4-1-2014

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Nucleic Acids Research Advance Access published February 7, 2014

Nucleic Acids Research, 2014, 1–8
doi:10.1093/nar/gku111

Thioflavin T as a fluorescence light-up probe for G4 formation

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Received November 20, 2013; Revised January 13, 2014; Accepted January 14, 2014

ABSTRACT

Thioflavin T (ThT) becomes fluorescent in the presence of the G-quadruplex structure such as that formed by the human telomeric motif. In this report, we extend and generalize these observations and show that this dye may be used as a convenient and specific quadruplex probe. In the presence of most, but not all, G4-forming sequences, we observed a large increase in ThT fluorescence emission, whereas the presence of control duplexes and single strands had a more limited effect on emission. This differential behavior allowed us to design a high-throughput assay to detect G4 formation. Hundreds of different oligonucleotides may be tested in parallel for G4 formation with a simple fluorescence plate reader. We applied this technique to a family of aptamers not previously recognized as G4-forming sequences and demonstrated that ThT fluorescence signal may be used to predict G4 formation.

INTRODUCTION

G-quadruplexes (G4s) are nucleic acid structures consisting of stacked G-quartets. Each quartet is composed of four guanines linked together by Hoogsteen hydrogen bonding. G4s can be formed by one to four G-rich strands and are stabilized by the presence of monovalent cations. They are polymorphic structures, as one G-rich sequence can adopt different G4 conformations depending on experimental conditions, flanking sequences, concentration and preparation protocol. One of the best examples is the human telomeric sequence, which can fold into at least 10 different conformations (1). Interest in these structures is growing: the G4 research community is diverse, from theoreticians to chemists and biologists (2). Computational approaches have revealed a high number of genomic regions compatible with quadruplex formation. Intramolecular G4s can be formed with DNA and RNA; sequences with the potential to form G4 structures are found at eukaryotic telomeres (3), in oncogene promoters (4), in minisatellites (5) and in 5′ and 3′ untranslated (UTR) regions of messenger RNAs (6). Consequently, some G4s, such as the human telomeric sequence and those found in the promoters of *c-myc*, *c-kit* and *Kras*, are considered as therapeutic targets.

Not all G-rich sequences form stable G4s; even sequences matching the generally accepted consensus may fail to form G4 structures *in vitro* (7). Therefore, it is essential to experimentally verify whether these sequences actually adopt this structure. A variety of techniques that detect the G4 structure is available, such as melting temperature determination (8), thermal or isothermal difference spectra (TDS and IDS, respectively) (9), nuclear magnetic resonance (NMR) and circular dichroism, but all are relatively time-consuming and require specialized equipment. Several ongoing projects that relate to drug discovery require that hundreds of sequences be analyzed in parallel, making these techniques impractical. Therefore, we were looking for a fast and simple screening method for detection of G4 structures.

Thioflavin T (ThT) is a benzothiazole used to detect amyloid fibrils (10–12). Recently, Mohanty *et al.* (13) reported that ThT recognizes the human telomeric motif and may be used to discriminate this structure from duplexes or single strands. This probe has also been used as a label-free turn-on fluorescent sensor for biothiol detection in the laboratory of B. Tang (14). In this article, we used this compound to develop a rapid and inexpensive test to determine whether an oligonucleotide adopts a G4 conformation (Figure 1). We also used ThT as a dye to visualize quadruplexes in an acrylamide gel. We validated the test on a variety of sequences with known structures.

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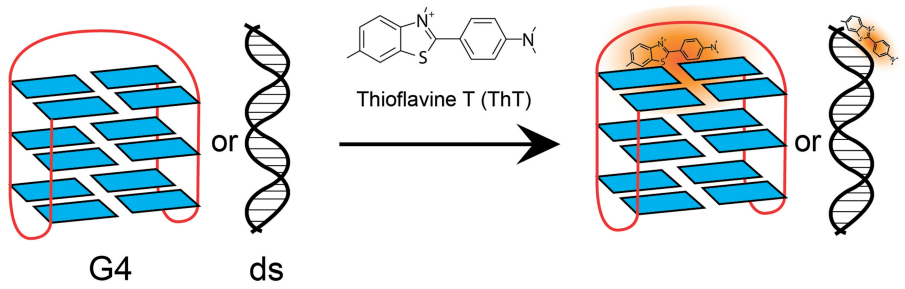

Figure 1. Principle of the ThT assay. ThT is added to nucleic acid structures preformed in 50 mM Tris/HCl, pH 7.5, and 50 mM KCl. ThT binds specifically to G4, and its fluorescence is enhanced. In contrast, when the oligonucleotide is single- or double-stranded, a lower fluorescence increase is observed.

MATERIALS AND METHODS

Oligonucleotides and compounds

Oligonucleotides were purchased from Eurogentec (Belgium). All were purified over a Reverse-Phase Cartridge Gold (Eurogentec) and then dissolved in bidistilled water and stored at −20°C. Concentrations of all oligonucleotides were determined by ultraviolet (UV) spectrometry using extinction coefficients provided by the manufacturer.

