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Harnessing the G-Tetrad Scaffold for Fluorescent Detection Strategies Within G-Quadruplex Forming Aptamers

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G-Tetrads are essential structural components required for the formation of G-quadruplexes. Replacement of G nucleobases within G-tetrads with fluorescent 8-aryl-dG residues provides diagnostic handles that are universally applicable to antiparallel G-quadruplex aptamers, as they can stabilize quadruplex folding and maintain aptamer function.

SELEX is an iterative process for searching libraries of partially random nucleic acids to afford aptamers with desired properties.[1, 2] Its utilization has lead to a large number of diverse oligonucleotide sequences with affinity for a variety of targets such as organic dyes, amino acids, antibiotics, proteins, viruses and whole cells.[3] This affinity is provided by their capacity for folding: they can incorporate small molecules into their nucleic acid structure or integrate into the structure of larger molecules such as proteins.[4] A significant proportion of DNA aptamers display G-quadruplex structures which enables molecular recognition of their ligands.[5]

Chemically modified aptamers have become ideal tools for the development of analytical methods and biotechnological applications.[6, 7] These modifications aim to address the intrinsic limitations of regular RNA and DNA oligomers to accomplish the following objectives: (1) impart chemical diversity to the nucleobase for molecular target selection;[8] (2) improve the pharmacokinetic profile of the aptamer through sugar modifications;[9, 10] and (3) create a scenario that produces a measurable response or signal upon aptamer function.[11-13] With regard to objective (3) the most popular modifications for G-quadruplex folding aptamers include externally bound fluorophores[11-14] modified pyrimidine-based probes[15] or commercially available fluorescent base analogues which are restricted to loop regions and typically destabilize the quadruplex structure.[16]

We recently reported on the synthesis, incorporation and structural implications of two 8-aryl-dG modifications (8-furyl-dG (^{Fur}dG) and 8-4-cyanophenyl-dG (^{CNPh}dG)) within the thrombin binding aptamer (TBA).[17] The 8-aryl-dG probes have no impact on the H-bonding sites of the nucleoside, and have the added benefit of being able to be incorporated within the G-tetrad of the aptamer. The electronic properties of the 8-aryl-dG probes resulted in contrasting diagnostic fluorescence, as ^{Fur}dG "turns on" upon quadruplex formation while ^{CNPh}dG "turns off." These probes were shown to effectively monitor duplex-quadruplex exchange reactions driven by K^+.[17] However, K^+ is not the intended molecular target of TBA.

Herein we show that the ^{Fur}dG and ^{CNPh}dG modifications within TBA are able to detect thrombin binding. The TBA positions that were modified are shown in Figure 1 and include G_5 (syn), G_6 (anti) and G_8 (TGT loop). The structure and placement of these analogues results in important implications in the aptamers ability to bind thrombin, as will be shown. To the best of our knowledge this is the first instance describing the incorporation of fluorescently modified dG analogues within a G-tetrad of TBA for thrombin detection. These findings are significant as they provide a universally applicable detection strategy for modifying antiparallel G-quadruplex aptamers utilizing internal modifications.

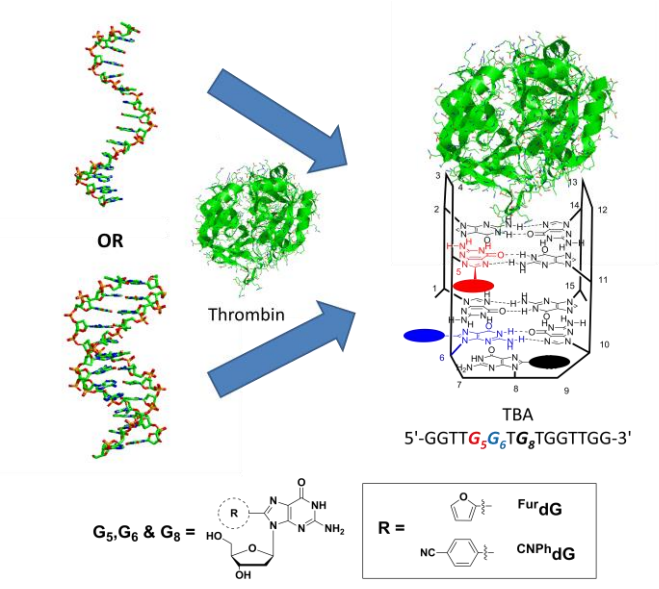

Figure 1. Depiction of the thrombin binding aptamer with ^{Fur}dG and ^{CNPh}dG modifications at G_5, G_6 and G_8. Oligonucleotides starting as their ssDNA or dsDNA forms assemble into the G-quadruplex upon introduction of thrombin.

