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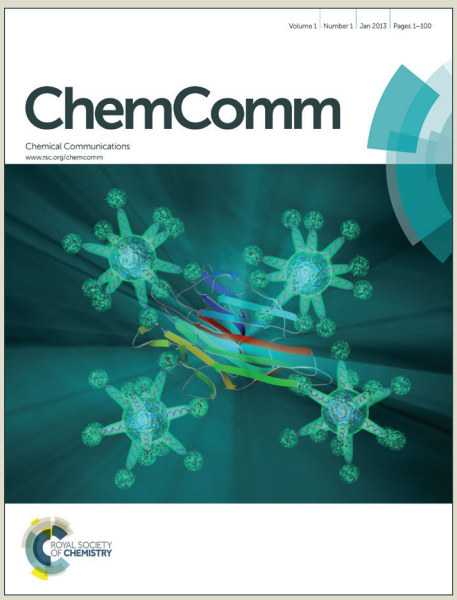

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Dual fluorescent deoxyguanosine mimics for FRET detection of G-quadruplex folding

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Replacement of deoxyguanosine (dG) nucleobases within G-tetrads of G-quadruplex folding oligonucleotides with donor (D)/acceptor (A) fluorescent 8aryldG residues provides diagnostic FRET signalling for G-quadruplex detection.

Fluorescence-based strategies are widely used to explore biomolecular recognition. For nucleic acids the natural nucleobases are essentially nonemissive[1] and thus fluorescent labels are required. Here, dual-labelled oligonucleotides have significant advantages over singly-labelled ones.[2,3] Common strategies for their use include molecular beacons[4-6] or G-quadruplex (GQ) exchange systems.[7-10] In molecular beacons, donor (D)/acceptor (A) dyes are typically attached *via* flexible linkers to the opposing ends of the oligonucleotide. In the stem-loop (hairpin) structure the two probes are close in space permitting fluorescence resonance energy transfer (FRET) from an excited fluorescence D to a ground state A through dipole-dipole interactions.[11] Ligand binding to the loop of the hairpin separates the FRET-pairs in the stem to signal target detection. In GQ exchange, the D/A probes are relatively far apart in the random coil or duplex, while intramolecular GQ folding brings the probes together to provide a FRET signal.[7,8]

Fluorescent nucleobase analogs may also be employed for FRET-based detection strategies. Compared to end-labelled bulky fluorescent dyes, isomorphic fluorescent nucleobases can be internally inserted with minimal perturbation to the helical structure.[12,13] In this regard, Börjesson and coworkers developed a FRET-pair consisting of two cytosine analogs that were employed in a DNA duplex to accurately distinguish distance- from orientation-changes using FRET.[14] Thus, we speculated that it would also be feasible to employ 8-aryl-deoxyguanosine (8aryldG) analogs for FRET detection in antiparallel GQ structures. Fluorescent 8aryldG probes can destabilize a B-form duplex because they prefer to adopt the *syn*-conformation, which disrupts Watson-Crick H-bonding with the opposing cytosine.[15] However, in antiparallel GQ structures they can occupy *syn*-G positions without disturbing H-bonding interactions.[16]

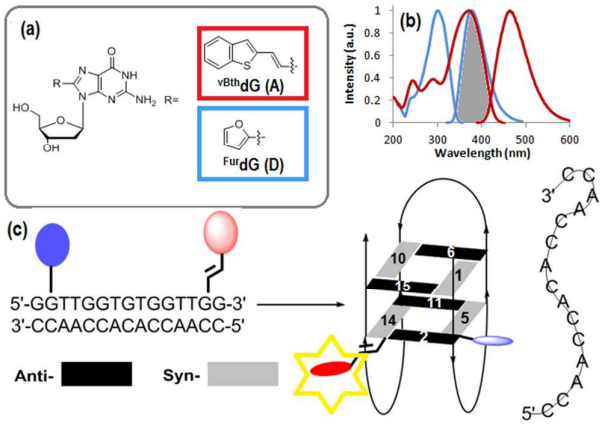

Fig. 1 (a) Structures of ^{vBth}dG (A) and ^{Fur}dG (D). (b) Excitation and emission spectra of ^{Fur}dG (blue trace) and ^{vBth}dG (red trace) recorded in MeOH, region of D/A spectral overlap is shaded grey. (c) Schematic for duplex/GQ exchange for TBA modified with the D/A guanine probes.

