Supporting Information

Inducing defects in ^{19}F-nanocrystals provides paramagnetic-free relaxation enhancement for improved *in-vivo* hotspot MRI

Reut Mashiach[1], Dana Cohen[1], Liat Avram[2], Talia Harris[2], Iddo Pinkas[2], Lothar Houben[2], Hyla Allouche-Arnon[1], Amnon Bar-Shir[1]*

[1]Department of Organic Chemistry, [2]Department of Chemical Research Support, Weizmann Institute of Science, Rehovot, 7610001, Israel

*Corresponding Author: amnon.barshir@weizmann.ac.il

Materials and Methods

Solvothermal Synthesis

Solvothermal synthesis of nanocrystals was performed using a compact micro-reactor (Parr Instruments Company, model 5500) with an internal thermocouple in addition to the internal stirrer. The reaction takes place inside a 100 ml Teflon liner. In a typical synthesis, 4.2 ml of oleic acid or 4.8 ml of oleyl phosphate, 12 ml of ethanol, and 0.1 gr of sodium hydroxide were mixed together until a homogeneous milky solution was formed. Then, 5 ml of an aqueous solution with 2 mmol $Ca(NO_3)_2$ of the appropriate stoichiometric amount of NaF was added to the mixture under vigorous stirring in a 50 ml flask. After 1 h, the mixture was transferred to the Teflon liner in the reactor. The reactor was sealed and heated to 160°C or 120°C (OA and OP, respectively) for 16 h. Then, the system was cooled to room temperature and the solution was centrifuged to obtain the powder products. This powder was washed in ethanol/cyclohexane two times to remove impurities. The obtained NCs were capped with a long-alkyl chain outside and were dispersed in cyclohexane organic solvent or vacuumed dried for further analysis.

Ligand Incorporation

For water dispersed PL-OP-CaF_2 and PL-OA-CaF_2 NCs phospholipids were incorporated with a hydrophilic head facing to the aqueous solution. 30mg of vacuumed dried NCs were dissolved in 2 ml chloroform, to this solution we added a solution of 30 mg of Myristoyl hydroxy phosphatidylcholine (MHPC), 2 mg cholesterol, 5 mg of 18:0 PEG1000_PE and 0.15 mg of 18:1 Lactosyl PE (for sugar modified NCs) dissolved in 5 ml of a 20:1 chloroform: methanol solvent mixture. The resulting lipid-nanoparticle solution was added dropwise to 30 ml deionized water at 80°C, under vigorous stirring. After the mixture had cooled down naturally to room temperature, it was centrifuged to remove large aggregates or unsuspended nanoparticles. The supernatant was transferred to a centrifugal filter unit (MWCO 10 kDa), in order to concentrate the sample, and centrifuged at 5000 g for several 15 min cycles, until the total volume reached 500 µl. Then, the sample went through a few dialysis cycles, either in ultra-purified water, to remove excess unbound phospholipids.

Powder XRD

Vacuum-dried powders of OP-CaF_2 and OA-CaF_2 were studied with XRD. Diffraction measurements were carried out with reflection geometry using an Ultima III (Rigaku, Japan) diffractometer equipped with a sealed Cu anode X-ray tube operating at 40 kV and 40 mA. A bent graphite monochromator and a scintillation detector were aligned in the diffracted beam. $\theta/2\theta$ scans were performed under specular conditions in the Bragg-Brentano mode with variable slits. The 2θ scanning range was 10–120 degrees, with a step size of 0.025 degrees and a scan speed of 0.5 degrees per minute.

Raman Spectroscopy

Vacuum-dried samples of OP-CaF_2 and OA-CaF_2 were analyzed by Raman spectroscopy (785 nm excitation) and collected by a Horiba LabRAM HR Evolution (Horiba, France) spectrometer equipped with four laser lines (325 nm, 532 nm, 633 nm, and 785 nm). The system has an 800 mm focal length spectrograph with interchangeable gratings and is mounted with an open electrode, front illuminated, cooled CCD detector. The sample is placed under a modular microscope (Olympus BX-FM) with a suitable objective. A 600 gr/mm grating was used with a pixel spacing of < 1 wavenumber. For this work, a x50 objective was used (Olympus Japan).

TEM and HRTEM

TEM images were acquired with a JEOL JEM 2100 high-resolution electron microscope (Gatan Digital Micrograph) with a bottom-mounted CCD camera (Gatan Ultra scanXP 2k x 2k). The accelerating voltage was 200 kV, and the beam source was LaB6 thermal emission. The powder was dispersed in a cyclohexane solution and deposited on a carbon film. Atomic-resolution images were taken on the spherical and chromatic double aberration-corrected FEI 60-300 Ultimate3 ('PICO') at the Ernst Ruska-Centre for Microscopy and Spectroscopy with Electrons (ER-C). Images were acquired on a Gatan OneView IS CMOS camera at 80 kV acceleration voltage with a spherical aberration value of -3 μm and a chromatic aberration value smaller than 1 μm. Optimized phase contrast for TEM images was achieved under NCSI conditions, with a point resolution better than 0.8 Å. Dry samples were prepared by drop-cast deposition on an ultra-thin carbon support foil acquired with a Themis-Z transmission electron microscope (Thermo Fisher Scientific Microscopy Solutions, Hillsboro, USA) equipped with a Super-X SSD detector at an

acceleration voltage of 200 kV. Dry samples were prepared by drop-cast deposition on an ultra-thin carbon support foil.

