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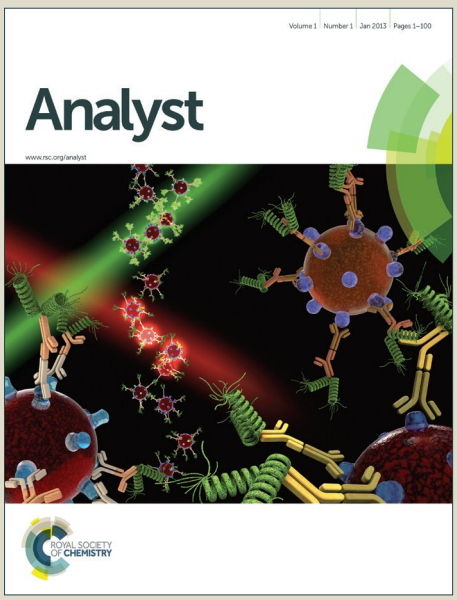

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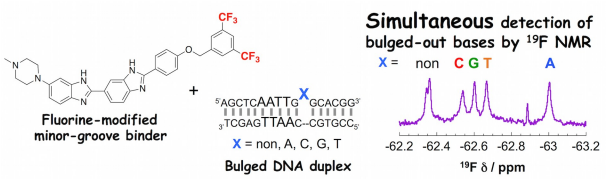
Single-nucleotide polymorphisms on the bulged-out nucleobase in dsDNA can be detected simultaneously in a ^{19}F NMR spectrum using 3,5-bis(trifluoromethyl)benzene-modified bisbenzimide H33258.



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Simultaneous detection of single-nucleotide polymorphisms in a DNA bulge structure using fluorine-modified bisbenzimide derivative

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Using 3,5-bis(trifluoromethyl)benzene-modified bisbenzimide H33258, bulged-out nucleobases on a DNA strand can be detected by the chemical shift change in ^{19}F NMR. Based on this technique, single-nucleotide polymorphisms on a DNA sequence can be analyzed simultaneously.

With their highly transparent signals, ^{19}F NMR-based molecular probes have wide potential for detecting and/or imaging biomolecules in cells and also *in vivo*. Various molecular probes that can detect biomolecules, such as amyloid beta plaque,[1] enzymes,[2] proteins,[3] and nucleic acids,[4] have been reported, which has opened the door to the analysis of endogenous biomolecules by ^{19}F NMR/MRI. The narrow ^{19}F NMR signal is also an advantage of ^{19}F NMR-based molecular probes because different molecules or the states of a molecule can be discriminated easily in a ^{19}F NMR spectrum. This provides a simultaneous detection method for various nitrile compounds,[5] neutral and anionic species,[6] different ubiquitin chains[7] and various structures of nucleic acids.[8] Further development of these methods would enable us to analyze simultaneously the disease-related biomarkers and the states of cells. This might contribute largely to diagnosis and the basic research of bioscience. Single-nucleotide polymorphism (SNP(s)) is one of the effective biomarkers for evaluating morbidity risk of various diseases, however, there is no report that applying ^{19}F NMR-based molecular probe for the simultaneous analysis of SNPs. Here we describe a proof-of-concept study on simultaneous detection of SNPs in DNA strand by ^{19}F NMR.

In our previous work, we developed an ^{19}F NMR-based molecular probe consisting of bisbenzimide H33258 and 3,5-bis(trifluoromethyl)benzyl group (Fig. 1a) for DNA detection, which can detect double-stranded DNA (dsDNA) having AATT sequence in a ^{19}F NMR/fluorescence bimodal manner.[9] Since the chemical shift of the probe that binds with dsDNA is changed and the chemical shift change is characteristic of the adjacent base-pair of the AATT binding site, the probe can clearly discriminate the DNA sequence around the AATT binding site and can detect simultaneously dsDNAs having different sequences by ^{19}F NMR. The unique characteristics of the probe might enable novel strategies for designing ^{19}F NMR based DNA probes. However, one problem remains; i.e. the binding orientation cannot be regulated because of the palindromicity of the probe-binding site (Fig. 1b). This prevents the precise detection of the adjacent base pair of the binding site in dsDNA.