Based on our previous studies using fluorescent 8aryldG bases to monitor GQ formation,[17-19] the emissive properties of ^{Fur}dG (Fig. 1a) suggested that it could serve as the D nucleoside. Its emission (~ 380 nm, Φ_{fl} = 0.49[20]) is strongly quenched in duplex DNA, but turns on in the GQ due to effective energy-transfer from the natural dG bases in the G-tetrad.[17-19] For the A, an 8-vinyl-aryldG[21,22] derivative could be designed with an absorbance ~ 380 nm for effective spectral overlap with the emission of ^{Fur}dG. As shown in Fig. 1b, the excitation spectrum of 8-vinyl-benzo[*b*]thienyl-dG (^{vBth}dG, red trace) overlaps with the emission of ^{Fur}dG (blue trace, region of D/A spectral overlap is shaded grey). The ^{vBth}dG probe also provides visible blue emission (λ_{em} = 473 nm, Φ_{fl} = 0.29, see ESI† for details). Thus, to test probe performance in a GQ-based sensor platform, the two 8aryldG derivatives were incorporated into various positions within the 15-mer (5′-$GG_2TTG_5G_6TGTG_{10}GTTG_{14}G$) thrombin binding aptamer (TBA),[23]

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† Electronic Supplementary Information (ESI) available: Experimental section, NMR spectra of ^{vBth}dG derivatives, MS spectra of mTBA, and CD spectra (Fig. S1). See DOI: 10.1039/x0xx00000x

which folds into an antiparallel GQ structure (Fig. 1c). The goal of this study was to determine whether excitation of ^{Fur}dG at ~ 315 nm could give rise to ^{vBth}dG emission at ~ 470 nm with enhanced intensity in the GQ compared to the emission of ^{vBth}dG in the random coil or duplex. Our studies are the first to describe FRET in a GQ through the use of internal D/A fluorescent dG mimics and provide insight for potential applications in aptasensor development.

Solid-phase DNA synthesis was used to generate modified TBA (mTBA) (see ESI† for experimental details, NMR spectra of synthetic samples, UV-vis spectra, HPLC traces and MS spectra of mTBA), which were studied using UV-thermal denaturation, circular dichroism (CD) and fluorescence spectroscopy. For the dual-labelled D/A mTBA samples, the D (^{Fur}dG) was inserted at *syn*-Gs (G_{14} or G_{10}), while the A (^{vBth}dG) was placed in both *anti*- (G_2 and G_6) and *syn*-G positions (G_5, see Fig. 1c for G numbering in TBA). Thus, in the antiparallel GQ structure produced by TBA, the D/A probes were either in the same (14;2 and 10;6) or separate G-tetrads (10;2, 14;6, and 10;5).

Thermal melting and photophysical parameters of the mTBA GQ structures are presented in Table 1. Under the conditions employed, the unmodified TBA duplex has a T_m of 64.5 °C in Na^+-solution, while the GQ gives a T_m ~ 53 °C in K^+-solution.[17] For the singly-labelled mTBA samples, ^{Fur}dG (D) at *syn*-G positions (10 and 14) had a stabilizing influence on the GQ (ΔT_m = 6.0 and 8.0 °C) and a slight destabilizing influence on the duplex (ΔT_m = −5.0 and −3.0 °C). The more lipophilic ^{vBth}dG (A) probe strongly decreased duplex stability at positions 6 and 5 (ΔT_m = −10.4 and −14.4 °C) and GQ stability at *anti*-G_6 (ΔT_m = −10.9 °C). However, at the *syn*-G_5 position, ^{vBth}dG only slightly diminished GQ stability (ΔT_m = −1.7 °C). For the doubly-labelled mTBA samples, duplex stability (ΔT_m ~ −20 °C) was strongly perturbed, as was the GQ with the A probe at *anti*-G_2 (ΔT_m ~ −9.0 °C). However, *anti*-G_6 insertion of the A probe (10;6, 14;6) produced T_m values similar to unmodified TBA (ΔT_m = −1.3 and −2.1 °C). When both probes were placed at *syn*-Gs (10;5), the GQ structure was stabilized (ΔT_m = 9.3 °C).