3,6-Dimethyl-2-(4-dimethylaminophenyl) benzothiazolium cation (ThT) was obtained from Sigma-Aldrich (Ref. T3516) and used without further purification. The concentration was calculated using the molar extinction coefficient at 412 nm in water of 36 000 M^{-1} cm^{-1}.

Preparation of oligonucleotides

Before analysis, oligonucleotides were heated at 90°C for 5 min at 4 μM concentration in water, then diluted into 100 mM Tris/HCl, pH 7.5, 100 mM KCl and slowly cooled to room temperature over 2 h. The triplex sample is preformed in the same conditions but with 5 mM MgCl added.

Fluorescence single-wavelength measurements

Experiments were performed with 96-well microplates from Greiner (Flat Bottom Black Polystyrol). Each condition was tested at least in triplicate in a volume of 20 μl for each DNA or RNA sample. Measurements were performed at room temperature. Oligonucleotides and ThT were mixed at 1 and 0.5 μM final concentrations, respectively. Under these conditions, the 1:1 highly fluorescent complexes should predominate (15). Samples were incubated in 50 mM Tris/HCl, pH 7.5, and 50 mM KCl. Fluorescence emission was collected at 490 nm after excitation at 425 nm in a microplate reader (Infinite M1000Pro, Tecan).

Fluorescence scans

Experiments were performed under the same conditions used for the fluorescence single-wavelength measurements except that the fluorescence emission was collected between 440 and 700 nm every 2 nm with a microplate reader (Infinite M1000Pro, Tecan).

Gel staining

Nondenaturing polyacrylamide gel electrophoresis was used to visualize oligonucleotides. DNA samples were heated at 90°C for 5 min in water, and then buffer was added to a concentration of 50 mM Tris/HCl, pH 7.5, and 50 mM KCl. The samples were heated at 90°C for 5 min and slowly cooled (over 2 h) to room temperature. Sucrose was added to a final concentration of 15% before loading. Oligothymidylate markers (dT_9, dT_{15}, dT_{22}, dT_{30}, dT_{45} and dT_{57}) were also loaded on the gel.

After electrophoresis, the gels were visualized by UV shadowing with a DNR Bio-imaging, MF-ChemiBIS 3.2 system. Gels were then incubated in a 0.5-μM ThT solution for 15 min under gentle agitation and briefly washed in water before visualization on a Typhoon Trio$^+$ Imager (GE Healthcare). Finally, the same gels were stained with 1× SYBR Gold (Ref. S11494 from Life Technologies) diluted in 1× Tris-borate-ethylenediaminetetraacetic acid for 40 min before visualization on a Typhoon Trio$^+$ Imager.

Isothermal difference spectra

IDS were obtained by taking the difference between the absorbance spectra from unfolded (in the absence of salt) and folded (in the presence of 100 mM KCl) oligonucleotides recorded in a 10-mM lithium cacodylate buffer, respectively, at 20°C. IDS provided specific signatures of different DNA structural conformations. IDS is preferred over TDS as quadruplexes that are highly thermally stable do not unfold at high temperature, and TDS is not informative for these molecules. Spectra were recorded on an Uvikon XL spectrophotometer.

Nuclear magnetic resonance

The concentration of each sample was typically 100 μM. Samples were heated at 90°C in water; 20 mM of potassium phosphate buffer, pH 6.9, 70 mM KCl and 10% D_2O were then added. The samples were slowly cooled to room temperature for 2 h, and spectra were recorded on a 700-MHz Bruker spectrometer at 25°C.

