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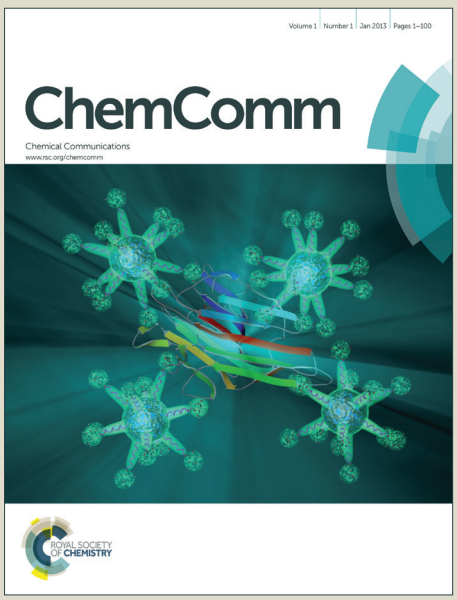

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Fluorescent G-quadruplex probe for the assay of base excision repair enzyme activity

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A sensitive and selective assay of uracil DNA glycosylase (UDG) activity is developed by utilizing G-quadruplex probe incorporating 2-aminopurine (2-AP), based on a novel design that excision reaction promoted by UDG triggers the formation of G-quadruplex structure with significant fluorescence enhancement of 2-AP within the probe.

Exogenous and endogenous agents cause many types of lesions in DNA such as deamination, methylation, oxidation and the complete loss of bases.[1, 2] The base excision repair (BER) pathway regulated by many different types of enzymes including DNA glycosylases, abasic endonucleases, deoxyribophosphodiesterases, DNA polymerases, and DNA ligases, ameliorates spontaneous DNA damage, consequently blocking mutagenesis and maintaining genomic integrity.[3]

In the BER pathway, DNA glycosylases are responsible for initiating the repair process by recognizing and cleaving the damaged or mismatched bases. Among the DNA glycosylases, uracil DNA glycosylase (UDG) specifically recognizes the uracil base that is produced by hydrolytic deamination of cytosine or by the misincorporation of dUTP during DNA replication, and excises the N-glycosidic bond between the deoxyribose and uracil base, leaving an abasic site and triggering subsequent damage repair processes.[3, 4] In this regard, UDG plays pivotal roles in maintaining genomic integrity, which is closely associated with the etiology of various diseases including cancer, aging, and neurodegenerative diseases.[2, 5, 6] Therefore, it is highly desirable to develop efficient methods to determine the UDG activity.

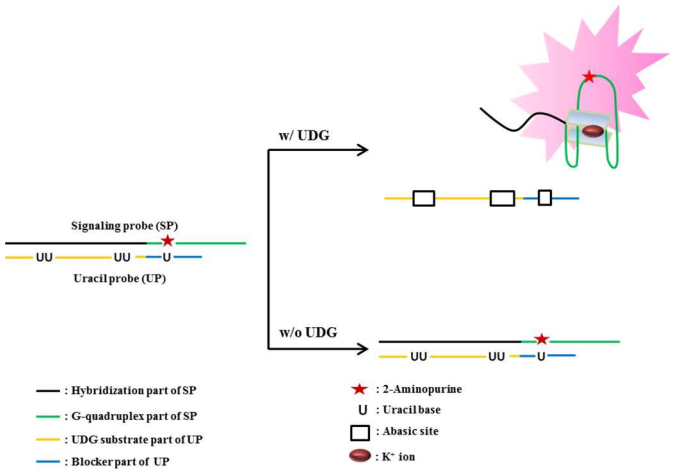
Previously, there have been classical efforts for the detection of the UDG activity, which are based on gel electrophoresis, radioisotopic labeling and streptavidin paramagnetic bead capture techniques.[7-9] However, these assays have some limitations including restricted availability, the additional requirement of a time-consuming separation step, and stringent safety regulations. To overcome these drawbacks, intense interest has recently grown in the development of fluorescence based strategies. Recently developed fluorescent methods could achieve sensitive, simple, and robust assays for UDG activity in a homogenous phase, but there still remain disadvantages to be resolved such as external labeling with both fluorophore and quencher and synthesis of signaling molecules which require high-cost, time-consuming, laborious, and complicated efforts, seriously hampering their practical use.[10-13]

By eliminating the disadvantages of the previous methods, we herein developed a simple yet effective strategy to accurately determine the UDG activity by exploiting G-quadruplex probe incorporating 2-aminopurine (2-AP), a fluorescent nucleobase analog. We particularly focused on the fact that significantly enhanced fluorescent emission of 2-AP occurs upon the formation of the G-quadruplex. This unique property of 2-AP was successfully utilized to achieve a novel strategy to determine the UDG activity, which enables a very convenient operation with a mix-and-detect manner in a homogenous phase.

