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Intelligent fluorescent nucleoside in sensing cytosine base: Importance of hydrophobic nature of perylene fluorophore

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Abstract—Fluorescence response upon hybridization of perylene labeled oligonucleotide probes depends on the microenvironment experienced by the perylene fluorophore. In mismatched duplex (^{Per}**U**-C), enhanced fluorescence was observed while in matched duplex (^{Per}**U**-A) fluorescence intensity decreased considerably. This observation will be a promising research effort in giving rise to a new powerful tool in detection of SNP.

The completion of the Human Genome Project, after 50 years of Watson–Crick, has set the stage to the biomedical researchers toward the conceptual basis of development of personalized medicine through the screening of genetic mutations.[1] Among all other mutations, single nucleotide polymorphisms (SNPs) are the most common form of genetic mutation. These genetic polymorphisms are often diagnostic of particular genetic predispositions toward diseases and drug responses.[2] The detection and targeting of such SNPs in an array of disease related genes provides an avenue for the rational design and the realization of new diagnostics and chemotherapeutics. Recently, a number of methods based on oligonucleotide probes sequence have been developed for SNP typing,[3] and all the research efforts are aimed to detect the fluorescence signal generated upon hybridization with complementary fully matched target oligonucleotide sequences. Since last several years, we have also devoted research efforts in designing base discriminating fluorescence (BDF) nucleobases for the discrimination of mismatched base and they are successfully utilized in the detection of SNPs in homogeneous assay.[4]

The fluorescence behavior of 3-perylene carboxaldehyde and 3-acetyl perylene toward the solvent polarity is just opposite to that of the corresponding pyrene or anthracene derivatives.[5] Being inspired by the hydrophobic nature of the perylene fluorophore,[6,7] we thought that it will be worthwhile to tether perylene carboxamide into oligonucleotides and follow up the fluorescence behavior in presence of complementary sequences. We hypothesized that upon binding with its fully matched complementary sequence, the fluorescence of the perylene chromophore would be quenched as a result of exposing toward more polar aqueous environment. But in the presence of mismatched sequence, however, it would involve in intercalative stacking interaction in the mispaired position (because of the lack of Watson–Crick base pairing) of the duplex, facing more hydrophobic organic microenvironment and thus mismatch containing duplex detection will be accomplished by fluorescence enhancement. This concept is completely opposite to the case of pyrene chromophore which upon hybridization with mismatched target oligonucleotide showed only weak fluorescence.[4a] Thus, if we are able to utilize a novel concept of detecting mismatch-containing duplexes with our designed perylene labeled nucleoside, it will be a promising research effort in giving rise to a new powerful tool in the detection of SNP and in designing quencher-free molecular beacon.[8]

To explore our concept, we tethered perylene to deoxyuridine and incorporated it into two different oligonucleotide sequences. We synthesized perylene carboxamide containing nucleoside **2** bearing a rigid

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acetylenic linker via Sonogashira coupling of 5-iodo-2′-deoxyuridine and the corresponding perylene carboxamide **1** (Fig. 1). Nucleoside **2** was ultimately incorporated into two oligonucleotide sequences ODN **3** and **4**, differ only by flanking bases near the labeled nucleoside [ODN **3** contains C (G–C rich sequence), while **4** contains T (A–T rich sequence) as the flanking bases] via standard DNA synthesis protocol, and were analyzed by MALDI-TOF mass spectrometry (Table 1).

Prior to incorporation into oligonucleotides, photophysical properties of nucleoside **2** were examined in different organic solvents of varying polarity. Increasing the solvent polarity has only little effect on their absorption maxima but a blue shifted pattern was observed. In contrast both the fluorescence emission intensity and the wavelength are markedly affected by solvent polarity (E_T^{30} as a polarity parameter). In 1,4-dioxane, least polar solvent tested nucleoside **2** displays relatively strong emission with a maximum at 467 nm, while in methanol, the most polar organic solvent tested, showed a weak emission at around 486 nm. Solvents of intermediate polarity display an intermediate behavior with a clear bathochromic and hypochromism effects with increase in solvent polarity.[9]

The single stranded ODN **3** (5′-CGCAAC^{Per}UCA ACGC-3′) and **4** (5′-CGCAAT^{Per}UTAACGC-3′) showed strong fluorescence emission at around 496 and 498 nm, respectively, when excited at 452 nm in sodium phosphate buffer (pH 7.0). When hybridized to their perfectly matched complementary ODNs to form duplexes **3/5** and **4/9**, the fluorescence intensity decreased considerably. The fluorescence intensities also quenched in the case of duplex containing T or G base opposite to ^{Per}**U** in the complementary strands for both A–T and G–C rich sequences. Most importantly, we observed a strong fluorescence enhancement in case of heteroduplexes, **3/8** and **4/12**, containing mismatched base cytosine opposite to the labeled base, ^{Per}**U**, in the target oligonucleotide sequences (Fig. 2).