Table 1. Selected oligonucleotides used in the fluorescence enhancement assay

Name	type/origin	Sequence (from 5′ to 3′)
Bom17	G4-*Bombyx* telomere	$G_2TTAG_2TTAG_2TTAG_2$
Oxy3.5	G4-*Oxytricha* telomere	$G_4TTTTG_4TTTTG_4TTTTG_4$
Asc20	G4-*Ascaris* telomere	$G_2CTTAG_2CTTAG_2CTTAG_2$
Plas24	G4-*Plasmodium* telomere	$G_3TTCAG_3TTCAG_3TTCAG_3$
22Ag	G4-Human DNA telomere	$AG_3TTAG_3TTAG_3TTAG_3$
45Ag	G4-Human DNA telomere	$G_3TTAG_3TTAG_3TTAG_3TTAG_3TTAG_3TTAG_3TTAG_3$
22AgR	G4-Human RNA telomere	$AG_3UUAG_3UUAG_3UUAG_3$
21CTA	G4-Human telomere variant	$G_3CTAG_3CTAG_3CTAG_3$
c-myc	G4-Promoter	$TGAG_3TG_3TAG_3TG_3TAA$
c-kit1	G4-Promoter	$G_3AG_3CGCTG_3AGGAG_3$
c-kit2	G4-Promoter	$G_3CG_3CGCGAG_3AGG_3$
Insulin a2	G4-Promoter	$ACAG_4TGTG_4ACAG_4TGTG_4$
AKT1	G4-Promoter	$G_3CG_3CGGCTCCG_3CGCG_3$
VEGF	G4-Promoter	$G_3AG_3TTG_4TG_3$
VAV1	G4-Promoter	$G_3CAG_3AG_3AACTG_3$
35B1 (Kras)	G4-Promoter	$AG_3CGGTGTG_3AAGAG_3AAGAG_5AGGCAG$
32Kras	G4-Promoter	$AG_3CGGTGTG_3AAGAG_3AAGAG_5AGG$
27Kras	G4-Promoter	$G_3CGGTGTG_3AAGAG_3AAGAGG_3$
TBA	G4-Aptamer	$G_2TTG_2TGTG_2TTG_2$
Ceb25	G4-Minisatellites	$AG_3TG_3TGTAAGTGTG_3TG_3T$
25DDX	G4-5′UTR	$G_3CG_3AUAGAGAGCGUG_3CG_3$
CT4	G4/Mixed quartets	$G_3CT_4G_3C$
CGG12	Trinucleotide	$(CGG)_{12}$
CTG12	Trinucleotide	$(CTG)_{12}$
CAG12	Trinucleotide	$(CAG)_{12}$
ds26	Duplex	CAATCGGATCGAATTCGATCCGATTG
dx12	Duplex	(a)GCGTGAGTTCGG (b)CCGAACTCACGC
19AT	Duplex	(a) ACGTCGATTATAGACGAGC (b) GCTCGTCTATAATCGACGT
PS1c	Parallel-duplex	(a) TTTTTTTTTTATTAAAATTTATAA (b) AAAAAAAAAATAATTTTAAATATT
GCdx	Stem-loop	$GCGCGCGCT_4GCGCGCGC$
Dx	Stem-loop	TATAGCTATAA(HEG)TATAGCTATA
dT30	Single strand	T_{30}
Triplex	Triplex	(a) TTTTTTTTTTTTTTTTTTTT (b) AAAAAAAAAAAAAAAAAAA (c) TTTTTTTTTTTTTTTTTTT

HEG: hexaethylene glycol.

RESULTS

G4 structures induce ThT fluorescence

We compared ThT fluorescence intensity alone or in the presence of a variety of sequences. ThT was directly added to oligonucleotide solutions, and the fluorescence signal was recorded on a microplate reader. We first compared the fluorescence intensity of 10 oligonucleotides of known structure: six G4s, three duplexes and one 'single strand' in the presence of ThT relative to the signal of ThT alone. DNA oligonucleotides 45Ag and 22Ag and RNA 22AgR contain several repeats of the human telomeric sequence. Oxy3.5 corresponds to 3.5 repeats of the of *Oxytricha nova* (a ciliate protozoa) telomeric sequence. C-myc is the G-rich region of the human promoter of this proto-oncogene. Finally, TBA is the thrombin-binding aptamer. 22AgR was the only RNA in this test. All listed sequences form G4 structures. dx12 and 19AT are two duplexes formed by two complementary DNA strands, whereas ds26 is a 'self-complementary' strand. dT_{30} is a 'single-stranded' DNA. Finally, the 'Triplex' sample is formed with two different strands in a 2:1 stoichiometry, in which a dT_{20} third strand binds to the major

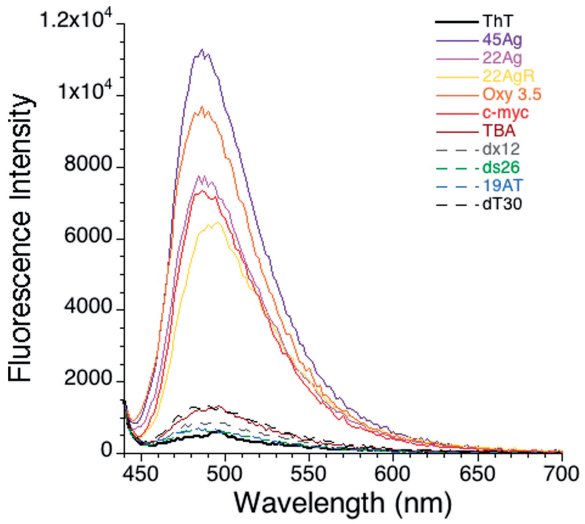

Figure 2. Fluorescence emission spectra (a.u.) of ThT in the presence of various oligonucleotides. Fluorescence emission of ThT alone is shown in black. Fluorescence in the presence of 10 different oligonucleotides is also shown. Samples were prepared at 1 μM strand in 50 mM Tris/HCl, pH 7.5, 50 mM KCl, and ThT was added to a concentration of 0.5 μM.

