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Supporting Information

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

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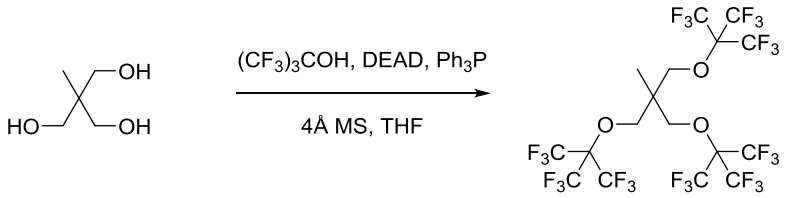
Contents:

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^{TM} 500 instrument (Billerica, MA, USA) at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR using tetramethylsilane 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.

Synthesis of 1,1,1-tris(perfluoro-*tert*-butoxymethyl)ethane (TPFBME)

TPFBME was synthesized according to a previous report.[S1] 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_2O (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) δ 0.90 (s, 3H), 3.81 (s, 6H); ^{13}C NMR (100 MHz, neat) δ 14.7, 41.7, 69.3, 79.7 (m), 120.6; ^{19}F NMR (376 MHz, neat) δ 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 ºC 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_2O as deuterium lock. The ^{19}F NMR spectra of PFC@SiO_2 were measured in H_2O containing 5% D_2O.

^{19}F MRI measurement of PFC@SiO_2

The nanoparticles dispersed in H_2O 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 × 128, field of view was 8 × 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 × 64, field of view was 8 × 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_{PFC} = 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_{PFC} = 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_{PFC} = 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 × 128, field of view was 7.0 × 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. ^{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 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:

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

Calculation of concentration of PFC (C_{PFC}) in nanoparticles

The PFC@SiO_2 was dispersed in water containing 10% D_2O and 1 mM sodium trifluoroacetate (TFANa), and ^{19}F NMR spectrum was obtained. The concentration of PFC in the nanoparticles was calculated as follows:

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

where C_{PFC} is the molarity of PFC, C_{TFANa} is the molarity of TFANa, m is the integral value of PFC calculated from the ^{19}F NMR spectrum when the integral value of TFANa is 1, n_{TFANa} is the number of fluorine atoms in TFANa (n_{TFANa} = 3), and n_{PFC} is the number of fluorine atoms in PFC.

