Supporting Information

**Perfluorocarbon-Based $^{19}$F MRI Nanoprobes for In Vivo Multicolor Imaging**

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1. Materials and Instruments

General reagents were of the best grade available, supplied by Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), Watanabe Chemical Industry (Hiroshima, Japan), Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and Peptide Institute, Inc. (Osaka, Japan). They were used as received without further purification.

NMR spectra were recorded on a JEOL JNM-AL400 instrument (Tokyo, Japan) at 400 MHz for $^1$H NMR and 100.4 MHz for $^{13}$C NMR; on a Bruker Ascend\textsuperscript{TM} 500 instrument (Billerica, MA, USA) at 500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR using tetramethysilane as an internal standard; and at 376 MHz for $^{19}$F NMR using sodium trifluoroacetate as an internal standard. Magnetic resonance imaging (MRI) was performed on a Bruker BioSpec 117/11 system equipped with a 35 mm inner diameter volume coil at a frequency of 500 MHz for $^1$H and 471 MHz for $^{19}$F measurements. Image acquisition and processing were carried out using the ParaVision software (Bruker BioSpin) and ImageJ software (NIH, Bethesda, MD, USA). Transmission electron microscopy (TEM) images were acquired with a HITACHI H-9000 (at 300 kV; Tokyo, Japan). Fluorescence spectra were measured using a HITACHI F7000 spectrometer (Tokyo, Japan).

2. Synthesis

![Scheme S1. Synthesis of 1,1,1-tris(perfluoro-tert-butoxymethyl)ethane.](image)

Scheme S1. Synthesis of 1,1,1-tris(perfluoro-tert-butoxymethyl)ethane.
Synthesis of 1,1,1-tris(perfluoro-tert-butoxymethyl)ethane (TPFBME)

TPFBME was synthesized according to a previous report.\(^{[51]}\) Briefly, to trimethylolethane (600 mg, 4.99 mmol), triphenylphosphine (5.90 g, 22.5 mmol, 4.5 eq.) and 4 Å molecular sieves (600 mg) in tetrahydrofuran (30 mL) at 0 °C under Ar atmosphere were added dropwise diethylazodicarboxylate (3.54 mL, 22.5 mmol, 4.5 eq.). Afterward, the reaction solution was stirred at 20 °C for 1 h, followed by addition of perfluoro-tert-butanol (3.14 mL, 22.5 mmol, 4.5 eq.). The resulting reaction mixture was stirred at 45 °C for 27 h under Ar atmosphere. H\(_2\)O (3 mL) was added to the reaction mixture and stirred at room temperature for 10 min. The solution after filtration of molecular sieves was transferred to a separate funnel and the lower phase was collected to afford TPFBME (1.55 g, 2.00 mmol, y. 40%). \(^1\)H NMR (400 MHz, neat) \(\delta 0.90\) (s, 3H), 3.81 (s, 6H); \(^{13}\)C NMR (100 MHz, neat) \(\delta 14.7, 41.7, 69.3, 79.7\) (m), 120.6; \(^{19}\)F NMR (376 MHz, neat) \(\delta 3.36\).

Preparation of PFCE@SiO\(_2\), PFTBA@SiO\(_2\), and TPFBME@SiO\(_2\)

Rhodamine B isothiocyanate (RITC) (5.3 mg, Aldrich), Fluorescein-4 isothiocyanate (FITC) (3.9 mg, Dojindo), or sulfo-Cyanine 5 NHS ester (sulfo-Cy5) (7.6 mg, Lumiprobe) were reacted with 24 μL of 3-aminopropyltriethoxysilane in 0.30 mL of ethanol under dark conditions for 48 h at 30 °C.

