Supplementary data for

Naphthalene-facilitated self-assembly of Gd-chelate as a novel T_2 MRI contrast agent for visualization of stem cell transplants

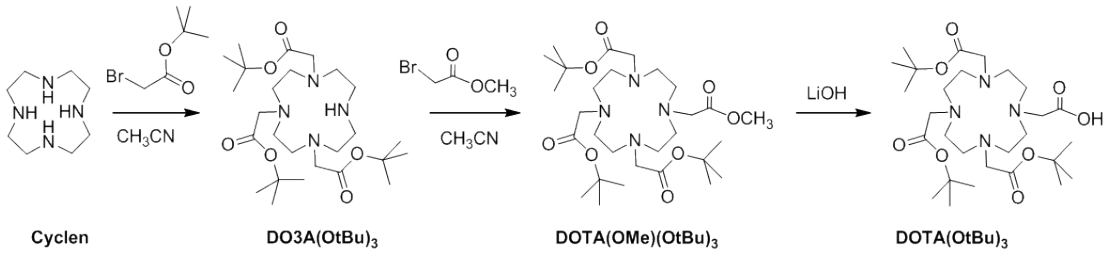
Yanhui Zhang,[a,b] Hailu Zhang,[b] Dehua Huang,[b] Bo Tan[b] Chenxing Zhang,[b] Zongwu Deng,[a,b]*

[a]*School of Nano-Tech and Nano-Bionics, University of Science and Technology of China, Hefei 230026, P. R. China*

[b]*CAS Key Laboratory of Nano-Bio Interface, Suzhou Institute of Nano-tech and Nano-bionics, Chinese Academy of Sciences, Suzhou 215123, P. R. China*

Text S1 Synthesis of DOTA(OtBu)$_3$:

1-(acetic acid)-4,7,10-tris(tert-butoxycarbonyl methyl)- 1,4,7,10- tetraazacyclododecane [DOTA(OtBu)$_3$] was synthesized from 1,4,7,10-tetraazacyclododecane tetrahydrochloride with an optimized multiple steps according to the literature[32].



Scheme S1 Synthetic procedure of 1-(acetic acid)-4,7,10-tris(tert-butoxycarbonyl methyl)- 1,4,7,10-tetraazacyclododecane [DOTA(OtBu)$_3$]

The optimized synthetic procedure (Scheme S1) was briefly described as following.

Cyclen: 1,4,7,10-tetraazacyclododecane(cyclen) was obtained by adding four molar ratio of sodium hydroxide into its tetrahydrochloride salt neutralizing the hydrochloric acid. The product of cyclen may contain one molecular water as the yield is more than 100%. ^{1}H NMR (in $CDCl_3$, 400 MHz): δ 2.69(s, 16H, CH_2), 2.13(br, 4H, N-H).

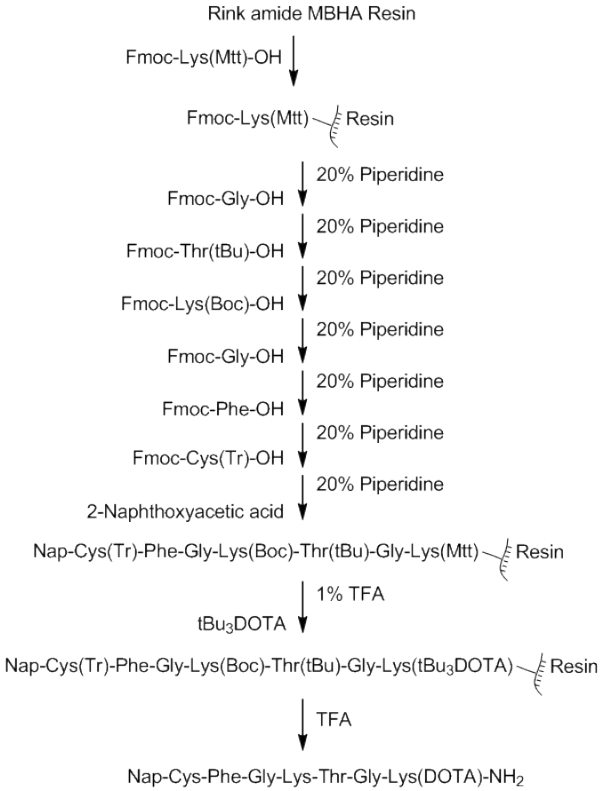
DO3A(OtBu)$_3$: 1,4,7-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (DO3A(OtBu)$_3$). Cyclen was dissolved in acetonitrile, to this solution 5 molar ratio of sodium bicarbonate was added in and 3 molar ratio of tert-butyl 2-bromoacetate in acetonitrile were added slowly dropwise at room temperature, and stirred for 24 hours. Then the solvent acetonitrile was evaporated, the residue was added dichloromethane and washed several times with brine(about 5%). The organic phase was dried with anhydrous Na_2SO_4 and evaporated to yield the desired product DO3A(OtBu)$_3$. ^{1}H NMR($CDCl_3$, 400 MHz): δ 10.05(s, 1H, N-H), 3.38(s, 4H, CH_2), 3.28(s, 2H, CH_2), 3.11(s, 4H, CH_2), 2.93(m, 8H, CH_2), 2.88(m, 4H, CH_2), 1.46(s, 27H, CH_3).

