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Label-Free Luminescent Switch-on Detection of Endonuclease IV Activity Using a G-Quadruplex-Selective Iridium(III) Complex

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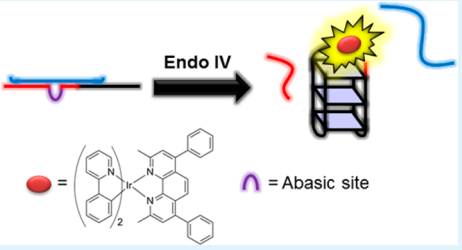
Supporting Information

ABSTRACT: We report herein the synthesis and application of a novel G-quadruplex-selective luminescent iridium(III) complex $[Ir(ppy)_2(bcp)]^+$ (where ppy = 2-phenylpyridine and bcp = 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) for the sensitive detection of apurinic/apyrimidinic (AP) endonuclease activity. Using endonuclease IV (Endo IV) as a model enzyme, a duplex DNA substrate containing a G-quadruplex-forming sequence is cleaved by Endo IV at the abasic site. This releases the G-quadruplex sequence, which folds into a G-quadruplex and is recognised by the G-quadruplex-selective iridium(III) complex with an enhanced luminescence response. The assay achieved high sensitivity and selectivity for Endo IV over other tested enzymes.



KEYWORDS: *G-quadruplex, iridium(III) complex, label-free, AP endonuclease, luminescent*

INTRODUCTION

The base excision repair (BER) pathway functions throughout the cell cycle to remove damaged bases from DNA.[1] It plays a critical function in the repair of DNA lesions and has been associated with both individual and population disease susceptibility.[2] Apurinic/apyrimidinic (AP) endonucleases play an important role in the BER pathway.[3,4] To remove damaged bases, AP endonucleases recognize and cleave abasic AP sites in DNA, creating a nick in the sugar-phosphate backbone of the AP site.[5−7] Therefore, it is desirable to develop simple and rapid methods for the detection of AP endonuclease activity.

Existing analytical methods for the detection of BER enzyme activity include gel-based assays, radioisotopic labeling and streptavidin paramagnetic bead capture techniques.[8,9] However, these methods tend to be unwieldy, labor-intensive, and time-consuming and may necessitate stringent safety measures to control radiographic exposure. In recent years, luminescent oligonucleotide-based sensing platforms for the detection of BER activity have been reported.[10−15] However, there have been no luminescent oligonucleotide-based assays for the detection of AP endonuclease activity reported in the literature.

Luminescent transition metal complexes have emerged as attractive candidates for G-quadruplex-sensing applications because of the following characteristics: (i) the properties of metal complexes can be easily tuned by adjustment of the auxiliary ligands, (ii) the relatively large Stokes shifts of luminescence helps to prevent self-quenching, (iii) metal complexes can be synthesized by simple synthetic protocols, and (iv) the long lifetime of the triplet metal-to-ligand charge transfer (^{3}MLCT) phosphorescence enhances image signal stability and reduces background autofluorescence noise.[16−20] Our group has recently demonstrated that iridium(III) complexes can be exploited as highly specific luminescent probes for the detection of G-quadruplex DNA.[23,24] G-quadruplex DNA is formed from guanine-rich sequences, and its secondary structure consists of square-planar arrangements of guanine nucleobases stabilized by Hoogsteen hydrogen bonding and monovalent cations.[26] The rich polymorphism of G-quadruplexes has rendered them as attractive signal-transducing elements for the development of oligonucleotide-based sensing platforms.[27−35]

We report herein the synthesis and application of a novel G-quadruplex-selective luminescent iridium(III) complex $[Ir(ppy)_2(bcp)]^+$ (**1**, where ppy = 2-phenylpyridine and bcp = 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) (Figure 1) for the sensitive detection of AP endonuclease activity. The prokaryotic endonuclease IV (Endo IV) was chosen as a model enzyme for this study. The proposed mechanism of the Endo IV activity assay is outlined in Scheme 1. An AP site-containing DNA oligonucleotide (P1, 5′-TATCTGCA-C*AGTG$_3$TAG$_3$CG$_3$T$_2$G$_2$-3′, where * = AP site), which also contains a G-quadruplex-forming sequence at the 3′-terminus, was obtained by removing uracil from a uracil-containing DNA oligonucleotide (P1U, 5′-TATCTGCA-CUAGTG$_3$TAG$_3$CG$_3$T$_2$G$_2$-3′) using uracil-DNA glycosylase