Distearoyl-sn-glycero-3-phosphocholine (DSPC: 5.0 mg, 6.3 μmol) and PAP (0.33 mg, 0.79 μmol) were dissolved in 3 mL of chloroform at 65 °C. The organic solvent was evaporated in a rotary evaporator at 65 °C to obtain a thin film; solvent traces were removed by maintaining the lipid film under vacuum for 12 h. The film was hydrated with 3 mL of water using a bath-type sonicator (Branson 1250) for 10 min at 60 °C. Then, 30 μL of perfluoro-[15] crown-5 ether (PFCE), perfluorotributylamine (PFTBA), or TPFBME was added to the emulsion, followed by homogenization (T10 basic ULTRA TURRAX, IKA) for 10 min and sonication using a bath-type sonicator for 120 min at 60 °C. The emulsion was filtered with a 0.45 μm filter (hydrophilic PFPE, Millipore). Water (12 mL) and tetraethyl orthosilicate (0.10 mL) were added to the emulsion, and then the mixture was stirred for 48 h at 25 °C. For fluorescence detection of the particles, 10 μL of RITC-conjugate, FITC-conjugate, or sulfo-Cy5-conjugate 3-aminopropyltriethoxysilane solution was then added and the solution was stirred for 24 h at 25 °C. The product of PFC@SiO\(_2\) was purified by centrifugation (14,000 × g, 4 °C, 30 min) and washed 3 times with ethanol (20 mL). Finally, PFC@SiO\(_2\) was dispersed in water (15 mL) for storage.

Preparation of amine-modified PFC@SiO\(_2\)

500 μL of 3-aminopropyl triethoxysilane (APTES) was added to PFC@SiO\(_2\) in 2-propanol (30 mL) and stirred at 80 °C for 3 h. The amine-modified PFC@SiO\(_2\) was purified by centrifugation (14,000 × g, 4 °C, 30 min) and washed 3 times with ethanol (20 mL) and dry N,N-dimethylformamide (DMF; 10 mL), respectively.
Preparation of carboxylated PFC@SiO$_2$
Amine-modified PFC@SiO$_2$ was dispersed in dry DMF (5 mL) under N$_2$ atmosphere. Next, succinic anhydride (1 g, 10 mmol) and dry triethylamine (1 mL) were added to the solution, then the mixture was stirred for 36 h at 40 °C. The product was purified by centrifugation (14,000 × g, 4 °C, 30 min) and washed 3 times with DMF (20 mL) and water (20 mL), respectively. Finally, carboxylated PFC@SiO$_2$ was dispersed in water (15 mL) for storage.

3. Experimental Procedures

$^{19}$F NMR measurement of PFCs and PFC@SiO$_2$
The $^{19}$F NMR spectra of PFCs were measured with glass capillary containing D$_2$O as deuterium lock. The $^{19}$F NMR spectra of PFC@SiO$_2$ were measured in H$_2$O containing 5% D$_2$O.

$^{19}$F MRI measurement of PFC@SiO$_2$
The nanoparticles dispersed in H$_2$O were transferred to a 384-well microplate. Then, $^1$H/$^{19}$F MRI measurements were performed according to the following methods. Acquired images were converted to DICOM format and rendered in red hot, cyan hot, and green hot color for PFCE@SiO$_2$, TPFBME@SiO$_2$, and PFTBA@SiO$_2$, respectively. $^1$H MRI RARE method: the image matrix was $256 \times 128$, field of view was $8 \times 4$ cm, and slice thickness was 1.5 mm. $T_R$ was 1000 ms. $T_{E,eff}$ was 32 ms. The number of averages was 2. The acquisition time was 32 s. $^{19}$F MRI RARE method: the image matrix was $128 \times 64$, field of view was $8 \times 4$ cm, and slice thickness was 30 mm. $T_R$ was 1000 ms. $T_E$ was 13 ms. The number of averages was 32. The acquisition time was 34 min 21 s.

DLS measurement
The particle size, size distribution, and ζ-potential of the obtained nanoparticles were measured at 25 °C with a 580 nm laser at a scattering angle of 90° for size measurements and 173° for ζ-potential measurements. For the size measurements, FLAME nanoparticles were suspended in water or ethanol. Suspensions of each material were prepared in water for ζ-potential measurements.

