Paramagnetic nanoemulsions with unified signal for sensitive $^{19}$F MRI cell tracking

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1. General information

$^1$H, $^{19}$F and $^{13}$C NMR spectra of compounds were recorded on a 400 MHz NMR spectrometer. Chemical shifts are in ppm and coupling constants ($J$) are in Hertz (Hz). $^1$H NMR spectra were referenced to tetramethylsilane (d, 0.00 ppm) using CDCl$_3$ or acetone-D$_6$ as solvent. $^{13}$C NMR spectra were referenced to solvent carbons (77.16 ppm for CDCl$_3$ and 29.84 ppm for acetone-D$_6$). $^{19}$F NMR spectra were referenced to 2% perfluorobenzene (s, -164.90 ppm) in CDCl$_3$. The splitting patterns for $^1$H NMR spectra are denoted as follows: s (singlet), d (doublet), q (quartet), m (multiplet).

Unless otherwise indicated, all reagents were obtained from commercial supplier and used without prior purification. All solvents were either analytical or HPLC grade. Deionized water was used unless otherwise indicated. DMF and THF were dried and freshly distilled prior to use. Column flash chromatography was performed on silica gel (200-300 mesh) with the eluent as indicated in procedures. The fluorescent dye BODIPY used here is a fluorinated BODIPY developed in this lab.

Ultrasound bath of 500 W was used for formulation. During the chelation, solutions of chloride or triflate salt of the metal ions were added to the solution of emulsions. For the convenience of discussion, two kinds of concentrations for the emulsions were used in the study: the concentration of imaging agent 1 (C (I, mM)) and the concentration of fluorines (C (\(^{19}\)F, mM)). They can be converted as: C (I, mM) = C (\(^{19}\)F, mM)/27.

2. $^{19}$F NMR of $^{19}$F MRI imaging agent 1 and chelator 2 mixture (Figure S1)

![Figure S1. $^{19}$F NMR of imaging agent 1 and chelator 2 mixture](image-url)
3. Emulsions stability study (Figure S2)

**Figure S2.** Emulsions stability study at room temperature through DLS-measured particle size (a: Eml-2; c: Eml-3) and PDI (b: Eml-2; d: Eml-3).

4. PRE-effect of ions on $^{19}$F relaxation times and peaks of Eml-3 (Figure S3)

**Figure S3** PRE-effect of ions on $^{19}$F relaxation times (a) and peaks (b) of Eml-3

5. Quantification of fluorine content in the emulsion (Figure S4)

Routine $^{19}$F-NMR spectra were obtained by diluting the emulsion sample with water and an inserted capillary containing 120 mM sodium triflate in D$_2$O was used as the internal standard (Peak 1) to calibrate the concentration of the emulsion sample (Figure S3). Comparing the integrals of the peaks, it was possible to assay the fluorine content of the sample.
6. PRE-effect of Eml-4 with the addition of chelator EDTA (Figure S5)

![Graph showing 1/T₁ (s⁻¹) vs. Incubation time (day)]

**Figure S5.** PRE-effect of Eml-4 with the addition of chelator EDTA (left) and picture of the sample (right, I: 0 day, II: 1 day, III: 8 days)

7. PRE-effect of emulsions at different temperature (Figure S6)

![Graphs showing 1/T₁ (s⁻¹) and 1/T₂ (s⁻¹) vs. Temperature (K)]

**Figure S6.** PRE-effect of emulsions at different temperature (a & b for Eml-4, c & d for Eml-6)
Cell cultures, cytotoxicity assay and cell uptake assessment (Figure S7)

Murine fibroblasts (L929) and the non-small-cell lung cancer cells (A549) were cultured in MEM-Alpha (HyClone) and IMDM (HyClone) medium, respectively, and the murine macrophage cells (RAW264.7) were cultured in DMEM-High glucose (HyClone) medium, supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin in a humidified air with 5% CO₂ at 37 °C.

About $1 \times 10^4$ cells (L929, A549 and RAW264.7, respectively) were seeded per well in 96-well plates and cultured for 24 h. Nanoemulsions (*Eml-2*, *Eml-4*, *Eml-5*, *Eml-6*) were diluted with the medium to certain concentration and added to each well, respectively. After incubated for 24 h, cells were washed with PBS (pH 7.4) three times. MTT was added to the culture medium to the final concentration of 0.5 mg/mL and the resulting mixture was incubated for 4 h. Then, 200 mL DMSO was added to each well after removal of the medium. The optical density at 490 nm for each well was measured by plate reader.

