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Visual observation of G-quadruplex DNA with the label-free fluorescent probe silole with aggregation-induced emission

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ABSTRACT

AIE molecule silole **1** could be used to detect G-quadruplex formation using an exonuclease I hydrolysis assay. This visual observation of G-quadruplexes has been successfully used in investigating multiple G-quadruplexes, including the one-stranded telomeric, *c-myc*, *c-kit*, *VEGF* G-quadruplexes, and a d($G_4T_4G_4$) inter-molecular G-quadruplex. The detection of G-quadruplex can be also applied to G-quadruplex isomers induced by small molecules.

1. Introduction

One, two, or four guanine-rich strands, forming a cyclic, hydrogen-bonded, square, planar alignment of four guanines, have the potential to fold into inter- or intra-molecular G-quadruplex scaffolds.[1] These guanine-rich segments are observed in biologically significant genome regions, such as telomeric regions and the *c-myc*, *c-kit*, and *VEGF* gene promoters.[2–4] The study of the structure and function of this peculiar DNA arrangement has implications for biology and potential applications in nano-technologies.[5–7]

G-rich motifs can be stabilized by the inorganic alkali metal cations K^+ or Na^+. In the past two decades, there has been considerable interest in small organic molecules targeting G-quadruplex nucleic acids.[8–17] The G-quadruplex triggers several biological dysfunctions that selectively alter the integrity of cancer cells.[18–20] Small organic molecules, which are able to stabilize the G-quadruplex structure, could provide a mechanism for the discovery of novel anti-cancer agents.[21] For efficient screening of G-quadruplex ligands, it is important that G-quadruplexes can be reliably detected.

Many approaches, including NMR spectroscopy, mass spectrometry, circular dichroism (CD), UV melting profile analysis, surface plasmon resonance, and polyacrylamide gel electrophoresis have been used in the study of G-quadruplex structures.[22–26] Fluorescent resonance energy transfer (FRET) is one of the most useful methods for indicating the presence of G-quadruplexes,[27–30] but its requirement for a dual-labeled fluorophore is uneconomical. For this reason, label-free bioprobes are an attractive alternative. Some of the most striking label-free biosensors are water-soluble aggregation-induced emission (AIE) molecules, for their application in detecting biomacromolecules.[31–34] This kind of non-emissive molecule can light up upon aggregation. The advantages of these biosensors are that they do not require pre-labeling with a fluorophore, and that label-free fluorescence probes could also provide high sensitivity, low background signals and a wide dynamic working range.[35,36] Although some molecules have been described as fluorescence turn-on DNA sensors,[31,34,37,38] the application of AIE molecules to indicate DNA secondary structure has been limited, especially investigation of the G-quadruplex. Recently, the Tang and co-workers reported that TTAPE (Scheme 1) was used as a label-free fluorescent probe to detect G-quadruplex conformation with AIE.[37] However, this method was restricted to the detection of K^+-induced G-quadruplex. Herein, we establish silole **1** as a new fluorescent light-up probe to detect G-quadruplex structure formation with an exonuclease I hydrolysis assay. This method was adapted to both the inter- and intra-molecular G-quadruplexes formed by different G-rich strands. Moreover, the visible observation of the G-quadruplex could be applied not only to G-quadruplexes stabilized by monovalent cations, but also to G-quadruplex isomers induced by small molecules.

The mechanism of the colorimetric biosensor for detecting G-quadruplex DNA is shown in Scheme 2. The silole derivatives were first reported as AIE molecules by Tang and co-workers.[39] Later, the