Fluorescence spectroscopy
Fluorescence spectra were measured at 37 °C after nanoparticles were dispersed in water. Excitation wavelengths were 483 nm for PFTBA@SiO$_2$ with FITC, 556 nm for PFCE@SiO$_2$ with RITC, and 647 nm for TPFBME@SiO$_2$ with sulfo-Cy5.
Fluorescence imaging of RAW264.7 cells after incubation with PFCE@SiO$_2$, TPFBME@SiO$_2$, or PFTBA@SiO$_2$

RAW264.7 cells were cultured in Dulbecco’s modified eagle medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco), and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin, Gibco). Cells were grown on glass dishes. The cells were washed with HBSS (Gibco) three times. Next, the cells were incubated with PFCE@SiO$_2$, TPFBME@SiO$_2$, or PFTBA@SiO$_2$ ($C_{PFCE} = 0.5$ mM) in DMEM for 1 h at 37 °C. After incubation, the cells were washed with HBSS three times. Then, fluorescence and phase contrast images were acquired using a confocal laser-scanning microscope (FLUOVIEW FV10i; Olympus, Tokyo, Japan) with excitation at 473 nm (FITC), 559 nm (RITC), and 635 nm (sulfo-Cy5).

Mouse experimental procedure using carboxylated PFCE@SiO$_2$, TPFBME@SiO$_2$, and PFTBA@SiO$_2$

All animal experimentation and handling was approved by the local ethics review board and was performed in accordance with the guidelines of the Animal Care and Use Committee of Osaka University. C57BL/6Jcl mice were obtained from CLEA Japan (Tokyo, Japan), anesthetized with sevoflurane, and subjected to MRI for data acquisition. $^1$H/$^{19}$F MRI images were acquired after subcutaneous injection of carboxylated PFCE@SiO$_2$, TPFBME@SiO$_2$, and PFTBA@SiO$_2$ ($C_{PFCE} = 10$ mM, 25 µL), (Figure 4). $^1$H MRI RARE method: the image matrix was 256 × 128, field of view was 6 × 3 cm, and slice thickness was 1.2 mm. $T_R$ was 500 ms. $T_E$ was 8 ms. The number of averages was 2. The acquisition time was 1 min 4 s. $^{19}$F MRI RARE method: [Sagittal and Coronal] the image matrix was 256 × 128, field of view was 6 × 3 cm, and slice thickness was 40 mm. $T_R$ was 1000 ms. $T_E$ was 16 ms. The number of averages was 128. The acquisition time was 17 min 4 s.

Evaluation of hepatic uptake using PFCE@SiO$_2$-PEG, TPFBME@SiO$_2$-COOH, and PFTBA@SiO$_2$-OH

In vivo $^1$H/$^{19}$F MRI images were acquired at 3, 12, and 24 h after intravenous injection of PFCE@SiO$_2$-PEG, TPFBME@SiO$_2$-COOH, and PFTBA@SiO$_2$-OH ($C_{PFCE} = 3.3$ mM, 300 µL).

[Tube] $^1$H MRI RARE method: the image matrix was 256 × 128, field of view was 7.0 × 3.5 cm, and slice thickness was 2.0 mm. $T_R$ was 500 ms. $T_E$ was 8 ms. The number of averages was 2. The acquisition time was 1 min 4 s. $^{19}$F MRI RARE method: the image matrix was 128 × 64, field of view was 7.0 × 3.5 cm, and slice thickness was 40 mm. $T_R$ was 1000 ms. $T_E$ was 64 ms. The number of averages was 16 (PFCE@SiO$_2$-PEG and TPFBME@SiO$_2$-COOH) or 32 (PFTBA@SiO$_2$-OH). The acquisition time was 2 min 8 s (PFCE@SiO$_2$-PEG and TPFBME@SiO$_2$-COOH) or 4 min 16 s (PFTBA@SiO$_2$-OH).
[Mouse] $^1$H MRI RARE method: the image matrix was $256 \times 128$, field of view was $7.0 \times 3.5$ cm, and slice thickness was 1.5 mm. $T_R$ was 500 ms. $T_E$ was 8 ms. The number of averages was 2. The acquisition time was 1 min 4 s. $^1$F MRI RARE method: the image matrix was $128 \times 64$, field of view was $7.0 \times 3.5$ cm, and slice thickness was 40 mm. $T_R$ was 1000 ms. $T_E$ was 12 ms. The number of averages was 128 (PFCE@SiO$_2$-PEG and TPFBME@SiO$_2$-COOH) or 256 (PFTBA@SiO$_2$-OH). The acquisition time was 17 min 4 s.

