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Calcium Fluoride Nanocrystals: Tracers for In Vivo ¹⁹F Magnetic Resonance Imaging

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Abstract: Inorganic nanocrystals (NCs) have been extensively developed for a variety of uses. The ability to obtain highresolution NMR signals from the core nuclei of NCs in solution could offer new opportunities in materials sciences and MR imaging. Herein, we demonstrate that small, water-soluble ¹⁹Fionic NCs can average out homonuclear dipolar interactions, enabling one to obtain high-resolution ¹⁹F NMR signals in solution that reflect the MR properties of F^- in the crystal core. Decorating ¹⁹F-NC surfaces with a biocompatible poly(ethylene glycol) coating maintains colloidal stability in water while preserving the NC high-resolution ¹⁹F NMR properties, even after further functionalization. The high content and magnetic equivalence of the fluorides within the NCs enable their use as imaging tracers for in vivo ¹⁹F MRI by facilitating a "hot-spot" display of their distribution.

 \mathbf{P} olymers and phospholipids^[1] are important components in nanoformulations designed and used in nanomedicine, but inorganic nanocrystals (NCs) have several advantages over these "soft" materials. The chemical composition, crystallinity, size, shape, surface properties, and physical characteristics of inorganic NCs^[2] can be controlled, providing them with unprecedented versatility and enabling their use as nanosensors for in vivo imaging.^[3] Nanoformulations designed for molecular and cellular magnetic resonance imaging (MRI) can be classified as either contrast agents or tracers.^[4] Their use enables accurate coregistering of high-resolution anatomical views with subcellular information, and they are being translated into the clinic.^[5] Metal-oxide-based NCs (i.e., contrast agents) exhibit supreme sensitivity^[6] with controllable size,^[7] shape,^[8] surface modifiability,^[9] metal content,^[10] and variable contrast.^[11,12] Nevertheless, their lack of specificity (relaxation-based MRI signal alternation) and high background signals call for background-free alternatives.

Capitalizing on the fact that ¹⁹F nuclei do not exist in soft biological tissues, together with the high sensitivity of ¹⁹F MRI, the ¹⁹F MR signal of an introduced ¹⁹F tracer can be directly monitored and presented as a quantitative "hotspot" map over anatomical ¹H MRI.^[13] Specifically, ¹⁹F-based perfluorocarbon (PFC) nanoemulsions have been proposed as ¹⁹F MRI tracers and have been successfully used in a wide

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range of applications,^[14] including in clinical settings.^[15] However, PFCs do not have the beneficial properties of inorganic NCs. For example, PFCs cannot be prepared as small (<10 nm) formulations, and thus cannot benefit from the improved delivery and clearance capabilities of such particles.^[16] Moreover, although silica coatings of PFCs have been proposed,^[17] the surfaces of inorganic NCs can be readily modified through diverse and well-established synthetic procedures. The growing field of nanofluoride-based inorganic materials with unique characteristics^[18] and controllable content, sizes, and shapes, creates new possibilities for ¹⁹Fbased formulations. However, one limiting feature of NCbased formulations is the restricted mobility of the elements within the crystal, which frequently results in NMR line broadening due to strong dipole-dipole interactions^[19] and hampers their use as MRI tracers. Herein we present synthetic, water-soluble, small (< 10 nm) fluoride-based NCs that average out homonuclear dipolar interactions and thus allow high-resolution ¹⁹F NMR spectroscopy of the NCs in aqueous solutions. These NCs were used for in vivo ¹⁹F MRI, thus demonstrating that they combine the advantages of NCs (small, high ¹⁹F equivalency, maximal ¹⁹F density, surface modifiability) with the merits of ¹⁹F MRI tracers.

