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FRET Templated by G-Quadruplex DNA: A Specific Ternary Interaction Using an Original Pair of Donor/Acceptor Partners

Clémence Allain, David Monchaud, and Marie-Paule Teulade-Fichou*

Contribution from the Laboratoire de Chimie des Interactions Moléculaires,Collège de France, CNRS UPR 285, 11 place Marcelin Berthelot, 75005 Paris, France

Received March 31, 2006; E-mail: mp.teulade-fichou@college-de-france.fr

Abstract: G-quadruplex represents a suitable scaffold for FRET (fluorescence resonance energy transfer) since its two external quartets offer two well-defined binding sites for concomitant trapping of donor/acceptor partners. Combining selective G-quadruplex binders (macrocyclic bis(quinacridine) BOQ_1 or monomeric quinacridine MMQ_1, donor) with a highly fluorescent DNA probe (thiazole orange, acceptor), we designed a structure-specific FRET-system based on an unprecedented noncovalent ternary complex. This system could be potentially usable as a signature for quadruplex-DNA conformation in solution, but also might offer a unique means for observing cation and ligand binding influence on quadruplex topology.

Introduction

DNA polymorphism exerts a fascination on a large scientific community due to its direct involvement in gene expression, its importance for drug targeting, and its DNA-based material engineering applications.[1] In addition to analysis by optical spectroscopies and biochemical methods, the structural diversity of DNA is commonly probed by the covalent incorporation of fluorescent motifs. In particular the labeling of oligonucleotides with donor/acceptor (D/A) pairs for FRET (fluorescence resonance energy transfer) has been largely employed for the conformational sensing of stem−loop hairpins and G-quadruplexes.[2] In contrast, the use of noncovalent fluorescent probes for direct detection of nucleic acid structures is still underdeveloped. This is essentially due to the challenging task of designing fluorescent ligands that combine a high specificity for DNA structure and that display a significant binding-induced change in emission quantum yield. The intercalator thiazole orange (TO, Figure 1A), belongs to the limited group of dyes that displays a strong increase in fluorescence when bound to DNA, but it does not exhibit a marked structural selectivity.[3]

With regards to both the exceptional photophysical properties of TO and our previous experience in the molecular recognition of nucleic acids,[4] we envisioned that sensitization of TO by a structure-specific DNA binder through FRET interaction could constitute an interesting novel system for probing DNA conformations. This system is based on conditions wherein TO and the sensitizer are trapped or bound into DNA in relative positions suitable for energy transfer. Numerous studies have shown that DNA provides a one-dimensional matrix for FRET interactions between dyes covalently attached at each extremity of a duplex.[5] However, G-quadruplex DNA represents an even more attractive scaffold for FRET than does duplex DNA since the two external quartets of quadruplexes offer two well-defined binding sites for positioning a pair of molecules with D/A characteristics.[6] Furthermore, most biological sequences forming G-quadruplexes are composed of three to four stacks of guanine quartets which provide a distance close to the lower range of the Förster distance (10−15 Å). G-quadruplex DNA is currently the subject of intense interest due to potential applications for controlling strategic genomic regions and for performing DNA-programmed assembly.[1,7] Despite the fact that G-quadruplex DNA has been used for templating reactions between ligands in a dynamic combinatorial strategy,[8] it has never been used as a noncovalent matrix for FRET partners. Also, studies on fluorescent labeling of G-quadruplex DNA are still sparse.[9]

Here we report on the FRET sensitization of TO templated by G-quadruplex DNA using a G-quadruplex specific binder as donor. This effect is based on the generation of an

