Electronic Supplementary Material (ESI) for Nanoscale.

Tumor-targeted superfluorinated micellar probe for sensitive in vivo ^{19}F-MRI

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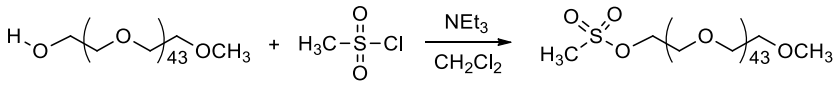
1) General

Unless otherwise specified, chemicals were purchased from Sigma–Aldrich and used without further purification. Reactions were carried out under nitrogen using dry solvents, unless otherwise stated. Flash chromatography was carried out on Kieselgel 60 (230–240 mesh, Merck) and analytical TLC was performed on Merck precoated silica gel (60 F254). NMR spectra were recorded on a Bruker AVANCE DPX 400 spectrometer. ^{1}H NMR spectra were recorded at 400 MHz, ^{13}C NMR at 100 MHz, and ^{19}F at 376 MHz. Mass spectra were recorded using a Waters Micromass ZQ 2000 ESI spectrometer. Dynamic Light Scattering size measurements were performed on a VascoFlex instrument from Cordouan Technologies equipped with a 450 nm laser diode. The Critical Micelle Concentration was measured using a K10 Krüss tensiometer. Static Light Scattering measurements were performed on a Zetasizer Nano S from Malvern.

2) Synthesis of PFTD-PEG amphiphile (1)

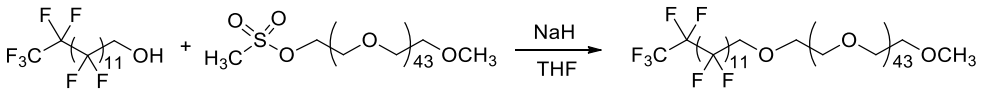
a. Activation of the PEG unit.



Under N_2, polyethylene glycol monomethyl ether (Mn 2000, 2 g, 1 mmol, 1 equiv.) in 20 mL of CH_2Cl_2 was treated sequentially with NEt_3 (0.69 mL, 5 equiv.) and methanesulfonyl chloride (0.4 mL, 5 equiv.). The solution was stirred for 48 h at room temperature and treated with 5% HCl (3 × 20 mL) and brine (30 mL). The organic phase was collected, dried over $MgSO_4$, filtered and evaporated under vacuum. Purification was carried out by precipitation of the product with Et_2O at −20 °C for 30 min. The mesylated PEG_{2000} monomethyl ether was obtained as a white solid (90% yield).

^{1}H-NMR (400 MHz, $CDCl_3$) δ2.41 (s, 3 H), 3.35 (s, 3 H), 3.44–3.70 (m, 10 H), 4.15 (t, *J* = 4.6 Hz, 2 H), 7.33 (d, *J* = 8.2 Hz, 2 H), 7.82 (d, *J* = 8.2 Hz, 2 H) ppm. ^{13}C-NMR (100 MHz, D_2O) δ69.5 (multiple C), 58.0, 36.5 ppm.

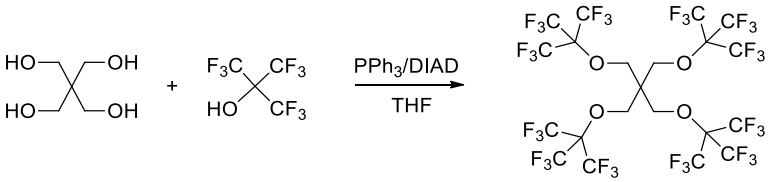
b. Coupling of the PEG unit to the fluorinated chain.



Under N_2, NaH (75 mg (60% in oil), 4 equiv.) in 8 mL of THF was slowly added to perfluorotetradecanol (1.33 g, 4 equiv.) in 10 mL of THF. The reaction mixture was stirred to reflux for 30 min before the above mesylated PEG_{2000} monomethyl ether (1 g, 0.48 mmol, 1 equiv.) was added. The reaction mixture was stirred for 2 days at 70 °C. THF was evaporated, the residual oil was solubilized in CH_2Cl_2 and filtered over Celite. The PFTD-PEG amphiphile **1** was obtained as a white solid (68% yield) by precipitation with Et_2O at −20 °C for 30 min.

^{1}H-NMR (400 MHz, D_2O) δ4.07 (t, 2H), 3.55 (s, 186H), 3.23 (s, 3H) ppm. ^{13}C-NMR (100 MHz, D_2O) δ69.5 (multiple C) ppm. ^{19}F-NMR (376 MHz, D_2O) δ −83.38, −123.16 ppm. MS-ESI^{+}, m/z 1327 $[M + H]^{2+}$.

3) Synthesis of PERFECTA (2)



This compound was synthesized according to the procedure reported by Tirotta et al. (*J. Am. Chem. Soc.* **2014**, *136*, 8524). At 0 °C and under N_2, to a stirred mixture of pentaerythritol (0.14 g, 1.03 mmol, 1 equiv.) and triphenylphosphine (1.6 g, 6 equiv.) in 10 mL of anhydrous THF, was added dropwise diisopropylazodicarboxylate (DIAD, 1 mL, 6 equiv.). After 30 min at room temperature, perfluoro-*tert*-butanol (0.86 mL, 6 equiv.) was added in one portion. The mixture was stirred at 45 °C for 72 h, until a fluorinated layer separated. The mixture was then cooled to 0 °C, filtered and washed with THF, acetone and water. The product was obtained as a white solid (45%). NMR characterization was performed by adding little amounts of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to the deuterated solvent in order to solubilize PERFECTA.

^{1}H-NMR (400 MHz, $CDCl_3$/HFIP) δ 3.59 (s, HFIP), 4.17 (s), 4.45-4.36 (m, HFIP) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$) δ 120.6 (q, J = 280 Hz), 119.1 (HFIP), 79.5 (m), 69.5 (HFIP), 65.5, 46.0 ppm. ^{19}F-NMR (376 MHz, $CDCl_3$/HFIP) δ −70.85 (s), −76.14 (s, HFIP) ppm.

