Highly selective fluorescent nucleobases for designing base-discriminating fluorescent probes*

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Abstract: There is increasing interest in single nucleotide polymorphism (SNP) typing since they can be used as markers to identify the genes that underlie complex diseases and to realize the full potential of pharmacogenomics in analyzing variable response to drugs. Among the different methodologies for SNP genotyping, the homogenous assay is more amenable than the heterogeneous one.

In this article, we will describe some of our most recently developed novel base-discriminating fluorescent (BDF) nucleosides useful for homogenous SNP typing. Our novel concept led to the investigation of a new type of pyrene-labeled BDF nucleosides PyU, PyC, 8pyA, and MePydA, which emitted strong fluorescence only when the bases opposite the BDF bases are A, G, T, and C, respectively. The DNA probes containing four different BDF bases enabled us to distinguish single-base alterations by simply mixing with a sample solution of target DNA. An example of SNP typing of c-Ha-ras SNP sequence has also been demonstrated. Detection of base insertion in insertion/deletion (indel) polymorphisms using pyrene excimer fluorescent probe has also been explored.

Keywords: SNPs; BDF; nucleosides; DNA; probes.

INTRODUCTION

Recently, there has been growing interest in single nucleotide polymorphisms (SNPs) due to their biological and clinical importance [1]. Various research groups have exerted a great deal of effort on the development of SNP genotyping technologies over the last few years. As a result, a lot of different SNP typing protocols have become available for us, however, there is no single protocol that meets all research needs. Different aspects should be taken into account to determine which technology is the most suitable, such as the sensitivity, the reproducibility, the accuracy, the capability of multiplexing, and the level of throughput. It is also important to have in mind the flexibility of the technology, the time consumption, and the cost, considering both the equipment required and the cost per genotype [2]. The
available methods generally consider the difference in hybridization efficiency or in enzymatic recognition between the nucleotide probes.

Fluorescence-labeled DNA probes play an important role in recent development of the detection of single-base alterations. The single-base discrimination in nearly all reported methods is achieved directly or indirectly on the basis of different hybridization efficiency between matched and mismatched target DNA/probe DNA duplexes. However, as far as the detection relies on the hybridization events, such DNA probes have inherent limitations in their selectivity. The differences in the hybridization efficiency are varied with sequence context, and often very small for the detection of a single-base mismatch in long-target DNA. In order to attain enough signal-to-noise ratio, the hybridization and washing conditions are carefully selected to minimize undesirable responses from the mismatched hybridization probes. From these points of view, the alternative probes that do not rely on the hybridization events are highly demanded [3].

In a series of work we have demonstrated a novel strategy to discriminate a single-base alteration by base-discriminating fluorescent (BDF) oligonucleotides probes [4]. The concept of the BDF probes is based on the fluorescence change of the BDF base itself in response to the bases on a complementary strand, not on whether the probe is hybridized as illustrated in Fig. 1.

**RESULTS AND DISCUSSION**

**Pyrene-labeled BDF probes: A/G-specific fluorescence enhancement**

The fluorescence of pyrene-carbonyl derivatives such as pyrene-1-carboxyaldehyde shows a strong dependency on solvent polarity [5]. For example, the fluorescence in nonpolar solvents such as in n-hexane is very weak (quantum yield <0.001), whereas the fluorescence quantum yield in polar methanol is 0.15. We assumed that if the pyrene-carbonyl fluorophore is attached to uracil at the C-6 position, via rigid acetylenic linker, the fluorophore would be extruded to the outside of the groove, a highly polar aqueous phase, due to the base pairing with A (matched). In such a case, the pyrene-labeled BDF probe should exhibit a strong fluorescence that is selective for A. When the pyrene fluorophore is intercalated into a DNA duplex due to the lack of base pairing (mismatched), the BDF base would not emit fluorescence due to the location of the fluorophore at a hydrophobic site in the groove.

On the basis of this concept, we designed a new type of pyrene-labeled BDF nucleosides, $\text{PyU}$ and $\text{PyC}$. These BDF bases, $\text{PyU}$ and $\text{PyC}$, exhibited unique fluorescence properties depending on the nature of the base on the complementary strand, and are capable of distinguishing A and G opposite to the BDF base, respectively, by a sharp change in fluorescence intensity (Fig. 2) [6].