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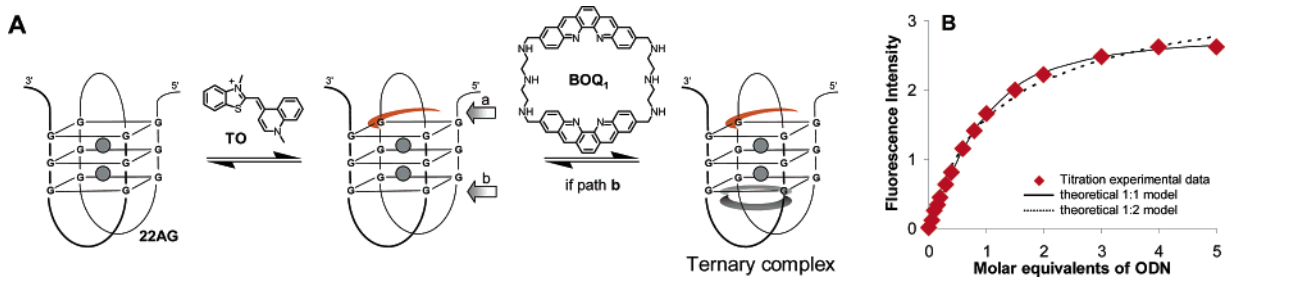

Figure 1. (A) Schematic representation of the possible interaction pathways between TO, BOQ_1, and 22AG quadruplex. (B) Fluorescence intensity (λ_{ex} = 501 nm) of TO under addition of increasing amounts of 22AG: experimental (red diamonds) and calculated (plain and dotted lines) titration curves (*Specfit32*, version 3.0; Spectrum Software Associates: Marlborough, MA).

unprecedented specific ternary complex between the quadruplex-DNA and the new D/A pair composed of TO and quinacridines (Figure 1A).[4] Additionally, we demonstrate that the efficiency of the FRET effect represents a signature for G-quadruplex conformations in solution.

Experimental Section

Materials and Chemicals. The syntheses of the quinacridine BOQ_1 and MMQ_1 have been described previously.[4] TO is purchased from Aldrich and used without further purification. Oligonucleotides are purchased from Eurogentec (Belgium). 22AG is an oligonucleotide mimicking the human telomeric repeat: [5′-$AG_3(T_2AG_3)_3$-3′]. The 17-bp duplex is a biological sequence used in previous studies.[4] The sequences of the two complementary strands are the following: [5′-CCAGTTCGTAGTAACCC-3′]/[5′-GGGTTACTACGAACTGG-3′]. The thrombin binding aptamer sequence (TBA) is: [5′-$G_2T_2G_2TGTG_2T_2G_2$-3′].