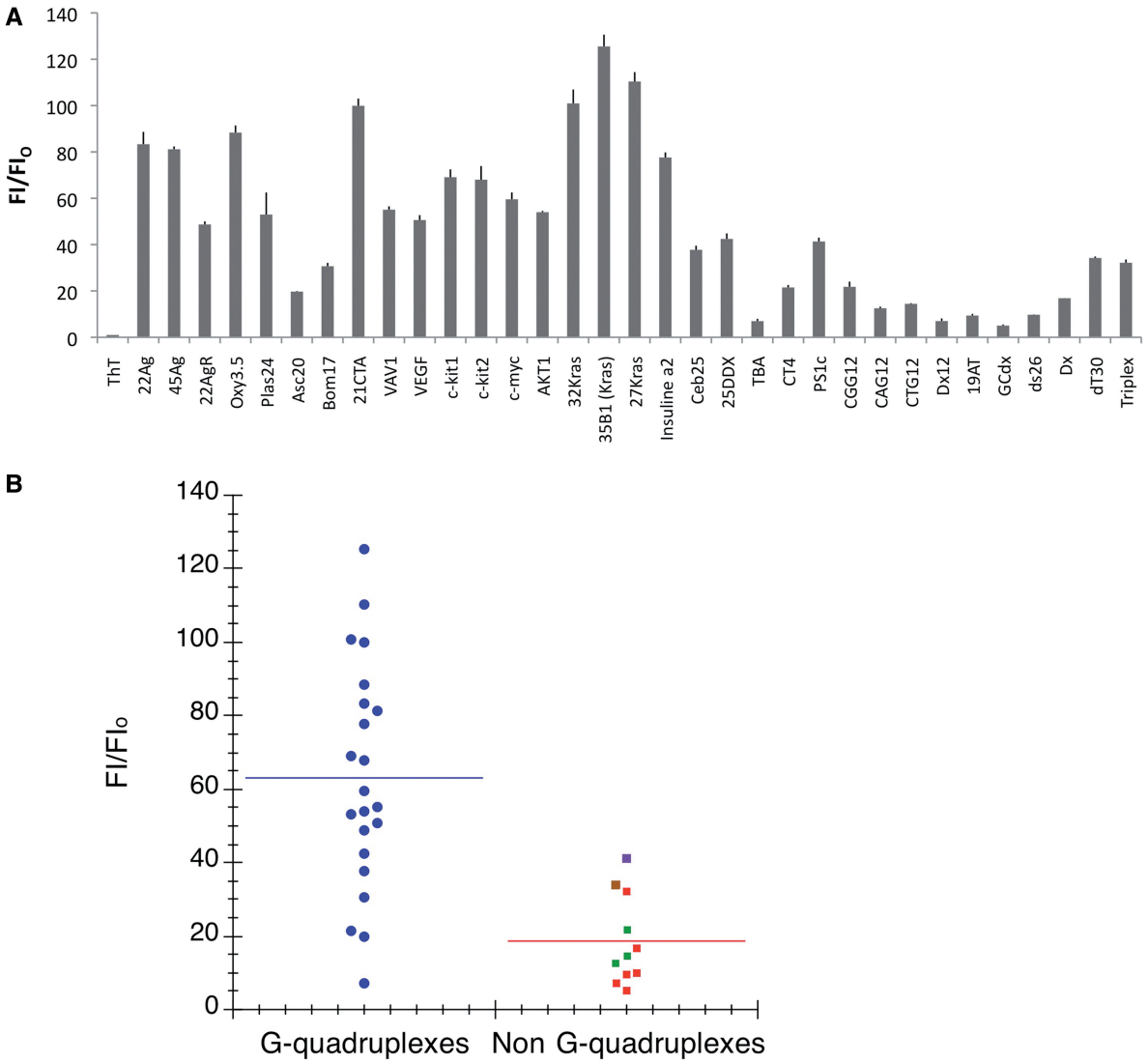

Figure 3. Bar graph and dot plot of fluorescence enhancement of ThT in the presence of a variety of nucleic acid structures. Oligonucleotides are described in Table 1. (**A**) Each bar of the graph corresponds to the fluorescence enhancement in the presence of the indicated sequence. Error bars correspond to S.D. (**B**) Each point corresponds to fluorescence enhancement in the presence of a different oligonucleotide. The change in fluorescence emission is plotted for DNA and RNA quadruplexes on the left (in blue) and non-G4 structures on the right. Green dots correspond to trinucleotides, purple dot to parallel-duplex, brown dot to the triplex and red to other nonquadruplex-forming sequences. The difference between these two distributions is highly significant ($P < 0.001$); however, note that Student's *t*-test assumes a normal distribution, which is unproven here (and actually unlikely).

groove of a dT_{20}–dA_{20} Watson–Crick duplex. All sequences are listed in Table 1.