2-AP is a fluorescent analog of the adenine nucleobase that hybridizes with thymine like natural adenine in Watson-Crick base pairing.[14] The fluorescence of 2-AP is significantly quenched due to the stacking interaction with surrounding bases when it is incorporated into a DNA duplex. Therefore, the dissociation of the DNA duplex into single-stranded DNAs enhances the fluorescence of 2-AP by reducing the stacking interaction. However, the existing interaction with intrastrand neighboring bases still causes slight quenching, leading to the weak fluorescence enhancement of 2-AP. Meanwhile, it has been recently found that the fluorescence intensity of 2-AP, which is site-specifically incorporated into G-rich sequence (i.e., G3T-15), enormously increases and becomes comparable to that of free 2-AP upon the formation of G-quadruplex because the π-stacking within the loops is distorted and electron transfer quenching of 2-AP by guanine cannot occur effectively.[15-17]

Focusing on this unique property of 2-AP, we developed a novel strategy to determine the UDG activity with G-quadruplex probe incorporating 2-AP, a fluorescent nucleobase analog. As schematically illustrated in Scheme 1, the duplex DNA used in this strategy is composed of a signaling probe (SP) and a uracil probe (UP) that are rationally designed to achieve sensitive assay for the UDG activity. The SP consists of a hybridization part (black region) complementary to the UDG substrate part of the UP and the G-quadruplex part (green region with red star) containing the 2-AP in a G-rich sequence. The UP consists of the UDG substrate part (yellow region) containing four uracil residues and the blocker part (blue region) which partially binds to the G-quadruplex part in the SP and prevents the formation of the G-quadruplex.

In the absence of UDG, the G-rich sequence of the SP remains



Scheme 1 Schematic illustration of the UDG activity assay based on the excision induced G-quadruplex formation with significant fluorescence enhancement of 2-AP.

hybridized with the UP, and its formation of the G-quadruplex is blocked, consequently resulting in a weak fluorescence emission from 2-AP. However, in the presence of UDG, it catalyzes the hydrolysis of the uracil bases in the UP, resulting in the dissociation of the duplex DNA by reducing the stability of the double-stranded (ds) state. The blocker part of the UP can no longer cage the G-quadruplex part of the SP, thus facilitating the formation of the G-quadruplex upon interaction with K^+ ions. As a consequence, 2-AP in the G-quadruplex generates a remarkably enhanced fluorescence signal. The formation of the G-quadruplex structure in the dissociated strand in the presence of K^+ ions occurs very quickly within a few minutes (Fig. S1, ESI†).

The effect of modification of 2-AP on the stability of G-quadruplex probe incorporating 2-AP (SP1-UP3 duplex) was first investigated through melting curve analysis (Fig. S2, ESI†). The optimal conditions required to efficiently analyze the UDG activity were then determined by examining the fluorescence intensities resulting from the 2-AP. The results of experiments in which the length of the blocker part in the UP and the concentration of the K^+ ions were varied, show that UP3 with a 7-bp long blocker part and a K^+ ion concentration of 10 mM are ideal for the efficient analysis of the UDG activity (Fig. S3 and S4, ESI†).

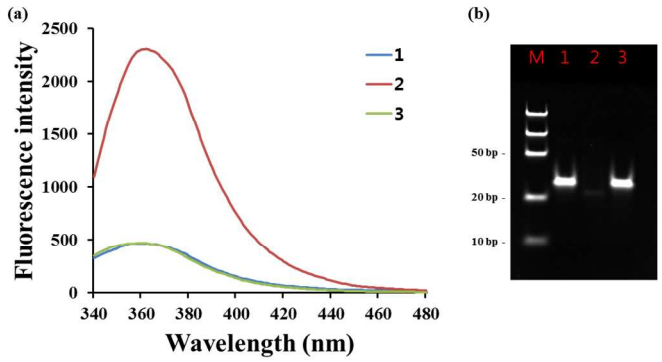


Fig. 1 UDG activity assay based on the excision induced G-quadruplex formation. (a) Fluorescence emission spectra of 2-AP in the UDG activity assay (1: SP1-UP3 duplex without UDG, 2: SP1-UP3 duplex with UDG, 3: SP1-TP3 duplex with UDG). (b) Polyacrylamide gel electrophoresis image of the resulting products after UDG treatment (lane M: DNA size marker, 1: SP1-UP3 duplex without UDG, 2: SP1-UP3 duplex with UDG, 3: SP1-TP3 duplex with UDG). The final concentrations of the SP1-UP3 duplex, SP1-TP3 duplex, and UDG are 500 nM, 500 nM, and 5 U/ml, respectively.

