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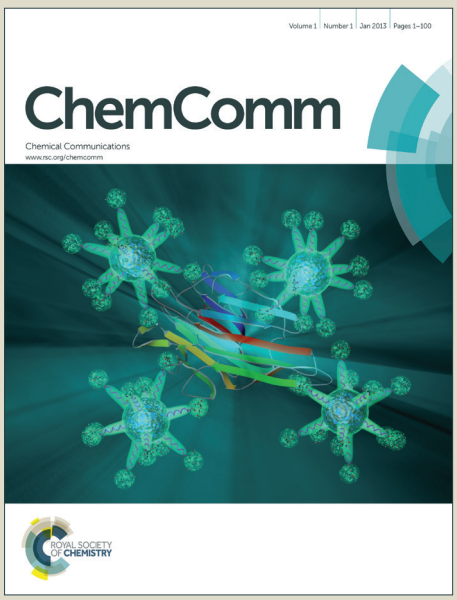

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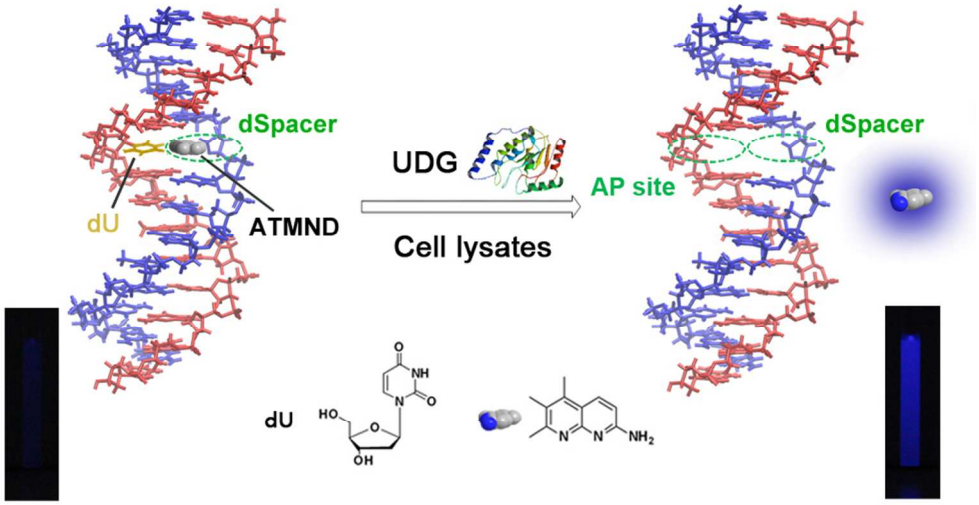

A label-free fluorescent method has been developed for sensitive detection of uracil-DNA glycosylase activity as well as UDG inhibitors.
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A label-free and sensitive fluorescent method for the detection of uracil-DNA glycosylase activity

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth XXX 201X,
Accepted ooth XXX 201X

DOI: 10.1039/xoxxoooooox

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The activity of uracil-DNA glycosylase (UDG), an enzyme in the base excision repair, is detected at a high sensitivity by a DNA substrate containing only one uracil through a label-free fluorescent approach, which is also successfully applied for the measurement of UDG inhibitors.

Enzymes participating in the repair of DNA lesions are essential for organisms to maintain the integrity of their genetic information.[1, 2] Of particular interest are DNA glycosylases in the base excision repair (BER) pathway that removes damaged DNA bases and generates apurinic/apyrimidinic (AP) sites in DNA for the downstream BER repair processes by other enzymes.[1, 3] Uracil-DNA glycosylase (UDG) is one of the most important BER enzymes responsible for the exclusion of uracil from cellular DNA.[4, 5] Because of the indispensable roles of UDG in BER pathway and gene regulation, as well as relevant human diseases such as Bloom syndrome and chemotherapy resistance,[6, 7] the methods for the detection of UDG activity are highly demanded for its biochemical studies and the development of drugs for UDG-related diseases.

