Glyconanofluorides as Immunotracers with a Tunable Core Composition for Sensitive Hotspot Magnetic Resonance Imaging of Inflammatory Activity

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ABSTRACT: Nature-inspired nanosized formulations based on an imageable, small-sized inorganic core scaffold, on which biomolecules are assembled to form nanobiomimetics, hold great promise for both early diagnostics and developed therapeutics. Nevertheless, the fabrication of nanobiomimetics that allow noninvasive background-free mapping of pathological events with improved sensitivity, enhanced specificity, and multiplexed capabilities remains a major challenge. Here, we introduce paramagnetic glyconanofluorides as small-sized (<10 nm) glycomimetics for immunotargeting and sensitive noninvasive in vivo 19F magnetic resonance imaging (MRI) mapping of inflammation. A very short $T_1$ relaxation time (70 ms) of the fluorides was achieved by doping the nanofluorides' solid crystal core with paramagnetic Sm$^{3+}$, resulting in a significant 8-fold enhancement in their 19F MRI sensitivity, allowing faster acquisition and improved detectability levels. The fabricated nanosized glycomimetics exhibit significantly enhanced uptake within activated immune cells, providing background-free in vivo mapping of inflammatory activity, demonstrated in both locally induced inflammation and clinically related neuropathology animal models. Fabricating two types of nanofluorides, each with a distinct chemical shift, allowed us to exploit the color-like features of 19F MRI to map, in real time, immune specificity and preferred targetability of the paramagnetic glyconanofluorides, demonstrating the approach’s potential extension to noninvasive multitarget imaging scenarios that are not yet applicable for nanobiomimetics based on other nanocrystal cores.

KEYWORDS: biomimetic, glyconanoparticles, nanocrystals, inflammation, 19F MRI, multicolor MRI

Small (<10 nm) inorganic nanocrystals (NCs) offer well-defined solid scaffolds for the “bottom-up” engineering of nanostructured biomimetic formulations.1 Decorating their solid core with natural moieties, such as protein nanocages,2 viral capsids,3 spherical nucleic acids,4 multivalent glycans,5 or lipoproteins,6 endows these fabrications with both the structure and function of a natural nanosized formulation. Their further engineering with imageable solid cores6–13 adds the option of noninvasive imaging of biological processes in deep tissues of live subjects, with improved biodistribution profiles and enhanced tissue accessibility and targetability.14 Among the biomedical imaging modalities for which these nanostructured biomimetics have been designed, magnetic resonance imaging (MRI) stands out due to its ability to provide target-specific information from the nanostructure agent that can be accurately localized on high-resolution anatomical images of soft tissues.15 Moreover, the ability to obtain complementary physiological16 and functional17 information from biodegradable MRI agents makes this modality preferable for molecular imaging applications. Yet, although highly sensitive,18 the currently used NCs for MRI are based

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on metal-oxide cores that generate nonspecific, strong background signals, which are not quantifiable and cannot be mapped in a “hotspot” background-free display fashion, as demonstrated for nanoformulation designs proposed for CEST-MRI\textsuperscript{19–21} or heteronuclear MRI\textsuperscript{22,23} applications.

As an alternative to magnetic NCs used for $^1$H MRI, it was recently demonstrated that fabricated small (<10 nm) inorganic nanofluorides can serve as nano tracers for $^{19}$F MRI.\textsuperscript{24} Combining the advantages of NCs\textsuperscript{25} with the benefits of $^{19}$F MRI,\textsuperscript{26} these nanofluorides offer MRI-detectable inorganic NCs that can be used as a small-sized solid scaffolds for the fabrication of nature-inspired nanomaterials. Contrary to $^1$H MRI, $^{19}$F MRI agents provide a platform for background-free MR signals that have the potential to be quantified as well as displayed as hotspot maps.\textsuperscript{23,27–32} Moreover, the $^{19}$F MR signal can be perfectly co-registered on high-resolution $^1$H MRI data, with the potential to be further designed and presented in a multicolor fashion,\textsuperscript{33–36} retarding the need for hybrid multimodal imaging technologies. Nevertheless, although nanofluorides offer the small-sized solid core needed for engineering nanobiometrics, a feat not possible with large-sized (100–200 nm) fluorine-based emulsions, their $T_1$ relaxation times are relatively long.\textsuperscript{27} This limits signal averaging and, thus, the ability to detect low-concentration targets at a given imaging time.

Here, we demonstrate the fabrication of paramagnetic nanofluorides that possess extremely short $T_1$ values for enhanced $^{19}$F MRI sensitivity, much shorter than that achieved by fabricating polycrystalline nanofluorides.\textsuperscript{37} Inspired by glyconanoparticles\textsuperscript{38} that display carbohydrate-based structures for improved recognition and enhanced affinity to inflammatory cells,\textsuperscript{39–41} we designed paramagnetic glyco nanofluorides as a tunable platform for noninvasive hotspot MRI mapping of inflammation. Specifically, we demonstrate that nanofluorides (CaF$_2$ or SrF$_2$) doped with paramagnetic elements for enhanced $^{19}$F MRI sensitivity and further coated with multivalent lactose moieties can function as imageable tracers with enhanced immunotargetability. Contrary to magnetic glyconanoparticles,\textsuperscript{42,43} which rely on the identification and interpretation of MRI signal voids,\textsuperscript{15,44} glyco nanofluorides offer background-free MRI signals from targeted immune cells. The fabricated paramagnetic glyco nanofluorides enable specific, in vivo $^{19}$F MRI mapping of inflammatory activity, both in a locally induced inflammation model and in a model of neuroinflammation. Our demonstration of enhanced immune targetability in a multicolor fashion when using two types of nanofluorides (CaF$_2$ and SrF$_2$) emphasizes another feature of these nanotracers, representing the potential for their further development with other targets and applications where small-sized inorganic NCs are advantageous.

