Molecular Imaging

DOI: 10.1002/anie.200806328

Dual-Function Probe to Detect Protease Activity for Fluorescence Measurement and ^{19}F MRI**

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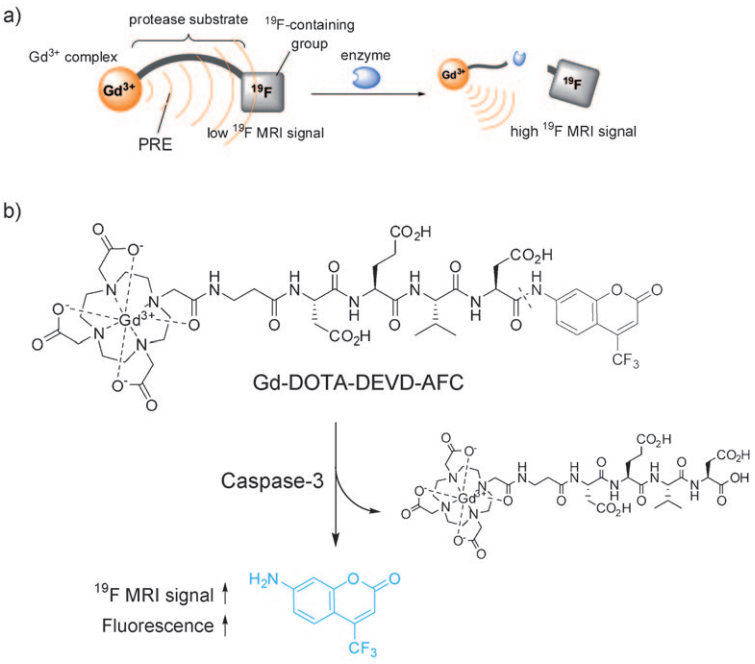
Noninvasive molecular imaging techniques are important for understanding the actual mechanisms of biological systems. In biological sciences, especially those involving cellular systems, the most widely used imaging technique is fluorescence microscopy, because of its high sensitivity, high spatiotemporal resolution, and simple experimental procedure.[1] On the other hand, magnetic resonance imaging (MRI) is one of the most successful imaging techniques in the field of clinical diagnosis. As MRI can visualize deep regions of animal bodies,[2] application of MRI to in vivo imaging of biomolecules is attracting attention.[3] Several ^{1}H MRI probes have been developed to investigate pH values,[4] metal ions,[5] and enzyme activities.[6]

Recently, heteronuclear MRI has been attracting considerable attention as an alternative molecular imaging technique. One of the most promising nuclides for MRI is ^{19}F,[7] which has a high NMR sensitivity that is comparable to that of ^{1}H, and almost no intrinsic ^{19}F signals can be observed in living animals. ^{19}F MRI does not have the drawback of background signals from intrinsic biomolecules, which interfere with the probe signals. Very recently, we developed a novel design strategy for ^{19}F MRI probes that can detect protease activity.[8] We exploited the paramagnetic relaxation enhancement (PRE) effect to achieve off/on switching of the probe MRI signals (Scheme 1a). Using the ^{19}F MRI probe Gd-DOTA-DEVD-Tfb (Tfb = *para*-trifluoromethoxybenzyl) based on this mechanism, we were successful in detecting caspase-3 activity by ^{19}F MRI.



Scheme 1. a) Representation of ^{19}F MRI detection of protease activity. b) Chemical structure of Gd-DOTA-DEVD-AFC and its reaction scheme for detecting caspase-3 activity.