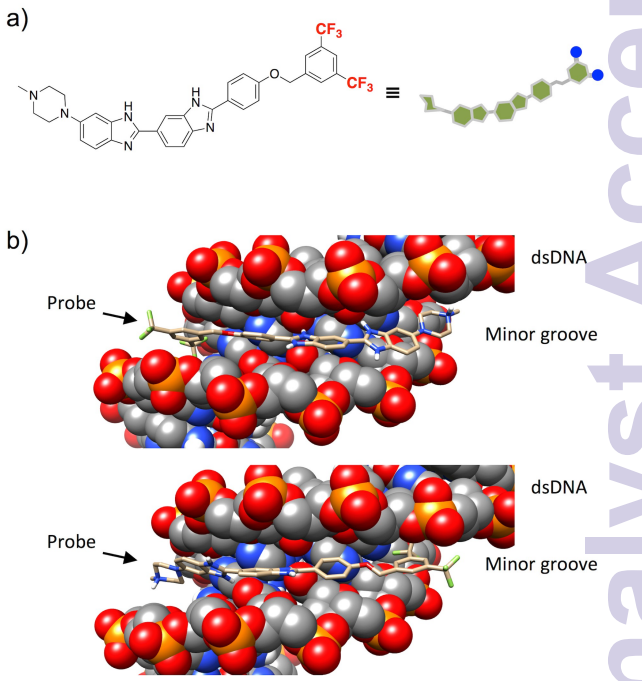

Figure 1. Structure of 3,5-bis(trifluoromethyl)benzene-modified bisbenzimide H33258 (a) and the predicted two binding models of the probe for AATT region in dsDNA. As the palindromicity of AATT binding site, the probe can bind with two different orientations.

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Electronic Supplementary Information (ESI) available: experimental details, fluorescence spectra, fluorescence titration curves, and partition coefficients (P_{OW}(s)) and reversed-phase ultra-high performance liquid chromatographs of 2'-deoxyribonucleosides. See DOI: 10.1039/x0xx00000x