**RESULTS AND DISCUSSION**

**Oleate-Coated Ln:CaF$_2$ Fabrications.** We created modifiable NCs that allow both paramagnetic doping (for enhanced $^{19}$F MRI sensitivity) and surface functionalization (for multivalent glycan presentation) by first synthesizing small-sized CaF$_2$ NCs using the phase transfer and separation synthetic approach, with oleic acid (OA) serving as the NCs’ capping ligand (Figure 1a).\textsuperscript{45} The obtained OA-CaF$_2$ NCs were small enough (<10 nm, by dynamic light scattering (DLS), Figure S1) to average out homonuclear dipolar interactions,\textsuperscript{24} providing a typical high-resolution $^{19}$F NMR signal at −109 ppm (Figure 1b). As the very long $T_1$ (16 s, Figure S2) of the fluoride content within OA-CaF$_2$ NCs restricts their applicability for in vivo $^{19}$F MRI studies, as it imposes a long data acquisition time frame, we next doped them with lanthanide cations (Ln$^{3+}$), which have an ionic radius relatively similar to that of Ca$^{2+}$. This allowed us to introduce paramagnetic elements that induce a paramagnetic relaxation enhancement (PRE) effect on the neighboring fluorides (F$^-$) in the crystal. The Ln$^{3+}$-doped CaF$_2$ (OA-Ln:CaF$_2$) NCs were synthesized by adding calculated amounts of Ln(NO$_3$)$_3$ to the reaction mixture (for further details, see the Methods section). To that end, 5 mol % of Ln$^{3+}$-doped oleate-coated CaF$_2$ NCs were synthesized, purified, and characterized, resulting in a series of OA-Ln:CaF$_2$ NCs (Figure 1b). In order to examine the PRE induction capabilities of the dopants, four representative Ln$^{3+}$ cations were used: La$^{3+}$ as a diamagnetic dopant, Sm$^{3+}$ and Ce$^{3+}$ as Ln$^{3+}$ cations with the mildest PRE induction capabilities, and Gd$^{3+}$ with the largest PRE induction capabilities. Of note, we obtained for all OA-Ln:CaF$_2$ NCs small-sized (<10 nm) monodispersed nanofluorides (DLS, Figure S1) that yielded a clearly resolved high-resolution $^{19}$F NMR signal while in solution (Figure 1b).

**Ln$^{3+}$ Dopants Induce a PRE Effect in CaF$_2$ NCs.** The ability of a PRE induction to improve the performances of $^{19}$F MRI was previously demonstrated in large-sized (150–200 nm) perfluorocarbon (PFC) emulsions in which the chelated paramagnetic elements were embedded in the fluorocarbon phase of the formulation.\textsuperscript{46–48} As expected, we observed a mild effect on $^{19}$F NMR line-broadening with the diamagnetic lanthanide, La$^{3+}$ (calculated from the full width at half-maximum, fwhm), and no effect on the $T_1$ relaxation properties (La:CaF$_2$ compared CaF$_2$; Figure 1b and Figure S2). A more pronounced line-broadening effect was, however, obtained when the paramagnetic lanthanide cations were examined (i.e., Sm$^{3+}$, Ce$^{3+}$ and Gd$^{3+}$) as dopants. Note that the “shoulder” peak, which resonates at ~105 ppm, is assigned to fluorides at the surface of the CaF$_2$ NCs, while the main peak, which resonates at ~109 ppm, represents the fluorides at their core.\textsuperscript{49} Upon the addition of dopants, these peaks tend to coalesce, probably due to NMR line-broadening but also due to a smaller deviation in

![Figure 1. Paramagnetic OA-CaF$_2$ NCs. (a) Schematic representation of the synthetic route used for the fabrication of paramagnetic OA-CaF$_2$ and OA-Ln:CaF$_2$ NCs. (b) $^{19}$F NMR spectra of OA-Ln:CaF$_2$ NCs with different Ln$^{3+}$ dopants (5 mol % in the synthesis).](https://doi.org/10.1021/acsnano.1c01040)
the chemical environment of the fluorides in the core and the shell of the NC. This observation is in agreement with phenomenon detected when highly crystalline CaF₂ NCs were compared to ones with a defected crystal core.²⁷

In contrast to the pronounced ¹⁹F NMR line-broadening obtained upon CaF₂ doping with Ce³⁺ and Gd³⁺, which reduces the SNR of the resultant ¹⁹F MRI,³⁶ doping the nanofluorides with Sm³⁺ dramatically shortens the T₁ relaxation time of the ¹⁹F content compared to that with OA-CaF₂ or OA-La:CaF₂ (compare inversion recovery plots, Figure S2) while having only a mild effect on ¹⁹F NMR line-broadening and T₂ characteristics (Figure 1b and Figure S2). The very broad line and the relatively poor SNR obtained for OA-Gd:CaF₂ did not allow us to determine its T₁ and T₂ values using the experiments used to evaluate these relaxation times for the other nanofluorides. These observations are in good agreement with previous reports that “NMR visibility” and, thus, the quantification of adjacent atoms in Ln³⁺-doped crystals is not significantly affected by Sm³⁺ as compared to crystals doped with lanthanides with more pronounced PRE capabilities.⁵⁰ It should be noted here that paramagnetic dopants may reduce the NMR signal of neighboring nuclear spins due to relaxation, paramagnetic broadening, and/or shielding. When such an effect by the dopant is strong, a “blind sphere” radius at which the NMR signal of neighboring nuclei is nullified can be defined.⁵¹ Sm³⁺ induces a negligible blind sphere radius compared to other lanthanides;⁵² thus, although it induces a significant PRE effect in nanofluorides (Figure S2), it should allow the detection of NMR signals even from atoms that are very close to the paramagnetic center of the NC lattice. Nevertheless, increasing the amount of Sm³⁺, which does not affect the size or dispersity of the obtained Sm:CaF₂ NCs (Figures S3–S5), does, indeed, lead to an even shorter T₁ of the fluoride within the NCs but also leads to ¹⁹F NMR line-broadening, resulting in a poor SNR, which may cancel out the T₁-shortening gain in ¹⁹F MRI studies (Figure S6). The transmission electron microscopy (TEM) images of OA-Sm:CaF₂ (Figure 2a) clearly indicate that the addition of Sm³⁺ dopant through the fabrication of OA-Sm:CaF₂ does not affect the NCs’s small size (6 ± 0.8 nm vs 5.5 ± 1.3 nm obtained for OA-CaF₂), with homogeneous distribution of the doping element deduced from energy-dispersive X-ray spectroscopy (EDS) elemental maps (Figures 2b). Notably, atomic-resolution scanning transmission electron microscope (STEM) images of a single Sm:CaF₂ NC revealed bright spots, which are associated with the strong high-angle scattering of the heavy Sm atoms, demonstrating their incorporation into the lattice sites of the CaF₂ crystal (Figure 2c and Figure S7). The quantification of the EDS maps allowed us to determine the atomic ratio in the Sm:CaF₂ NCs, which was found to be (31 ± 1):(65 ± 1):(3.5 ± 0.5) for Ca:F:Sm (Figure 2d).

