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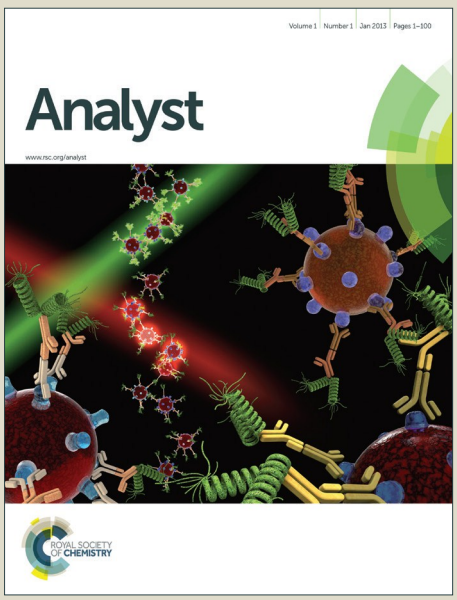

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Sensitive and Selective Detection of Uracil-DNA Glycosylase Activity with a New Luminescent Switch-on Molecular Probe

Yu-Jing Lu[a,*], Dong-Ping Hu[a], Qiang Deng[a], Zheng-Ya Wang[a], Bao-Hua Huang[a], Yan-Xiong Fang[a], Kun Zhang[a,b,*], Wing-Leung Wong[c,*]

[a] Institute of Natural Medicine and Green Chemistry, School of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, P.R. China.

[b] School of Chemical and Environment Engineering, Wuyi University, Jiangmen 529020, P.R. China.

[c] Department of Science and Environmental Studies, Centre for Education in Environmental Sustainability, The Hong Kong Institute of Education, 10 Lo Ping Road, Tai Po, Hong Kong SAR, P.R. China.

E-mail: luyj@gdut.edu.cn; Tel: +86-20-39322235
E-mail: kzhang@gdut.edu.cn; Tel: +86-20-39322235
E-mail: wingleung@ied.edu.hk; Tel: +852-2948-8401
*Corresponding author

Abstract

Uracil-deoxyribonucleic acid glycosylase (UDG) is known to function as an important base-excision repair enzyme and eliminate uracil from DNA molecules for maintaining genomic integrity. A new small organic molecule (**DID-VP)** with interesting structural properties was synthesized as a G-quadruplex selective ligand and was demonstrated to be a sensitive luminescent switch-on probe in a convenient luminescent assay specifically for UDG detection in fetal bovine serum samples under rapid and simple conditions. This newly developed analytical method is based on the UDG enzymatic activity to unwind a duplex DNA substrate, which comprises with a G-quadruplex-forming sequence (ON1) and uracil-containing DNA strand (ON2) to generate a remarkable fluorescence signal through the specific interaction of **DID-VP** with ON1. This luminescent switch-on assay is able to

achieve high sensitivity and specificity for UDG over other enzymes. The application range of the present analytical system is found from 0.05 to 1.00 U/mL UDG with a very low detection limit of 0.005 U/mL. The recovery study of UDG in real samples gave very good performance with 75.05% - 102.7% recovery. In addition, an extended application of the assay in screening of UDG inhibitors is demonstrated. A good dose-dependence of the luminescence response with respect to the concentration of UDG inhibitors in samples was observed.

Keywords: *Uracil- Deoxyribonucleic acid glycosylase; G-quadruplex Deoxyribonucleic acid; G-quadruplex ligand; Fluorescence probe*