Table 1 Thermal melting and photophysical parameters of mTBA GQs

Site (D;A)	ΔT_m[a] (GQ/d)	λ_{ex} (I)[b]	λ_{em} (I)[b]	I_{rel}[c]	FRET[d]
D, 10	6.0/−5.0	315 (187)	381 (187)	10.4	/
D, 14	8.0/−3.0	318 (85)	380 (85)	5.0	/
A, 6	−10.9/−10.4	388 (598)	471 (598)	1.4	/
A, 5	−1.7/−14.4	380 (600)	471 (600)	0.6	/
10;2	−9.0/−20.3	310 (37)	470 (171)	5.6	80%
14;2	−8.6/−17.6	310 (26)	471 (106)	3.0	68%
10;6	−1.3/−18.3	312 (28)	469 (193)	3.7	85%
14;6	−2.1/−17.3	315 (27)	470 (259)	4.4	68%
10;5	9.3/−20.0	313 (21)	470 (219)	2.4	88%

[a]Calculated relative to the T_m values of unmodified TBA duplex (d) in Na^+-solution or GQ in K^+-solution, as previously published.[16] [b]Excitation and emission wavelength in nm, intensity (I, a.u.) for the GQ in K^+-solution; for dual-labeled mTBA samples, excitation wavelength and intensity of D (^{Fur}dG) and emission wavelength and intensity of A (^{vBth}G) in the GQ. [c]I_{rel} is relative emission intensity of GQ versus duplex. [d]FRET efficiency = 1-(I_{DA}/I_D), where I is the emission intensity of the donor (^{Fur}dG) at 390 nm following excitation at 315 nm in the absence (I_D) and presence of A (I_{DA}).

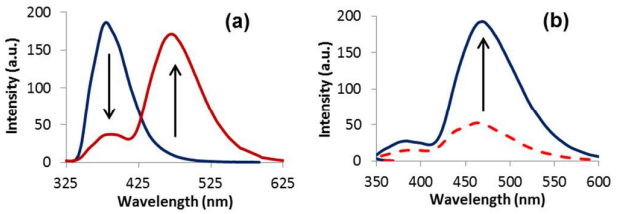

Fig. 2 (a) Emission spectra (λ_{ex} = 315 nm) of mTBA in K^+-solution at 10 °C with ^{Fur}dG (D, *syn*-G_{10}) in the absence (blue trace) and presence (red trace) of ^{vBth}dG (A, *anti*-G_6). (b) Emission spectra at 10 °C of mTBA (D/A, 10;6) duplex (dashed red trace) and GQ (solid blue trace) in Na^+- and K^+-solution, respectively.

TBA is known to form a chair-type antiparallel GQ structure in K^+-solution with a characteristic CD spectrum showing negative and positive bands at ~ 265 and 295 nm, respectively.[24] All the mTBA samples displayed antiparallel CD spectra in K^+-solution (Fig. S1, ESI), confirming that insertion of the D/A probes did not disrupt GQ folding. In Na^+-solution, the mTBA duplex samples showed characteristic B-form sigmoidal CD curves with positive (275 nm) and negative (244 nm) bands with a crossover at approximately 260 nm (Fig. S1, ESI).[25]

In terms of mTBA fluorescence response, the A emission at ~ 470 nm in the GQ structure following excitation of the D at ~ 315 nm was compared to the corresponding A emission in the duplex. In addition, the energy transfer efficiency in the GQ structures was determined by comparing emission intensities of the donor in the presence and absence of the acceptor (FRET values in Table 1).[14] Representative emission spectra of mTBA with the D/A pair at positions *syn*-G_{10} and *anti*-G_6 are shown in Fig. 2. In the absence of the A, excitation (315 nm) of ^{Fur}dG (D) at *syn*-G_{10} provided emission at 381 nm (I = 187, blue trace, Fig. 2a). However, in the presence of ^{vBth}dG (red trace, Fig. 2a), the emission for ^{Fur}dG at 381 nm was strongly quenched, while the emission for ^{vBth}dG at 470 nm increased in intensity (indicated by arrows, Fig. 2a). From this change in donor emission intensity the FRET efficiency was determined to be 85% (Table 1). Energy transfer from the D to the A also occurred in duplex (dashed red trace, Fig. 2b), but the A emission intensity at 470 nm was 3.7-fold (I_{rel} value, Table 1) lower than the corresponding A emission in the GQ. This observation can be explained by the reduced quantum yield of ^{Fur}dG (D) in the B-form duplex.[17-19]