Phospholipid-Coated Ln:CaF₂ NCs. NCs capped by hydrophobic ligands (such as the OA-CaF₂ NCs in Figure 1) can be subsequently coated with a monolayer of phospholipids by exploiting the strong hydrophobic interactions between the tails of the capping ligand (OA) and the phospholipid (PL).⁵⁸ Such a procedure (schematically shown in Figure 3a), which endows the NCs with the desired water solubility, can be used to incorporate into these NC targeting ligands and/or fluorescently labeled PLs. In an effort to obtain small, stable, water-soluble PL-Sm:CaF₂ NCs, we added 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (14:0 Lyso:PC), PEGylated PL (18:0 PEG1000-PE), and cholesterol to a solution of OA-Sm:CaF₂ NCs (relevant amounts are summarized in Table S1). The monodispersity of the resultant PL-Sm:CaF₂ NCs was maintained in aqueous solution, as confirmed by both TEM images (Figure S8) and DLS measurements, with the expected larger hydrodynamic diameter (11.3 ± 2.8 nm) (Figure 3b) and increased organic-coating mass (Figure S9) compared to the one measured prior to the PL incorporation. PL-Sm:CaF₂ NCs were found to be stable for at least 1 month in an aqueous buffer, with no observable changes in their size and monodispersity (Figure S10). Importantly, we found the T₁ relaxation time of the PL-Sm:CaF₂ NCs to be extremely short, 70 ms, more than 2 orders of magnitude shorter (210³ shorter) than that obtained for diamagnetically doped PL-La:CaF₂ NCs (15 s, Figure 3c) in water. This short T₁ value, which was found to be very short at different magnetic fields (Table S2), is comparable to the one obtained for paramagnetic fluorinated nanoemulsions.⁴⁶ Surprisingly, the evaluated T₁ value of the PL-Sm:CaF₂ NCs in aqueous solution was found to be even shorter than the one calculated for OA-Sm:CaF₂ dispersed in cyclohexane (Figure S2). This observation might be explained by the different correlation times of the NCs in water, which could be affected by several factors, such their larger size (Figure 3b and Figure S10), the existence of hydration shell only in water, different viscosity of the dispersing solution, hydrogen bonds, or even charge differences.

Next, a phantom composed of three samples (containing similar fluorine concentrations) of non-, diamagnetic-, or paramagnetic-doped nanofluorides (PL-CaF₂, PL-La:CaF₂, or PL-Sm:CaF₂ NCs, respectively, all three are not fluorescent, Figure 3d) was studied in order to evaluate the gain in sensitivity upon T₁ shortening (from 15 s to 70 ms) when using them for ¹⁹F MRI. The short T₁ of PL-Sm:CaF₂ NCs (Figure 3c) allows the repetition time of the acquisition to be reduced to only 4 ms with an excitation flip angle of 5°.
(fulfilling the Ernst angle condition and complying with the hardware limitations) and, thus, a $^{19}$F MR signal to be obtained from PL-Sm:CaF$_2$ NCs within a few seconds (Figure S11). The very short $T_1$ of PL-Sm:CaF$_2$ NCs leads to an 8-fold higher SNR compared to the two types of diamagnetic CaF$_2$ NCs (non-doped PL-CaF$_2$ and PL-La:CaF$_2$) at a given scan time (Figure 3e). Impressively, PL-Sm:CaF$_2$ NCs (Figure 3f) yield a SNR of $\sim 6$ at a concentration of only 0.22 $\mu$M, which is 1.25 mM of $^{19}$F content. This represents a more than 40-fold improvement in CaF$_2$ detectability over previously proposed PEG-coated CaF$_2$ NCs$^{24}$ and a 5-fold improvement over defected CaF$_2$ NCs.$^{37}$

