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A symmetrical fluoruous dendron-cyanine dye conjugated bimodal nanoprobe for quantitative ^{19}F MRI and NIR fluorescence bioimaging

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Abstract

^{19}F MRI and optical imaging are two powerful non-invasive molecular imaging modalities in biomedical applications. ^{19}F MRI has great potential for high resolution *in vivo* imaging, while fluorescent probes enable ultracontrast cellular/tissue imaging with high accuracy and sensitivity. We, thus, developed a bimodal nanoprobe integrating the merits of ^{19}F MRI and fluorescence imaging into a single synthetic molecule, which was further engineered into nanoprobe, by addressing shortcomings of conventional contrast agents to explore the quantitative ^{19}F MRI and fluorescence imaging and cell tracking. Results showed that this bimodal imaging nanoprobe presented high correlation of ^{19}F MR signal and NIR fluorescence intensity *in vitro* and *in vivo*. Additionally, this nanoprobe enabled quantitative ^{19}F MR analysis, confirmed by complementary fluorescence analysis. This unique feature can hardly be obtained by traditional ^{19}F MRI contrast agents. We envision that this nanoprobe would hold great potential for quantitative and sensitive multi-modal molecular imaging.

Keywords

^{19}F MRI; fluorescence imaging; nanoparticles; stem cells; biodegradable polymer

1. Introduction

Magnetic resonance imaging (MRI) is a powerful non-invasive imaging tool for excellent anatomical illustration of soft tissue. It also enables unbiased assessment of tissue physiology and pathology.^[1] Generally, MRI can be employed directly for imaging the ubiquitous ^1H in the body.^[2] MRI contrast agents, however, are required for enhanced imaging contrast in most circumstances. Numerous MRI contrast agents, such as superparamagnetic iron oxide (SPIO), which are used to modulate the relaxation time of surrounding protons in body fluid, have been developed over the past years.^[3] Nevertheless, these contrast changes via indirect modulation by contrast agents to protons could be a challenge to quantitatively identify and interpret in practically complex body conditions.^[4] It is, therefore, necessary to develop a robust MRI contrast agent through direct manipulation of the agent's relaxation time under magnetic field for quantitative spectrum interpretation and MR imaging.

Fluorine (^{19}F) is regarded as a potent moiety for quantitative MRI. It presents comparable magnetic resonance sensitivity to proton (^1H), while exhibiting minimal, if any, tissue background interference.^[1] In addition, the ^{19}F spectrum manifests a range of more than 200 ppm which permits specific identification of ^{19}F containing compounds by ^{19}F MR.^[5] Given the merits of fluorine, the development of ^{19}F MRI contrast agents has been a hot pursuit for years. Current ^{19}F MRI contrast agents focus on perfluorocarbon (PFC) emulsions^[6] using perfluoropolyether (PFPE) in either linear or macrocyclic structures as the ^{19}F containing agent. Unfortunately, several shortcomings remain in these PFC emulsions: the split multiple fluorine signals of linear PFPE may result in lower signal intensity and imaging artifacts; the fluorophilic feature of macrocyclic PFPE makes it insoluble in most solvents, which hampers its quantitative analysis in biological samples. In addition, the breakdown of macrocyclic structure for any chemical conjugation can lead to asymmetrical multiple fluorine signals, which is deleterious for quantitative analysis and MR imaging. Thus, macrocyclic PFC can hardly be modified with other imaging modality for multimodality imaging. ^{19}F MRI has great potential for high resolution *in vivo* imaging, while fluorescent probes enable ultracontrast *in vitro* cellular/tissue imaging with high accuracy and sensitivity.^[7] Hence, the combination of ^{19}F MRI and fluorescence imaging will significantly benefit prognosis and diagnosis in medicine.^[8] In this report, we developed a bimodal contrast probe integrating the merits of ^{19}F MRI and fluorescence imaging into single organic molecule for dual-modality quantitative ^{19}F MRI and fluorescence imaging.

2. Results and discussion

We adopted a proportionate branching method^[9] to prepare a set of symmetrically structured fluorous dendrons tethered with near infrared (NIR) cyanine dyes as bimodal molecules with single fluorine spectral peak and intense fluorescence emission.

After screening the synthesized molecules, one promising lead molecule was further processed into an engineered polymeric nanoparticle to enhance the aqueous dispensability, reduce possible non-specific interaction in complex body fluid, and improve the biocompatibility for biomedical applications. This nanoparticulate fluorous dendron-cyanine dye bimodal nanoprobe realized the quantitative ^{19}F spectrum and imaging analysis in biological samples, and enabled consistent *in vivo* dynamic tracking of transplanted stem cell progression by ^{19}F MRI and NIR fluorescence imaging.

