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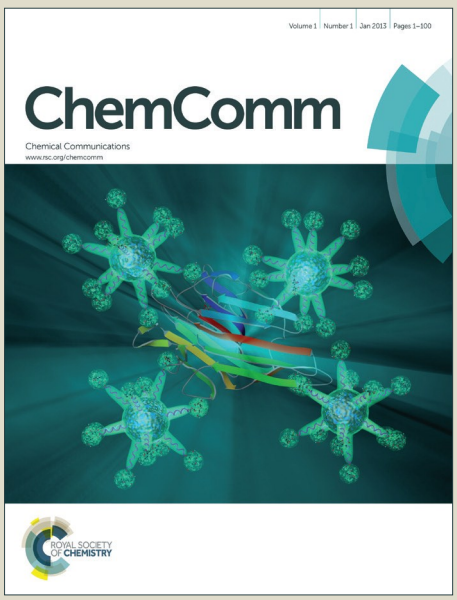

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Luminescence switch-on assay of interferon-gamma using a G-quadruplex-selective iridium(III) complex

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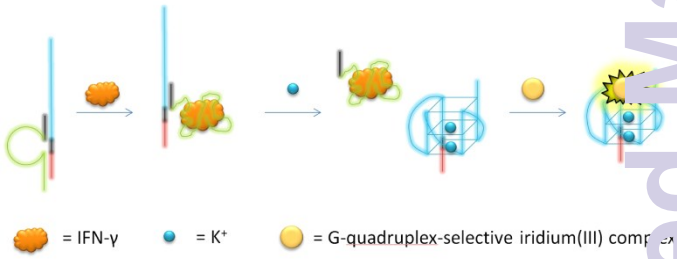
In this study, we synthesized a series of 9 luminescent iridium(III) complexes and studied their ability to function as luminescent probes for G-quadruplex DNA. The iridium(III) complex 8 $[Ir(pbtz)_2(dtbpy)]PF_6$ (where pbtz = 2-phenylbenzo[*d*]thiazole; dtbpy = 4,4'-di-*tert*-butyl-2,2'-bipyridine) showed high selectivity for G-quadruplex DNA over single-stranded and double-stranded DNA, and was subsequently utilized to for the development of a label-free oligonucleotide-based assay for interferon-gamma (IFN-γ), an important biomarker for a range of immune and infectious diseases, in aqueous solution. We further demonstrated that this assay could monitor IFN-γ levels even in the presence of cellular debris. This assay represents the first G-quadruplex-based assay for IFN-γ detection described in the literature.

A biomarker is a measurable indicator of biological states or diseases. As one kind of biomarker, interferon-gamma (IFN-γ) is an important inflammatory cytokine that is released by immune cells such as T-helper ($CD4^+$) cells and cytotoxic T-lymphocytes [1] in response to invading pathogens,[2] and is involved in the regulation of differentiation, proliferation, and immunity.[3] Consequently, the level of IFN-γ is highly related to various diseases such as viraemia, acquired immune deficiency syndrome (AIDS) and latent tuberculosis.[4] Thus, the detection and quantification of IFN-γ can be used to investigate the roles of immune cells and to assess the vigor of the immune response.[5]

At present, antibody (Ab)-based immunoassays are regarded as the standard for cytokine detection.[6] While several approaches for the optimization and miniaturization of Ab-based cytokine immunoassays have been explored in the literature,[7] these strategies are still limited by the relatively high cost and low stability of Abs. Moreover, Ab-based immunoassays tend to involve multiple washing steps, which reduces their utility in monitoring dynamically changing cytokine levels.[8]



Scheme 1. Schematic representation of the G-quadruplex-based luminescence sensing platform for IFN-γ detection. The green line represents the IFN-γ-binding aptamer sequence.