Sample Preparation and Measurements. Quadruplexes from 22AG and TBA are prepared by heating the corresponding oligonucleotides at 90 °C for 5 min in a 10 mM sodium cacodylate buffer (pH 7.3) with 100 mM KCl and cooling in ice to favor the intramolecular folding by kinetic trapping. For 22AG in Na^+ buffer, the same protocol is applied in a 10 mM sodium cacodylate buffer (pH 7.3) with 100 mM NaCl. The 17-bp duplex is prepared by heating the two corresponding complementary strands at 90 °C for 5 min in a 10 mM sodium cacodylate buffer (pH 7.3) with 100 mM KCl and then slowly cooling overnight. Concentrations are evaluated by UV (after thermal denaturation, 5 min at 85 °C) before use. Fluorescence measurements are performed on a FluoroMax-3 spectrophotometer (Jobin-Yvon). Temperature is kept constant at 20 °C with thermostated cell holders. Each experiment is performed in a 3-mL cell, in 3 mL of 10 mM sodium cacodylate buffer (pH 7.3) with 100 mM KCl or 100 mM NaCl, depending on the experiments. A fluorescence emission spectrum is recorded at 0 and 5 min after each addition of DNA (only spectra recorded after 5 min are presented), for both excitation wavelengths, 328 and 501 nm. Titrations experiment is performed in a 10 mM sodium cacodylate (buffer pH 7.3) with 100 mM KCl. Spectra are recorded in the following order: (1) addition of TO (1.0 μM) and then addition of 22AG; (2) 0.05 μM; (3) 0.1 μM; (4) 0.15 μM; (5) 0.2 μM; (6) 0.3 μM; (7) 0.4 μM; (8) 0.6 μM; (9) 0.8 μM; (10) 1.0 μM; (11) 1.5 μM; (12) 2.0 μM; (13) 3.0 μM; (14) 4.0 μM, and (15) 5.0 μM. FID experiments are performed in a 10 mM sodium cacodylate buffer (pH 7.3) with 100 mM KCl. FID experiments are carried out with BOQ_1; spectra are recorded in the following order: (1) addition of 22AG (0.25 μM); (2) addition of TO (0.5 μM) and then addition of BOQ_1; (3) 0.025 μM; (4) 0.05 μM; (5) 0.1 μM; (6) 0.15 μM; (7) 0.25 μM; (8) 0.375 μM; (9) 0.50 μM; (10) 0.625 μM; (11) 0.75 μM; (12) 1.0 μM; (13) 1.25 μM; (14) 1.5 μM, and (15) 2.0 μM. For each FRET experiment, spectra are recorded in the following order: (1) addition of BOQ_1 or MMQ_1 (0.5 μM); (2) addition of TO (0.5 μM) and then addition of oligonucleotides; (3) 0.1 μM; (4) 0.2 μM; (5) 0.3 μM; (6) 0.4 μM; (7) 0.5 μM; (8) 0.75 μM; (9) 1.0 μM; (10) 1.25 μM; (11) 1.5 μM; (12) 2.0 μM, and (13) 2.5 μM.

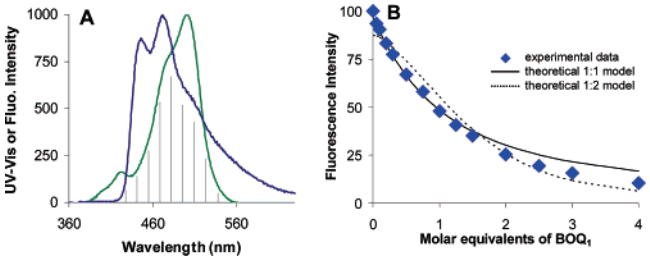

Figure 2. (A) Spectral overlap between fluorescence emission (blue line) of BOQ_1 (λ_{ex} = 328 nm) and UV−vis absorption of TO (green line). (B) Fluorescence induced displacement (FID) of TO by BOQ_1 onto 22AG quadruplex: experimental (blue diamonds) and calculated (plain and dotted lines) curves (Specfit32, version 3.0; Spectrum Software Associates: Marlboro, MA).

Results and Discussion

First, we investigated the G-quadruplex DNA-binding characteristics of TO through a fluorimetric titration using the quadruplex-forming oligonucleotide 22AG which mimics the human telomeric repeats.[4c] Upon addition of 22AG to a solution of TO, a strong increase of TO emission (~500 fold) is observed, which is in the range reported for the binding of the dye to DNA duplexes.[3,10] Processing the data by least-squares fitting evidenced the formation of a 1:1 complex (Figure 1B), the 2:1 stoichiometry being clearly excluded. A K_a value of 3 $\times$ 10^6 M^{-1} was found for this unique binding site which is close to the value determined for duplex binding.[3,10] Importantly, this unambiguous 1:1 binding is consistent with the existence of one tetrad of higher affinity that has been characterized for intra- and bimolecular G-quadruplexes.[11] As a donor molecule, the macrocyclic bis(quinacridine) BOQ_1 was selected on the basis of two criteria. First, its photophysical characteristics are highly favorable for FRET interaction with TO due to a remarkably large spectral overlap (Figure 2A). Second, BOQ_1 is a high-affinity quadruplex binder (K_a = 1.2 $\times$ 10^7 M^{-1}) with a 10-fold preference for quadruplex *over* duplex DNA. 22AG is able to accommodate two BOQ_1 via stacking on its two external