Given that the A emission intensity in the GQ could be clearly distinguished from its emission intensity in the duplex following D excitation at 315 nm (Fig. 2b), the ability of the mTBA sample to signal thrombin binding through FRET was determined. For these titration experiments, the mTBA sample with ^{Fur}dG (D) at *syn*-G_{10} and ^{vBth}G (A) at *syn*-G_5 was utilized. This mTBA sample was the only oligonucleotide to preferentially stabilize the GQ over the duplex, providing a T_m value of 62.3 °C for the GQ versus 44.5 °C for the duplex. Titration experiments were carried out as previously described for mTBA containing ^{Fur}dG in the absence of ^{vBth}dG,[18] in which duplex-GQ exchange was driven by thrombin binding. Thus,

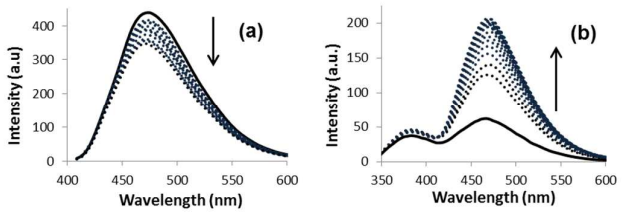

Fig 3 Thrombin emission titrations carried out with mTBA (D/A, 10;5) duplex (3 μM). Initial emission of duplex depicted by solid line, while dotted lines depict emission traces upon successive addition of thrombin. (a) Excitation wavelength = 380 nm. (b) Excitation wavelength = 315 nm.

the mTBA sample was annealed to its complementary strand (5'-CCAACCACACCAACC) in 100 mM sodium phosphate buffer to afford the duplex prior to thrombin addition.

To demonstrate the impact of the D on the A fluorescence response to thrombin binding, the emission titration was carried out in two ways (Fig 3). During one titration, changes in ^{vBth}dG (A) emission at 470 nm was monitored with λ_{ex} = 380 nm to directly excite the A fluorophore (Fig. 3a), while the second titration was monitored with λ_{ex} = 315 nm to highlight FRET (Fig 3b). When ^{vBth}dG was directly excited at 380 nm, its emission at 470 nm was slightly quenched (I_{rel} = 0.7) upon thrombin binding (Fig. 3a). The ^{vBth}dG (A) probe is more emissive in the duplex than in the GQ at *syn*-G_5 and acts as a relatively poor turn-off probe upon duplex-GQ exchange. In contrast, exciting ^{Fur}dG at 315 nm provided turn-on 470 nm emission for ~ 4-fold increase in fluorescence intensity upon thrombin binding (Fig 3b). Clearly the FRET response of the A probe was much more effective at signalling thrombin binding. The titration data provided a 1:1 association constant (K_a) of 2.4 x 10^5, which is in the same range determined previously for the singly-labelled mTBA samples containing ^{Fur}dG (D).[18]

Combining 8aryldG nucleobases with 8-vinyl-aryldG derivatives clearly provides applications for aptasensor development. In GQ folding oligonucleotides, 8aryldG bases can be employed at *syn*-Gs to preferentially stabilize the GQ and destabilize the duplex. They display quenched emission in the duplex that turns-on in the GQ. The ^{vBth}dG acceptor used in the current study exhibits relatively bright emission at ~ 470 nm in both duplex and GQ structures. By itself, ^{vBth}dG is not particularly useful for monitoring duplex-GQ exchange because its emission lacks sensitivity to the change in DNA topology. However, by pairing ^{vBth}dG with ^{Fur}dG, the donor ^{Fur}dG can act as a switch, turning on the visibly emissive signal from ^{vBth}dG. The ability to insert D/A 8aryldG probes at defined sites within the G-tetrad should pave the way for new opportunities in monitoring GQ folding dynamics and for studying GQ-ligand binding interactions. The level of precision offered by internal placement of D/A nucleobase probes should complement existing flexible-linker end-labelled fluorescence quench[26] and pyrene excimer assays[27] for screening GQ-ligand binding[26] and GQ detection in living cells.[27] Current efforts are focused on the optimization of emissive 8aryldG probes that undergo excitation with visible light and can be employed in *anti*-G positions for applications within parallel GQ structures.

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