The SELEX (Systematic Evolution of Ligands by Exponential Enrichment) strategy [9] is a method to discover suitable aptamers for small molecules, proteins, metal ions and even cancer cells.[10] Aptamers are nucleic acid molecules that bind to their target species with high affinities and selectivities.[11] Consequently, DNA is an attractive element for the construction of sensing platforms due to its facile synthesis and easy modification,[12] and DNA-based assays have received increasing attention as viable alternatives to Ab-based immunoassays.[13] Using the previously described IFN-γ-binding aptamer,[14] Rezvin and co-workers have developed IFN-γ assays using fluorescence resonance energy transfer (FRET) and electrochemical methods.[15] Meanwhile, the G-quadruplex is a non-canonical DNA motif which is formed by guanine-rich sequences under the stabilization of monovalent cations such as potassium ions. The G-quadruplex structure contains planar stacks of guanine tetrads stabilized by Hoogsteen-type hydrogen bonding.[16] Due to its fascinating structural diversity, the G-quadruplex structure has found extensive use for the development of analytical assays.[17]

Organic fluorophores have been widely investigated as probes for biomolecules, and many of these have been used in imaging or oligonucleotide-based sensing applications. However, their application is still limited by their short lifetimes, which reduces their utility in highly auto-fluorescence backgrounds. To circumvent this issue, pioneering researchers have developed long-lifetime phosphorescent transition metal complexes as luminescent probes.[17, 18] Transition metal complexes also benefit from simple synthetic protocols that allow their photophysical properties to be easily tuned. Furthermore, transition metal complexes possess large Stokes shift values which help to avoid self-quenching. Among the reported luminescent transition metal complexes, some iridium(III) and ruthenium(II) complexes have been demonstrated to possess selectivity towards G-quadruplex over other DNA structures.[19] Compared to the relatively narrow range of emission wavelengths of

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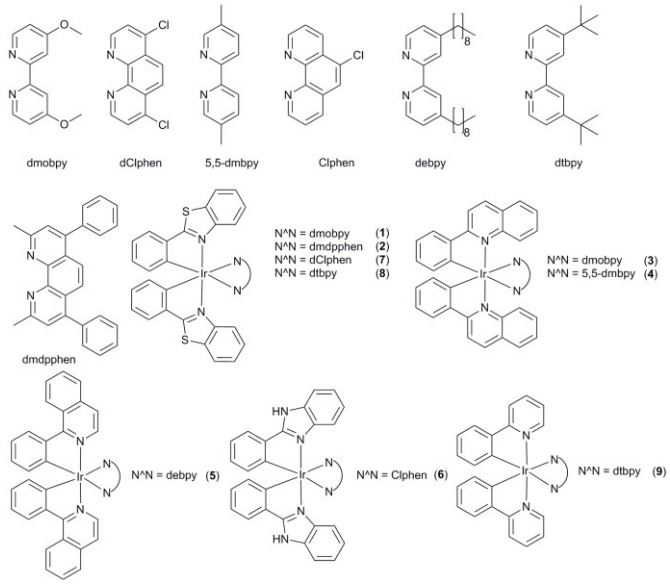

Fig. 1 Chemical structures of cyclometallated iridium(III) complexes **1**–**9**.

ruthenium(II) complexes, iridium(III) complexes can be fine-tuned to display different emission colors, from green to red, by modification of the auxiliary ligands. Additionally, iridium(III) complexes usually have longer lifetimes with higher relative quantum yields.[20] And in recent years, different iridium(III) complexes have been used for the detection of various substances including metal ions, small molecules, enzymes and proteins.[19d, 20b] However, the application of iridium(III) complexes for the detection of disease-related protein biomarkers has been rarely explored. Therefore, we present a luminescent switch-on detection platform for IFN-γ by using a G-quadruplex-selective iridium(III) complex from a focused library of 9 iridium(III) complexes. As far as we know, the application of iridium(III) complex for the detection of IFN-γ has not yet been reported in the literature.

The principle of the present G-quadruplex-based assay for IFN-γ is depicted schematically in Scheme 1. In the initial state, the IFN-γ-binding aptamer sequence (green line, **D**) is partially hybridized with a G-rich sequence (blue and red line, **L**), generating a double-stranded DNA structure with a loop region. The addition of IFN-γ induces a structural transition in the aptamer sequence due to the formation of the IFN-γ–aptamer complex. This unmasks the G-quadruplex-forming sequence, which folds into a G-quadruplex structure in the presence of K^+ ions. The nascent G-quadruplex structure is subsequently bound by the G-quadruplex-selective iridium(III) complex, resulting in a "switch-on" luminescent response to IFN-γ.

