours at room temperature. The final concentration of NP-Sia was 0.083 mg/mL and final concentration for series of Aβ solutions were 0.033, 0.130, 0.521, 2.083, 8.333, 33.333 µM. The remaining two tubes were considered blanks (contained 3 mL of NP-Sia with a final concentration of 0.083 mg/mL only). To evaluate the T2* characteristics of the nanoparticles in phantoms, the following parameters were used: head coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 echo times (TEs) = 2.1 ms, 4.6 ms, 7.0 ms, 9.4 ms, 11.8 ms, 14.3 ms, 16.7 ms, 19.1 ms, 21.5 ms, 24.0 ms, 26.4 ms, 28.8 ms, 31.2 ms, 33.7 ms, 36.1 ms, and 38.5 ms, time of repetition = 41.9 ms, receiver bandwidth = ± 62.5 kHz, field of view = 16 cm, slice thickness = 1.5 mm, number of slices = 16, acquisition matrix = 256 × 256, and number of excitation = 1.

LIVE/DEAD® Cytotoxicity Assay

SH-SY5Y cells were plated into 24-well plates at a density of 1 x 10^4 cells per well in cell culture medium for 24 hours at 37 °C and 5% CO_2. Then the culture medium was replaced with non-serum DMEM media of 0.78, 3.125, 6.25, 12.5, 25, and 50 µM Aβ solution (100 µL/well). After 24 hour incubation at 37 °C and 5% CO_2, the supernatant was carefully collected (but not discarded due to the fact that it might contain dead cells). To detach cells 200 µL trypsin was added to each well. Then 500 µL of serum containing DMEM media was added. Cells from each well were collected and added to the corresponding supernatant, and after gentle swirling the tubes were centrifuged for 5 minutes at 2500 rpm at 4°C. Supernatants were discarded and

the cells were washed with serum-free DMEM media. This process was repeated twice. After the final wash the cells were resuspended in 500 µL LIVE/DEAD® solution and transferred to FACS tubes on ice. Tubes were removed from the ice container and incubated at room temperature for 10 minutes and they were analyzed with the flow cytometry. Flow cytometry was set to measure the green fluorescence emission of calcein at 530 nm, 30 nm bandpass (emission 488 nm) and red fluorescence emission of ethidium homodimer-1 (EthD-1) at 660 nm, 20 bandpass. Standard compensation was performed using single color stain cells.

Preparation of the LIVE/DEAD solution was done by adding 10 µL of supplied 2 mM EthD-1 stock solution (component B) and 2 µL of the supplied 4mM calcein AM stock solution (component A) to 5 mL of DMEM.

The controls used for this assay were unstained cells, Aβ untreated cells, and dead cells (50% ethanol fixed).

Rescue of SH-SY5Y cells by NP-Sia as determined by the LIVE/DEAD® Assay

SH-SY5Y cells were plated into 24-well plates at a density of 1 x 10^4 cells per well in cell culture medium for 24 hours at 37 °C and 5% CO_2. The culture medium was replaced with non-serum DMEM media of 2 µM Aβ (250 µL/well) in addition to 250 µL/well of different concentration of NP-Sia (0.97, 1.95, 7.81, 15.62, and 31.25 µg/mL). After 24 hour incubation at 37 °C and 5% CO_2, the supernatant was carefully collected (but not discarded due to the fact that it might contain dead cells). To detach cells 200 µL trypsin was added to each well. 500 µL of serum containing DMEM media was added. Cells from each well were collected and added to the corresponding supernatant, and after gentle swirling the tubes were centrifuged for 5 minutes at 2500 rpm at 4°C. Supernatants were discarded and the cells were washed with serum-free DMEM media. This process was repeated twice. After the final wash the cells were

resuspended in 500 µL LIVE/DEAD® solution and transferred to FACS tubes on ice. Tubes were removed from the ice container and incubated at room temperature for 10 minutes and they were analyzed with the flow cytometry. Flow cytometry was used to measure the green fluorescence emission of calcein at 530 nm, 30 nm bandpass (emission 488 nm) and red fluorescence emission of ethidium homodimer-1 (EthD-1) at 660 nm, 20 nm bandpass. Standard compensation was performed using single color stain cells.

Preparation of the LIVE/DEAD solution was done by adding 10 µL of supplied 2 mM EthD-1 stock solution (component B) and 2 µL of the supplied 4mM calcein AM stock solution (component A) to 5 mL of DMEM.

The controls used for this assay were unstained cells, Aβ untreated cells, dead cells (50% ethanol fixed), 2 µM Aβ treated cells, and only-NP-Sia treated cells.