Under optimal conditions, we first verified the feasibility of the present strategy by measuring the fluorescence signals coming from the formation of the G-quadruplex induced by excision. As expected, in the absence of UDG, a weak fluorescence emission was exhibited (1, Fig. 1(a)). In contrast, a significant increase in fluorescence signal at 360 nm, which is the peak emission wavelength of 2-AP, was observed in the presence of UDG which facilitates the formation of the G-quadruplex by removing the uracil bases within the UP and dissociating the SP1-UP3 duplex into ss DNAs (2, Fig. 1(a)). This greatly enhanced fluorescence signal is also quite beneficial to mask and overcome the possible influence of undesired autofluorescence from biomolecules present in the biological samples.[18]

To demonstrate that the detection principle relies on the catalytic activity of UDG hydrolyzing the uracil bases, a SP1-TP3 duplex in which the uracil residues in the SP1-UP3 duplex are replaced with thymine residues, was designed and treated with UDG. In this case, a weak fluorescence signal comparable to that of the SP1-UP3 duplex in the absence of UDG (1, Fig. 1(a)) was emitted despite the presence of UDG (3, Fig. 1(a)). This result indicates that the remarkable enhancement in fluorescence intensity originates exclusively from the catalytic activity of UDG.

Fig. 1(b) shows the results from polyacrylamide gel electrophoresis (PAGE) analysis of the products obtained after treatment with UDG. Without UDG, the ds form of the SP1-UP3 duplex was maintained, as evidenced by a single intense band (Lane 1, Fig. 1(b)). Additionally, the SP1-TP3 duplex was not dissociated even in the presence of UDG, and a single intense band appeared at the same position with that of lane 1 (Lane 3, Fig. 1(b)). In the case of the SP1-UP3 duplex, however, the presence of UDG dissociated the SP1-UP3 duplex, and the band corresponding to the duplex DNA disappeared. Instead, a weak band was observed at a lower position, which corresponds to the dissociated ss DNAs (Lane 2, Fig. 1(b)). In addition, circular dichroism (CD) spectra were also recorded to examine the conformational changes of probes after treatment with UDG (Fig. S5, ESI†). The results from PAGE and CD analyses are quite consistent with the spectral data and again ensure the feasibility of the proposed strategy.

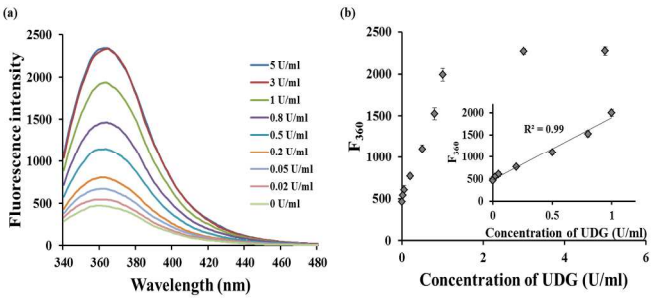


Fig. 2 Sensitivity of the UDG activity assay. (a) Fluorescence emission spectra from 2-AP after treatment with UDG at varying concentrations. (b) Fluorescence intensities at 360 nm from 2-AP in the presence of UDG at varying concentrations. Inset: linear relationship between fluorescence intensity and UDG concentration. The final concentration of SP1-UP3 duplex is 500 nM.