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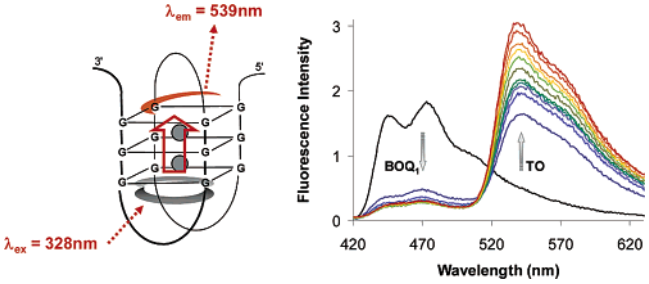

Figure 3. Schematic representation of the putative ternary complex and fluorescence spectra of the FRET pair BOQ_1/TO recorded at increasing concentration of 22AG (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 equiv, from black to red curves), λ_{ex} = 328 nm (10 mM sodium cacodylate buffer, pH 7.3, 100 mM KCl).

G-quartets. Consequently, in the presence of TO, the fixation of BOQ_1 on the opposite external tetrad of 22AG is plausible (Figure 1A, path b) along with a competitive binding for the TO occupied tetrad (Figure 1A, path a). To evaluate this, a fluorescence intercalator displacement (FID) assay was carried out.[10] As shown in Figure 2B the displacement of TO by BOQ_1 is observed, but fitting of the curve failed either with a 1:1 or 1:2 model, consistent with the existence of two binding sites for BOQ_1, whereas only one site is sensed by TO. However, the curve shows that the acceptor probe is only partially evicted at a 1/1 or lower TO/BOQ_1 ratio, indicating that the formation of a 1:1:1 ternary complex suitable for FRET (Figure 1A) can be expected in this concentration range.

On this basis a FRET DNA-templated assay was devised as follows. The two dyes were mixed in a 1:1ratio, and DNA was added in increasing concentration. As seen in Figure 3, excitation of the ligand mixture at 328 nm (BOQ_1 absorbance) does not induce the emission of TO (black curve), confirming that no detectable interaction occurs between the two cationic dyes. In contrast when 22AG is added, a spectacular decrease of the emission of BOQ_1 is observed together with the strong emission of TO, both increasing with the increasing concentration in DNA. This evidences that BOQ_1 is able to transfer its excitation energy to TO in the presence of the oligonucleotide, suggesting that the DNA acts as a template to position favorably the two dyes for FRET interaction. Consistent with our structural model, the efficiency of FRET decreases when the concentration in BOQ_1 increases above a 1:1 to 2:1 D/A threshold (Figure S1), reflecting the displacement of the acceptor probe.

Interestingly, this effect is highly sensitive to the structure of the DNA matrix. As seen in Figure 4A when the experiment is carried out in the presence of a 17-bp duplex (red triangles), a significantly lower level of FRET is observed as compared to that induced by 22AG (blue squares). The difference between the two curves strongly suggests that G-quadruplex DNA provides a more favorable template for FRET than duplex as anticipated. In the case of the duplex, the intercalation of TO is expected with the nonspecific electrostatically driven repartition of donor BOQ_1 along the double helix, resulting in a less favorable situation for the energy transfer.

Most importantly, in the same conditions, no significant differences are observed for the fluorescence of TO when induced by direct excitation (501 nm, Figure 4A′). Altogether, these results strongly support that the structural discrimination observed by excitation of TO via FRET reflects the specific positioning of the pair of dyes by the G-quadruplex template.

