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Detection of 3′ → 5′ exonuclease activity using a metal-based luminescent switch-on probe

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ABSTRACT

A luminescent iridium(III) complex has been discovered to be selective for G-quadruplex DNA, and was employed in a label-free G-quadruplex-based detection assay for 3′ → 5′ exonuclease activity in aqueous solution. A proof-of-concept of this assay has been demonstrated by using prokaryotic exonuclease III (ExoIII) as a model enzyme. In this assay, a G-quadruplex-forming hairpin oligonucleotide (hairpin-G4 DNA, 5′-GAG$_3$TG$_4$AG$_3$TG$_4$A$_2$GCAGA$_2$G$_2$ATA$_2$CT$_2$C$_4$AC$_3$TC$_4$AC$_3$TC-3′) initially exists in a duplex conformation, resulting in a low luminescence signal due to the weak interaction between the iridium(III) complex and duplex DNA. Upon digestion by ExoIII, the guanine-rich sequence is released and folds into a G-quadruplex, which greatly enhances the luminescence emission of the iridium(III) probe. This method was highly sensitive for 3′ → 5′ exonuclease over other DNA-modifying enzymes.

1. Introduction

DNA-modifying enzymes play vital roles in fundamental biological processes such as replication, recombination, repair, and transcription, and are important for maintaining genomic stability and integrity [1–3]. In particular, enzymes containing 3′ → 5′ exonuclease activities are involved in many important biological processes, such as DNA proofreading and repair [4–7]. 3′ → 5′ exonuclease inhibitors have the potential to potentiate the action of DNA-alkylating antitumor drugs by inhibiting DNA repair [7–9]. At the same time, 3′ → 5′ exonucleases have been utilized to provide signal amplification in oligonucleotide-based sensing platforms for the detection of various analytes [10–12]. Radioactive labeling in conjunction with gel electrophoresis is the most commonly-used technique for assaying 3′ → 5′ exonuclease activity [13,14]. However, these protocols are generally discontinuous, time-consuming, require multiple steps, and necessitate the use of stringent safety procedures to control radiographic exposure. Consequently, it is desirable to develop efficient strategies to assay 3′ → 5′ exonuclease activity, which could facilitate the screening of modulators of such enzymes as potential drugs and biochemical tools.

DNA oligonucleotides have attracted tremendous interest for the construction of sensing platforms due to their low cost, ease of synthesis, high solubility, biocompatibility and stability in aqueous solution and biological media [15–37]. In particular, G-quadruplexes have attracted intense attention for the development of various analytical assays. The G-quadruplex is a non-canonical DNA secondary structure that consists of planar stacks of four guanines stabilized by Hoogsteen hydrogen bonding [38–40]. G-quadruplexes show a rich diversity in structural topologies that can be sensitive to several factors, such as base sequence, loop connectivity, or cations in solution [41]. The extensive structural polymorphism of G-quadruplexes has rendered them as versatile biological sensing elements for the construction of colorimetric, chemiluminescent, or fluorescent DNA-based sensing platform for metal ions, small molecules, and biomolecules [42–44]. In recent years, a number of luminescent DNA-based assays for 3′ → 5′ exonuclease activity have been reported. For example, our group has developed a label-free, G-quadruplex-based switch-on fluorescence assay for 3′ → 5′ exonuclease activity by using the organic dye crystal violet as G-quadruplex-binding probe [45]. Zhao and co-workers reported a fluorescence assay for monitoring 3′ → 5′ exonuclease activity in living cells by employing a photoinduced electron transfer process between stacked guanine bases and a fluorescent probe [46]. Min and co-worker developed a fluorescence assay for the detection of 3′ → 5′ exonuclease activity by

Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ctDNA, calf-thymus DNA; ExoIII, exonuclease III; T7 Exo, T7 exonuclease; λ Exo, lambda exonuclease; T4 PNK, T4 polynucleotide kinase; UDG, uracil-DNA glycosylase; BSA, bovine serum albumin.