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

$$C_{PFC} = \frac{1 \times m \times 3}{n_{PFC}}\ (\text{mM})$$

4. Supporting Figures and Tables

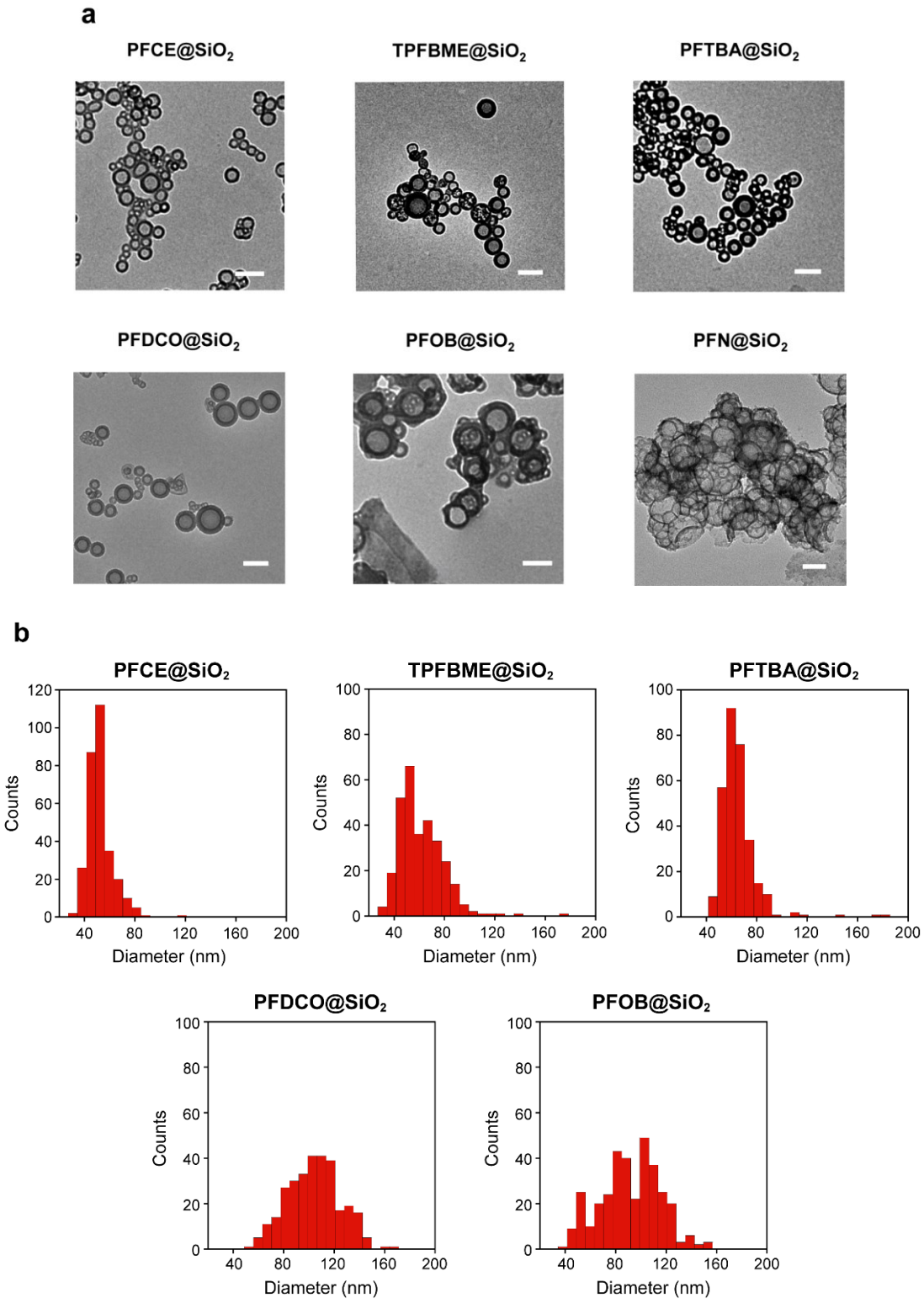


Figure S1. (a) TEM images of PFC@SiO_2. Scale bars represent 100 nm. (b) Particle size distribution histogram measured by TEM images (n = 300).

