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A luminescence switch-on probe for terminal deoxynucleotidyl transferase (TdT) activity detection by using an iridium(III)-based i-motif probe†

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An iridium(III) complex exhibiting higher responce towards i-motif DNA over dsDNA and ssDNA was employed for the construction of a TdT activity detection platform. The assay exhibited a linear signal enhancement for TdT in the concentration range of 0 to 8 U mL^{-1}, and the limit of detection for TdT was 0.25 U mL^{-1}.

Terminal deoxynucleotidyl transferase (TdT) is a template-free polymerase that can catalyze the random addition of deoxyribonucleoside triphosphates (dNTP) to the 3′-OH terminus of single-stranded DNA (ssDNA) and blunt-ended or 3′-protruding double-stranded DNA (dsDNA) fragments.[1] TdT generates DNA sequences that are largely dependent on the composition of the substrate dNTP pool, thus making it widely used as a versatile tool for the detection of target DNA,[2] RNA,[3] protein,[4] metal ions[4] and DNA modifying enzymes.[5] Moreover, TdT is of pathological significance since it is a biomarker for acute leukemia.[1a,6]

Therefore, it is highly important to explore sensitive and simple TdT activity detection methods. Traditional TdT activity detection methods have been based on immunological, gel electrophoresis and biochemical assays.[1b,6a,7] However, these methods require radioactive or fluorescently-labeled oligonucleotides and/or the preparation of antibodies, making them relatively time-consuming and expensive. Meanwhile, DNA oligonucleotides have emerged as viable alternatives for sensing applications compared to protein antibodies because they are generally more robust at a wider range of temperatures and pH values, and can be cheaply synthesized on a large scale. In recent years, several luminescent oligonucleotide-based sensing platforms for TdT activity detection have been developed. For example, Yuan and co-workers[5] have reported a label-free and turn-on fluorescence assay for TdT activity detection by employing long-chain C-rich oligonucleotide-encapsulated silver clusters (DNA–AgNCs). Liu and co-workers[8] used random G-rich DNA sequences catalyzed by TdT itself to monitor TdT activity, and the signal was transduced into colorimetric response by a peroxidase-mimicking DNAzyme, or into a fluorescent signal by thioflavin T (ThT). However, to our knowledge, no luminescent i-motif-based assay for TdT activity detection has been reported.

The i-motif is a non-canonical DNA secondary structure comprised of two parallel duplexes hydrogen-bonded together by intercalated C–C$^+$ base pairs.[9] The formation of the i-motif is favored at low pH, due to protonation of N3 in cytosine. Compared to the well-known G-quadruplex structure, the i-motif has not yet found significant use in the development of label-free luminescent detection platforms.[10] Moreover, few i-motif-binding molecules (especially iridium(III) complexes) have been reported as probes in i-motif-based detection platforms. Although organic dyes such as crystal violet, TmPyP4 and ThT have been employed as i-motif probes,[11] they can bind to multiple DNA configurations, and their synthesis is relatively complicated.[9a] On the other hand, transition metal complexes have attracted interest as alternatives to organic dyes due to their large Stokes shift, long lifetimes, simple synthetic procedures and tunable emission and excitation maxima in the visible range.[12] In recent years, our group has discovered that cyclometallated iridium(III) complexes can be employed for the monitoring of specific DNA secondary structures, such as G-quadruplex DNA.[13] Therefore, we envisaged that certain iridium(III) complexes could also act as i-motif monitoring probes, and that these complexes could be used to construct a label-free detection platform for TdT activity detection.

In the present study, iridium(III) complexes **1**–**5** (Fig. 1a and Fig. S1, ESI†) carrying the same C^N ligand 1-phenylisoquinoline (piq) and different N^N ligands based on the 1,10-phenatheroline (phen) core were evaluated. Complexes **1**–**5** were initially tested for their ability to discriminate the i-motif (HIF-1α) from dsDNA (ds17) and ssDNA (CCR5-DEL). Interestingly, the novel complex **1**

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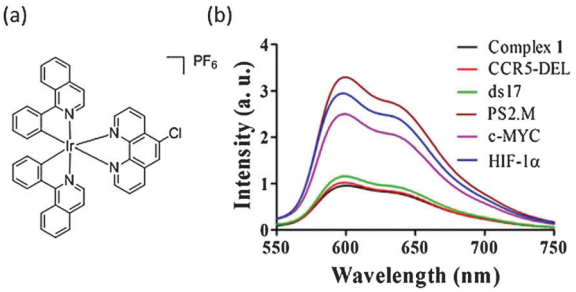


Fig. 1 (a) Chemical structure of iridium(III) complex **1**. (b) Emission spectra of complex **1** (0.5 μM) in the presence of 5 μM of ssDNA (CCR5-DEL), dsDNA (ds17), G-quadruplex (PS2.M) and two kinds of i-motif DNA (c-MYC and HIF-1α).