4) Assembly, characterization and loading of the PFTD-PEG micelles

a. Assembly of the PFTD-PEG micelles

A solution of compound **1** (60 mg) in 0.9 % NaCl (3 mL) was sonicated with an ultrasonic probe (300 ms pulse per second, output 45%) for 10 min leading to a final micelle concentration of 20 mg mL^{-1}. The size of the PFTD-PEG micelles was measured by dynamic light scattering at 20°C, which indicated a mean hydrodynamic diameter of 13 nm.

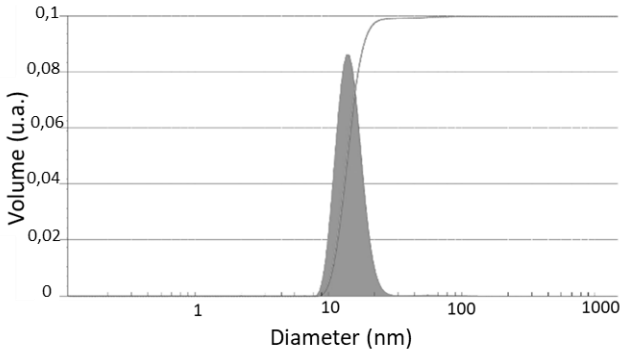


Figure S1. DLS analysis of the PFTD-PEG micelles

b. Determination of the Critical Micelle Concentration

The CMC was evaluated by measuring the surface tension of micelles solution. Solutions at different concentrations of amphiphilic units (from 1 mg mL^{-1} to 7.8 µg mL^{-1}) were prepared in water and the surface tension was measured (in triplicate), using the du Noüy ring method. The CMC value was obtained at the intersection of the two lines and was found to be 0.051 mg mL^{-1} for the empty micelle (Figure S2a) and 0.046 mg mL^{-1} for the PERFECTA-loaded micelles (Figure S2b).

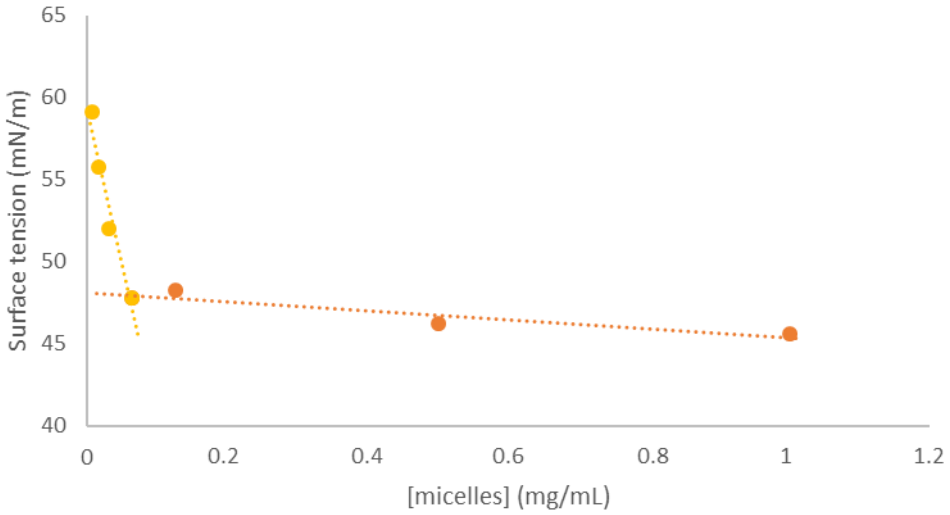


Figure S2a. Calculation of the CMC value for PFTD-PEG amphiphiles

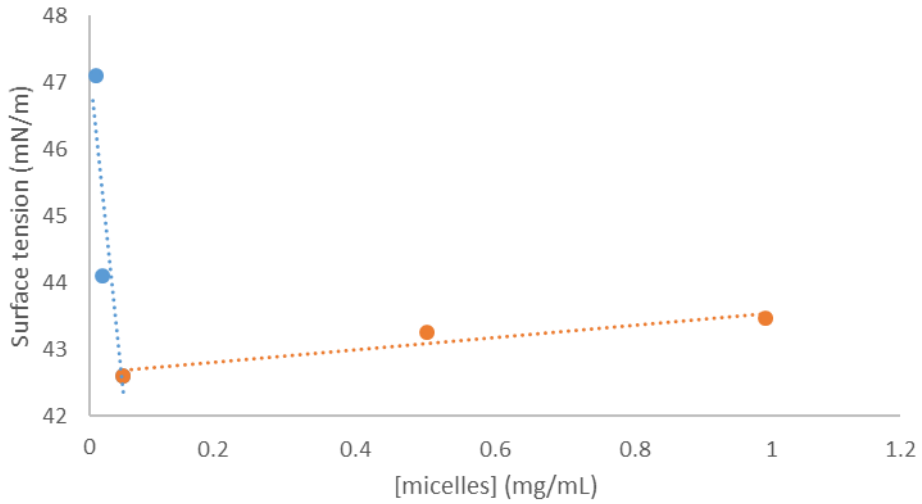


Figure S2b. Calculation of the CMC value for PFTD-PEG amphiphiles in the presence of PERFECTA

c. Loading of the PFTD-PEG micelles with PERFECTA

PERFECTA (20 mg) was added to a solution of PFTD-PEG micelles in 0.9% NaCl (1 mL, 20 mg mL^{-1}) and the mixture was sonicated with an ultrasonic probe (300 ms pulse per second, output 45%) for 20 min. The suspension was then filtered on a 0.22 µm membrane to remove insoluble aggregates. Encapsulation efficiency (amount of PERFECTA loaded in micelles/total amount of PERFECTA used × 100) is (14 mg/20 mg) × 100 = 70%.

d. Measurement of PERFECTA concentration by ^{19}F-NMR spectroscopy

The measurement of PERFECTA concentration in micelles was performed on different samples of PERFECTA@PFTD-PEG micelles that were prepared by varying the loading of PERFECTA from 2.5 to 20 mg in a 20 mg mL^{-1} PFTD-PEG micelles solution. Potassium fluoride (KF) was used as internal standard. For each experiment, 400 µL of a 0.082 M KF solution in D_2O (corresponding to 32.74 µmol) was introduced in a NMR tube containing 100 µL of PERFECTA@PFTD-PEG micelles. The amount of PERFECTA in the sample was measured by comparative integration of the peak at −76.17 ppm (corresponding to PERFECTA) and that of the KF internal standard set at −125.3 ppm.