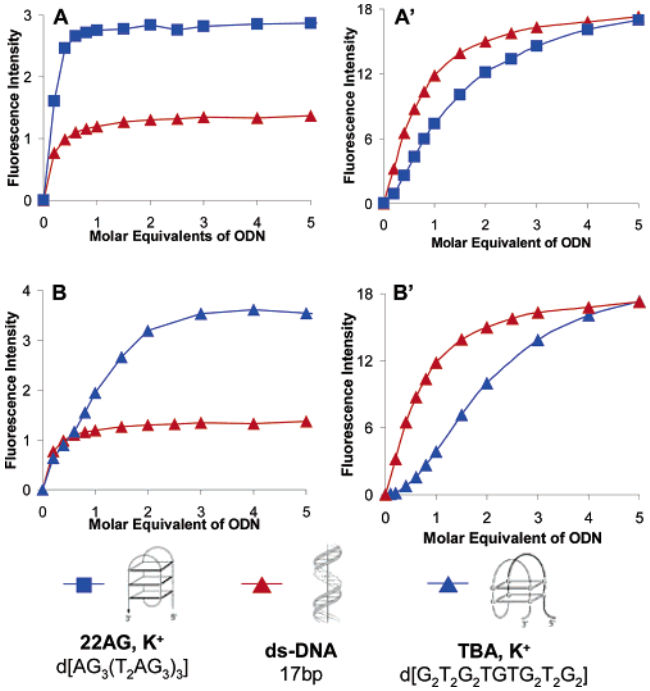

Figure 4. FRET experiments with BOQ_1: Fluorescence emission of TO recorded at increasing concentration of oligodeoxynucleotides (ODN) 17-bp (λ_{em} = 529 nm), 22AG (λ_{em} = 539 nm), and TBA (λ_{em} = 535 nm). (A/B) FRET-mediated excitation (λ_{ex} = 328 nm). (A′/B′) Direct TO excitation (λ_{ex} = 501 nm).

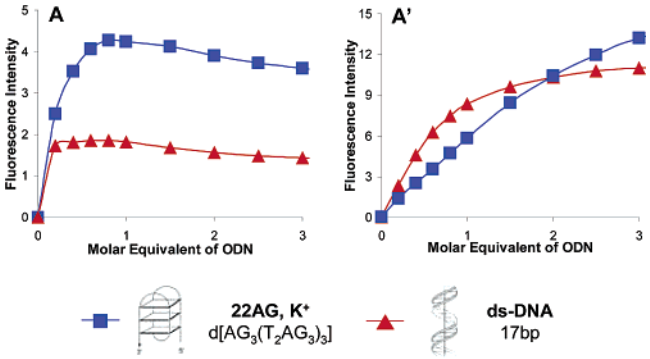

Figure 5. FRET experiments with MMQ_1: Fluorescence emission of TO recorded at increasing concentration of oligodeoxynucleotides (ODN) 17-bp (λ_{em} = 529 nm) and 22AG (λ_{em} = 539 nm). (A) FRET-mediated excitation (λ_{ex} = 328 nm). (A′) Direct TO excitation (λ_{ex} = 501 nm).

Additionally, to further prove that the high FRET level is specific for the quadruplex matrix and not associated with a particular feature of 22AG, the thrombin-binding aptamer (TBA) which forms a well-defined intramolecular quadruplex was used as template.[12] A preliminary fluorimetric titration also evidenced the formation of an unambiguous 1:1 complex (Figure S2). The same trend was observed (Figure 4, B and B′), thus confirming the occurrence of a quadruplex-related effect.

We were then interested in investigating how the FRET effect can be affected by the characteristics of the donor. For that purpose, the same set of experiments was conducted with the monomeric quinacridine MMQ_1 as the donor (structure in Figure 6) which is also highly favorable for FRET interaction with TO (Figure S3).[4b] MMQ_1 is an efficient quadruplex interactive agent ($K_a \approx 10^7\ M^{-1}$) but with a slightly lower quadruplex vs