Dynamic Light Scattering

The diameter and size distribution of the obtained nanoparticles were evaluated by dynamic light scattering (Malvern Nano-ZS). For cyclohexane-dispersed NCs, measurements were taken in a 12.5 mm diameter cylindrical quartz cuvette according to the appropriate solvent viscosity. For water-dispersed NCs, a disposable cuvette was used.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) experiments were performed on a 9.4 T NMR spectrometer (Bruker, Germany), at 298 K. Prior to the ^{19}F-NMR experiments (376 MHz), ^{1}H-NMR spectra (400 MHz) were acquired for all samples. All ^{19}F spectra were acquired with 64 scans. The longitudinal (T_1) and transverse (T_2) relaxation times were calculated using inversion recovery (IR) and Carr-Purcell-Meiboom-Gill (CPMG) experiments, respectively.

Magnetic Resonance Imaging

Both the *in-vitro* and the *in-vivo* studies were performed on a Bruker BioSpec 15.2 Tesla AVANCE III HD imaging spectrometer equipped with a three-channel gradient amplifier. A dual ^{1}H/^{19}F, 25 mm RF coil was used to acquire both the ^{1}H and ^{19}F-MR images. ^{1}H and ^{19}F images were acquired with either a RARE or a 3D UTE sequence using different parameters.

***In-Vitro* MRI**

Two 5 mm glass tubes containing 150 mM PL-OP-CaF_2 and PL-OA-CaF_2 in aqueous solution were placed in a 25 mm plastic tube containing a 4% gelatin (w/w) solution in water. For ^{1}H-MRI, a rare sequence was used with TR/TE=1000/7.6 ms, 1 slice of 1 mm in thickness, FOV=3.2×3.2 cm^2, matrix size=256×256, NA=1 for a total scan time of 32 sec. For ^{19}F- MRI data, a 3D-UTE sequence was used with the following parameters: TR/TE=4.2/0.008 ms, flip angle of 5°, FOV=3.2×3.2×6.4 cm^3, matrix size=32×32×32, NA=30, scan time of 6.6 min (Fig.4a and Fig.4b). Five 5 mm glass tubes containing varied concentrations of PL-OP-CaF_2 in aqueous solution were placed in a 25 mm plastic tube containing a 4% gelatin (w/w) solution in water. For ^{1}H-MRI, a rare sequence was used

with TR/TE=2000/5 ms, 1 slice of 1 mm in thickness, FOV=3.2×3.2 cm^2, matrix size=128×128, NA=2, scan time of 1 min. For ^{19}F-MRI data, a 3D-UTE sequence was used with the following parameters: TR/TE=4.5/0.008 ms, flip angle of 5°, FOV=3.2×3.2×12.8 cm^3, matrix size=32×32×32, NA=300, scan time 71 min. To improve the SNR, consecutive slices were summed, resulting in a final matrix size of 32x32x16 with the same FOV (Fig. 4d,e).

Comparison of PL-OP-CaF_2 and PFC (VS-1000, Celsense Inc.). Two 5 mm glass tubes containing 20mM ^{19}F-content of PL-OP-CaF_2 and PFCs' nanoemulsions (VS-1000, celsense Inc., 19F-content concentration was calculated for the major peak of the material's ^{19}F-NMR spectrum) in aqueous solution were placed in a 25 mm plastic tube containing a 4% gelatin (w/w) solution in water. For ^{1}H-MRI a rare sequence was used with TR/TE= 220/4 ms, 1 slice of 4mm thickness, FOV= 4.8×4.8cm^2, matrix size= 128×128, NA=1for a total scan time of and scan time 28sec. For the detection of PL-OP-CaF_2 ^{19}F- MRI data a 3D-UTE sequence was used with the following parameters: TR/TE=4/0.12 ms, flip angle of 5°, FOV= 4.8×4.8×12.8cm^3, matrix size= 32×32×32 NA=95 and scan time 20min. For the detection of PFCs nanoemulsions ^{19}F- MRI data a RARE sequence was used with the following parameters: TR/TE=2000/1835 ms, FOV= 4.8×4.8, matrix size= 32×32×32 NA=150 and scan time 20min. O1 was set to the studied material frequency (-109ppm for NCs and -91ppm for PFC).

Cell Toxicity Studies

CCK8 Assay: HeLa cells (12 × 10^3 cells/ml) were cultured in a 96-well microplate with Dulbecco Modification of Eagle medium (DMEM, High Glucose) for 24 h at 37 °C and 5% CO_2. Then, cells were treated with water-soluble PL-OP-CaF_2 NCs over a range of concentrations (0 (living cells), 3.9, 7.8, 15.6, 31, 62, 124.5, or 498 µg/ml). After 1.5 hrs of incubation, the incubating medium was replaced with 100 µl fresh medium and followed by the addition of WST-8 solution (10 µl) to each well for additional 3 hrs of incubation at 37°C. The absorbance at 460 nm was measured using a plate reader. Cell viability was calculated as a percentage of the treated cells from that of the control (non-treated living cells). For each concentration of NCs, the average absorbance value was calculated from 8 biological replicates. Cells treatment with 50% DMSO were used as positive control.

