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Signal turn-on probe for nucleic acid detection based on ^{19}F nuclear magnetic resonance

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ABSTRACT

To image gene expression in vivo, we designed and synthesized a novel signal turn-on probe for ^{19}F nuclear magnetic resonance (MR) imaging based on paramagnetic relaxation enhancement. The stem-loop structured oligodeoxyribonucleotide (ODN) having a molecular beacon sequence for point mutated K-ras mRNA was doubly labeled with bis(trifluoromethyl)benzene moiety and Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid chelate moiety at the each termini of the ODN probe, respectively. We found that the ^{19}F MR signal of the bis(trifluoromethyl)benzene moiety tethered at the 5′ termini of the probe turned on by the addition of complementary ODN. The probe has the potential to image gene expressions in vivo.

In vivo imaging technologies, such as X-ray computed tomography, positron emission tomography, and nuclear magnetic resonance imaging, are promising technologies for diagnosis of various disorders and follow up after surgical treatments. To image biological events in greater detail in vivo, molecular probes targeting biomolecules, such as disease marker enzymes[1] or receptors,[2] have been developed. Imaging probes that can image enzyme activity,[3–5] protein,[6] or protein agrregate[7] by ^{19}F nuclear magnetic resonance (MR) imaging technology have been reported. Due to the low background signal of ^{19}F MR in vivo, these ^{19}F MR based imaging probes have a potential for high contrast and specific imaging of target biomolecules. However, no molecular probes that can image genome activity in vivo have been reported. In this study, we tried to develop a novel hybridization probe that can image gene expression in vivo based on ^{19}F MR imaging technology. Such a probe will enable us to image invasively genomic activity related to various disorders and to investigate directly the genome function in vivo.

We designed and synthesized a stem-loop structured oligonucleotide probe having a fluorine and paramagnetic compound at the 5′ and 3′ termini, respectively (Fig. 1). In the absence of target nucleic acid, it is expected that the distance dependent paramagnetic relaxation enhancement (PRE) effect[8] of the paramagnetic compound at the 3′ termini quenches the ^{19}F MR signal of the fluorine compound at the 5′ termini. The addition of target nucleic acid causes change in the distance between 5′ and 3′ termini, and then the PRE effect is reduced. The ^{19}F MR signal is expected to be turned on by the addition of target nucleic acid.

We adopted bis(trifluoromethyl)benzene moiety, as a fluorine compound, and Gd-DOTA chelate, as a paramagnetic compound. Firstly, we synthesized phosphoramidite unit of the fluorine compound (**3**) according to Scheme 1. These compounds were introduced to the 5′ termini of oligonucleotide (CCTACGCCAACAGCTCCgtagg) according to Scheme 2. As the use of standard phosphoramidite chemistry, bis(trifluoromethyl)benzene moiety could be labeled quantitatively with the 5′ termini of the oligonucleotide. And the phosphoramidite unit could be labeled in multiples with the 5′ termini of the oligonucleotide as shown in Fig. S1 (See Supplementary data). As the ^{19}F MR signal of the multiply **3** labeled oligonucleotide was linearly increased with the number of **3** introduced (Fig. S2), it was suggested that the sensitivity of the probe could potentially enhance by increase the number of **3** introduced to the probe. DOTA was easily labeled with the 3′ termini of the probe by standard NHS-ester chemistry in aqueous solution. Gd was quantitatively chelated to DOTA at the 3′ termini of the probe. As the conversions of each labeling reaction were quantitative (Fig. S3), purification of all products except for the phosphoroamidite coupling of the fluorine compound was easily performed by gel filtration using the NAP-5 column. These highly labeling yields and easy purification procedures might contribute to mass production of the probe for practical usage.