The proportionate branching method adopted to synthesize the fluorous dendrons is illustrated in scheme 1. Different from the conventional dendrimer synthesis method,^[10] the proposed proportionate branching method avoided the steric congestion during dendron synthesis to make the growth of the branch number and length of the dendrimer exponentially but in the opposite direction, where branches close to the core are longer than those near to the periphery of a dendrimer molecule. This hypothesis was proven to be successful in synthesizing high generation defect free dendrimers in the previous report.^[9]

As ^{19}F MR imaging agent candidates, we prepared three cyanine dye conjugated molecules with different fluorine contents. F3-Cy5.5 compound **3** and F9-Cy5.5 compound **6** were

obtained by introducing the fluorine unit and dye unit successively. Selective reaction of one of the four hydroxyl groups in pentaerythritol with *tert*-butyl acrylate afforded compound **7**. Highly symmetric 27 fluorine atoms were grafted onto **7** by classical Mitsunobu reaction. Conversion of the ester group in compound **8** to free amine afforded compound **9**, which was subjected to reaction with cyanine dye to yield F27-Cy5.5 **10** (Fig. 1A). All the synthesized fluorinated dyes showed single/sharp ^{19}F signal, indicating no breakdown of symmetry during reactions (Fig. 1B). F27-Cy5.5 compound **10** (FC) exhibited the highest S/N ratio and fluorine intensity under the equivalent concentration (Fig. 1C, d and S1) with highest relative fluorine content in molecule. FC also presents the highest compatibility for incorporation into engineering polymeric nanoparticle with nearly 40% (by weight) loading efficiency and 35% (by weight) loading content. However, fluorine dendrons of higher generation were not able to form stable nanoparticles or the loading efficiency was too low to be useful for ^{19}F MRI imaging. Recent advances in engineered nanoparticles encapsulating imaging probes have evidenced the superiority of contrast agent sequestered nanoparticles, especially the biodegradable nanoparticles, for molecular imaging.^[11] In this study, we employed a simple but robust nanoprecipitation method^[12] with modification to load FC into a biocompatible and biodegradable polymeric nanoparticle (FCNP) (Fig. 2A). The size of this particle is determined to be about 130 nm with a polydistribution index (PDI) < 0.2 by dynamic light scattering (DLS) measurement (Fig. 2B). Transmission electron microscopy (TEM) image (Fig. 2A) shows a homogenous and spherical morphology of this nanoprobe with relatively uniform sizes. Additionally, this FCNP is fairly stable at 4 °C for over a month without noticeable aggregation.

To prove that FCNP can function as a quantitative imaging probe in complex biological systems, we intravenously administered FCNP in different dosages to healthy mice. After 24 hour circulation, blood samples were withdrawn, weighted and analyzed for fluorine signals and fluorescence intensity by ^{19}F NMR and fluorescence spectrometry, respectively. Results show that both ^{19}F NMR signal and S/N ratio are proportional to the dose of administered FCNP (Fig. S2). The fluorescence intensity is linearly proportional to the administered doses of FCNP. Fig. 2C demonstrates the high linear correlation ($R^2 = 0.9942$) of fluorine signal to the fluorescence intensity of FCNPs. Thus, FCNP is stable under physiological condition and its dual modality signals can be analyzed and mutually correlated by ^{19}F NMR and spectrometry analytical methods.

Molecular imaging of stem cell behavior has drawn much attention over the past decade^[13], especially in the evaluation of transplanted stem cells.^[14] We thus used the FCNP bimodal nanoprobe to label mesenchymal stem cells (MSCs) prior to their transplantation into mice. Fig. 3A presents the confocal imaging of FCNP uptake by MSCs after 24 h incubation. The time dependent MSC uptake of FCNP showed that more than 90% of MSCs were completely stained in the cytoplasm by FCNP in 24 h (Fig S3 and S4). We then used this incubation time in our further studies. Moreover, this FCNP is fairly biocompatible as no significant cytotoxicity was shown by cell migration study (Fig. 3B) and MTT assay (Fig. S5). In addition to being safe for stem cell labeling, the contrast agent should also have sufficient retention time in the cytoplasm for longitudinal cell tracking and monitoring. We employed time lapse fluorescence microscopy and flow cytometry to quantify the FCNP

retention in cytoplasm over time. As shown in Fig. 3C and S6, FCNPs show high retention (by flow cytometry analysis) in MSC cytoplasm over 9 days. Although the MSCs were proliferating, by which the FCNPs could be diluted for every passage of proliferation, FCNPs remained high fluorescence intensity in MSCs. The high stability and strong fluorescence intensity of FC in engineered nanoparticle may contribute to this long-term stem cell labeling efficiency. This is consistent with other reports regarding the long-term cell labeling and tracking by engineered nanoparticles.^[15]

Since we will apply FCNP for quantitative *in vivo* bimodal imaging and analysis, the MRI spectroscopy detection limit of FC in organic solvent and FCNP labeled MSCs in buffer was quantified. As shown in Fig. 4, ¹⁹F signals from both FC in tetrahydrofuran (THF) (Fig. 4A, B and S7) and FCNP in labeled MSCs (Fig. 4C, D and S8) demonstrate high linear relationship with the corresponding fluorine concentrations. Figure S9 illustrates the calculated MRI detection thresholds of FC in THF at 0.0833 mg/mL and FCNP labeled MSCs at 4.58×10^4 cells. As an MR imaging probe, we also experimentally determined the T_1 and T_2 values of FC in THF and FCNP in cells (Fig. S10). The shorter T_1 value of FCNP in cells than that of PFC could reduce the data collection time and potentially increase signal intensity.^[16]