1. Introduction

The uracil residues in DNA can be readily removed by uracil-excision repair enzyme uracil-DNA glycosylase (UDG).[1-3] Many evidences have shown that UDG involves in the cell cycle regulation, apoptosis, tumor development, and virus proliferation.[4-8] Classical strategies for UDG activity investigation including gel electrophoresis, mass spectrometry, and radioactive labeling[9-13] have long been well established; however, these methodologies are known to be time-consuming and indirect due to the requirements of sophisticated, additional multi-step separation techniques, and costly labeled reagents. To overcome the drawbacks of the traditional assays, fluorescence-based assay techniques performing in homogeneous phases have been developed to realize rapid assay of UDG activity under simple conditions.[14-23] For example, Qu's group developed a label-free fluorescence turn-on method based on the unique strong interaction between *N*-methyl mesoporphyrin IX and the folded G-quadruplex.[21] The G-quadruplex motif is a DNA secondary structure consisting of square-planar arrangements of guanine nucleobases stabilized by Hoogsteen hydrogen bondings and monovalent cations.[24-26] The double quadruplex DNA has also been found applications in biology and nanotechnology under specific conditions;[27] however, the G-quadruplex DNA represents a more versatile sensing platform for bioanalytical assays because the specific recognition can be done by luminescent sensing probes.[28-31] More recently, Liu and Chen, have also developed a novel nicking enzyme assisted signal amplification method based on G-quadruplex structures for sensitive label-free colorimetric analysis of UDG activity.[32] In general, among these reported systems, the molecular structure design and the specific functionality of the incorporated moieties of the sensor probe are

found very critical for G-quadruplex selectivity, sensitivity, and signaling enhancements in the luminescent assays.

Styryl dyes are widely used as fluorescent probes for cellular organelles or macromolecules in medicinal analysis, such as nuclear-selective staining.[33-35] The mechanism of nuclear staining of these dyes may involve an increase of their fluorescence intensity upon binding to DNA.[36] However, there are only a few reports available in the literature in which the styryl dye could be specifically binding to G-quadruplex. In this study, a new charged organic molecule (**DID-VP**) with a rotatable structure and the tunable fluorescent on-off property was synthesized specifically for G-quadruplex detection. The small molecule shows a very strong binding affinity and good selectivity with the secondary-macromolecular G-quadruplex DNA and gives remarkable luminescence enhancement in the assay.

Structurally, **DID-VP** is a new pyridinium-based organic molecular probe with a positive charge, which is able to enhance aqueous solubility;[37] also, the compound is inexpensive, easily prepared and purified, and easy-to-use for bioassay or bio-analytical applications in aqueous medium. More importantly, its molecular structure is simple but it shows very specific and strong fluorescence response signal upon binding with G-quadruplex DNA. This is the key advantage of this dye, which is able to give better performance than other systems. By taking the merit properties of **DID-VP**, we developed a sensitive and selective UDG detection method based on the specific interaction of **DID-VP** with G-quadruplex to form an emissive adduct complex. A mechanism was proposed on the basis of unwinding of a duplex DNA substrate by UDG, followed by the formation of a G-quadruplex-forming sequence (ON1) and uracil-containing DNA strand (ON2). The fluorescence signal is attributed to the

specific G-quadruplex sequence (ON1)/**DID-VP** interaction. The present method shows excellent sensitivity and specificity for UDG detection in fetal bovine serum samples.

Experimental

2.1 Reagents and instruments

All solvents were obtained from commercial suppliers and were used without further purification. The chemical reagents for synthesis were purchased from Alfa Aesar or Sigma Aldrich Co. Escherichia coli uracil DNA glycosylase (UDG) and 10×UDG reaction buffer (200 mM Tris–HCl, 10mM EDTA, 100 mM NaCl, pH 8.2) were purchased from Fermentas China Co., Ltd.. All oligonucleotides used in this work were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The detailed sequences were listed in Table 1. ON1 and ON2 were used as provided and were diluted individually with 10 mM Tris–HCl buffer solution (pH 7.4, containing 60 mM KCl) as the stock solutions (100 μM). Each oligonucleotide was heated to 95 ℃ and the solution was maintained at this temperature for 10 min and then was allowed to cool to room temperature gradually. The oligonucleotide was kept at 4 ℃ for overnight incubation. The fetal bovine serum was purchased from biological industries (US).

UV-Vis spectroscopic studies were performed on a Lambda 25 UV/Vis spectrophotometer (Waltham, USA) using a 2.5 mm path length quartz cuvette. Fluorescence studies were performed on a LS45 luminescence spectrophotometer (Waltham, USA). A quartz cuvette with 2 mm x 2 mm path length was used for the spectra recorded at 10 nm slit width for both excitation and emission unless otherwise specified. Circular dichroism (CD)

spectra were carried out using a Chirascan spectrophotometer (Surrey, UK). FRET assay was determined with a Roche LightCycler 96 real-time PCR (Basque, Switzerland).