To confirm the ^{19}F MR signal turn-on manner of the mono-**3** labeled probe, ^{19}F NMR spectra were measured[11] in the presence or absence of the target oligonucleotide (Kras Mut; gtagttGGAGCTGTTGGCGTAGGcaag). As shown in Figure 2, the ^{19}F MR signal of bis(trifluoromethyl)benzene moiety at the 5′ termini of the probe (62.9 and 63.0 ppm) appeared by the addition of Kras Mut ODN having a complementary sequence for the probe. The appeared two peaks were assigned to each enantiomer of the

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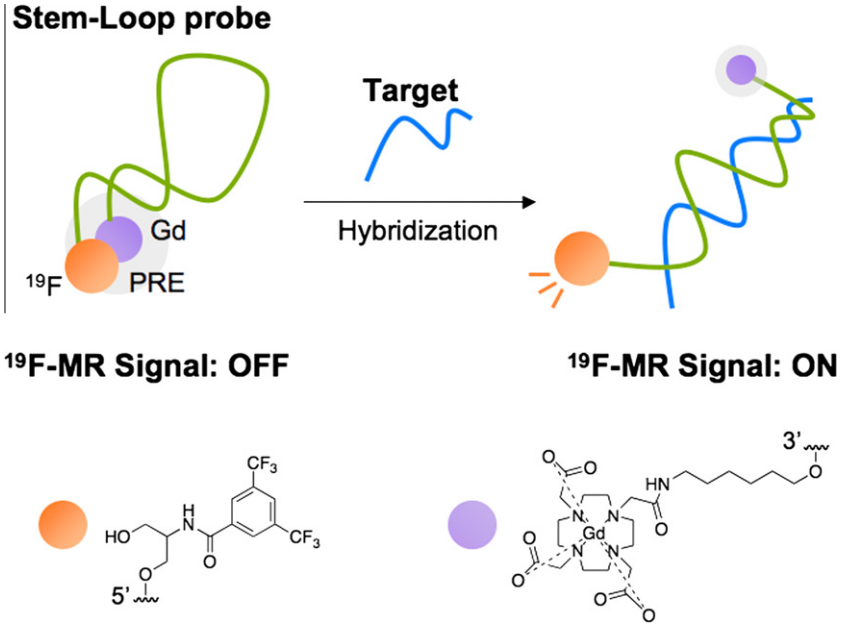
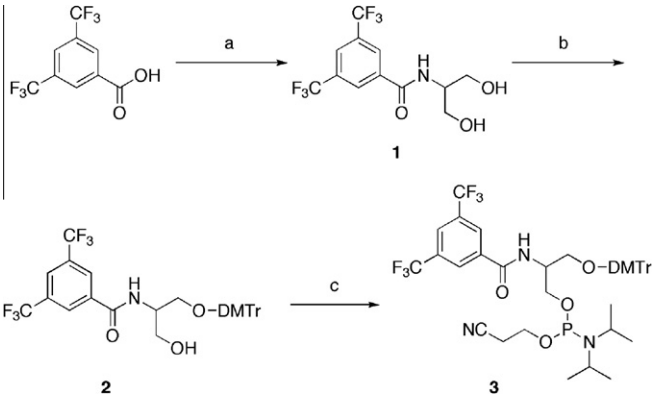


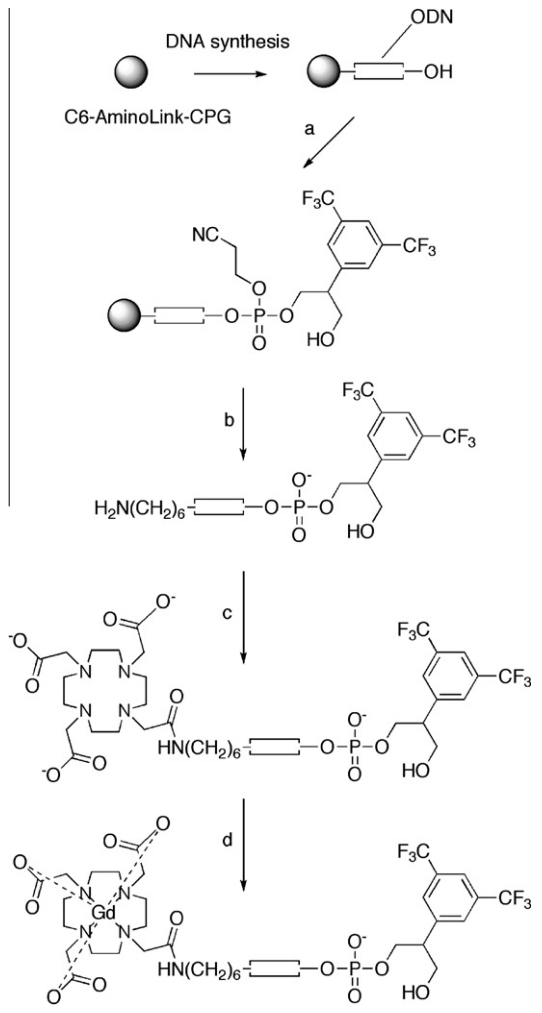
Figure 1. Schematic drawing of the mechanism of target nucleic acid detection using stem-loop typed oligonucleotide probe having ^{19}F and Gd-DOTA at the each termini of the probe.