We initially tested the selectivity of six iridium(III) complexes (**1**–**6**, Fig. 1) for binding to c-kit87 G-quadruplex DNA over double-stranded DNA (ds26) and single-stranded DNA (ssDNA). The top candidate from the primary screen was the cyclometallated iridium(III) complex **2**, which contains the 2-phenylbenzo[*d*]thiazole (pbtz) C^N ligand and the 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (dmdpphen) N^N ligand, as it possessed $I_{c\text{-}kit87}/I_{ds26}$ and $I_{c\text{-}kit87}/I_{ssDNA}$ luminescent intensity ratios of 2.99 and 1.84, respectively (Fig. S1a). We then used the structure of complex **2** to design a concentrated library of 3 other iridium(III) complexes that were enriched in the beneficial motifs for G-quadruplex-binding as revealed from the primary screening (**7**–**9**, Fig. 1). Complexes **1**, **7**, **8** bear the same pbtz C^N ligand as complex **2**, but differ in their phenanthroline-based or bipyridine-based N^N ligands. Meanwhile, complex **9** carry the same dtbpy N^N ligand as **2**, but possess different C^N ligands. In the secondary screening campaign, the novel iridium(III) complex **8** (Fig. 2a) showed the greatest selectivity for G-quadruplex DNA over dsDNA and ssDNA (Fig. S1b). Structurally, complex **8** which bears the pbtz C^N ligand and the 4,4'-di-tert-butyl-2,2'-bipyridine (dtbpy) N^N ligand.

Preliminary structure-activity relationships can be derived from comparing the DNA-binding behaviors of these iridium(III) complexes. Since complexes **1**, **2** and **7** all possess the same pbtz C^N ligand, the superior G-quadruplex-selectivity of complex **8** could be due to the dtbpy N^N ligand that it carries. Interestingly, although the N^N ligands of complexes **1**, **5** and **8** all share the same basic 2,2'-bipyridine (bpy) framework, the superior performance of **8** suggests that the t-butyl substituents at the 4 and 4' positions of the bpy scaffold are important for G-quadruplex affinity, possibly due to their ability to facilitate additional contact interactions with the loop or groove regions of the G-quadruplex.[21] Among the complexes in the focused library, although **9** bears the same dtbpy N^N ligand as **8**, its lower G-quadruplex-binding selectivity suggests that the pbtz C^N ligand of **8** is superior to the 2-phenylpyridine C^N ligand carried by complex **9**.

Out of all of the complexes tested, complex **8** showed the highest selectivity for G-quadruplex DNA over ssDNA and dsDNA. The luminescence of complex **8** was increased by *ca.* 6.0-fold enhancement in the presence of 5 μM of c-kit87 G-quadruplex DNA (Fig. 2b). Complex **8** was also selective for G-quadruplex DNA over the natural calf-thymus duplex DNA (ct-DNA, Fig. S2).[22]

We further utilized fluorescence resonance energy transfer (FRET) melting and G-quadruplex fluorescent intercalator displacement (G4-FID) assays study the G-quadruplex-selectivity of complex **8**. In the G4-FID assay, complex **8** displaced thiazole orange (TO) from c-kit87 G-quadruplex DNA with a $^{G4}DC_{50}$ value of 5.5 μM, while less than 30% was displayed from dsDNA even at 7.0 μM of **8** (Fig. 2c). In FRET-melting assays, 3 μM of complex **8** increased the melting temperature (ΔT_m) of the F21T G-quadruplex by about 6.0 ℃ (Fig. 2d), whereas the melting temperature of F10T dsDNA was increased by only 1.5 ℃ increase under the same conditions (Fig. 2e). Finally, the ability of complex **8** to