Table 1. Oligonucleotides used in this study.

Entry	Abbreviation	Sequence	Structure
1	dA21	AAAAAAAAAAAAAAAAAAAAA	Single stranded
2	dT21	TTTTTTTTTTTTTTTTTTTTT	Single stranded
3	4A4T	AAAATTTT	Duplex
4	4C4G	CCCCGGGG	G-quadruplex
5	LQ1	TGGGGT	G-quadruplex
6	Oxy28	GGGGTTTTGGGGTTTTGGGGTTTTGGGG	G-quadruplex
7	4-Telo	GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG	G-quadruplex
8	bcl-2	GGGCGCGGGAGGAAGGGGGCGGG	G-quadruplex
9	c-kit1	AGGGAGGGCGCTGGGAGGAGGG	G-quadruplex
10	c-kit2	GGGCGGGCGCGAGGGAGGGG	G-quadruplex
11	pu18	AGGGTGGGGAGGGTGGGG	G-quadruplex
12	pu27	TGGGGAGGGTGGGGAGGGTGGGGAAGG	G-quadruplex
13	RET	GGGGCGGGGCGGGGCGGGGG	G-quadruplex
14	VEGF	GGGGCGGGCCGGGGGCGGGG	G-quadruplex
15	HTG21	GGGTTAGGGTTAGGGTTAGGG	G-quadruplex
16	HTG-imotif	CCCTAACCCTAACCCTAACCC	G-quadruplex
17	ON1	GGGTAGGGAAATTCTTAAGTGCGGGTTGGG	G-quadruplex
18	ON1m	TAGTAGAGAAATTCTTAAGTGCGAGTTGTA	Single stranded
19	ON2	CGCACUUAAGAAUUTC	Single stranded
20	F21T	FAM-d(GGG[TTAGGG]$_3$)-TAMRA	G-quadruplex
21	F10T	FAM-dTATAGCTA-HEG-TATAGCTATAT-TAMRA	Duplex

2.2 Synthesis of DID-VP

The synthetic route of the target compound is shown in Scheme 1. *N*-methyl-2, 6-lutidinium iodide (1.72 g, 0.69 mmol), indole-3-carbaldehyde (1.00 g, 0.69 mmol), 4-methylpiperidine (0.3 mL) were reacted in n-butanol (15 mL) at reflux conditions (120 ℃ approximately) for 1.5 h. After cooling in an iced bath, the precipitate was filtrated and the residue was rinsed with n-butanol. The residue collected was purified by re-crystallization in acetone to afford the compound (**DID-VP**) as the orange solids (0.75 g, 69.4%): m.p.209-211 ℃; ^{1}H NMR (400 MHz, DMSO-d6): δ 11.95 (s, 2H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.13(d, *J* = 11.0 Hz, 6H), 8.06 (s, 1H), 8.02 (s, 1H), 7.53 (d, *J* = 7.9 Hz, 2H), 7.34 (s, 1H), 7.30 (s,1H), 7.29 - 7.21 (m, 4H), 4.25 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d6): δ 154.39,141.69, 137.70, 136.90, 131.82, 125.55, 123.24, 121.52, 121.01, 120.44, 113.81, 112.98, 112.84, 41.43; ESI-MS m/z: 376.2 $[M-I]^{+}$

2.3 Assay of UDG activity

50 μL 1×UDG reaction buffer (20 mM Tris–HCl, 1 mM EDTA, 10 mM NaCl, pH 8.2) and a known concentration of UDG (5.0 U/mL) were added to the solution containing a duplex DNA substrate. The mixture was incubated at 37 ℃ for 30 min to allow the base cleavage reaction taking place. The mixture was cooled to room temperature and then was diluted with Tris buffer solution (50 mM Tris, 20 mM KCl, 50 mM NH_4OAc, pH = 7.0) to final volume of 500 μL. Finally, the **DID-VP** solution (2 μM in Tris buffer) of was added to the mixture. Emission spectra were recorded in the wavelength ranges of 500−700 nm with excitation wavelength at 486 nm.