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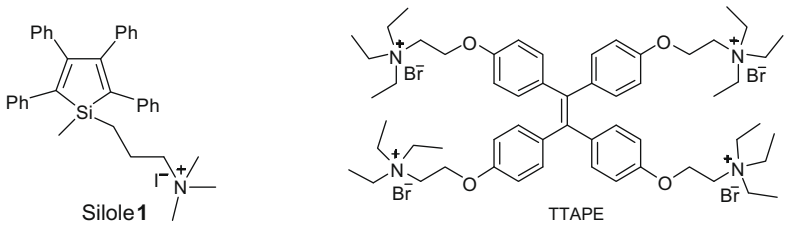
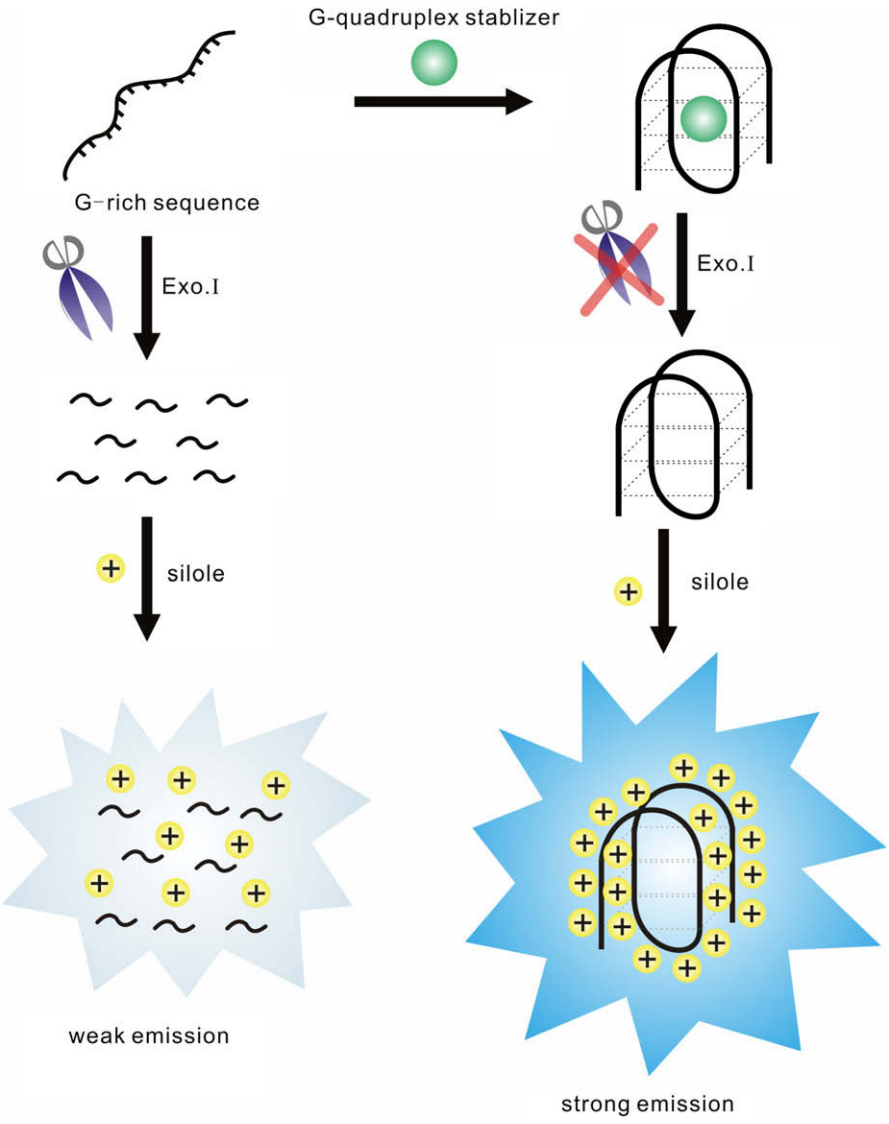


Scheme 1. Constitutional formula of silole **1** and **TTAPE**.



Scheme 2. Schematic representation colorimetric strategy for G-quadruplex DNA detection with silole **1**.