Lactose-Modified CaF$_2$ NCs for Immune Targeting. In addition to improved sensitivity (i.e., shorter $T_1$), robust mapping of $^{19}$F MRI tracers requires extensive and efficient nanofluoride accumulation at the region-of-interest (ROI) to ensure a detectable number of $^{19}$F atoms. Therefore, with an eye to enhance $^{19}$F MRI detectability of inflammation, we synthesized glyconanofluorides by coating Sm:CaF$_2$ NCs with lactose moieties to obtain lactose multivalency on the surface of the nanofluoride and to prompt their cellular accumulation (i.e., immunolabeling) within sites of inflammation.$^{12}$ By applying the same synthetic procedure described in Figure 3a, Sm:CaF$_2$ NCs were coated with glycosylated phospholipids (18:1 lactosyl-PE). Additionally, fluorescently labeled phospholipids (14:0 Liss Rhod PE) were integrated into the NC’s surface for cellular uptake validation by fluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis. Of note, the intrinsic fluorescent properties of the Sm:CaF$_2$ NCs were found to be negligible, with no effect on the fluorescent characteristics of the rhodamine dye (Figure S12). High-resolution mass spectrometry (Figures S13 and S14) confirmed the attachment of the introduced ligands to obtain both lactose-coated, fluorescently labeled nanofluorides (LPL-Sm:CaF$_2$) and their nonglycosylated control, PL-Sm:CaF$_2$ NCs. Both NC solutions (i.e., LPL-Sm:CaF$_2$ and PL-Sm:CaF$_2$) showed a similar, small hydrodynamic diameter, were transparent, exhibited the strong characteristic color of the rhodamine dye (Figure S15), and had the identical, typical, high-resolution $^{19}$F NMR signal of CaF$_2$ NCs (−109 ppm, Figure S16). In good agreement with previous reports of increased cellular uptake of lactose-modified, small-sized NCs,$^{32-34}$ incubating a mouse monocyte-derived macrophage cell line (RAW 264.7) with lactose-coated nanofluorides (LPL-Sm:CaF$_2$) or nonglycosylated control, PL-Sm:CaF$_2$ NCs. Both NC solutions (i.e., LPL-Sm:CaF$_2$ and PL-Sm:CaF$_2$) showed a similar, small hydrodynamic diameter, were transparent, exhibited the strong characteristic color of the rhodamine dye (Figure S15), and had the identical, typical, high-resolution $^{19}$F NMR signal of CaF$_2$ NCs (−109 ppm, Figure S16). In good agreement with previous reports of increased cellular uptake of lactose-modified, small-sized NCs,$^{32-34}$ incubating a mouse monocyte-derived macrophage cell line (RAW 264.7) with lactose-coated nanofluorides (LPL-Sm:CaF$_2$) or nonglycosylated nanofluorides (PL-Sm:CaF$_2$) led to a relative increase in the uptake of the former (Figure S17). Furthermore, a toxicity evaluation of the NCs found no evidence of enhanced toxicity by either the Sm$^{3+}$ dopant (3.5 ± 0.5%) or the lactose moiety (Figure S18).

It is important to mention that the in vitro toxicity assay does not mimic the situation in vivo (due to the dilution of the injected material in the blood, its immediate washout as compared to a lengthy passive incubation and more), and a maximum tolerated dose was evaluated prior to the in vivo...
experiment (and found to be 50 mg/kg body weight). Then, the biodistribution profile of the fluorescent LPL-Sm:CaF₂ and PL-Sm:CaF₂ was assessed following their intravenous administration into two respective groups of mice (N = 9 for each group). The organs of the mice were excised 30 min (N = 3 for each group), 2 h (N = 3 for each group), or 24 h (N = 3 for each group) after NC administration, and their fluorescent intensity was quantified (Figure S19). No significant difference between the biodistribution profiles and NC clearance could be detected between the LPL-Sm:CaF₂ and PL-Sm:CaF₂ groups. A histopathological evaluation of the kidneys of the examined mice showed no evidence of pathological damage 24 h following LPL-Sm:CaF₂ administration (Figure S20). However, in light of recent concerns regarding GD₃ involvement in nephrogenic systemic fibrosis induction, additional long-term studies of the effect of the proposed NCs are needed to evaluate their biological and physiological safety prior to considering them for human studies. It is important to note here that, although the obtained fast clearance from all the examined organs without evident damage indicates a very important feature, considering the prolonged half-life of other fluorine-containing contrast agents, this property should be further investigated. Given that the biodistribution evaluation was based on the fluorescent signal of the rhodamine-labeled phospholipid, and in light of previous observations of the high probability of dynamic lipid exchange between nanoparticles and cells’ lipids, future studies to fully characterize fluoronanoconjugates’ biodistribution and clearance profiles should likewise be undertaken.

**Glyconanoconjugates for Immune Targeting of Inflamed Lymph Nodes in Vivo.** To assess whether the LPL-Sm:CaF₂ NCs can serve as background-free imaging tracers for the noninvasive mapping of inflammatory activity, we used an *in vivo* animal model of inflammation. First, the footpads of mice were subcutaneously injected with 50 μL of an immunogenic emulsion, inducing a local inflammatory response in both right and left hind legs. Ten days post-immunization, when extensive inflammatory activity was observed in the lymph nodes (LNs) in the proximity of both immunized hinds, fluorescently labeled lactose-presenting (LPL-Sm:CaF₂) and control (PL-Sm:CaF₂) NCs (Figure 4a) were simultaneously injected into the right and left hinds, as noted in the figure. Two hours post-injection, LN cells were harvested and analyzed by FACS (Figure 4b–d). We found the paramagnetic nanoconjugates to have no toxic effect on the excised lymphatic cells as compared to the control, even when injected at relatively high concentrations (Figure S21).

Our FACS assessment of the LNs of an inflamed mouse following the nanoconjugate injections clearly shows an accumulation of LPL-Sm:CaF₂ NCs more extensive and much higher than that of PL-Sm:CaF₂ NCs (Figure 4b), with the quantitative analysis of this data set demonstrating a...
greater than 6-fold difference between the two studied groups (Figure 4c, N = 5). Such a significant accumulation (p value = 0.017) of lactose-modified CaF$_2$ NCs confirms that glyconano-
fluorides offer improved immune targeting of inflammatory tissue in vivo, even upon their systemic injection. Note here, as summarized in Figure 4d, CD11b leukocytes (7.1-fold higher, p value = 0.008, for LPL-Sm:CaF$_2$), CD19 B-cells (6.3-fold higher, p value = 0.007, for LPL-Sm:CaF$_2$), and CD8 T-cells (2.7-fold higher, p value = 0.006, for LPL-Sm:CaF$_2$) showed significantly higher accumulation of the targeted lactose-coated
fluorides, representing their lymphatic distribution. These results are in agreement with previous reports that showed that several types of immune cells, including CD11b leukocytes, B-
cells, and T-cells, can recognize and bind synthetic carbohydrate formulations.35,38,59

**Paramagnetic Glyconanoparticles Allow in vivo 19F MRI Mapping of Inflammatory Activity.** To examine the ability to use the obtained glyconanoparticles as nanotracers for background-free in vivo mapping of inflammation with 19F MRI, we exploited in situ labeling of circulating immune cells, an approach frequently used by PFC-based formulations.27,28 To do so, 10 days after being immunized, mice (N = 4, Figure 5) were subcutaneously injected with 20 μL (25 mg/mL of
labeled as “R”) compared to the leg injected with the control nanofluorides (PL-Sm:CaF$_2$, labeled as “L”). This significant (p value = 0.033) 2-fold higher 19F MR signal (Figure 5d) confirms our hypothesis that paramagnetic glyconano-
fluorides can be used as small (<10 nm), sensitive (T$_1$ = 70 ms), and specific (lactose-coating) nanotracers for in vivo background-
free mapping of inflammatory activity with 19F MRI.