The relative intensity (Figure S5) was calculated as follows:

$$\text{Relative intensity} = \frac{S/N \text{ ratio (Mouse liver)}}{S/N \text{ ratio (Tube)}} \text{ (a.u.)}$$

**Calculation of concentration of PFC ($C_{\text{PFC}}$) in nanoparticles**

The PFC@SiO$_2$ was dispersed in water containing 10% D$_2$O and 1 mM sodium trifluoroacetate (TFANa), and $^1$F NMR spectrum was obtained. The concentration of PFC in the nanoparticles was calculated as follows:

$$C_{\text{PFC}} = \frac{C_{\text{TFANa}} \times m \times n_{\text{TFANa}}}{n_{\text{PFC}}}$$

where $C_{\text{PFC}}$ is the molarity of PFC, $C_{\text{TFANa}}$ is the molarity of TFANa, $m$ is the integral value of PFC calculated from the $^1$F NMR spectrum when the integral value of TFANa is 1, $n_{\text{TFANa}}$ is the number of fluorine atoms in TFANa ($n_{\text{TFANa}} = 3$), and $n_{\text{PFC}}$ is the number of fluorine atoms in PFC.

When $C_{\text{TFANa}}$ is 1 mM, $C_{\text{PFC}}$ was calculated as follows:

$$C_{\text{PFC}} = \frac{1 \times m \times 3}{n_{\text{PFC}}} \text{ (mM)}$$
4. Supporting Figures and Tables

**Figure S1.** (a) TEM images of PFC@SiO$_2$. Scale bars represent 100 nm. (b) Particle size distribution histogram measured by TEM images ($n = 300$).
Figure S2. Chemical structures and $^{19}$F NMR spectra of PFCs. The peaks of colored fluorine atoms in the chemical structures are indicated by arrows in each spectrum.

Figure S3. Emission spectra of PFTBA@SiO$_2$ with FITC ($\lambda_{ex}$: 483 nm, $\lambda_{em, max}$: 516 nm), PFCE@SiO$_2$ with RITC ($\lambda_{ex}$: 556 nm, $\lambda_{em, max}$: 580 nm), and TPFBME@SiO$_2$ with sulfo-Cy5 ($\lambda_{ex}$: 647 nm, $\lambda_{em, max}$: 664 nm).
**Figure S4.** Fluorescence images of RAW264.7 cells after incubation with PFCE@SiO$_2$, TPFBME@SiO$_2$, and PFTBA@SiO$_2$, respectively. Scale bars = 20 µm. Excitation: 473 nm (FITC), 559 nm (RITC), and 635 nm (sulfo-Cy5).
Table S1. Hydrodynamic diameters of emulsions and PFC@SiO$_2$ as measured by DLS. Data are presented as mean ± SD ($n = 3$).

| Name of PFC | PFCE  | PFOB  | PFDCO | PFTBA  | TPFBME | PFN  |
|-------------|-------|-------|-------|--------|--------|------|
| Emulsion (nm) | 61 ± 5 | 71 ± 2 | 87 ± 5 | 89 ± 6 | 79 ± 6 | 133 ± 3 |
| SiO$_2$-OH (nm) | 138 ± 0.3 | 165 ± 1 | 104 ± 1 | 144 ± 2 | 147 ± 2 | 372 ± 11 |
| SiO$_2$-COOH (nm) | 148 ± 3 | – | – | 148 ± 2 | 165 ± 3 | – |