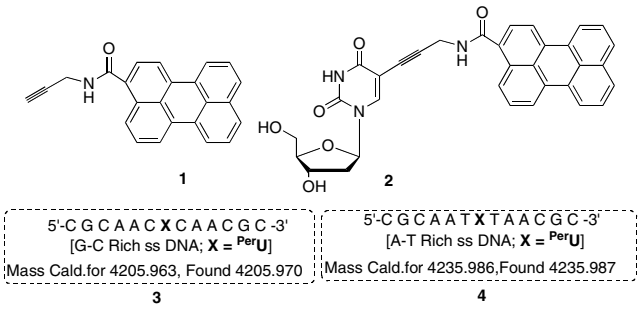

Figure 1. Synthesized perylene labeled nucleoside and oligonucleotides.

Three possible factors may be considered all of which can explain these photophysical observations. These are: (i) the intercalation,[10] (ii) stacking interaction,[10] and (iii) the intercalative stacking interaction and the hydrophobic[6] nature of perylene fluorophore. There are several reports in which fluorescence of matched duplex is quenched via intercalation/stacking between the fluorophore and the DNA bases inside the duplex.[11] This is only possible in case of flexible ethylenic linker that can allow the fluorophore to come inside the duplex. But in the present case the linker is rigid acetylene containing amide bond and thus the fluorophore is unable to come in between the bases inside the duplex for matched case. Thus, the only possible way is that perylene may reside outside the duplexes along the major groove and hence more exposed toward the polar aqueous microenvironment, and fluorescence quenching is the result, which was exactly the case of completely matched duplex (Fig. 2). This explanation is supported by the nature of monomer fluorescence and absorption behavior in solvents of different polarity as described above.[9]

On the other hand, the mismatched duplexes **3/8** and **4/12** showed a strong fluorescence emission that can be explained if we consider a possible more hydrophobic environment faced by perylene. As the opposite base is cytosine, hence in that position of the duplex because of impossible H-bonding between ^{Per}**U** and C bases, it is quite reasonable that the rigid fluorophore upon getting little flexibility and may slightly exposed itself to the mispaired position and bind strongly via intercalative stacking interaction and thus experienced more hydrophobic organic microenvironment. As a result, fluorescence enhancement was observed (Fig. 2).

The UV absorption of monomer ^{Per}**U** and ODNs, and the thermal melting temperature behavior of duplex ODNs support our proposed explanation. The melting temperature experiments showed that the mismatched duplexes **3/8** and **4/12** are almost as stable as (T_m = 56.4 °C, for **3/8** and T_m = 52.0 °C, for **4/12**) the fully matched duplexes **3/5** and **4/9** (T_m = 59.9 °C, for **3/5** and T_m = 52.3 °C, for **4/9**). Such stability is only possible if the perylene unit comes closer to the DNA bases on the opposite strand and takes part in stacking interaction. The absorbance behavior of the mismatched duplexes compared to that of the fully matched one also supports such stacking interaction within the duplexes

Table 1. The sequences of oligonucleotides (ODNs)

(ODNs)	G–C Rich sequences	(ODNs)	A–T Rich sequences
3	5′-d(CGCAAC^{Per}UCAACGC)-3′	**4**	5′-d(CGCAAT^{Per}UTAACGC)-3′
5	5′-d(GCGTTG **A** GTTGCG)-3′	**9**	5′-d(GCGTTA **A** ATTGCG)-3′
6	5′-d(GCGTTG **T** GTTGCG)-3′	**10**	5′-d(GCGTTA **T** ATTGCG)-3′
7	5′-d(GCGTTG **G** GTTGCG)-3′	**11**	5′-d(GCGTTA **G** ATTGCG)-3′
8	5′-d(GCGTTG **C** GTTGCG)-3′	**12**	5′-d(GCGTTA **C** ATTGCG)-3′