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using the graphene oxide and a singly-labeled oligonucleotide substrate [47]. These reports demonstrate that DNA oligonucleotides can be integrated as useful functional and structural elements for the construction of sensitive detection platforms for 3′ → 5′ exonuclease activity.

Meanwhile, luminescent transition metal complexes have found use in various chemical and biological sensors in view of their useful photophysical properties such as, (i) tunable excitation and emission maxima over the visible region; (ii) large Stokes shift for facile separation of excitation and emission wavelengths and elimination of self-quenching; and (iii) relatively long phosphorescent lifetimes that can be distinguished from a short-lived autofluorescence background through the use of time-resolved spectroscopy [48–52]. Luminescent transition metal complexes have been used to detect DNA [53], RNA [54], protein [55], small molecules [56], and metal ions [57]. For example, the group of Zhang utilized the "molecular light switch" complex $[Ru(phen)_2(dppz)]^{2+}$ (where phen = phenanthroline and dppz = dipyrido[3,2-a:2',3'-c]phenazine) for mercury ion detection [58].

Our previous 3′ → 5′ exonuclease activity assay was limited by the significant affinity of crystal violet for duplex DNA [59,60], which led to a high fluorescent background signal in the absence of enzyme. We envisaged that the conformational change of oligonucleotides induced by 3′ → 5′ exonuclease could be more effectively monitored by luminescent transition metal complexes displaying a greater selectivity for the G-quadruplex motif over other DNA conformations. In this work, the luminescent cyclometallated iridium(III) complex $[Ir(phq)_2(BPhen)]^+$ (**1**, where phq = 2-phenylquinoline and BPhen = bathophenanthroline, Fig. 1) was discovered to be selective for G-quadruplex DNA. We therefore employed complex **1** to develop a "mix-and-detect" assay for 3′ → 5′ exonuclease activity that is simple, convenient, label-free, and that does not require expensive instrumentation or extensive sample preparation.

2. Materials and methods

2.1. Chemicals and materials

Reagents were purchased from Sigma–Aldrich (St. Louis, MO) and used as received. Iridium chloride hydrate ($IrCl_3{\cdot}xH_2O$) was purchased from Precious Metals Online (Australia). All oligonucleotides were synthesized by Techdragon Inc. (Hong Kong, China) (Table 1).

2.2. General experimental

Mass spectrometry was performed at the Mass Spectroscopy Unit at the Department of Chemistry, Hong Kong Baptist University, Hong Kong (China). Melting points were determined using a Gallenkamp melting apparatus and are uncorrected. Deuterated solvents for NMR purposes were obtained from Armar and used as received.

^1H and ^{13}C NMR were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz (^1H) and 100 MHz (^{13}C). ^1H and ^{13}C chemical shifts were referenced internally to solvent shift (CD_3CN: ^1H δ 1.94, ^{13}C δ 118.7; d_6-DMSO: ^1H δ 2.50, ^{13}C δ 39.5). Chemical shifts (δ) are quoted in ppm, the downfield direction being defined as positive. Uncertainties in chemical shifts are typically ±0.01 ppm for ^1H and ±0.05 for ^{13}C. Coupling constants are typically ±0.1 Hz for ^1H–^1H and ±0.5 Hz for ^1H–^{13}C couplings. The following abbreviations are used for convenience in reporting the multiplicity of NMR resonances: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. All NMR data was acquired and processed using standard Bruker software (Topspin).

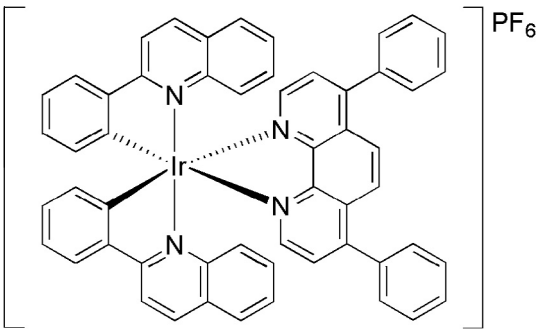

Fig. 1. Chemical structure of the cyclometallated luminescent iridium(III) complex **1**.

