Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Visualising brain capillaries on magnetic resonance images *via* supramolecular self-assembly

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Methods

Synthesis

8-arm PEG-FGd_3 was synthesised in accordance with previously described methods. [21] Briefly, hexaglycerol octa(aminopropyl) polyoxyethylene (8-arm PEG; Mw = 15,000; NOF, Tokyo, Japan) was incubated with fluorescein isothiocyanate (FITC, Sigma-Aldrich, Tokyo, JAPAN) and 1,4,7,10-Tetraazacyclododecane-1,4,7,10,-tetraacetic acid mono (*N*-hydroxysuccinimidyl ester) (DOTA-NHS-ester, Macrocyclics, Dallas, TX). The feed ratios of FITC and DOTA-NHS-ester to amino groups of PEGs were 1.1:1 and 30:1, respectively. Following incubation, the samples were purified via membrane dialysis (Spectra/pore 6; molecular weight cut-off (MWCO): 1.0 kDa, Los Angeles, CA). The chemical structures were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy (Gemini 2000/300; Varian Inc., CA). $GdCl_3$ was added to fluorescein- and DOTA-conjugated 8-arm PEG solutions at a 3-fold molar ratio to DOTA, and the solution was maintained at a pH of 6.6 to 7.7 via the addition of 1 M NaOH solution. The reaction mixture was dialysed against distilled water three times and lyophilised to produce 8-arm PEG-FGd_3. The concentration of Gd into the PEGs was determined by inductively coupled plasma-mass spectrometry ICP-MS (ICPM-8500, Shimadzu Co., Kyoto, Japan).

Safety of MR contrast agent

The safety of 8-arm PEG-FGd_3 was evaluated by in vitro cell viability assay. Human umbilical vein endothelial cells (HUVECs, KE4109, Kurabo, Biomedical Business, Japan) were cultured on a polystyrene surface using endothelial basal medium (EBM-2; Lonza, Switzerland) supplemented with EGM-2 and a growth factor kit (Lonza, Switzerland). The 3.0×10^3 cells were seeded on the 96 well plate (Iwaki glass Inc., Tokyo, Japan), and incubated for 24h. The medium was changed, and the cells were incubated with 8-arm PEG-FGd_3 at the concentration of 0.1 mg/mL, 1.0 mg/ml, and 10 mg/mL. The cell viability was measured at 1, 3, 6, and 24 h by using CCK-8 assay (Dojin Laboratories, Kumamoto, Japan).

Stability of gadolinium ion of 8-arm PEG-FGd_3

The 8-arm PEG-FGd_3 solution in saline at the concentration of 10 mg/mL was closed in a dialysis membrane (Spectra/pore Biotech CE Tubing; molecular weight cut-off (MWCO): 100-500Da, Los Angeles, CA). The membranes were placed in the PBS at 37°C and incubated for 24 h. The solutions were collected at 1, 3, 6, and 24 h. The concentration of the gadolinium ion in 8-arm PEG-FGd_3 was determined by ICP-MS.

DLS and spectroscopic analysis

The hydrodynamic radius was determined using a Malvern Zeta Nanosizer (Malvern Instruments Ltd, Worcester, UK). The measurement protocols were conducted in accordance with the manufacturer's manuals. Briefly, 8-arm PEG-FGd_3 was dissolved in saline solution at a concentration of 0.5 to 200 mg/mL and incubated at 25˚C for 5 min. Absorption spectra were measured using a UV spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). The CD spectrum was acquired using a J-1500 CD spectrometer (JASCO) equipped with a water jacket cell holder connected to a circulating water bath (CA-115, EYELA, Tokyo, Japan). The fluorescence intensity was recorded using a spectrophotometer (RF-5300PC, Shimadzu Co., Kyoto, Japan) equipped with a polariser (Polarizer (UV-VIS), Shimadzu Co., Kyoto, Japan). The excitation wavelength was set to the peak wavelength in the excitation spectra around 490 nm. Fluorescence anisotropy (A) was then defined as a function of the observed parallel (I_{VV}: VV denotes vertical excitation, vertical emission) and perpendicular intensities (I_{VH}: VH denotes vertical excitation, horizontal emission). The G-factor (G) was used to correct for polarisation bias (I_{HH} denotes horizontal excitation, horizontal emission; I_{HV} denotes horizontal excitation, horizontal emission).