LDH Assay*: HeLa cells (12 × 10^3 cells/ml) were cultured in a 96-well microplate with Dulbecco Modification of Eagle medium (DMEM, High Glucose) for 24 hrs at 37 °C and 5% CO_2. Then cells were treated with water-soluble PL-OP-CaF_2 NCs over a range of concentrations (7.8, 15.6, 31, 62, 124.5, or 498 µg/ml) for 1.5 h followed by the transfer of 5 µl of supernatant of each well into a new 96 well plate containing 95 ul of reaction mixture (LDH Substrate Mix (2ul), Picoprobe (4ul) and LDH Assay Buffer (89ul)). The plate was then wrapped with foil and mixed by gently shaking at room temperature for 10 min. The fluorescence of each well was measured using $\lambda_{ex}/\lambda_{em}$ of 535nm/587nm using a dedicated plate reader. Cell viability was calculated according to: $Cell\ Viability\ [\%] = (1 - \frac{Test\ sample - Neg\ control}{Lysate\ control - Neg\ Control}) \times 100$ where the test sample are the cells treated with NCs at a range of concentrations (see above) and *the negative control are cells that were treated with a minimum concentration of NCs of 3.9 ug/ml that showed no cytotoxicity. Lysate control are the cells treated with cell lysis solution (10 ul/well) and used as positive control. For each concentration of NCs, the fluorescence intensity (λ_{em} 587nm) was calculated and averaged over from eight wells representing eight biological replicates.

MTT Assay: HeLa cells (12 × 10^3 cells/ml) were cultured in a 96-well microplate with Dulbecco Modification of Eagle medium (DMEM, High Glucose) for 24 hrs at 37 °C and 5% CO_2. Following replacing the incubating medium with a fresh one, cells were treated with water-soluble PL-OP-CaF_2 NCs over a range of concentrations (0 (living cells), 3.9, 7.8, 15.6, 31, 62, 124.5, or 498 µg/ml). After 1.5 h of incubation, the media was discarded from cell cultures followed by the addition of MTT Reagent solution (50 µl) and DMEM medium (50 µl) to each well. Following 3 hrs of incubation at 37°C the mixture was replaced by MTT Solvent (150 µl). The plate was then covered with foil and shacked on an orbital shaker for 15 minutes. The absorbance at OD=590 nm was measured using a dedicated plate reader. Cell viability was calculated as a percentage of the treated cells from that of the control (non-treated living cells). For each concentration of NCs, the average value and the standard deviation was obtained from eight different wells representing eight biological replicates. Cells treatment with 50% DMSO were used as positive control.

***In-Vivo* MRI**

All experiments involving vertebrate animals were conducted following the approval of the Weizmann Institute of Science's Committee on Animal Care.

Male C57BL/6 inbred mice (Envigo, Israel) were anesthetized using 75 mg/kg Ketamine, 1 mg/kg Dexmedetomidine in 0.9% saline and intramuscularly injected with 20 μl 150 mM PL-OP-CaF_2 and 20 μl 150 mM PL-OA-CaF_2 to the right and left legs, respectively. MRI studies were conducted while the animal respiration rate was monitored and the animal body temperature was maintained stable using a heating pad.

The ^{19}F-ISIS spectra were acquired from the right leg (PL-OP-CaF_2 injection) using TR=3 s, NA=50, total ISIS averages=400 and scan time of 20 min. The ^{19}F-ISIS spectra from the left leg (PL-OA-CaF_2 injection) was acquired using TR=30 s, NA=10, total ISIS averages=80 and scan time of 40 min. Anatomical ^{1}H-MRI of a live mouse was acquired with a 3D-UTE protocol with the following parameters: TR/TE=4/0.008 ms, flip angle of 5°, FOV=3.2×2.5×12.8 cm^3, matrix size=128×128×128 NA=1, 1 slice of 32 mm in thickness, scan time 3 min 25sec (Fig. 5d). The ^{19}F-MRI image was acquired with a 3D-UTE sequence with the following parameters: TR/TE=4.2/0.008 ms, flip angle of 5°, FOV=3.2×2.5×3.2 cm^3, matrix size=32×32×32 NA=150, scan time of 33min (Fig. 5e).