Although MRI can visualize deep regions of living bodies, its sensitivity is inferior to that of fluorescence measurement. The lower sensitivity requires longer accumulation time for imaging. If the probes are multifunctional, we can choose the appropriate imaging method in accordance with the experimental conditions. Higuchi et al. developed the dual-function probe (*E*,*E*)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy)-styrylbenzene (FSB), which aggregates to amyloid β (Aβ) plaques, for ^{19}F MRI and fluorescence measurements.[9] ^{19}F MRI signals localized on Aβ plaques were observed in living mice in vivo, and fluorescence signals in brain slices ex vivo.[9] As such complementary experiments have resulted in more reliable conclusions, development of multimodal imaging probes is very important.[10] Herein we report a dual-function probe to detect protease activity by fluorescence measurement and ^{19}F MRI that is based on the development of Gd-DOTA-DEVD-Tfb.[8]

We chose 7-amino-4-trifluoromethylcoumarin (AFC) as a reporter group that is active in both ^{19}F MRI and fluorescence measurement. AFC is strongly fluorescent in polar solvents,

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[**] This work was supported in part by MEXT of Japan. MRI = magnetic resonance imaging.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200806328.

and the fluorescence properties of 7-aminocoumarin derivatives depend on the electron-donating ability of the 7-amino group.[11] Usually, the peptide modification on the 7-amino group induces a blue shift of the fluorescence spectrum with a decrease in fluorescence intensity. Thus, AFC has been utilized as the fluorophore for protease activity detection.[12] Furthermore, the ^{19}F NMR spectrum of AFC shows only a singlet peak without any coupling to intramolecular protons. AFC is thus appropriate for ^{19}F MRI.

We designed a bimodal probe Gd-DOTA-DEVD-AFC (Scheme 1), in which the probe consists of mainly three parts: Gd^{3+}-DOTA complex (DOTA = 1,4,7,10- tetraazacyclododecane-1,4,7,10-tetraacetate), caspase-3 substrate peptide (DEVD), and ^{19}F-containing fluorophore (AFC). When caspase-3 cleaves the C terminus of the DEVD sequence, AFC is produced. After the enzyme is cleaved, the ^{19}F MRI signal is increased in much the same manner as in Gd-DOTA-DEVD-Tfb (Scheme 1a). Simultaneously, the fluorescence spectrum of AFC is increased. Thus, Gd-DOTA-DEVD-AFC is expected to work as a bimodal probe that detects caspase-3 activity.

The Gd-DOTA-DEVD-AFC probe was synthesized using Fmoc solid-phase chemistry, followed by complex formation with the Gd^{3+} ion (Scheme 2). The excitation peak of Gd-DOTA-DEVD-AFC is at 340 nm, and irradiation at 400 nm results in little fluorescence emission. The incubation of the probe with caspase-3 at 37 °C induced the excitation spectral shift toward longer wavelengths. Therefore, when the probe was excited at 400 nm, the emission at around 500 nm was substantially increased (Figure 1). From the fluorescence measurements, the kinetic parameters for hydrolysis of Gd-DOTA-DEVD-AFC by caspase-3 were measured. The V_{max}/K_m value of Gd-DOTA-DEVD-AFC is 7.61×10^{-3} s^{-1}. On the other hand, V_{max}/K_m of Ac-DEVD-AMC, the commercially available fluorescent substrate (AMC = 7-amino-4-methylcoumarin), is 9.91×10^{-4} s^{-1}. This result indicates that Gd-DOTA complex does not hinder the enzyme reaction at all. Thus, Gd-DOTA-DEVD-AFC can be used as a superior fluorogenic probe for detecting caspase-3 activity.