The detection sensitivity of this sensing system was then determined by measuring the fluorescence intensities at 360 nm as a function of target UDG concentrations (Fig. 2). The data show that the fluorescence intensities increase with increasing concentrations of UDG up to 3 U/ml, but reach a plateau at concentrations greater than 3 U/ml (Fig. 2(a)). Quantitative analysis with triplicate experiments exhibits that an excellent linear relationship exists in the

range of 0~1 U/ml with relatively low standard deviations, and the limit of detection (LOD) (3σ/slope) is ca. 0.02 U/ml, a value that is lower than or comparable to those from previous reports for the determination of the UDG activity (Fig. 2(b)).[10, 11, 13]

Next, the specificity of the new detection system towards UDG was investigated. For this purpose, the abilities of other DNA repair enzymes (hAAG and hOGG1) and nuclease (Exo I) to induce a high fluorescence enhancement were examined and compared with that of UDG. As seen in Fig. 3, a significant fluorescence enhancement is

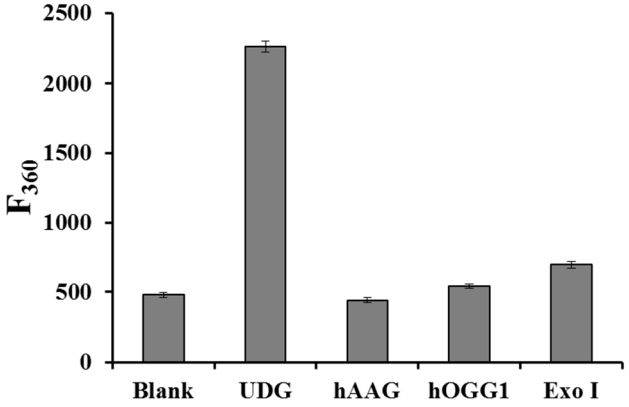


Fig. 3 Specificity of the UDG activity assay. Fluorescence responses after treatment with UDG (5 U/ml), other DNA repair enzymes (50 U/ml), and nuclease (50 U/ml). The final concentration of the SP1-UP3 duplex is 500 nM.

observed only when the sample is treated with UDG. In contrast, the other enzymes result in relatively low signals even though they are present at ten times higher concentrations than that of the UDG. This excellent selectivity is attributed to the specific recognition and removal of uracil bases by UDG, which leads to the subsequent formation of the G-quadruplex.

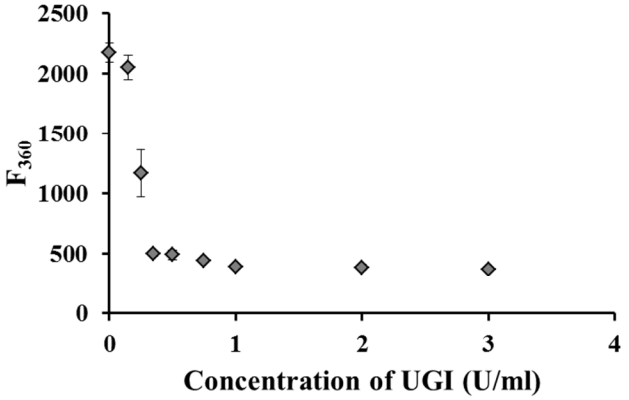


Fig. 4 Screening assay for the UDG inhibitor. Fluorescence intensities at 360 nm from 2-AP in the presence of UGI at increasing concentrations (0, 0.15, 0.25, 0.35, 0.5, 0.75, 1, 2, and 3 U/ml). The final concentrations of the SP1-UP3 duplex and UDG are 500 nM and 5 U/ml, respectively.

Finally, we demonstrated the capability of our strategy to screen candidate UDG inhibitors, which is of great importance because UDG inhibitors could be used as therapeutic agents in the treatment of several diseases including various cancers and AIDS.[19, 20] In this experiment, uracil DNA glycosylase inhibitor (UGI) was selected as a model inhibitor of UDG. Fig. 4 shows that the intensities of the fluorescence signal decrease with increasing concentrations of UGI. This result confirms that the developed strategy has great potential to be employed as a cost-effective and rapid platform for screening UDG inhibitors.

Conclusions

In this work, we developed a novel strategy for the sensitive determination of UDG activity by utilizing G-quadruplex probe incorporating 2-AP, a fluorescent nucleobase analog. The use of 2-AP as a signaling unit enables a cost-effective and convenient assay for UDG activity, and thus renders this method more substantially advantageous than previous ones which require laborious time-consuming external labeling and synthesis of signaling molecules. In addition, this approach is highly sensitive and selective due to the significant fluorescence enhancement of 2-AP upon the formation of the G-quadruplex and the specific catalytic activity of UDG. To the best of our knowledge, this study is the first report which exploits 2-AP as a G-quadruplex selective switch-on fluorescent molecule to develop biosensors. Furthermore, this strategy has a great potential to serve as a universal detection platform for the analysis of other BER enzyme activities by rationally designing the DNA probes to have the corresponding damaged bases (Fig. S6, ESI†).

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Notes and references

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