Supporting Figures

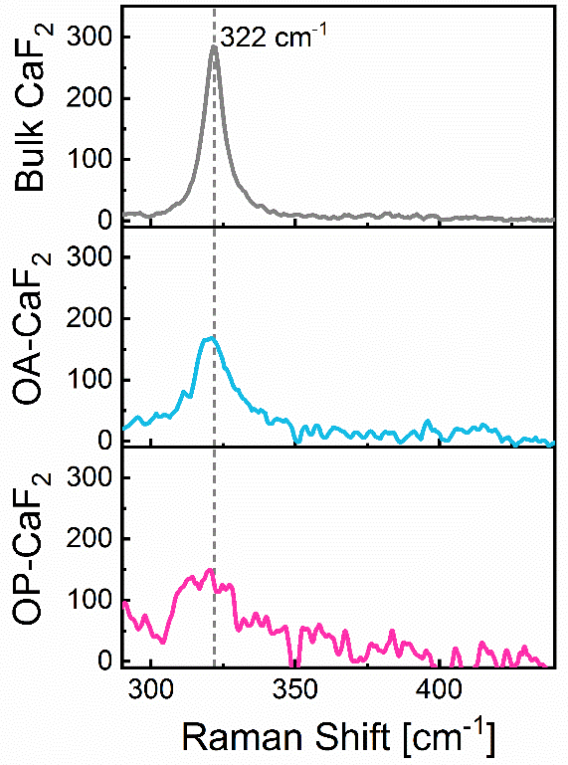

Figure S1. Raman spectra of CaF_2 NCs. Commercially available CaF_2 (grey), OA-CaF_2 (light blue) and OP-CaF_2 (pink) NCs. Dashed line is located at U=322 cm^{-1} for the Raman peak specific for CaF_2 [1].

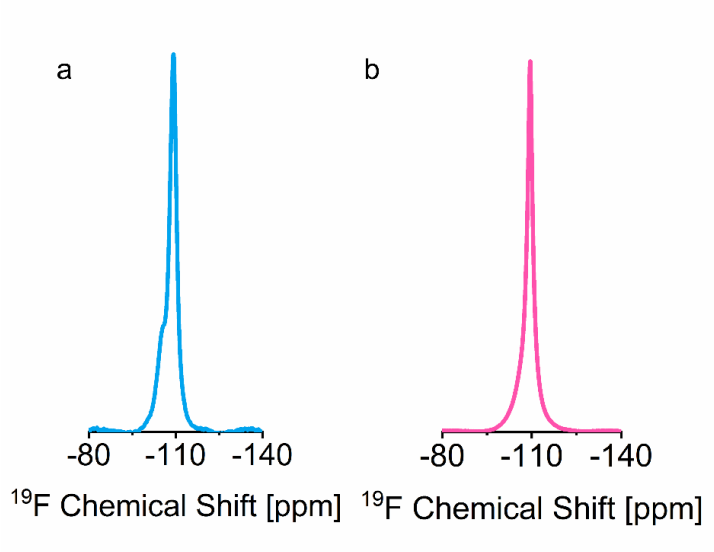

Figure S2. High-resolution ^{19}F-NMR of cyclohexane-dispersed CaF_2. Shown are the ^{19}F-NMR spectra of (a) OA-CaF_2 and (b) OP-CaF_2.