$$\text{Fluorescence anisotropy (A)} = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2 \cdot G \cdot I_{VH})$$

$$\text{G-factor (G)} = I_{HV} / I_{HH}$$

Scanning probe microscopy

The supramolecular structure was evaluated using a JSPM-5200 apparatus (JEOL Ltd., Tokyo, Japan). An AIO Cantilever from Budget Sensors was used in non-contact-mode AFM, and the spring constants and free resonance frequencies were 3 $N \cdot m^{-1}$ and 75 kHz, respectively. The reported images were acquired at 25°C. All sample images were acquired on a highly oriented pyrolytic graphite (HOPG) surface. The AFM images were analysed using Image J (ver. 1.52a).

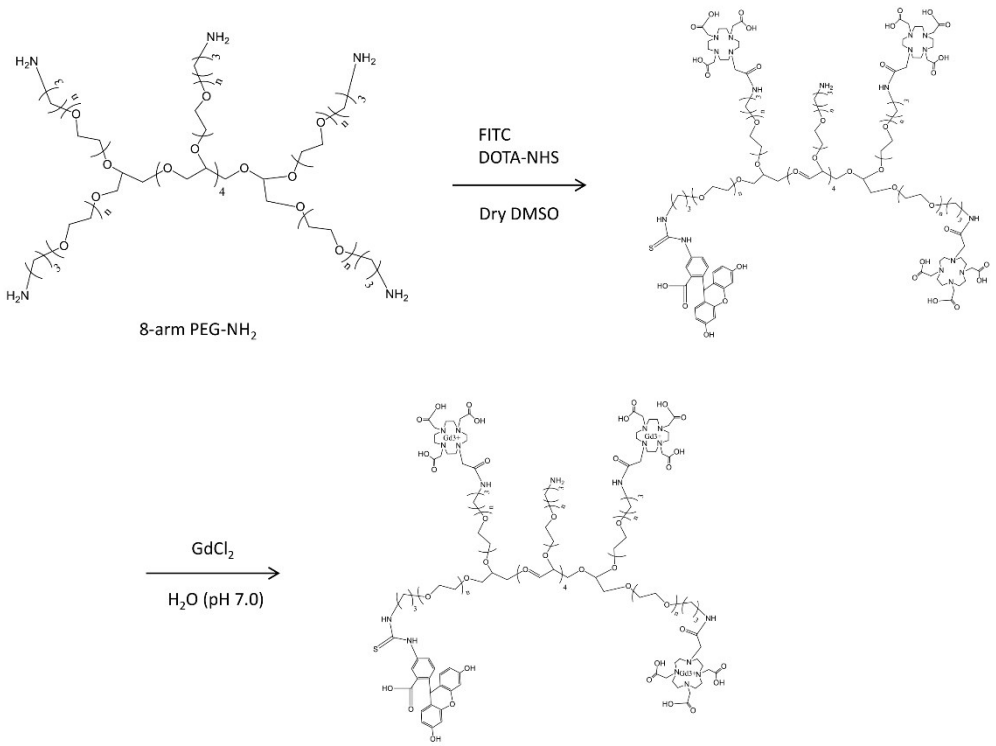
Rat brain imaging on MRA scan

The animal study was performed in accordance with experimental guidelines provided by the National Cerebral and Cardiovascular Center Research Institute. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Cerebral and Cardiovascular Center Research Institute (Permit Number: 009017). We used healthy 7-to-9-week-old male Sprague Dawley rats for the present study. The imaging procedure was almost the same as the previous reports,[21] and the