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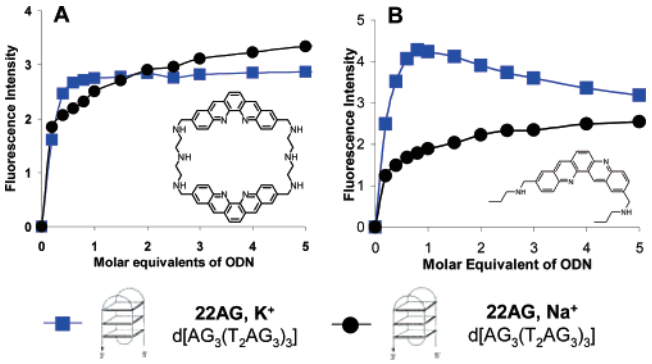

Figure 6. Fluorescence emission of TO upon FRET-mediated excitation ($\lambda_{ex} = 328$ nm) in 100 mM K^+ (blue curve) or Na^+ (black curve) buffer in the presence of (A) BOQ_1 and (B) MMQ_1.

duplex selectivity as compared to BOQ_1. As depicted in Figure 5, identical conclusions arose since FRET-mediated TO emission is significantly higher in the presence of 22AG than with duplex DNA (Figure 5A), whereas no significant difference is observed for the TO fluorescence profiles upon direct excitation (Figure 5A′).

Finally, we decided to turn our attention to a critical issue, i.e. the polymorphism of intramolecular G-quadruplexes, which is currently intensively debated.[13] The influence of counterions (K^+, Na^+) has been particularly studied, but no general rules emerged. It was thus of interest to test if our FRET system could be responsive to the presence of various forms of 22AG differing by loop arrangement. In the case of BOQ_1/TO (Figure 6A), although the curve shape is different, no striking variation in FRET efficiency was observed when the experiment was performed in Na^+ buffer (black circles) as compared to the initial experiment in K^+-doped buffer. In contrast, when MMQ_1 is used as donor, the FRET-induced signal of TO appears remarkably sensitive to ionic environment (Figure 6B), showing a much higher level in the presence of K^+.

Hence, the TO/MMQ_1 system seems to reflect the conformational differences of 22AG with regard to the added cation. In particular the lower signal observed in the presence of Na^+ could indicate a lower accessibility of the tetrads in these conditions. On the other hand the poor sensitivity of the TO/BOQ_1 system to the cationic environment might indicate that binding of BOQ_1 modifies the topology of the quadruplex in inducing the conversion to a predominant form as it has already been observed for other macrocyclic quadruplex binders.[14] The differences in the sensitivities of the TO/MMQ_1 and TO/BOQ_1 systems may also originate in the variations of the photophysical properties of the bound dyes (quantum yield, dipole orientation) which furthermore might be affected by the cation nature. However, interpretations of short-range FRET are difficult due to the numerous parameters involved, and extended investigations with other quadruplex-forming oligonucleotides are currently being undertaken to address this point. On the other hand, the covalent grafting of either the donor or the acceptor to quaruplex-forming oligonucleotides should be of great help to gain insight into these interactions; synthetic efforts dedicated to that purpose are also currently in progress.

Conclusions

In conclusion, we have designed a novel set of FRET partners, based on quinacridine G-quadruplex binders as donor and the highly fluorescent probe TO as acceptor. TO is remarkable for its enhancement of fluorescence upon binding to DNA; however, this enhancement is independent of the nature of the DNA matrix. We have thus used the selectivity of quinacridines for quadruplex DNA to specifically FRET-sensitize TO bound to the quadruplex architecture. This system leads to a structure-specific FRET response, potentially usable as a signature for quadruplex DNA in solution. This response is based on an unprecedented noncovalent ternary interaction, proposed to result from the concomitant occupancy of the two external tetrads of the quadruplex structure by the D/A partners.

Additionally, we have shown that, depending on the donor used, the FRET effect is sensitive to the presence of Na^+ and K^+ cations. This system offers, then, a novel and valuable means for observing the influence of cation and ligand binding on quadruplex topology.

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Supporting Information Available: Additional titration curves, UV and fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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