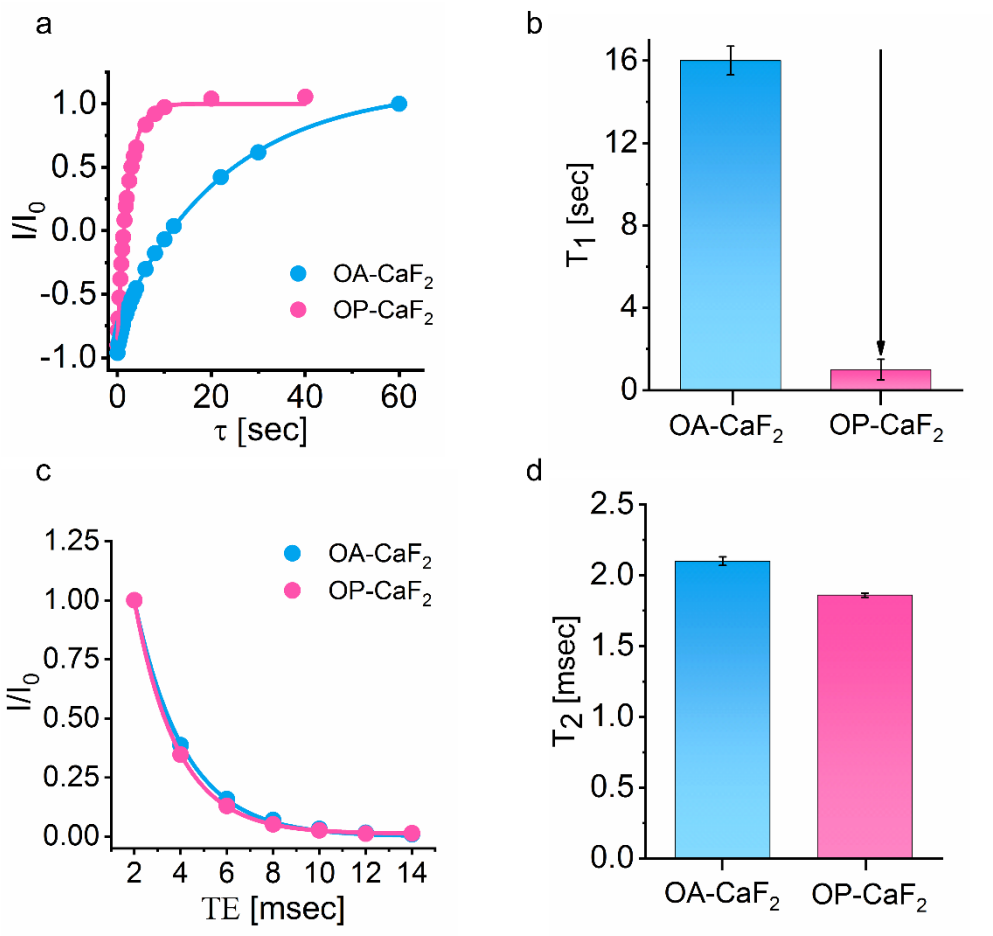

Figure S3. Spin-lattice relaxation ^{19}F-NMR measurements of of cyclohexane-dispersed CaF_2 NCs. (a) ^{19}F-signals of CaF_2 NCs as a function of inversion time obtained from ^{19}F-NMR inversion-recovery experiments performed for OA-CaF_2 (light blue) and OP-CaF_2 (pink) NCs. (b) Spin-lattice relaxation time values measured for OP-CaF_2 (T_1=1±0.5 sec) and OA-CaF_2 (T_1=16±0.7 sec) NCs (N=3 experiments). (c) ^{19}F-signals of CaF_2 NCs as a function of echo time (TE) from CPMG ^{19}F-NMR experiments performed for OA-CaF_2 (light blue) and OP-CaF_2 (pink) NCs (d) Spin-spin relaxation time values measured for OA-CaF_2 (T_2=2.1±0.03 msec) and OP-CaF_2 (T_2=1.9±0.02 msec) NCs.

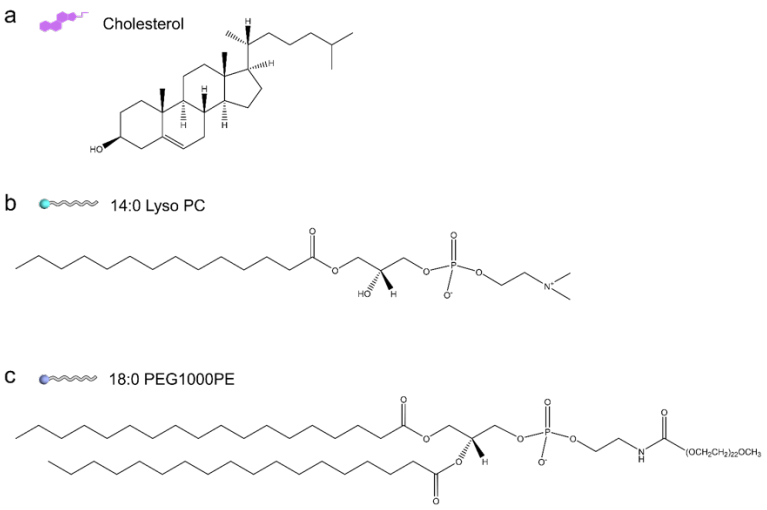

Figure S4. Molecular structures of agents used for ligand incorporation. (a) Cholesterol ((3β)-cholest-5-en-3-ol). (b) 14:0 Lyso PC (1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine). (c) 18:0 PEG1000 PE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000]).

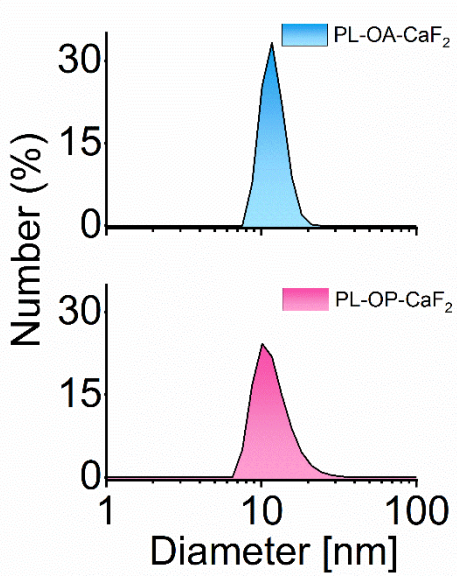

Figure S5. DLS measurements of water-dispersed CaF_2 NCs. The hydrodynamic diameter of water-dispersed PL-OA-CaF_2 (D=11.9±2.0nm) and PL-OP-CaF_2 NCs (D=11.9±3.5nm).

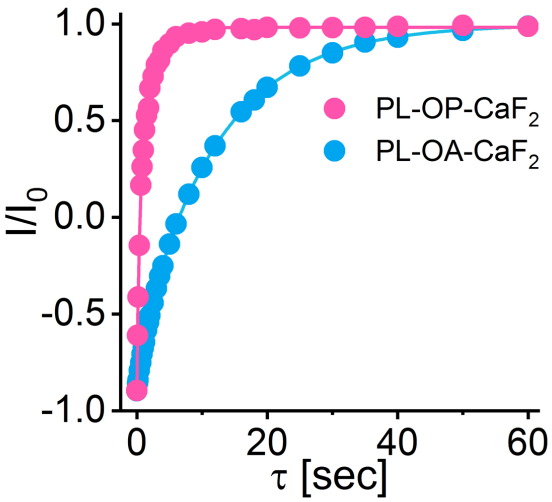

Figure S6. Spin-lattice relaxation ^{19}F-NMR measurements of water-dispersed CaF_2 NCs: ^{19}F-signals of CaF_2 NCs as a function of the inversion time obtained from ^{19}F-NMR inversion-recovery experiments performed for PL-OA-CaF_2 (light blue) and PL- OP-CaF_2 (pink) NCs.