**Multiplexed In Vivo 19F MRI of Immune Specificity of Glyconanofluorides.** Capitalizing on the ability to classify different types of synthetic nanofluorides and present them in a “multicolor” fashion, we demonstrated the immune specificity of glyconano-
fluorides (i.e., LPL-Sm:CaF$_2$, colored light blue in Figure 6) over nonglycosylated nanofluorides (i.e., PL-Sm:SmF$_2$, colored yellow in Figure 6), in real time, in the same inflamed tissue. This entailed synthesizing Sm$^{3+}$-doped SrF$_2$ NCs using the same approach employed for the synthesis of Sm:CaF$_2$
NCs. The resultant small-sized, monodispersed Sm:SmF$_2$ NCs provided a single high-resolution 19F NMR peak that resonated at −88 ppm, as expected,24 with the expected short T$_1$ values (Figure S22). We dispersed Sm:SmF$_2$ in water using the synthetic approach shown in Figure 3a, without adding the lactose-modified PLs to obtain PL-Sm:SmF$_2$. The nonglycosy-
lated PL-Sm:SmF$_2$ was of a similar size and shape to that of the glycosylated LPL-Sm:CaF$_2$ (Figure 6a, left) but with well-
resolved high-resolution 19F NMR peaks that differ from one another by more than ~20 ppm (Figure 6b), allowing their spatial distribution to be mapped with 19F MRI (Figure 6c) and presented in a multicolor manner. The specificity for detecting each of the agents (Sm:SmF$_2$ and Sm:CaF$_2$) shown in Figure 6c was evaluated by determining the contrast-to-noise ratio at each of the examined frequency offsets (−88 ppm vs −109 ppm) for each of the studied particles and showed negligible signals overlapping and thus ultimate specificity (Table S3).

This capability to monitor the two types of nanofluorides (glycosylated vs nonglycosylated) simultaneously in the same imaging frame, without overlapping signals and without affecting the two detectable 19F MR signals (Figure 6c, bottom tube, displayed as green color and summarized in Table S3), was used to demonstrate the immune specificity of glyconano-
fluorides in vivo. In this case, an aqueous solution containing similar concentrations of the two types of nanofluorides (PL-Sm:SmF$_2$ and LPL-Sm:CaF$_2$) was injected into the footpads of inflamed mice (shown schematically in Figure 6d). After the injected mouse was localized in the MRI scanner and a 1H MR image acquired to identify the inflamed LN of interest (white arrow in Figure 6e), a 19F NMR spectrum was acquired from the whole imaging frame. Importantly, an identical 19F NMR signal intensity was obtained (Figure 6f) from the injected PL-Sm:SmF$_2$ (−88 ppm) and LPL-Sm:CaF$_2$ (−109 ppm) NCs, reflecting their similar concentration in the tissue of the live subject following their administration. Note that, while 19F MRI detection is limited to the number of 19F spins in the imaging voxel (LPL-Sm:CaF$_2$, Sm:SmF$_2$, PL-Sm:SmF$_2$ are expected to accumulate in the LNs in higher levels as compare to PL-Sm:SmF$_2$), the 19F NMR spectrum averages out the signal of all the spins from the whole imaging volume (similar levels of LPL-Sm:CaF$_2$ and PL-Sm:SmF$_2$). Therefore, sufficient accumulation of nanofluorides in the inflamed LN is required to reach the detectability level in 19F MRI.

Indeed, although the two types nanofluorides were injected as a mixture with equal concentrations (Figure 6f), a much

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**Figure 5. In vivo 19F MRI study of inflamed mice.** Ten days post-
immunization, mice (N = 4) were subcutaneously injected with LPL-Sm:CaF$_2$ NCs (right leg, labeled as “R”) and PL-Sm:CaF$_2$ NCs (left, labeled as “L”). Then, 2 h post-injection mice were anesthetized and scanned with MRI. (a) 1H MRI, (b) 19F MRI, and (c) 1H/19F MRI overlay of a representative mouse. (d) SNR of 19F MRI at the LNs ROIs (N = 4, Student’s t test, * represents a p value <0.05). The in-plane resolutions of the 1H MR and 19F MR images are 0.35 × 0.2 mm$^2$ and 1.4 × 0.78 mm$^2$, respectively; the slice thickness is 1 mm in 1H MRI and 0.78 mm in 19F MRI.

NCs) of LPL-Sm:CaF$_2$ NCs and PL-Sm:CaF$_2$ NCs in the right (labeled “R”) and left (labeled “L”) footpads, respectively. Two hours later, mice were anesthetized and scanned with both 1H MRI (Figure 5a) and 19F MRI (Figure 5b). The localization of the detected nanofluoride-derived 19F MR signal was confirmed by overlaying the obtained 19F MR images on the high-resolution anatomical 1H MR images (Figure 5c). In high correlation with the results obtained from the FACS experiments (Figure 4b,c), massive accumulation in the inflamed LN was clearly observed in the leg in which the paramagnetic glyconano-fluorides were injected (LPL-Sm:CaF$_2$,

