Pyrene-functionalized triazole-linked 2′-deoxyuridines—probes for discrimination of single nucleotide polymorphisms (SNPs)†

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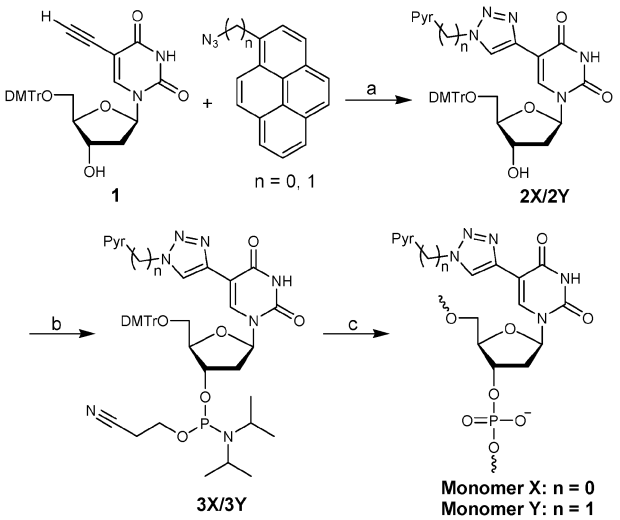
Oligonucleotides modified with pyrene-functionalized triazole-linked 2′-deoxyuridines display remarkable hybridization-induced increases in fluorescence emission and enable efficient fluorescent discrimination of SNPs *via* G-specific quenching.

Single nucleotide polymorphisms (SNPs) are the most prevalent mutation type in the human genome (~10 million SNPs) and commonly linked to diseases and individual responses to therapeutics. Development of SNP typing technologies has accordingly been an area of much recent focus.[1] The use of oligonucleotides (ONs) modified with base discriminating fluorescent (BDF) nucleotides is a particularly interesting approach toward this end, as complementary targets are discriminated from singly base pair mismatched sequences by differences in fluorescence emission.[2] Attachment of polarity-sensitive fluorophores to the C5-position of 2′-deoxyuridines *via* alkynyl linkages has been a very successful strategy toward development of BDFs.[3] Interestingly, while the Cu(I) catalyzed azide alkyne Huisgen 1,3-dipolar cycloaddition[4] has been extensively utilized in nucleotide chemistry[5] to, for example, introduce moieties at the C5-position of pyrimidines,[6] it has not been utilized to generate BDF nucleotides. Motivated by this, the general interest in pyrene-modified ONs,[7] and our own interest in C5-functionalized[8] and pyrene-functionalized ONs[9] for potential biomedical applications, we set out to synthesize and study C5-pyrene-functionalized triazole-linked 2′-deoxyuridine monomers **X** and **Y** (Scheme 1). We surmised that attachment of pyrene fluorophores to the C5-position of 2′-deoxyuridine *via* short linkers would lead to precise positional control of the label and development of new BDF nucleotides.

C5-Ethynyl 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine **1**[10] is a readily attainable starting material (63% over three steps from 5-iodo-2′-deoxyuridine) for the synthesis of target amidites **3X**/**3Y**. Coupling of **1** with either 1-azidopyrene[11] or 1-azidomethylpyrene[12] in a "click reaction" afforded



Scheme 1 Synthesis of monomers **X** and **Y**. *Reagents and conditions*: (a) sodium ascorbate, $CuSO_4$, THF : H_2O : *t*BuOH (**2X**: 52% yield; **2Y**: 80% yield), (b) 2-cyanoethyl *N*,*N*′-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2 (**3X**: 73% yield; **3Y**: 66% yield), and (c) DNA synthesizer. Pyr = pyren-1-yl.