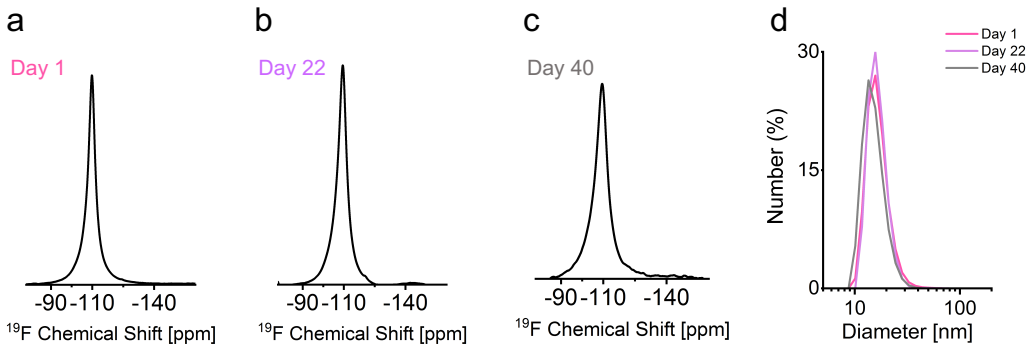

Figure S7. Long-term stability of PL-OP-CaF_2 NCs dispersed in water. (a-c) Longitudinal ^{19}F -NMR signal of PL-OP-CaF_2 NCs dispersed in water acquired 1 day (a), 22 days (b) and 40 days (c) following their preparation. (d) The hydrodynamic diameter of PL-OP-CaF_2 NCs in aqueous solution 1 day, 22 days and 40 days following their preparation.

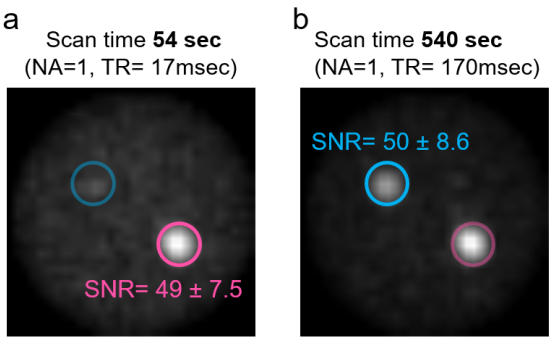

Figure S8. ^{19}F-MRI of CaF_2 NCs (single scan). Single scan ^{19}F-MRI of a phantom composed of two tubes containing either PL-OA-CaF_2 or PL-OP-CaF_2 NCs acquired at 9.4T MRI scanner. For PL-OP-CaF_2 NCs the TR (based on 10^o Ernst angle) was set to 17 msec, resulted in a 54 sec of total acquisition time. For PL-OA-CaF_2 NCs the TR (based on 10^o Ernst angle) was set to 170 msec, resulted in a 540 sec of total acquisition time. The obtained SNR was calculated for each of the formulations at the relative optimal acquisition parameters as noted in pink (For PL-OP-CaF_2 NCs acquired with TR=17 msec) and in light blue (For PL-OA-CaF_2 NCs acquired with TR=170 msec). ^{19}F-MRI were performed on 9.4T MR spectrometer; 3D-UTE protocol with a flip angle of 10^o, TR/TE=17/0.03 msec, FOV=3.2×3.2×3.2 cm3, matrix=32×32×32

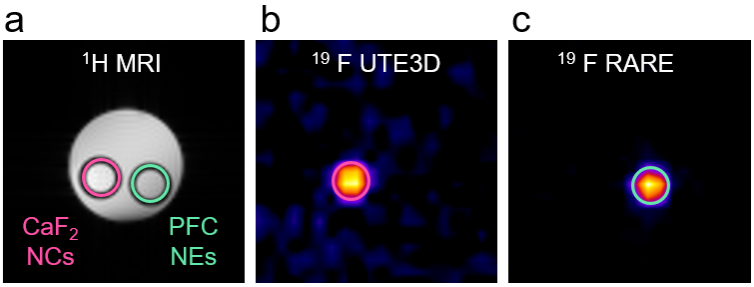

Figure S9. ^{19}F-MRI of PL-OP-CaF2 NCs (10 nm) and PFC nanoemulcions (NE). Raw data for MRI comparing the signal from PL-OP-CaF_2 NCs to a PFC nanoemulsion (VS-1000, CelSense Inc.). A phantom composed of two water tubes containing either PL-OP-CaF_2 NCs or a PFC nanoemulsion (VS-1000, CelSense Inc.) with a comparable ^{19}F-content of 20 mM. (a) ^{1}H-MRI, (b) UTE ^{19}F-MRI (O_1 set to the frequency offset of CaF2, -109 ppm). (c) RARE ^{19}F-MRI (O_1 set to the frequency offset of the main fluorine-19 peak of the PFCs, -91 ppm). The acquisition parameters and the obtained SNR values for each of the studied samples are summarized in Table S1.