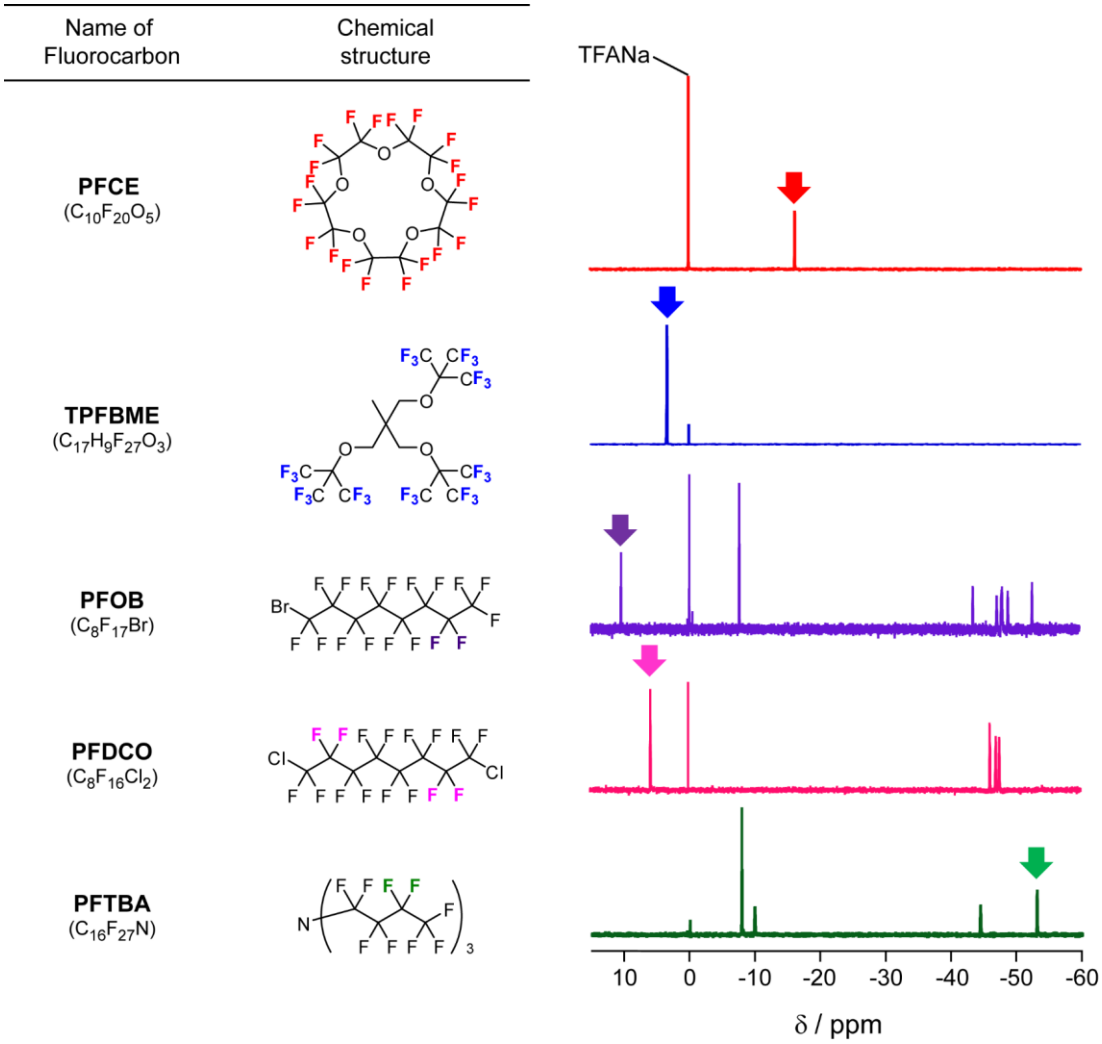


Figure S2. Chemical structures and ^{19}F NMR spectra of PFCs. The peaks of colored fluorine atoms in the chemical structures are indicated by arrows in each spectrum.

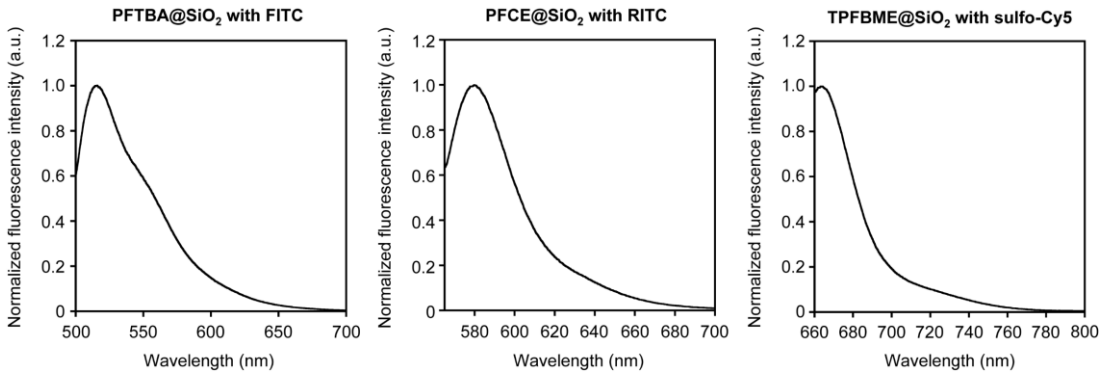


Figure S3. Emission spectra of PFTBA@SiO_2 with FITC (λ_{ex}: 483 nm, $\lambda_{em,\ max}$: 516 nm), PFCE@SiO_2 with RITC (λ_{ex}: 556 nm, $\lambda_{em,\ max}$: 580 nm), and TPFBME@SiO_2 with sulfo-Cy5 (λ_{ex}: 647 nm, $\lambda_{em,\ max}$: 664 nm).