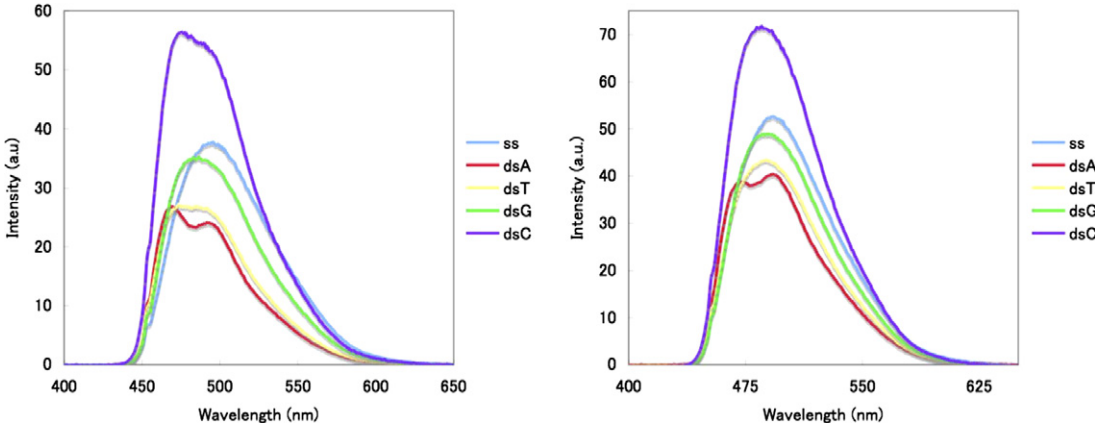

Figure 2. Fluorescence behavior of 2.5 μM solution in a buffer (pH 7.0) containing different ODNs; ss denotes single stranded ODN and dsA, dsT, dsG, and dsC denote double stranded ODNs containing A, T, G, and C bases opposite to ^{Per}**U**, respectively. Left side is for G–C and right side is for A–T rich sequences.

along the major groove. The higher T_m value for ^{Per}**U**-C mismatched duplexes suggests that perylene is involved in stronger stacking interaction than in the case of other mismatched duplexes (N = T and G) in both sequences.[12]

Further support to our explanation comes from the molecular modeling study. The optimized structures of the ^{Per}**U** containing homo- and hetero-duplexes 5′-d(CGCAAC^{Per}**U**CAACGC)-3′/5′-d(GCGTTGNGTTGCG)-3′ (N = A, C) and 5′-d(CGCAAT^{Per}**U**TAACGC)-3′/5′-d(GCGTTANATTGCG)-3′ (N = A and C) were obtained using AMBER* force field in water employing MacroModel vs. 9.0.[9] In the energy minimized structures for the fully matched duplexes in both the A–T and G–C rich sequences, the perylene carboxamide chromophore was extruded to the outside of the duplexes and thus exposed to a highly polar aqueous microenvironment. On the other hand, the duplexes containing ^{Per}**U**-C mismatched pairs bound strongly via site-specific intercalative stacking interaction between the DNA bases and the aromatic rings of perylene from the major groove side of the helix. The intercalated perylene itself resembles another base pair, stacked between neighboring base pairs. Thus, in case of mismatched duplexes, perylene fluorophore is exposed to a more hydrophobic microenvironment. Thus, it is clear that the microenvironment around ^{Per}**U** is quite different in matched (^{Per}**U**-A) and mismatched (^{Per}**U**-C) cases irrespective of flanking base pairs. As discussed earlier, perylene emits fluorescence more strongly in hydrophobic environment, thus, from both study it is quite obvious that ^{Per}**U** shows cytosine selectivity in fluorescence irrespective of the sequences because of hydrophobic nature of the perylene fluorophore. Although our present result is just opposite to our previously reported pyrene-labeled BDF nucleoside,[4] it will open a new dimension in SNPs typing.

In conclusion, both G–C and A–T flanking base pairs stabilize the ^{Per}**U**-C mispair containing duplexes that is clear from the melting temperature behavior. We have developed a novel perylene containing fluorescent oligonucleotide which upon hybridization with the complementary strands containing a mismatched or fully matched sequence opposite to the base labeled with fluorophore is able to sense ^{Per}**U**-C mispair containing duplex through the generation of enhanced fluorescence signal. We explored, for the first time, that the hydrophobic nature of the perylene fluorophore is mainly responsible for the selection of a particular duplex containing ^{Per}**U**-C mismatch base pair. Selection of particular mismatched pair irrespective of sequence is highly promising for the detection of SNPs. Discrimination based on mismatched base pair recognition offers an opportunity for selective targeting of mutated genes. Efforts are underway to find even more efficient hydrophobic probes with longer emission wavelength for SNPs genotyping.

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Supplementary data

Synthetic Scheme, photophysical properties, Energy minimized Structures. This material is available free of charge via the Internet at http://www.sciencedirect.com. doi:10.1016/j.bmcl.2006.09.039.

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