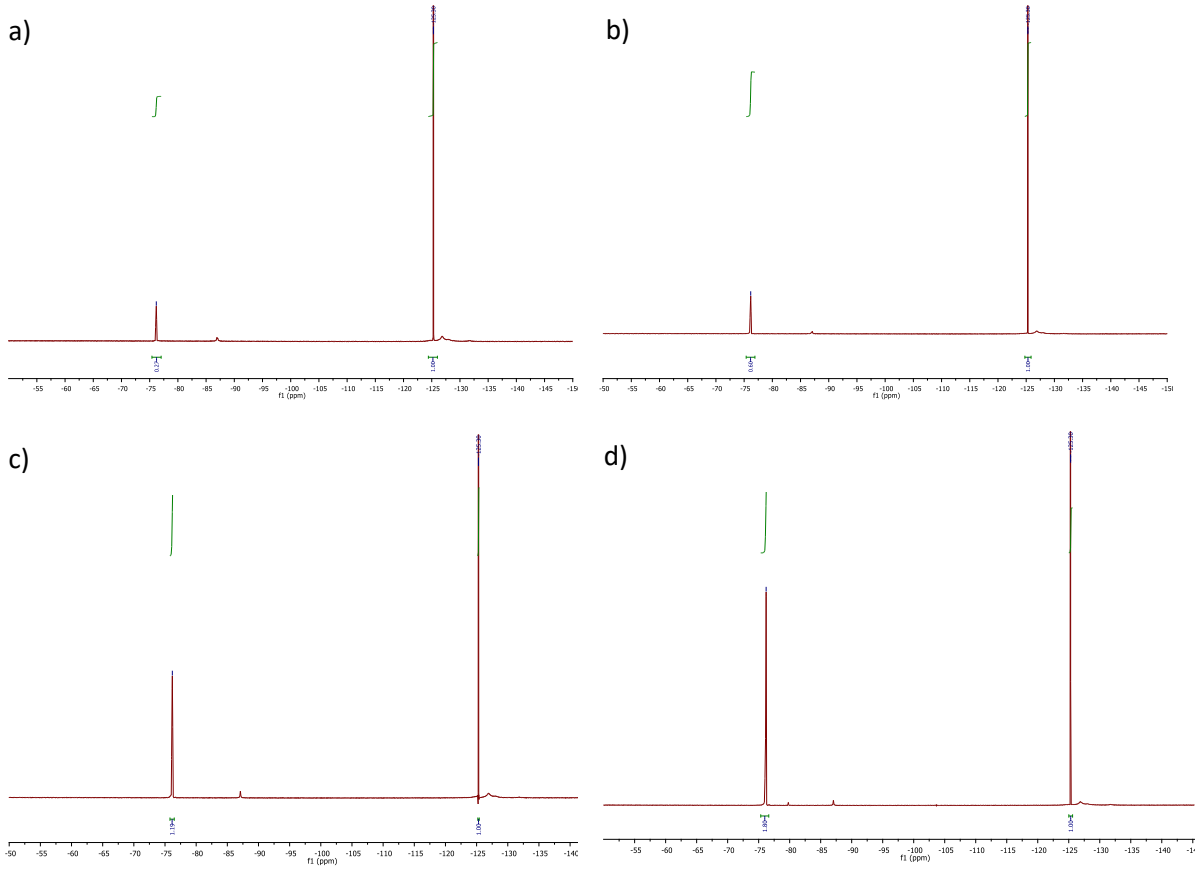


Figure S3. ^{19}F-NMR of PERFECTA@PFTD-PEG micelles in D_2O using KF as internal standard. PFTD-PEG micelles (20 mg mL^{-1}) were sonicated in the presence of: a) 2.5 mg, b) 5 mg, c) 10 mg and d) 20 mg of PERFECTA.

e. Determination of the number of amphiphilic units per micelle

The number of amphiphilic units per micelle was evaluated by static light scattering (SLS) experiments. Solutions of PFTD-PEG micelles were prepared at different concentrations ranging from 1 mg mL^{-1} to 10 mg mL^{-1} and analyzed. A molecular mass of 80.3 ± 5.77 kDa corresponding to the PFTD-PEG micelle was extrapolated, which corresponds to approximately 30 amphiphilic units per micelle.

f. Stability assessment of the loaded micelles over time

Stability of the PERFECTA@PFTD-PEG micelles solution was evaluated by measuring the hydrodynamic size distribution of the samples (20 mg mL^{-1} in 0.9% NaCl at 25 °C) over time. We did not observe any aggregation of the sample over 1 month.

g. Stability assessment of the loaded micelles in serum and at acidic/basic pH

Stability of PERFECTA@PFTD-PEG micelles in serum and at different pH was assessed by ^{19}F-NMR. In brief, PERFECTA@PFTD-PEG micelles (20 mg/mL of micelles containing 14 mg of PERFECTA) were incubated in either 10% FBS/D_2O, pH 5 or pH 9 D_2O solutions. KF was introduced as internal standard (see above section *d.*), and ^{19}F-NMR were recorded at different time intervals. Intensity of the ^{19}F-NMR signal of PERFECTA was used as a probe to look at the colloidal integrity of the system. The PERFECTA signal remained constant for more than 96 h, which suggests that neither leaching of the payload nor precipitation of the micelle had taken place and that the colloidal system remained unaffected under the conditions studied.

h. Cryo-electron microscopy of PFTD-PEG micelles and PERFECTA@PFTD-PEG micelles

PFTD-PEG and PERFECTA@PFTD-PEG micelles samples were vitrified using a Thermofisher Vitrobot Mark IV. Around 4 µL of the sample ([PFTD-PEG] = 20 mg/mL, [PERFECTA@PFTD-PEG] = 20 mg/mL of micelle containing 14 mg of PERFECTA) was deposited onto an electron microscopy grid with a lacey support film, with or without additional graphene oxide films in the Vitrobot chamber at room temperature with 100% relative humidity. Imaging was performed on a Thermofisher Tecnai F20 microscope at 200 keV in a low-dose setting and images were recorded with a Ceta camera.