Figure 2 shows the emission spectra of ThT in the presence of a variety of DNA sequences and structures. An emission of maximum ~490 nm was observed in all cases. In the presence of five G4s (45Ag, 22Ag, 22AgR, Oxy3.5 and c-myc), we observed a large fluorescence increase relative to ThT alone, respectively, 23-, 15-, 13-, 19- and 14-fold. In contrast, TBA did not induce a significant ThT fluorescence enhancement; the signal was similar to the one obtained with the single-stranded control dT_{30}. The emission intensities in the presence of either of the duplex forming oligonucleotides were close to the one of ThT alone.

We subsequently performed this assay on a larger set of 31 sequences. Structures of these oligonucleotides have been characterized, and these oligonucleotides have been used in the G4-Fluorescence Intercalator Displacement (FID) assay (16). All sequences are listed in Table 1, and results are presented in Figure 3. Twenty-one of the 31 sequences are likely to form G4s. We used a 96-well plate format, and oligonucleotides were tested in triplicate. The test is inexpensive, as only 1 µM of oligonucleotide in 20 µl (i.e. 20 pmol) was used in each well. Three wells were dedicated to ThT alone; this allowed us to define the fluorescence enhancement as the ratio between ThT fluorescence in the presence of oligonucleotide (FI) and background fluorescence of ThT alone (FI_0) after subtraction of the buffer fluorescence. Figure 3 demonstrates that ThT is a general G4 light-up probe: With the exception of TBA, all sequences known to form G4s lead to a FI/FI_0 >20. In contrast, none of the other

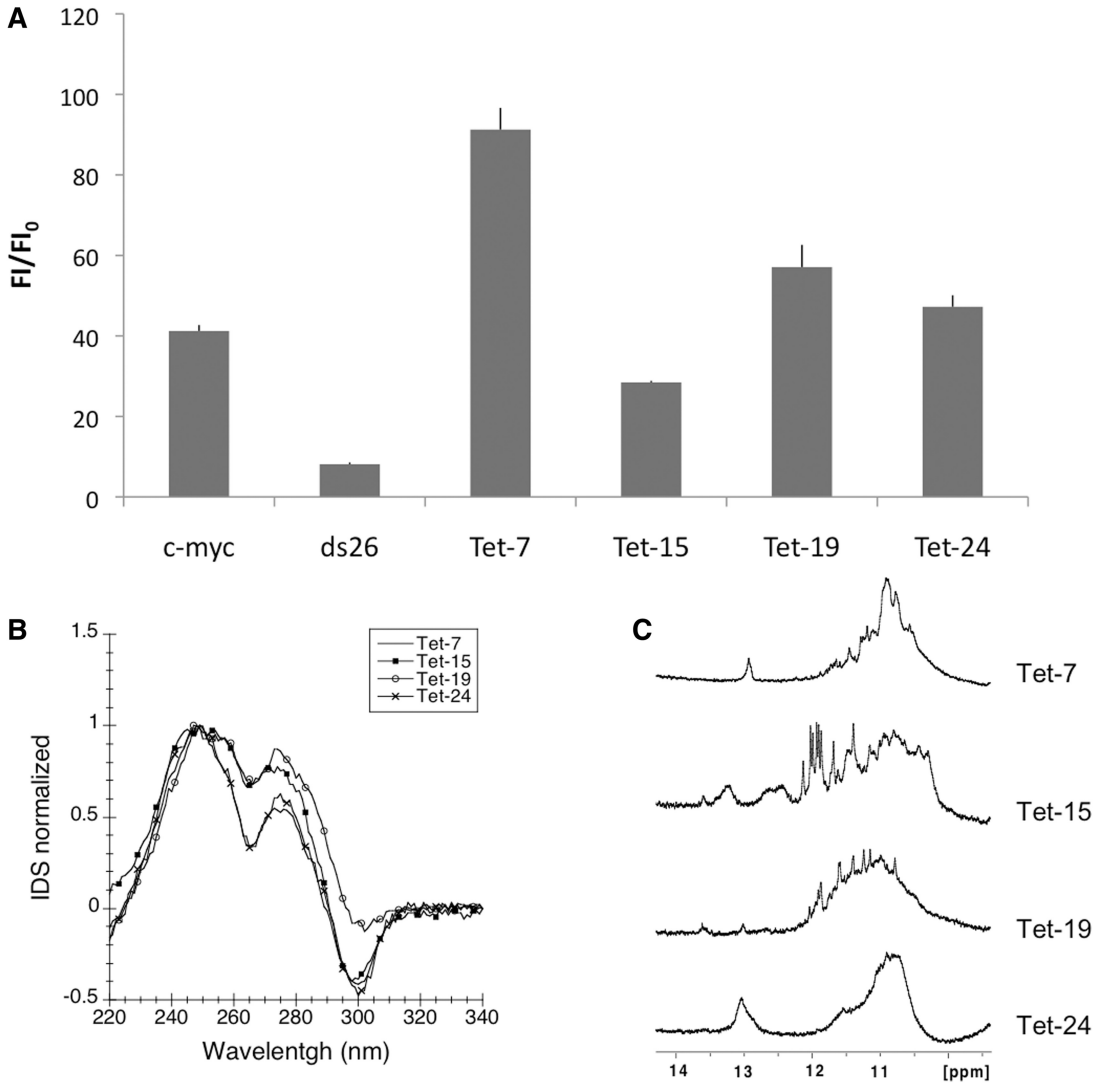

Figure 4. Evidence for G4 formation for the Tet aptamers. (**A**) Bar graph of fluorescence enhancement with 1 μM indicated oligonucleotide and 0.5 μM ThT. (**B**) IDS of the four aptamers tested. (**C**) Representative imino proton spectrum of the four aptamers tested. The presence of peaks between 10.5 and 12 suggests G4 formation.

