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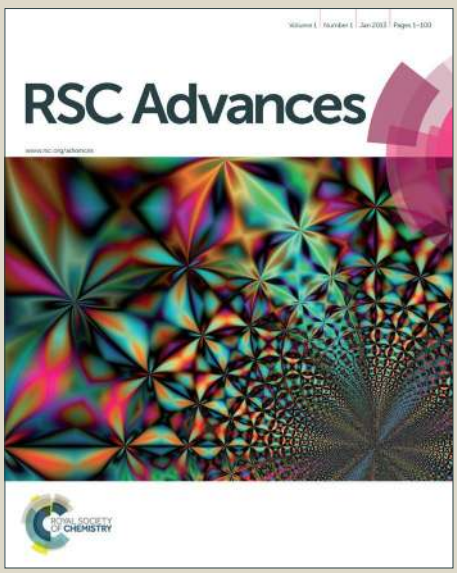

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COMMUNICATION

A Turn-On Dual Emissive Nucleobase Sensitive to Mismatches and Duplex Conformational Changes†

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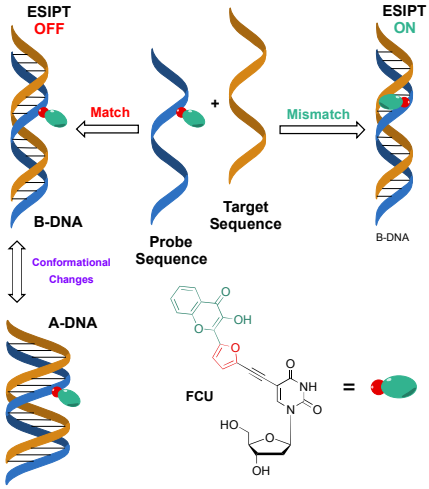
Herein, a 2-furyl-3-hydroxychromone-based deoxyuridine analogue was synthesized and incorporated in DNA. Its ESIPT mechanism, investigated by steady-state and time-resolved spectroscopy, was found to be highly sensitive to hydration through its ratiometric response. As a result, this analogue demonstrated a unique on-off dual emissive behavior which allows monitoring DNA hybridization as well as discriminating matched from mismatched duplexes, and B from A conformations.

Nucleic acids are the genomic templates, crucial for encoding proteins that are essential for all cellular functions. Mutations or variations in the genome sequence, known as single nucleotide polymorphisms (SNPs), produce malfunctioned proteins and cause several diseases including cancers.[1] SNPs occur at every few hundred to thousand base pairs in genomic DNA, and serve as markers for identifying disease-causing genes, allowing an early diagnosis and treatment. Among various methods developed for analysing SNPs, fluorescence-based assays have received strong interest owing to their simple, rapid, inexpensive and accurate detection.[2]

Unlike proteins that show intrinsic fluorescence from aromatic amino acids (e.g., tryptophan), nucleic acids exhibit very low inherent fluorescence. Hence, design of fluorescent nucleobase analogues is an important area of research in nucleic acid chemistry. The majority of hitherto discovered emissive nucleosides are single-band emitters that respond to environment by changes in their fluorescence intensity or λ-shifts in their emission maximum.[3] However, many of these dyes also suffer from quenching by neighbouring nucleobases or poor sensitivity to perturbations in the DNA structure.[4] Therefore, designing novel emissive nucleosides with unique fluorescence properties remains a key challenge. Recently, 3-hydroxychromone (3HC)-based fluorophores have received tremendous interest due to their distinctive dual emissive character.[5] These molecules feature two well-resolved fluorescence bands, namely the normal (N*) and tautomer (T*) maxima resulting from an excited-state intramolecular proton transfer (ESIPT) process. Since their ratiometric emission is highly sensitive to polarity and hydration, 3HC derivatives have been widely used to probe biomolecules *in vitro* and *in vivo*.[6] In order to take advantage of these ultrasensitive probes in nucleic acid chemistry, we started synthesizing and evaluating 3HC-based nucleobases.[7] In continuation of our efforts, herein we report a newly-designed emissive nucleobase based on a 2-furyl-3HC conjugated electronically to uracil moiety (**FCU**). When incorporated in oligonucleotides (ODNs), this ESIPT-based nucleoside was found to be highly sensitive to mismatches and DNA conformation (Scheme 1).



Scheme 1. Structure of **FCU** and schematic representation of ESIPT on-off switching mechanism upon hybridization with matched or mismatched sequences and upon helix conformational changes.

