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An oligonucleotide-based label-free luminescent switch-on probe for RNA detection utilizing a G-quadruplex-selective iridium(III) complex†

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We report herein the synthesis and application of a novel G-quadruplex-selective luminescent iridium(III) complex for the construction of an oligonucleotide-based, label-free, rapid and convenient luminescent RNA detection platform.

Ribonucleic acid (RNA) plays fundamental roles in transcription and translation processes in living cells. mRNA is responsible for the delivery of genetic information from the nucleus to the ribosomes of the cell, where it is translated into proteins. A special type of noncoding RNA, microRNA, is involved in the transcriptional and post-transcriptional regulation of gene expression, via hybridisation with complementary mRNA molecules. The abnormal expression or mutation of various types of RNA has been associated with a range of human diseases, such as cancer, mitochondrial disease, muscle disease and neurodegenerative disease.1-6 Commonly-used detection methods for RNA include Northern blotting,7 microarray technology8 and real-time polymerase chain reaction (RT-PCR).9, 10 However, these methods tend to be unwieldy, labor-intensive, time-consuming, costly or may necessitate stringent safety measures to control radiographic exposure. Therefore, the development of rapid, simple and low-cost detection platforms for RNA detection is highly desirable. In recent years, DNA-based techniques utilising luminescent,11-18 colorimetric19 or electrochemical20 signals for the construction of RNA detection platforms have been reported.21

The G-quadruplex is a DNA secondary structure consisting of square-planar arrangements of guanine nucleobases stabilized by Hoogsteen hydrogen bonding and monovalent cations.22-25 Since the rich structural polymorphism of the G-quadruplex motif,26-28 this structure widely act as a versatile signal transducing element in the construction of DNA-based detection platforms for metal ions,29 DNA,30-33 small molecules,34-36 protein,37, 38 and enzyme activity.39-42 Meanwhile, luminescent heavy metal complexes have attracted tremendous interest in sensory applications33-47 due to their following advantages: (i) their long-lived phosphorescence allows them to be distinguished in highly fluorescent environments with the use of time-resolved spectroscopy, (ii) their large Stokes shifts can prevent self-quenching, and (iii) their modular synthesis allows their photophysical and molecular recognition properties to be easily tuned without labour-intensive synthetic protocols.48-51

Fig. 1 Chemical structures of cyclometallated iridium(III) complexes 1, 2 and 3.

We report herein the synthesis and application of a novel G-quadruplex-selective luminescent iridium(III) complex 1 [Ir(ptpy)(dmph)]PF6 (where ptpy = 2-(p-tolyl)pyridine; dmph = 2,9-dimethyl-1,10-phenanthroline) for the sensitive detection of RNA. The mechanism of this assay is depicted in Scheme 1. Two oligonucleotides, P1 and P2, each contain a split G-quadruplex-forming sequence (black line) and a complementary sequence (blue line) that hybridizes with the target RNA (orange line). Upon addition of the target RNA, the two split G-quadruplex sequences will be brought into close proximity, and an intermolecular G-quadruplex can be formed. 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 RNA.

The spectroscopic and photophysical characterization of the novel iridium(III) complex 1 are presented in the Electronic Supporting Information (Table S1, Fig. S1 and S2). We first investigated the luminescence behavior of the iridium(III) complex 1 towards different types of DNA. Complex 1 was weakly emissive in aqueous buffered solution. Encouragingly, the luminescence of complex 1 was significantly enhanced in the presence of various G-quadruplex DNA sequences (PS2.M, Pu27 and Pu22). A ca. 5.5-fold enhancement in the luminescence of complex 1 was observed in the presence of 5 µM of PS2.M, while ca. 4-fold enhancements were observed for Pu27 or Pu22 G-quadruplex DNA. In contrast, no significant changes in the luminescence of complex 1 was observed upon the addition of 5 µM of ssDNA, dsDNA or DNA/RNA hybrid duplex (Fig. 2a and 2b).
Fig. 2 (a) Emission spectrum of complex 1 (1 μM) in the presence of 5 μM of ssDNA, ctDNA or various G-quadruplexes. (b) Luminescence enhancement of complex 1 (1 μM) in the presence of 5 μM of ssDNA, dsDNA or various G-quadruplexes. (c) Luminescence enhancement of complexes towards intermolecular G-quadruplex. (d) G4-FID titration curves of DNA duplexes (ds17 and ds26) or G-quadruplexes (Pu27, and c-kit87up). (e) Inhibition activity of complex 1 (1 or 10 μM) on the peroxidase activity of G-quadruplex/hemin complexes (1 μM of G-quadruplex, 1 μM of hemin, 2 mM of ABTS, and 2 mM of H2O2).