oligonucleotides, with the exceptions of PS1c (a parallel-stranded duplex), dT30 (oligothymidylate) and the triplex induced significant fluorescence. According to the statistical analysis of the data (Figure 3B), ThT fluorescence enhances >60 times in the presence of G4 and <20 times on average in the presence of other structures.

Example of application

We next used this assay to characterize aptamers of unknown structure. In 2008, Niazi and coworkers reported a new family of seven aptamers selected against tetracycline (Table 2) (17). The authors used MFold to predict the secondary structures of these oligonucleotides; this software did not have the ability to predict G4 formation. Given that all seven aptamers are G-rich, it seemed likely that they form G4 structures. We used the ThT assay to investigate this possibility for four of these aptamer sequences. Two oligonucleotides were used as controls: (i) The c-myc oligonucleotides was the G4-forming positive control; its FI/FI_0 in the first test was approximately the average of all the G4-forming sequence signals. (ii) ds26 was a duplex-forming negative control. Interestingly, the four aptamers tested here (Figure 4A) exhibited FI/FI_0 responses much higher than ds26; three of them actually led to signal higher than the one found for c-myc, our positive control. This large fluorescence enhancement suggests that these sequences adopt a G4 fold. We performed IDS and 1D NMR (Figure 4B and C) to confirm this: data from the three techniques led us to conclude that these sequences form G4s.

Gel staining

To further evaluate the specificity of ThT, we used it to stain oligonucleotides separated on a nondenaturing gel. We chose one well-known G4 (22Ag) as a positive control and one duplex (19AT) as a negative control (Table 1). The oligonucleotides were loaded at eight different

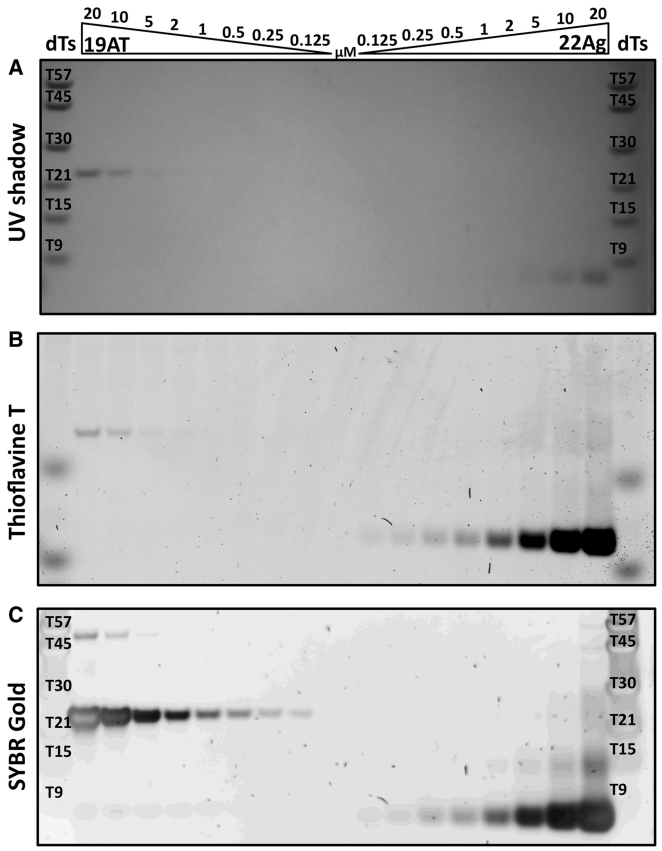

Figure 5. Oligonucleotide migration on a nondenaturing gel revealed by three different methods. Two oligonucleotides, a duplex on the left (19AT) and a quadruplex on the right (22Ag), were tested at eight different concentrations (20, 10, 5, 2, 1, 0.5, 0.25 and 0.125 μM). Samples were prepared in a 50 mM Tris/HCl, pH 7.5 buffer with 50 mM KCl and loaded on a nondenaturing 15% acrylamide gel supplemented with 10 mM KCl. The gel was electrophoresed at 18°C. Migration markers are single-stranded dT_n. The gel was visualized by (**A**) UV shadowing, (**B**) staining with 0.5 μM of ThT and (**C**) staining with SYBR Gold.