$([Ir(phq)_2(Cl\text{-}phen)]PF_6$ (where Cl-phen = 5-chloro-1,10-phenanthroline)) showed an excellent discrimination ability for i-motif DNA, with the highest $I_{i\text{-motif}}/I_{dsDNA}$ and $I_{i\text{-motif}}/I_{ssDNA}$ values among the five complexes screened (Fig. 2). Complex **1** showed a *ca.* 3.0-fold luminescence response to i-motif DNA, while only slight luminescence changes were observed for ssDNA (*ca.*1.1-fold) and dsDNA (*ca.*1.1-fold) (Fig. S2, ESI†). On the other hand, complexes **2–5** showed little or no specificity towards i-motif DNA (Fig. S2, ESI†).

Based on the analysis of these results, a brief structure-activity relationship can be concluded. Large N^N ligands such as 4,7-diphenyl-1,10-phenanthroline (in **4**) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (in **5**), both of which have two pendant phenyl groups attached to the phenanthroline motif, appeared to detract from the luminescence enhancement ratio, indicating that the size of the phenanthroline-based N^N ligand may be an important determinant for i-motif binding selectivity. Complex **2**, which contains the N^N 1,10-phenanthroline ligand, also showed lower luminescent enhancement ratios compared to **1**, indicating that the presence of the chlorine atom (in **1**) is desirable for i-motif binding selectivity. Moreover, complex **3**, which contains the 5,6-dimethyl-1,10-phenanthroline N^N ligand, also displayed low luminescent enhancement ratios, suggesting that the two methyl groups do not improve the i-motif binding activity of these types of complexes. We presume that the enhanced luminescence of the complex **1** in the presence of i-motif DNA is due to the selective interaction of complex **1** with the i-motif.[9b] This shields the metal centre from solvent interactions and suppresses non-radiative decay of the excited state, thus enhancing its ^{3}MLCT emission.[12a] On the other hand, complex **1** weakly interacts with double-stranded or single-stranded DNA, thus lower luminescence enhancement was observed for dsDNA or ssDNA. To validate the i-motif selectivity of complex **1**, the UV/vis titration experiment was performed. The results revealed that the binding constant of complex **1** towards the HIF-1α i-motif is 1.53×10^5 M^{-1} (Fig. S3a, ESI†), which is greater than that for dsDNA (0.74×10^5 M^{-1}, Fig. S3b, ESI†) or ssDNA (0.68×10^5 M^{-1}, Fig. S3c, ESI†), respectively.

Additionally, **1** also bound to G-quadruplex DNA with a similar luminescent enhancement (*ca.* 3.2-fold) compared to i-motif DNA. This result is similar to other previously reported DNA probes that have displayed strong luminescence towards two or more types of DNA secondary structures. For example, the well-known "molecular light switch" $Ru(phen)_2dppz$ (phen = phenanthroline, dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine) was first reported as an intercalating probe for dsDNA.[14] However, this complex has more recently been discovered to also show luminescent enhancement towards G-quadruplex and i-motif DNA.[15] Hence, further optimization is required to develop more specific probes for DNA secondary structures. The high luminescence response of **1** towards the i-motif and the G-quadruplex indicates that **1** may be potentially applied in an i-motif-based or G-quadruplex-based label-free sensing platform. To the best of our knowledge, no i-motif-based probe for detection of TdT activity has been reported in the literature. This work thus demonstrates the utility of complex **1** for the construction of an i-motif-based TdT activity detection platform for the first time.

As reported by Hu and Liu,[4,8] primers in the presence of a G-rich dNTP (60% dGTP, 40% dATP) substrate pool can be elongated into G-rich DNA sequences by TdT, which can then fold into G-quadruplex structures in the presence of K^+. Due to the analogous nature of i-motif and G-quadruplex-forming sequences, we envisaged that the primers could be elongated into C-rich DNA sequences by TdT by using a C-rich dNTP pool, and that the C-rich sequences could fold into i-motif structures in the presence of H^+. Thus, the mechanism of the proposed i-motif-based TdT activity assay is outlined in Scheme 1. The DNA primer (5′-GTTAACCTAGCCAG-3′) is incubated with TdT in the presence of a C-rich dNTP substrate pool. The nascent C-rich oligonucleotide will fold into the i-motif structure under acidic conditions, which can then be recognized by the luminescent iridium(III) complex with an enhanced luminescence response. Thus, this system can function as a switch-on luminescent detection platform for TdT activity assay.