First, we synthesized small, water-soluble, citrate-coated CaF_2 (CF-Cit) NCs (Figure 1) to examine the hypothesis that high-resolution ¹⁹F NMR spectra can be obtained by sufficient averaging of homonuclear dipolar interactions of ¹⁹F nuclear spins within freely tumbling fluoride-containing NCs. The obtained CF-Cit NCs were highly crystalline (Figure 1a)





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and monodisperse (see Figure S1 in the Supporting Information). Energy-dispersive X-ray spectroscopy (EDS) analysis revealed an approximately 1:2 ratio of Ca²⁺ to F⁻ ions (see Figure S2), and the NCs preserved monodispersity in water, as determined by dynamic light scattering (DLS, Figure 1b). The XRD pattern of the synthetic CF-Cit NCs (Figure 1c) features a typical cubic-phase, fluorite-type structure, which was supported by FFT analysis of the TEM image of a single particle (see Figure S3). Then, the water-soluble CF-Cit NCs were studied by high-resolution ¹⁹F NMR spectroscopy in water (Figure 1 d). In a face-centered cubic (fcc) structure of CaF₂, all fluorides were expected to be magnetically equivalent, as reflected by the first coordination sphere scheme (inset, Figure 1 c). Indeed, a singlet peak was observed in the high-resolution ¹⁹F NMR spectrum at -109.6 ppm, similar to the frequency observed for CaF2 powder by solid-state NMR spectroscopy.^[20] This singlet peak is an advantage when proposing novel ¹⁹F-based tracers for ¹⁹F MRI applications, for which high magnetic equivalence of the ¹⁹F source is desired for maximizing ¹⁹F MR signal intensity.^[14a,21]

To confirm that the fcc structure of fluoride NCs is crucial for obtaining a highly intense singlet peak in high-resolution ¹⁹F NMR experiments, two additional water-soluble nanofluorides were synthesized (Figure 2). Citrate-coated watersoluble SrF₂ NCs (Figure 2a), with a similar fcc crystal structure to that of CaF₂ (Figure 2b), also feature a clear, single high-resolution ¹⁹F NMR peak in water (Figure 2c). Conversely, an aqueous solution of small monodispersed citrate-coated LaF₃ NCs (Figure 2d), with a tysonite-type crystal structure (Figure 2e), exhibited a more complex highresolution ¹⁹F NMR spectrum in water. This spectrum (Figure 2 f) depicts more than one ¹⁹F resonance with the



Figure 2. Water-soluble SrF₂ and LaF₃ NCs. a, d) DLS histograms of Cit-SrF₂ (a) and Cit-LaF₃ (d). b, e) XRD patterns of Cit-SrF₂ (b) and Cit-LaF₃ (e); schematic illustrations of the first coordination sphere of Sr²⁺ and the La³⁺ are shown as insets in (b) and (e), respectively. c, f) High-resolution ¹⁹F NMR spectra of Cit-SrF₂ and Cit-LaF₃ NCs, respectively, in aqueous solution.

expected chemical shifts obtained in solid-state ¹⁹F NMR studies of LaF₃ crystals, reflecting fluorides at nonequivalent crystallographic sites.^[20,22]

To facilitate the use of nanofluorides in vivo, we developed and synthesized poly(ethylene glycol) (PEG)-coated CaF_2 NCs (Figure 3) with the aim of providing solubility,



Figure 3. Water-soluble PEGylated CaF₂ NCs. a) Illustration of the PEGylated CaF₂ NCs (CFP). b) DLS histograms. c) High-resolution ¹⁹F NMR spectrum in aqueous solution. d–f) Fluorescence spectra and high-resolution ¹⁹F NMR spectra of functionalized CFP NCs. Green and red stars represent FITC and SCY3 functional groups conjugated to the CFP NC, respectively.

stability, monodispersity, and surface modifiability.^[23] The CaF₂-PEG (CFP) NCs were synthesized by a solvothermal approach with a mixture of PEG-hydroxyl and PEG-carboxvlate (average MW 600), purified, and characterized by TEM, XRD, EDS, ¹H and ¹³C NMR spectroscopy, FTIR spectroscopy, and thermogravimetric analysis (TGA: see Figures S4-S10). The DLS histograms of CFP NCs in aqueous solution indicate that the small particles are monodisperse with colloidal stability retained for at least 40 days (Figure 3b). High-resolution ¹⁹F NMR experiments of CFP NCs in aqueous solution revealed a clear singlet peak at the expected chemical shift of CaF_2 (Figure 3c). To demonstrate the feasibility of chemically modifying the CFP for future applications, we further functionalized the CFP NCs with fluorescent moieties (for a description of the synthesis, see the Supporting Information). Both fluorescein isothiocyanate (FITC) and sulfo-cyanine3 (SCY3) were used to demonstrate the feasibility of CFP functionalization (Figure 3d-f). In all cases, the high-resolution ¹⁹F NMR properties of the modified NCs were preserved along with high fluorescence (Figure 3 fh), used to monitor uptake of NCs by live cells (see Figure S11). The DLS histograms of all functionalized CFP NCs in aqueous solution showed colloid monodispersity that was stable for several weeks (see Figure S12 and Table S1 in the Supporting Information).