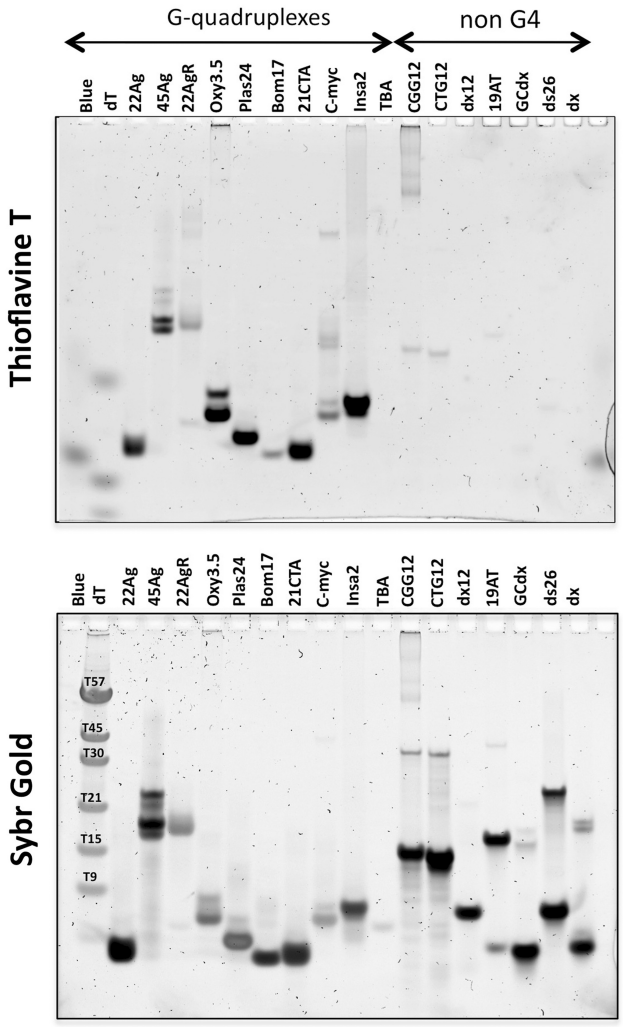

Figure 6. G4 structures detected on nondenaturing polyacrylamide gel electrophoresis by ThT staining. Eighteen different oligonucleotides were tested at 2 μM concentration. Samples were prepared in a 50 mM Tris/HCl, pH 7.5, 50 mM KCl and loaded on a nondenaturing 15% acrylamide gel supplemented with 10 mM KCl. The gel was electrophoresed at 20°C. Migration markers were single-stranded dT_n. The gel was visualized by (Top) staining with 0.5 μM of ThT and (Bottom) staining with SYBR Gold. Preferential staining of G4 structures by ThT is observed (upper panel), whereas SYBR Gold reveals all oligonucleotides (duplexes, single strands and quadruplexes).

concentrations (20, 10, 5, 2, 1, 0.5, 0.25 and 0.125 μM) in 50 mM Tris/HCl, pH 7.5, supplemented with 50 mM KCl. Single-stranded dT_n oligonucleotides were used as migration markers. The gel was first visualized after migration by UV shadowing (Figure 5A), and then was stained in a bath of 0.5 μM ThT for 15 min under agitation, then briefly washed (Figure 5B). Finally, we restained the same gel with SYBR Gold (Figure 5C). Both UV shadowing and SYBR Gold stain for total DNA and show similar intensities for each 22Ag and 19AT. ThT allowed us to visualize G4 structures selectively; even at the lowest concentration tested, a faint band for 22Ag was visible. In contrast, duplexes were weakly stained even at high concentrations (10–20 μM) and were barely visible at 5 μM.

We decided to proceed at a fixed concentration of 2 μM to test a larger set of oligonucleotides. G4-forming oligonucleotides used in the 96-plate test (22Ag, 45Ag, 22AgR, Oxy3.5, Plas24, Bom17, 21CTA, c-myc, Insa2 and TBA) and negative controls (CGG12, CTG12, dx12, 19AT, GCdx, ds26 and dx) were loaded on a nondenaturing gel in 50 mM Tris/HCl, pH 7.5, buffer supplemented with 50 mM KCl. After electrophoresis, the gel was stained in a bath of 0.5 μM ThT for 15 min under agitation, then briefly washed (Figure 6A). The same gel was then restained with SYBR Gold (Figure 6B). The comparison