The previously reported thermal melting analysis of the modified TBA (mTBA) showed the ability of the probes to preferentially stabilize the G-quadruplex structure at *syn*-G_5 ($\Delta T_m \geq +7^oC$) within the tetrad and their acceptance within the TG_8T loop despite their *syn*-preference ($\Delta T_m \geq -6^oC$). Substitution at

anti-G_6 resulted in large destabilization for ^{Fur}dG (ΔT_m = -11°C) and inhibition of quadruplex formation for ^{CNPh}dG.[17]

The choice of using TBA as the aptamer scaffold was based on the extensive amount of information pertaining to its overall structure and details concerning its molecular interactions with its target molecule thrombin.[18] The interactions between thrombin and TBA involve residues of the TT loops with a further contribution of G_5. The metal ion also plays a key role in the stability of the TBA quadruplex, with K^+ markedly stabilizing TBA (T_m = 53°C and 24°C in the case of TBA-K and TBA-Na, respectively).[18] Given that the emission of ^{Fur}dG and ^{CNPh}dG responds to G-quadruplex folding, our titration experiments were carried out in the presence of Na^+ to avoid quadruplex formation prior to the introduction of thrombin.

Thrombin binding was probed in the following way: 1 mL mTBA solutions were prepared (6 μM) in 100 mM sodium phosphate buffer pH 7.0 with 0.1 M NaCl. This solution was titrated with aliquots (0.4 μM) of thrombin at 20 min intervals and monitored by fluorescence. Titrations were carried out until no further changes in fluorescence were detected upon additions of thrombin. Alternatively, some samples were designed to undergo duplex-quadruplex exchange, driven by thrombin. For these titrations, duplexes (6 μM) were prepared by mixing mTBA with its complementary sequence (5'-CCAACCACACCAACC) in the aforementioned buffer prior to titrations. Representative titrations are shown in Figure 2, while the remaining spectra are presented in the Electronic Supplementary Information (ESI). Association constants (K_a) for thrombin binding by the mTBA oligonucleotides are presented in Table 1. The stoichiometry of the binding reaction indicated a 1:1 aptamer/thrombin interaction. In general, K_a values are ~1 order of magnitude below previously reported values for thrombin binding by unmodified TBA in K^+ buffer.[19] This correlates with the lower stability of the thrombin-TBA-Na^+ compared to that of thrombin-TBA-K^+.[18]

The probes are capable of signalling thrombin binding. Specificity was confirmed by control titrations with bovine serum albumin (BSA), which resulted in less than 1% change in emission intensity (ESI). Probe performance based on location within the aptamer was well depicted within the ^{Fur}dG series of modifications (representative titration shown in Figure 2a). Overall K_a correlates with the stability of the quadruplex structure and increases from ^{Fur}dG@6 < ^{Fur}dG@8 < ^{Fur}dG@5 (Table 1). All ^{Fur}dG probes displayed enhanced fluorescence emission upon transitioning from ssDNA to the quadruplex. ^{Fur}dG@5 provided the largest relative emission intensity (I_{rel}) for the series of modifications (I_{rel} =2.07) followed by ^{Fur}dG@8 (I_{rel} =1.41) and ^{Fur}dG@6 (I_{rel} = 1.26). In titrations with ^{Fur}dG mTBA, thrombin binding was accompanied by the appearance of the diagnostic quadruplex folding 290 nm peak in the excitation spectra.[17]

Fluorescence response was further improved for ^{Fur}dG@5 by performing a dsDNA to quadruplex transition titration (Figure 2c). This resulted in larger relative intensities (I_{rel} = 7.43), since the emission of ^{Fur}dG is strongly quenched in the duplex structure. The titration generated K_a values on the same order of magnitude as the ssDNA to quadruplex titration even though the duplex exhibited a much larger T_m (58°C) than the quadruplex in Na^+ (T_m = 40°C). Introduction of thrombin significantly stabilizes the quadruplex as the stability of the ternary thrombin-