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ACS Nano 2021, 15, 7563–7574
larger accumulation of LPL-Sm:CaF₂ (light blue frame) in the LN was observed, in contrast to a much lower ¹⁹F MRI signal of PL-Sm:SrF₂ (yellow frame). This ability to map both the targeted and nontargeted nanotracers and present their spatial distribution and targetability in a “multicolor” fashion is demonstrated in Figure 6h, and the 2-fold increase in ¹⁹F MRI SNR when comparing glyconano-fluorides (LPL-Sm:CaF₂) to nonglycosylated nano-fluorides (PL-Sm:SrF₂) is depicted in Figure 6i. The same 2-fold difference was observed when comparing LPL-Sm:CaF₂ to PL-Sm:CaF₂ injected in two different legs and imaged with “unicolor” ¹⁹F MRI (Figure 5d). This demonstrates the desired capability of spatially mapping multiple nanofabrications, with the added benefit of no unwanted background signal. Further, using simultaneously the same imaging modality, we showcase the potential of using nano-fluorides with their targetability and “multicolor” imaging capabilities for studying biological multiplexity in scenarios where simultaneous mapping of multiple targets is required. Note here that the use of an MRI scanner operating at 15.2 T like the one used here is beneficial for “multicolor” MRI studies that yield better spectral resolution in addition to the higher sensitivity obtained at higher B₀. Nevertheless, the preserved short T₁ at lower magnetic fields (Table S2) implies that designed paramagnetic nanofluorides will be applicable also for studies performed with MRI scanners operating at lower, more common magnetic fields.

**Paramagnetic Glyconano-fluorides Detect Activated Immune Cells at Sites of Neuroinflammation.** Finally, we also examined our synthesized glyconano-fluorides’ ability to map inflammatory processes within different (nonlymphatic) bioenvironments and pathologies. To this end, we employed glyconano-fluorides and ¹⁹F MRI to visualize the recruitment of activated immune cells to a stroke region in the central nervous system (CNS), which typically peaks between 1⁶¹,⁶² and 2 weeks post-ischemic onset. Thus, the injection of the LPL-Sm:CaF₂ and the ¹⁹F MRI experiments were performed 2 weeks after the stroke induction (14 days, Figure 7a), mice were retro-orbitally injected with 20 μL of a LPL-Sm:CaF₂ (25 mg/mL) solution...
strengthen the conclusion that the $^{19}$F MRI signal obtained at the stroke region is a result of neuroinflammation activity.

**CONCLUSION**

Small-sized glyconanoparticles imitate natural glycann-based structures of pathogens for enhanced immune targeting and can alter their properties (i.e., multivalency, size, charge, density, etc.) for a desired purpose. The paramagnetic nano-fluorides introduced here, which exhibit enhanced $^{19}$F MRI sensitivity, offer a small-sized inorganic NC-based platform for the synthesis of nanosized glycomimetics. Using the paramagnetic dopant Sm$^{3+}$, which induces a significant PRE effect for shortening the $T_2$ of nano-fluorides by more than 200-fold without affecting their already short $T_2$ values, an 8-fold enhancement in their $^{19}$F MRI SNR was obtained. By modifying the surface of the paramagnetic nano-fluorides with multivalent lactose moieties, we obtained paramagnetic glyconanotfuorides that could detect activated immune cells at sites of inflammation following their systemic administration, which we were able to present as hotspot MRI maps. The proposed approach for immune targeting and spatial in vivo mapping of inflammatory activity, demonstrated here in both footpad-induced inflammation and in neuroinflammation following ischemic stroke models, could be extended to other diseases in which inflammation plays key roles. In light of the recent advances in engineering small-sized (~10 nm), nature-inspired mimetics, we envision that the proposed paramagnetic nano-fluorides could be further evolved to yield a wider range of designs and applications.

**METHODS**

**Synthesis of Oleate-Coated CaF$_2$/Doped-CaF$_2$/Doped-SrF$_2$ NCs.** In a typical synthesis, 4.2 mL of oleic acid, 12 mL of ethanol, and 0.1 g of sodium hydroxide were mixed under vigorous stirring in a round-bottom flask at room temperature (RT) for 6 h. To the resultant homogeneous milky mixture were added once 5 mL of an aequous solution containing 2 mmol M(NO$_3$)$_2$·4H$_2$O (M = Ca$^{2+}$, Sr$^{2+}$), the relevant mol % of Ln$^{3+}$ precursor (added as Ln(NO$_3$)$_3$ salt), and 5 mL of a 4 mmol NaF aqueous solution. The obtained mixture was vigorously stirred for 1 h and then heated to 160 °C in a Teflon-lined sealed autoclave reactor for 16 h. The reaction mixture was left to cool at RT and then centrifuged at 8500 rpm for 10 min. The obtained precipitate was washed three times with cyclohexane/ethanol (1:10 ratio), followed by 10 min centrifugation at 6500 rpm. The final product was obtained as a white solid, which was dispersed in 2 mL of cyclohexane, followed by an additional centrifugation for 3 min at 2000 rpm in order to remove any remaining impurities and aggregates. The resultant clear solution was kept in a glass vial for further characterization.