Table 2. Aptamers with high affinity for tetracyclines

Name	Sequence (from 5′ to 3′)
Tet-7	GGGCAGCGGTGGTGTGGCGGGATCTGGGGTTGTGCGGTGT
Tet-15	GGAGGAACGGGTTCCAGTGTGGGGTCTATCGGGGCGTGCG
Tet-19	CGGGAGGGCGGGGTGTGGTATGTATTGAGCGTGGTCCGTG
Tet-20	CCCCCGGCAGGCCACGGCTTGGGTTGGTCCCACTGCGCGT
Tet-22	GGGCGGACGCTAGGTGGTGATGCTGTGCTACACGTGTTGT
Tet-23	GGGGGCACACATGTAGGTGCTGTCCAGGTGTGGTTGTGGT
Tet-24	GGGCGGGGGTGCTGGGGGTGGAGTGCTGCGTGCTGCGG

of both panels is instructive: ThT selectively stained the G4 structures, whereas SYBR Gold was a general light-up probe for the nucleic acids. As noted during our solution measurements, ThT does not stain all G4 structures equally well (e.g. TBA was not stained in solution or on the gel). The relative staining intensities in the gel correlated relatively well with the solution emission measurements. Of interest, the two non-G4 structures stained by ThT are trinucleotide repeats. CGG repeats are known to form both mismatched hairpins and G4 structures; the slow migrating bands we observed in the gel may correspond to G4 higher-order structures. CTG12 can form duplexes with T–T mismatches, which may be a site for binding of ThT (18).

DISCUSSION

In this report, we demonstrated that ThT fluorescence enhancement may be used as a diagnostic tool to determine whether a sequence forms a G4 structure. Here we expanded on the recent reports by the teams of Bhasikuttan (13), Tang (14) and Miyoshi (15) to show that a large fluorescence enhancement of ThT is a hallmark of most G4-forming sequences. The assay we developed and presented here may therefore be used as a first-in-line assay to identify G4 formation.

This is far from being the first report of a light-up fluorescent probe for G4 structures [e.g. (19–23); for a review (24–26)]. However, most of the previously reported probes suffer from one or more of the following problems: (i) a limited specificity for quadruplexes over duplexes (examples are thiazole orange and, as illustrated here, SYBR Gold); (ii) a low quantum yield, Stoke's shift or extinction coefficient; and (iii) difficult synthesis and lack of commercial availability. ThT, because of its specificity, high relative increase in emission, low cost and commercial availability does not suffer from these limitations. Furthermore, its maximum wavelengths for excitation and emission are compatible with most experimental settings. One drawback is that the ThT assay both in solution and on a gel is not 100% accurate, as at least one sequence (TBA) would have been missed. Using an arbitrary threshold of 20-fold induction of fluorescence, 27 of 28 quadruplexes were accurately predicted to form G4 structures based on the solution assay for a false-positive rate of ~4%. TBA was the only exception of the known G4-forming oligonucleotides we evaluated and is an outlier for a number of tests. For example, this aptamer selected against thrombin has a weak affinity for thiazole orange, another fluorescent probe used in FID experiments (27). Regarding a possible selectivity for one G4 conformation (parallel, antiparallel, 3 + 1 . . .), we cannot draw clear conclusions at this stage, as the exact conformation(s) is only known for a subset of these sequences, and not always under the experimental conditions chosen here. Results obtained with Bom17 and TBA suggest that G4 structures involving only two quartets lead to a lower increase in ThT fluorescence. One possible explanation would be that, assuming ThT binds to the G4 grooves, such G4s do not offer an optimal site for ThT sequestration. The affinity of ThT for non-nucleic acid structures such as amyloid aggregates would be a problem for cellular or *in vivo* applications but is clearly not an issue for the test developed here.

In addition to false negatives such as TBA, some false positives were observed: these sequences that do not form G4 but that lead to a high ThT fluorescence enhancement form noncanonical structures. This is the case for sequences such as PS1c, which adopts a parallel-stranded duplex structure or, to a lesser extent, to the dT_{20}–dA_{20}–dT_{20} triplex. These motifs are rare and A–T-rich; they would not have been suspected to form G4 base on primary sequence. Moreover, in the case of these particular sequences, another ligand named 9944, which has specificity for G4, also bound to PS1c (28) and triplexes. One should also note that the thresholds we chose for the fluorescence enhancement cutoff will depend on the experimental settings and instrumental response; one should include a few positive and negative controls in each experiment.

Despite these shortcomings, this assay nicely complements the other tests currently used to establish G4 formation. In our hands, none of the methods we routinely use is perfect: inferring G4 formation *in vitro* should be based on at least two independent techniques. ThT fluorescence is simple to monitor, yields a fast response and is low cost. Furthermore, this assay may be performed in solution and read with a spectrofluorimeter or a simple fluorescence plate reader, or ThT may be used to stain gel that is scanned by a camera or a Typhoon instrument. Our current recommendation for evaluation of G4 formation *in vitro* for any given short sequence is to combine the ThT fluorescence assay presented here with 1D NMR and isothermal differential spectra analysis. Hundreds of nucleic acid sequences have been investigated using these three independent methods.

FUNDING

Funding for open access charge: ANR (Quarpdiem, T-Kinet and Oligoswitch), Nanobiotech [ANR-10-NANO-04], Conseil Régional d'Aquitaine ('Chaire d'accueil' to J.L.M. and Aquitaine-Midi Pyrénées call) and Nanobiotech / Vibbnano [ANR-10-NANO-04].

Conflict of interest statement. None declared.

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