DOTA(OMe)(OtBu)$_3$: 1-(Methoxycarbonyl methyl)-4,7,10-tris(tert-butoxycarbonyl methyl)- 1,4,7,10- tetraazacyclo-dodecane [DOTA(OMe)(OtBu)$_3$]: DO3A(OtBu)$_3$ and 3 molar ratio of potassium carbonate was added into acetonitrile, and 1.2 molar of methyl 2-bromoacetate in acetonitrile solution was added dropwise at room temperature and stirred for 4 hours at 40°C. Then acetonitrile was evaporated and the residue was dissolved in dichloromethane, the organic phase was washed with brine(about 5%) for several times, dried with anhydrous sodium sulfate and evaporated to obtain a yellowish viscous liquid. The crude product was purified by column chromatography CH_2Cl_2/MeOH (20:1) and the yellowish viscous foam like product was obtained with a yield of up to 75%. DOTA(OMe)(OtBu)$_3$: ^{1}H- NMR($CDCl_3$, 400 Hz) δ：3.69(s, 3H), 3.43(s, 2), 3.31-3.28(br, 6H), 2.82(s,16H), 1.43(s, 27H).

DOTA(OtBu)$_3$: 1-(acetic acid)-4,7,10-tris(tert-butoxycarbonyl methyl)- 1,4,7,10- tetraazacyclododecane [DOTA(OtBu)$_3$]: DOTA(OMe)(OtBu)$_3$ was dissolved in the mixed solvent of methanol and water (2:1), and hydrolyzed with 2 molar ratio of lithium hydroxide monohydrate for 4 hours. Methanol was evaporated and the solution was neutralized with hydrochloric acid to pH 5 ~ 6, then extracted with dichloromethane, dried with anhydrous sodium sulfate, then the solvent was evaporated to yield viscous foam like product DOTA(OtBu)$_3$ about 70%. ^{1}H NMR ($CDCl_3$, 400 MHz, Figure S1): δ 3.67(s, 2H), 3.59(br, 4H), 3.37(s, 2H), 3.29(s, 4H), 3.03(t, 4H, J=5.6Hz), 2.75(br, 8H), 1.43(s, 27H).

Text S2 Synthesis of Nap-CFGKTGK-DOTA-Gd (Nap-Gd)

The synthesis procedure of Nap-CFGKTGK(DOTA) was described as following and showed in Scheme S2:



Scheme S2 Synthesis procedure of Nap-CFGKTGK(DOTA).

General process: Nap-CFGKTGK(DOTA) was Synthesized by a standard solid phase N-9-Fluorenylmethoxycarbonyl (Fmoc) peptide synthesis strategy according to the designed peptide sequence from C to N terminal.

Rink amide MBHA Resin (0.5 mmol/g) as the solid support was swelled well in DMF for 2 h. Fmoc-Lys(Mtt)-OH was added to form the amide bond by condensation of carboxyl and amino groups. The molar ratio of amino acid(AA) and the condensing agents was AA : DIC : HOBT : Resin = 3 : 3 : 3 : 1. Condensation efficiency of about 99 % was determined with the quantitative ninhydrin assay.