We also compared the luminescence response of complex 1 towards the G-quadruplex formed by the association of P1 and P2 in the presence of the target RNA, with two other G-quadruplex-selective iridium(III) complexes (2 and 3). We found that complex 1 showed the highest luminescence response to the intermolecular G-quadruplex out of all the complexes tested, suggesting that complex 1 is the ideal choice for this sensing platform (Fig. 2c). To further validate the suitability of complex 1 as a G-quadruplex-selective probe, G-quadruplex fluorescent intercalator displacement (G4-FID) and fluorescence resonance energy transfer (FRET) melting assays were employed to evaluate the binding affinity and selectivity of complex 1 for G-quadruplex DNA. Although the emission region of thiazole orange (TO) (510−750 nm) and complex 1 (500−700 nm) overlap, complex 1 is not expected to interfere with the excitation wavelength of TO (501 nm). The results of the G4-FID assay indicated that complex 1 could displace TO from G-quadruplex structures with G4-DC90 values (half-maximal concentration of compound required to displace 50% TO from DNA) of < 9 μM, while the displacement of TO from dsDNA was less than 50% even at the highest concentration of 1 tested (Fig. 2d). The selective binding of complex 1 towards G-quadruplex DNA was further analysed by FRET-melting assays. The melting temperature (ΔT_m) of the F21T G-quadruplex was increased by about 6 °C upon the addition of complex 1 (Fig. 3a). By comparison, no significant change in the melting temperature of F10T dsDNA was observed at the same concentration of complex 1 (Fig. 3b). Additionally, the stabilization effect of complex 1 towards the F21T G-quadruplex was not significantly perturbed by the addition of 50-fold higher concentration of unlabeled competitor dsDNA (ds26) or ssDNA (Fig. 3c). This indicates that complex 1 binds selectively to G-quadruplex DNA over dsDNA or ssDNA. However, the addition of a 50-fold excess of an unlabeled G-quadruplex competitor Pu27 abrogated the stabilizing effect of complex 1 on the F21T G-quadruplex, as it is able to sequester complex 1 and prevent its interaction with the labeled G-quadruplex (Fig. 3d). Taken together, these results demonstrate the ability of complex 1 to discriminate between G-quadruplex DNA and dsDNA or ssDNA.