We initially investigated whether the C-rich DNA sequences generated by the C-rich dNTP (60% dCTP, 40% dTTP) substrate pool are able to fold into i-motif structures under acidic conditions. The iridium(III) complex **1** was used to monitor the formation of the i-motif. We found that complex **1** showed a greater luminescence enhancement to the product formed from a C-rich dNTP (60% dCTP, 40% dTTP) substrate pool compared to that

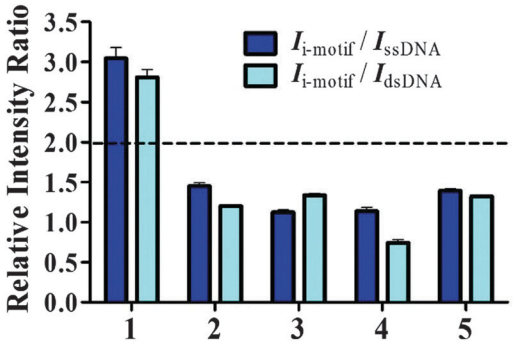
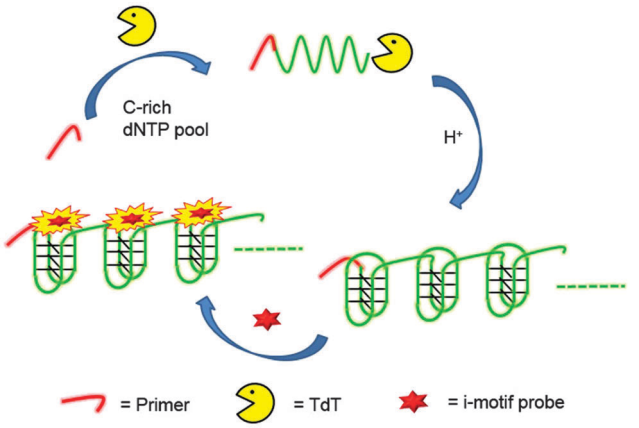


Fig. 2 Diagrammatic bar representation of the relative intensity ratio of complexes **1–5** for i-motif DNA (HIF-1α) over dsDNA (ds17) and ssDNA (CCR5-DEL). Error bars mean the standard deviations of the results from 3 independent experiments.



Scheme 1 Schematic diagram of the TdT detection assay using an i-motif-selective luminescent probe.

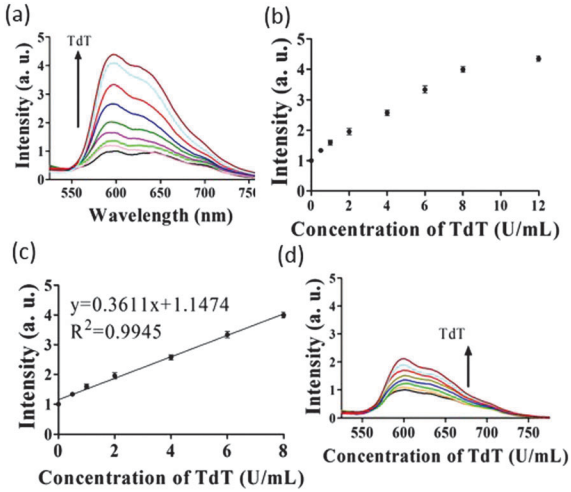


Fig. 3 (a) Luminescence spectra of **1** in response to various concentrations of TdT: 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 U mL^{-1} in the C-rich pool (60% dCTP, 40% dTTP). (b) The relationship between luminescence intensity at λ = 600 nm and TdT concentration. (c) Linear plot of the change in luminescence intensity at λ = 600 nm *vs.* TdT concentration. Error bars show the standard deviations of the results from three independent experiments. (d) Luminescence spectra of 1 in response to various concentrations of TdT: 0, 0.5, 1, 2, 4, 6, 8 and 12 U mL^{-1} in the unbiased dNTP pool.

formed by an unbiased dNTP (25% dCTP, 25% dATP, 25% dTTP, 25% dGTP) substrate pool (Fig. S4, ESI†). This result suggests that the i-motif structure could be formed under the conditions used in our study. Circular dichroism (CD) spectroscopy was used to further confirm the formation of the i-motif structure in the presence of the C-rich dNTP pool (Fig. S5, ESI†). The CD spectrum of the DNA product formed in the C-rich dNTP pool under acidic conditions exhibited a stronger positive peak at around 290 nm and a negative peak at 245 nm, which are the typical peaks for i-motif.[16] In contrast, the product formed from the unbiased dNTP pool revealed a weaker positive peak at around 290 nm and a weak negative band at 240 nm, which are consistent with random-coil DNA.[11c] Taken together, these results suggest that the C-rich dNTP substrate pool generated C-rich DNA sequences, which subsequently folded into i-motif structures under acidic conditions.

