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Fluorescence detection of guanine–adenine transition by a hydrogen bond forming small compound

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In combination with abasic site-containing oligodeoxynucleotides, 2-amino-4-oxopteridine (pterin) can selectively recognize guanine base over other nucleobases accompanied by fluorescence quenching, which allows clear detection of a guanine–adenine transition with the naked eye.

The need for the rapid discovery and detection of single-nucleotide polymorphism (SNP) has led to a number of available methods that include high-density arrays,[1a] primer extension methods,[1b] real time PCR,[1c] and Invader assay.[1d] These methods, however, require several time-consuming steps, use of several kinds of fluorophore-labeled oligodeoxynucleotides (ODNs), and/or special enzymes. Mass spectroscopy has recently been applied to genotyping,[1e] but careful treatments are required to ensure purity of the sample. A quick, simple and cost-effective method for the routine detection of SNPs is highly desirable.[2]

Among the variety of approaches to this end,[3] of particular interest to us is use of synthetic and/or biotic small ligands possessing hydrogen-bonding groups suitable for nucleotide recognition.[4,5] Nakatani *et al.*[4] have recently synthesized a dimeric naphthyridine derivative that specifically binds to the guanine–guanine (G–G) mismatch, and they succeeded in developing a non-gel-based scanning method to detect the ligand-bound SNPs, based on an SPR (surface plasmon resonance) assay with a sensor chip carrying the G–G mismatch-binding molecule on its surface. We have also demonstrated that hydrogen bond-mediated nucleotide recognition in water is successfully achieved by the use of an abasic site (AP site) as a molecular recognition field.[5] While naturally occurring AP sites are one of the most common forms of DNA damage,[6] we intentionally constructed the AP site in an ODN duplex so as to orient the AP site toward a target nucleobase, by which hydrophobic microenvironments are provided for ligands to recognize nucleotides through hydrogen-bonding (*cf.* Fig. 1).[5] From the examination of the binding behaviors of 2-amino-7-methylnaphthyridine (AMND) with nucleotides at the AP site, we found AMND was selectively bound to cytosine (C) base with high affinity (K_d < 1 μM), and this was accompanied by remarkable quenching of its fluorescence. AMND would therefore be applicable to selective, visible detection of single-nucleotide mutation related to C.[5] In this work, our system is further developed for the detection of G-related single-nucleotide mutation by the use of a biotic fluorescence compound, 2-amino-4-oxopteridine (pterin,[7] *cf.* Fig. 1), as a hydrogen-bonding ligand. Potential use of pterin is presented for the fluorescence detection of G/A (adenine) transition of the cancer repression gene p53.

Firstly, we examined the complexation between pterin and 11-meric model ODN duplexes (5′-TCCAG**X**GCAAC-3′/3′-AGGTC**Y**CGTTG-5′, **X** = dSpacer, **Y** = G, C, A, T) by melting temperature (T_m) measurements.† As given in Table 1, increases in T_m of AP site-containing ODNs are observed upon addition of pterin, whereas hardly any changes in T_m are observed for normal ODN duplexes that have no AP sites (ΔT_m = + 0.2 °C for 5′-TCCAGCGCAAC-3′/3′-AGGTCGCGTTG-5′). The increase in T_m depends on the nucleotides opposite the AP site, and follows in the order of G (+ 2.7 °C) > C (+ 0.9 °C), T (+ 0.9 °C) > A (+ 0.1 °C). Apparently, pterin is incorporated into the AP site by binding to nucleotides with selectivity for G, which results in an increase in the thermal stability of AP site-containing ODN duplexes.

In accordance with these results of T_m measurements, the useful selectivity for G over other nucleotides is clearly seen from the fluorescence binding studies. As is shown in Fig. 2a, pterin shows significant fluorescence quenching upon binding with G opposite the AP site. The fluorescence intensity at 445 nm is quenched by as much as 40% in the presence of 60 μM of an ODN duplex. A non-linear fitting shows that the changes in fluorescence spectra can be explained by the formation of a 1 : 1 complex, and the binding constant between pterin and G is calculated as 1.2×10^4 M^{-1} (Fig. 2b).[8] It seems likely that pterin binds to G *via* a three-point hydrogen-bonding motif as is shown in Fig. 2b. By contrast, no evidence is obtained for the interaction between pterin and normal duplexes containing no AP sites, supporting our proposal that the observed response is due to the binding event at the AP site. In addition, as is shown in Fig. 2a, pterin shows only slight responses for other nucleobases. The difference in fluorescence responses of pterin is indeed evident with the naked eye between G and other nucleobases (*cf.* Fig. 3), indicating that pterin works as a fluorescence probe for the detection of SNP related to G.

Our system was therefore applied to the the G/A mutation sequence at codon 175 of the cancer repression gene p53.[9] This missense mutation, named R175H, is one of the hot spots of p53, which results in an amino acid change from arginine to histidine. As is shown in Fig. 3, the fluorescence of pterin is significantly quenched in the case of the wild type sequence (5′-GAGGC**G**CTGCC-3′) while no quenching is observed for the

Fig. 1 Schematic illustration of single-nucleotide recognition by a hydrogen-bonding ligand (pterin) in water, in combination with AP site-containing ODN duplexes. Target base and AP site containing ODN are colored red and blue, respectively.