Scheme 1. Synthetic method for compounds **1**–**3**;[9] (a) 2-amino-1,3-propanediol, HOBt, WSC, DMF; (b) 4,4′-dimethoxytritylchloride, *N*,*N*-dimethylaminopyridine, pyridine; (c) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite, benzylthio-1*H*-tetrazole, acetonitrile.

fluorine compound tethered to 5′ termini. The addition of GAPDH ODN having an uncomplimentary sequence (aactttggtatcgtggaaga-actcatgacc) did not affect the ^{19}F MR signal. These results indicate that the probe could discriminate the sequence of the target ODNs. In the case of the addition of Kras Wt ODN, which has one base mismatch sequence for the probe (gtagttGGAGCTGgTGGCGTAGG-caag), the ^{19}F MR signal was increased to the same intensity as in the case of the addition of Kras Mut ODN. This indicates that the probe could not discriminate one base mismatch sequence in the present condition, and that the concentration of the probe and target ODN was too high to discriminate one base mismatch. Indeed, the melting temperature of each duplex was not so different at the present condition (57 °C for probe/Kras Mut and 52 °C for probe/Kras Wt). These results suggest that the probe has the potential to target DNA or RNA by ^{19}F MR imaging technology, although further improvement of the sensitivity of the probe may be required.

Change in the chemical shift of the probe was observed by the addition of Kras Mut ODN. As the chemical shift of ^{19}F compound was changed dependent on the environment around ^{19}F



Scheme 2. Synthetic method for the probe;[10] (a) compound 3, benzylthio-1*H*-tetrazole, acetonitrile, 999 s; (b) 28% NH_3 aq, 55 °C, 18 h; (c) DOTA-NHS, 50 mM Na–borate buffer (pH 9.0); (d) $Gd(Cit)_2$, 50 mM Tris–HCl buffer (pH 7.6).

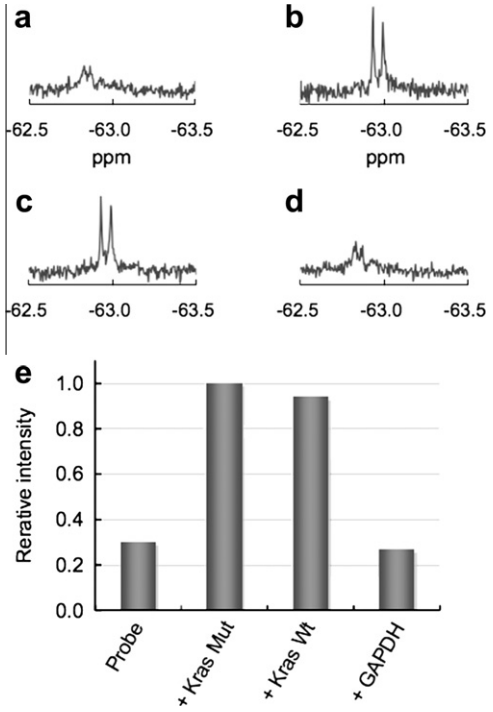


Figure 2. ^{19}F NMR spectra and signal intensity of the probe in the presence of various oligonucleotides. (a) Probe alone; (b) + Kras Mut; (c) + Kras Wt; (d) + GAPDH; (e) relative signal intensity of the probe. [probe] = [ODN] = 10 μM in 50 mM Tris–HCl (pH 7.6), 10% (v/v) D_2O, 10 μM TFA (as internal standard), 25 °C.

compound,[12] this result also might cause by the change in the environment around the ^{19}F compound, for example, the ^{19}F compound possessed blunt end of the stem in the stem-loop structured state (probe alone) or sticky end in the hybridized state (+ Kras Mut). This result suggests that the probe has a potential to become a chemical shift ratio-imaging sensor for hybridization detection.