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Synthesis of **FCU** amidite was adapted and optimized from our reported protocol (Schemes S1 & S2).[7e] The probe was incorporated in 15-mer model ODNs d(CGT TTT **XMX** TTT TGC), where **X** = **A**, **C**, **G** or **T** and **M** = **FCU**, by solid-phase synthesis using phosphoramidite chemistry (Figures S1-S4). Each labelled ODN was then annealed with its complementary ODN *d(GCA AAA YXY AAA ACG)*, in which ***Y*** is ***A***, ***C***, ***G***, or ***T*** and ***X*** is ***A***, ***C***, ***G***, ***T*** or ***Ab*** (abasic), resulting in duplexes with different compositions.

Photophysical characterizations of **FCU** in solvents and within single- and double-stranded DNA are depicted in Figures 1 and S5, and Tables S1-S3. Generally, 3HC derivatives exhibit a single absorption band and two well-separated emission bands due to ESIPT reaction.[5a] The N*/T* ratio of the two emission bands strongly depends on the polarity and proticity of the solvent (Figure 1). The T* band is largely dominant in low polar and aprotic solvents, where the ESIPT process is highly efficient. In contrast, **FCU** shows a single N* emission band in bulk water, likely as a result of the inhibition of ESIPT by surrounding H_2O molecules that form intermolecular hydrogen bonding interactions with the α-hydroxy ketone of **FCU**.[7e,8]

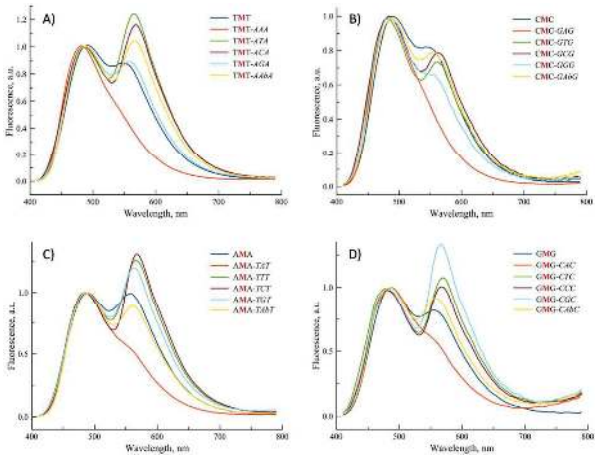

Figure 1. Emission spectra of various matched and mismatched dsDNA in pH 7 phosphate buffer (20 mM phosphate, 150 mM NaCl, 1 mM EDTA). Excitation wavelength was 390 nm.

Interestingly, when **FCU** is incorporated in single strand ODNs, the tautomer band is observed in all ssDNAs irrespective of the adjacent nucleobases. This T* band may be attributed to the partial shielding of **FCU** from water by stacking with the neighbouring bases, which facilitates the progress of ESIPT. While the intensity ratio (N*/T*) is similar in all ssDNAs, its quantum yield (QY) depends on the nature of the neighbouring bases (Table S1). As for many other emissive nucleobases,[3,4] the lowest quantum yields (QY$\sim$2%) were observed when **FCU** is flanked by G residues, which act as efficient fluorescence quenchers. In all other cases, the QY was between 9-14 %, comparable to the value of the non-incorporated **FCU** in bulk water (13 %).[7e]

Next, we investigated the dependence of **FCU** fluorescence properties upon formation of matched and mismatched duplexes. To this end, the synthesized **FCU**-labelled oligonucleotides were annealed to their complementary sequences with varying opposite nucleobases to **FCU**. In a first step, the thermal stability of the duplexes was investigated by monitoring the temperature-induced absorbance changes at 260 nm (Figure S6-S7). The melting temperatures of the labelled duplexes paralleled well those of the corresponding non labelled ones being decreased by less than 6 °C (Table S3). It suggests that **FCU** adopts similar base pairing as U or T with the opposite base in the matched and mismatched duplexes and that the 3HC fluorophore only destabilizes at minima the duplexes. Noticeably, when an abasic site was opposite to **FCU**, the duplex was 5-6 °C more stable than the corresponding one with natural U, suggesting that the 3HC dye may stack with the surrounding bases to stabilize the helix. Circular dichroism studies further indicated that **FCU** preserved the right-handed helical B-form of the duplexes (Figure S8).