Table S2. ζ-potential values of emulsions and PFC@SiO$_2$ as measured by DLS. Data are presented as mean ± SD ($n = 3$).

| Name of PFC | PFCE  | PFOB  | PFDCO | PFTBA  | TPFBME | PFN  |
|-------------|-------|-------|-------|--------|--------|------|
| Emulsion (mV) | 19 ± 1 | 20 ± 0.4 | 23 ± 0.1 | 27 ± 2 | 11 ± 1 | 25 ± 3 |
| SiO$_2$-OH (mV) | −13 ± 1 | −34 ± 0.3 | −5 ± 2 | −12 ± 4 | −23 ± 1 | – |
| SiO$_2$-COOH (mV) | −61 ± 0.3 | – | – | −77 ± 1 | −73 ± 1 | – |

Table S3. The average diameters of PFC@SiO$_2$ as measured by TEM images. Data are presented as mean ± SD ($n = 300$).

| Materials | PFCE @SiO$_2$ | PFOB @SiO$_2$ | PFDCO @SiO$_2$ | PFTBA @SiO$_2$ | TPFBME @SiO$_2$ |
|-----------|---------------|---------------|---------------|---------------|----------------|
| Average diameter (nm) | 53 ± 17 | 92 ± 24 | 104 ± 23 | 66 ± 20 | 61 ± 18 |

Figure S5. Relative $^{19}$F signal intensities of PFCE@SiO$_2$-PEG, TPFBME@SiO$_2$-COOH, and PFTBA@SiO$_2$-OH in the liver (Detailed calculation were presented in experimental procedures).
Table S4. $T_1$ and $T_2$ values of PFC liquids as measured by $^{19}$F MRI.

| Name of PFC | PFCE @SiO$_2$ | PFOB @SiO$_2$ | PFDCO @SiO$_2$ | PFTBA @SiO$_2$ | TPFBME @SiO$_2$ |
|-------------|----------------|----------------|----------------|----------------|----------------|
| $T_1$ (ms)  | 543            | 590            | 309            | 305            | 399            |
| $T_2$ (ms)  | 502            | 412            | 164            | 106            | 240            |

Table S5. $T_1$ and $T_2$ values of PFC@SiO$_2$ as measured by $^{19}$F MRI.

| Name of PFC@SiO$_2$ | PFCE@SiO$_2$ | PFOB@SiO$_2$ | PFDCO@SiO$_2$ | PFTBA@SiO$_2$ | TPFBME@SiO$_2$ |
|---------------------|--------------|--------------|---------------|---------------|---------------|
| $T_1$ (ms)          | 463          | 348          | 360           | 204           | 375           |
| $T_2$ (ms)          | 284          | 108          | 106           | 96            | 117           |

$T_1$ was measured using the inversion-recovery pulse sequence on an 11 T MR scanner. $T_2$ was measured using the spin-echo pulse sequence on an 11 T MR scanner.

The center frequencies of the TPFBME peak (at approximately $\delta = 3.3$ ppm), PFCE peak (at approximately $\delta = -16.4$ ppm), PFOB and PFDCO peaks (at approximately $\delta = -47.7$ ppm), and PFTBA peak (at approximately $\delta = -53.0$ ppm) were excited for $T_1$ and $T_2$ measurements.

Table S6. Comparison of $T_1$ and $T_2$ values among the different $^{19}$F MRI nanoprobes.

| Materials | PEO-b-P (DPA$_{48}$r-TEF$_{12}$)$_{[S2]}$ | Citrate-coated CaF$_2$$_{[S3]}$ | Poly(OEGMA-co-PFPEMA)$_{[S4]}$ | DOX-loaded fluorinated liposome, L1$_{[S5]}$ |
|-----------|------------------------------------------|-------------------------------|-----------------------------------|-----------------------------------|
| $T_1$ (ms) | --                                       | 7020                          | 410                              | 606                              |
| $T_2$ (ms) | 44                                       | 3                             | 60                               | 14                               |

The data are reproduced from the references.$^{S2-S5}$

5. Supporting References

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