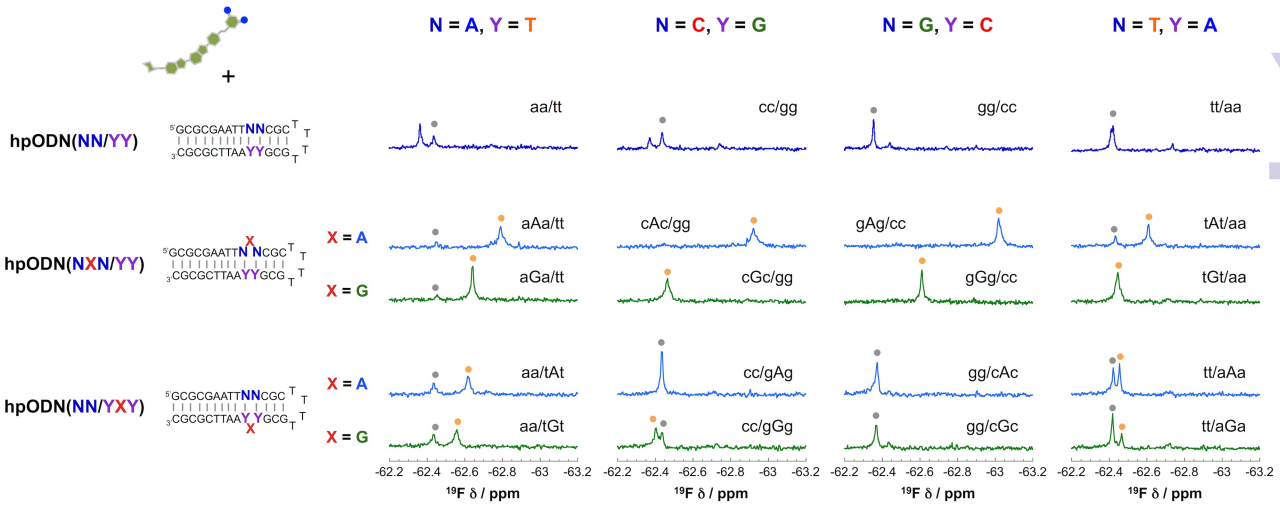

Figure 2. ^{19}F NMR spectra of the probe in the presence of various hpODNs. [probe] = [hpODN] = 10 μM in 50 mM Tris-HCl (pH 7.6) containing 100 mM NaCl and 10% (v/v) D$_2$O. Measurements were performed at 25°C. New peaks appeared by the addition of a bulge structure as marked with orange circles.

Table 1. Dissociation constant (K_D) of the interaction between the probe and hpODNs

	hpODN(gg/cc)	hpODN(gAg/cc)	hpODN(gGg/cc)
K_D / nM[a]	6.9 ± 0.5	1.9 ± 0.1	6.0 ± 0.9

[a]Values of K_D were determined by fluorescence titration analysis with nonlinear least-squares curve-fitting (Fig. S1).

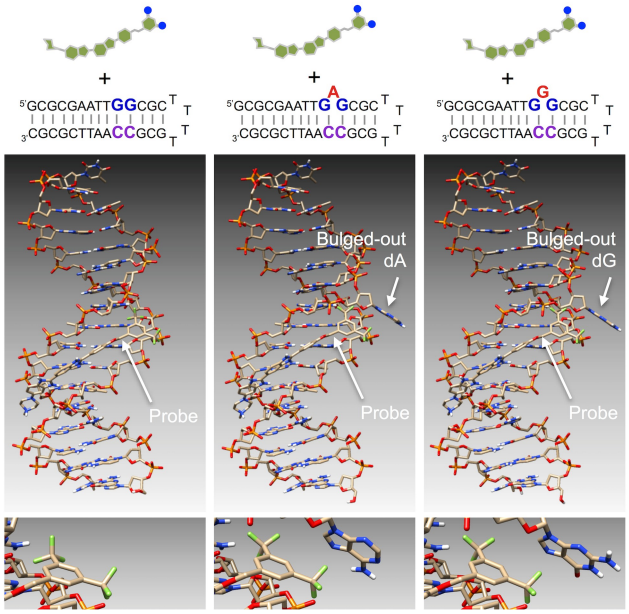

Figure 3. Energy minimized structure of the complex consisting of the probe and hpODN(gXg/cc) estimated by MacroModel ver. 8.1. Calculations were performed with AMBER* force field and water as a solvent.

In this study, we focused on the binding orientation of 3,5-bis(trifluoromethyl)benzene-modified bisbenzimide H33258 with dsDNA, a bulge structure was adopted for regulating the binding orientation. The feasibility of the probe for the simultaneous detection of bulged-out nucleobases and SNPs in target DNA was also evaluated.