Cell viability (%) was calculated as the formula:

$$\text{Cell viability} (\%) = \left( \frac{A_{\text{Test}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}} \right) \times 100\%$$

$A_{\text{Test}}, A_{\text{Control}}$ and $A_{\text{Blank}}$ represented the absorbance of cells with different treatments, untreated cells and blank culture media, respectively.

The cellular uptake of nanoemulsions was measured with $^{19}\text{F}$ NMR and confocal microscope.

About $2 \times 10^6$ cells (L929, A549 and RAW264.7) were plated in 10 cm dishes and allowed to attach overnight. Nanoemulsions (*Eml-2*, *Eml-4*, *Eml-5*, *Eml-6*) were diluted with the medium and added to the dish, respectively. After incubation for 19 h at 37 °C, the medium was removed, and cells were washed three times with phosphate-buffered saline (PBS), detached by trypsinization, washed again with PBS, and resuspended in 1 mL of PBS. To assay nanoemulsion uptake, cells were pelleted and resuspended in 0.2 mL of lysis solution. The solution was transferred to a 5 mm NMR tube, adding a capillary containing a 20 mM solution of sodium triflate in D₂O and $^{19}\text{F}$ NMR spectra were obtained to measure the $^{19}\text{F}$ uptake.

About $1 \times 10^5$ cells (A549 and RAW264.7) were seeded on cell culture dishes. The regular culture mediums were removed when the cell confluence reached to 40–50%. Culture medium containing the BODIPY labeled nanoemulsions (*Eml-6*) was added. After 5 h of incubation at 37 °C,
cells were incubated with DAPI for 10 min for nuclear staining. All the cells were washed at least three times with PBS. The fluorescence images were taken by confocal laser scanning microscope.

**Figure S7.** Cytotoxicity assay of emulsions on L929 cells (a) and cell uptake assessment of emulsions on L929 cells (b) and A549 cells (c)

9. **19F MRI Phantom experiments (Figure S8)**

All MRI experiments were performed on a 400 MHz MRI system. The temperature of the magnet room was maintained 25 °C during the entire MRI experiment.

Phantom images of nanoemulsions (Eml-2, Eml-4, Eml-5, Eml-6): An emulsion of 512 mM $^{19}$F was serially diluted $1\times$, $2\times$, $4\times$, $8\times$, $16\times$, $32\times$, $64\times$, $132\times$ times by ultra-pure grade water, resulting nanoemulsions with $^{19}$F concentrations of 512 mM, 256 mM, 128 mM, 64 mM, 32 mM, 16 mM, 8 mM, 4 mM, respectively. The $^{19}$F phantom images were acquired using a RARE sequence, RARE factor = 8, TR = 2000 ms, TE = 5.37 ms, matrix = 32×32, number of average = 8, FOV = 30 mm×30 mm, slice thickness = 20 mm, scan time = 64 s.

For T$_1$-weighted MRI, a RARE sequence was used with the following parameters (RARE factor = 1, TR = 150 ms, TE = 5.37 ms, matrix = 32×32, number of average = 4, FOV = 30 mm×30 mm, slice thickness = 20 mm, scan time = 19.2 s).

For T$_2$-weighted MRI, a RARE sequence was performed with the following parameters (RARE factor = 1, TR = 2000 ms, TE = 100 ms, matrix = 32×32, number of average = 4, FOV = 30 mm×30 mm, slice thickness = 20 mm, scan time = 256 s).
10. In vitro $^{19}$F MRI (Fig. S9)

About $5 \times 10^6$ A549 cells were plated in T75 cell culture dishes and allowed to attach overnight. $\text{Eml-2}$ was diluted with the medium to $^{19}$F concentrations of 27 mM and added to the dish. After incubation of 12 h at 37 °C, the medium was removed, and cells were washed three times with phosphate-buffered saline (PBS), detached by trypsinization, washed again in PBS, and resuspended in 1 mL of PBS. The solution was transferred to 1.5 mL centrifuge tube and cells were pelleted.

$^{19}$F MRI were acquired using a FLASH sequence with the following parameters: TR = 500 ms, TE = 2.3 ms, matrix = 64×64, number of average = 8, FOV = 37 mm×37 mm, slice thickness = 37 mm, scan time = 256 s). $^1$H MRI: method = RARE, matrix size = 128×128, FOV = 30 mm×30 mm, TR = 3000 ms, TE = 50 ms, number of average = 1, scan time = 96 s.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_s9.png}
\caption{In vitro $^{19}$F MRI of A549 cells [$C^{(19)F} = 27$ mM, incubation for 12 h] in Eppendorf tube.}
\end{figure}
11. Mice $^{19}\text{F}$ MRI experiments

5-week old male Balb/c nude mice were used in the in vivo $^{19}\text{F}$ MRI study. In this study, mice were anesthetized by isoflurane. RAW264.7 cells were labelled ex vivo with Eml-2 and Eml-4 at an imaging agent concentration of 1 mM for 12 h, respectively. RAW 264.7 cells labeled with Eml-2 were injected subcutaneously in the left hind leg region (6×10⁶ cells in 100 µL of physiologic solution). RAW 264.7 cells labeled with Eml-4 were injected s.c. in the right hind leg region (6×10⁶ cells in 100 µL of physiologic solution). $^{19}\text{F}$ MRI was measured 24 h after the injection.