The most striking and obvious difference between the duplexes turns out to be the T* band in emission spectra that is clearly observed in mismatched duplexes but nearly disappears in matched duplexes (Figure 1). As the T* band is present in ssDNA, the ESIPT process is believed to be switched off along the formation of matched duplexes, and turned on in mismatched cases. The switching off of the ESIPT process was noticed with all tested sequences, but was more efficient with flanking pyrimidines than purines. As the emission spectrum of **FCU** in matched duplexes is similar to the spectrum of the free nucleoside in water, it may be concluded that whereas the uracil moiety base-pairs with A, the 3HC fluorophore is projected towards the major groove and directly exposed to water. The accessibility to water causes the inhibition of the ESIPT reaction through intermolecular H-bonding, and thus, favours the N* form. In contrast, the emergence of T* band in mismatched duplexes indicates that the 3HC fluorophore resides in a hydrophobic/less-polar environment with a limited access to water. It is thus likely that in mismatched cases, the 3HC fluorophore is at least partly stacked with the neighbouring bases. This can be explained by the fact that the base conformation flips from anti to syn, with the uracil projecting away from the opposite strand and the 3HC projected inside the duplex (Figure 2). The N*/T* value was found to depend on the nature of both the opposite nucleobase and the flanking ones in mismatches. This suggests that the degree of intercalation of the dye in the double helix is dependent on the steric constraints imposed by the opposite base and its ability to stack with the adjacent nucleobases. Although no clear trend emerged from the comparison of the N*/T* mismatch values, preventing the identification of their nature, **FCU** remains able to easily detect a single nucleotide polymorphism (SNP). Through its large change in the N*/T* ratio, the new label could be applied for the detection of A-alleles, which are challenging to detect in common hybridization DNA assays because of the limited difference in the thermal stability between matched and mismatched duplexes for a single base mutation. In this sense, **FCU** is

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comparable to the base-discriminating fluorescent probes first developed by Saito and co-workers[2f] for genotyping of SNP.

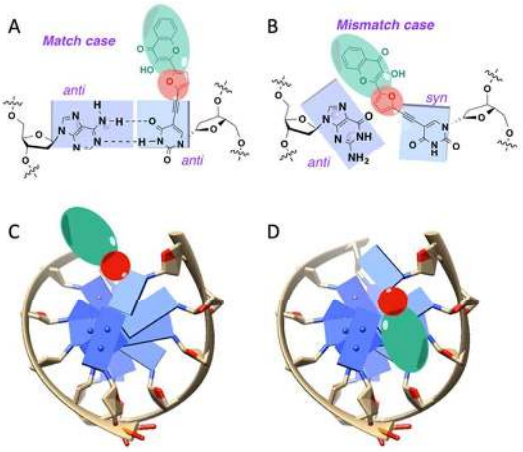

Figure 2. Schematic representation of the M-Y base pair in matched (A/C: anti/anti) and mismatched (B/D: syn/anti) double strand DNA.

Due to the turn-off of ESIPT reaction upon hybridization, this feature could in principle be used to monitor the melting of the duplex and determine its Tm. To corroborate this hypothesis, we monitored for the matched **TMT**-*AAA* duplex, the dependence of the fluorescence emission *vs* the temperature (Figure 3). We found that the T* band progressively emerges by increasing the temperature. When the T*/N* ratio *vs* T was plotted, the obtained transition temperature exactly matched with the T_m calculated by absorption measurements. Therefore, this ESIPT on-off switching process appears as a novel convenient way to monitor the annealing or melting of duplexes, and obtain accurately their Tm value.

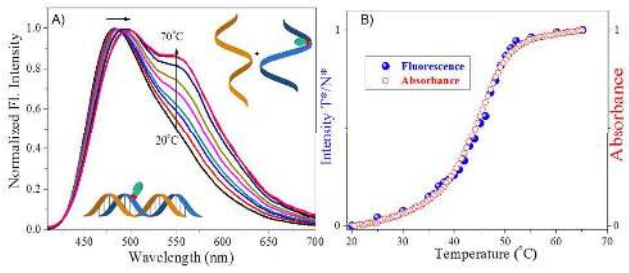

Figure 3. A) Emission spectra of **TMT**-*AAA* dsODN with increase in temperature. B) Overlay of thermal melting curves calculated from fluorescence and absorbance measurements. Buffer and excitation wavelength were as in Figure 1.

Next, to extend the scope of the **FCU** probe, its ability to sense ratiometrically the helical conformation was investigated. For that purpose, a **FCU**-labelled ODN was annealed with its complementary RNA target sequence and the resulting emission was monitored (Figure 4).