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The longitudinal (T_1) and transverse (T_2) relaxation times of the ¹⁹F within the CFP NCs were determined to optimize the acquisition parameters for future ¹⁹F MRI experiments (Figure 4a; for similar measurements with CF-Cit, see Fig-



Figure 4. CFP relaxation properties and ¹⁹F MRI. a) ¹⁹F NMR signal of CFP NCs as a function of inversion time (TI, upper panel) or echo time (TE, bottom panel), used to calculate T_1 and T_2 values, respectively. b) ¹⁹F NMR signal as a function of the flip angle (α) obtained from 3 min experiment time. c–e) MRI phantom containing CFP NCs with different concentrations (total F⁻): 1) 250, 2) 75, 3) 25, 4) 5, and 5) 2.5 mM; c) ¹H MRI; d) ¹⁹F MRI (TE = 25 µs); e) display of ¹⁹F data as a "hot-spot" map on a ¹H MR image.

ure S14 and Table S2). The relatively long T_1 time of CFP may be less favorable for MRI applications owing to the long repetition time (TR) needed to fulfil the $5 \times T_1$ condition for maximizing the MR signal. However, by using low-flip-angle excitation pulses it was possible to significantly shorten the TR used, allowing increased averaging and thus a higher signal-to-noise-ratio (SNR) for a given experiment time. The ¹⁹F MR signal of CFP could be maximized at low flip angles (Ernst angle) at a fixed experiment time, while a very low SNR was obtained when a 90° pulse was used (TR = $5 \times T_1$ = 48.5 s; Figure 4b). The addition of a paramagnetic dopant into the NCs should be considered for the purpose of shortening T_1 , as proposed for PFC nanoemulsions.^[25] Although until recently nuclear spins with relatively short T_2 values could not be monitored with conventional MRI acquisition schemes, ultrashort echo time (UTE) and zero echo time (ZTE) pulse sequences enable MRI of nuclear spin pools with an extremely short T_2 value,^[24] including metallated PFCs with ¹⁹F MRI.^[25]

Next, the feasibility of spatially mapping the distribution of our newly developed water-soluble nanofluorides with ¹⁹F MRI was examined. A phantom composed of samples containing CFP at a range of concentrations was prepared and imaged with a 9.4 T MRI scanner (Figure 4c–e). As expected, no difference was observed between the four tubes using ¹H MRI (Figure 4c). However, by using the UTE sequence, which enables MRI of nuclear spin pools with an extremely short T_2 value, a clear ¹⁹F MR signal could be observed from the CFP-containing tubes (Figure 4d). These data were acquired with an echo time (TE) of 25 µs (TR = 50 ms with a flip angle of 5.7° to achieve Ernst angle conditions) and allow a "hot-spot" representation of the distributions of nanofluorides, thus demonstrating their potential to be used as imaging tracers for MRI applications (Figure 4e). Importantly, ¹⁹F MR images acquired with a gradient-echo-based sequence (TE = 3.1 ms; see Figure S16) did not reveal any ¹⁹F signal from the CFP samples, thus reflecting the necessity of using extremely short echo times when acquiring ¹⁹F MR images of nanofluorides. This ability to monitor extremely short T_2 values with conventional MRI setups (UTE-based acquisition schemes) offers new opportunities for the design and development of novel inorganic NCs as X-nuclei MRI tracers.