Firstly, to evaluate the ability for regulating the binding orientation of the probe, the ^{19}F NMR of the probe was measured with various hairpin oligodeoxyribonucleotides (hpODN(s)) with or without a purine-bulge structure beside the AATT binding site (Fig. 2). In most cases, the insertion of the bulge structure caused a peak-shift in ^{19}F NMR (orange circles), suggesting that these bulge structures can be detected clearly by the change in the ^{19}F chemical shift. Two peaks appeared in the case of hpODN(aXa/tt), (aa/tXt), (cc/gXg), (tAt/aa) and (tt/aXa), and either one of the peaks was identical to the peaks that appeared in the case of non-bulge hpODN (gray circle), suggesting that two binding modes having different orientations still remained. On the other hand, in the case of hpODN(cXc/gg), (gXg/cc) and (tGt/aa), only one peak appeared and the chemical shift differed from the case of non-bulge hpODN. These results suggest that the probe bound to hpODN with only one orientation in which the 3,5-bis(trifluoromethyl)benzene moiety was close to the bulge region. To prove this hypothesis, the binding affinity of the probe with hpODN(gg/cc), (gAg/cc) and (gGg/cc) was evaluated by fluorescence titration. As shown in Table 1, the dissociation constant (K_D) of the probe with hpODN(gAg/cc) or (gGg/cc) was lower than that with hpODN(gg/cc), suggesting strongly that the probe binds hpODN(gXg/cc) preferentially with an orientation in which 3,5-bis(trifluoromethyl)benzene moiety was close to the bulge region.

The chemical shift of the peaks shifted was different depending on the bulged-out base particularly in the case of hpODN(gXg/cc). To elucidate why this difference occurred, the energy minimized structures of the complex consisting of the probe and hpODN(gXg/cc) were estimated by MacroModel ver. 8.1. As shown in Fig. 3, 3,5-bis(trifluoromethyl)benzene moiety possesses nearby the bulged-out nucleobase, suggesting that the difference in the chemical shift was caused by the character of the bulged-out nucleobases, such as hydrophobicity or dipole moment.