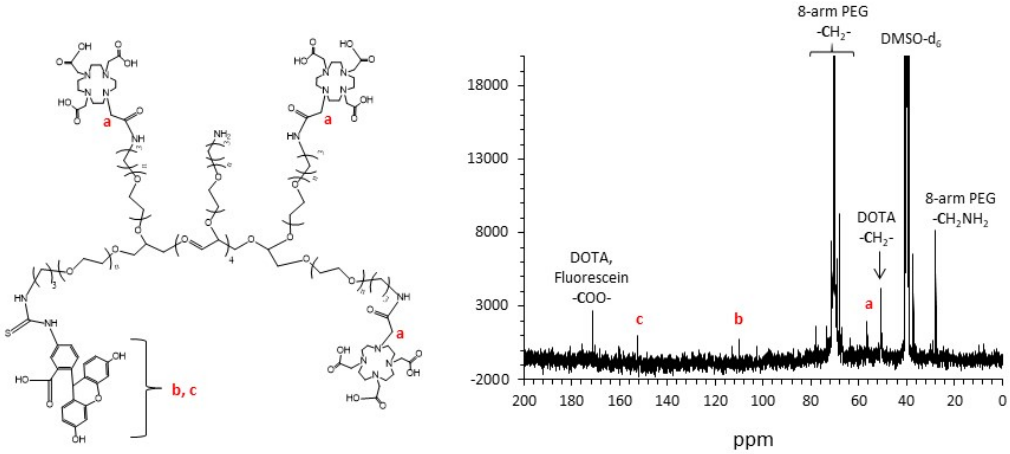
resolution setting was changed. Three-dimensional (3D) TOF-MRA images were acquired before and after tail vein injection of the contrast agents. We injected a total volume of 1 mL at the injection speed of 1 mL/min, and the concentration of the contrast agent was 200 mg/mL (10 μmol Gd per 100 g body weight). Post-contrast brain MRA scans were initiated 1 min after injection. MRA scans were obtained using a 7 T horizontal bore imaging system (PharmaScan 70/16 US; Bruker BioSpin, Ettlingen, Germany). Brain MRA was performed using a 40-mm inner diameter quadrature volume coil for radiofrequency transmission and signal reception. The rats were anaesthetised using isoflurane (5.0% for induction and 1.5–2.0% for maintenance). Animals were placed in the prone position. For brain MRA, the head was secured with a bite bar and ear bars. Heart rate and respiratory rate were continuously monitored during scans, and rectal temperature was continuously monitored during brain MRA only. The body temperature of the rats was maintained with warm air or warm water heating pad. We used a fast low-angle shot (FLASH) sequence with a tilted optimised non-saturating excitation pulse and flow compensation to acquire 3D TOF-MRA images. The scan parameters for brain MRA were stetted as follows: repetition time (TR)/echo time (TE): 15.1/2.6 ms; number of averages: 2; matrix size: 457 × 457 × 411; field-of-view (FOV): 3.20 × 3.20 × 2.88 cm^3; reconstruction matrix size: 914 × 914 × 823; spatial resolution: 35 × 35 × 35 μm^3; and scan time: 1 h 11 min.

Statistical analysis

Quantitative data are expressed as the mean ± standard deviation (SD) of the mean. One-way analyses of variance (ANOVA) followed by Tukey's honest significant difference (HSD) *post hoc* tests were used for parametric analyses, while Kruskal-Wallis tests followed by Stell-Dwass tests were used for non-parametric analyses. All statistical analysis was performed by R version 3.6.1.