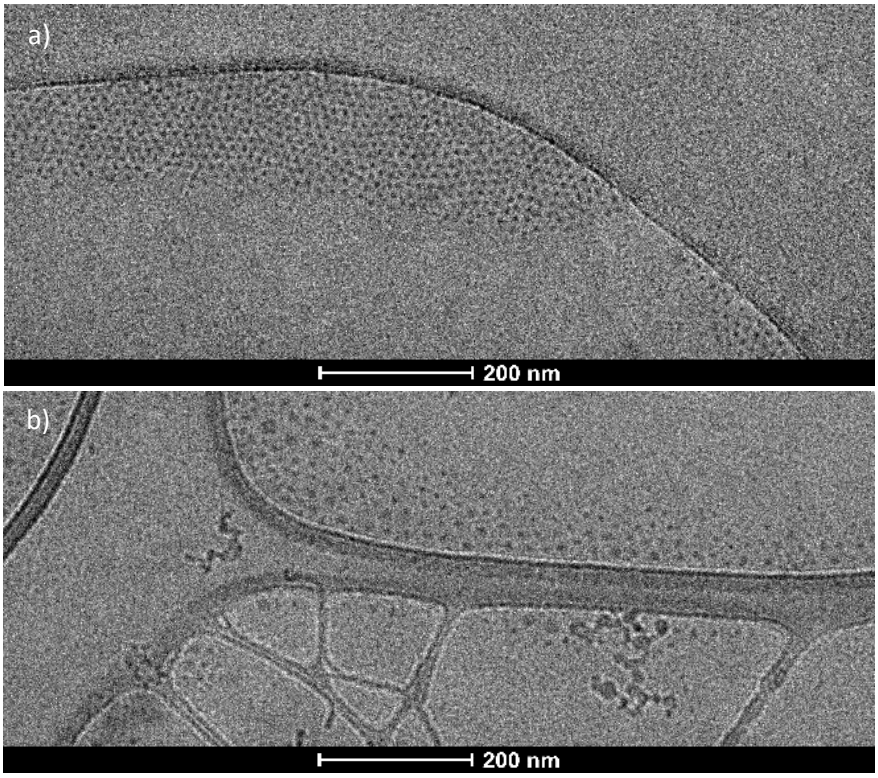


Figure S4. Cryo-transmission electron microscopy images of a) empty PFTD-PEG micelles and b) PFTD-PEG micelles loaded with PERFECTA (PERFECTA@PFTD-PEG).

5) Cell proliferation/survival assay on HEK-293 and MC38 cells

Immortalized human embryonic kidney HEK-293 cells (ATCC) were routinely maintained in DMEM supplemented with 10% (v/v) Fetal Bovine Serum (PAA), Penicllin (100 U mL^{-1}) and Streptomycin (100 µg mL^{-1}). Murine colon carcinoma MC38 cells were routinely maintained in DMEM supplemented with 10% (v/v) Fetal Bovine Serum (PAA), glutamin (2 mM), non essential amino acids (0.1mM), sodium pyruvate (1 mM), Hepes (10 mM), gentamycin sulfate (50 µg mL^{-1}), Penicllin (100 U mL^{-1}) and Streptomycin (100 µg mL^{-1}). Cell proliferation/survival assay of cells exposed to PFTD-PEG micelles was conducted in the same way as previously described (see M.-D. Hoang et al., *Nanoscale Adv.* **2019**, *1*, 4331–4338). In short, HEK-293 or MC38 cells suspended in culture medium were seeded into the wells of an optical 96-well culture plate (Costar #3904) at a final density of 1000 (MC38) and 1500 cells/well (HEK-293). Plates were then incubated for 6 h at 37 °C, 5% CO_2 to allow cell adhesion. Micelles (or PBS as vehicle) were then diluted in PBS and immediately layered on top of the culture wells to achieve the indicated final concentrations, and plates were incubated for 72 h at 37 °C, 5% CO_2. Cells were then fixed by addition of paraformaldehyde (4% [w/v] final) and nuclear DNA was fluorescently labeled by Hoechst 33342 (2 µg mL^{-1} final). After an overnight incubation at 4 °C, supernatants were removed by aspiration, replaced by 100 µL PBS, and plates were imaged on a high-content imaging device (Operetta, Perkin Elmer) in the blue channel (Ex. 360–400 nm, Em. 410–480 nm, 10 × magnification, 9 fields acquired/well). Using a high-content imaging analysis software (Harmony 3.0, Perkin-Elmer), DNA-labeled nuclei were segmented, and the absolute amount of nuclei per condition was quantitated. Results are expressed as the relative amount of cells in micelle-treated wells relative to the average amount of cells in the controls treated with vehicle (PBS).