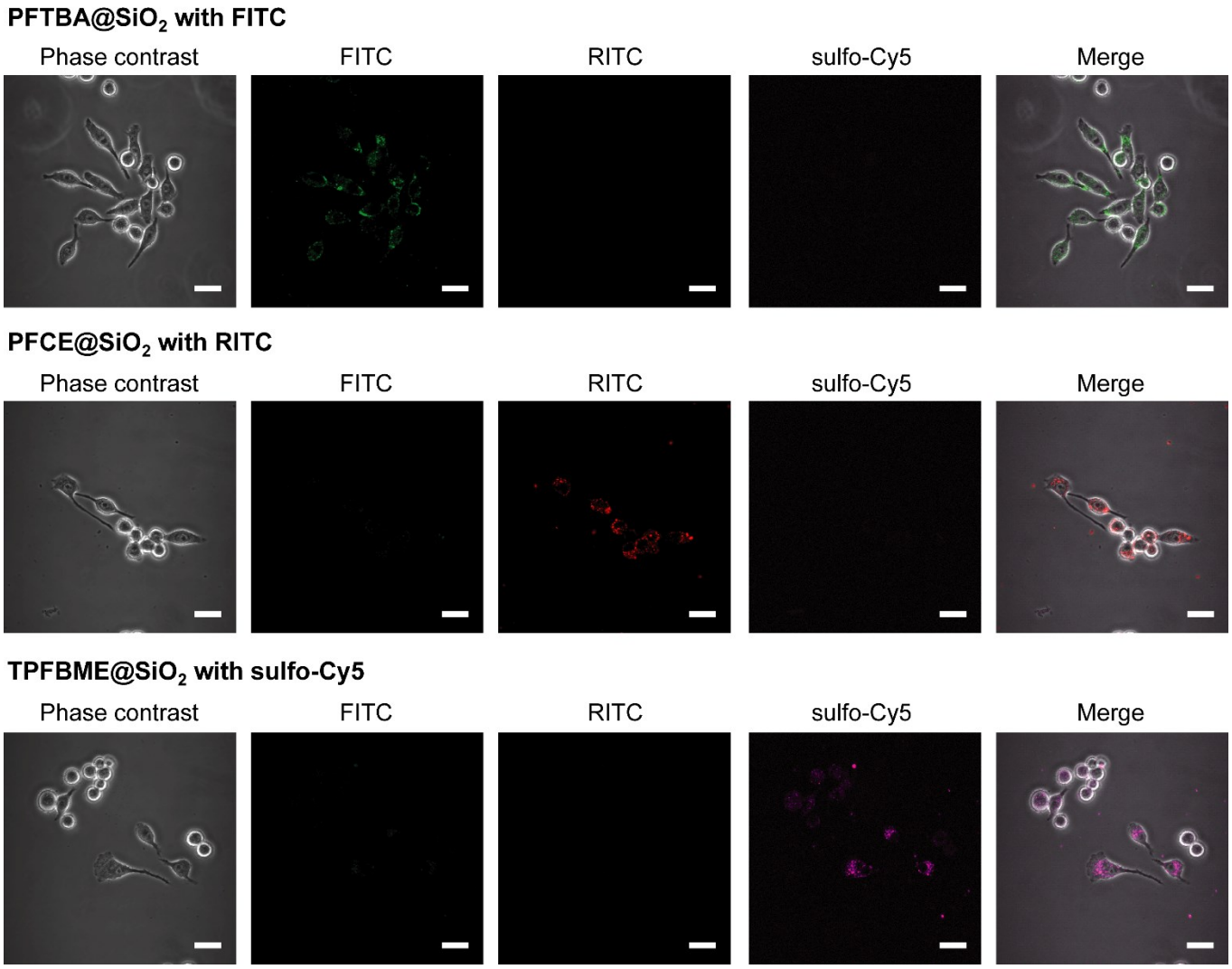


Figure S4. Fluorescence images of RAW264.7 cells after incubation with PFCE@SiO_2, TPFBME@SiO_2, and PFTBA@SiO_2, respectively. Scale bars = 20 μm. Excitation: 473 nm (FITC), 559 nm (RITC), and 635 nm (sulfo-Cy5).

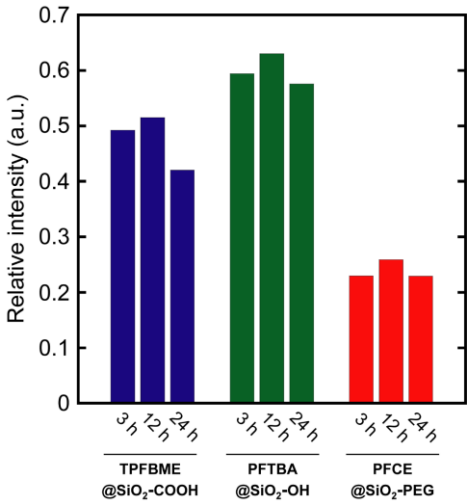


Figure S5. Relative ^{19}F signal intensities of PFCE@SiO_2-PEG, TPFBME@SiO_2-COOH, and PFTBA@SiO_2-OH in the liver (Detailed calculation were presented in experimental procedures).

Table 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

Table S4. T_1 and T_2 values of PFC liquids as measured by ^{19}F MRI.

Name of PFC	PFCE	PFOB	PFDCO	PFTBA	TPFBME
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 δ = 3.3 ppm), PFCE peak (at approximately δ = –16.4 ppm), PFOB and PFDCO peaks (at approximately δ = –47.7 ppm), and PFTBA peak (at approximately δ = –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*-TFE_{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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