5-(1,2,3-triazol-4-yl)-2′-deoxyuridines **2X** and **2Y** in 52% and 80% yield, respectively. Subsequent O3′-phosphitylation provided phosphoramidites **3X** and **3Y** (73% and 66%),‡ which were incorporated once into the center of mixed sequence 13-mer ONs[3a] using automated solid phase DNA synthesis (0.2 μmol scale). The nucleotides flanking the **X**/**Y** monomers were systematically varied to study the influence of neighboring nucleotides on biophysical properties. Coupling yields of >98% were observed for unmodified monomers as well as for phosphoramidites **3X** and **3Y** (15 min coupling time; DCI as activator).† The identity and purity of purified ONs was verified by MALDI-TOF MS analysis (Table S2, ESI†) and RP-HPLC (>80%), respectively.†

Incorporation of monomer **X** into ONs (**ON5–ON8**) results in decreased thermal denaturation temperatures (T_m's) of the corresponding duplexes with complementary DNA relative to reference strands **ON1–ON4** (ΔT_m between −6.5 °C and −9.0 °C, Table 1, Fig. S1, ESI†). These results are in agreement with previous observations where incorporation of bulky C5-substituted pyrimidine monomers leads to decreased thermal affinity toward DNA targets.[6,13] While reference strands **ON1–ON4** display the expected pattern of discrimination with singly mismatched DNA targets, **ON5–ON8** almost invariably exhibit poor mismatch discrimination when the mismatched

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† Electronic supplementary information (ESI) available: General experimental section, protocols for synthesis, purification and characterization of nucleosides and ONs (including NMR-spectra and MS-data), details of thermal denaturation and fluorescence studies, representative T_m-curves, additional fluorescence emission spectra, data for studies against RNA targets. See DOI: 10.1039/c0cc01133a

Table 1 Thermal denaturation temperatures of duplexes between **ON1–ON12** and complementary (bold) or mismatched DNA targets[a]

ON	Sequence	T_m (ΔT_m)[b]/°C B = A	Mismatch ΔT_m[c]/°C C	G	T
1	5′-CG CAA ATA AAC GC	**48.5**	−10.0	−5.0	−9.0
2	5′-CG CAA CTC AAC GC	**55.5**	−13.5	−9.5	−9.0
3	5′-CG CAA GTG AAC GC	**55.5**	−13.0	−9.5	−10.0
4	5′-CG CAA TTT AAC GC	**48.5**	−11.0	−9.0	−11.0
5	5′-CG CAA A**X**A AAC GC	**42.0** (−6.5)	+1.0	+2.0	−1.0
6	5′-CG CAA C**X**C AAC GC	**48.5** (−7.0)	−1.5	0.0	−1.5
7	5′-CG CAA G**X**G AAC GC	**47.5** (−8.0)	0.0	−10.5	−3.5
8	5′-CG CAA T**X**T AAC GC	**39.5** (−9.0)	+0.5	−1.0	−1.0
9	5′-CG CAA A**Y**A AAC GC	**43.5** (−5.0)	−5.0	−4.5	−5.5
10	5′-CG CAA C**Y**C AAC GC	**54.5** (−1.0)	−11.0	−6.5	−6.0
11	5′-CG CAA G**Y**G AAC GC	**50.0** (−5.5)	−5.0	−10.0	−7.0
12	5′-CG CAA T**Y**T AAC GC	**43.5** (−5.0)	−6.5	−5.5	−7.5

[a] T_m's measured as maximum of first derivative plot of melting curves (A_{260} *vs.* T) recorded in medium salt buffer solution ([Na^+] = 110 mM, [Cl^-] = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4), [EDTA] = 0.2 mM) using 1.0 μM concentrations of each strand. T_m's are an average of at least two measurements. [b] (ΔT_m) = change in T_m-value relative to unmodified reference duplex *e.g.* **ON5**:DNA *vs.* **ON1**:DNA. [c] Mismatch ΔT_m = difference in T_m-value between mismatched duplex and complementary duplex; mismatched sequences: 3′-GC GTT T**B**T TTG CG-5′ (for **ON1**/**ON5**/**ON9**), 3′-GC GTT G**B**G TTG CG-5′ (for **ON2**/**ON6**/**ON10**), 3′-GC GTT C**B**C TTG CG-5′ (for **ON3**/**ON7**/**ON11**) and 3′-GC GTT A**B**A TTG CG-5′ (for **ON4**/**ON8**/**ON12**) where **B** is **A**, **C**, **G** and **T**.