A titration study of the target ODN, Kras Mut, was performed to demonstrate the concentration dependency of the signal turn-on manner of the probe. As shown in Figure 3, the ^{19}F MR signal was increased in a concentration dependent manner of the target ODN and reached a plateau at the equimolar concentration of the probe, indicating that the probe can quantitatively detect a target nucleic acid strand. As the addition of the excess amount of DOTA (2 mM) to the 1:1 mixture of the probe (10 μM) and Kras Mut ODN (10 μM) did not cause any change in the ^{19}F MR signal (Fig. S4), the quenched ^{19}F MR signal that was caused by the stem-loop structure of the probe was completely recovered by the conformational change of the probe caused by the hybridization with the target DNA strand.

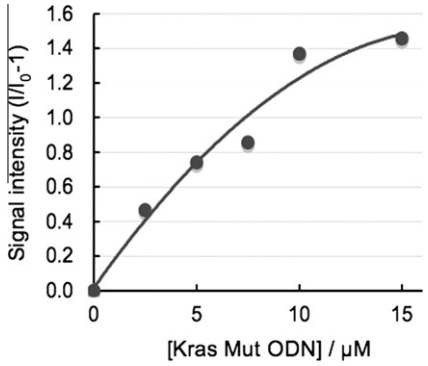


Figure 3. ^{19}F NMR signal intensity of the probe as a function of Kras Mut ODN. [probe] = 10 μM in 50 mM Tris–HCl (pH 7.6), 10% (v/v) D_2O, 10 μM TFA (for an internal standard), 25 °C.

In summary, we developed a novel sensor DNA molecule that can detect complementary nucleic acid by ^{19}F MR signal based on the PRE effect. This molecular beacon type probe can detect target nucleic acid by its ^{19}F MR signal turn-on manner and has the potential to become a nucleic acids imaging probe by ^{19}F MR imaging technology. To improve the selectivity and sensitivity of the probe, fine-tuning of the number and structure of fluorine compound for 5′ label is now underway.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.013.