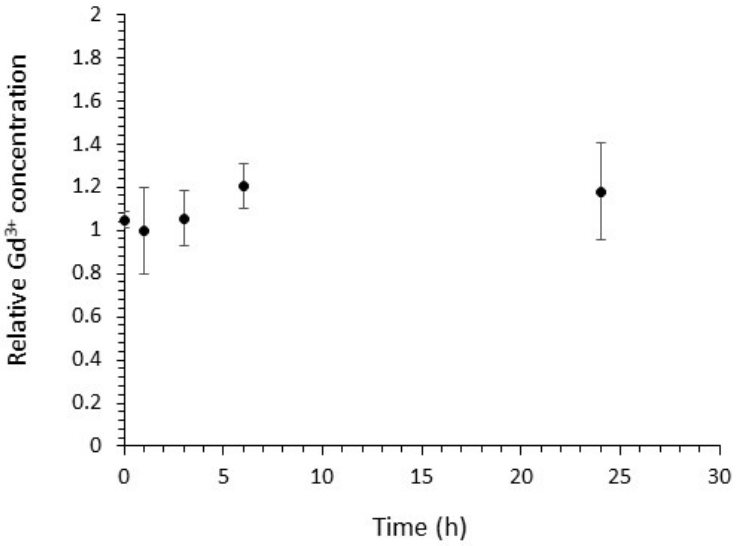


Supplementary Figure S1. Schema for synthesising fluorescein and Gd-chelate conjugated 8-arm PEG.

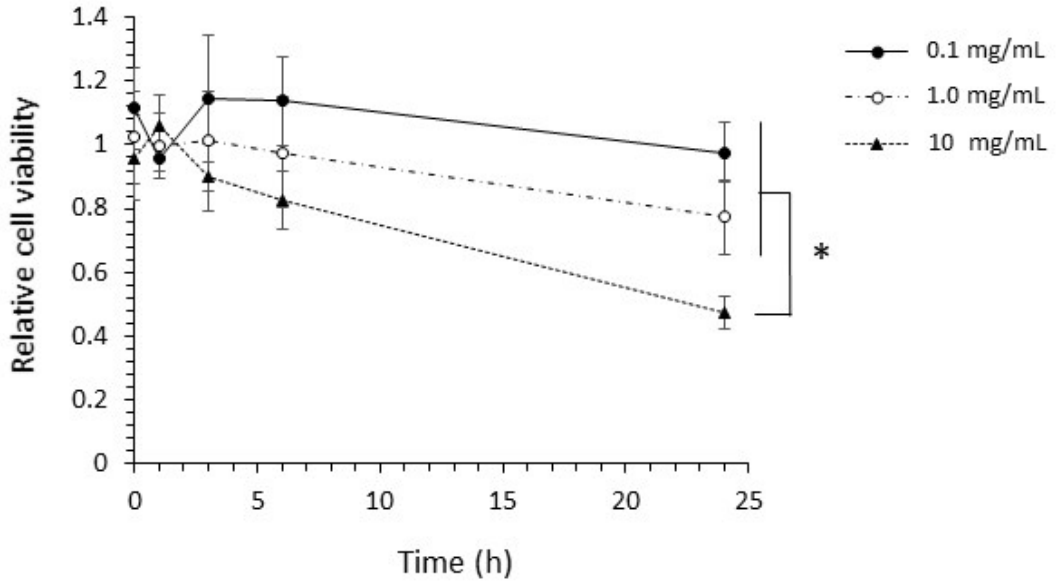


Supplementary Figure S2. ^{13}C-NMR analysis of DOTA conjugated 8-arm PEG-F.