AIE biosensor silole **1** (Scheme 1) was published as a probe for fluorescence turn-on detection of DNA.[38] The enhancement of its emission depends on the length of oligonucleotide, thus it can also be employed to monitor DNA cleavage by nucleases. This property initiated the development of a visual observation method for G-quadruplexes. Exonuclease I degrades single-stranded DNA in the 3′ to 5′ direction in a stepwise manner. Tan and co-workers reported an exonuclease I hydrolysis assay for evaluating G-quadruplex stabilization by small molecules.[40] In the presence of K^+ or other G-quadruplex stabilizers, the induction of a G-quadruplex structure at the 3′ end of the DNA strand blocks the hydrolysis reaction. In this case, the aggregation complex of silole **1** and undigested DNA exhibits strong fluorescence. In the absence of G-quadruplex binder, DNA is digested by exonuclease I, and silole **1** is incapable of aggregating with small fragments of DNA, quenching the emission.

2. Results and discussion

2.1. Fluorescence detection of intra-molecular G-quadruplex DNA with silole 1

In order to test our hypothesis, we performed a label-free fluorescence exonuclease I hydrolysis assay. The fluorescence intensity of silole **1** caused by AIE was used as an indicator of the presence of

a G-quadruplex. It is well known that the human telomere sequence 21G adopts a hybrid G-quadruplex structure in K^+ solution.[41–44] As shown in Figure 1, in the absence of K^+, the fluorescence intensity of silole **1** containing telomere sequence 21G became gradually weak by prolonging the hydrolysis reaction time. This is simply due to the fragmentation of DNA in the presence of exonuclease I, and as a result the aggregation of silole **1** cannot take place efficiently. However, after addition of KCl, the fluorescence of silole **1** decreased very slightly as shown in Figure 1 (right). This is because of the formation of the corresponding G-quadruplex that can inhibit the hydrolysis reaction. In fact, the CD (circular dichroism) spectra indicate that this oligodeoxynucleotide is prone to fold into a hybrid in the presence of KCl in hydrolysis reaction buffer (Supplementary data, Fig. S1A).

G-quadruplex formation prevents 21G DNA digestion in K^+ concentration-dependent manner. Figure 2A shows the enzymatic activity at various K^+ concentrations (from 0 to 100 mM). The fluorescence emission of silole **1** increased gradually after adding KCl into the hydrolysis system. It indicates that the increased ratio of G-quadruplex by various K^+ concentration cease enzymatic hydrolysis and provide a template for silole **1** aggregation. Furthermore, the fluorescence decreases in the presence of 100 mM LiCl (Fig. 2B). This confirms that Li^+ cannot stabilize G-quadruplex and prevent the enzymatic activity.

2.2. Visual fluorescence turn-on detection of various intramolecular G-quadruplex DNA

Visual observation of G-quadruplexes now became feasible (Fig. 3). Under UV light (365 nm), a 21G oligomer hydrolysis sample emitted visible blue-fluorescence. This phenomenon indicated formation of a K^+-induced G-quadruplex. In a parallel experiment with the random oligomer ssDNA20, which cannot form a G-quadruplex, the fluorescence intensity rapidly disappeared during the cleavage reaction in 100 mM K^+ buffer. The remarkable disparity in fluorescence intensity directly depends on DNA secondary structure. This fluorescent light-up detection of G-quadruplexes has also been employed for other intra-molecular G-quadruplexes in K^+ buffer, such as the *c-myc*, *c-kit*, and *VEGF* G-quadruplexes. The results are consistent with those of the human telomere hybrid G-quadruplex (Supplementary data, Figs. S2 and S3). The strong fluorescence emission of silole **1** implies that G-quadruplex inhibit the cleavage reaction. Two random coil oligomers ssDNA18 and ssDNA23 were also used in this experiment (Supplementary data, Fig. S2D). All the sequences are shown in Table 1. The single-stranded ssDNA 18, 20, and 23 could not fold up by hydrogen bonds in the presence of some metal cations (potassium, sodium, and lithium) and some ligands. The random structures were further characterized and clarified by CD spectra (Supplementary data, Fig. S4). There is little change in the CD signal upon adding