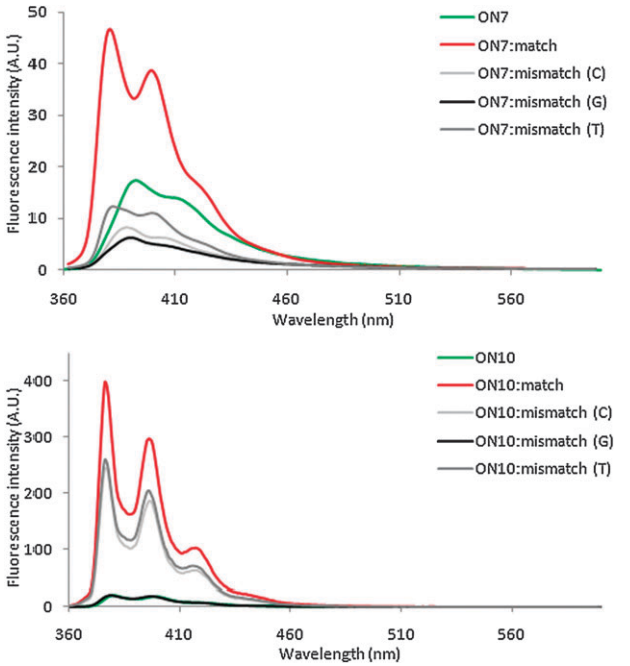

Fig. 1 Steady state fluorescence spectra of single stranded **ON7** (upper panel) or **ON10** (lower panel) and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite to modification in parenthesis). Buffers and concentrations are as described for T_m experiments (see footnote, Table 1); λ_{ex} = 344 nm; T = 5 °C.

nucleotides are positioned centrally opposite of monomer **X** (compare mismatch ΔT_m-values for **ON1** and **ON5**, Table 1).

ONs that are singly modified with monomer **Y** (**ON9–ON12**) display less pronounced decreases in thermal affinity toward complementary DNA (ΔT_m between −1.0 °C and −5.5 °C, Table 1, Fig. S2, ESI†) and more efficient mismatch discrimination than **ON5–ON8** (compare mismatch ΔT_m values for **ON1**, **ON5**, and **ON9**, Table 1). These differential trends suggest that the subtle change in linker chemistry between the pyrene and triazole units in monomers **X** and **Y** markedly influences the binding mode of the C5-substituent.

Next, steady-state fluorescence emission spectra of **ON5–ON12** and the corresponding duplexes with matched or singly mismatched DNA targets were recorded at 5 °C using an excitation wavelength of λ_{ex} = 344 nm. Duplexes between **ON5–ON8** (monomer **X**) and complementary DNA exhibit two broad maxima at 382 and 400 nm. Hybridization is accompanied by substantial increases in fluorescence intensity at λ_{em} = 382 nm (2- to 5-fold, Fig. 1 upper panel, Fig. 2 upper panel and Fig. S3–S5, ESI†). In contrast, hybridization of **ON6** or **ON7** to DNA strands with mismatched nucleotides opposite of monomer **X** results in significantly lower fluorescence output than with matched duplexes (Fig. 1 upper panel and Fig. 2 upper panel). Flanking nucleotides influence the fluorescence properties of monomer **X** as evidenced by the much more efficient fluorescent discrimination of SNP targets when monomer **X** is flanked by cytidines or guanosines (**ON6**/**ON7**) than when flanked by adenosines or thymidines (**ON5**/**ON8**, Fig. 2 and Fig. S3–S5, ESI†). The results demonstrate that monomer **X** is a base-discriminating fluorescent nucleotide similar to C5-pyrenecarboxamide-modified propargyl-linked 2′-deoxyuridines,[3a] which have been used to discriminate SNP sites in human breast cancer.[14] We speculate that the fluorophore of monomer **X** is: (a) positioned in the polar major groove and highly emissive when ONs are hybridized to complementary targets, and (b) intercalating when ONs are hybridized to mismatched targets leading to reduced thermal discrimination[15] and variable fluorescence emission that depends on the nature of nearby nucleotides—guanines are efficient quenchers of pyrene monomer fluorescence.[16] Direct comparison with C5-pyrenecarboxamide-modified propargyl-linked 2′-deoxyuridines in identical sequence contexts reveals that SNP discrimination by **ON6**/**ON7** is equally efficient.[3a]

