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## EDGE ARTICLE

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## Label-free luminescence switch-on detection of hepatitis C virus NS3 helicase activity using a G-quadruplex-selective probe†

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A series of luminescent Ir(III) complexes were synthesised and evaluated for their ability to act as luminescent G-quadruplex-selective probes. The Ir(III) complex **9**, [Ir(phq)<sub>2</sub>(phen)]PF<sub>6</sub> (where phq = 2-phenylquinoline; phen = 1,10-phenanthroline), exhibited high luminescence in the presence of G-quadruplex DNA compared to dsDNA and ssDNA, and was employed to construct a label-free G-quadruplex-based assay for hepatitis C virus NS3 helicase activity in aqueous solution. Moreover, the application of the assay for screening potential helicase inhibitors was demonstrated. To our knowledge, this is the first G-quadruplex-based assay for helicase activity.

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### Introduction

Helicases unwind dsDNA and dsRNA, and displace nucleic acid-binding proteins by using energy from ATP hydrolysis.<sup>1,2</sup> Helicase is an essential enzyme in cells for the reading, replication, and repair of genomes. However, helicases are also implicated in a number of viral diseases due to their critical role in facilitating viral replication and proliferation.<sup>3</sup> Viral helicase inhibitors have been developed for the treatment of hepatitis C and herpes simplex viral infections.<sup>4</sup> Due to its biological and medical importance, the development of efficient assays for monitoring the nucleic acid unwinding activity of helicase is of great interest.

Conventional techniques for the detection of helicase activity typically involve radioactive labeling in conjunction with gel electrophoresis.<sup>5–7</sup> However, this method is discontinuous, time-consuming, inefficient, and necessitates the use of stringent safety procedures to control radiographic exposure, thus limiting the scope of its application. Meanwhile, rapid advances in the field of DNA technology over the past several years have highlighted the potential use of oligonucleotides as attractive signal transducing units for the detection of biologically and environmentally important analytes.<sup>8–17</sup> Oligonucleotides offer salient advantages in bio-sensing applications, such as their relatively small size, low

cost, facile synthesis and modification, good thermal stability, and reusability.<sup>18–26</sup> The G-quadruplex motif, which is a non-canonical DNA secondary structure composed of planar stacks of four guanines stabilized by Hoogsteen hydrogen bonding,<sup>27–30</sup> has attracted particular interest in sensing applications.<sup>31,32</sup> The extensive structural polymorphism of G-quadruplexes has rendered them versatile signal-transducing elements for the development of DNA-based probes.<sup>33–48</sup>

In recent years, a number of luminescent oligonucleotide-based sensing platforms for helicase activity have been developed.<sup>49–53</sup> For example, Min and co-workers reported a fluorescent assay for hepatitis C virus (HCV) NS3 helicase activity and severe acute respiratory syndrome coronavirus (SARS-CoV, SCV) helicase nsP13 activity by utilizing graphene and a fluorescently labeled dsDNA substrate.<sup>54,55</sup> Ali and co-workers used SYBR Green dye, which is strongly fluorescent in the presence of dsDNA but not ssDNA, for the detection of helicase activity.<sup>56</sup> A similar principle was utilized by Kowalczykowski and co-workers to construct a switch-off platform for helicase activity using ethidium bromide, ethidium homodimer, bis-benzimide (DAPI), Hoechst 33258 or thiazole orange. The groups of Frick and Boguszevska-Chachulska reported an approach for monitoring helicase activity using molecular beacons.<sup>57,58</sup> Recently, Balci and co-workers used single-molecule Forster resonance energy transfer (FRET) imaging to monitor helicase activity.<sup>59–61</sup> These reports demonstrate that DNA oligonucleotides can be integrated as useful functional and structural elements for the construction of sensitive “switch-on” luminescent platforms for the detection of helicase.

In recent years, luminescent transition metal complexes have arisen as viable alternatives to organic dyes for sensory

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applications due to their notable advantages.<sup>62–70</sup> Firstly, metal complexes generally emit in the visible region with a long phosphorescence lifetime, allowing them to be readily distinguished from a fluorescent background arising from endogenous fluorophores in the sample matrix by the use of time-resolved fluorescence spectroscopy. Secondly, the precise and versatile arrangement of co-ligands on the metal centre allows the interactions of metal complexes with biomolecules to be fine-tuned for maximum selectivity and sensitivity. Thirdly, these metal complexes often possess interesting photophysical properties that are strongly affected by subtle changes in their local environment. For example, Pt(II)<sup>71–73</sup> and Ru(II)<sup>74–77</sup> complexes have been extensively investigated as “molecular light switches” for nucleic acids, including G-quadruplex DNA. However, luminescent complexes based on the Ir(III) center have been comparatively less explored. In this study, a series of luminescent Ir(III) complexes were synthesised and evaluated for their ability to act as G-quadruplex-selective luminescence switch-on probes. The iridium(III) complex **9**, [Ir(phq)<sub>2</sub>(phen)] PF<sub>6</sub> (where phq = 2-phenylquinoline; phen = 1,10-phenanthroline), (Fig. 1) was employed as a G-quadruplex probe for the construction of a label-free luminescent detection platform for helicase activity in aqueous solution, utilizing hepatitis C virus NS3 helicase as a model enzyme. To our knowledge, no luminescent G-quadruplex-based assay for the detection of helicase activity has yet been reported in the literature.