![Fig. 1 Schematic illustration for homogeneous SNP typing using BDF probe.](image)
Such a sharp change in fluorescence intensity is possibly because of the difference in polarity of the microenvironments near PyU pyrenecarboxamide moieties. The difference in polarity was also supported by the energy-minimized structures for the duplex containing a PyU/A base pair. It was found that the pyrenecarboxyamide chromophore of PyU was extruded to the outside of the duplex, and exposed to a highly polar aqueous phase. In contrast, the duplex containing a PyU/G mismatched base pair showed a structure in which the glycosyl bond of uridine was rotated to the syn conformation. The propargyl linker of PyU was stacked into the minor groove, and the pyrenecarboxyamide unit was bound to the duplex along the minor groove. In this conformation, the pyrenecarboxyamide group was located at a hydrophobic site of the duplex (Fig. 3).
Since PyU shows a highly A-selective fluorescence emission, we expected that the analogous C derivative (PyC) would exhibit a G-selective fluorescence emission. Actually, the fluorescence emission from 5'-d(CGCAACPyCCACGC)-3'/5'-d(GCGTTGNGTTGCG)-3' was highly G-selective [6]. BDF nucleoside that emits fluorescence selectively for G is unusual. We also designed BDF nucleosides 8PyA and MePydA, which emitted strong fluorescence when the opposite bases are T and C, respectively (Fig. 4) [7]. Thus, we were able to discriminate four DNA bases (A, T, G, C) by using four different BDF nucleosides.

Fig. 3 The energy-minimized structures of the duplexes containing a PyU/A or PyU/G base pair.

Fig. 4 Structures of four BDF nucleobases.
These BDF probes enabled us to distinguish single-base alterations by simply mixing with a sample solution of target DNA. For example, we examined the discrimination of SNPs in human c-Ha-ras SNP sequence, which possesses a C/A SNP site, using PyU-containing BDF probes [6]. On hybridization of the BDF probe, 5'-d(GGCGCCGPyUCGGTG)-3', with the target sequence, the BDF probe showed an A-allele-specific fluorescence emission at 398 nm when excited at 344 nm. The fluorescence of nonhybridized oligodeoxynucleotide (ODN) or an ODN probe hybridized with a C-allele sequence was negligible. The homogeneous SNP typing method using these four BDF nucleosides is a powerful alternative to conventional SNP typing methods.

Pyrene-labeled polarity-sensitive BDF probes

Fluorescent molecules, whose emission spectra and quantum yields are markedly sensitive to solvent polarity, are widely used as reporter probes for investigating chemical, biochemical, and biological phenomena [8]. Such fluorescent nucleosides would be valuable for monitoring the microenvironment of DNA duplex, such as for sensing the difference in polarities between the inside and outside of DNA duplexes. In our continuous efforts to develop BDF nucleosides, we devised a new homogeneous assay that provides a clear distinction of the base on the complementary strand by polarity-dependent fluorescence change. Thus, we have developed novel alkanoylpyrene-labeled BDF nucleosides, \(\text{AMPyU} \) and \(\text{MPyU} \) (Fig. 5) [4f]. The nucleosides described above exhibit strong fluorescence emission at long wavelength that is highly sensitive to solvent polarity. BDF probes containing \(\text{AMPyU} \) selectively emit fluorescence only when the base opposite BDF nucleoside is adenine and acts as effective reporter probe for homogeneous SNP typing. The homogeneous SNP typing method using \(\text{AMPyU} \)-containing BDF probes is a powerful alternative to conventional SNP typing as well as gene detection.

![Fig. 5](image)

(a) Fluorescence spectra of ODN\(_{\text{ALDH2}}\) (AMPyU) hybridized with ODN\(_{\text{ALDH2}}\) (A or G) and (b) corresponding fluorescence image. (c) Fluorescence spectra of ODN\(_{\text{bcr/abl}}\) (AMPyU) hybridized with ODN\(_{\text{bcr/abl}}\)(A) or ODN\(_{\text{bcr/abl}}\)(G) and (d) corresponding fluorescence image.
Pyrene-labeled BDF probe: G-specific fluorescence quenching

It is interesting to note that sometimes the neighboring nucleobases can quench the fluorescence of fluorophores [9]. In particular, G exhibits exceptionally high quenching efficiency with many different types of fluorescent labels. Although, in general, by the use of BDF probes, this quenching may be considered as a drawback for SNP typing protocol, this process can be used to advantage in single-base discrimination, if this G-specific quenching could be switched on and off through base pairing with a BDF base. Thus, with this idea, we have developed a new strategy for the detection of single-base alterations through fluorescence quenching by G.