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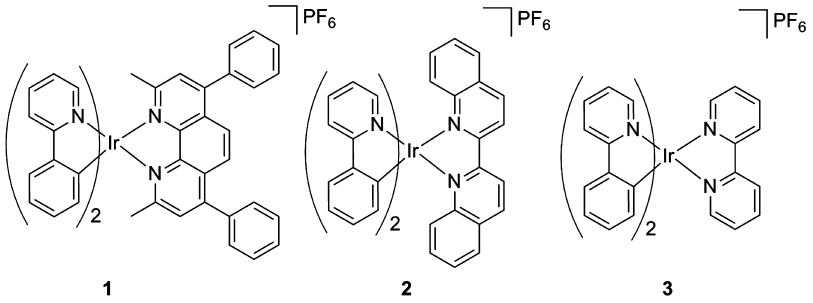
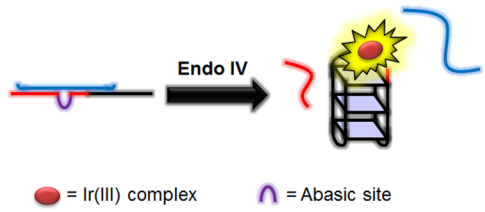


Figure 1. Chemical structures of the iridium(III) complexes investigated in this study.

Scheme 1. Schematic illustration of the G-Quadruplex-Based Assay for Endonuclease IV Activity Detection



(UDG). The AP site-containing sequence P1 was then hybridized with a partially complementary DNA strand (P2, 5′-$TC_2TAC_3ACTAGTGCAGAT_2$-3′) to form a duplex substrate. Treatment of the P1−P2 duplex substrate with Endo IV will result in cleavage of the AP site. The two resulting fragments of P1 will dissociate from P2, and the released G-quadruplex-forming fragment at the 3′-end (black line in Scheme 1) will fold into a G-quadruplex in the presence of K^+ ions. The formation of the G-quadruplex structure is recognized by the G-quadruplex-selective iridium(III) complex with an enhanced luminescence response, allowing the system to function as a switch-on luminescent probe for Endo IV activity.

EXPERIMENTAL SECTION

Materials. Reagents, unless specified, were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received. Iridium chloride hydrate ($IrCl_3{\cdot}xH_2O$) was purchased from Precious Metals Online (Australia). UDG and Endo IV were purchased from New England Biolabs Inc. (Beverly, MA, USA). All oligonucleotides were synthesized by Techdragon Inc. (Hong Kong, China). The oligonucleotides, which represent the various forms of DNA, are indicated as follows. ssDNA, 5′-$C_2AGT_2CGTAGTA_2C_3$-3′. dsDNA: ds26, 5′-$CA_2TCG_2ATCGA_2T_2CGATC_2GAT_2G$-3′ (palindromic sequence); ds17, 5′-$C_2AGT_2CGTAGTA_2C_3$-3′/5′-$G_3T_2ACTACGA_2CTG_2$-3′. G-quadruplex DNA: PS2.M, 5′-$GTG_3TAG_3CG_3T_2G_2$-3′; c-*kit*87up, 5′-$AG_3AG_3CGCTG_3AG_2AG_3$-3′; Pu27, 5′-$TG_4AG_3TG_4AG_3TG_4A_2G_2$-3′; and thrombin binding aptamer (TBA), 5′-$G_2T_2G_2TGTG_2T_2G_2$-3′. P1U, 5′-TATCTGCA-$CUAGTG_3TAG_3CG_3T_2G_2$-3′; P2, 5′-$TC_2TAC_3ACTAGTGCAGAT_2$-3′; mutant P1U lacking uracil bases: $P1_{mut}$, 5′- $TATCTGCACTAGTG_3TAG_3CG_3T_2G_2$-3′.

