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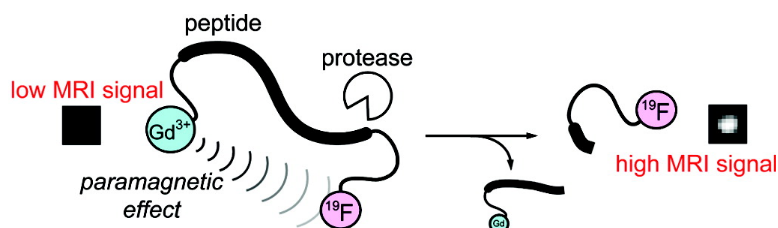

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Paramagnetic Relaxation-Based ^{19}F MRI Probe To Detect Protease Activity

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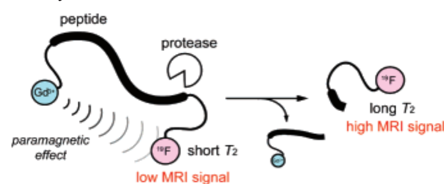
Real-time imaging of enzyme activities in vivo offers valuable information in understanding living systems and in developing medicine for various types of diseases. Currently, a variety of fluorescent probes for detecting enzyme activities are generally used for their high-sensitivity characteristics. However, general fluorescence imaging methods are not suitable for in vivo studies because visible fluorescence scarcely transmits through animal bodies. Although near-infrared fluorophores are useful for in vivo imaging for tissue surfaces, they cannot be applied to the deep section of the organ. On the other hand, magnetic resonance imaging (MRI) is known as an imaging technique adequate for in vivo studies.

^{1}H is a highly NMR-sensitive nuclide and abundant in living bodies. Thus, ^{1}H MRI is used as a powerful diagnostic imaging technique for identifying many human pathologies or medical conditions. Paramagnetic contrast agents such as several types of Gd complexes are in clinical use for their abilities to enhance the signal intensity by shortening the longitudinal relaxation time (T_1) of water protons. Presently, many scientists are interested in modifying the structures of the contrast agents to be functional in molecular imaging of biomolecules.[1] MRI probes for pH,[2] metal ions,[3] enzyme activities,[4] and so on have been developed. However, ^{1}H MRI often suffers from interference or low contrast due to the background signals from intrinsic ^{1}H, which hamper interpretation of the resultant images. Therefore, non-proton MRI is currently drawing a fair amount of attention.

One of the most promising nuclides for MRI is ^{19}F.[5] This nuclide has a high gyromagnetic ratio (γ) of 40.05 MHz/T and a 100% natural isotopic abundance ratio. Thus, the NMR sensitivity of ^{19}F is 0.83 relative to ^{1}H. Conveniently, due to their close γ values, ^{19}F NMR can be measured with most ^{1}H NMR instruments by appropriately tuning the RF coils. In our bodies, ^{19}F atoms are concentrated in the form of solid salts mostly in bones and teeth. Thus, the transverse relaxation time (T_2) of the intrinsic ^{19}F is extremely shortened,[6] and the MRI signal is hardly detectable. When ^{19}F-containing compounds are treated in human or animals, only the extrinsic ^{19}F MRI signals can be monitored without interference from background signals. For these above reasons, functional probes for ^{19}F MRI are very attractive for in vivo molecular imaging.

Known ^{19}F MRI probes are roughly categorized into two groups; one is the group of ^{19}F-containing compounds which accumulate in specific sites. Higuchi and co-workers synthesized a ^{19}F-containing thioflavin derivative that accumulates in amyloid β (Aβ) aggregates and visualized Aβ plaques in living animals by ^{19}F MRI.[7]

Scheme 1. Design Principle of ^{19}F MRI Probe Detecting a Protease Activity



Another class of ^{19}F MRI probes is active agents.[5] They undergo chemical modification by the target molecules and then change their NMR parameters. Mason and co-workers have developed ^{19}F MRI probes detecting reporter enzyme activities by the chemical shift change.[8] These probes are hydrolyzed by the reporter enzyme and change the ^{19}F chemical shift. Although this approach is promising, this is highly dependent on the magnitude of chemical shift changes coupled with the target reaction, where sometimes the ranges of the chemical shift changes are limited. We here propose a novel design strategy for ^{19}F MRI probes in detecting protease activity.