**Fabrication of Glyconanofluorides.** In order to provide the suspended hydrophobic lanthanide-doped NCs (either Ln:CaF$_2$ or Ln: SrF$_2$) with the required water solubility for biological applications, their surface was covered with phospholipids, which contain both hydrophobic and hydrophilic regions. In a typical synthesis, 30 mg of myristoyl hydroxyphosphatidylcholine, 2 mg of cholesterol, 5 mg of 18:0 PEG1000-PE, and 0.15 mg of 18:1 lactosyl-PE (for the lactose-modified NCs) were dissolved in 5 mL of a 20:1 chloroform/methanol solvent mixture. This solution was then added to 2 mL of chloroform containing 30 mg of the dissolved desired NCs. The resultant lipid-coated NC solution was then added dropwise to 30 mL of deionized water at 80 °C under vigorous stirring and left to cool to RT. Next, it was centrifuged at 2000 rpm for 3 min, and the sediment (large aggregates or unsuspended nanoparticles) was discarded. To concentrate the supernatant, it was transferred to a centrifugal filter unit (MWCO 10 kDa) and centrifuged at 5000g for several 15 min cycles until the total volume reached 500 μL. Then, the sample went and then, 1 h later, anesthetized for further $^1$H and $^{19}$F MRI examination. As hypothesized, the glyconanoparticles accumulated in the lesioned area, as clearly shown for a representative mouse in Figure 7b,c (the examination of all four mice is presented in Figure S24). A localized $^{19}$F MR spectroscopy of the lesioned region revealed a characteristic $^{19}$F NMR spectrum of CaF$_2$ with the expected $-109$ ppm resonance (Figure 7d), which further confirmed the accumulation of intact glyconanofluorides in the stroke area. Importantly, using confocal microscopy on brain sections collected from these mice, we observed a massive accumulation of the glycanofluorides in Iba-1-positive cells recruited to the vicinity of the stroke region (Figure 7e and Figure S25). These observations corroborate our previous results, showing that glycopolymers, such as dextran, accumulate in activated Iba-1-expressing cells 2 weeks after a stroke when their numbers increase around the infarcted area and in the penumbra nearby. Interestingly, when administered 2 h post-stroke, both fluorescently labeled dextran and LPL-Sm:CaF$_2$ were found mostly in the blood vessels and not in Iba-1 expressing cells (Figure S26). Nevertheless, 2 weeks after the stroke, both the glycopolymer (dextran) and the glyconanoparticles (LPL-Sm:CaF$_2$) could be found mostly in Iba-1 expressing cells that populated the areas mentioned above (Figure S27). Based on our finding that glyconanofluorides massively accumulate in activated immune cells of inflamed lymph nodes (Figures 4–6), the results shown in Figure 7 and Figures S23–S27...
through several dialysis cycles, either in ultrapureified water, to remove excess unbound phospholipids, or in phosphate buffer saline (PBS), for further biological usage.

**Animal Studies.** All animal studies were performed in accordance with the Weizmann Institute’s Institutional Animal Care and Use Committee (IACUC) guidelines and regulations.

**Local Inflammation Induction.** Eight-week-old female SJL/J mice were immunized by the subcutaneous injection of 50 μL of an immunogenic emulsion (in PBS) composed of complete Freund’s adjuvant containing 150 μg of Mycobacterium tuberculosis H37Ra. Mice were then placed in their cage for 10 days to develop inflammation.

**Stroke Induction.** This stroke model was previously described by Karatas et al. Briefly, mice were anesthetized with 4% isoflurane in an induction chamber, placed in a stereotactic frame, and kept on 1% isoflurane. The body temperature was maintained at 37 ± 0.1 °C using a homeothermic blanket control unit (TC-1000, CWE INC, USA). Surgery was performed under an operating biomicroscope. The scalp was opened, and the cranial sutures and bregma were exposed. The right temporal muscle was pushed aside until the squamous part of the temporal bone was exposed. The area just above the junction between the zygomatic arch and the squamous bone was thinned, using a high-speed drill, and cooled with saline. The trace of MCA was visualized through the thinned temporal bone. The thinned bone was removed carefully to avoid damaging the MCA. A piece of 30% FeCl₃-saturated filter paper was removed and a clot was observed in the MCA, under the operating biomicroscope. The right temporal muscle was placed back, and the scalp was closed using Vetbond 3M (3M, Minneapolis, USA). Surgery was performed 10 days post-immunization when maximum inflammatory activity is expected in the above-mentioned animal model. Mice were sacrificed 2 h post-nanoliposome injection (20 μL of 25 mg/mL of NCs), and the cells of their popliteal LN’s were harvested and suspended in PBS for FACS analysis using a LSR II flow cytometer (BD Biosciences). Quantitative analysis of fluorescent cells was performed using Flowjo software (version 10, TreeStar, Oregon, USA).

**Toxicity Determination of Paramagnetic Nano- fluorides.** Lymphatic cells were harvested at day 10 post-immunization from two groups of mice (N = 6 mice in each group) following the subcutaneous injection (20 μL of 75 mg/mL of NCs) of either LPL-Sm:CaF₂ NCs or PL-Sm:CaF₂ NCs. The viability of nonfixed lymphatic cells was evaluated by a commonly used DNA-binding dye assay using 4’,6-diamidino-2-phenylindole (DAPI). Excised cells were resuspended in PBS and stained for 20 min at 4 °C with 1 μg/mL DAPI solution. Cells were then washed twice with cold PBS and immediately analyzed by flow cytometry using a UV laser (355/450 nm).

**Evaluation of Lymphatic Accumulation of Nanofluorides.** Ten days post-immunization, five mice were simultaneously injected with 20 μL of glycan-presenting (LPL-Sm:CaF₂) and control (PL-Sm:CaF₂) NCs in their right and left hind, respectively. Two hours post-injection, mice were sacrificed and popliteal lymph node cells were immediately harvested and suspended in PBS for FACS analysis of the fluorescence of NC accumulation.

**Magnetic Resonance Imaging.** All MRI experiments were performed on a 15.2 T horizontal scanner (Biospec, Bruker) using a dual resonator ¹H/¹⁹F 23 mm volume coil. For ¹⁹F imaging, standard UTE-3D sequence (provided by Bruker) was used, using the following parameters: dummy scans = 250, dummy delay = 1000 ms, bandwidth = 10000 Hz, excitation pulse-length = 0.0043 ms, excitation pulse bandwidth = 300000 Hz, receiver gain = 203.

**Phantom Experiments (In Vitro).** In order to evaluate the gain in SNR upon the doping of CaF₂ NCs (5 mol % of dopant), water-soluble nanofluorides (PL-CalF₂, PL-La:CaF₂, and PL-Sm:CaF₂) were placed in 5 mm NMR tubes at a final fluoride concentration of 70 mM for each of the studied samples. First, ¹H MRI was acquired using the RARE (rapid acquisition with relaxation enhancement) sequence to obtain the localization of the tubes with the following parameters: RARE factor = 8, TR/TE = 1000/20 ms, 1 mm thick slice, FOV = 20 × 20 cm², matrix size = 128 × 128, spatial resolution = 0.015 × 0.015 cm², with an average and experiment time of 16 s. The ¹H MRI was acquired using a three-dimensional ultrashort TE (3D-UTE) acquisition scheme with the following parameters. For Figure 2c, TR = 4 ms, TE = 8 μs, FOV = 2.0 × 2.0 × 3.0 cm³, matrix size = 32 × 32 × 32, spatial resolution = 0.062 × 0.062 × 0.093 cm³. The ¹⁹F MRI acquisition was completed in 25 min using 128 number of averages. For Figure 2e, TR = 4 ms, TE = 8 μs, FOV = 3.2 × 3.2 × 3.2 cm³, matrix size = 32 × 32 × 32, spatial resolution = 0.1 × 0.1 × 0.1 cm³. The signal was an average of 128 times, and the ¹⁹F MRI data were acquired in 1 h.