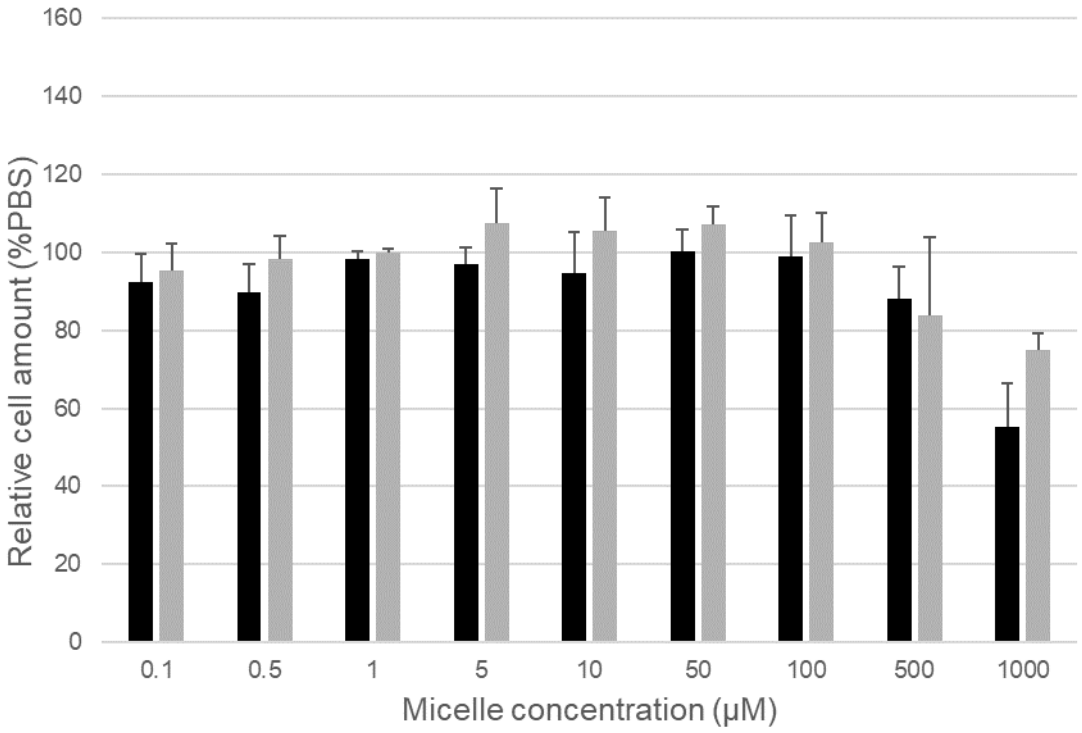


Figure S5a. Proliferation/survival of HEK293 cells treated with micelles for 72 h. Grey: empty PFTD-PEG micelles, black: PERFECTA-loaded PFTD-PEG micelles.

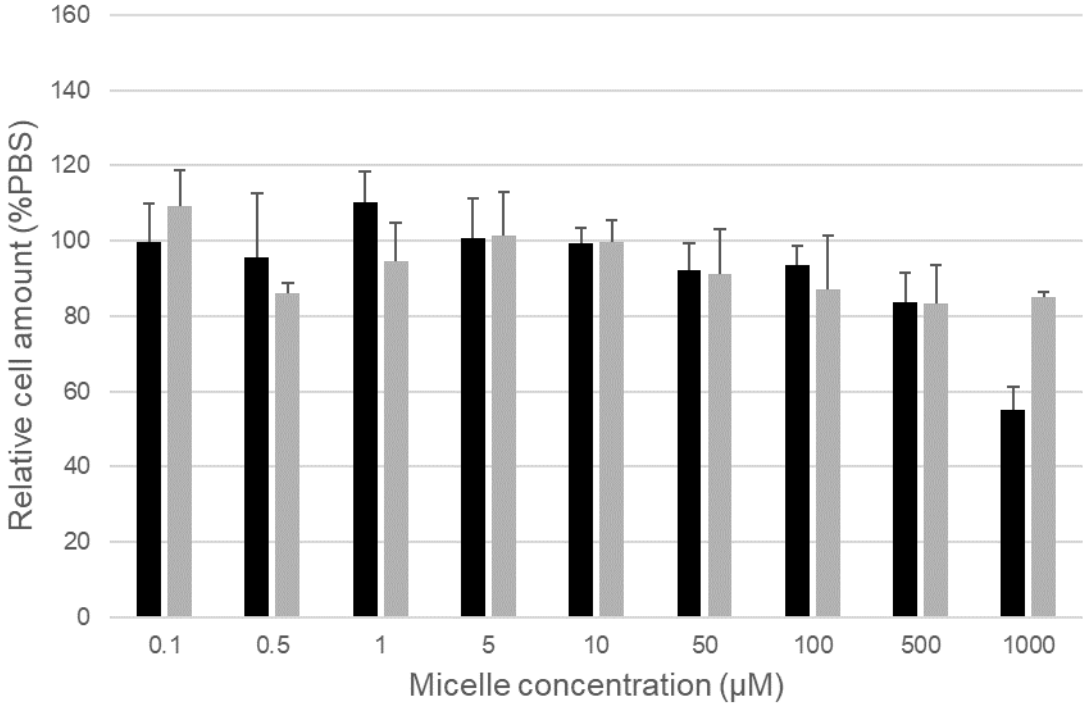


Figure S5b. Proliferation/survival of MC38 cells treated with micelles for 72 h. Grey: empty PFTD-PEG micelles, black: PERFECTA-loaded PFTD-PEG micelles.

6) Cell internalization studies

Fluorescently labelled micelles were prepared as follows: 20 µL of a 1 mg mL^{-1} solution of DiD (1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine) in EtOH were added to PFTD-PEG micelles (20 mg mL^{-1}, 1 mL). The mixture was tip sonicated for 3 × 10 min. The clear colloid was filtered through a 0.22 µm membrane and stored in the dark.

MC38 cells suspended in culture medium were seeded into the wells of an optical 96-well culture plate (Costar #3904) at a final density of 4000 cells/well. Plates were then incubated for 24 h at 37 °C, 5% CO_2 to allow cell adhesion. PFTD-PEG micelles alone or encapsulating DiD were then diluted in PBS and immediately layered on top of the culture wells to achieve the indicated final micelle concentrations, and plates were incubated for 18 h at 37 °C, 5% CO_2. Cells were then fixed and DNA stained with Hoechst 33342 as described above. Plates were imaged on a high-content imaging microscope (Operetta, Perkin Elmer) in the blue (Hoechst) channel (λ_{Ex} 360–400 nm, λ_{Em} 410–480 nm) and in the red (DiD) channel (λ_{Ex} 620–640 nm, λ_{Em} 650–760 nm) at 20 × magnification.