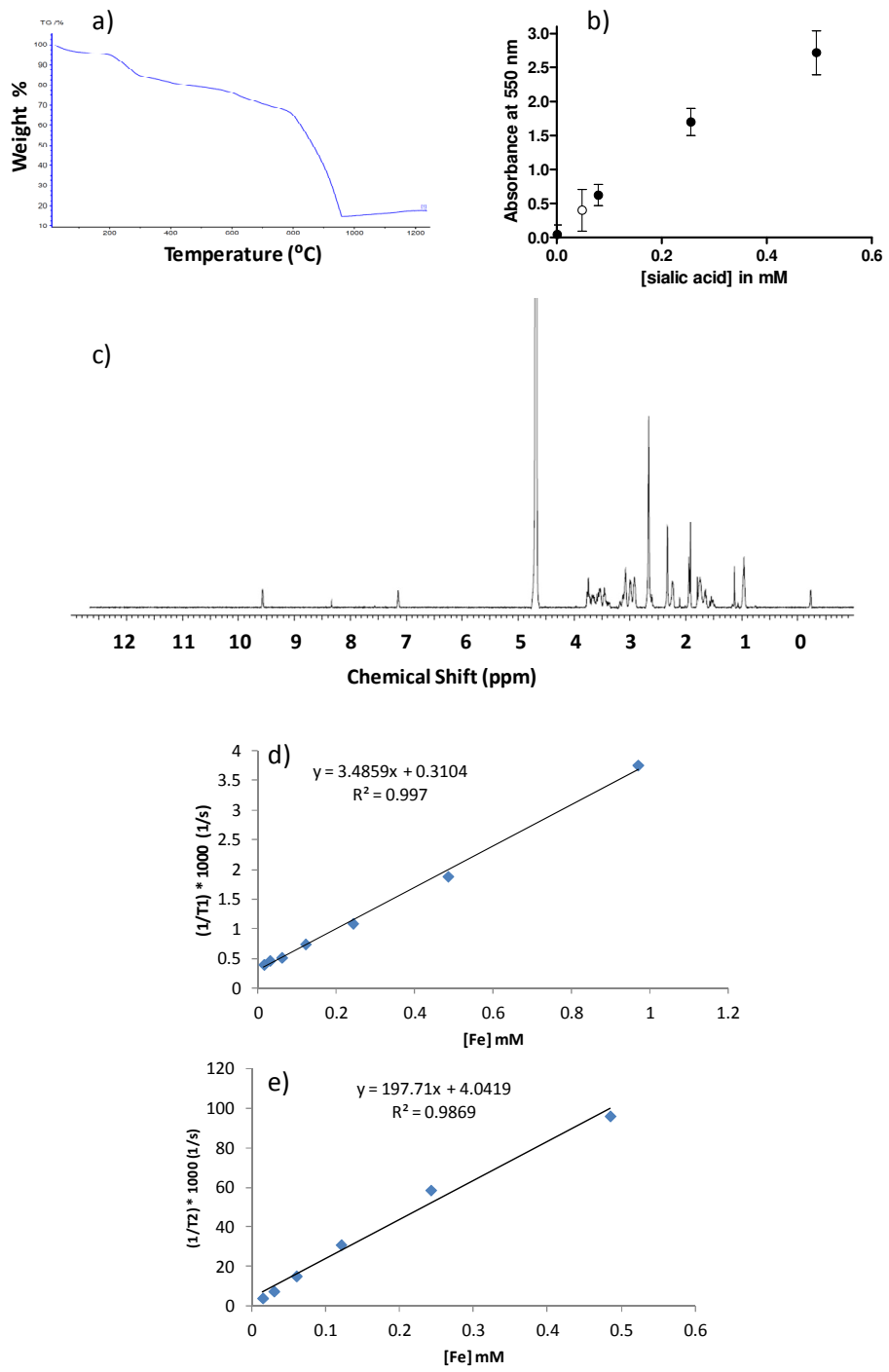

Figure S1. Characterization of NP-Sia a) thermogravimetric analysis (TGA) of the NP-Sia; b) thiobarbituric acid assay (TBA) showed the amount of sialic acid was 0.05 mM, which corresponded to 4% weight of the NP-sia; c) high-resolution magic angle spinning nuclear magnetic resonance (HRMAS-NMR) of the NP-Sia; d) T1 and e) T2 relaxivity characterization of NP-Sia. The R1 and R2 values are 3.5 $mM^{-1}s^{-1}$ and 198 $mM^{-1}s^{-1}$ respectively.