After accomplishing the amide bond formation, Fmoc was deprotected with adding 20v/v% piperidinein/DMF for 30 min and washing thoroughly six times with DMF.

The designed peptide with protection groups was prepared by repeating above two steps in the order of Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Cys(Tr)-OH, and 2-Naphthoxyacetic acid. After accomplishing attaching 2-Naphthoxyacetyl group to the N terminal, 1% TFA of dichloromethane solution was used to deprotect the Mtt group at the side chain of Lysine at C terminal, followed by washing six times with DMF. Then NMM and HATU were used to conjugate DOTA(OtBu)$_3$ with a molar ratio of DOTA(OtBu)$_3$: HATU : NMM = 2 : 1.9 : 4. The condensation efficiency was determined by measuring free residual amine group with the quantitative ninhydrin assay.

After the success of DOTA(OtBu)$_3$ conjugation, the crude product was cleaved off the resin by treatment with a mixture solution of 90% TFA, 5% EDT, 2.5% thioanisole and 2.5% H_2O for 3 h at room temperature. Meanwhile, all protective groups were removed. After filtration to remove the resin, the solution was placed into cold ether of ten times in volume to precipitate the product. The sediment was centrifuged and washed three times with cold ether followed by purification with semi-preparative HPLC (with a gradient of 0.1%TFA CH_3CN from 20% to 40% in 20 min, an elution speed of 10 mL/min, and UV detection at 220 nm). The product was lyophilized to yield Nap-Cys-Phe-Gly-Lys-Thr-Gly-Lys(DOTA)-NH_2 (Nap-CFGKTGK(DOTA) in short) as a white amorphous solid with a purity of more than 95%. Nap-CFGKTGK(DOTA) structure was confirmed by Electron Stimulated Ionization-Mass Spectrometry (ESI-MS, Agilent 6120 Single-Quadrupole LC/MS System).

Text S3 Aqueous MRI experimental parameters

Aqueous longitudinal (T1) relaxation times of different Gd concentration were measured using a saturation recovery method with TE = 7 ms, TR = 40, 70, 100, 180, 300, 500, 750, 1000, 1500, 3000, 5000 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice thickness = 0.8 mm, and number of average = 2. Aqueous transverse (T2) relaxation times were measured using a multi-slice multi-echo (MSME) sequence with 20 echoes. The experimental parameters were TR = 3000 ms, TE = 8~160 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice thickness = 0.8 mm, and number of average = 1.

Text S4 *In vitro* cellular MRI experimental parameters

Multi-Slice Multi Echo (MSME) pulse sequence was used to acquire *in vitro* T1- and T2-weighted images. The experimental parameters for acquiring T1-weighted images were TR = 500 ms, TE = 5.2 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice thickness/gap= 0.8 / 0.2 mm and number of average = 4. The experimental parameters for acquiring T_2-weighted images were TR = 3000 ms, TE = 40 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice thickness / gap= 0.8 / 0.2 mm and number of average = 2. These experimental parameters were used throughout the experiments for ease of MRI signal calibration of different experiments.

In vitro T_1-relaxation times were measured using a Rapid Acquisition with Refocused Echoes **(**RARE) pulse sequence with effective TE = 7 ms, TR = 40, 70, 100, 180, 300, 500, 750, 1000, 1500, 3000, 5000 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice / gap thickness = 0.8 / 0.2 mm, and number of average = 2. *In vitro* T2-relaxation times were measured using MSME sequence with 60 echoes. The experimental parameters were TR = 3000 ms, TE = 8~480 ms, FOV = 12 × 12 mm^2, matrix = 96 × 96, slice / gap thickness = 0.8 / 0.2 mm, and number of average = 2. They are reported in relaxation rates.