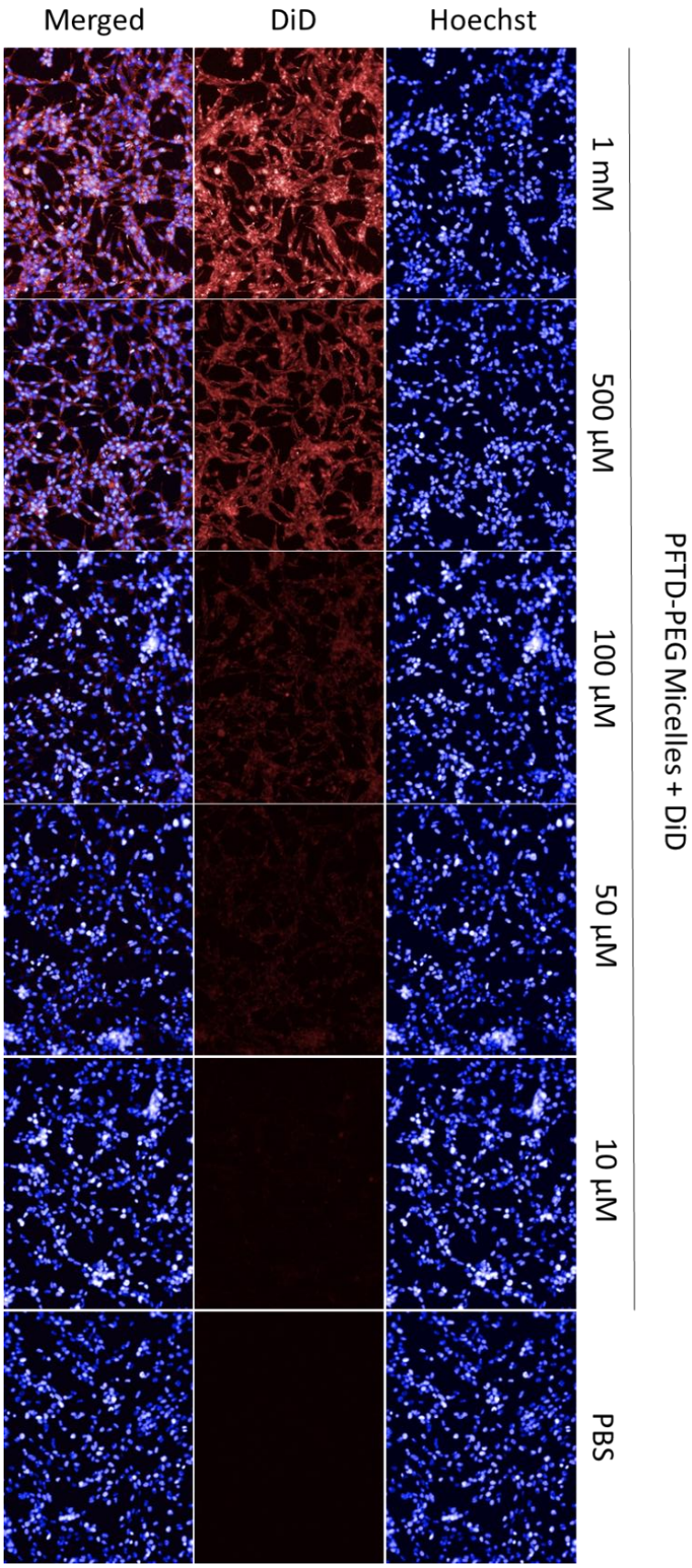


Figure S6. MC38 cellular uptake of PFTD-PEG micelles fluorescently labelled with DiD.

7) ^{19}F MRI experiments

^{19}F-MRI was performed *in vitro* with a 7 T Biospec preclinical scanner (Bruker) using fluorinated micelles at different concentrations of PERFECTA (0.660, 0.155, 0.074, 0.046, 0.044, 0.037, 0.026, 0.022 M as determined by ^{19}F-NMR). This experiment allowed the evaluation of the linearity between the concentration in fluorine and the signal obtained by ^{19}F-MRI, as well as to determine detection thresholds in terms of fluorine concentration. For MRI acquisition, two sequences were used. The first one was a Multi-Slice Multi-Echo sequence (MSME) with the following parameters: TR = 2500 ms; TE = 8 ms; 40 echoes spaced by 8 ms; voxel size = 0.3 mm × 0.3 mm × 12 mm; matrix = 88 × 88 × 1; acquisition time = 73 min 20 s; 20 averages. The second one was a Rapid Acquisition with Refocused Echoes sequence (RARE) with the following parameters: TR = 2500 ms; effective TE = 15 ms; RARE factor = 8; voxel size = 0.3 mm × 0.3 mm × 12 mm; matrix = 88 × 88 × 1. This sequence was run with 2 averages (RARE2, acquisition time = 55 s), 10 averages (RARE10, acquisition time = 4 min 35 s) and 20 averages (RARE20, acquisition time = 9 min 10 s).

	SNR Echo 1 MSME	*[^{19}F]*
A	70	0.660
B	16.43	0.155
C	7.87	0.074
D	4.93	0.046
E	4.7	0.044
F	3.9	0.037
G	2.74	0.026
H	2.32	0.022

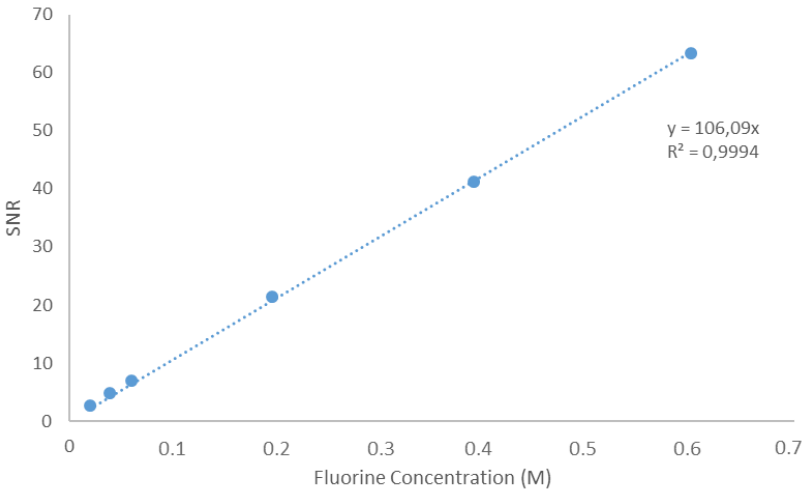


Figure S7. ^{19}F-MRI performed on phantoms containing PERFECTA@PFTD-PEG micelles in 0.9% aqueous NaCl at different concentrations and assessment of SNR linearity.

To determine the T_1, T_2 and T_2^* relaxation times of PERFECTA-loaded micelles, several parametric sequences were acquired on a tube filled with fluorinated micelles at 0.5 M.