Synthesis of Complexes. The following complexes were prepared according to modifications of previously reported methods: $[Ir_2(ppy)_4Cl_2]$ (precursor complex),[23] $[Ir(ppy)_2(biq)]PF_6$ (**2**),[23] and $[Ir(ppy)_2(bipy)]PF_6$ (**3**) (where biq = 2,2′-biquinoline and bipy = 2,2′-bipyridine).[38]

Synthesis of $Ir(ppy)_2(bcp)]PF_6$ (**1**): a suspension of the precursor complex (0.2 mmol) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (0.41 mmol) in a mixture of DCM:methanol (1:1, 20 mL) was refluxed overnight under a nitrogen atmosphere. The resulting solution was allowed to cool to room temperature, and was filtered to remove unreacted cyclometalated dimer. An excess of aqueous ammonium hexafluorophosphate was added to the filtrate and the mixture was reduced in volume by rotary evaporation until precipitation of the crude product occurred. The precipitate was then filtered and washed with water (50 mL × 2) followed by diethyl ether (50 mL × 2). The product was recrystallized by acetonitrile: diethyl ether vapor diffusion to yield the titled compound as a yellow solid.

Luminescence Response of Complexes Towards Different forms of DNA. The G-quadruplex DNA-forming sequences (PS2.M, c-*kit*87up, Pu27 and TBA) were annealed in Tris-HCl buffer 1 (20 mM Tris-HCl pH 7.2, 100 mM KCl) and were stored at −20°C before use. ssDNA, dsDNA, or G-quadruplex DNA (5 μM) was mixed with complex (1 μM) in Tris-HCl buffer 1. Emission spectra were recorded using an excitation wavelength of 360 nm.

Detection of Endo IV Activity. The P1U oligonucleotide (100 μM) was mixed with 12.5 units of UDG in 50 μL of 2× reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$). After incubation at 37 °C for 20 min, 50 μL of P2 (100 μM) was added and the mixture was heated to 95 °C for 10 min and then allowed to cool to 25 °C. To 20 μL of this solution was added different concentrations of Endo IV. After incubation at 37 °C for 30 min, 1 μL of 2 M KCl was added, and the volume was adjusted to 100 μL with 1× reaction buffer. The mixture was heated to 95 °C for 10 min and allowed to cool to 25 °C. Next, 399.5 μL of Tris-HCl buffer 1 (20 mM Tris-HCl pH 7.2, 100 mM KCl) was added, along with 0.5 μL of complex **1** (0.5 mM). Emission spectra were recorded in the 450−700 nm range using an excitation wavelength of 360 nm.

RESULTS AND DISCUSSION

The spectroscopic and photophysical characterization of the novel iridium(III) complex **1** are presented in the Supporting Information (Table S1, Figure S1). Iridium(III) complexes **1**−**3** (Figure 1) each contain two ppy *C*^*N* co-ligands, but vary in the nature of the *N*^*N* substituent. Complexes **1** and **2** possess the relatively large bcp or biq *N*^*N* ligand, whereas complex **3** contains the smaller bipy *N*^*N* donor ligand. We hypothesized that larger *N*^*N* ligands might allow the resulting complex to form superior π-stacking interactions with the G-quadruplex tetrads or loops, thus contributing to a stronger luminescence enhancement. We first investigated the luminescence behavior

of iridium(III) complexes **1**−**3** toward different types of DNA. The complexes were weakly emissive in aqueous buffered solution (20 mM Tris, pH 7.2, 100 mM KCl). However, the luminescence of complexes **1** and **2** was significantly enhanced in the presence of the various G-quadruplex DNA sequences (PS2.M, Pu27 and c-*kit*87up). A *ca.* 5-fold enhancement in the luminescence signal of complex **1** or **2** was observed in the presence of 5 μM of PS2.M, Pu27 or c-*kit*87up G-quadruplex DNA (Figure 2 and Figure S2 in the Supporting Information).

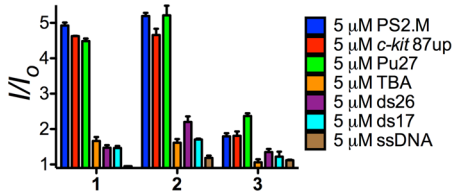


Figure 2. Luminescence enhancement of complex **1**, **2** and **3** (1 μM) in the presence of 5 μM of ssDNA, dsDNA, or various G-quadruplexes.

In contrast, the addition of 5 μM of single-stranded DNA (ssDNA) or double-stranded DNA (ds17, ds26) did not induce significant changes in the luminescence of complexes **1** or **2**. This result indicates that complexes **1** and **2** are able to discriminate G-quadruplex DNA from dsDNA or ssDNA. On the other hand, complex **3** was found to be nonselective for G-quadruplex DNA. The above result suggests that the selectivity of these iridium(III) complexes for the G-quadruplex motif may be fine-tuned by adjustment of the auxiliary *N*^*N* ligand.