Phantoms of FCNP labeled MSCs by ¹⁹F MRI and fluorescence imaging (Fig. 5A) and quantitative analysis (Fig. 5B and S11) demonstrate the high linear correlation of ¹⁹F signal with fluorescence emission intensity. Fig. 5C presents subcutaneous *in situ* injection of FCNP labeled MSC into nude mice along with quantitative analysis of the corresponding imaging signal (Fig. 5D). The almost linear correlation of ¹⁹F MRI signal and fluorescence imaging intensity implies the potency of FCNP for *in vivo* stem cell tracking and quantitative analysis.

In recent years, stem cell therapies have drawn considerable attention as promising treatments for various brain pathologies, such as traumatic brain injury (TBI), stroke and neurodegenerative diseases. Thus, it is crucial to develop reliable imaging agents for monitoring transplanted stem cells *in vivo* prior to the clinical translation of stem cell therapies.^[17] In this study, we transplanted FCNP labeled MSCs into TBI mice, and tracked the progression of MSCs for 7 days. Figure 6A shows the fluorescence imaging (upper panel) and ¹⁹F MRI (lower panel) of FCNP labeled *in situ* transplanted MSCs. The imaging results reveal that the MSCs mainly concentrated at the injury site with small number of cells migrated to the periphery area of injury site starting at day 3. The imaging signals from both ¹⁹F and fluorescence decreased at day 7. The harsh microenvironment of TBI area may damage the exogenous MSCs as indicated by the lower ¹⁹F and fluorescence imaging signals at day 7. In addition to the monitoring of intracranially injected stem cells, we also conducted the bimodal tracking of FCNP labeled MSCs after intraperitoneal injection (Fig. S12). Results show that majority of MSCs were located in the intestines and kidneys close to the injection site with scattered cells located in the lymph nodes at 24 h after MSC administration. Thus, it is clear that the developed fluororous dendron-cyanine dye bimodal nanoprobe can be used as a robust quantitative imaging contrast agent for bimodal cell tracking *in vivo*.

3. Conclusion

We applied the proportionate branching method to synthesize a series of defect-free fluororous dendrons tethered with widely used cyanine dye to form a set of bimodal molecules. These molecules showed single ^{19}F spectral peak and high solubility in most organic solvents. These merits make those molecules suitable for ^{19}F MR imaging and quantitative analysis. One promising lead compound with 27 fluorine atoms was selected to be incorporated into an engineered polymeric nanoparticle, which was used as a bimodal nanoprobe, for quantitative stem cell labeling and imaging analysis *in vitro* and *in vivo*. We envision that the developed bimodal nanoprobe would hold great potential for quantitative and sensitive multi-modal molecular imaging.

4. Experimental Section

Synthesis of fluorine dendrons with different fluorine content

Please refer to the supporting information for detailed synthetic information and compound characterizations.

Preparation and characterization of FCNP

The nanoparticle was prepared by modified lipid assisted nanoprecipitation method. In brief, the synthesized compound **10** was dissolved in 1 mL of acetonitrile along with 20 mg of poly(lactic-co-glycolic acid) (PLGA) (Resomer® 502H). 4 mg 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG2K) (Avanti polar lipid) and 1 mg 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (DSPC) were dissolved in 5 mL 4% ethanol aqueous solution, and aqueous solution was adjusted to pH 7.4 by diluted sodium hydroxyl solution, then the solution was heated to 70 °C. 10 min after the aqueous solution was heated at 70 °C, the acetonitrile solution was added dropwise into the heated solution under mild magnetic stirring, followed by vigorous vortexing for 5 min before magnetic stirring for another 4 h for nanoparticle self-assembly. The resultant nanoparticle was washed extensively with PBS (pH 7.4) by ultrafiltration method (Amicon® Ultra, M.W.C.O.= 10 k) until there is no trace amount of UV/Vis absorption of the dye under spectrometer measurement. The concentrated FCNP was kept in PBS (pH 7.4) in the refrigerator for further use. The size and distribution of the FCNP was monitored by dynamic light scattering (Marvin Nano90, U.K.), and the dried morphology of FCNP was observed with transmittance electron microscope (TEM) (Philips/FEI CM200).

Mesenchymal stem cell (MSC) isolation and culture

MSCs were isolated from bone marrow of 3-4 week old FVB mice. The isolated MSCs were cultured in MesenCult™ MSC Basal Medium (Mouse) with MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements (Mouse) (Stemcell technologies, Vancouver, Canada), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C with 5% CO₂. MSCs of passages 3-8 were used in the entire experiment.