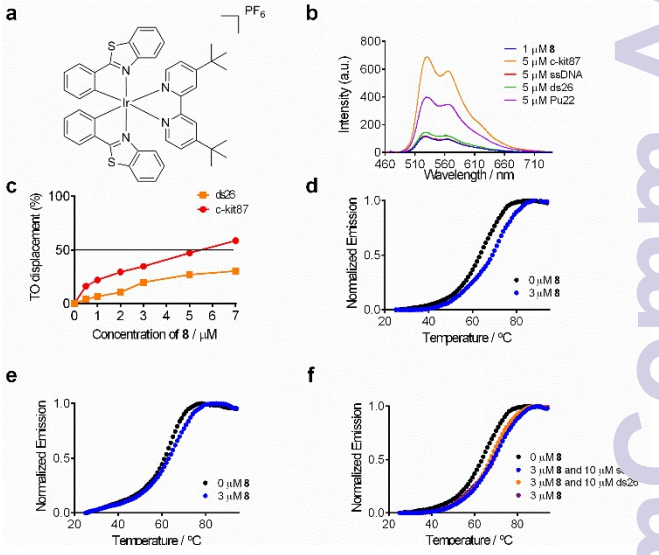

Fig. 2 (a) Chemical structure of complex **8**. (b) Emission spectrum of complex **8** (1.0 μM) in the presence of 5 μM of ssDNA, ds26 or various G-quadruplexes. (c) G4-FID titration curves of complex **8** with duplex (ds26) or G-quadruplex (c-kit87) DNA. (d) Melting profile of F21T G-quadruplex DNA (0.2 μM) in the absence and presence of **8** (3 μM). (e) Melting profile of F10T dsDNA (0.2 μM) in the absence and presence of **8** (3 μM). (f) Melting profile of F21T G-quadruplex DNA (0.2 μM) in the absence and presence of **8** (3 μM) and ds26 (10 μM) or ssDNA (10 μM).

stabilize the F21T G-quadruplex was unaffected by the addition of 50-fold higher concentrations of unlabeled dsDNA (ds26) or ssDNA (Fig. 2f). In summary, complex **8** is able to selectively discriminate G-quadruplex DNA from dsDNA or ssDNA.

We next investigated the interaction of complex **8** towards G-quadruplex DNA structures with different loop sizes[23] using a competitive FRET-melting assay in order to understand the role of the loop regions in the binding of complex **8** to G-quadruplex DNA. The results showed that the highest increases in melting temperature induced by complex **8** occurred for G-quadruplexes bearing a 3-nucleotide 5'-side loop, central loop or 3'-side loop (Fig. S3). The observation that the change in melting temperature is loop size and location-dependent indicates that complex **8** may make significant interactions with the G-quadruplex loop regions and is consistent with previous studies.[24]

Based on the demonstrated selectivity of complex **8** for G-quadruplex DNA, we harnessed complex **8** as a G-quadruplex-selective probe for the luminescent detection of IFN-γ using the assay mechanism depicted in Scheme 1. In the absence of IFN-γ, the G-quadruplex-forming sequence is hybridized to its complementary sequence, forming a duplex substrate that interacts only weakly with complex **8**. However, in the presence of IFN-γ, the emission intensity of **8** was increased by *ca.* 6.0-fold (Fig. 3a). We presume that the increase in luminescence of complex **8** is due to the binding of nascent G-quadruplex structure that is produced following the formation of the IFN-γ–aptamer complex. We also performed a control experiment that utilized a mutant G-quadruplex sequence that is unable to fold into a G-quadruplex. No luminescent enhancement was observed in response to IFN-γ when the mutant sequence was used (Fig. 3a), indicating that the **8**-G-quadruplex interaction is critical for the functioning of the IFN-γ assay. We also conducted circular dichroism (CD) spectroscopy to verify the conformational transition of DNA in response to IFN-γ. Upon addition of IFN-γ, a positive peak at 265 nm and negative peak at 240 nm appeared in the CD spectrum (Fig. 3b), which are consistent with the formation of a parallel G-quadruplex structure. Finally, the luminescent intensity of complex **8** was not enhanced by IFN-γ when DNA was absent, indicating that complex **8** does not interact directly with IFN-γ (Fig. 3c).

In order to achieve the best performance of the assay, various experimental conditions are then optimized, including the concentrations of DNA and complex **8**, incubation temperature, and incubation time (Fig. S4). Under the optimized conditions, the luminescence signal of **8** was enhanced as the concentration of IFN-γ was increased (Fig. 4a). Using the 3σ method, the