We constructed a design principle whereby the intramolecular paramagnetic effect for T_2 of the ^{19}F NMR signal can be modulated by protease activities. T_2 is an important contrast factor for MRI, as the apparent intensity of the MRI signal directly depends on T_2 values. Generally, paramagnetic metal ions such as Fe^{3+}, Gd^{3+}, or the paramagnetic molecules such as O_2 shorten the T_2 of samples by paramagnetic relaxation enhancement (PRE).[9] In particular, Gd^{3+} has a very strong relaxivity (T_2-shortening activity) because of its large electron spin quantum number. When ^{19}F nuclei and a Gd^{3+} ion are attached to a short peptide, the ^{19}F nuclei exhibit a strong paramagnetic effect from the Gd^{3+}. Thus, the T_2 of the compound in the ^{19}F NMR would be shortened, and the MRI signal would be attenuated. If the peptide has a substrate sequence which can be cleaved by a certain protease, incubation of the compound with the protease should induce the extension of the T_2 and the enhancement of the ^{19}F MRI signal (Scheme 1).

According to the above strategy, we synthesized a ^{19}F MRI probe, Gd-DOTA-DEVD-Tfb, for detecting caspase-3 activity (Figure 1). Caspase-3 is a marker enzyme of apoptosis and is used in the evaluation of anticancer agents inducing apoptosis of tumor cells. The probe consists mainly of three parts, which are a Gd^{3+} complex, an enzyme substrate peptide, and a ^{19}F-containing group. The peptide sequence is DEVD because caspase-3 selectively cleaves the C-terminal peptide bond of the sequence DXXD (X: optional).[10] A macrocyclic metal ligand, DOTA, was attached via a β-alanine linker with the N-terminus of ^{19}F-containing peptide DEVD-Tfb, which was synthesized by Fmoc solid-phase peptide synthesis. The ligand-conjugated peptide, DOTA-DEVD-Tfb, was complexed with Gd^{3+} ion and purified with a reversed-phase HPLC to yield Gd-

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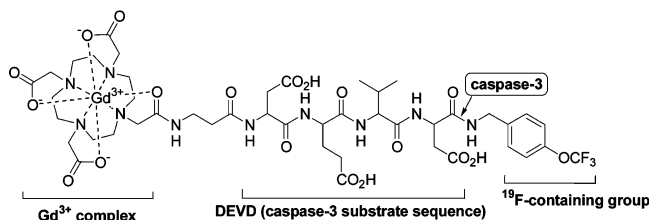

Figure 1. Structure of Gd-DOTA-DEVD-Tfb.

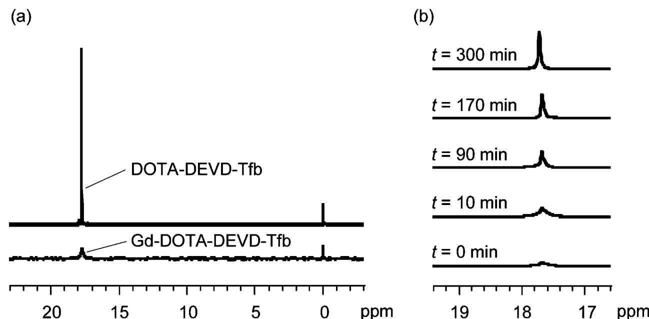

Figure 2. (a) ^{19}F NMR spectra of DOTA-DEVD-Tfb (1 mM) and Gd-DOTA-DEVD-Tfb (1 mM). Sodium trifluoroacetate was added as an internal standard (0 ppm). (b) Time-dependent ^{19}F NMR spectral change of Gd-DOTA-DEVD-Tfb with caspase-3 at 37 °C.

DOTA-DEVD-Tfb. The probe structure was identified with ESI-TOF MS, and its purity was confirmed by HPLC and ^{19}F NMR.