## Results and discussion

### Principle of the luminescent G-quadruplex-based probe for HCV NS3 helicase activity

HCV NS3 helicase is able to unwind dsDNA into ssDNA, and is one of the essential enzymes required for the replication of HCV.<sup>78</sup> The proposed mechanism of the HCV NS3 helicase activity assay is outlined in Scheme 1. We designed a double-stranded oligomer consisting of a G-quadruplex-forming sequence (ON1, 5'-GTG<sub>3</sub>TAG<sub>3</sub>CG<sub>3</sub>T<sub>2</sub>G<sub>2</sub>TG<sub>2</sub>CGACG<sub>2</sub>CAGCGAG<sub>2</sub>CAGAG<sub>2</sub>AGCAGAG<sub>3</sub>AGCA-3') and its complementary cytosine-rich sequence (ON2, 5'-GC<sub>2</sub>TCGCTGC<sub>2</sub>GTCGC<sub>2</sub>AC<sub>2</sub>A<sub>2</sub>C<sub>3</sub>GC<sub>3</sub>-3'), which acts as a substrate for helicase. In the absence of helicase, the double-stranded oligonucleotide substrate is not unwound, and remains as a duplex structure that interacts only weakly with the luminescent Ir(III) complex. However, in the presence of helicase, unwinding of the duplex DNA substrate by helicase generates two ssDNA fragments. After the reaction is stopped by the addition of EDTA,<sup>51</sup> the G-quadruplex-forming oligomer ON1 is able to fold into a G-quadruplex motif in the presence of K<sup>+</sup> ions. The nascent G-quadruplex structure is then recognized by the luminescent Ir(III) complex with an enhanced emission response, allowing the system to function as a switch-on luminescent probe for helicase activity.

### Screening of iridium(III) complexes as G-quadruplex-selective probes

In the present study, a library of seven luminescent Ir(III) complexes (**1–7**, Fig. 1) were initially examined for their emission response to different forms of DNA, including G-quadruplex, ssDNA and dsDNA (Table S1†). Of these seven complexes, only complex **7** bearing the N<sup>N</sup> ligand chlorophen (5-chloro-1,10-phenanthroline) and the C<sup>N</sup> ligand phq showed a selective response for G-quadruplex DNA (Fig. S1†), while not showing any luminescence enhancement in the presence of helicase (data not shown). On the other hand, complexes **3**, **5**, and **6** were found to be non-selective for G-quadruplex DNA (Fig. S1†), whereas the luminescence of **1**, **2**, and **4** was enhanced in the presence of helicase only (data not shown). Based on the structure of complex **7**, a focused library of eleven Ir(III) complexes (**7–17**, Fig. 1) containing phq and chlorophen derivatives as ligands were designed and



Fig. 1 Chemical structures of the luminescent Ir(III) complexes **1–17** that were synthesised and evaluated in this study.



Scheme 1 Schematic diagram of the luminescent switch-on assay to monitor the duplex-DNA unwinding activity of helicase using a G-quadruplex-selective probe.





Fig. 3 (a) Luminescence spectra of the 9/G4-quadruplex system in response to increasing concentrations of helicase: 0, 0.09, 0.18, 0.27, 0.36, 0.45, 0.54, 0.72, and 0.9  $\mu\text{M}$ . (b) The relationship between luminescence intensity at  $\lambda = 571$  nm and helicase concentration. Inset: linear plot of the change in luminescence intensity at  $\lambda = 571$  nm vs. helicase concentration.

### Selectivity of the G-quadruplex-based HCV NS3 helicase activity assay

The selectivity of our approach to an HCV NS3 helicase activity assay was evaluated by investigating the response of the system to S1 nuclease (S1), endonuclease IV (Endo), DpnI, exonuclease I (ExoI), EcoRI, RNase, DNase and single-stranded DNA binding protein (SSB). The results showed that only helicase could significantly enhance the luminescence of the complex 9/G-quadruplex DNA system (Fig. 4a). No significant change in emission intensity was observed upon addition of the nucleases, while a relatively low emission enhancement was observed for single-stranded DNA binding protein. These results indicate that the system displays selectivity for helicase over nucleases or single-stranded DNA binding proteins, which presumably originates from the unwinding of the duplex DNA substrate by helicase.

### Application of the HCV NS3 helicase activity detection assay in biological samples

To evaluate the robustness of the system, we investigated the performance of our G-quadruplex-based sensing platform for helicase activity in the presence of cellular debris. In a reaction system containing 0.5% (v/v) cell extract, the 9/G-quadruplex DNA system exhibited a gradual increase in luminescence intensity as the concentration of helicase increased (Fig. 4b). This result demonstrates that this assay could potentially be further developed for the detection of helicase unwinding activity in biological samples.