Traditional methods for UDG detection require complicated radioactive labelling, time-consuming electrophoresis or surface-based separations.[8-11] In contrast, fluorescent probes developed in recent years for UDG activity assays based on uracil-containing oligonucleotides are simpler, faster and capable of monitoring UDG-catalyzed reactions in real time.[12] For instance, pairs of fluorophores and quenchers were attached at the ends of uracil-containing DNA substrates to measure UDG activity via fluorescence enhancement.[13-15] Nanomaterials including graphene oxide and gold nanoparticles were also utilized as quenchers and colorimetric reporters, respectively, for optical UDG assays.[16, 17] Besides, the activation of uracil-containing DNAzymes by UDG was applied for more sensitive detection of UDG activity through DNAzyme-based signal amplifications.[18, 19] Particularly, by incorporating an artificial nucleotide such as 2-aminopurine[20, 21] or pyrene[22, 23] adjacent to uracil in DNA substrates, fluorescent monitoring of UDG activity was achieved without the use of quenchers, and remarkably the pyrene-based fluorescent probe was successfully applied for the imaging of UDG activity inside living cells.[22, 23]

Except for the above methods that require labelling signal reporters covalently, label-free fluorescent approaches have also been developed for the detection of UDG.[24] Compared to the labelled counterparts, the label-free probes do not need covalent attachment of fluorophores or quenchers to DNA, thus making the detection even simpler, low-cost, and minimizing the effect of labels on the activity of UDG. For example, uracil-containing DNA substrates that underwent UDG-induced formation of G-quadruplex in the presence of porphyrin or metal complex were utilized for the detection of UDG activity based on fluorescence enhancement,[25-27] which was resulted from the binding of the organic compounds to the G-quadruplex.

Nevertheless, these label-free fluorescent approaches for UDG activity detection,[25, 26] as well as many labelled probes reported before,[13-18] are based on simultaneous removal of multiple uracil bases to induce the conformational changes of DNA for signal transduction. Such DNA substrates containing multiple uracil sites are efficient but may make the estimation of UDG activity relatively complicated, because it can give little signal even if some (not all) of the uracil sites in each DNA substrate are removed but insufficient to induce the DNA conformational changes.

In this work, to overcome the above challenges, a label-free fluorescent method for the detection of UDG activity based on the removal of one uracil in each DNA substrate has been developed (Figure 1A). A dSpacer[28-33] (1',2'-Dideoxyribose, an artificial non-base spacer in DNA, abbreviated as "dSp" and shown in the blue structure in Figure 1A) binding fluorophore, named 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND), is allowed to bind the dSpacer opposite to a uracil site in the DNA duplex (DNA-U/DNA-X) through 3 complementary hydrogen bonds and π-π stacking.[28-33] ATMND undergoes fluorescence quenching by adjacent bases when binding to the dSpacer. Upon the removal of uracil by UDG to generate an AP site (2'-deoxyribose, shown in the yellow structure in Figure 1A), ATMND dissociates from the dSpacer due to the disruption of

hydrogen bonds in the absence of the uracil, thus the quenched fluorescence will recover to induce fluorescence enhancement.