Text S5 *In vivo* MRI experimental parameters

In vivo T1-weighted images were acquired by using MSME sequence with TR = 500 ms, TE = 5.7 ms, number of average = 8, FOV = 2.0 × 2.0 cm^2, matrix = 128 × 128 with slice thickness of 0.5 mm. The corresponding voxel size is 156 × 156 × 500 μm^3. *In vivo* T2-weighted images were acquired by using RARE sequence with TR / effective TE = 3000 / 22 ms, number of average = 8, FOV = 2.0 × 2.0 cm^2, matrix = 128 × 128 with slice thickness of 0.5 mm. The corresponding voxel size is 156 × 156 × 500 μm^3.

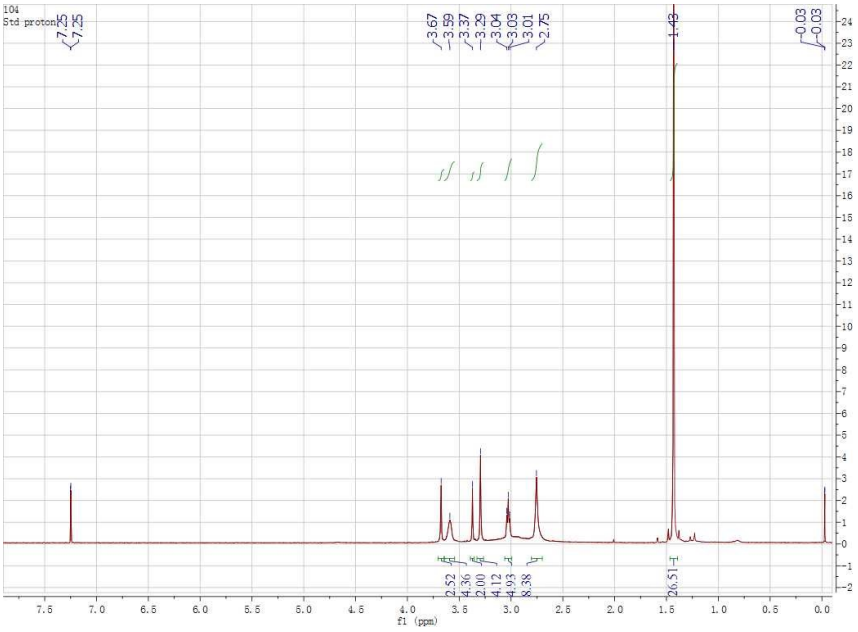


Fig. S1 ^1H NMR ($CDCl_3$, 400 MHz) of $DOTA(OtBu)_3$.