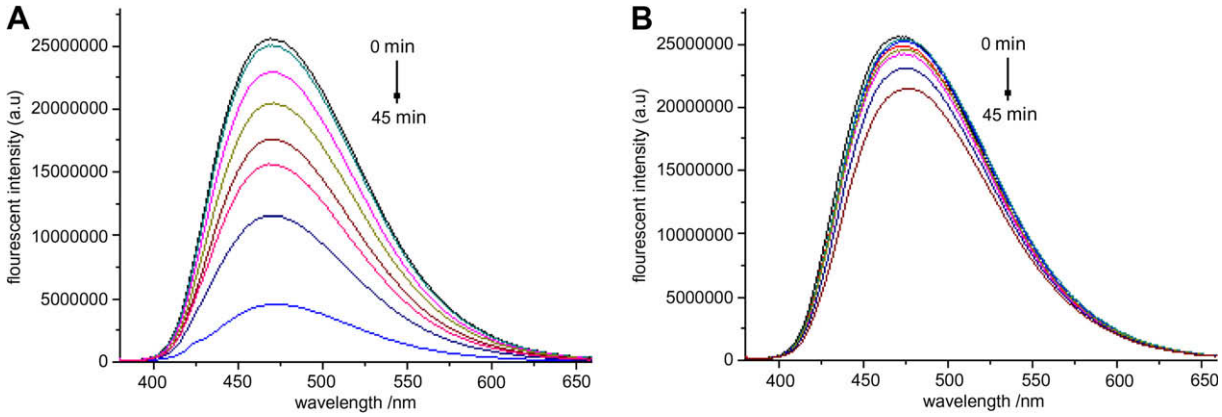


Figure 1. Fluorescence intensity of silole **1** (20 μM in 20 mM, pH 7.4 Tris buffer) containing 2.5 μM 21G versus the exonuclease I hydrolysis reaction time (from 0 to 45 min) in the absence (A) or presence (B) of 100 mM KCl.

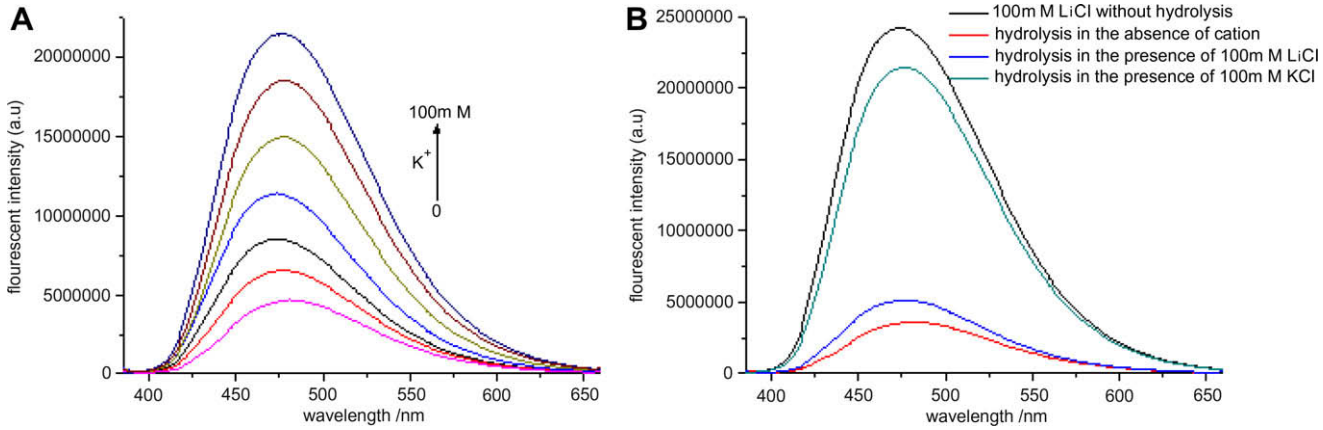


Figure 2. Fluorescence spectra of silole **1** (20 μM in 20 mM, pH 7.4 Tris buffer) containing 2.5 μM 21G hydrolyzed by exonuclease I for 45 min (A) in the presence of different amounts of KCl (from 0 to 100 mM); (B) in the presence of LiCl and KCl.