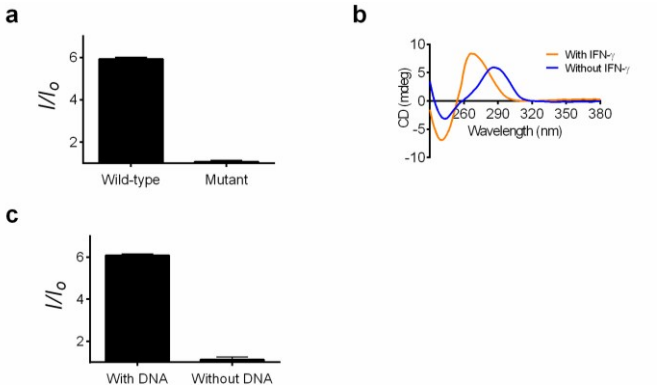

Fig. 3 (a) Luminescence of the system in the presence of wild-type and mutant G-quadruplex DNA. (b) CD spectra with and without 10 nM IFN-γ. (c) Luminescence enhancement of the system in response to IFN-γ (10 nM) in the presence or absence of hairpin DNA. Unless otherwise stated, the concentration of complex **8** was 1.0 μM, the concentration of DNA was 0.14 μM, and Tris buffer (10 mM Tris, pH 7.2) was used.

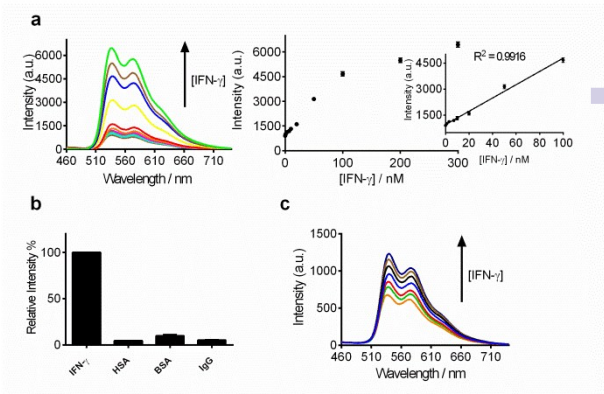

Fig. 4 (a) Luminescence spectra and the relationship between luminescence intensity of the **8**/G4-quadruplex system at λ = 532 nm in response to various concentrations of IFN-γ: 0, 1, 3, 7, 10, 20, 50, 100, 200 and 300 nM. Inset: linear plot of the change in luminescence intensity at λ = 532 nm vs. IFN-γ concentration. (b) Relative luminescence intensity of the system ([**8**] = 1.0 μM, [DNA] = 0.14 μM) in the presence of 10 nM or 5-fold excess of other proteins. (c) Luminescence spectra of the **8**/G-quadruplex system in a reaction system containing 0.5% (v/v) cell extract in response to various concentrations of IFN-γ: 0, 1, 3, 7, 10, 20 and 50 nM.

detection limit of this assay was determined to be 0.12 nM, while a linear relationship between the luminescent intensity and concentration of IFN-γ can be established from 1 to 100 nM with the dynamic detection range from 1 to 300 nM.

The selectivity of this assay for IFN-γ is over other proteins such as human serum albumin (HSA), bovine serum albumin (BSA) and immunoglobulin G (IgG). The luminescence enhancement of the assay in response to IFN-γ was considerably higher compared to 5-fold excess amounts of the other proteins (Fig. 4b).

The ability of our IFN-γ assay for to function in the presence of cellular debris was next investigated. In a solution containing 0.5% (v/v) cell extract from malignant melanoma A375 cells, the assay exhibited a dose-dependent increase in luminescence intensity in response to IFN-γ (Fig. 4c). We envisage that our G-quadruplex-based detection platform could be further optimized for the accurate detection of IFN-γ levels in biological matrices.

In this work, we screened a total of 9 luminescent iridium(III) complexes containing various C^N and N^N ligands for their ability to discriminate G-quadruplex DNA over ssDNA and dsDNA. After two rounds of screening, the iridium(III) complex **8** emerged as the top candidate, and was utilized to construct a label-free G-quadruplex-based detection platform for IFN-γ. Compared to previously reported antibody-based immunoassays that require multiple steps and/or the use of expensive reagents, our strategy is more efficient as tedious washing steps and expensive antibodies are circumvented. Moreover, the luminescent transition metal complexes utilized in our assay exhibit a number of advantages compared to more commonly-used organic probes, such as large Stokes shifts, facile syntheses and long-lived phosphorescence lifetimes (> 2.9 μs, Table S1). Additionally, the assay could perform efficiently in diluted cell extract. We envisage that this label-free oligonucleotide-based luminescent detection method for IFN-γ utilizing the iridium(III) complex **8** could be further developed into a useful technique in associated disciplines.

Acknowledgments

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