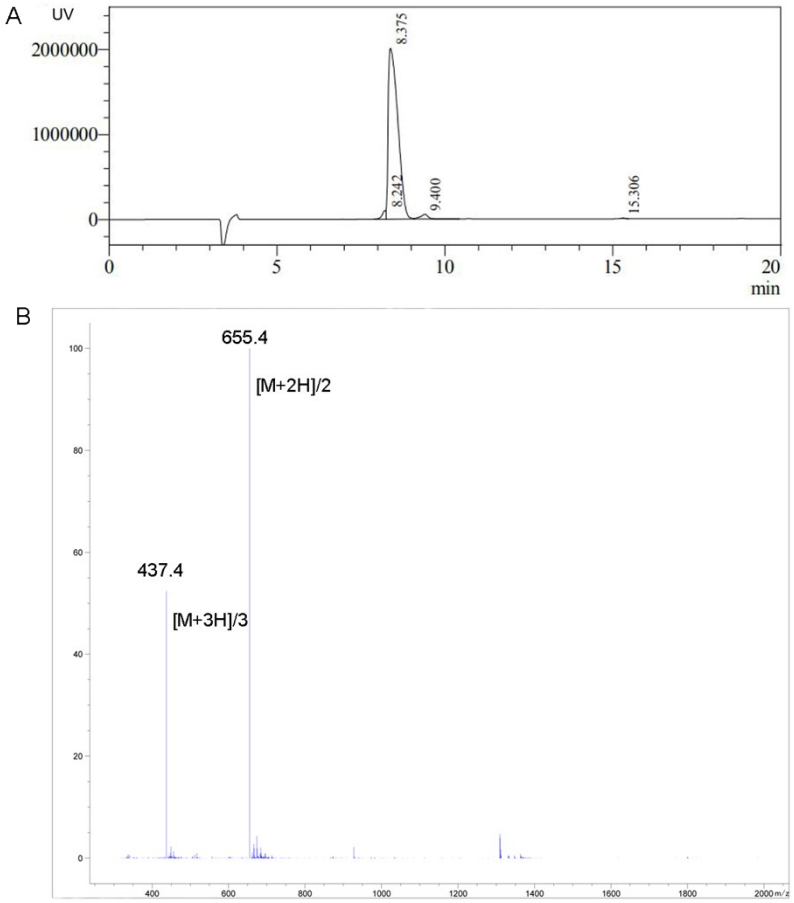


Fig. S2 LC-MS of Nap-CFGKTGK-(DOTA).

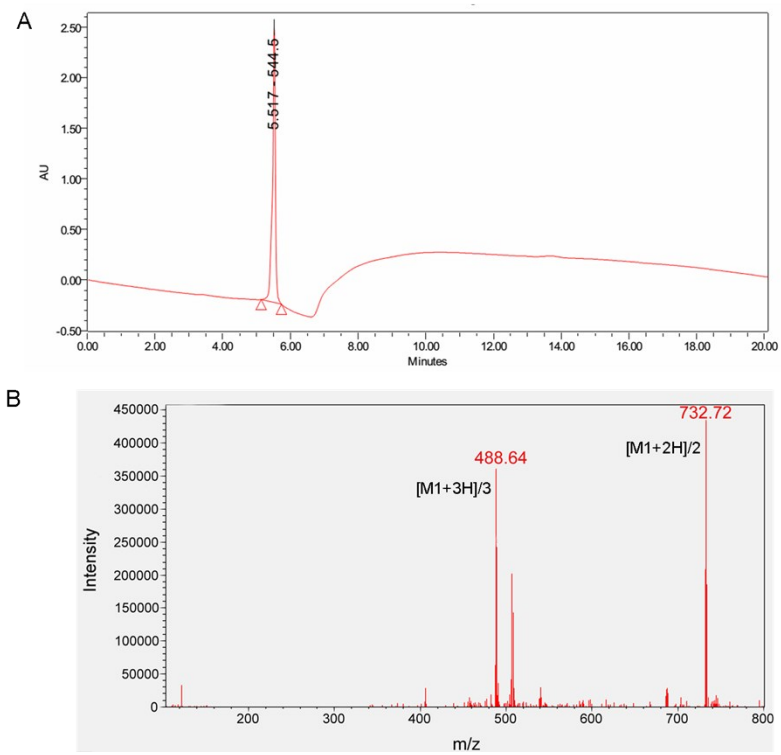


Fig. S3 LC-MS of Nap-Gd.

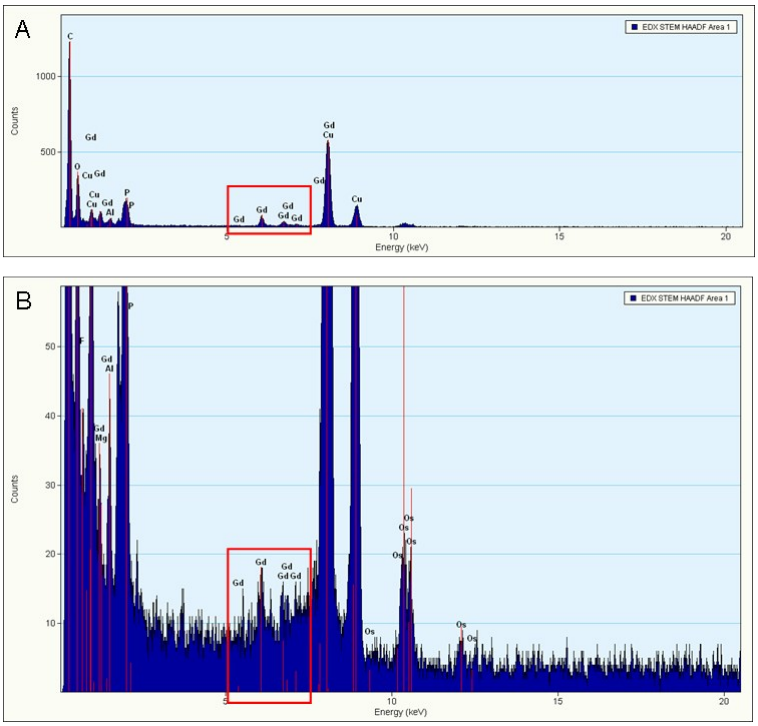


Fig. S4 EDX of celluar TEM: (A) nanocluster (B) nanofiber.