2.4 Assay of UDG inhibitor screening

For UDG inhibitor screening, the duplex substrate was incubated with a known concentration of UDG (2.5 U/mL) and with a various concentration of UDG inhibitor (0, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, and 3.00 U/mL). The mixture was heated to 37 ℃ for 30 min and then was cooled to room temperature. After that, the mixture was diluted to a final volume of 500 μL with Tris buffer (50 mM Tris, 20 mM KCl, 50 mM NH_4OAc, pH = 7.0). The **DID-VP** solution **(**0.5 μM in Tris buffer) was subsequently added to the mixture. Emission spectra were recorded in the wavelength range of 500-700 nm using an excitation wavelength of 486 nm.

2.5 PAGE (20%) for the heteroduplex unwinding demonstration

For the 20 % polyacrylamide gel electrophoresis (PAGE) analysis of UDG detection, the process of uracil excision by UDG was conducted as aforementioned, except the concentration of ON1 and ON2 was 15 μM, and the UDG concentration was 5 U/mL. The samples were loaded into a 20% polyacrylamide gel and the electrophoresis was carried out in tris-borate-EDTA (TBE) buffer (90 mM Tris, 90 mM boric acid, 2mM EDTA, and 12.5 mM $MgCl_2$ at pH 8.0) at 45 V for 1 h and 100V for 2 h. The gel was stained with SYBR Green I or DID-VP for 20 min and scanned by a Gel imaging system (AlphaImager HP).

2. Results and discussion

3.1 Synthesis and characterization of DID-VP

The butterfly-shaped organic probe, 2,6-bis((E)-2-(1H-indol-3-yl)vinyl)-1-methylpyridin-1-ium iodide (**DID-VP**), was prepared

by N-methylation of 2,6-lutidine (**1**) with iodomethane to generate a positive charged on the pyridine ring (**2**) and followed by the reaction with indole-3-carbaldehyde as shown in Scheme 1. **DID-VP** is easily purified and is obtained in good yields from the reaction using 4-methylpiperidine as a base. The compound was characterized with ^{1}H & ^{13}C NMR and electrospray ionization-MS (**Fig. S1**)**.** The positively charged methylpyridinium is able to enhance the solubility in aqueous medium. In addition, the two symmetrical 3-methylindole units are designed to be rotatable in solution; however, when it interacts and stacks with G-quadruplex, free rotation is no longer feasible and the **DID-VP** molecular structure becomes planar. Thus, it renders strong luminescent signal.

3.2 DID-VP as a selective G-quadruplex fluorescent probe

By taking the unique structural property of the compound, the use of **DID-VP** as a luminescent G-quadruplex-selective probe was investigated in detailed under various conditions. We employed fluorimetric titration, UV-Vis absorption titration, and fluorescence resonance energy transfer (FRET) melting assay to evaluate the binding affinity and specificity of **DID-VP** with G-quadruplex DNA. The fluorescence responses of **DID-VP** towards DNA substrates with different configurations listed in Table 1 are summarized in **Fig. 1** and **Fig. S2**, respectively. In general, the results show that the fluorescence intensity of **DID-VP** is enhanced greatly upon binding with G-quadruplex DNA substrates. Among of DNA substrates examined, c-kit1, Pu18 and Pu27 induce the highest emission enhancement (approximately 30-fold signal enhancement compared to **DID-VP** alone). Whereas, **DID-VP** exhibits very weak fluorescence when it is treating with the non-G-quadruplex DNA substrates. Using UV-Vis absorption titration, the intrinsic binding affinity of **DID-VP** with

G-quadruplex DNA (Telo21) was determined to be 5.02×10^6 M^{-1}. The binding affinity is 6-fold higher than that of ssDNA and 4-fold higher than that of dsDNA. FRET-melting analyses (**Fig. S3**) reveal that **DID-VP** is able to stabilize the G-quadruplex structure effectively with a 6 °C increase in the melting temperature (ΔT_m). However, the stabilization effect of **DID-VP** found on dsDNA is not obvious ($\Delta T_m < 1$ °C). These experimental results may lead to conclude that **DIP-VP** is an effective ligand which shows excellent binding selectivity to G-quadruplex DNA and the specific binding thus induces a strong emission signal at λ_{em} = 550 nm due to increasing the structural rigidity of **DIP-VP** upon interacting with G-quadruplex DNA in solution.