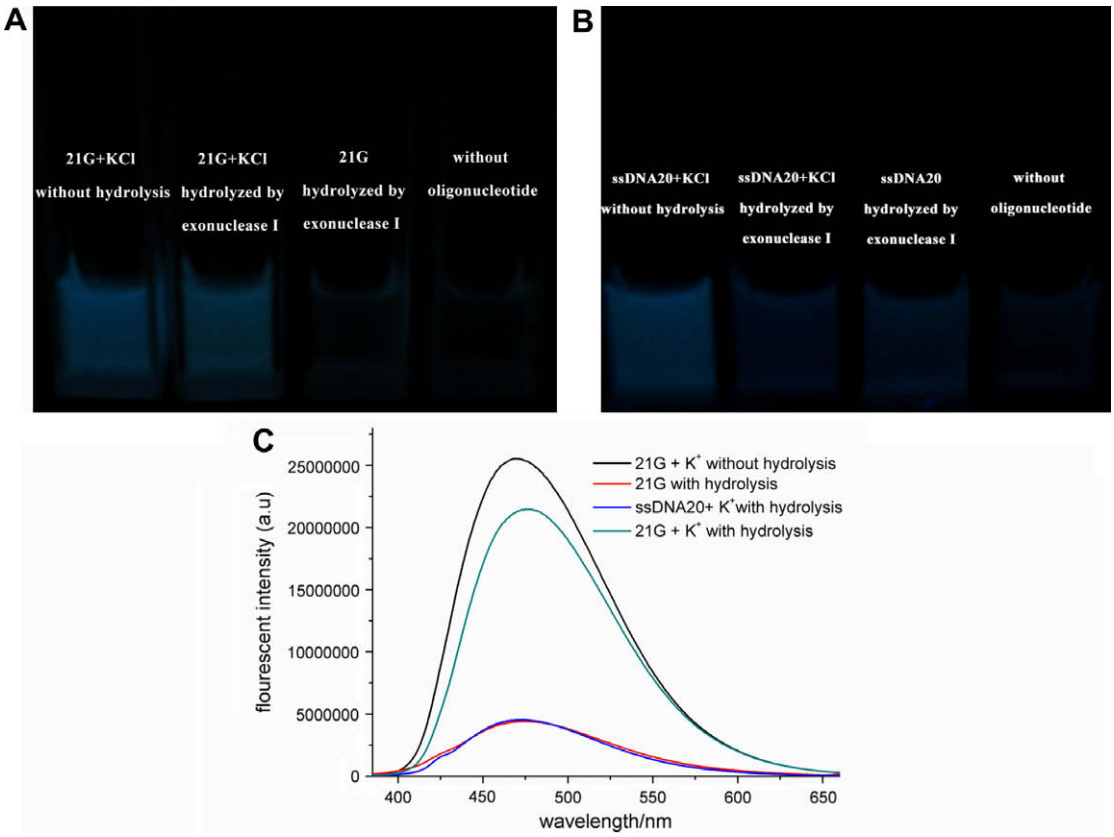


Figure 3. G-quadruplex detection assay based on aggregation-induced emission of silole **1** (20 μM) in 20 mM Tris–HCl buffer. (A) The photos of silole **1** in the presence or absence of 21G hydrolysis products (B) The photos of silole **1** in the presence or absence of ssDNA20 hydrolysis products. All the photos were taken under illumination with UV light (365 nm). (C) Fluorescence spectra of silole **1** with or without hydrolysis products.

Table 1
The oligodeoxynucleotides used in this study

ODN	Sequence
21G	5′-d(GGGTTAGGGTTAGGGTTAGGG)-3′
c-myc22	5′-d(GGGGAGGGTGGGGAGGGTGGGG)-3′
c-kit20	5′-d(GGGCGGGCGCGAGGGAGGGG)-3′
VEGF18	5′-d(GGGCGGGCCGGGGGCGGG)-3′
G4T4G4	5′-d(GGGGTTTTGGGG)-3′
ssDNA18	5′-d(ATCGCTTCTCGGCCTTTT)-3′
ssDNA20	5′-d(GCATTGGTAACTGTCAGACC)-3′
ssDNA23	5′-d(CGCGATTGTTAAATATTCGTCTT)-3′

potassium, sodium, and lithium cations. As a negative control, all the single-stranded DNA of ssDNA 18, 20, and 23 are unable to form DNA secondary structure and prevent hydrolysis. Upon addition of silole **1**, well-dispersed silole **1** was faintly emissive (Supplementary data, Fig. S5). The results concurred with those of the G-quadruplex observations by visible fluorescence.