With the exception of **ON12**, hybridization of ONs modified with monomer **Y** to complementary DNA results in remarkable increases in fluorescence intensity (9- to 23-fold relative to SSP at λ_{ex} = 377 nm, Fig. 1 lower panel, Fig. 2 lower panel and Fig. S6–S8, ESI†) to give emission spectra with three sharp peaks with maxima at 377, 397 and 417 nm§ (Fig. 1). Interestingly, brighter fluorescence was observed for these probes as compared to **ON5–ON8** (monomer **X**).¶ Very few singly modified pyrene-labelled ON probes have been reported to exhibit such levels of hybridization-induced increases in fluorescence intensity,[9a,17] rendering monomer **Y** as an interesting building block for diagnostic applications. Unlike ONs modified with monomer **X**, **ON9–ON12** do not efficiently discriminate SNP targets except those resulting in **Y**:G mismatches (Fig. 1 lower panel, Fig. 2 lower panel and Fig. S6–S8, ESI†). This, along with thermal denaturation studies, suggests that the fluorophore of monomer **Y** is less

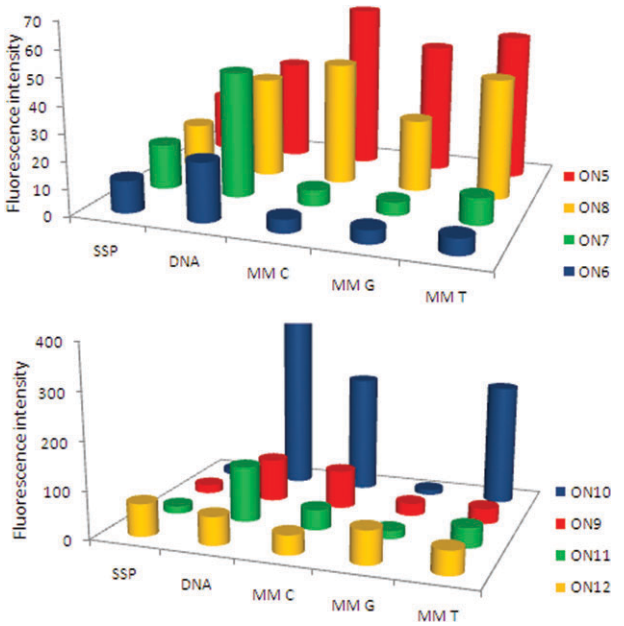

Fig. 2 Fluorescence intensity of single stranded probes (SSPs) and corresponding duplexes with complementary DNA or mismatched DNA targets. Intensity observed at λ_{em} = 382 nm for **ON5–ON8** and λ_{em} = 377 nm for **ON9–ON12** at T = 5 °C. Note that different y-axis scales are used.

prone to intercalate upon hybridization to mismatched targets than that of monomer **X**. The different binding modes are related to the subtle change in linker chemistry between **Y** and **X** monomers, but are not fully understood at the molecular level. Diagnostic assays employing monomer **Y** will—unlike assays employing monomer **X**—require precise temperature control to minimize undesired duplex formation with non-targets and concomitant false positives.

Finally, given the importance of probes for detection of RNA targets to elucidate biological roles of RNA in living organisms,[18] we recorded thermal denaturation curves and fluorescence emission spectra of the representative probes **ON6** and **ON10** and their duplexes with complementary or singly mismatched RNA targets (Table S3, Fig. S9 and S10, ESI†). Similar trends were observed for **ON6** (monomer **X**; efficient SNP discrimination) and **ON10** (monomer **Y**; pronounced hybridization-induced increases, discrimination of **Y**:G mismatches) as when targeting the corresponding DNA strands, which emphasizes the generality of these probes.

To sum up, two fluorescent nucleotide monomers have been prepared in five steps from commercially available 5-iodo-2′-deoxyuridine. ONs modified with monomer **X** allow efficient fluorescent discrimination of SNPs *via* a G-specific quenching mechanism, while ONs modified with monomer **Y** display remarkable hybridization-induced increases in emission and discrimination of **Y**:G-mismatches. Evaluation of these building blocks in diagnostic assays is ongoing.

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Notes and references

‡ The identity of the reported compounds was fully ascertained by NMR (^{1}H, ^{13}C, ^{31}P, COSY, HSQC and/or DEPT) and MALDI-HRMS, while purity was determined by 1D NMR.
§ Single stranded probes and duplexes with mismatched targets generally result in red-shifting of fluorescence emission peaks by up to 5 nm.
¶ Relative fluorescence emission quantum yield of **ON10**:DNA = 0.16.

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