3.2 Design of the selective UDG sensing system

3.2.1 Working mechanism of UDG detection assay

The proposed mechanism of the UDG activity assay is outlined in Scheme 2. First, the G-quadruplex sequence (ON1, 5'-G3TAG3A3T2CT2A2GTGCG3T2G3-3') is hybridized with a partially complementary uracil-containing DNA strand (ON2, 5'-CGCACU2A2GA2U2TC-3') to form a duplex substrate. And then, the treatment of ON1–ON2 duplex substrate with UDG removes uracil bases to generate basic sites on ON2 and then ON1 is liberated. Finally, ON1 is able to fold into a G-quadruplex conformation that is subsequently recognized by fluorescent probe **DID-VP** selectively and giving an enhanced luminescence response.

3.2.2 Optimization of influential factors

Having established the selectivity of **DID-VP** for G-quadruplex motif, we applied the compound in the development of the DNA-based UDG detection assay. In the absence of UDG, **DID-VP** is only slightly emissive due to the weak interaction of the compound with ON1–ON2 duplex substrate. The luminescence signal of **DID-VP** is significantly enhanced in the presence of UDG (**Fig. 2A**) due to the release of ON1 and the subsequent formation of the G-quadruplex structure that interacts with the probe. Nonetheless, the luminescence enhancement of **DID-VP** is not observed under the same conditions for the duplex DNA substrate formed from ON2 and a mutant sequence ON1m (5'-T AGTAGAGA$_3$T$_2$CT$_2$A$_2$GTGCGAGT$_2$GTA-3', base mutants underlined) because the substrate does not have consecutive guanine bases and cannot fold into a G-quadruplex structure after liberation (**Fig. S4**). The results evidently indicate that the luminescence enhancement of the system is dependent on the ability of ON1 folding into a G-quadruplex motif to bind with the probe specifically in the presence of UDG.

A series of control experiments were performed to further validate the mechanism of luminescent switch-on UDG assay. The emission titration experiments (**Fig. S5**), studying the effects of UDG concentration with the **DID-VP** probe in the absence of the duplex substrate, indicate that no significant luminescence enhancement occurred due to no specific interactions between UDG and **DID-VP** probe. In addition, non-denaturing polyacrylamide gel electrophoresis (PAGE) confirms that the duplex substrate is dissociated into discrete bands, which is perfectly matched with the isolated ON1 and ON2 in the control lanes, in the presence of UDG (**Fig. S6**). Circular dichroism (CD) spectroscopy (**Fig. S7 and S8**) also confirmed the formation of ON1 G-quadruplex structure in the assay after the treatment with

UDG.

The assay conditions including **DID-VP** probe concentration, duplex DNA substrate (ON1-ON2) concentration, KCl concentration, ammonium buffer concentration, and pH were further optimized of the assay (**Fig. S9 - S13**). Under the optimized conditions, the relationship on the luminescence response (enhanced emission intensity) of the system towards various UDG concentrations was investigated by using 0.25 μM ON1-ON2 duplex DNA substrate and 2 μM **DID-VP**. As shown in **Fig. 2A** and **B**, the emission intensity is increasing with respect to the concentration of UDG added in the assay and it was found that the emission intensity reaches a plateau when the concentration of UDG used is more than 1.00 U/mL. A good linear correlation for the emission intensity generated and the UDG concentration over the range from 0.05 to 1.00 U/mL is obtained (R^2 = 0.985). The detection limit estimated is 0.005 U/mL based on 3σ. The results exhibit comparable or even better performance compared to the recent reported fluorescent or colorimetric methods.[14, 21]