2.3. The observation of inter-molecular G-quadruplex DNA with silole 1

All of the above detectable G-quadruplexes were one-stranded intra-molecular monomers. G-quadruplexes can be formed by two or four strands as an inter-molecular biopolymer.[45] We found that this method was also applicable to inter-molecular G-quadruplex systems. As previously reported, the oligomer d($G_4T_4G_4$) is prone to form a dimeric, antiparallel G-quadruplex in K^+ or Na^+ buffer.[46–48] In these experiments, Na^+ was used as a G-quadruplex stabilizer. The CD spectra show that d($G_4T_4G_4$) forms an antiparallel G-quadruplex in Na^+ buffer (Fig. 4B). The fluorescence intensity of silole **1** with d($G_4T_4G_4$) hydrolysis products in the presence of Na^+ was much stronger than that of the products without Na^+ (Fig. 4A). The strong fluorescence emission, indicating the presence of G-quadruplexes, can be easily seen under irradiation at 365 nm (Supplementary data, Fig. S2E). In contrast, there was no fluorescence in the absence of Na^+.

2.4. Detection of G-quadruplex isomers induced by small molecules

We investigated whether small molecule-stabilized G-quadruplexes could also be identified by a visual fluorescence signal. In addition to monovalent cations, a number of small molecules have been reported to be G-quadruplex ligands.[8–17] **TMPyP$_4$** is one of the most extensively studied G-quadruplex ligands.[49–52] **TMPyP$_4$** can induce and stabilize G-quadruplex at certain conditions, such as in K^+ buffer. In the presence of Li^+ or absence of cation, **TMPyP$_4$** shows little stabilization potentiality. Here, we used **TMPyP$_4$** to assess this novel G-quadruplex detection system. However, the ligand could compete with the silole for binding to the quadruplex, preventing the fluorescence response, and the fluorescence from **TMPyP$_4$** may also induce fluorescence signal interference. In this case, the ligand was removed using a QIA quick nucleotide purification kit (Qiagen) after the hydrolysis assay. The results show that colorimetric cell containing oligomer 21G exhibits weak emission intensity (Supplementary data, Fig. S6). This was identical to the cell containing the non-quadruplex-forming oligomer ssDNA20 in the presence of **TMPyP$_4$**. It suggests that **TMPyP$_4$** is unable to induce the formation of a G-quadruplex in the absence of cation. In the presence of Li^+, **TMPyP$_4$** also cannot form G-quadruplex, displaying the low fluorescence signal (Fig. 5). This result is identi-