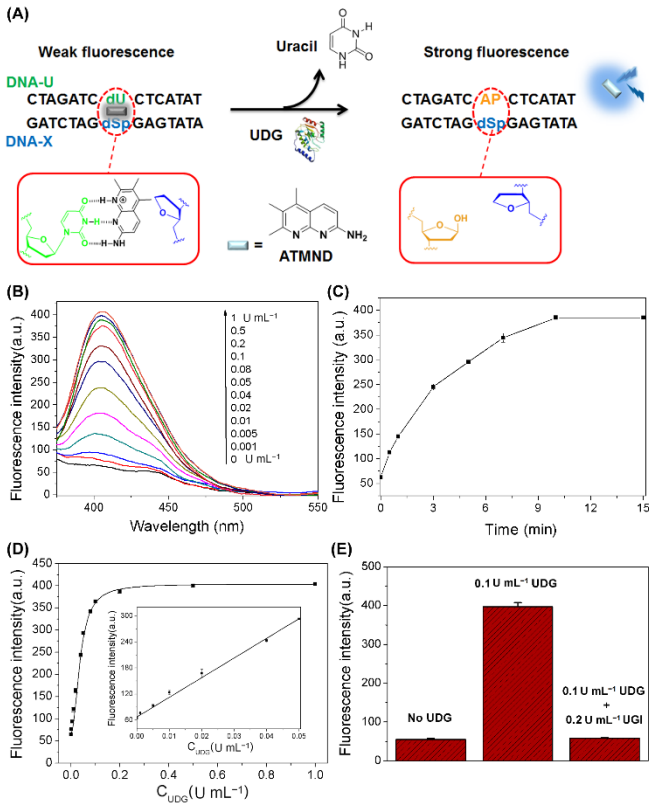

Figure 1. (A) Scheme of the label-free fluorescent method for the detection of UDG activity in solution and cell lysates. (B) Fluorescence spectra of ATMND binding to DNA-U/DNA-X in the presence of different amounts of UDG. (C) Kinetics of fluorescence enhancement of ATMND binding to DNA-U/DNA-X in the presence of 0.1 U mL^{-1} UDG. (D) Measurement of UDG activity in solution by fluorescence enhancement. (E) Inhibitory effect of UGI on the fluorescence enhancement of ATMND binding to DNA-U/DNA-X in the presence of UDG.

To test our hypothesis, the design in Figure 1A was used for the detection of UDG activity in solution by monitoring the fluorescence enhancement of ATMND. In a reaction buffer containing 50 mM NaCl, 0.5 mM EDTA and 50 mM Tris-HCl at pH 7.0, ATMND (0.5 μM) was added to the DNA duplex DNA-U/DNA-X (1 μM) made of a uracil-containing DNA-U (1 μM) and a dSpacer-containing complementary DNA-X (1 μM). After the addition of UDG at different concentrations to the mixture solution and followed by incubation at 37 °C (the optimal temperature for UDG activity) for 10 min, the fluorescence was then measured at 5 °C (to quench UDG activity). As shown in Figure 1B, in the absence of UDG, the fluorescence of ATMND was weak because of the binding of ATMND to the DNA duplex. However, in the presence of increasing amounts of UDG up to 1 U mL^{-1}, the uracil removal caused the dissociation of ATMND from the DNA duplex and enhanced the fluorescence to about 8 folds. UDG alone was found to affect little of the ATMND fluorescence (Figure S1, ESI†). The kinetics of the fluorescence enhancement in the presence of 0.1 U mL^{-1} UDG were studied within 15 min (Figure 1C), indicating the completion of reaction in 10 min. The effects of pH and ionic strength on the fluorescence enhancement were also investigated, suggesting the maximum fold of enhancement was reached at pH 7.0 and 50 mM NaCl (Figure S2 and S3, ESI†). Under this optimal condition, the detection of UDG activity was carried out. According to the standard curve established from the results shown in Figure 1D, the method was found capable of detecting UDG activity as low as 0.0008 U mL^{-1} based on 3σ_b/S, in a range of at least 0.0008~0.1 U mL^{-1}. This sensitivity was comparable or even better than those in the previously reported fluorescent methods for UDG activity assays,[13-21, 25-27, 34] most probably ascribed to its characteristics of one uracil removal in each DNA substrate without the need of DNA conformational changes. The results of polyacrylamide gel electrophoresis (PAGE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis on the DNA duplex in the absence and presence of UDG clearly showed that the uracil in DNA-U was removed by UDG regardless of the presence of DNA-X or ATMND (Figures S4~S6, ESI†), supporting our proposed mechanism in Figure 1A. To confirm the fluorescence enhancement was indeed induced by UDG, a uracil glycosylase inhibitor protein (UGI) was added to the solution to eliminate the activity of UDG before the addition of UDG to the DNA substrate. No fluorescence enhancement was observed for a solution containing ATMND and the DNA duplex when UGI and UDG were present at 0.2 and 0.1 U mL^{-1} respectively (Figure 1E), demonstrating the indispensable role of UDG activity for the fluorescent enhancement. The design was also successfully simplified by using a "vacant site"[32] approach, where two DNA substrates (DNA-Hairpin and DNA-Vac) without any dSpacer (e.g. all natural nucleotides) displayed 2-fold fluorescence enhancement of ATMND in the presence of UDG (Figure S7, ESI†).