The hybrid DNA/RNA duplex was found to exhibit dual emission, indicating that the ESIPT process is switched on in DNA/RNA hybrid whereas in matched DNA/DNA context, it turns off. The dual emission in DNA/RNA hybrid might be attributed to the lower exposure of the probe to water in A-DNA, in line with the shallower major groove in A-DNA as compared to B-DNA.[9] These data indicate that the **FCU** dye does not only sense hybridization or SNPs, but also distinguishes A from B helical forms.

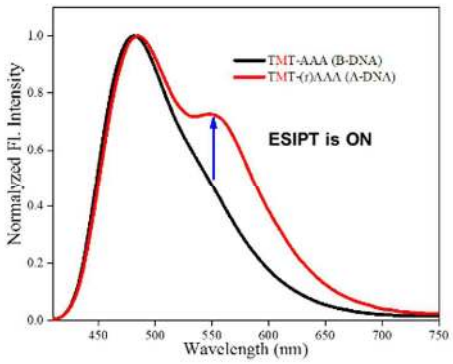

Figure 4. Emission spectra overlay of DNA/DNA (B-form, black) and DNA/RNA (A-form, red) duplexes (**TMT**-*AAA*). Buffer and excitation wavelength were as in Figure 1.

To further understand the photophysics of **FCU** and notably, its excited-state dynamics, pico-second time-resolved measurements were performed on the free **FCU** (Table S4) as well as on the **TMT** single strand and its duplexes with matched and mismatched complementary sequences (Table S5). The emission decays were collected at both N* and T* bands. The obtained fluorescence decays are depicted in Figure 5A. The emission decay of the single-stranded **TMT** collected at the N* band could be well fitted using a triexponential function with lifetime components of ~200 ps, 860 ps and 1.83 ns. The first two components are major, representing ~90 %. When the decays are collected at T* band, the ssDNA showed a biexponential decay with a ~250 ps rise component and a 2.26 ns decay component. The rise component at T* band matches well with the short decay component observed at N* band and can thus be assigned to the ESIPT process.[10] The long lifetime component (2.26 ns) was significantly longer than that in methanol (1.3 ns) or ethanol (1.5 ns), suggesting that an additional factor contributed to its increased lifetime. As previously described,[11] the restricted environment provided by the flanking nucleobases likely limits the rotation between the aromatic moieties of the 3HC fluorophore, favouring a flatter conformation of the probe with a longer lifetime.

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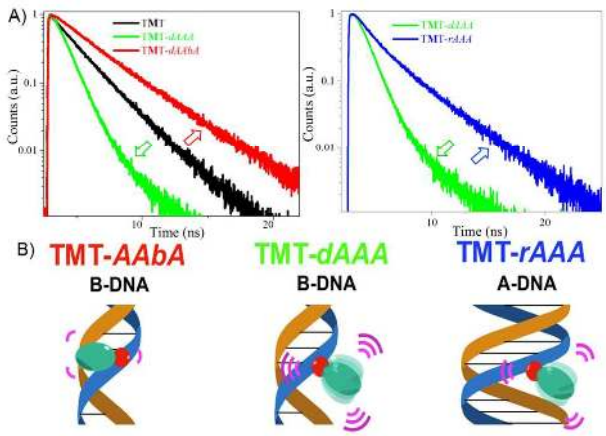

Figure 5. Fluorescence decays (A) and scheme (B) of **FCU**-labelled matched and mismatched B-DNA, DNA/DNA (B-DNA) and DNA/RNA (A-DNA) duplexes (**TMT**-AAA). The decays were obtained with λ_{em}=485 nm and λ_{em}=580 nm. Excitation wavelength was 420 nm.