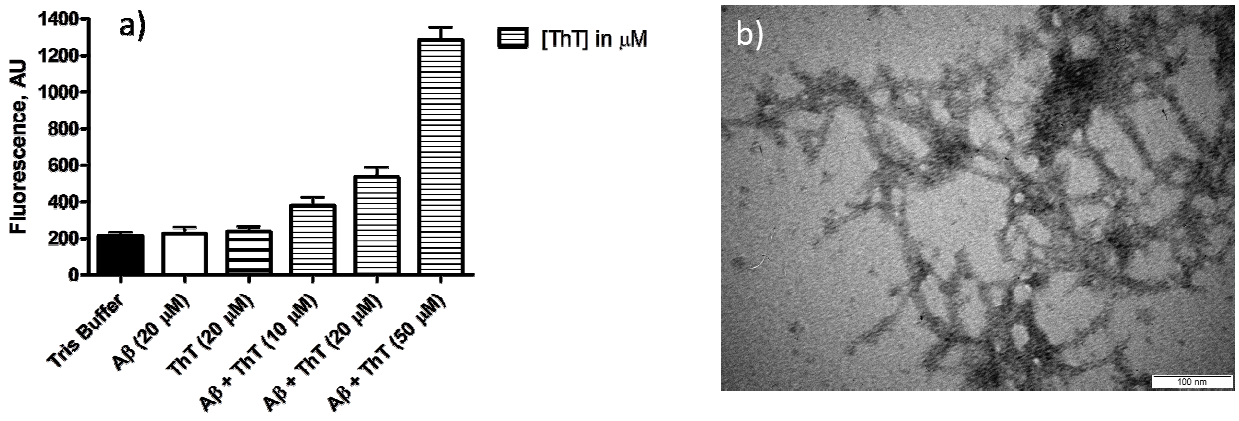

Figure S2. a) Enhancement of ThT fluorescence (excitation 440 nm and emission at 489 nm) upon binding to β-sheet rich Aβ fibrils; b) TEM images of Aβ fibrils with 2% uranyl acetate staining.

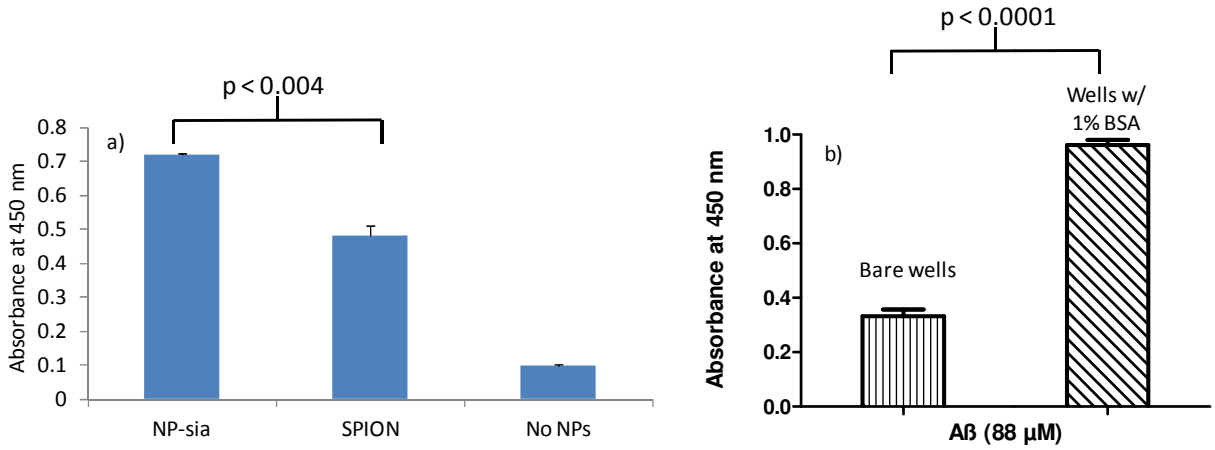

Figure S3. a) Absorbance of Aβ binding to wells containing NP-Sia, SPION and bare wells suggesting the importance of sialic acid in Aβ recognition ($p < 0.004$ based on t test); b) Bare ELISA wells showed significantly less Aβ binding compared to wells incubated with 1% BSA ($p < 0.0001$ based on t test). This suggests that BSA can bind with Aβ.