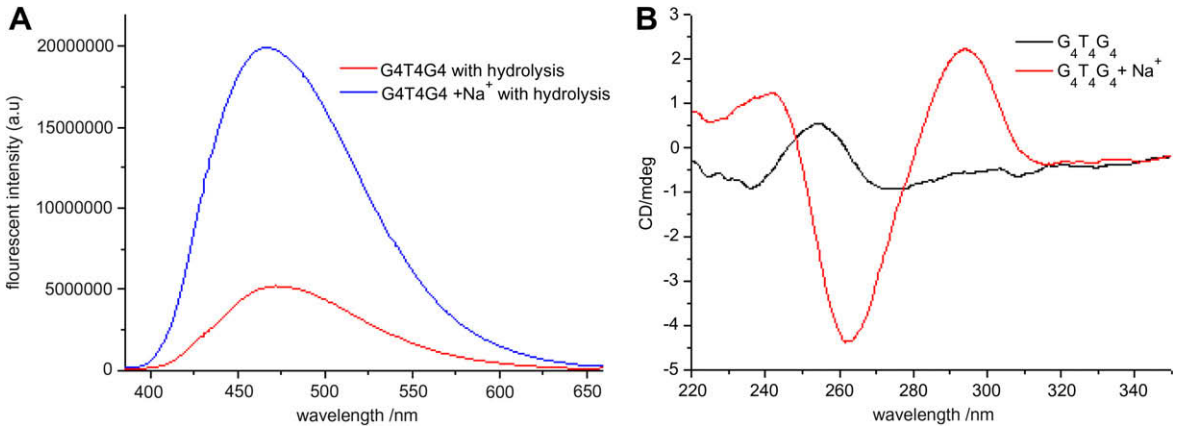


Figure 4. (A) The fluorescence spectra of silole **1** (20 μM) in 20 mM Tris–HCl buffer with hydrolysis products of d($G_4T_4G_4$). (B) CD spectrum of d($G_4T_4G_4$) in the presence or absence of 100 mM Na^+.

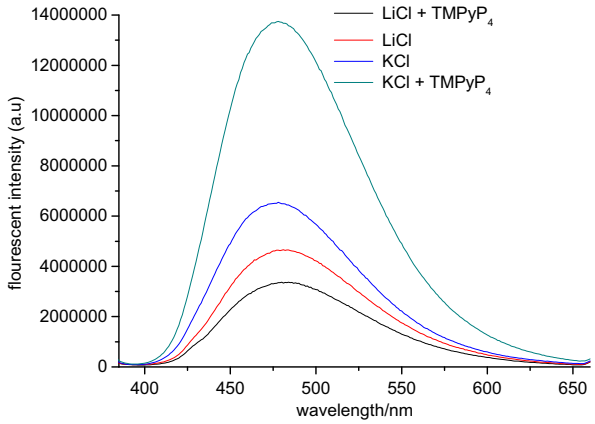


Figure 5. The fluorescence spectra of silole **1** (20 μM) in 2 mM K^+ and Li^+ buffer with hydrolysis products of 21G DNA in the presence or absence of 2.5 μM **$TMPyP_4$**.

cal to that in Tan's report that **$TMPyP_4$** failed to block the hydrolysis of telomeric DNA in Li^+ solution.[40] In the presence of 2 mM K^+, the K^+ shows weak quadruplex stabilization potential. When **$TMPyP_4$** was added, fluorescence emission increased, by comparison with that in the absence of **$TMPyP_4$**. It illustrates that **$TMPyP_4$** stabilize the G-quadruplex in K^+ buffer.

Recently, bis(benzimidazole)aryl (**BBP**) has been proposed to be a novel G-quadruplex binding ligand.[53] It was shown that the induction of a G-quadruplex structure conferred the ability to resist digestion. Figure 6 shows the results of **BBP** under irradiation with UV light (365 nm). The difference in fluorescence intensity for silole **1** between oligomers 21G and ssDNA20 can be distinguished easily with the naked eye. This result indicates that **BBP** can induce and stabilize formation of G-quadruplexes. We used CD to confirm this result (Supplementary data, Fig. S7). Upon addition of **BBP** to the human telomeric sequence 21G, a positive peak at around 295 nm was observed in the CD spectrum, suggesting the presence of G-quadruplex. It is not surprising that there are no significant changes to ssDNA in the presence of **BBP**, implying that **BBP** specifically bind the G-quadruplex.

3. Conclusion

In conclusion, the AIE molecule silole **1** was designed to detect G-quadruplex formation using an exonuclease I hydrolysis assay. The visual observation of G-quadruplexes has been successfully used in investigating multiple G-quadruplexes, including the one-stranded telomeric, *c-myc*, *c-kit*, and *VEGF* G-quadruplexes, and a d($G_4T_4G_4$) inter-molecular G-quadruplex. Significantly, G-quadruplexes stabilized by small molecules are visible to the naked eye.