Cell uptake assay

Cells were seeded in Lab-Tek® chambers (Nalgene, Nunc Inc., Denmark) at a density of 2×10^4 per well, and allowed to attach overnight. Cells were incubated with 0.1 mM FCNP over pre-determined time period, followed by 3 times PBS washing and fixed in 4% neutralized formalin solution for 10 min and 0.1% Triton X-100 (TX-100) for 5 min. Then, Alex® 488 conjugated phalloidin pre-diluted solution (0.5% BSA in PBS) was incubated on cell monolayer for 30-40 min before washing 3 times with PBS, and mounted with Vectashield mounting media with DAPI. To study the FCNP retention in cytoplasm over 9 days, MSCs pre-labeled by FCNP was cultured on cover slip in 6-well plate at 40-50% confluence. The cells were monitored at pre-determined time points by epifluorescence microscopy (Olympus X71) with GFP channel and Cy5.5 channel over 9 days.

Flow cytometry assay

To evaluate the time-dependent cellular uptake of FCNP, MSCs were incubated with 0.1 mM FCNP for 24 h before being washed and harvested by 0.25% Trypsin-EDTA up to 1×10^6 cells/mL into FACS tubes. The cells were washed twice with PBS, centrifuged at 300 ref for 5 min and the supernatant was decanted. FACS buffer (R&D systems) was used to dilute the cell pellet into 0.5×10^6 cells/mL. The flow cytometry analysis was conducted on BD Accuri C6 flow cytometer with channel 4, which covers Cy5.5 fluorophore detection. Gated 10,000 cells were selected for each running. Data were analyzed with accompanied software. For the study of FCNP retention in cultured MSC over 9 days, cells were cultured in 100 mm poly-lysine coated Petri dish at 40% confluence before incubation with 0.1mM FCNP for 24 h. On the next day, cells were harvested up to 1×10^6 cells/mL into FACS tubes. Similar procedure was conducted as that of cellular uptake experiment above for data collection and analysis.

Transwell® assay to evaluate labeled MSC migration

MSCs were pre-labeled by FCNP (0.1 mM) for 24 h before being plated on transwell upper insert at 2×10^4 cells/vial density in serum/nutrition free culture medium. The lower level of transwell plate was filled with complete cell culture medium with overnight incubation at 37 °C, 5% CO₂ incubator. The next day, the insert was washed 2 times with PBS, followed by fixation by 4% neutralized formaldehyde solution for 10 min. Cotton swab was applied to the inner side of the insert to get rid of non-migrated cells. Then, the membrane was peeled off from insert and attached to slide with cover slip for imaging under epifluorescence microscopy (Olympus X71). MSCs without any labeling were used as control.

Traumatic brain injury surgery

Traumatic brain injury (TBI) model was induced in adult, female balb/c mice (6-8 weeks) by a controlled cortical impact model (CCI). Briefly, mice were anesthetized by isoflurane (3% for induction and 1.5% for maintenance) and then placed in a stereotaxic device. After drilling a hole (4 mm $\times$ 4 mm) on the right area of skull, an impact tip with a diameter of 2 mm was used to contuse the brain tissue. The tip penetration depth was 2.0 mm and the velocity was 5m/s. A warming pad was used to keep body temperature around 37 °C during the surgery. All the animal studies were conducted in accordance with the principles and

procedures outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Clinical Center, NIH.

Magnetic Resonance Imaging

All MRI studies were performed on a 7.0 Tesla small animal scanner (Pharmascan, Bruker, Germany) with a double tuned $^1\text{H}/^{19}\text{F}$ coil. The “hot iron” color lookup table (ParaVision, Bruker) was applied to ^{19}F images for superimposing the images of both ^1H and ^{19}F images. For ex vivo phantom study, EP tubes with different concentrations of MSCs labeled with FCNP were mixed with 2% agarose gel on ice. Morphological ^1H images were acquired with a multi spin echo sequence. The parameters were as follows: TR = 500 ms, TE = 12 ms, NEX = 2, slice thickness = 3 mm, Matrix = 256×256 . And then ^{19}F images were performed in the matching position with a fast low-angle shot (FLASH) sequence (TR = 500 ms, TE = 2.1 ms, NEX = 32, slice thickness = 3 mm, Matrix = 64×64).

Mice were anesthetized by 1.5% isoflurane and the body temperature was maintained at 37 °C during the experiment. For subcutaneous injection study, anatomical ^1H images were first acquired with a respiratory-gated multislice multi spin echo sequence (TR = 1000 ms, TE = 10 ms, NEX = 1, slice thickness = 1 mm, Matrix = 256×128). Then the resonator was tuned to ^{19}F , and anatomically matching ^{19}F images were recorded with a FLASH sequence (TR = 500 ms, TE = 2.1 ms, NEX = 32, slice thickness = 10 mm, Matrix = 64×64 , total acquisition time = 17 min 4 s). For TBI model study, a multislice rapid acquisition with relaxation enhancement (RARE) sequence was used to acquire ^1H images (TR = 3000 ms, TE = 70 ms, NEX = 4, RARE factor = 6, slice thickness = 1 mm, Matrix = 256×256), for injury area localization. Corresponding ^{19}F images were obtained using a FLASH sequence with similar parameters except for NEX = 128 and slice thickness = 5 mm.