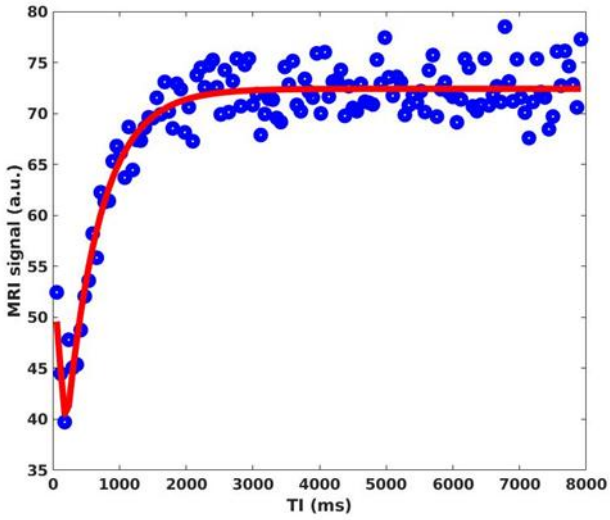
For T_1 estimation, an Inversion Recovery Fast Gradient Echo sequence (IR-FGE) with the following parameters was used: TR_1 = 5 ms; TR_2 = 10 s; TE = 2.5 ms; 6 segments; flip angle = 5°; 132 inversion times spaced by 60 ms from 60 ms to 7920 ms; voxel size = 0.4 mm × 0.4 mm × 8 mm; matrix = 72 × 72 × 4; acquisition time = 6 h; 90 averages. After images reconstruction, the T_1 relaxation time was computed by fitting the signal intensity versus echo time using Bloch equations.

For T_2 estimation, a MSME sequence with the following parameters was used: TR = 2500 ms; TE = 8 ms; 40 echoes spaced by 8 ms from 8 ms to 320 ms; voxel size = 0.4 mm × 0.4 mm × 8 mm; matrix = 72 × 72 × 4; acquisition time = 4 h 30 min; 90 averages. After images reconstruction, the T_2 relaxation time was computed by fitting the signal intensity versus echo time using Bloch equations.

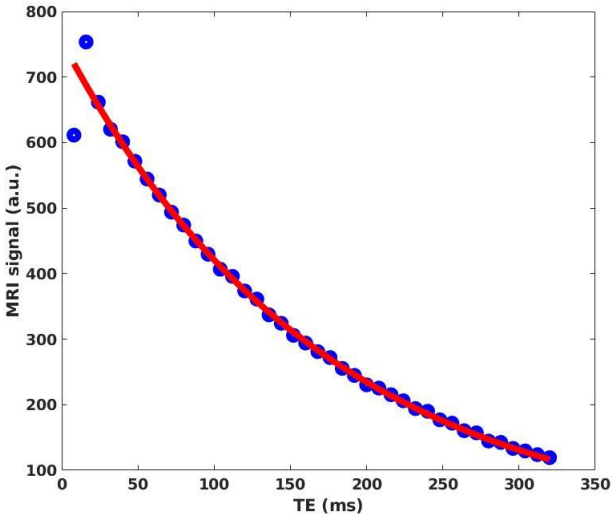
For T_2^* estimation, a Multi Gradient Echo sequence (MGE) with the following parameters was used: TR = 800 ms; TE = 2.5 ms; 30 echoes spaced by 2.5 ms from 2.5 ms to 75 ms; voxel size = 0.4 mm × 0.4 mm × 8 mm; matrix = 72 × 72 × 4; acquisition time = 21 min 36 s; 30 averages. After images reconstruction,

the T_2^* relaxation time was computed by fitting the signal intensity versus echo time using Bloch equations.

Fit T_1 → T_1 = 248.22 ms



Fit T_2 → T_2 = 171.12 ms



Fit T_2^* → T_2^* = 5.99 ms

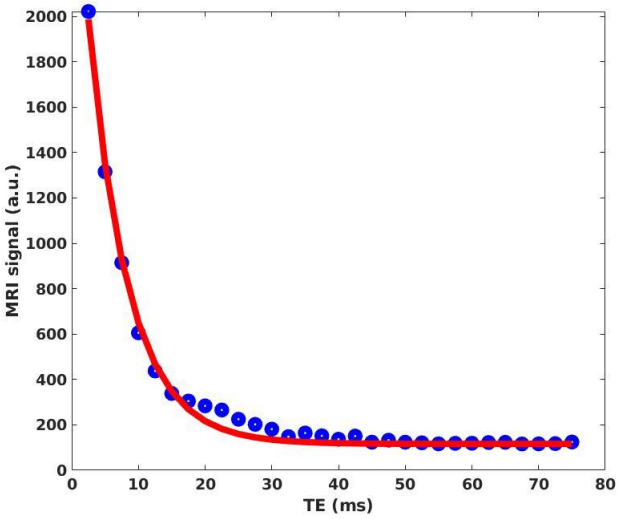


Figure S8. Measurement of T_1, T_2 and T_2* relaxation times of PERFECTA-loaded micelles ([$^{19}F_{PERF}$] = 0.5 M)

8) *In vivo* experiments

a. Animal model

MC38 cell line was grown in Dulbecco's modified MEM with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, 50 µg mL^{-1} gentamycin sulfate, pen/strep and sub-cultured twice a week at 1:10 during 3 weeks after defrost. Two-month old female mice (C57Bl/6, 20 g, purchased from Janvier Labs) were subcutaneously implanted with 10^6 MC38 cells in 100 µL of DPBS.

b. In vivo *MRI investigation of PERFECTA-loaded micelles biodistribution*

Ten days after MC38 cells implantation (mean tumor volume = 138 ± 26 mm^3, N = 9), a solution of PERFECTA@PFTD-PEG micelles was injected intravenously (150 µL, [$^{19}F_{PERF}$] = 0.5 M) in the tail of the anesthetized animals (C57Bl/6-MC38 mice, N = 9) every day during 4 days. $^1H/^{19}F$-MRI images of the mice abdomen (from the heart to the bladder) were acquired immediately (0+) and 6 hours after injection (6+), then 1 day (N = 6) and 3 days (N = 3) after the last injection.