Next, we measured the ^{19}F T_1 and T_2 values of the probe free from and in complex with Gd^{3+}. The T_1 and T_2 of DOTA-DEVD-Tfb (1 mM) were 1.910 and 0.326 s, respectively. In contrast, the values of Gd-DOTA-DEVD-Tfb could not be measured, due to markedly shorter relaxation times and the low signal intensity of the ^{19}F resonance. This result indicates that the ^{19}F nucleus of Gd-DOTA-DEVD-Tfb undergoes strong PRE by Gd^{3+}. This effect was explicitly shown in the one-dimensional ^{19}F NMR spectrum, in which the peak intensity of Gd-DOTA-DEVD-Tfb was largely decreased with broadening as compared with that of DOTA-DEVD-Tfb (Figure 2a). When we treated the Gd-DOTA-DEVD-Tfb with caspase-3 at 37 °C, the ^{19}F NMR peak became sharper and higher in a time-dependent manner (Figure 2b). This indicated the intramolecular paramagnetic effect from Gd^{3+} to ^{19}F was cancelled by the cleavage of the probe. The T_1 and T_2 of the 1 mM cleaved product were extended to 0.122 and 0.032 s, respectively. These values are still shorter than those of DOTA-DEVD-Tfb. An additional experiment suggests that this is because of the intermolecular PRE by the Gd^{3+} complex (Figure S4 in Supporting Information).

Finally, an attempt was made to visualize caspase-3 activity using a ^{19}F MRI phantom to verify the practical applicability of this probe and its sensing principle. As was expected, Gd-DOTA-DEVD-Tfb showed no signal on the ^{19}F MRI phantom image. Time course of the density-weighted ^{19}F MR images of Gd-DOTA-DEVD-Tfb with and without caspase-3 are shown in Figure 3. Caspase-3 activity induced a noticeable signal enhancement. This result is compatible with the one-dimensional ^{19}F NMR data shown in Figure 2b. We used a relatively high probe concentration for MRI because of the sensitivity limitation due to our current instrument setup. However, the intrinsically high sensitivity for the ^{19}F signal of the probe is demonstrated by the NMR spectra measured at 1−5 μM by a well-tuned spectrometer (Figure S5 in Supporting Information). This result indicates the possibility that a suitable instrument enables in vivo ^{19}F MR imaging in the future.

In conclusion, a novel design principle of ^{19}F MRI probes detecting protease activity was developed. This principle is based

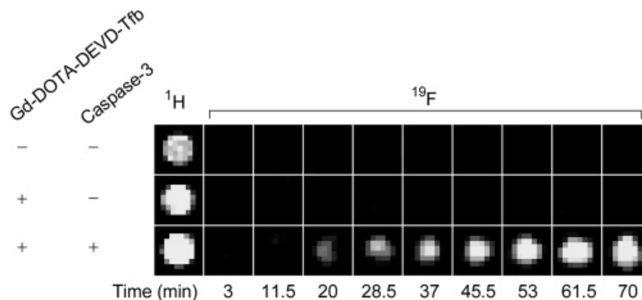

Figure 3. Time course of density-weighted ^{19}F MR images of Gd-DOTA-DEVD-Tfb (1 mM) with or without caspase-3 at 37 °C.

on MRI signal quenching from the intramolecular paramagnetic effect of Gd^{3+}. The intramolecular Gd^{3+} made the T_2 of the probe too short to be measured, with the paramagnetic effect which can be cancelled by the probe hydrolyzation by caspase-3. T_2 is a parameter that can be used to generate contrasts in MR images. Using this probe as a positive contrast agent, we demonstrated that the probe could detect caspase-3 activity spatially from the phantom image using ^{19}F MRI. This method could be applied to the sensing of not only other kinds of proteases but also other hydrolases such as nucleases and phosphodiesterases. It is expected that this sensing strategy might become the basis for the next stage of in vivo molecular imaging techniques.

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Supporting Information Available: Detailed experimental procedures and supplementary results. This material is available free of charge via the Internet at http://pubs.acs.org.

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