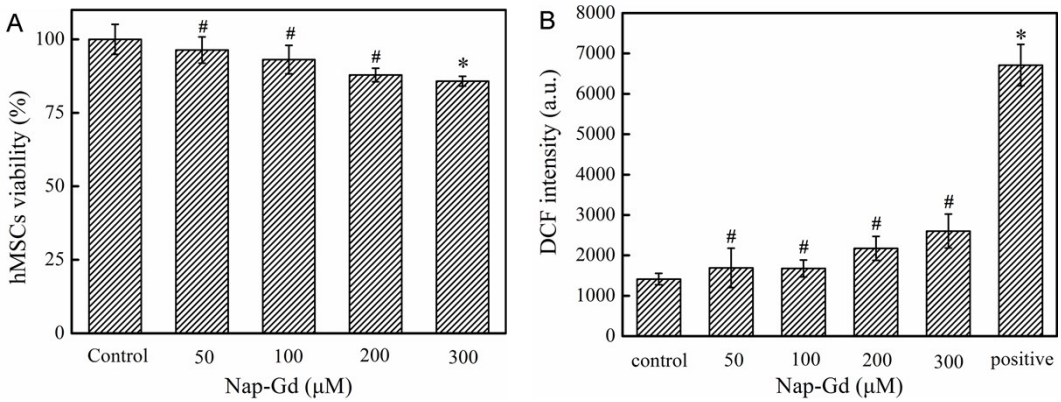


Fig. S5 Cell viability (A) and ROS (B) of hMSCs incubated with Nap-Gd at concentrations up to 300 μM. Statistical significance was determined by unpaired two-tailed student's t-test and one way ANOVA. $^{\#}P > 0.01$ means no statistical difference, $^{*}P < 0.01$ means statistical difference.

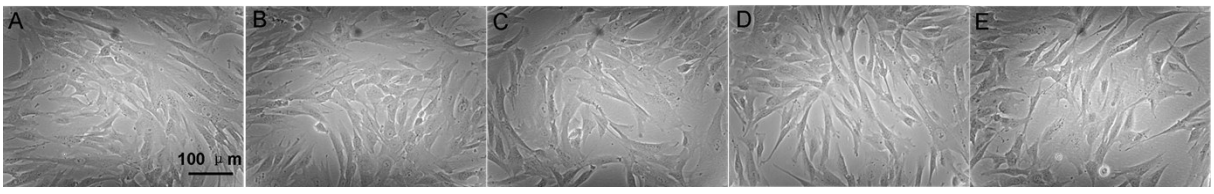


Fig. S6 Morphological images of hMSCS incubated with different concentration of Nap-Gd (A) control (B) 50 μM (C) 100 Mm (D) 200 μM (E) 300 μM.