3.3 Selectivity of the UDG activity assay

The specificity of the present method towards UDG against a number of nucleases including ExoI, ExoIII and T7 was also examined. From the results shown in **Fig. 3A**, the presence of other nucleases, even at a 10-fold higher concentration compared to UDG, there was only very small changes observed on the luminescence signal enhancement of **DID-VP**. This indicates that **DID-VP** is selectively response to G-quadruplex DNA that is produced specifically by the enzymatic activity of UDG. Because of the high sensitivity and specificity of the system, this **DID-VP**/G-quadruplex-based assay can also be further utilized for rapid

screening UDG inhibitors. For a typical study, the uracil-DNA glycosylase inhibitor (UGI) was chosen as a model inhibitor of UDG activity assay. As expected, the luminescence of the system was diminished in the presence of UGI and the relationship was found in a dose-dependent manner (**Fig. 3B**). The results demonstrate that the present method can be further developed as a rapid and low-cost high throughput screening platform for UDG inhibitors for biological applications.

3.4 Detection of UDG content in real serum samples

To further demonstrate the potential of the present method for practical applications, the **DID-VP**/G-quadruplex-based switch-on system was applied to direct detection of trace UDG contents in fetal bovine serum. From **Fig. 4**, the luminescence intensity response with respect to various concentrations of UDG is found to be a good linear correlation over the UDG concentration range from 0.10 U/mL to 1.00 U/mL. Each recovery of serum was determined by comparing the results obtained before and after the addition of standard UDG to the diluted serum samples. The results are listed in Table 2. The recoveries of UDG in the three samples were found 75.07% - 102.70%, which validated the reliability and practicality of this method.

Table 2. Determination of UDG concentration in fetal bovine serum samples (n = 3).

Samples	Spiked (U/mL)	Found (U/mL)	Recovery (%)	R.S.D. (%)
1	0.20	0.15	75.07	8.09
2	0.40	0.37	92.93	6.58
3	0.90	0.92	102.70	3.18

3. Conclusion

In conclusion, we have synthesized a new G-quadruplex-selective compound **DID-VP** and demonstrated its utility by developing a switch-on luminescence system to detect UDG contents in serum samples. This new methodology is rapid, simple and convenient to use. It is able to detect down to 0.005 U/mL of UDG without any signal amplification or fluorescent labeling of oligonucleotides. The sensitivity of this assay is comparable to the recently reported DNA-based detection methods for UDG. Furthermore, we have demonstrated that this method is potentially employed as a screening platform for the identification of UDG inhibitors. This method shows great potential to provide a promising platform for convenient and visualized analysis of trace UDG or other biomolecules.

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Electronic Supplementary information

ESI data including characterizations of new compounds and optimizations of Uracil-DNA glycosylase activity are associated with this article.

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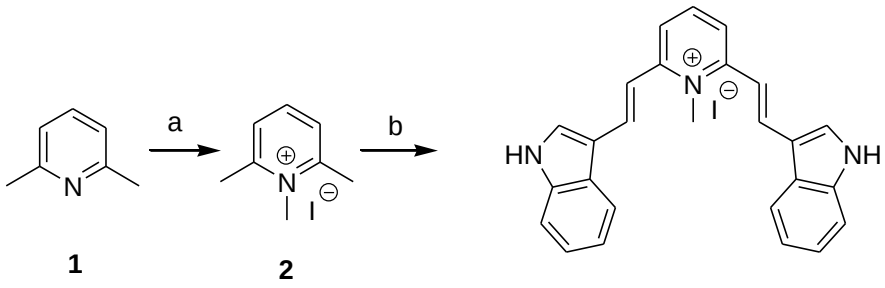
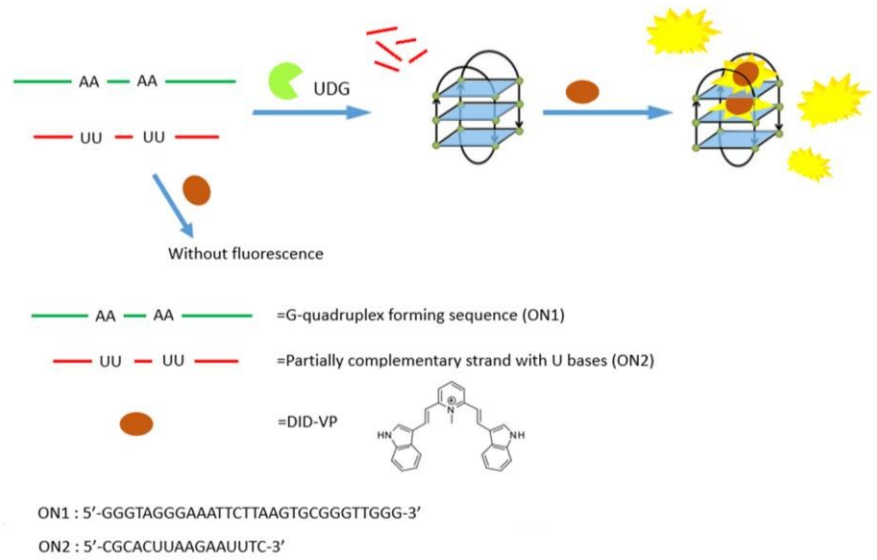
List of Schemes and Figures