Significant changes in the fluorescence decays were observed when this ssDNA was annealed with its complementary target sequences. These changes strongly depended on the nucleobase opposite to **FCU**. Interestingly, a faster fluorescence decay is observed when the opposite nucleobase is adenine, while with other nucleobases the decay becomes slower (Figure 5A). In the matched duplex, the fluorescence decay of the N* form is devoid of longer lifetime component. The lifetime was found highly similar to that of the free probe in water strongly suggesting that the 3HC probe is an unrestricted environment where the aromatic moieties can freely rotate (Figure 5). This confirms that the 3HC fluorophore of **FCU** is not intercalated in the matched duplex, but is likely projected towards the major groove, where it has full access to the bulk water (Figure 5B). In sharp contrast to the matched duplexes, the fluorescence decay of the T* band of all mismatched duplexes was dominated by a 3.3–3.4 ns lifetime component. Such a long lifetime suggests that **FCU** is in a highly restricted environment, favouring the more emissive flat conformation of the probe. This confirms the hypothesis that **FCU** may be intercalated between the base pairs in the mismatched DNA strands, while it is projected towards the solvent in the matched duplex (Scheme 1, Figure 5B). The values of the short component attributed to the ESIPT process were also found to differ in the matched and mismatched duplexes, being 2- to 3-fold higher in the former. This indicates that there may be a higher energy barrier, which slows down the formation of the T* form, resulting in limited accumulation of this form, as observed for the free probe in water. The time-resolved data of **FCU** can also distinguish the A from B conformations of the duplexes (Figure 5 and Table S6). Indeed, the fluorescence decay of the N* form of the DNA/RNA hybrid (A-DNA) was observed to differ from that of the DNA/DNA duplex (B-DNA) by a much shorter ESIPT lifetime, in line with a lower energy barrier between the N* and T* forms, and thus an efficient formation of the T* form in the A conformation. The decays differed also by the 1.59 ns lifetime component that was only present for the A conformation. This additional lifetime of the N* form as well as the 3.42 ns lifetime of the T* form are likely due to the restricted environment of the shallower major groove in the A form that favours the planar conformation of the 3HC probe.

As 3HC emission depends on pH, we monitored the pH dependent absorption and emission properties of FCU in its free form and included in ss and ds DNA (Figures S16-S19). By increasing the pH, we observed a new band with a maximum at ~450 nm in the absorption spectrum as well as a corresponding new emission band with a ~540 nm maximum, corresponding to the anionic form of the probe. From this dependence on pH, the pK_a values of FCU could be determined (Figure S20). The free FCU exhibited a pK_a of 8.3 whereas its pK_a was found to be increased to 8.8 in ss and ds DNA. For mismatched DNA the pK_a value was even higher (~9.2), clearly indicating that the acid-base properties of the probe are modified in the context of oligonucleotides. This increase of pKa when the probe is incorporated in DNA is consistent with the fact that the proton density around DNA molecules is up to two orders of magnitude larger than that of the bulk solvent.[12] As the pKa value of **FCU** in oligonucleotides is close to 9, only minimal amounts of the anionic form could contaminate the spectra recorded at pH7.

In conclusion, we reported the dramatic sensitivity of a dual emissive deoxyuridine analogue **FCU**, towards SNPs and DNA conformations using steady-state and time-resolved spectroscopy. Solvent-dependent emission study of the free nucleoside displayed a single band emission in water and two-well resolved bands in other solvents due to modulation of the ESIPT process. When incorporated in DNA, the probe exhibited different photophysical properties in ss and dsDNA. The T* band, not at all observed for the free **FCU** in water, appeared in ssDNA indicating a switching on of the ESIPT process. When the labelled oligonucleotide was annealed with its complementary target sequence presenting various bases opposite **FCU**, the T* band intensity revealed to be highly sensitive to the nature of its opposite nucleobase. While the T* band emerged in all mismatched duplexes, it was nearly absent in the matched duplexes, so that SNPs can be easily detected by measuring the N*/T* intensity ratio or the T* fluorescence decay. The on-off switching of ESIPT on DNA annealing can also be used to sensitively determine the melting temperature of matched duplexes. Interestingly, the T* band was also observed in the A-form of DNA/RNA hybrid duplexes, indicating that **FCU** was also capable to sense the helical conformation. The on-off ESIPT mechanism observed with **FCU** appears thus, as a new approach for monitoring DNA annealing as well as detecting SNPs and changes in nucleic acid conformation.

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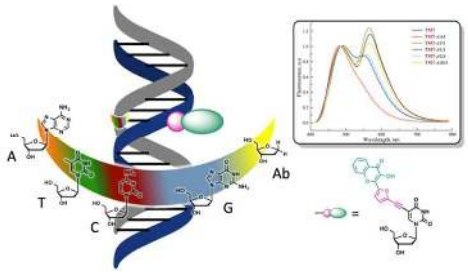

Herein, we demonstrate on-off dual emissive behaviour of a fluorescent nucleoside sensitive towards DNA hybridization and conformational changes as well as detection of single nucleotide polymorphisms.