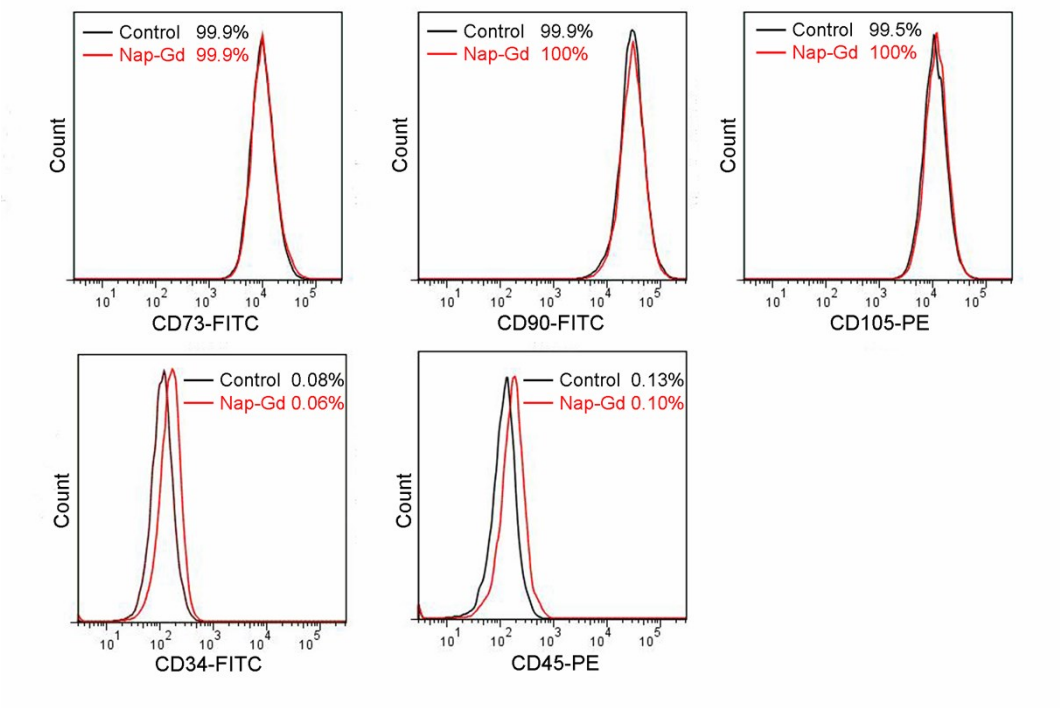


Fig. S7 Analysis of surface biomarkers of hMSCs labeled (red line) and unlabeled (black line) with Nap-Gd by flow cytometry.

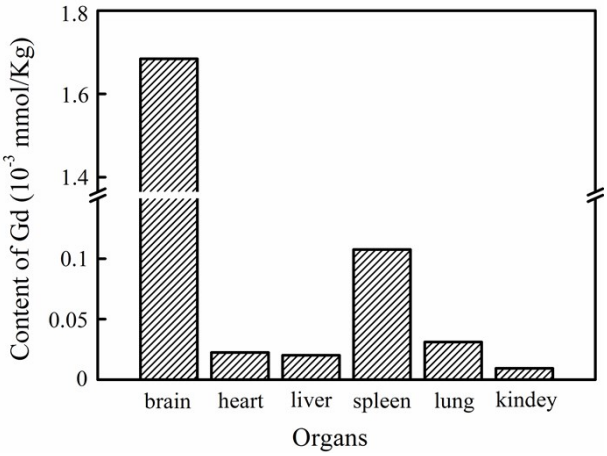


Fig. S8 Residual Gd content in the tissues of brain, heart, liver, spleen, lung and kidney of a mouse with its brain receiving injection of ~ 3×10^5 hMSCs incubated with Nap-Gd at 200 μM for 24 hours.

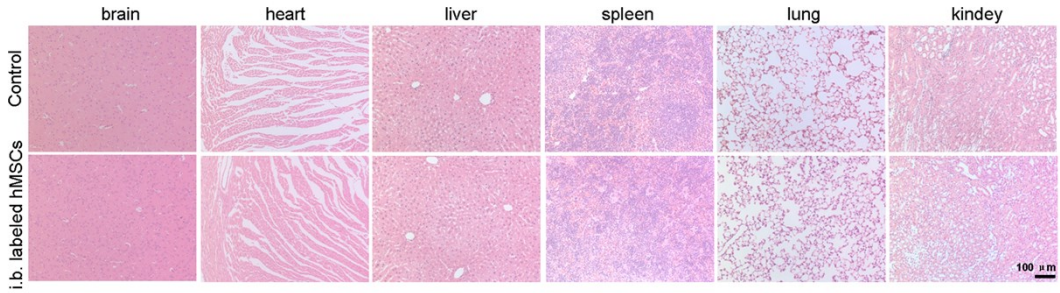


Fig. S9 H&E staining of brain, heart, liver, spleen, lung and kidney sections of a mouse with its brain receiving injection of ~ 3×10^5 hMSCs incubated with Nap-Gd at 200 μM for 24 hours for histopathological evaluation.