We next optimized the composition of the C-rich dNTP pool in order to improve the performance of the sensing assay. We tested the luminescence response of complex **1** to the DNA products formed from dNTP pools consisting of >50% dCTP, with the remaining nucleotides being either dTTP or dATP. In general, we found that the dCTP/dTTP substrate pool resulted in a higher signal enhancement compared to the dCTP/dATP pool (Fig. S6, ESI†). According to Chilkoti and co-workers,[17] dTTP promotes the greatest extension in the TdT-catalyzed polymerization reaction. Based on this phenomenon, we reasoned that the dCTP/dTTP pool generated longer C-rich DNA sequences compared to the dCTP/dATP pool, therefore allowing for the formation of more i-motif structures. A mixture of 60% dCTP and 40% dTTP was found to result in the strongest luminescence intensity of complex **1**. Further optimization experiments showed that the concentration of primer and complex **1** was optimal at 1 μM and 0.5 μM, respectively (Fig. S7 and S8, ESI†). Additionally, the best results were obtained at a pH value of 5.0 (Fig. S9, ESI†) and a reaction time of 120 min (Fig. S10, ESI†).

Under the optimized conditions, the luminescence response of the system to different concentrations of TdT (0 to 12 U mL^{-1}) was investigated. The system exhibited a *ca.* 4.5-fold enhancement in luminescence intensity at [TdT] = 12 U mL^{-1} (Fig. 3a), with a linear range of detection for TdT from 0 to 8 U mL^{-1} (Fig. 3c). The limit of detection (LOD) of this assay for TdT was estimated to be 0.25 U mL^{-1} at a signal-to-noise ratio (S/N) of 3 (Fig. S11, ESI†). Compared to previously reported label-free TdT detection platforms, this limit of detection is superior to an assay utilizing water-soluble conjugated polymers (LOD = 6 U mL^{-1}),[18] and comparable to other assays utilizing long-chain C-rich oligonucleotide-encapsulated silver clusters (LOD = 0.038 U mL^{-1})[5] and randomly arrayed G-quadruplexes (LOD = 0.04 U mL^{-1}).[8] A further experiment was carried out by using an unbiased dNTP substrate pool under the same reaction conditions (Fig. 3d). The luminescence enhancement was only about half that with the C-rich substrate pool, suggesting that complex **1** interacted less strongly with the random-coil DNA sequences generated by the unbiased dNTP pool. Repeating the experiment 12 times with the C-rich dNTP pool under the same conditions showed that the system exhibited good reproducibility (relative standard deviation (RSD) = 7.26%) (Fig. S12, ESI†), indicating that this detection platform is a reliable method for TdT activity detection.

The selectivity of the i-motif-based TdT activity detection platform was tested by investigating the response of the assay to four kinds of polymerases and six other DNA-modifying enzymes (Fig. S13, ESI†). The results indicate that the system displays superior selectivity to TdT over other DNA-modifying enzymes, which presumably originates from the specific generation of C-rich DNA by TdT in the detection system.

The ability of this system to detect TdT in a diluted cell extract was investigated. In a reaction system containing 0.5% (v/v) cell extract, the system experienced a gradual linear increase in signal enhancement as more TdT was added (Fig. 4a and b). This result showed that the detection system could potentially be adapted for TdT detection in real samples.

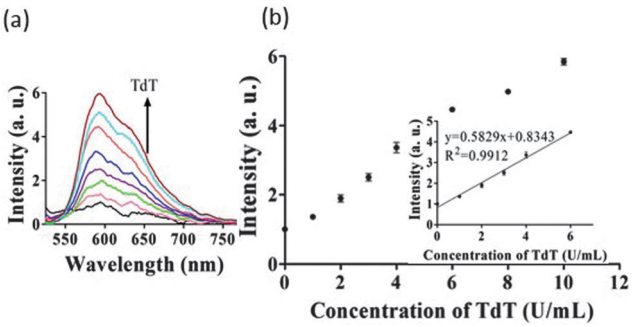


Fig. 4 (a) Luminescence spectra of **1** in the system containing 5% (v/v) cell extract in response to various concentrations of TdT: 0, 1, 2, 3, 4, 6, 8 and 10 U mL^{-1}. (b) The relationship between luminescence intensity at λ = 600 nm and TdT concentration. Inset: linear plot of the change in luminescence intensity at λ = 600 nm *vs.* TdT concentration. Error bars show the standard deviations of the results from three independent experiments.

In summary, a novel cyclometallated iridium(III) complex **1** has been found to display higher response to i-motif DNA over dsDNA and ssDNA, and was utilized for the construction of a label-free i-motif-based detection platform for TdT. Moreover, the application of the assay for the detection of TdT in diluted cell extracts was demonstrated. This label-free i-motif-based luminescence switch-on platform is simple and cost-effective compared to conventional biochemical or immunological assays.

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