Table 1
DNA sequences.

	Sequence
Hairpin-G4 DNA	5-$GAG_3TG_4AG_3TG_4A_2GCAGA_2G_2ATA_2CT_2C_4AC_3TC_4AC_3TC$-3
PS2.M	5-$GTG_3TAG_3CG_3T_2G_2$-3
ss DNA	5-$GA_3T_2CT_2A_2GTGCGATCGAG$-3
Pu27	5-$TG_4AG_3TG_4AG_3TG_4A_2G_2$-3
Pu22	5-$GAG_3TG_4AG_3TG_4A_2G$-3
Hairpin-G4 DNA_{m1}	5-$T_2AGT_3GTA_4GCAGA_2G_2ATA_2CT_4ACA_3CTA_2$-3

2.3. Synthesis

$[Ir(phq)_2(BPhen)]PF_6$ was synthesized according to the reported literature method [61] and was characterized by ^1H NMR, ^{13}C NMR and high resolution mass spectrometry (HRMS).

$[Ir(phq)_2(BPhen)]PF_6$ **(1)**. A suspension of $[Ir_2(ppyr)_4Cl_2]$ (0.2 mmol) and bathophenanthroline (0.44 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 filtered to remove unreacted dimer. To the filtrate was added an aqueous solution of ammonium hexafluorophosphate in excess, and the filtrate was reduced in volume by rotary evaporation until precipitation of the crude product occurred. The precipitate was then filtered and washed with several portions of water (2 × 50 ml) followed by diethyl ether (2 × 50 ml). The product was recrystallized by acetonitrile:diethyl ether vapor diffusion to yield the titled compound as an orange solid.

Yield: 67%. ^1H NMR (400 MHz, CD_3CN): 8.61 (d, J = 8.0 Hz, 2H), 8.37 (q, J = 8.0 Hz, 4H), 8.22 (d, J = 8.0 Hz, 2H), 7.86 (s, 2H), 7.78 (d, J = 8.0 Hz, 2H), 7.75 (d, J = 8.0 Hz, 2H), 7.58–7.55 (m, 6H), 7.47–7.44 (m, 4H), 7.33–7.22 (m, 6H), 6.94–6.86 (m, 4H), 6.67 (d, J = 8.0 Hz, 2H); ^{13}C NMR (400 MHz, CD_3CN): 171.6, 152.6, 151.9, 149.8, 149.1, 148.6, 147.7, 141.5, 137.0, 136.0, 132.1, 131.9, 131.1, 130.5, 130.4, 129.9, 129.2, 128.9, 128.4, 128.1, 127.0, 125.6, 124.4, 119.5; MALDI-TOF-HRMS: Calcd for $C_{54}H_{36}IrN_4[M-PF_6]^+$: 933.2568. Found: 933.2606. Anal. Calcd for $C_{54}H_{40}F_6IrN_4O_2P$: C, 58.22; H, 3.62, N, 5.03. Found: C, 58.47; H, 3.50; N, 5.09.

2.4. Emission response of *1* toward different forms of DNA

The G-quadruplex-forming sequences (PS2.M, Pu22, and Pu27) were annealed in Tris–HCl buffer (20 mM Tris, 100 mM KCl, pH 7.0) and were stored at −20 °C before use. Complex **1** (1 μM) was added to 5 μM of ssDNA, ctDNA or G-quadruplex DNA (PS2.M, Pu22, and Pu27) in Tris–HCl buffer (20 mM Tris, pH 7.0). Emission spectra were recorded in 510–700 nm range using an excitation wavelength of 360 nm.