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A highly sensitive strategy for base excision repair enzyme activity detection based on graphene oxide mediated fluorescence quenching and hybridization chain reaction

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A novel fluorescent nanosensor has been developed by combining super fluorescence quenching ability of graphene oxide and hybridization chain reaction amplification, which enables highly sensitive detection of base excision repair enzyme activity with a wide dynamic range from 0.0001 to 100 U/mL and a detection limit of 0.00006 U/mL.

Base excision repair (BER) enzymes play vital roles in maintaining the integrity of the genomes. BER is the major pathway that acts throughout the cell cycle to remove damaged DNA bases and generate abasic site (AP site). Then the repair process is completed by coordinating with AP endonucleases, deoxyribophosphodiesterases, DNA polymerases and DNA ligases. Uracil-DNA glycosylase (UDG) is one of the most important BER enzymes responsible for removing uracil residues from DNA strand by catalyzing the hydrolysis of the N-glycosyl bond joining the uracil base to the deoxyribose phosphate backbone. Since the uracil excision repair process plays an essential role in genetic integrity and related diseases such as type 1 human immunodeficiency virus and Bloom syndrome, strategies for detecting the activity of UDG sensitively and selectively are in urgent need, especially for the study of mechanisms and functions of many fundamental biochemical processes.

Traditional approaches for activity screening of UDG include radioactive labeling, gel electrophoresis, chromatography and mass spectrometry. However, these methods are time-consuming, operationally tedious and indirect due to the requirement of sophisticated instrumentation or additional separation techniques. In order to overcome these limitations, fluorescence-based strategies have attracted great attention for their intrinsically high sensitivity, simplicity, and ease of operation. In the setting, there are some emerging methods based on fluorescence resonant energy transfer (FRET) via using molecular beacon or fluorophore-labeled oligonucleotides for activity screening of UDG. Performed in homogeneous phases, these methods are generally simple, robust and scalable for high-throughput parallel analysis of hundreds of samples. However, these UDG assays, because of the lack of signal amplification steps, typically show limited sensitivity. Coupling of enzymatic amplification with UDG reactions, though affording enhanced sensitivity, may have difficulty in the compatibility of enzymes in a single reaction. The pursuit of enzyme-free amplification for highly sensitive UDG assays has been rarely explored.

The concept of hybridization chain reaction (HCR) has attracted significant research interest in the sensor design because of its capability of signal amplification with no need of enzymes. By coupling HCR signal amplification with various molecular probes, including functional nucleic acid probes, antibodies, and functionalized nanoparticles, sensitive biosensors have been successfully applied to the detection of various targets such as molecules, nucleic acids and metal ions. Recent development of using graphene oxide (GO) for selective fluorescence quenching of HCR products have provided a simple, cost-efficient and high signal-to-background platform for sensing HCR amplification cascades. Our group has also made effort in developing HCR-based biosensors for diverse biological applications.

Herein, we report for the first time the development of a novel approach for highly sensitive and selective detection of UDG activity by combining HCR amplification and GO-based fluorescence quenching platform, as illustrated in Scheme 1. This approach shows superior sensitivity with a detection limit of 0.00006 U/mL. To our knowledge, it affords the most sensitive approach to UDG assay. In this sensing system, we designed three probes, including a helper hairpin probe (HP), a second hairpin probe, hairpin probe 1 (H1) and fluorophore-labeled hairpin probe 2 (H2), for HCR amplification. The helper hairpin probe HP contains four uracil bases in its stem which can be excised by UDG. In the absence of UDG, the initiator for the HCR is blocked in the HP probe, and no HCR amplification is achieved between two other hairpin probes, as illustrated in Scheme 1.
The concentration of HP as an initiator for HCR. In contrast, uracil excision of the HP probe, with a fluorophore (FAM), can be adsorbed on the GO surface via its loop and sticky end through π-π stacking interaction, thereby displaying a substantially quenched fluorescence signal. In contrast, upon uracil excision by UDG, AP sites are produced in the HP probe, which significantly decrease the self-folding stability of the probe and induce a single-stranded conformation for the probe. This conformation change activates the initiator, which, in turn, triggers the HCR between two other hairpin probes, a non-labeled probe H1 and a fluorophore-labeled probe H2, and generates a chain-like double-stranded DNA (dsDNA) assembly with multiple fluorophore labels. The dsDNA HCR product will not be adsorbed on GO nanosheets due to the nucleobases in dsDNA are effectively hidden in helical structure, which prevents the π-π stacking interaction of nucleobases with GO surface. Therefore, a strong fluorescence signal is obtained as a quantitative indicator for the activity of UDG.

Fig. 1 Typical fluorescence spectra of UDG analysis. (a) H2 + GO, (b) H1 + H2 + GO, (c) UDG + H1 + H2 + GO, (d) HP + H1 + H2 + GO, (e) UDG + HP + H1 + H2 + GO, (f) UDG + HP + H1 + H2 + GO, (g) HP. The concentrations of UDG, UGI, HP, H1, H2, and GO were 100 U/mL, 100 U/mL, 50 nM, 100 nM, 100 nM, and 20 µg/mL, respectively. That the fluorescence enhancement only depended on active UDG that mediated uracil removal. Further agarose gel electrophoresis analysis revealed that only the active UDG treatment gave a long dsDNA of HCR product while no HCR product was obtained without UDG reaction (Fig. S1, ESI†). These results implied that the HCR was specifically triggered by UDG-excised HP, which demonstrates that the proposed method can be used for selective detection of UDG activity.