Interestingly, complex 1 showed only weak luminescence enhancement in the presence of the thrombin-binding aptamer (TBA) G-quadruplex (Fig. 2a and 2b). TBA has previously been shown to readily accommodate planar aromatic ligands, but not ribbon-like molecules. This result suggests that complex 1 may bind outside the G-tetrad for the other G-quadruplexes. In order to explore whether complex 1 mainly bind to the G-quadruplex structure by end-stacking, we investigated the ability of complex 1 to inhibit the peroxidase activity of the G4/hemin ensemble. Hemin has been described as an end-stacking binding ligand that forms a peroxidase-like DNAzyme upon binding to G-quadruplex DNA. Therefore, ligands that also bind to the termini of the G-quadruplex motif will compete with hemin, and thereby decrease the generation of the colored product generated by the DNAzyme-catalysed oxidation of ABTS2−.
results showed that the peroxidase activity of the G4/hemin complex was only slightly decreased by the addition of up to 10 µM of complex 1 (Fig. 2e). This suggests that the end-stacking is not the predominant interaction between complex 1 and G-quadruplex DNA. The ability of complex 1 to displace TO but not hemin from G-quadruplex DNA could be due to the fact that the porphyrin-like hemin is a strong G-quadruplex binding ligand.\(^{56}\) Thus, complex 1, which exhibited a \( {^{19}DC} \) value of about 4–8 µM against TO in the G4-FID assay, may not have been able to displace hemin under the conditions used in the experiment. Alternatively, TO may bind to the G-quadruplex at multiple sites, as has been reported for the human telomeric G-quadruplex \((5^'G_3T_2G_2)_n3^'\) by Teulade-Fichou, Mergny and co-workers.\(^{55}\) Therefore, complex 1 may have been able to displace TO from different binding sites on the G-quadruplex, while not being an avid enough ligand to dislodge the strong end-stacking ligand hemin from the terminal G-tetrad. 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 topologies of the sequences utilized in this experiment were validated by Mergny et al.\(^{57}\) We explored G-quadruplex sequences containing a 5'-side loop \((5'^'G_5T_7G_5T_7G_6T_2G_23'^')\), a central loop \((5'^'G_5T_7G_5T_7G_5G_33'^')\) or a 3'-side loop \((5'^'G_3T_2G_2G_5T_7G_5G_33'^')\), 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 (Fig. 4). The luminescence enhancement of complex 1 increased from 2.0 to 6.5-fold for the 5'-side loop and central loop, and from 2.0 to 3.5-fold for the 3'-side loop, as the loop size increased from 1 to 15 nt. Interestingly, the maximal luminescence enhancement for both the central and side loops occurred at a loop size of about 9 to 12 nt. This result suggests that the G-quadruplex loop may play an important role in the G-quadruplex-complex 1 interaction, and 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.\(^{58}\) Encouraged by the superior selectivity of complex 1 for G-quadruplex DNA, we sought to utilize complex 1 for the development of the G-quadruplex-based RNA detection assay. A preliminary experiment showed that the luminescence of complex 1 was significantly enhanced when P1-P2 was annealed with the target RNA sequence. We presume that the luminescence enhancement of complex 1 was due to the formation of the DNA/RNA hybrid that brings the intermolecular G-quadruplex sequences into close proximity, leading to the subsequent formation of the split G-quadruplex structure that interacts strongly with complex 1. After optimization of the pH, concentration of complex 1 and KCl (Fig. 5a–c), we performed an emission titration experiment with increasing concentrations of target RNA. Encouragingly, the luminescence intensity of complex 1 was enhanced as the concentration of target RNA was increased (Fig. 6). The system exhibited a linear range of detection for RNA from 25 to 200 nM, with a maximal luminescence reached at 1 µM of the target RNA. Although the assay showed a modest 5-fold maximal luminescence enhancement at saturating RNA concentrations, it was highly sensitive for RNA, with a detection limit of 25 nM using the 3σ method. To validate the mechanism of our sensing platform, we utilized mutant split G-quadruplex sequences \((P1_{mut} and P2_{mut})\) that lack consecutive guanine bases, and are thus unable to fold into a G-quadruplex upon hybridization to the target RNA. No significant change in the luminescence of complex 1 was observed when \(P1_{mut} and P2_{mut}\) were incubated with the target RNA (Fig. 5d). We also conducted an emission
No enhancement was observed upon addition of 2 µM of target RNA (Fig. 5e), indicating that the luminescence response of corresponding DNA sequence, the system showed strong high luminescence enhancement was observed with the substitutions, or with random coil RNA. Although a relatively negligible effects on the luminescence of complex without any signal amplification or fluorescent labeling of oligonucleotides. It can detect down to 25 nM of target RNA without any signal amplification or fluorescent labeling of oligonucleotides and can effectively discriminate between RNA sequences differing by a single base.

In conclusion, we have synthesized a novel G-quadruplex-selective iridium(III) complex and have explored its utility for the construction of the first metal and G-quadruplex-based luminescent assay for RNA detection. Our “mix-and-detect” sensing platform is rapid, simple, convenient to use, cost-effective and does not require the covalently labeling of oligonucleotides. It can detect down to 25 nM of target RNA without any signal amplification or fluorescent labeling of oligonucleotides and can effectively discriminate between RNA sequences differing by a single base.

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Notes and references