$^{1}\text{H}$ MRI: method = RARE, matrix size = 256×256, FOV = 60 mm×40 mm, TR = 5000 ms, TE = 56 ms, RARE factor = 8, number of average = 1, scan time = 160 s. $^{19}\text{F}$ MRI: method = RARE, RARE factor = 8, TR = 2000 ms, TE = 5.37 ms, matrix = 32×32, number of average = 64, FOV = 74 mm×49 mm, slice thickness = 20 mm, scan time = 512 s.

For estimating $^{19}\text{F}$ T₁-weighted MRI, a RARE sequence was used with the following parameters (RARE factor = 8, TR = 500 ms, TE = 5.37 ms, matrix = 32×32, number of average = 64, FOV = 74 mm×49 mm, slice thickness = 20 mm, scan time = 128 s).

For estimating $^{19}\text{F}$ T₂-weighted MRI, a RARE sequence was performed with the following parameters (RARE factor = 8, TR = 2000 ms, TE = 30 ms, matrix = 32×32, number of average = 64, FOV = 74 mm×49 mm, slice thickness = 20 mm, scan time = 512 s).

12. Synthesis of library compounds

**Preparation of compound 1.** Under an argon atmosphere, to a stirred suspension of 1,1,1-tris(hydroxymethyl)-ethane (7.2 g, 60.0 mmol), triphenylphosphine (70.8 g, 270.0 mmol) and 4 Å molecular sieves (7.0 g) in tetrahydrofuran (300.0 mL) at 0 °C was added dropwise diethylazodicarboxylate (54.6 g, 270.0 mmol). Afterward, the reaction mixture was allowed to warm to room temperature and stirred for an additional 20 min. Then perfluoro-tert-butanol (63.7 g, 270.0 mmol) was added in one portion and the resulting mixture was stirred for 48 h at 45 °C in a sealed vessel. Water (30.0 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 1 as clear oil (32.5 g, 70% yield). $^{1}\text{H}$ NMR (400 MHz, Acetone-D₆) δ 4.19 (s, 6H), 1.22 (s, 3H). $^{19}\text{F}$ NMR (376 MHz, Acetone-D₆) δ -71.32 (s).
Preparation of compound 5. To a stirring solution of NaOH (5.2 g, 130.0 mmol) in H$_2$O (80.0 mL) at rt was added pentaerythritol (36.8 g, 270.8 mmol). Afterward, the reaction mixture was stirred for an additional 10 min. Then 3-bromo-1-propene (13.1 g, 108.3 mmol) was added dropwise and the resulting mixture was stirred for 6 h at 70 °C. The resulting solution was directly evaporated under vacuum to dryness and purified by flash chromatography on silica gel (EA/PE = 1:1) to give 5 as clear liquid (8.7 g, 46% yield). $^1$H NMR (400 MHz, CDCl$_3$) δ 5.91-5.84 (m, 1H), 5.28-5.19 (m, 2H), 3.97 (d, $J$ = 5.6 Hz, 2H), 3.70 (s, 6H), 3.46 (s, 2H), 3.34 (s, 3H).

Preparation of compound 6. Compound 6 was prepared from 5 by following the same procedure for 1, giving 6 as clear oil (18.3 g, 65% yield). $^1$H NMR (400 MHz, Acetone-D$_6$) δ 5.99-5.81 (m, 1H), 5.31-5.16 (m, 2H), 4.22 (s, 6H), 4.00 (d, $J$ = 5.6 Hz, 2H), 3.49 (s, 2H). $^{19}$F NMR (376 MHz, Acetone-D$_6$) δ -71.30 (s). $^{13}$C NMR (100 MHz, Acetone-D$_6$) δ 135.09, 121.33 (q, $J$ = 290.9 Hz), 117.27, 81.35-79.84 (m), 72.98, 66.57, 65.61, 47.27.