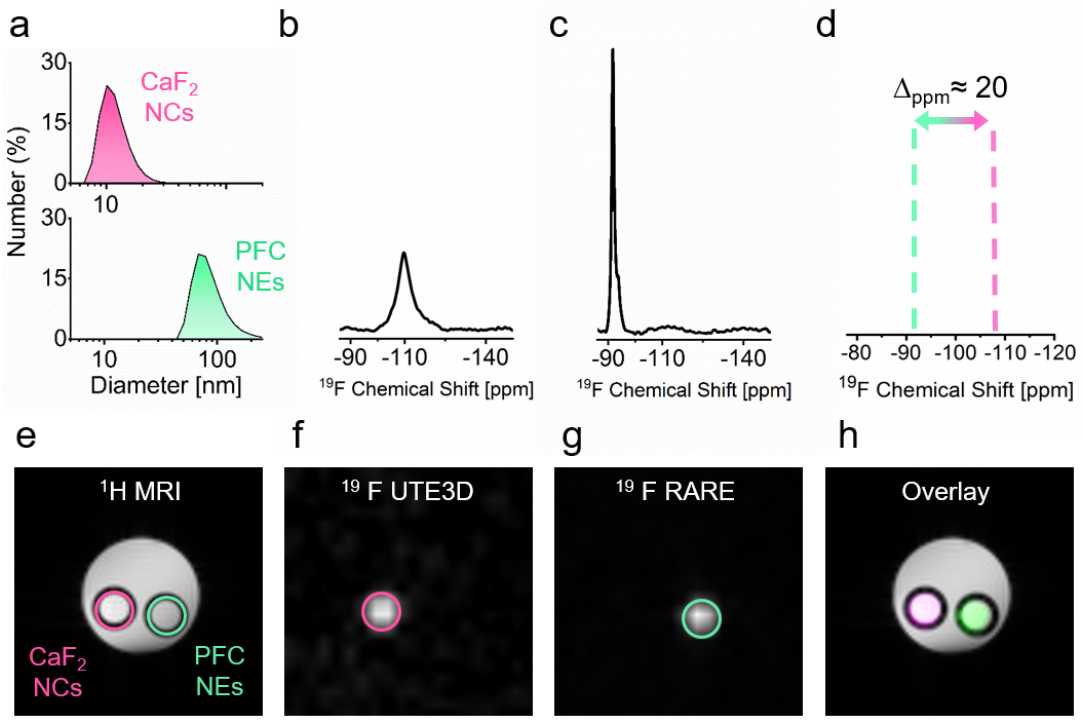

Figure S10. Multiplexed ^{19}F-MRI using PL-OP-CaF_2 NCs and a PFC nanoemulsion (NE). (a) The hydrodynamic diameter of the two studied nanoformulations in water, i.e., PL-OP-CaF_2 NCs (in pink) and PFC nanoemulsion (in green). (b) ^{19}F-NMR signal of PL-OP-CaF_2 NCs (20 mM ^{19}F-content) in water. (c) ^{19}F-NMR signal of PFC nanoemulsion (20 mM ^{19}F-content) in water. (d) Schematic representation of the chemical shift difference between the resonances of the ^{19}F-content of the two nanoformulations. (e) ^{1}H-MRI of a phantom composed of two tubes containing either PL-OA-CaF_2 or PFC nanoemulsions. (f) ^{19}F-MRI 3D-UTE detection of the signal from PL-OP-CaF_2 NCs (O_1 set to -109 ppm). (g) ^{19}F-MRI RARE detection of the signal from PFC nanoemulsions (O_1 set to -91 ppm). (h) Artificial multiplexed ^{19}F-MRI of PL-OP-CaF_2 NCs (in pink) and PFC nanoemulsion (in green) overlaid on a ^{1}H-MR image.

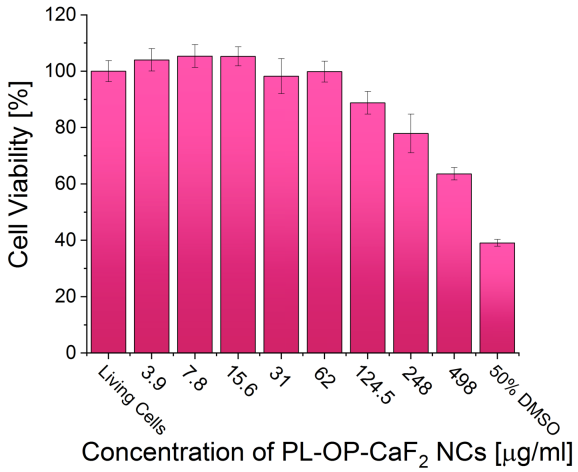

Figure S11. CCK-8 cytotoxicity assay. Concentration-response bar-graph of CCK-8 assay performed on HeLa cells representing cell viability as a function of the concentration of the PL-OP-CaF2 NCs in the medium. Cells were incubated with the noted concentration of the NCs for 1.5 h at 37 ºC. Treatment with 50% DMSO was used as a positive control. The data is shown as the percentile of live cells (relative to untreated cells) and expressed as mean ± SD of eight repetitions.