In addition to buffer solution, we performed the detection of UDG activity in eukaryotic cell lysates to investigate whether the method could also quantify UDG from eukaryotic cells compared with that from recombinant *E. Coli* in the above experiments, as well as whether the detection was tolerant toward cellular components such as large amounts of other proteins. MCF-7 and HeLa cells were washed to remove culture media and then lysed using a standard lysing buffer to prepare the lysates. Different amounts of such cell lysates were then diluted into large volumes of the reaction buffer to serve as the samples for measuring UDG activity therein. As shown in Figure 2A, both cell lysates induced fluorescence enhancement due to the presence of UDG activity, and UGI was also added to the cell lysates to confirm that the fluorescence enhancement was solely generated by UDG rather than any other component in the lysates. Because the dilution factor was large and the standard curve for the quantification of UDG activity was found independent on the amount of lysis buffer or proteins added to the reaction buffer (within the dynamic ranges of Figure 1D in this work), the activity of UDG in the cell lysates can then be measured and calculated based on the slopes of the stand curve in Figure 1D and the curves in Figure 2B and 2C. The total amounts of protein in the cell lysates were determined by a BCA protein assay kit to serve as references. We found that the UDG activities in lysed MCF-7 and HeLa cells were determined as 0.286 and 0.144 U mg^{-1} (U UNG per mg total protein) in our study, respectively. Moreover, another culture of HeLa cells were lysed and tested using the standard addition method (Figure 2D), which gave the results of 0.208 U mg^{-1}, close to the above 0.144 U mg^{-1} and with some understandable difference between different batches of cells. Since the UDG activity in cell lysates was rarely measured quantitatively in previous reports using either classic methods or fluorescent methods according to our literature search, currently we cannot find any known data of UDG activity in cells to compare them with the results from our method.

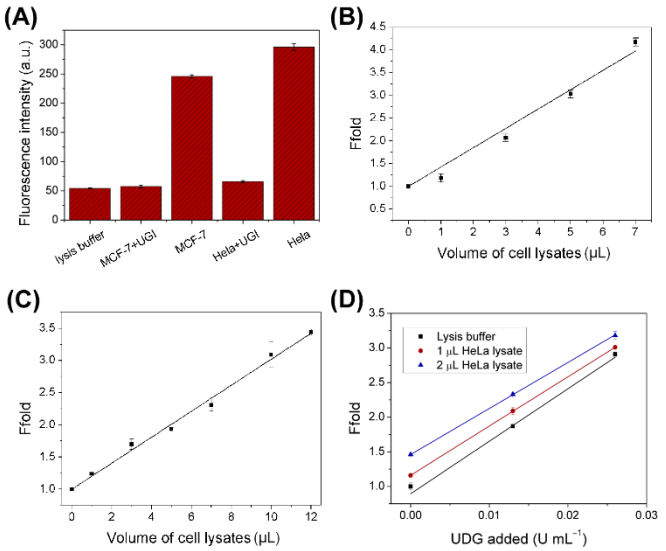

Figure 2. (A) Fluorescence enhancement of ATMND binding to DNA-U/DNA-X in the absence and presence of MCF-7 and HeLa cell lysates, and the inhibitory effect of UGI on the UDG activity in the cell lysates. (B) Fluorescence enhancement of binding to DNA-U/DNA-X in the presence of different amounts of MCF-7 cell lysates. (C) Fluorescence enhancement of binding to DNA-U/DNA-X in the presence of different amounts of HeLa cell lysates. (D) Fluorescence measurement of UDG activity in HeLa cell lysates by the standard addition method.