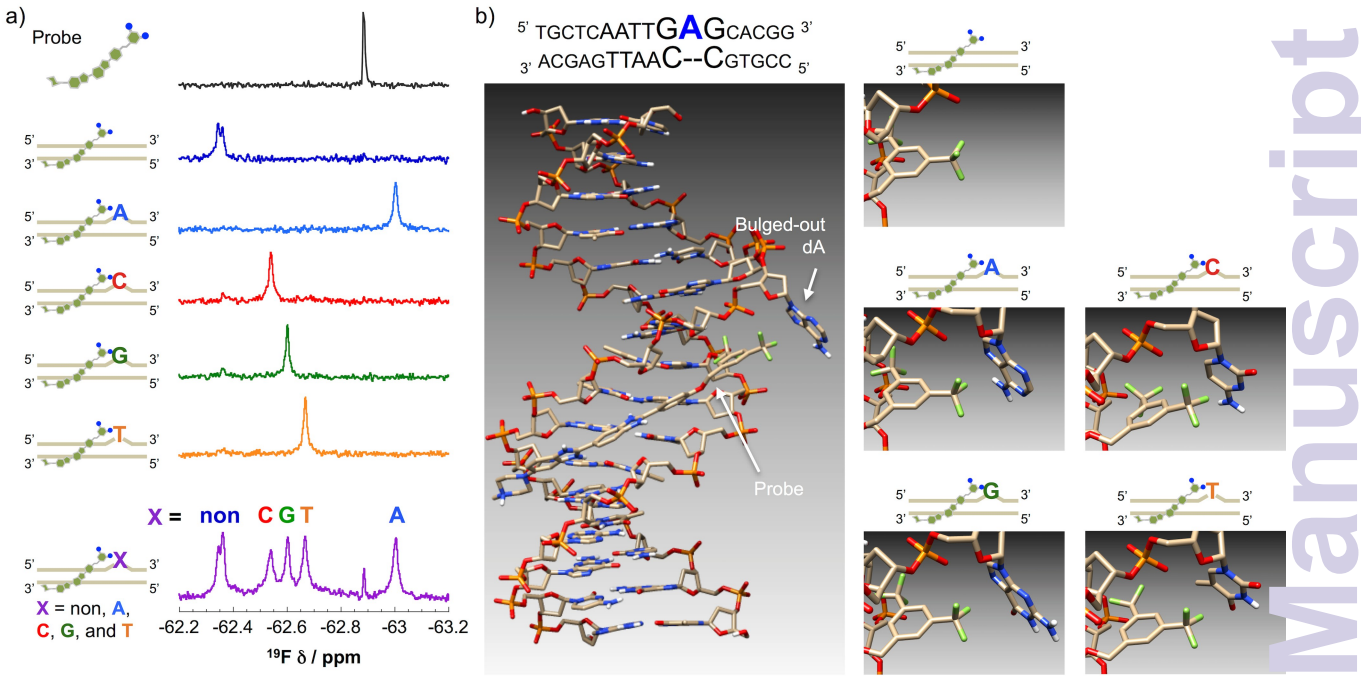

Figure 4. SNPs detection on the bulged-out bases in dsDNA. (a) ^{19}F NMR spectra of the probe in the presence of various dsDNA having X bulge structure. [probe] = 10 μM (or 26 μM for dsDNA mix), [dsDNA] = 10 μM (or 5 μM each) in 50 mM Tris-HCl (pH 7.6) containing 100 mM NaCl and 10% (v/v) D_2O. Measurements were performed at 25°C. (b) Energy minimized structure of the complex consisting of probe and dsDNA estimated by MacroModel ver. 8.1. Calculations were performed with AMBER* force field and water as a solvent.

If the chemical shift of the probe changes differently depending on the bulged-out nucleobases, SNPs in DNA sequence can be detected simultaneously in a ^{19}F NMR spectrum. To assess the concept, model target DNAs, 5'TGCTCAATTGXGCACGG3' (**X** = non, A, C, G, T), were designed and ^{19}F NMR spectra of the probe with the target DNAs and complementary DNA (5'CCGTGCCAATTGAGCA3') were measured. As shown in Fig. 4a, the initial peak was changed with the addition of five different DNAs and the chemical shifts were completely different in all cases. These results suggest that the chemical shift changed by the character of the bulged-out nucleobases. The order of the chemical shifts; C, G, T, A (lower to higher magnetic fields), was consistent with the elution order of the corresponding 2'-deoxyribonucleoside in the reversed-phase ultra-high performance liquid chromatography (Fig. S2), suggesting strongly that the hydrophobicity of the bulged-out nucleobases affects largely the chemical shift of 3,5-bis(trifluoromethyl)benzene moiety possessed nearby the bulged-out bases (Fig. 4b). The significantly higher octanol-water partition coefficient (P_{OW}) of 2'-deoxyadenosine compared to the other 2'-deoxyribonucleoside (Table S1) explains the large difference in the ^{19}F chemical shift for bulged-out A from the hydrophobicity of the nucleobases. Finally, to assess the feasibility of the simultaneous detection of SNPs in the DNA sequence, five target DNAs and complementary DNA were added to the probe, and then the ^{19}F NMR spectrum was measured. As shown in the bottom of Fig. 4a, six peaks identical to the non-bulged DNA, four bulge DNAs and the unbound probe were observed, and these peaks were clearly distinguishable, indicating that these four DNAs having different SNPs on the DNA bulge structure and the non-bulged DNA can be detected simultaneously on a ^{19}F NMR spectrum. Since the probe and complementary DNA are common to all cases, SNPs and 1 base deletion in target DNA can be simultaneously detected by this method.

In conclusion, we found that the binding orientation of the probe with dsDNA can be regulated by introducing a bulge structure at the position adjacent to the AATT binding site and the bulged-out nucleobases were clearly detected by the change in ^{19}F NMR chemical shift. Since the chemical shifts of the probe bound with non-bulged dsDNA and dsDNA having four different bulged-out bases were completely different, simultaneous detection of SNPs and 1 base deletion in the target single-stranded DNA was clearly demonstrated. Our unique and novel approach, which aims to read out the bases bulged-out in dsDNA, might contribute to realizing a novel design of ^{19}F NMR-based probe for detecting target DNA structures and/or sequences including SNPs. Now we are attempting to expand the method for the detection of the branched-out nucleobase of DNA and RNA. This would contribute to our method being applied to analyze SNPs in cellular RNAs.

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