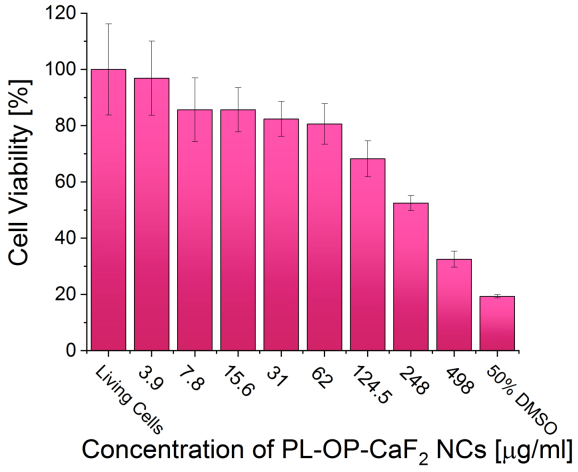

Figure S12. MTT cytotoxicity assay. Concentration-response bar-graph of MTT assay performed on HeLa cells representing cell viability as a function of the concentration of the PL-OP-CaF2 NCs in the medium. Cells were incubated with the noted concentration of the NCs for 1.5 h at 37 ºC. Treatment with 50% DMSO was used as a positive control. The data is shown as the percentile of live cells (relative to untreated cells) and expressed as mean ± SD of eight repetitions.

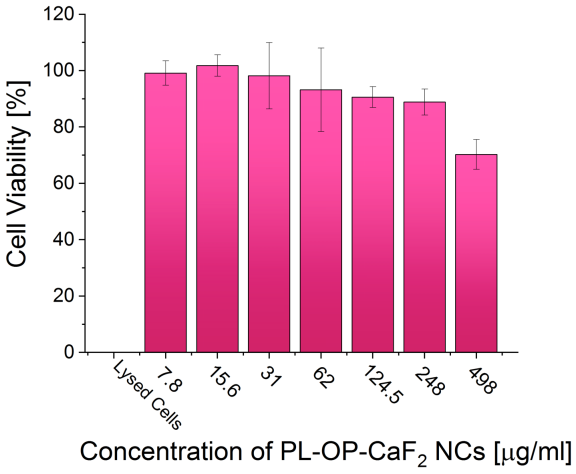

Figure S13. LDH cytotoxicity assay. Concentration-response bar-graph of LDH assay performed on HeLa cells representing cell viability as a function of the concentration of the PL-OP-CaF2 NCs in the medium. Cells were incubated with the noted concentration of the NCs for 1.5 h at 37 ºC. Treatment with 50% DMSO was used as a positive control. The data is shown as the percentile of live cells (relative to untreated cells) and expressed as mean ± SD of eight repetitions. * cells treated with the lowest concentration of PL-OP-CaF_2 NCs 3.9 ug/ml were used as negative control for the calculations.

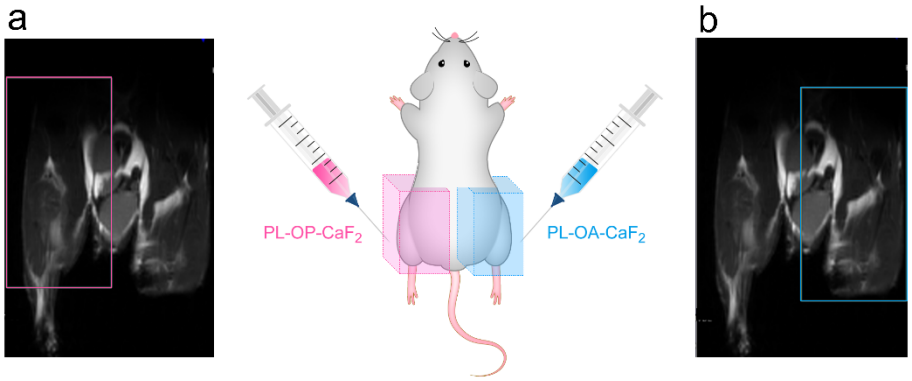

Figure S14. *Selected voxels for localized* ^{19}F-ISIS spectra of injected PL-OA-CaF_2 and PL-OP-CaF_2 NCs. (a) Selected area (pink box) overlaid on an anatomical ^{1}H-MRI of a live mouse, voxel size 10×20×20 mm, for the obtained ^{19}F-ISIS spectra acquired from the right leg (PL-OP-CaF_2 injection) with ISIS parameters TR=3 s, NA= 50 for 20 min. (b) Selected area (light blue box) overlaid on an anatomical ^{1}H-MRI of a live mouse, voxel size 10×20×20 mm, for the ^{19}F-ISIS spectra acquired from the left leg (PL-OA-CaF_2 injection) with ISIS parameters TR=30 s, NA=10 for 40 min.

Table S1. Parameters and calculated SNR for comparing PL-OP-CaF2 and PFC NE (Figure S9)

	PL-OP-CaF_2 NCs	PFC NEs
Concentration [^{19}F atoms]	20mM	20mM
Sequence	^{19}F UTE 3D	^{19}F RARE
Measurement Time	20min	20min
SNR	41	181
TR	4msec (flip=5^{o})	2000 msec
TE	0.12msec	1,835mesc
FOV	48×48×128	48×48
Matrix	32×32×32	32×32
Slice Thickness	4mm	4mm
NA	95	150

References

1. Koningstein, J. A.; Lucazeau, G., The Raman spectrum of tetragonal Eu3+CaF2. *Chem. Phys.* **1973,** *1* (2), 112-119.