Experimental procedures

tert-Butyl (3-(2,2,2-trifluoroacetamido)propyl)carbamate

To a 0 °C solution of compound **1** (174 mg, 1 mmol) in CH_2Cl_2 (5 mL) was added Et_3N (0.17 mL, 1.2 mmol), then trifluoroacetic anhydride (0.17 mL, 1.2 mmol) was added dropwise. Afterwards, the solution was stirred overnight at room temperature, concentrated through rotary evaporation and subjected to silica gel chromatography using hexane/ EtOAc as eluent to afford trifluoroacetyl compound (232 mg, 86% yield). ^1H NMR (300 MHz, CDCl_3) δ 8.09 (br, 1H), 5.16 (t, J = 6.0 Hz, 1H), 3.35 (q, J = 6.3 Hz, 2H), 3.14 (q, J = 6.0 Hz, 2H), 1.66 (q, J = 6.0 Hz, 2H), 1.39 (s, 9H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 157.8 (q, J = 36.2 Hz), 157.3, 116.1 (q, J = 287.7 Hz), 79.8, 37.0, 36.5, 29.5, 28.3; ^{19}F NMR (282 MHz, CDCl_3) δ -76.16 (s).

N-(3-Aminopropyl)-2,2,2-trifluoroacetamide hydrochloride (**2**)

To a solution of the above trifluoroacetyl compound (20 mg, 0.074 mmol) was added 4 M HCl in dioxane solution (0.5 mL), the solution was stirred at room temperature for 2 h and concentrated through rotary evaporation to afford compound **2** (11.8 mg, 77% yield). ^1H NMR (300 MHz, CD_3OD) δ 3.40 (s, 2H), 2.97 (s, 2H), 1.95 (s, 2H); ^{13}C NMR (75.5 MHz,

CD₃OD) δ 159.4 (q, J = 37.0 Hz), 117.4 (q, J = 289.2 Hz), 38.5, 38.0, 28.3; ¹⁹F NMR (282 MHz, CD₃OD) δ – 77.25 (s).

1,1-Dimethyl-3-(6-oxo-6-((3-(2,2,2-trifluoroacetamido)propyl)amino)hexyl)-2-((1E,3E,5E)-5-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium chloride (3)

To a solution of Cy5.5 NHS ester (1.5 mg, 2 μ mol) in DMF (0.1 mL) was added DIPEA (1.4 μ L, 8 μ mol), compound **2** (1.2 mg, 6 μ mol) successively, the mixture was stirred in dark at room temperature for 3 h, then diluted with water and subjected to semi-preparative HPLC to afford compound **3** (1.1 mg, 73% yield) as deep blue solid. For semi-preparative HPLC, a Beckman Ultrasphere C₁₈ column (10 $\times$ 250 mm) and a gradient elution profile were used with 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in CH₃CN (solvent B). The elution profile was 45% solvent B gradient to 100% solvent B over 35 min, and finally to 100% solvent B over the next 5 min. The major peak at about 25 min was collected and lyophilized. MS (ESI) m/z 736.25 [M + H]⁺.

2,2,2-Trifluoro-N-(3-((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propyl)acetamide

To a solution of compound **4** (86 mg, 0.2 mmol) in DMF (5 mL) was added sodium perfluoro-tert-butanoate (103 mg, 0.4 mmol). Afterwards, the solution was stirred overnight at 65 °C. The mixture was then quenched with water and extracted with DCM, washed successively with water and brine, concentrated through rotary evaporation and subjected to silica gel chromatography using hexane/EtOAc as eluent to afford the perfluoro-tert-butyl ether (65 mg, 67% yield). ¹H NMR (300 MHz, CD₃OD) δ 4.14 (t, J = 6.0 Hz, 2H), 3.72 (t, J = 6.0 Hz, 2H), 1.99 (t, J = 6.0 Hz, 2H), 1.52 (s, 18H); ¹³C NMR (75.5 MHz, CD₃OD) δ 154.0, 122.0 (q, J = 293.7 Hz), 84.1, 69.8, 44.5, 30.6, 28.4; ¹⁹F NMR (282 MHz, CD₃OD) δ – 71.65 (s).

3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propan-1-amine hydrochloride (5)

To a solution of above perfluoro-tert-butyl ether (20 mg, 0.041 mmol) was added 4 M HCl in dioxane solution (0.5 mL), the solution was stirred at room temperature for 2 h and concentrated through rotary evaporation to afford compound **5** (10.9 mg, 82% yield). ¹H NMR (300 MHz, CD₃OD) δ 4.25 (t, J = 5.7 Hz, 2H), 3.10 (t, J = 5.4 Hz, 2H), 2.13 – 2.07 (m, 2H); ¹³C NMR (75.5 MHz, CD₃OD) δ 118.2 (q, J = 287.7 Hz), 56.0, 44.0, 29.1; ¹⁹F NMR (282 MHz, CD₃OD) δ – 71.74 (s).