In order to achieve the best assay performance, we optimized reaction time of UDG, the HP concentration, as well as the concentration of GO nanosheets. It was observed that the fluorescence intensity ratio (F/F0, where F0 and F are the fluorescence signals in the absence and presence of UDG, respectively) increases with time and reaches a plateau at 60 min (Fig. S2, ESI†). Therefore, the optimal reaction time of UDG was chosen to be 60 minutes and used throughout subsequent experiments. According to the dependency of the F/F0 of the UDG assay on the concentration of HP (Fig. S3, ESI†), an optimal fluorescence signal ratio was obtained with the HP concentration of 50 nM. So, the optimal HP concentration was chosen to be 50 nM. It was also found that the F/F0 ratio gradually increased with an increasing concentration of GO nanosheets up to 20 µg/mL while became decreased with a further increasing concentration of GO nanosheets (Fig. S4, ESI†). This finding was attributed to the weak adsorption of dsDNA HCR product on the excessive amount of GO nanosheets. Therefore, the GO nanosheet concentration of 20 µg/mL was chosen for further experiments to reach the desired sensitivity and selectivity.

Under the optimized experimental conditions, the performance of the developed strategy for quantitative activity screening of UDG was further investigated. As shown in Fig. 2A, a dramatic increase in the fluorescence intensity was observed with the increasing concentration of UDG. A quite wide dynamic range from 0 to 100 U/mL was achieved for this assay. The calibration curve for detection of UDG activity is shown in Fig. 2B, the calibration equation was F = 2888 – 2771 / (1 + 4697 C)^1/2, with a squared correlation coefficient R^2 of 0.9982, where F is the fluorescence intensity and C is the concentration.
Fig. 2 (A) Typical fluorescence spectra upon addition of different concentrations of UDG (from bottom to top: 0, 0.0001, 0.0002, 0.0005, 0.001, 0.005, 0.01, 0.1, 1, 10, 100 U/mL). (B) Fluorescence peak intensities versus UDG concentrations. Inset: linear relationship between the fluorescence intensity and the logarithm of UDG concentration. Error bars are standard deviations of three repetitive experiments.

of UDG. Moreover, the fluorescence intensity F was found to have a linear correlation to the logarithm of UDG concentration C in the range from 0.0001 U/mL to 100 U/mL. The calibration equation was $F = 896 + 190 \lg C$ with a squared correlation coefficient $R^2$ of 0.9962. The detection limit was estimated to be 0.00006 U/mL according to the 3σ rule, which was lower than existing assays. These results indicated that the proposed method could be used for highly sensitive detection of the UDG activity.

Furthermore, we performed a series of contrast experiments using non-specific protein BSA and another BER enzyme 8-oxoguanine DNA glycosylase (hOGG1) to evaluate the specificity of the proposed UDG assay (Fig. S5, ESI†). It was found that only marginal fluorescence changes were obtained with the addition of these proteins compared to the blank. These results suggested that the proposed method had high selectivity for UDG detection.

In order to demonstrate the feasibility of this method for screening potential UDG inhibitors, uracil DNA glycosylase inhibitor (UGI) was selected as a model inhibitor for this study. UGI was reported to be produced by the Bacillus subtilis bacteriophage PBS1 and form an extremely specific protein complex with a 1:1 UDG:UGI stoichiometry. When binding to UGI, the activity of UDG was inhibited and it was unable to excise the uracil bases in HP. As shown in Fig. 3, the fluorescence signals were found to decrease with the increasing concentrations of UGI, the calibration equation was $F = 231 + (25 / C) + (-0.2 / C^2) + (0.0002 / C^3)$, with a squared correlation coefficient $R^2$ of 0.9976, where F is the fluorescence intensity and C is the concentration of UGI. The result gave direct evidence that the proposed assay held the potential for quantitative screening UDG inhibitors.

To determine whether the developed method could be applied in real complex biological samples, we measured extracts from HeLa cell lines (Fig. S6, ESI†). It was observed that when the HeLa cell lysates were incubated in the assay system, a dramatic increase in the fluorescence intensity was observed. Moreover, the fluorescence intensity gradually increased with increasing the reaction time as well as the number of the cell lines. In a control experiment, the HeLa cell lysates were incubated with UGI in the assay system, we obtained a very low fluorescence signal and had no remarkable change with increasing the reaction time. The results suggested that the fluorescence changes in the assay system were specifically responsive to UDG present in the cell lysates. These results indicated that the proposed method was capable of screening UDG activity sensitively in real complex samples.

In summary, we have developed a novel approach for highly sensitive and selective detection of UDG activity by combining HCR amplification and GO-based fluorescence quenching platform. Compared with other UDG assay methods, this method showed higher sensitivity and lowered fluorescence background because of the efficient signal amplification of HCR and the superb fluorescence quenching efficiency of GO nanosheets. The detection limit is 0.00006 U/mL, which affords the most sensitive approach to UDG assay. This strategy can also be used to evaluate the inhibition of the UDG activity. In virtue of these advantages, the proposed strategy is expected to provide an intrinsically robust, highly sensitive and selective assay platform for UDG activity assay and related biochemical researches.

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Notes and references
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