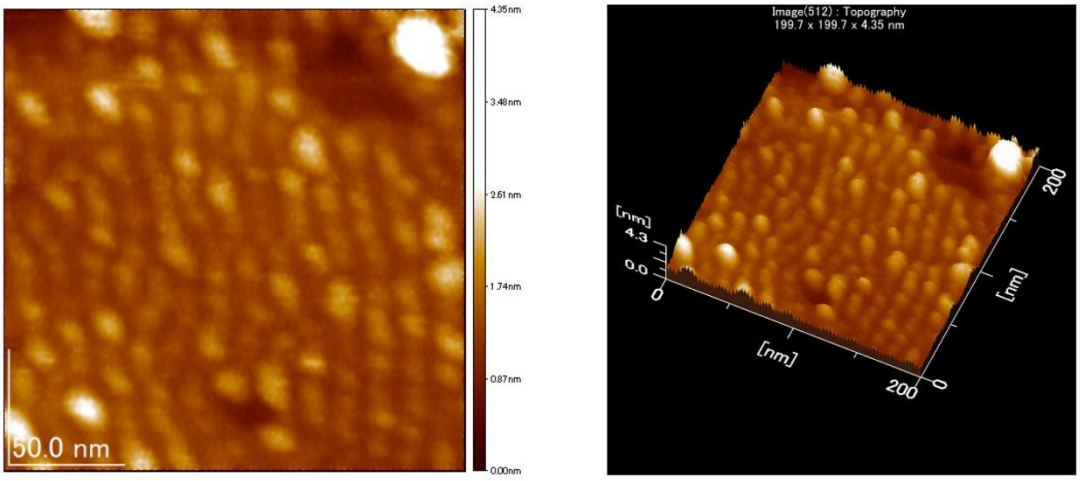
^{13}C-NMR spectra were acquired in DMSO-d6.



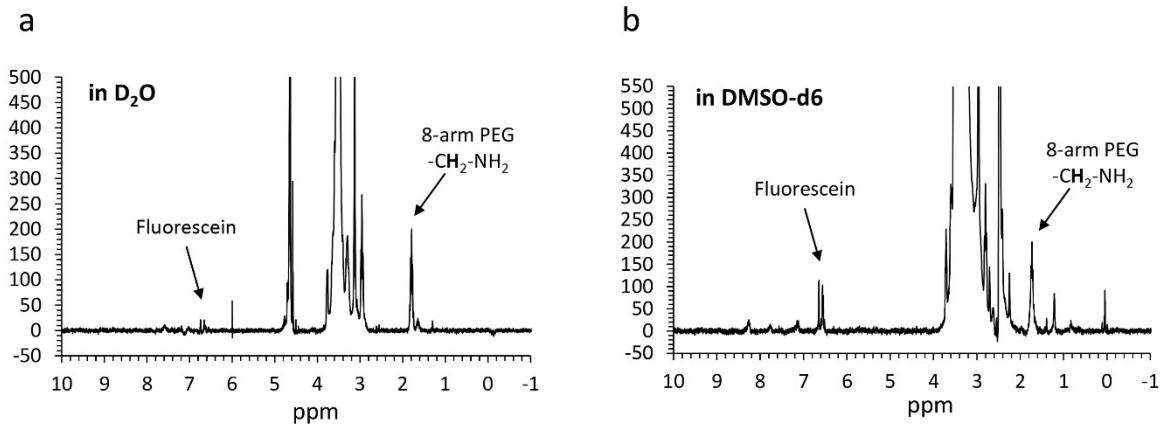
Supplementary Figure S3. Stability of the gadolinium ion chelated in the 8-arm PEG- FGd_3 in PBS at 37°C. A significant difference was not indicated by ANOVA ($p=0.46$).



Supplementary Figure S4. Relative cell viability of HUVECs incubated with the 8-arm PEG-FGd$_3$. The concentration of the 8-arm PEG-FGd3 were (filled circle and solid line) 0.1 mg/mL, (open circle and dash-dotted line) 1.0 mg/mL, and (filled triangle and dotted line) 10 mg/mL. An asterisk indicates a significant difference ($p<0.01$).



Supplementary Figure S5. AFM images of 8-arm PEG at a concentration of 5 mg/mL. The observed diameter and height were approximately 10 nm and 0.5 nm, respectively.



Supplementary Figure S6. ^{1}H-NMR analysis of DOTA conjugated 8-arm PEG-F at a concentration of 5 mg/mL. a-b, ^{1}H-NMR spectra were acquired in D_2O (**a**) and DMSO-d6 (**b**).