3-(6-((3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propyl)amino)-6-oxohexyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium chloride (6)

To a solution of Cy5.5 NHS ester (1.5 mg, 2 μ mol) in DMF (0.1 mL) was added DIPEA (1.4 μ L, 8 μ mol), compound **5** (2 mg, 6 μ mol) successively, the mixture was stirred in dark at room temperature for 3 h, then diluted with water and subjected to semi-preparative HPLC to afford compound **6** (1.4 mg, 78% yield) as deep blue solid. For semi-preparative HPLC, a

Beckman Ultrasphere C₁₈ column (10 × 250 mm) and a gradient elution profile were used with 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in CH₃CN (solvent B). The elution profile was 45% solvent B gradient to 100% solvent B over 35 min, and finally to 100% solvent B over the next 5 min. The major peak at about 27 min was collected and lyophilized. MS (ESI) *m/z* 859.30 [M + H]⁺.

tert-Butyl 3-(3-hydroxy-2,2-bis(hydroxymethyl)propoxy)propanoate (7)

To DMSO (100 mL) was added pentaerythritol (68 g, 0.5 mol), the heterogeneous suspension was heated to 80 °C until the system became clear, then aqueous NaOH (4 g NaOH in 9 mL H₂O) was added in one portion, tert-butyl acrylate (87 mL, 0.6 mol) was added to the solution dropwise, vigorous stirring continued overnight at 80 °C. After cooling, the solution was extracted with EtOAc. The combined organic phase was washed with H₂O and brine, concentrated through rotary evaporation, and the residue was subjected to silica gel chromatography using CH₂Cl₂/MeOH as eluent to give 7 (54.3 g, 0.21 mol, 41% yield) as a clear oil. ¹H NMR (500 MHz, CDCl₃) δ 3.63 (br, 3H), 3.56 (t, *J* = 5.5 Hz, 2H), 3.52 (s, 6H), 3.37 (s, 2H), 2.38 (t, *J* = 5.0 Hz, 2H), 1.37 (s, 9H); ¹³C NMR (126 MHz, CDCl₃) δ 171.7, 81.2, 72.1, 67.2, 63.6, 45.3, 36.1, 28.1; MS (APCI) *m/z* 209 (M – tBu + 2H)⁺, 265 (M + H)⁺, 287 (M + Na)⁺.

tert-Butyl 3-(3-((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)methyl)propoxy)propanoate (8-CO₂tBu)

To a stirred suspension of compound 7 (26.4 g, 100 mmol), triphenylphosphine (118 g, 450 mmol) and 4 Å molecular sieves (15 g) in tetrahydrofuran (700 mL) at 0 °C was added dropwise diisopropylazodicarboxylate (90 mL, 450 mmol). Afterwards, the reaction mixture was allowed to warm to room temperature and was stirred for an additional 20 min. Then perfluoro-tert-butanol (62.5 mL, 450 mmol) was added in one portion and the resulting mixture was stirred for 36 h at 45 °C in a sealed vessel. Water (30 mL) was added to the reaction mixture and stirred for an additional 10 min. Then the mixture was transferred to a separatory funnel and the lower phase was collected. Removal of the perfluoro-tert-butanol under vacuum gave the product 8-CO₂tBu (65 g, 70.8 mmol, 71% yield) as a clear oil. ¹H NMR (500 MHz, CDCl₃) δ 4.08 (s, 6H), 3.66 (t, *J* = 6.0 Hz, 2H), 3.45 (s, 2H), 2.46 (t, *J* = 6.0 Hz, 2H), 1.45 (s, 9H); ¹³C NMR (126 MHz, CDCl₃) δ 170.6, 120.4 (q, *J* = 293.3 Hz), 80.8, 80.4 – 79.2 (m), 67.4, 66.6, 66.1, 46.4, 36.0, 28.0; ¹⁹F NMR (470 MHz, CDCl₃) δ –73.50 (s); MS (APCI) *m/z* 863 (M – tBu + 2H)⁺, 791 (M – tBuOCOCH₂CH₂ + 2H)⁺.

3-(3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(tri-fluoromethyl)propan-2-yl)oxy)methyl)propoxy)propan-1-ol (8-CH₂OH)

To a 0 °C solution of compound 8-CO₂^tBu (12 g, 13 mmol) in THF (100 mL) was added of lithium aluminium hydride power (1 g, 26 mmol) in portions. Afterwards, the solution was stirred overnight at room temperature and quenched with dilute HCl carefully, concentrated through rotary evaporation and subjected to silica gel chromatography using hexane/EtOAc as eluent to afford alcohol 8-CH₂OH (10.6 g, 12.5 mmol, 95% yield) as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ 4.04 (s, 6H), 3.68 (t, *J* = 6.3 Hz, 2H), 3.51 (t, *J* = 6.3 Hz, 2H),

3.38 (s, 2H), 1.83 (br, 1H), 1.80 (t, $J = 6.3$ Hz, 2H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 120.4 (q, $J = 292.9$ Hz), 80.8 – 78.8 (m), 69.2, 66.2, 65.7, 60.3, 46.4, 32.5; ^{19}F NMR (282 MHz, CDCl_3) δ – 70.86 (s).