We have devised a novel BDF nucleoside, 4'-pyrenecarboxamide-modified thymidine (4'PyT), which exhibits intense fluorescence only when the 4'PyT is involved in a complementary base pair with A (Fig. 6). Pyrenecarboxamide was selected as the fluorophore, due to its intercalative activity and its reactive quenching by G [10].

Stable base-pairing with A locates the fluorophore in the minor groove, where the fluorophore escapes efficient quenching by the flanking G bases. The pyrene group cannot intercalate within the DNA in keeping with the base pairing, due to the short methylamide linker in the 4'-position [10c]. In contrast, when the complementary base of 4'PyT is mismatched (T, G, or C), then the hydrophobic pyrenyl group is likely to intercalate with the π-stacked DNA helix, breaking the weak hydrogen bonds. This intercalation enables the fluorophore to come into intimate contact with the flanking base pairs and results in the quenching of the fluorescence (Fig. 6). The explanation of our results is supported by optimized conformations of the duplexes and also from induced circular dichroism (CD) spectral measurements [4g].

These results confirm our concept that a single-nucleotide alteration can be distinguished through fluorescence quenching by flanking G bases. 4'PyT-containing DNA oligomer thus exhibits A-selective fluorescence, and can act as an effective probe for homogeneous SNP typing.

Detection of base insertion by pyrene excimer fluorescence

Insertion/deletion (indel) polymorphisms occupy approximately 10% of all the polymorphisms in the human genome [11] and lead to serious gene expression errors because they often cause a translational frame shift and create premature proteins. While numerous methods for SNP typing are available, a fluorescent ODN probe that is specific for indel detection has not been delineated. It is highly desirable to develop a new ODN probe that can easily detect indel polymorphisms at a specific site on the target DNA. The development of pyrene-labeled ODNs suitable for the detection of extra bases will make it

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possible to judge the presence/absence of indel polymorphisms located at a specific site on a target DNA by simply hybridizing with target DNA.

We designed a Py2Lys-containing ODN, 5'-d(GTGTTAAGCCPy2LysGCCAATATGT)-3' [12]. With excitation at 350 nm, the fluorescence of the single-stranded ODN was negligible. When the Py2Lys-containing ODN was hybridized with 5'-d(ACATATTGGCGGCTTAACAC)-3', which does not possess the base opposite Py2Lys, the fluorescence was still weak. In contrast, the fluorescence spectrum of the duplex with 5'-d(ACATATTGGCGGCTTAACAC)-3', where A is the base opposite Py2Lys, had a strong fluorescence peak at 495 nm that corresponds to the fluorescence wavelength from a pyrene excimer (Fig. 7).

The clear change in the fluorescence that depends on the presence/absence of the inserted base opposite Py2Lys (A bulge) is very useful for the detection of insertion polymorphisms. We tested the detection of an insertion mutation by hybridization of the Py2Lys-containing probe using the coding sequence of the epithelial sodium channel b subunit (bENaC) gene associated with Liddle’s syndrome, which is an autosomal dominant form of hypertension with variable clinical expression [13,14]. We prepared the Py2Lys-containing probe, 5'-d(CTCACTGGGGTAGGGCCCAGTPy2LysGTTGGGGCT)-3', and hybridized with bENaC gene sequences, 5'-d(AGCCCCAAC(G)nACTGGGCCCTACCCCAGT-GAG)-3' (wild type, n = 0; G-inserted mutant, n = 1). The fluorescence emission from the duplex with a G-inserted strand was very strong and clearly distinguishable from the poor fluorescence of the duplex with a wild-type strand. The hybridization of the Py2Lys-containing ODN with a target DNA facilitates the determination of the presence/absence of insertion polymorphisms located at a specific site on the target DNA by simply mixing.

![Py2Lys](image)

**Fig. 7** The structure of Py2Lys and the fluorescence spectra of the Py2Lys-containing probe hybridized with an ODN with/without A bulge; “ss” denotes a single-stranded probe.

**SUMMARY AND OUTLOOK**

In this paper, we have summarized our current efforts toward the development of concept and approaches to afford several BDF nucleosides valuable in homogeneous SNP typing. We have demonstrated their synthesis and photophysical properties. These BDF nucleosides exhibit a drastic change in fluorescence intensity only when the BDF-labeled probe hybridizes with a target sequence. The clear fluorescence change is very useful for SNP genotyping. The SNP genotyping using our BDF probes serve as a powerful alternative to conventional SNP typing methods. The BDF probes described here are now used on a DNA microarray and will be commercialized soon. Much effort is underway to find even more efficient BDF probes for SNP genotyping.

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