Interestingly, complex **1** showed only weak luminescence enhancement in the presence of the TBA G-quadruplex (Figure 2). TBA has previously been shown to readily accommodate planar aromatic ligands, but not ribbon-like molecules.[39] This result suggests that complex **1** may bind outside the G-tetrad for the other G-quadruplexes.[40] To examine the role of the G-quadruplex loops in the binding interaction of complex **1**, we investigated the luminescence response of complex **1** towards various G-quadruplex DNA structures with different loop sizes. The G-quadruplex folding of the sequences utilized in this experiment was extensively validated by Mergny et al.[41] We explored G-quadruplex sequences containing a 5′-side loop (5′-$G_3T_nG_3T_3G_3T_3G_3$-3′), a central loop (5′-$G_3T_3G_3T_nG_3T_3G_3$-3′) or a 3′-side loop (5′-$G_3T_3G_3T_3G_3T_nG_3$-3′), with loop sizes ranging from 1 to 15 nucleotides (n = 1, 2, 3, 4, 5, 6, 7, 9, 12, 15). The results showed that the luminescence intensity of complex **1** generally increased with greater loop size, regardless of the location of the loop (see Figure S3 in the Supporting Information). For the G-quadruplex structures containing a 5′-side loop, complex **1** showed a 2-fold emission enhancement when the loop size was 1 or 2 nt. The luminescence of complex **1** decreased to 1.5-fold with the 3-nt loop, but then increased uniformly to 2.7-fold as the loop size was increased to 15 nt. With G-quadruplexes containing central loops or 3′-side loops, the correlation between G-quadruplex loop size and luminescent enhancement was positive, but nonlinear. The luminescent enhancement of complex **1** increased from 1.0 to 3.1-fold for the central loop, and from 1.0 to 3.0-fold for the 3′-side loop, as the loop size was increased from 1 to 15 nt. Interestingly, the maximal luminescence enhancement for both the central and side loops occurred at a loop size of 12 nt. This result suggests that the G-quadruplex loop may play an important role in the G-quadruplex-complex **1** interaction, which is consistent with previous work by Qu and co-workers who showed that the nature of the loop region could affect the binding interaction between ligands and G-quadruplex DNA.[42]

Complexes **1** and **2** both displayed robust luminescence responses towards G-quadruplex DNA, but complex **1** displayed a superior selectivity for G-quadruplex DNA vs. dsDNA (Figure 3). Encouraged by the superior selectivity of complex **1** for G-quadruplex DNA, we sought to utilize complex **1** for the development of the oligonucleotide-based Endo IV activity detection assay. A preliminary experiment showed that the luminescence signal of complex **1** was significantly enhanced when P1−P2 was incubated with Endo IV. We presume that the luminescence enhancement of complex **1** was due to the release of the G-quadruplex-forming fragment of P1 upon cleavage of the AP site with Endo IV, leading to the subsequent formation of the G-quadruplex structure that interacted strongly with complex **1**. After optimization of the reaction times with UDG and with Endo IV, the pH of the solution, and the concentrations of complex **1**, DNA and KCl (see Figure S4 in the Supporting Information), we performed an emission titration experiment with increasing concentrations of Endo IV. Encouragingly, the luminescence intensity of complex **1** was enhanced as the

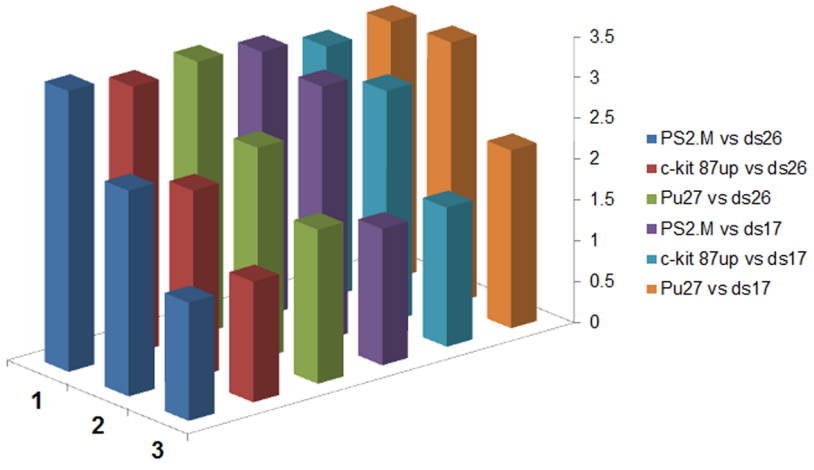


Figure 3. Diagrammatic bar array representation of the luminescence enhancement selectivity ratio of complexes **1**−**3** for G-quadruplex DNA over dsDNA.