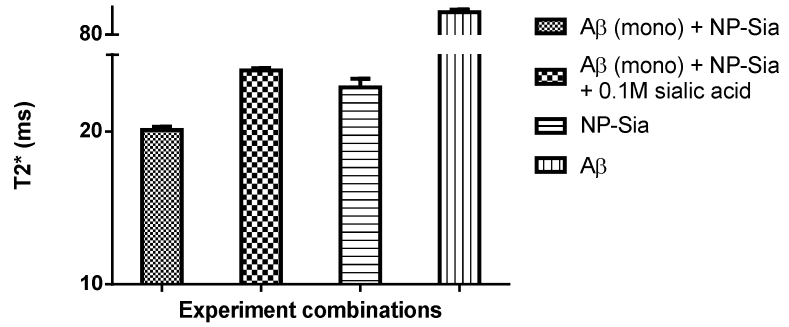

Figure S4. MRI generated T2* values of NP-Sia/Aβ binding. NP-Sia (0.1 mg/mL) incubated with Aβ monomers (30 μM) and also in the presence of free sialic acid (0.1 M). The error bars represent the standard deviation of three measurements.

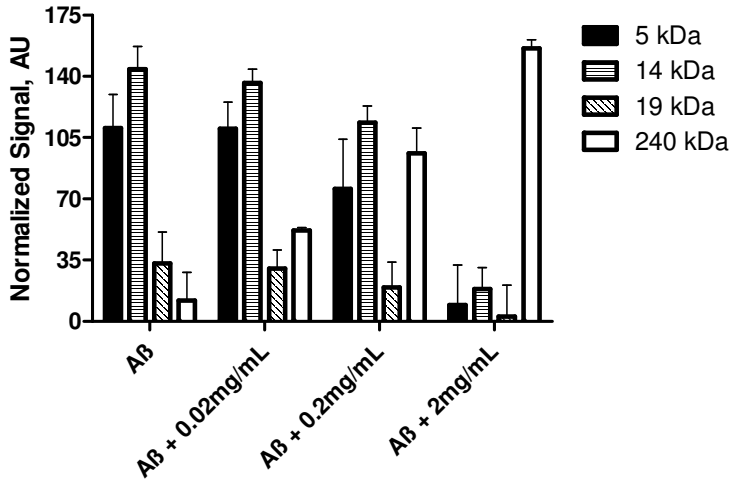

Figure S5. Quantification of the intensities of various bands on the four lanes of the PAGE gel shown in Figure 7. Aβ only (Lane 1); Aβ incubated with 0.02 mg/mL (Lane 2); 0.2 mg/mL (Lane 3); and 2 mg/mL (Lane 4) of NP-Sia. (5 kDa represents the Aβ monomer. 14 kDa and 19 kDa represent the Aβ oligomers. The bands around 250 kDa are Aβ fibrils.)

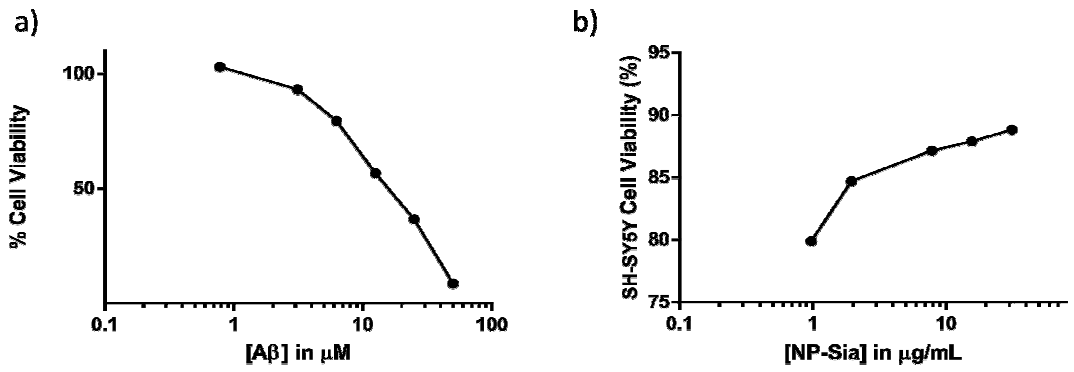

Figure S6. a) Viability of SH-SY5Y neuroblastoma cells decreased with increasing concentration of Aβ as determined by a LIVE/DEAD cell viability assay. b) Addition of NP-Sia to SH-SY5Y neuroblastoma cells incubated with Aβ (2 μM) protected cells from Aβ induced cytotoxicity using LIVE/DEAD cell viability assay.

References:

(1) Aminoff, D. (1961) Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids, *Biochem. J. 81*, 384-392.

(2) Santos-Lopez, G., Flores, E., Banos, R., Herrera-Camacho, I., and Reyes-Leyva, J. (2004) Purification of the Porcine rubulavirus attachment protein by liquid isoelectric focusing, *Protein Expression Purif. 35*, 120-125**.**