1 **2**

2,6-bis((*E*)-2-(1*H*-indol-3-yl)vinyl)-1-methylpyridin-1-ium iodide

Scheme 1. Synthesis of **DID-VP**. Conditions: (a) Iodomethane, Sulfolane, 50 °C, 4h; (b) Indole-3-carbaldehyde, 4-methylpiperidine, n-butanol, 120 °C, 1.5h.



Scheme 2. Schematic diagram of UDG activity assay based on a duplex substrate and a G-quadruplex selective luminescent probe **DID-VP**. U and A denote uracil and adenine nucleobases, respectively.

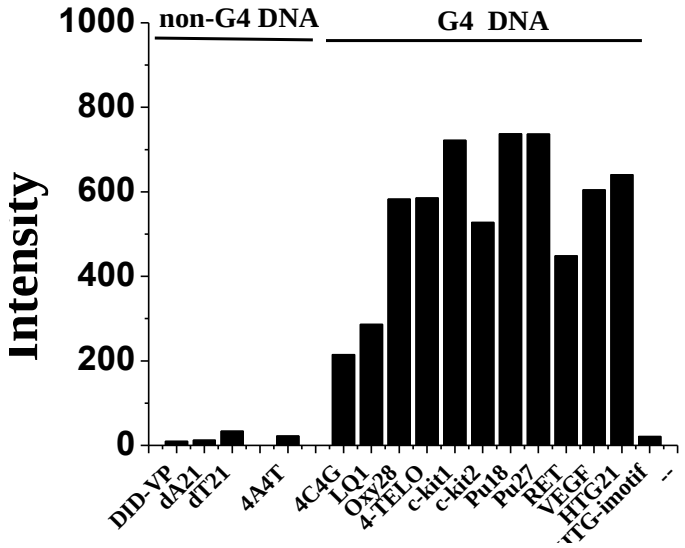


Fig. 1 Fluorescence spectra of 2.5 μM **DID-VP** binding with different common DNA substrates at 2.5 μM.