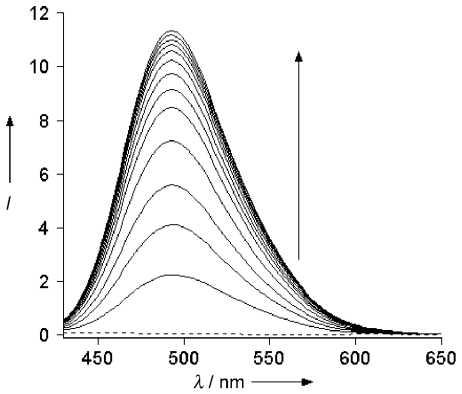
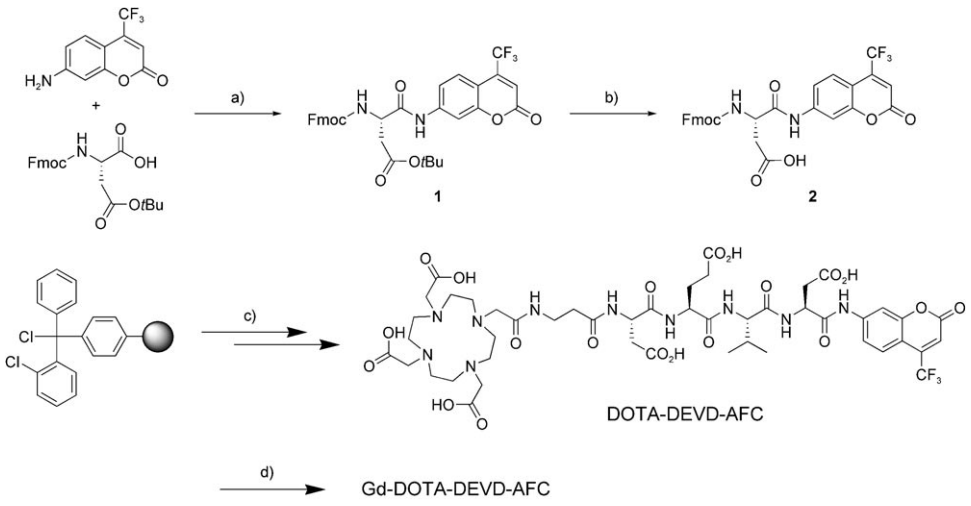


Figure 1. Time-dependent emission spectra of Gd-DOTA-DEVD-AFC (10 μM) with casapse-3 (0.84 mU) in the reaction buffer (pH 7.4) at 37 °C. The spectra were measured every 2 min after the addition of the enzyme. The dotted line indicates no caspase-3. The excitation wavelength: 400 nm. Reaction buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4, 50 mM) containing glycerol (10%), NaCl (100 mM), dithiothritol (DTT, 10 mM), ethylenediaminetetraacetic acid (EDTA, 1 mM), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, 0.1%).

We measured the ^{19}F NMR spectra of Gd-DOTA-DEVD-AFC and its metal-free analogue DOTA-DEVD-AFC. The NMR signal of Gd-DOTA-DEVD-AFC was broad and weak compared to that of the Gd^{3+}-free DOTA-DEVD-AFC (Supporting Information). This change in peak shape and intensity suggests that ^{19}F undergoes an intramolecular PRE effect from the Gd^{3+} ion. Longitudinal (T_1) and transverse (T_2) relaxation times of DOTA-DEVD-AFC (250 μM) were (0.479 ± 0.003) s and (0.152 ± 0.006) s, respectively (Table 1). In case of Gd-DOTA-DEVD-AFC, we could not estimate either T_1 or T_2, because these relaxation times were markedly shorter and the ^{19}F NMR signal intensity was low. From molecular modeling, the distance between the Gd^{3+} ion and the ^{19}F atom in the probe was estimated to be less than 25 Å. However, as the substrate peptide is flexible, the Gd^{3+} ion can be distributed in closer proximity to ^{19}F, such that the PRE effect works efficiently.



Scheme 2. Synthetic route to Gd-DOTA-DEVD-AFC. a) $POCl_3$, pyridine. b) trifluoroacetic acid. c) Fmoc peptide synthesis: **2**, Fmoc-Val-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-β-Ala-OH, tris-*t*Bu-DOTA, deprotection. d) $GdCl_3 \cdot 6H_2O$, 100 mM HEPES buffer (pH 7.4). Fmoc = fluorenylmethyloxycarbonyl.

Next, we performed an enzyme assay using ^{19}F NMR spectroscopy. When Gd-DOTA-DEVD-AFC was treated with caspase-3 in the reaction buffer at 37 °C, a sharper and a more intense ^{19}F NMR signal was observed, with a slight downfield shift (Figure 2). T_1 and T_2 of the cleaved product (250 μM) were elongated to (0.38 ± 0.04) s and (0.097 ± 0.004) s, respectively (Table 1). This finding indicates that the intramolecular PRE effect from the Gd^{3+} ion to the ^{19}F atom was cancelled owing to the cleavage of the probe. After complete cleavage by

Table 1: Longitudinal and transverse relaxation times of synthesized probes.