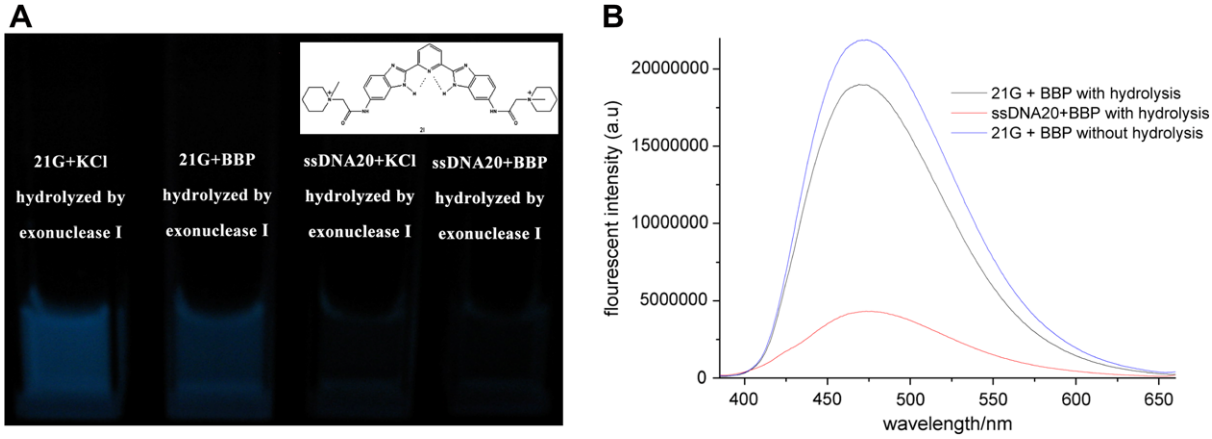


Figure 6. (A) The photos of silole **1** (20 μM) in 20 mM Tris–HCl buffer with different hydrolysis products. The inset shows the structure of **BBP**. (B) Fluorescence measurement of silole **1** (20 μM) in 20 mM Tris–HCl buffer with hydrolysis products. The **BBP** was removed before taking photos and fluorescence measurement.

4. Experimental section

Silole **1** was synthesized in light of previous reported procedure.[38] Exonuclease I was purchased from TaKaRa Biotech (Dalian, China). All the oligomers used in this research were purchased from Invitrogen (China). **$TMPyP_4$** was purchased from Sigma; **BBP** was synthesized in lab according to the previous report.[53]

4.1. Exonuclease I hydrolysis assay

Exonuclease I hydrolysis experiment was carried out in 40 μL reaction mixture containing 67 mM Tris–HCl, pH 7.4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 2.5 μM oligonucleotides. Before the addition of exonuclease I and reaction buffer, the oligonucleotides were annealed to form G-quadruplex. The hydrolysis was initiated by adding 5U exonuclease I at 37 °C and stopped by heating to 94 °C for 5 min.

4.2. Removal of G-quadruplex ligands

For organic molecules inhibitory exonuclease I hydrolysis assay, 2.5 μM **BBP** and **$TMPyP_4$** was added into exonuclease I hydrolysis mixture. After 45 min digestion at 37 °C, The hydrolysis products were processed by QIA quick nucleotide purification kit (Qiagen) to remove the compounds according to the kit manual, then the purified products was used for fluorescence spectral measurements and photograph.

4.3. Fluorescence spectra

Total volume of 40 μL hydrolysis products were mixed with 20 mM Tris–HCl, pH 7.4 buffer. The silole **1** was added before fluorescence spectral measurements. Fluorescence spectra were collected on Nanolog Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, France) from 380 to 660 nm with a excitation of 370 nm at room temperature. The excitation and emission slits were both 5 nm.

4.4. Photograph

Forty microliters hydrolysis products were dispersed in 20 μM silole **1**, 20 mM Tris–HCl solution, pH 7.4. The total 1 mL solution was added into fluorescence cell, and then the photos were taken under UV light illumination at 365 nm.

4.5. Circular dichroism measurement

CD spectra were recorded on a Jasco-810 spectropolarimeter (Jasco, Easton, MD) using a quartz cell of 1 mm optical path length at room temperature. The instrument scanning speed is 100 nm/min with a response time of 1 s, and over a wavelength range of 220–350 nm. All CD spectra were baseline-corrected for signal contributions due to the buffer. The sample used in CD experiment contains the same buffer with hydrolysis reaction.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.040.

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