**In Vivo MRI of Inflamed Mice.** In order to prevent any residual ¹⁹F MR signal of fluorinated anesthetics (i.e., isoflurane) that may cause a ¹⁹F MRI background signal, mice were anesthetized by an intraperitoneal injection of 1 mg/kg medetomidine (Dormitor) and 75 mg/kg ketamine. Immunized mice (N = 4) were injected subcutaneously with 20 μL (25 mg/mL) of LPL-Sm:CaF₂ NCs and PL-Sm:CaF₂ NCs in the right and left footpad, respectively. Two hours after the nanofluorides injections, the mice were placed on in the MRI scanner, and ¹H MRI was acquired to obtain high-resolution anatomical images of the scanned mice with both RARE and 3D-UTE protocols. ¹H RARE was acquired with the following parameters: RARE factor = 8, TR/TE = 1000/5 ms, 19 slices of 1 mm thickness, FOV = 4.5 × 2.5 cm², matrix size = 128 × 128, spatial resolution = 0.035 × 0.019 cm² with 1 average and an experiment time of 16 s. 3D-UTE was acquired with the following parameters: TR = 4 ms, TE = 8 μs, FOV = 4.5 × 2.5 × 2.5 cm³, matrix size = 128 × 128 × 128, spatial resolution = 0.035 × 0.019 × 0.019 cm³ with 1 average that resulted in a scan time of 3 min. Then, ¹⁹F MRI was acquired using a 3D-UTE sequence with TR = 4 ms, TE = 8 μs, matrix size = 32 × 32 × 32, and 150 averages resulted in a scan time of 30 min. It is important to mention that the FOV dimensions were adjusted to those used to acquire the ¹H MR image to allow an accurate overlay of the ¹⁹F MR images on the anatomical ¹H MR images.

**In Vivo Multiplexed MRI.** Immunized mice were injected subcutaneously with 20 μL of a mixture of Lac-PL-Sm:CaF₂ NCs and PL-Sm:SmF₂ NCs (25 mg/mL). Mice were then placed on the MRI scanner, and ¹H MRI was acquired to obtain high-resolution anatomical images of the scanned mice with the RARE protocol. ¹H RARE was acquired with the following parameters: RARE factor = 8, TR/TE = 1000/20 ms, 15 slices of 1 mm thickness, FOV = 4.5 × 2.5 cm², matrix size = 128 × 128 × 128, spatial resolution = 0.035 × 0.019 × 0.019 cm³ with 1 average that resulted in a scan time of 3 min. Then, ¹⁹F MRI was acquired using a 3D-UTE sequence with TR = 4 ms, TE = 8 μs, matrix size = 32 × 32 × 32, spatial resolution = 0.14 × 0.078 × 0.14 cm³, and 100 averages, resulting in a scan time of 20 min. The ¹⁹F signals were acquired separately, once with the RF excitation pulse set to the frequency of CaF₂ (δ = -109 ppm), bandwidth set to 16,000 Hz, based on the width of the ¹⁹F NMR signal at the baseline, derived from its ¹H spectrum, and once for the SmF₂ frequency (δ = -88 ppm) with the relevant bandwidth. It is important to mention that the FOV dimensions were adjusted to those used to acquire ¹H MRI to allow further accurate overlay of the ¹⁹F MR images on the anatomical ¹H MR images. The ¹⁹F NMR spectrum of the injected mice was acquired using a simple ¹⁹F single-pulse protocol, with the following parameters: TR = 200 ms, TE = 50 μs with 500 scans, which resulted in 2 a min scan time.

**In Vivo MRI of Mice after Stroke.** Fourteen days after stroke, mice (N = 4) were injected retro-orbitally with 20 μL (25 mg/mL) of LPL-Sm:CaF₂ NCs. One hour after the nanofluorides injection, the mice were placed in the MRI scanner and ¹H MRI was acquired to obtain high-resolution anatomical images of the scanned mice with both RARE and 3D-UTE protocols. ¹H RARE was acquired with the following parameters: RARE factor = 8, TR/TE = 1000/5 ms, 19 slices of 1 mm thickness, FOV = 4.5 × 2.5 cm², matrix size = 128 × 258
128, spatial resolution = 0.035 × 0.019 cm² with 1 average and an experiment time of 16 s. 19F MRI was acquired using a 3D-UTE sequence with TR = 4 ms, TE = 8 µs, FOV = 3.0 × 3.0 × 3.2 cm³, matrix size = 32 × 32 × 32, and 150 averages resulted in a scan time of 30 min. All other experimental procedures, characterization data, and supporting figures are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.1c01040.

Additional descriptions of the performed experiments and supporting figures (PDF)

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D.C. and A.B.-S. designed the study. D.C. and R.M. developed the synthesis. D.C. carried out the nanofluoride synthesis, purification, and characterization and performed high-resolution NMR experiments and MRI acquisitions. D.C. and L.H. performed all EM-related studies (TEM, EDS, and STEM). A.G. performed the biodistribution experiments including injections, imaging and data analysis. H.A.-A. performed the animal studies, including the inflammation induction and FACS. D.C., H.A.-A., A.L., and Y.A. performed the immunostaining and confocal microscopy. D.K. performed the stroke model. P.B. conceptualized the ischemia experiments and provided resources. D.C. and A.B.-S. wrote the manuscript.

Notes
The authors declare no competing financial interest.

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