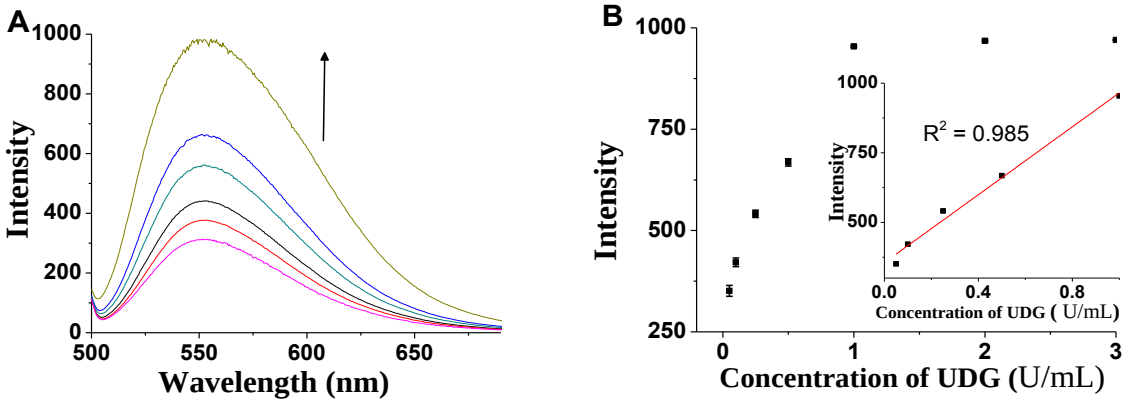


Fig. 2 (A) Fluorescence titration of the **DID-VP**/ON1-ON2 complex with increasing concentrations of UDG (0.05, 0.1, 0.25, 0.5, 1.0, 2.0). (B) Fluorescence change upon interaction of the **DID-VP**/ON1-ON2 complex with different concentrations of UDG. The inset shows the fluorescence signal change in the UDG concentration of 0.05-1.00 U/mL. The measurements were taken with excitation wavelength at λ_{ex} = 486 nm and emission wavelength at λ_{em} = 550 nm. Error bars represent the standard deviations of the results from three independent experiments.

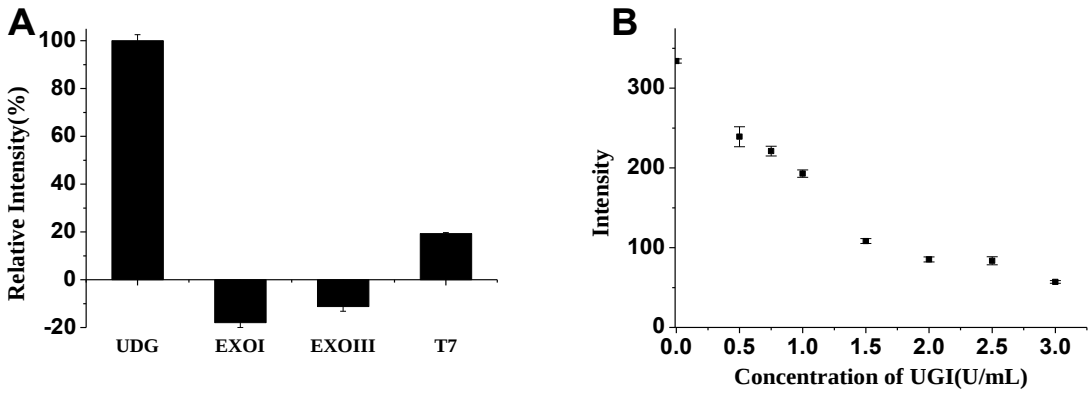


Fig. 3 (A) Luminescence response of the system ([ON1-ON2] = 0.25 μM, [**DID-VP**] = 2 μM) treated with UDG (2.5 U/mL) or 10-fold excess of Exo III, Exo I or T7 (25 U/mL) at 37 °C for 30 min. (B) Luminescence response of the system ([ON1-ON2] = 0.25 μM, [**DID-VP**] = 2 μM) treated with UDG (2.5 U/mL) in the presence of increasing concentrations of UGI (0, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, and 3.00 U/mL).

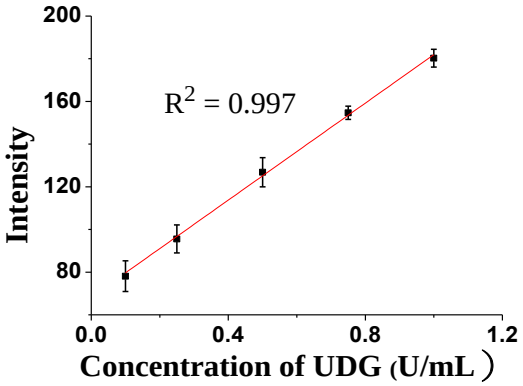


Fig. 4 Luminescence intensity of **DID-VP** at 550 nm versus various concentrations of UDG (0, 0.10, 0.25, 0.50, 0.75, and 1.00 U/mL). The figure shows the linear relationship between the emission signal intensity (I) and the concentration of UDG.