3-(3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(tri-fluoromethyl)propan-2-yl)oxy)methyl)propoxy)propyl methanesulfonate (8-CH₂OMs)

Triethylamine (Et_3N , 0.34 mL, 2.4 mmol) and methanesulfonyl chloride (0.2 mL, 2.4 mmol) were added to a solution of compound **8-CH₂OH** (1 g, 1.2 mmol) dissolved in CH_2Cl_2 (15 mL) at 0 °C. The reaction mixture was then stirred at r.t. overnight. The reaction was quenched with water and extracted with CH_2Cl_2 . Evaporation through rotary evaporation followed by flash chromatography on silica gel using hexane/EtOAc as eluent afforded product mesylate **8-CH₂OMs** (1.1 g, 1.2 mmol, quant. yield) as a colorless oil. ^1H NMR (300 MHz, CDCl_3) δ 4.26 (t, $J = 6.3$ Hz, 2H), 4.04 (s, 6H), 3.50 (t, $J = 6.0$ Hz, 2H), 3.39 (s, 2H), 2.96 (s, 3H), 1.98 (t, $J = 6.3$ Hz, 2H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 120.3 (q, $J = 292.2$ Hz), 81.7 – 78.7 (m), 67.3, 66.8, 66.3, 65.6, 46.4, 37.4, 29.6; ^{19}F NMR (282 MHz, CDCl_3) δ – 70.82 (s).

2-(3-(3-Azidopropoxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)methyl)propoxy)-1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propane (8-CH₂N₃)

To a solution of mesylate **8-CH₂OMs** (1 g, 1.1 mmol) in DMF (6 mL) was added sodium azide (0.14 g, 2.2 mmol). The reaction mixture was stirred at 70 °C overnight. The mixture was then extracted with DCM, washed successively with water and brine, concentrated through rotary evaporation and subjected to silica gel chromatography using hexane/EtOAc as eluent to afford the azide **8-CH₂N₃** (0.70 g, 0.8 mmol, 73% yield) as a light yellow liquid. ^1H NMR (300 MHz, CDCl_3) δ 4.10 (s, 6H), 3.49 (t, $J = 6.0$ Hz, 2H), 3.47 (s, 2H), 3.34 (t, $J = 6.6$ Hz, 2H), 1.88 – 1.80 (m, 2H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 120.6 (q, $J = 292.9$ Hz), 80.6 – 79.0 (m), 68.6, 66.4, 65.8, 48.6, 46.6, 29.1; ^{19}F NMR (282 MHz, CDCl_3) δ – 71.2 (s).

3-(3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)methyl)propoxy)propan-1-amine (9)

To the azide compound **8-CH₂N₃** (600 mg, 0.69 mmol) in dry THF (6 mL) was added Ph_3P (360 mg, 1.4 mmol). The reaction mixture was monitored by TLC. After the starting material has been completely consumed, water (0.1 mL) was added and the reaction mixture was stirred overnight at room temperature. After removal of the solvent in vacuum through rotary evaporation, the residue was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{TFA}$ as the eluent to give compound **9** as a TFA salt (610 mg, 0.63 mmol, 92% yield) as yellow oil. ^1H NMR (300 MHz, CD_3OD) δ 4.14 (s, 6H), 3.53 (t, $J = 6.0$ Hz, 2H), 3.45 (s, 2H), 3.00 – 2.95 (m, 2H), 1.98 – 1.89 (m, 2H); ^{13}C NMR (75.5 MHz, CD_3OD) δ 163.3 (q, $J = 34.7$ Hz), 125.9 (q, $J = 251.4$ Hz), 114.2 (q, $J = 292.2$ Hz), 82.1 – 80.1 (m), 69.7, 67.3, 67.1, 47.6, 38.6, 29.1; ^{19}F NMR (282 MHz, CD_3OD) δ – 71.1 (s, 9F), –76.6 (s, 3F).