Table 1 Melting temperature (T_m) of AP site-containing ODN duplexes in the presence ($T_{m(+)}$) and absence ($T_{m(-)}$) of pterin[a]

AP site-containing ODN 5′-TCCAG**X**GCAAC-3′/3′-AGGTC**Y**CGTTG-5′			
X	**Y**	$T_{m(-)}$	$T_{m(+)}$ (ΔT_m)
	G	34.8 ± 0.4	37.5 ± 0.2 (+ 2.7)
	C	31.6 ± 0.6	32.5 ± 0.4 (+ 0.9)
	A	34.4 ± 0.1	34.5 ± 0.2 (+ 0.1)
	T	29.5 ± 0.2	30.4 ± 0.1 (+ 0.9)

[a] [ODN duplex], 30 μM; [pterin], 290 μM; [NaCl], 100 mM; [EDTA], 1 mM; [sodium cacodylate], 10 mM, at pH 7.0; light-path length, 1 mm.

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mutation sequence (5′-GAGGC**A**CTGCC-3′). Under the examination conditions, it is indeed possible to judge the G/A SNP with the naked eye based on the changes in fluorescence of pterin.

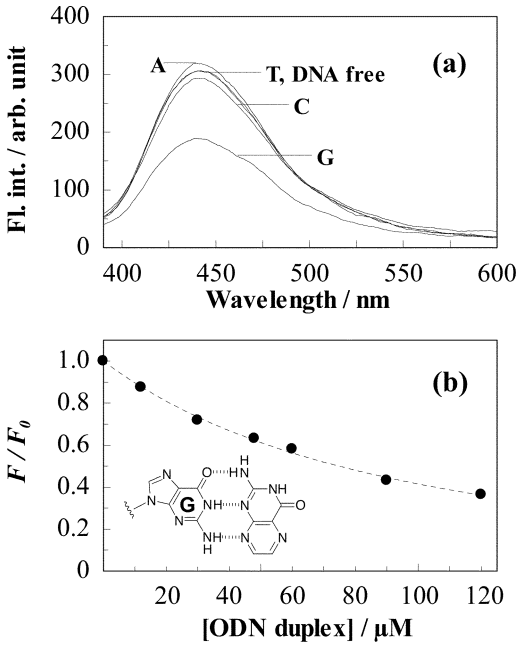

Fig. 2 (a) Fluorescence spectra of pterin (15 μM) with AP site-containing ODN duplexes (5′-TCCAG**X**GCAAC-3′/5′-GTTGC**Y**CTGGA-3′, **X** = dSpacer, **Y** = A, C, G, T, 60 μM) in water. Excitation wavelength: 360 nm.† (b) Nonlinear regression analysis of the changes in the fluorescence intensity ratio at 440 nm based on a 1 : 1 binding isotherm model. (F and F_0 denote the fluorescence intensities of pterin in the presence and absence of ODN duplexes (**Y** = G), respectively.)

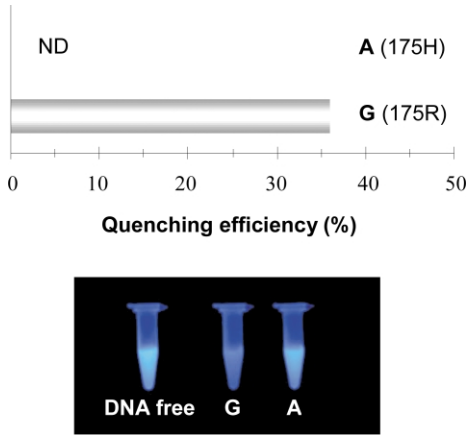

Fig. 3 Fluorescence detection of G/A SNP by pterin in combination with AP site-containing ODN duplexes (5′-GGCAG**X**GCCTC-3′, **X** = dSpacer). [pterin], 10 μM; [AP site-containing ODN], 100 μM; [target ODN], 100 μM; wild type (175R), 5′-GAGGC**G**CTGCC-3′; mutation type (175H), 5′-GAGGC**A**CTGCC-3′. The samples were excited with a UV lamp at 302 nm.†

In summary, we have demonstrated that, in combination with AP site-containing ODNs, pterin does selectively bind to G with useful fluorescent signaling. Pterin could be successfully utilized for detection of the G/A transition; it also seems applicable to other types of mutations such as the G/C transversion. Even though the improvement of the rather low binding affinity of pterin may be necessary for the detection of SNP present in the PCR amplification products, we expect that use of low-molecular-weight ligands offers a novel approach to a simple, low-cost assay for SNP typing. Some studies are in progress for further development of our ligand-based detection method.

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Notes and references

† All measurements were done in 10 mM sodium cacodylate buffer solutions (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. As for T_m measurements, absorbance of ODNs was measured at 260 nm as a function of temperature with a heating rate of 1.5 °C/min. The resulting absorbance *vs.* temperature curves were analyzed by MeltWin[10] to determine T_m values.

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