MRI acquisitions were performed with mice under isoflurane anesthesia (1–2%) with an air/O_2 mixture (50:50). Physiological parameters of the animal (respiratory frequency and temperature) were monitored during the whole imaging session. Two tubes containing known concentrations of PERFECTA@PFTD-PEG micelles ([$^{19}F_{PERF}$] = 0.044 and 0.66 M) were placed next to mice abdomen and used as reference for estimating fluorine concentration.

Then 150 µL of PERFECTA@PFTD-PEG micelles were injected in the tail vein and the animal was placed in a dedicated cradle inside a 7 T Biospec preclinical scanner (Bruker). ^1H high resolution MR images were first acquired for future segmentation of organs of interest using a flow-compensated Fast Low Angle Shot sequence (FcFLASH) with the following parameters: TR = 475 ms; TE = 3 ms; voxel size = 0.25 mm × 0.25 mm × 1 mm; matrix = 160 × 160 × 30; 6 averages; acquisition time = 7 min 36 s. Then ^{19}F MR images were acquired to detect PERFECTA-loaded micelles using a RARE sequence with the following parameters: TR = 2500 ms; effective TE = 15 ms; RARE factor = 8; voxel size = 1 mm × 1 mm × 5 mm; matrix = 40 × 40 × 6; 20 averages; acquisition time = 8 min 20 s. The total acquisition time for each mouse was around 30 min including calibration steps and respiratory triggering.

c. Extraction of PERFECTA from selected organs

Mice were sacrificed and exsanguinated after the MRI experiments and the organs of interest were collected (tumor, heart, liver, muscle) and kept at −80 °C. Frozen organs were grinded using a ball milling system in the presence of an extraction solvent (HFIP) which was added to reach a final concentration of 0.2 g of organs in 1 mL of solvent. The homogenate was centrifuged (20 000 ×*g*, 2 × 10 min, 5 °C), the supernatant was collected, filtered on Celite, and the solvent evaporated. $CDCl_3$ was added to the residue and ^{19}F-NMR was recorded. PERFECTA NMR signal was detected in liver and tumor tissues, but not in muscle tissues.

d. Post-processing of MR images

Images were reconstructed from raw data using home-made Matlab codes (The Mathworks). Regions of interest (ROIs), corresponding to the investigated organs (tumor, liver, heart) and the reference tube, were manually segmented on each slice of the ^1H images. ^{19}F images were oversampled to reach the spatial resolution and field-of-view of ^1H images. Then, ROIs were applied on oversampled ^{19}F images to quantify the fluorine signal. ^{19}F concentrations were calculated thanks to the signal measured in the reference tube with the highest concentration of fluorine (0.66 M).

9) *Ex vivo* experiments

Ex vivo quantification of PERFECTA@PFTD-PEG micelles was achieved by performing MRI acquisitions on resected liver, using a reference tube of known fluorine concentration ([^{19}F] = 660 mM). The resected liver was placed in a dedicated tube inside a 7 T Biospec preclinical scanner (Bruker). One ^{1}H high resolution MR image was first acquired for future segmentation of liver using a MSME sequence with the following parameters: TR = 4500 ms; TE = 5 ms; 24 echoes spaced by 5 ms; voxel size = 0.3 mm × 0.3 mm × 0.9 mm; matrix = 138 × 105 × 24; 1 average; acquisition time = 7 min 52 s. Then one ^{19}F MR image was acquired to detect PERFECTA-loaded micelles using a MSME sequence with the following parameters: TR = 4000 ms; TE = 4 ms; 48 echoes spaced by 4 ms; voxel size = 0.9 mm × 0.9 mm × 1.8 mm; matrix = 46 × 35 × 12; acquisition time = 59 h 44 min; 64 averages. *Ex vivo* measurements indicated [$^{19}F_{PERF}$] = 89 mM, which is in good agreement with *in vivo* data ([$^{19}F_{PERF}$] = 92 mM at 144 h, just before sacrifice).

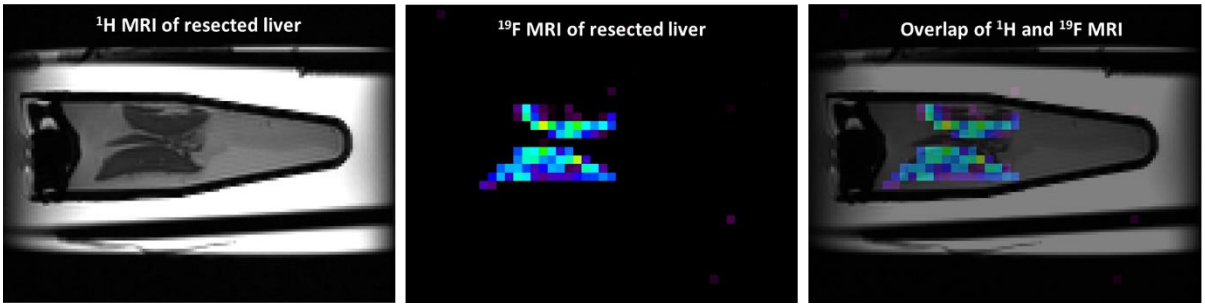


Figure S9. *Ex vivo* ^{1}H- and ^{19}F-MRI of PERFECTA@PFTD-PEG micelles in the liver

10) Table S1: Comparison of selected fluorinated probe formulations monitored by ^{19}F-MRI at 7 T

Fluorinated probe	Formulation	[^{19}F] (M)	NPs size (nm)	*In vivo* spatial resolution	*In vivo* acquisition time	Reference
PERFECTA	micelle	0.50	20	1 × 1 × 5 mm^3	8 min 20 sec	Current study
PFOB	nanocapsule	1.40	120	0.63 × 0.94 × 16 mm^3	4 min 30 sec	*Biomaterials* **2012**, *33*, 5593
PERFECTA	nanoemulsion	0.18	180	1.56 × 1.56 × 3 mm^3	10 min	*J. Am. Chem. Soc*. **2014**, *136*, 8524
PFCE	nanoemulsion	1.23	180	0.78 × 0.94 × 1 mm^3	36 min	*Radiology* **2019**, *291*, 351