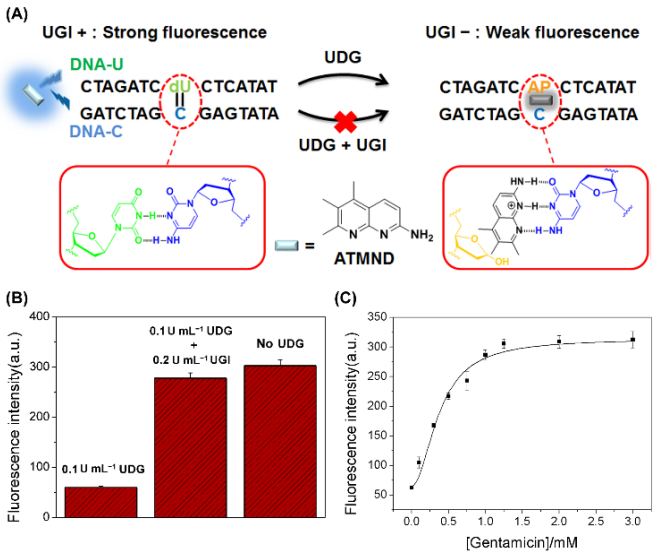

Figure 3. (A) Scheme of the method for the detection of UDG inhibitors via fluorescence enhancement of ATMND. (B) Fluorescence enhancement of ATMND induced by UGI for a sample containing DNA-U/DNA-C and UDG. (C) Measurement of IC_{50} for gentamicin as a small molecular UDG inhibitor.

Besides the detection of UDG, our method based on ATMND and dSpacer-containing DNA substrates was also converted into another design for the determination of UDG inhibitors, which are useful biochemical tools and potential drugs.[35-37] The original design in Figure 1A underwent fluorescence enhancement in the presence of UDG, thus it displayed fluorescence quenching accordingly when UDG inhibitor were present. A fluorescence enhancement response is usually more preferred in comparison with a quenching one, because the former is thought to be more resistant to sample matrix and fluorophore bleaching than the latter. To develop a label-free method for the detection of UDG inhibitors via fluorescence enhancement, we replaced the dSpacer opposite to the uracil (Figure 1A) by a cytosine (Figure 3A). In such a design, ATMND cannot bind to the DNA duplex until the uracil is removed by UDG to generate an AP site opposite to the cytosine. As shown in Figure 3A, upon the binding of ATMND to the AP site through three complementary hydrogen bonds with the cytosine and π-π stacking,[28-33] the fluorescence of ATMND was quenched. However, in the presence of UDG inhibitors, the activity of UDG was inhibited so that the binding of ATMND to DNA and the fluorescence quenching should be prohibited to rescue fluorescence. Based on this design, the inhibitory activities of UGI (a protein-based UDG inhibitor) as well as gentamycin (a small molecular inhibitor of UDG) were both successfully detected via a fluorescence enhancement response, as illustrated in Figure 3B and 3C, respectively. An IC_{50} of 0.42 mM was calculated from the result of UDG inhibition by different concentrations of gentamicin (Figure 3C), which is in the same order of magnitude compared with the findings in another report,[34] with some difference mainly because of the different buffer conditions or different DNA substrates. These results demonstrate the promise of our approach as a simple method for the determination of UDG inhibitors via fluorescence enhancement.

In summary, the label-free fluorescent method developed in this work has been successfully applied for the detection of UDG activity in both solution and cell lysates. The method is based on the removal of only one uracil in each DNA substrate by UDG, which perturbes the binding of ATMND to the DNA substrate and resulted in fluorescence enhancement for UDG detection. A high sensitivity capable of detecting 0.0008 U mL^{-1} UDG has been achieved by the method, owing to its advantage of label free and one uracil removal compared with other reported fluorescent methods. By replacing the dSpacer with a cytosine, the determination of UDG inhibitors via fluorescence enhancement is also successful. Because of the availability of other fluorophores that can non-covalently bind dSpacer sites opposite to different lesion bases in DNA substrates, the method should be generally applicable for the detection of other DNA glycosylases in the BER pathway.

Acknowledgement

We thank for the financial support from the National Natural Science Foundation of China (Nos. 2142200199, 21390410 and 21375074), the National Key Scientific Instrument and Equipment Development Plan (No 2012YQ030111), the Tsinghua University Initiative Scientific Research Program (No. 20131089220), and the Recruitment Program of Global Youth Experts of China.

Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental details and additional figures]. See DOI: 10.1039/c000000x/

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