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9. 2-Amino-1,3-propanediol (0.18 g, 1.95 mmol) and bis(trifluoromethyl)benzoic acid (0.50 g, 1.94 mmol) were added in dry DMF (30 mL) containing WSC (0.45 g, 2.33 mmol) and 1-hydroxybenzotriazole (0.36 g, 2.33 mmol). After the reaction mixture was stirred at room temperature for 24 h, the solvent was removed, and the remaining oil was subjected to silica gel column chromatography (0–5% MeOH/$CHCl_3$) to afford **1** (0.48 g, 75%). ^{1}H NMR (400 MHz, [D_6]DMSO): *d* = 8.61 (*d* (8.4 Hz), 1H, –N*H*CO–), 8.53 (s, 2H, *o*-Ar*H*), 8.30 (s, 1H, *p*-Ar*H*), 4.72 (t (6 Hz), 2H, –O*H*), 4.01 (m, 1H, –NH*CH*(CH_2OH)$_2$) and 3.53 (m, 4H, –NHCH(CH_2OH)$_2$). MALDI-TOF-MS: calcd 354.05 ([(M+Na)$^+$]), found 354.71. To a solution of **1** (0.33 g, 1.00 mmol) in dry pyridine (5 mL) was added a solution of 4,4′-dimethoxytritylchloride (0.41 g, 1.20 mmol) and *N*,*N*-dimethylaminopyridine (24 mg, 0.2 mmol) in dry pyridine (5 mL) on ice bath. After the reaction mixture was stirred at room temperature for 20 h, the solvent was removed, and the remaining oil was subjected to silica gel column chromatography ($CHCl_3$) to afford 2 (0.15 g, 23%). ^{1}H NMR (400 MHz, $CDCl_3$): *d* = 8.17 (s, 2H, *o*-Ar*H*), 8.02 (s, 1H, *p*-Ar*H*), 7.40–6.80 (m, 13H, Ar*H*), 4.29 (s, 1H, –O*H*), 3.99 (m, 1H, –NHC*H*(CH_2OH)(CH_2O-DMTr)), 3.79 (dd (11.2 Hz), 2H, –CH_2O-DMTr), 3.77 (s, 6H, (ArOCH_3)$_2$) and 3.49 (m, 2H, –CH_2OH). MALDI-TOF-MS: calcd 656.18 [(M+Na)$^+$], found 656.90. The residual trivial amount of water in **2** was removed by azeotropic distillation with dry acetonitrile (twice). Then, **2** (0.10 g, 0.16 mmol), 0.1 M solution of 5-benzylthio-1*H*-tetrazole in dry MeCN (0.63 mL, 0.16 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (50 mL, 0.16 mmol) were mixed in dry acetonitrile (0.8 mL) under nitrogen for 2 h. The crude mixture was dissolved in ethyl acetate. The solution containing **3** was washed with water, a saturated solution of $NaHCO_3$, and a saturated solution of NaCl. The solution was dried over $MgSO_4$, and then filtered and the ethyl acetate was removed. The yellow foam (0.14 g, quant.) was obtained and immediately used for DNA synthesis without further purification.
10. Bis(trifluoromethyl)benzene moiety and Gd-DOTA labeled oligonucleotide probe were synthesized according to Scheme 2. Oligonucleotide was synthesized by standard cyanoethyl phosphoroamidite chemistry with an automated DNA synthesizer (3400 DNA Synthesizer, Applied Biosystems) using 3′-PT-amino-modifier C6 CPG (Glen Research). The phosphoramidite monomer of fluorine compound **3** was coupled with the 5′ termini of the oligonucleotide by the DNA synthesizer with a coupling time of 999 s. After the cleavage with 28% aqueous ammonia (1 h, rt), the solution was incubated for 18 h at 55 °C and dried. The residual was solved in deionized water and purified by a reversed-phase HPLC system (JASCO) equipped with a column (COSMOSIL 5C18-AR-II, nacalai tesque, 4.6 × 150 mm); elution was with

0.05 M ammonium formate containing 2–32% CH_3CN, linear gradient (30 min) at a flow rate of 1.0 mL/min. To a solution of 5′ fluorine compound and 3′ amino modified oligonucleotide (^{19}F-ODN-NH^2) in 50 mM Na–Borate buffer (pH 9.0) (400 μM, 125 μL), the solution of DOTA-NHS in DMSO (40 mM, 50 μL) was added slowly on ice bath, and then incubated for 12 h at rt. The fluorine and DOTA labeled ODN (^{19}F-ODN-DOTA) was purified by gel filtration (NAP-5, GE Healthcare) according to the manufacturer's instructions, and the purity was checked by a reversed-phase HPLC. Fluorine and DOTA labeled ODN (300 μM, 40 nmol) was incubated with Gd citrate (3 mM, 400 nmol) in 50 mM Tris–HCl (pH 7.6) for 24 h at rt. The resultant fluorine and Gd-DOTA labeled ODN probe (^{19}F-ODN-Gd-DOTA) were purified by gel filtration according to the manufacturer's procedure, and the purity was checked by a reversed-phase HPLC. All ODN derivatives were identified by MALDI-TOF-MS analysis. ^{19}F-ODN-NH_2; calcd 7235.25 [$(M+H)^+$], found 7235.89, ^{19}F-ODN-DOTA; calcd 7621.43 [$(M+H)^+$], found 7621.79, ^{19}F-ODN-Gd-DOTA; calcd 7776.33 [$(M+H)^+$], found 7776.52.

11. ^{19}F NMR spectra were recorded on a Bruker AVANCE III 500 instrument with a 5 mm probe head (PA BBO 500S2 BBF-H-D-05 Z) at 470 MHz for ^{19}F. The solvent for ^{19}F NMR measurement was 50 mM Tris–HCl buffer (pH 7.6) containing 10 μM trifluoroacetic acid (for an internal standard, 75.6 ppm) and 10% (v/v) D_2O. Acquisition; 4096 times.
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