### Application of the HCV NS3 helicase activity detection assay in inhibitor screening

The utility of this G-quadruplex-based assay for screening potential helicase inhibitors was also studied. Here, ciprofloxacin was chosen as a model inhibitor of helicase.<sup>56</sup> The luminescence signal of the system was diminished in the presence of ciprofloxacin in a dose-dependent manner, with a decrease of about 40% observed at a ciprofloxacin concentration of 10  $\mu\text{M}$  (Fig. 4c). The inhibitory effect of ciprofloxacin on helicase activity in our assay is comparable to that reported by

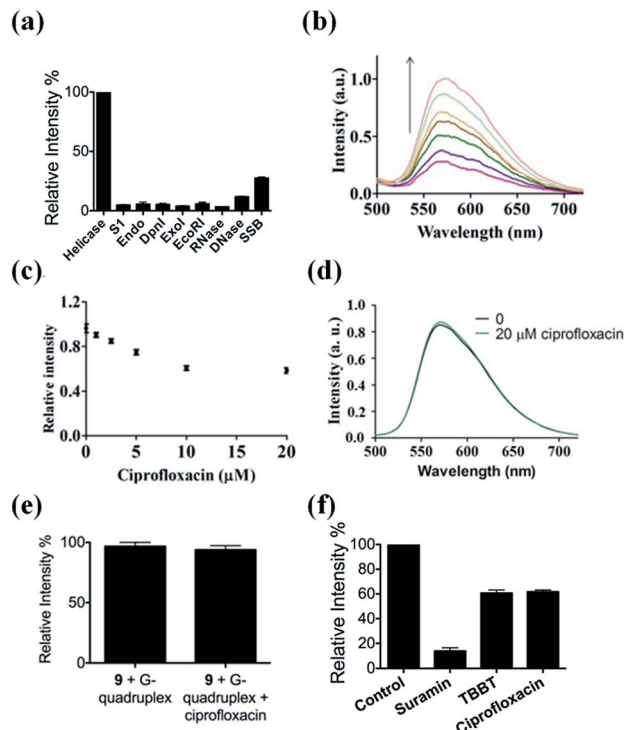


Fig. 4 (a) Luminescence response of the system in the presence of helicase, S1, Endo, DpnI, ExoI, EcoRI, RNase, DNase and SSB. (b) Luminescence spectra of the 9/G-quadruplex system in a reaction system containing 0.5% (v/v) cell extract in response to various concentrations of helicase: 0, 0.18, 0.36, 0.45, 0.54, 0.72, and 0.9  $\mu\text{M}$ . (c) Relative luminescence intensities of the system in the presence of increasing concentrations of ciprofloxacin: 0, 1, 2.5, 5, 10, and 20  $\mu\text{M}$ . (d) Emission spectra of complex 9 in the absence and presence of 20  $\mu\text{M}$  ciprofloxacin. (e) Relative luminescence response of the 9/G-quadruplex ensemble upon the addition of 20  $\mu\text{M}$  ciprofloxacin. (f) Luminescence response of the system treated with 0.8  $\mu\text{M}$  helicase and 10  $\mu\text{M}$  of suramin, TBET and ciprofloxacin.

Ali and co-workers.<sup>56</sup> Furthermore, ciprofloxacin has no direct quenching effect on the luminescence of 9 (Fig. 4d) or the 9/G-quadruplex ensemble (Fig. 4e). To further demonstrate the application of this platform for inhibitor screening, we investigated a group of well-known HCV NS3 helicase inhibitors.<sup>80</sup> The inhibitors tested displayed inhibitory activity towards HCV NS3 helicase in this platform, without having a direct quenching effect on 9 or the 9/G-quadruplex ensemble (Fig. 4f and S11†). These results further validate our detection platform as a screening tool for HCV NS3 helicase inhibitors.

## Conclusions

In conclusion, a library of 17 luminescent Ir(III) complexes containing various C<sup>N</sup> and N<sup>N</sup> ligands were screened for their ability to act as G-quadruplex probes. Ir(III) complex 9 was discovered to be a G-quadruplex-selective luminescent probe, and a label-free luminescent assay for helicase activity was developed utilizing the G-quadruplex-selective property of 9. Compared to previously reported radiographic or luminescent assays that require multiple steps and/or the use of isotopically





or fluorescently labeled nucleic acids, our label-free approach is less time consuming and more cost-effective, as expensive and tedious pre-labeling or immobilization steps are avoided. On the other hand, the labeling of an oligonucleotide with a fluorophore may disrupt the interaction between the oligonucleotide and its cognate target. Finally, our developed label-free DNA-based detection platform employs luminescent transition metal complexes, which offer several advantages compared to the relatively more popular organic fluorophores, such as long phosphorescence lifetimes, large Stokes shift values and modular syntheses. Additionally, the assay could function effectively in diluted cell extract, and its potential application in the screening of helicase inhibitors was also demonstrated, though further optimisation may be required. We envision that our novel switch-on, label-free G-quadruplex-based luminescent detection method for helicase activity could potentially be developed as a useful tool in biochemical and biomedical research.

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