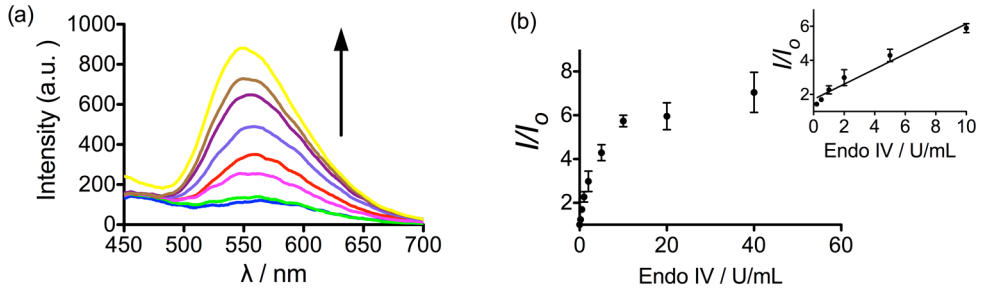


Figure 4. (a) Emission spectrum of the **1**/duplex system ([**1**] = 0.5 μM, [duplex DNA] = 2 μM, reaction temperature of Endo IV = 37°C) in the presence of 0, 0.2, 0.5, 1, 2, 5, 10, and 20 U/mL of Endo IV. (b) Luminescence response of the system vs. Endo IV concentration. Inset: linear regression of the luminescence intensity vs. Endo IV concentration.

concentration of Endo IV was increased. The system exhibited a linear range of detection for Endo IV from 0.2 to 10 U/mL, and the luminescence of the system plateaued at 40 U/mL of Endo IV (Figure 4). This assay was highly sensitive for Endo IV, with a detection limit of 0.2 U/mL using the 3σ method.

To validate the mechanism of our assay, we conducted an emission experiment with Endo IV and complex **1** in the absence of duplex DNA. No enhancement was observed upon addition of 20 U/mL Endo IV (see Figure S5 in the Supporting Information), indicating that the luminescence response of complex **1** was dependent on the presence of duplex DNA substrate. We also analyzed a mutant duplex substrate ($P1_{mut}$–P2) that does not have the potential to form an AP site and be cleaved by Endo IV, as $P1_{mut}$ lacks uracil bases. No significant change in the luminescence intensity of complex **1** was observed when $P1_{mut}$–P2 was incubated with Endo IV (see Figure S6 in the Supporting Information). This result indicates that the luminescence enhancement of the system is dependent on the ability of Endo IV to cleave the AP site in the P1–P2 DNA duplex.

The selectivity of the method for Endo IV was examined by testing the response of the system against various other enzymes, including exonuclease I (Exo I), lambda exonuclease (λ exo), *dam* methyltransferase (dam Meth), T7 exonuclease (T7), exonuclease III (Exo III), and DNase I. A moderate enhancement in the luminescence of complex **1** was observed in the presence of Exo III, which could be attributed to the AP endonuclease property of Exo III (see Figure S7 in the Supporting Information).[43] However, only minor effects on the luminescence of complex **1** were observed in the presence of the other enzymes. Therefore, the assay is selective for Endo IV over the other enzymes tested.

CONCLUSION

In summary, we have employed the novel iridium(III) complex **1** as a G-quadruplex-specific probe for the oligonucleotide-based luminescent switch-on detection of Endo IV activity. This strategy is rapid, simple, sensitive, and convenient and can detect down to 0.2 U/mL of Endo IV without any signal amplification or fluorescent labeling of oligonucleotides. Furthermore, we showed that the G-quadruplex selectivity of these iridium(III) complexes could be tuned by adjustment of the auxiliary ligands, with relatively larger *N^N* ligands conferring greater selectivity.

ASSOCIATED CONTENT

Supporting Information

Additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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