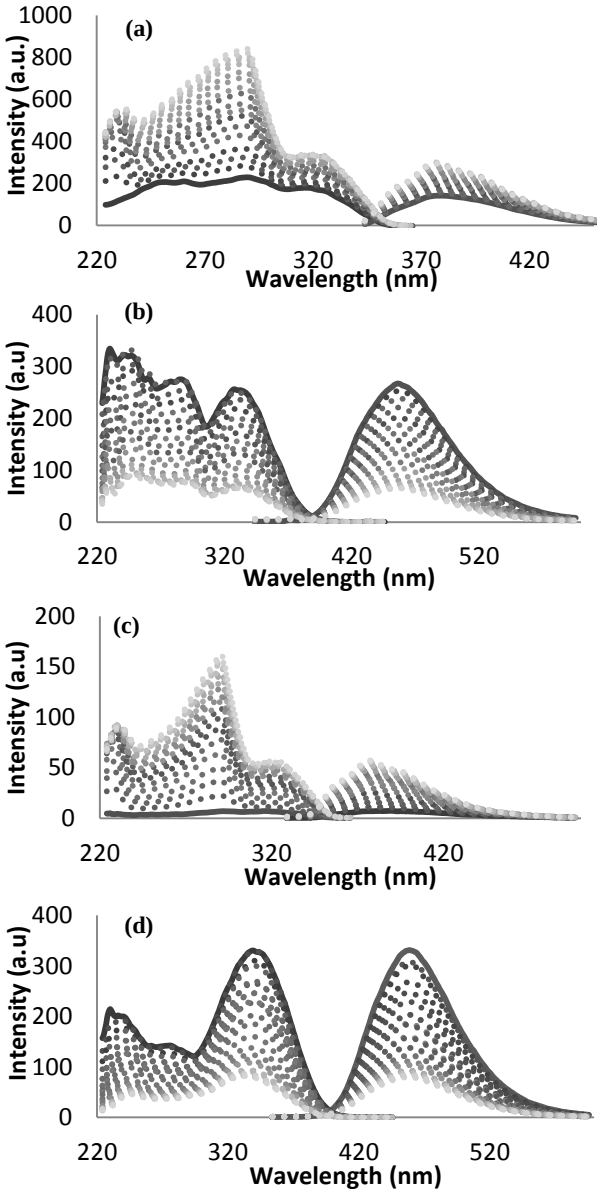

Figure 2. Fluorescence titrations carried out with thrombin. Initial excitation and emission traces are depicted by a solid line while dotted lines depict fluorescent traces upon successive introduction of thrombin. Titrations include: (a) ^{Fur}dG@5 ssDNA ➔ Quadruplex; (b) ^{CNPh}dG@5 ssDNA ➔ Quadruplex; (c) ^{Fur}@5 dsDNA ➔ Quadruplex; (d) ^{Fur}@5, ^{CNPh}@8 dsDNA ➔ Quadruplex.

TBA-Na^+ complex is considerably higher than the TBA-Na^+ binary complex.[18] Previous dsDNA to quadruplex titrations driven by K^+ utilized a truncated 10mer complementary strand in order to reduce the T_m of the duplex which accelerated the transition.[17] Truncated duplexes were unnecessary for thrombin titrations; unlike K^+, thrombin acts as a molecular chaperone which effectively performs duplex to quadruplex transitions.

In contrast to ^{Fur}dG, the fluorescence emission of ^{CNPh}dG was quenched upon thrombin binding (Figure 2b), as expected for G-quadruplex folding.[17] For ^{CNPh}dG@5 the emission was quenched 4-fold (I_{rel} 0.25) while I_{rel} = 0.4 for ^{CNPhd}G@8 (Table 1). Of the modifications studied the association constant was again related to overall quadruplex stability as ^{CNPh}dG@5 was larger than ^{CNPh}dG@8.

Table 1. Association Constants for thrombin binding by various ^{Fur}dG and ^{CNPh}dG mTBA oligonucleotides.

Starting Structure [a]	Modification(s)	K_a (x10^5) [b]	I_{rel} [c]	ΔT_m [d]
ssDNA	^{Fur}dG@5	3.00	2.07	9.1
ssDNA	^{Fur}dG@5; ^{CNPh}dG@8	2.62	0.50	6.7
dsDNA	^{Fur}dG@5; ^{CNPh}dG@8	2.27	0.28	/
ssDNA	^{Fur}dG@8	2.22	1.41	-9.5
ssDNA	^{Fur}dG@6	1.91	1.26	-11
dsDNA	^{Fur}dG@5	1.81	7.43	/
ssDNA	^{CNPh}dG@5	1.56	0.25	7.1
ssDNA	^{CNPh}dG@8	1.06	0.40	-6.3

[a] Starting structure refers to whether the titration was performed on the single stranded (ssDNA) or preformed double stranded (dsDNA) oligonucleotides. [b] All determined association constants calculated from K_d values obtained through SigmaPlot Simple Ligand Binding (see ESI for details). [c] I_{rel} calculated as fluorescence intensity of the starting oligonucleotide divided by fluorescence intensity of the fully bound TBA. [d] Calculated in relation to the unmodified TBA sequence in K^+, as previously published.[17]

The doubly modified ^{Fur}dG@5;^{CNPh}dG@8 (Figure 2d) oligonucleotide also showed quenched fluorescence throughout the titrations. For this mTBA derivative the K_a value is similar to the singly modified mTBA with ^{Fur}dG@5 (Table 1). In this case the ^{Fur}dG remained optically silent upon thrombin binding and the ^{CNPh}dG provided the fluorescent response. The titration showed a 2-fold decrease in emission intensity at 460 nm for the ssDNA to G-quadruplex transition, while a 4-fold decrease was observed for the dsDNA to G-quadruplex transition (Figure 2d).

Utilizing 8-aryl-dG modifications that can stabilize G-quadruplexes and provide diagnostic signals upon quadruplex folding provide numerous applications for aptamer development. G-Tetrad positions are much less variable than terminus, or loop regions of G-quadruplex folding aptamers. Incorporation of internal fluorescent G-mimics within G-tetrads can be advantageous for investigations of quadruplex folding where the level of detail needs to be high since the probe can be designed to be very close to the site of examination.[20] The most important implication of this study is the proposition that the utilization of 8-aryl-dG nucleobase analogues within G-tetrads can provide a probing scenario that is applicable to any G-quadruplex aptamer folding into an antiparallel topology.[17]

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Electronic supplementary information (ESI) available: Experimental section, additional titration data for mTBA.

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