3-(6-((3-(3-((1,1,1,3,3,3-Hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)-2,2-bis(((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)methyl)propoxy)propyl)amino)-6-oxohexyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1H-benzo- [e]indol-3-ium chloride (10)

To a solution of Cy5.5 NHS ester (4.3 mg, 6 μ mol) in DMF (0.3 mL) was added DIPEA (2.6 μ L, 15 μ mol), compound **9** (4.8 mg, 5 μ mol) successively, the mixture was stirred in dark at room temperature overnight, then diluted with water and subjected to semi-preparative HPLC to afford compound **10** (5.0 mg, 3.7 μ mol, 58% yield) as deep blue solid. For semi-preparative HPLC, a Beckman Ultrasphere C₁₈ column (10 $\times$ 250 mm) and a gradient elution profile were used with 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in CH₃CN (solvent B). The elution profile was 30% solvent B gradient to 100% solvent B over 35 min, and finally to 100% solvent B over the next 10 min. The major peak at about 34 min was collected and lyophilized. ¹H NMR (300 MHz, CDCl₃) δ 8.70 (s, 2H), 8.13 (d, *J* = 7.8 Hz, 2H), 8.04 (d, *J* = 5.4 Hz, 1H), 7.97 (d, *J* = 8.7 Hz, 4H), 7.68 – 7.62 (m, 2H), 7.54 – 7.50 (m, 2H), 7.42 – 7.37 (m, 2H), 6.80 (t, *J* = 13.5 Hz, 1H), 6.40 (d, *J* = 13.8 Hz, 1H), 6.31 (d, *J* = 13.5 Hz, 1H), 4.20 (t, *J* = 8.1 Hz, 2H), 4.06 (s, 6H), 3.75 (s, 3H), 3.44 (t, *J* = 6.3 Hz, 2H), 3.38 (s, 2H), 3.32 – 3.25 (m, 2H), 2.31 (t, *J* = 7.2 Hz, 2H), 2.02 (s, 12H), 1.94 – 1.88 (m, 2H), 1.82 – 1.76 (m, 4H), 1.62 – 1.54 (m, 2H); ¹³C NMR (75.5 MHz, CDCl₃) 174.4, 174.0, 173.4, 166.6, 152.3, 151.6, 140.1, 139.3, 134.0, 133.3, 132.0, 131.8, 130.9, 130.7, 130.19, 130.15, 128.2, 128.1, 127.8, 126.2, 125.3, 125.0, 122.0, 118.2, 114.3, 110.7, 110.2, 103.9, 103.2, 80.0 – 78.9 (m), 69.5, 65.8, 65.5, 51.2, 50.8, 46.2, 44.6, 36.5, 36.1, 31.9, 29.6, 27.7, 27.4, 26.4, 25.3, 22.7; ¹⁹F NMR (282 MHz, CD₃OD) δ – 70.40 (s); Mass (ESI) *m/z* 1413.98 (M + H)⁺.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Synthesis and characterization of a set of fluorous dendron-cyanine dye bimodal molecules.

a) Synthesis route of fluorous dendron-cyanine dye molecules; b) ^{19}F NMR spectra of synthesized bimodal molecules; c) Comparison of ^{19}F NMR spectral signal intensity of different bimodal molecules ($[c] = 1 \mu\text{M}$); d) Comparison of ^{19}F NMR S/N ratio of different bimodal molecules ($[c] = 1 \mu\text{M}$).

Figure 2.

A) Polyethylene glycol (PEG) protected FC incorporating engineered polymeric nanoprobe (FCNP) (components were not drawn in scale) and TEM image of FCNP with negative staining (Scale bar = 100 nm); B) DLS size measurement of FCNP; C) Linear regression analysis of ^{19}F NMR signal to fluorescence intensity of different dosages of FCNP after 24 h circulation in mouse blood. (n=3)

Figure 3.

A) Confocal microscopy image of MSC uptake of FCNP (0.1mM) after 24 h incubation. Inset shows the magnified single MSC labeled by FCNP; B) Transwell study of MSCs^{GFP+} migration after labeling with FCNP (0.1mM). Scale bar=50μm; C) Fluorescence microscopy and flow cytometry analysis of FCNP (0.1mM) labeling retention in MSC cytoplasm over 9 days. Green: MSCs^{GFP+}; Red: FCNP; Yellow/orange: overlay of MSCs and FCNP.

Figure 4.

A) Quantitative analysis of FC in THF at different fluorine concentrations by MR spectroscopy. External standard=1 μ l hexafluorobenzene; B) Signal to noise ratio analysis of FC in THF by MR spectroscopy; C) Quantitative analysis of FCNP labeled MSCs at different concentrations by MR spectroscopy. External standard=1 μ l hexafluorobenzene; D) Signal to noise ratio analysis of FC in THF by MR spectroscopy.

Figure 5.

A) Phantoms of ^{19}F MRI and fluorescence images of different number of FCNP labeled MSCs; B) Quantitative linear correlation analysis of phantom ^{19}F NMR signal to fluorescence intensity; C) Fluorescence imaging (left) and ^{19}F MRI imaging (right) of animal after *in situ* injection of different amount of FCNP labeled MSCs; D) Quantitative linear correlation of ^{19}F MRI signal with fluorescence intensity.

Figure 6. Fluorescence (upper channel) and ^{19}F MRI imaging (lower channel) of intracranially administered FCNP labeled MSCs to TBI mice at different time points.

Conventional dendrimer synthesis **Proportionate branching synthesis****Scheme 1.**

Comparison of conventional dendrimer synthesis method and proportionate branching method.