	T_1 [s][a]	T_2 [s][a]
DOTA-DEVD-AFC	0.479(3)	0.152(6)
Gd-DOTA-DEVD-AFC	–[b]	–[b]
Gd-DOTA-DEVD-AFC + caspase-3	0.38(4)[c]	0.097(4)[c]

[a] Parenthesis denotes standard deviation ($n=3$). [b] The relaxation time was too short to be determined. [c] The relaxation times were measured after the enzyme (250 μM) reaction was complete.

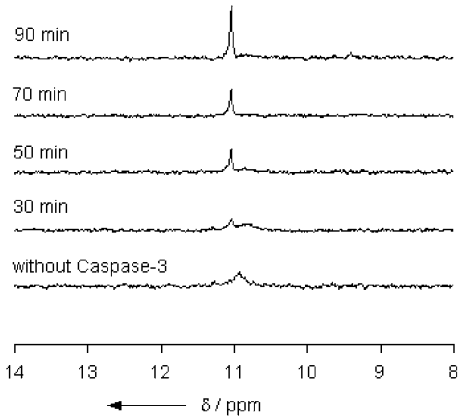


Figure 2. Time-dependent ^{19}F NMR spectra of Gd-DOTA-DEVD-AFC (250 μM) after addition of caspase-3 (1.25 mU) at 37 °C. Reaction buffer: As in Figure 1 plus D_2O (5%).

caspase-3 (confirmed by HPLC), the relaxation times T_1 and T_2 were lower than those observed for the metal-free ligand. These shorter relaxation times are most likely due to the intermolecular PRE of the cleaved Gd-DOTA (Supporting Information, Figure S4).

Finally, we attempted to visualize caspase-3 activity using a ^{19}F MRI phantom with Gd-DOTA-DEVD-AFC. Because of the extremely short relaxation time T_2, the ^{19}F MRI of Gd-DOTA-DEVD-AFC had no signals. When caspase-3 was added to the solution of Gd-DOTA-DEVD-AFC, augmentation of the ^{19}F MRI signal of the probe was observed (Figure 3).

In conclusion, we developed a novel dual-function probe, Gd-DOTA-DEVD-AFC, which detects caspase-3 activity by dual signal increase in fluorescence and in ^{19}F MRI. Because fluorescence measurement and MRI provide complementary information, such dual-mode probes should be quite useful for various biological experiments. Although several multimodal probes, such as fluorescence measurement and MRI, have been developed recently,[13] most probes are constructed by simple attachment of reporter moieties such as fluorescence dyes or MRI contrast agents. In contrast, multimodal probes accompanying plural signal enhancement have been scarcely reported. Such multimodal smart probes would be the next-generation probes in multimodal imaging for detecting enzyme activity.

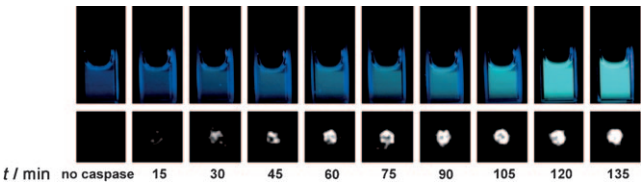


Figure 3. Time-dependent fluorescence images (top, λ_{ex}: 400 nm) and ^{19}F MR phantom images (bottom, diameter: approximately 2 mm) of Gd-DOTA-DEVD-AFC (10 μM for fluorescence measurement and 1 mM for ^{19}F MRI) with caspase-3 (60 nU for fluorescence measurement and 2 mU for ^{19}F MRI) at 37 °C. Reaction buffer: As in Figure 1. For ^{19}F MRI, [D_6]DMSO (20%) was introduced into the reaction buffer.

Received: December 26, 2008
Published online: April 7, 2009

Keywords: fluorescence · magnetic resonance imaging · molecular imaging · multimodal probe · proteases

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