It is frequently useful to obtain information from multiple imaging agents (or targets) simultaneously and present this information as a multicolor map. MRI signals of different nanofluorides (i.e. CaF₂ and SrF₂) can be color-encoded based on the difference between their ¹⁹F NMR chemical shifts (Figures 1 and 2) and displayed in a multiplexed manner (Figure 5; see also Figure S17). The ¹⁹F NMR chemical shift



Figure 5. Artificial "multicolor" ¹⁹F MR imaging. A phantom composed of two tubes containing water and two tubes containing either CaF₂ or SrF₂ NCs were used. From left to right: ¹H MRI, ¹⁹F-UTE MRI (O₁ set to the frequency offset of CaF₂), ¹⁹F-UTE MRI (O₁ set to the frequency offset of SrF₂), multicolor map overlaid on the ¹H MR image with CaF₂ (red) and SrF₂ (green) ¹⁹F MR signals.

of CaF₂ (ca. -109 ppm) differs from that of PFC-based nanoemulsions as well and therefore allows the combination of CaF₂ and PFC in future multicolor studies differentiating between these two nanoformulations while in the same field of view (see Figure S18). The favorable relaxation properties of PFCs for ¹⁹F MRI result in a higher SNR as compared to nanofluorides, when comparing the same ¹⁹F content within the studied samples (see Figure S18). However, by using nanofluorides a much higher ¹⁹F payload per nanoparticle can be achieved (27–71 % more ¹⁹F per particle for CaF₂ NCs as compared to PFC; see Table S4 and related equations and text). In principle, by using ¹⁹F-NCs other than CaF₂ (e.g., MgF₂) an even larger ¹⁹F/particle payload could be achieved (see Table S4).

Finally, the potential of using the proposed ¹⁹F-NCs (specifically, CFP-SCY3, Figure 3 f) as imaging tracers for in vivo ¹⁹F MRI was evaluated in a mouse model of inflammation. Cellular toxicity of the CFP-SCY3 NCs as well as their ability to be taken up by macrophages was evaluated prior to in vivo MRI experiments (see Figures S15 and S11, respectively). Ten days post-immunization, when extensive inflammatory activity is expected, mice were subjected to MRI sessions that acquired pre- and post-injection of CFP-SCY3 NCs.

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Figure 6. In vivo imaging of PEGylated CaF₂. Anatomical ¹H MRI of two representative mice (left-hand images) and matched ¹⁹F MRI (middle images) shown as pseudocolor maps overlaid on the anatomical ¹H MR images (right-hand images). Mice were subjected to injection of CFP-SCY3 (80 μ L into mouse footpads), and the ¹⁹F MRI scan time was 1 h (TR/TE=150/0.02 ms).

A clear ¹⁹F signal was observed at the region of the popliteal lymph node (LN) of mice injected with NCs (N = 4) within the same leg as the injection site (Figure 6) 1 h post-injection. Tracers of the ¹⁹F MR signal could be observed at the injection site (Figure 6, middle and right panels, bottom ¹⁹F MR signal) with no ¹⁹F MR signal observed from contralateral regions. These results, showing that injected nano-fluorides accumulate in the LN of inflamed mice, are in good agreement with previous observations of small inorganic NCs draining into the LN of the injected leg of inflamed subjects following subcutaneous injection.^[26]

In summary, we have demonstrated that small fluoridebased NCs freely tumbling in solution can be studied by highresolution ¹⁹F NMR spectroscopy and be used as nanotracers for direct and real-time in vivo ¹⁹F MRI. The growing interest in fluoride-based nanomaterials for biological sciences and nanomedicine,^[27] coupled with the strengths of ¹⁹F-MRI, called for novel fluorine imaging agents. The ¹⁹F-NC platform described herein fills this gap by introducing a new type of ¹⁹F tracer with advantageous size, maximal ¹⁹F density, and surface modifiability. The enhanced performance of these nanofluorides suggests utility in a wide range of applications.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cellular imaging · fluorine ·

magnetic resonance imaging \cdot nanofluorides \cdot nanoparticles

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Communications



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Calcium Fluoride Nanocrystals: Tracers for In Vivo ¹⁹F Magnetic Resonance Imaging



Core competency: Small, water-soluble, monodisperse inorganic-fluoride-based nanocrystals freely tumbling in aqueous solution showed characteristic high-resolution ¹⁹F NMR spectra of the ¹⁹F content of their core. The fabricated PEGcoated CaF₂ nanoparticles combine the advantages of nanocrystals as nanosized imaging agents with the merits of ¹⁹F MRI tracers (see picture).

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