Preparation of compound 7. To a stirring solution of 6 (5.0 g, 6.0 mmol) in a mixture of CH$_3$CN (50.0 mL), CCl$_4$ (50.0 mL) and water (75.0 mL) was added NaIO$_4$ (19.0 g, 88.8 mmol) and RuCl$_3$·3H$_2$O (31.5 mg, 0.12 mmol) sequentially and the resulting mixture was stirred for 2 h at rt. Afterward, the reaction mixture was allowed to warm to 35 °C and was stirred for an additional 4 h. The catalyst was filtered off, and the resulting solution was extracted with CH$_2$Cl$_2$, washed with saturated brine (3 × 30 mL). The organic layer was collected and concentrated under vacuum to give solid. Then, to a stirring solution of the above solid in DMF (30.0 mL) at rt was added K$_2$CO$_3$ (2.6 g, 18.0 mmol). Afterward, the reaction mixture was allowed to warm to 50 °C and was stirred for an additional 1 h. Then CH$_3$I (2.5 g, 18.0 mmol) was added and the resulting mixture was stirred at 50 °C overnight. The catalyst was filtered off, and the solution was concentrated under vacuum to give a residue which was dissolved with EtOAc (40 mL) and washed with saturated salt water (3 × 30 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and evaporated under vacuum to dryness. The residue was purified by flash chromatography on silica gel (CH$_2$Cl$_2$/PE = 1:10) to give 7 as clear oil (4.9 g, 96% yield). $^1$H NMR (400 MHz, CDCl$_3$) δ 4.11 (s, 6H), 4.05 (s, 2H), 3.74 (s, 3H), 3.58 (s, 2H). $^{19}$F NMR (376 MHz, CDCl$_3$) δ -73.56 (s). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.08, 120.32 (q, $J$ = 291.1 Hz), 80.46-78.96 (m), 68.65, 67.41, 65.95, 51.78, 46.33.
**Preparation of compound 2.** Under an argon atmosphere, to a stirring solution of 4-acetylanisole (360.4 mg, 2.4 mmol, in 10.0 mL THF) at rt was added 7 (1.4 g, 1.6 mmol, in 5.0 mL THF). Afterward, the reaction mixture was stirred for an additional 10 min. Then Potassium tert-butoxide (359.0 mg, 3.2 mmol) was added and the resulting mixture was stirred for 3 h at 55 °C. The reaction mixture was cooled to rt, quenched with water (10.0 mL), and concentrated under vacuum to give a residue which was dissolved with EtOAc (30 mL) and washed with brine (3 × 20 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and evaporated under vacuum to dryness. The residue was purified by flash chromatography on silica gel (EA/PE = 1:20) to give 2 as a white solid (705 mg, 45% yield). $^1$H NMR (400 MHz, Acetone-D$_6$) $\delta$ 7.94 (d, $J$ = 9.0 Hz, 2H), 7.06 (d, $J$ = 9.0 Hz, 2H), 6.52 (s, 1H), 4.32 (s, 6H), 4.23 (s, 2H), 3.91 (s, 3H), 3.73 (s, 2H). $^{19}$F NMR (376 MHz, Acetone-D$_6$) $\delta$ -71.25 (s). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 190.40, 184.40, 163.65, 129.3, 127.13, 120.3 (q, $J$ = 289.8 Hz), 114.16, 92.51, 80.14-79.24 (m), 72.97, 67.10, 65.74, 55.58, 46.46. HRMS (ESI) calcd for C$_{28}$H$_{19}$F$_{12}$O$_7$Na$^+$ ([M + Na]$^+$), 1003.0597; found, 1003.0616.

13. **$^{19}$F NMR of Eml-4 and Eml-6 at 280 K, 298 K, and 310 K**

Eml-4 (280K)
Eml-6 (280K)
**Eml-6 (298K)**

![Graph of Eml-6 at 298K](image)

**Eml-6 (310K)**

![Graph of Eml-6 at 310K](image)
14. Copies of $^1$H/$^{13}$C/$^{19}$F NMR and HRMS spectra of compounds

$^1$H NMR of compound 1
$^{19}$F NMR of compound 1

$^1$H NMR of compound 5
${}^{1}H$ NMR of compound 6

${}^{19}F$ NMR of compound 6
$^{13}$C NMR of compound 6

![13C NMR spectrum](image)

$^1$H NMR of compound 7

![1H NMR spectrum](image)
$^{19}$F NMR of compound 7

$^{13}$C NMR of compound 7
$^1$H NMR of compound 2

$^{19}$F NMR of compound 2
$^{13}$C NMR of compound 2

HRMS of compound 2
15. $^{19}\text{F} \text{ NMR of different emulsions}$

$^{19}\text{F} \text{ NMR of Eml-1}$

$^{19}\text{F} \text{ NMR of Eml-2}$
$^{19}$F NMR of Eml-3

$^{19}$F NMR of Eml-4
$^{19}$F NMR of **Eml-5**

$^{19}$F NMR of **Eml-6**