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Real time monitoring uracil excision using uracil-containing molecular beacons

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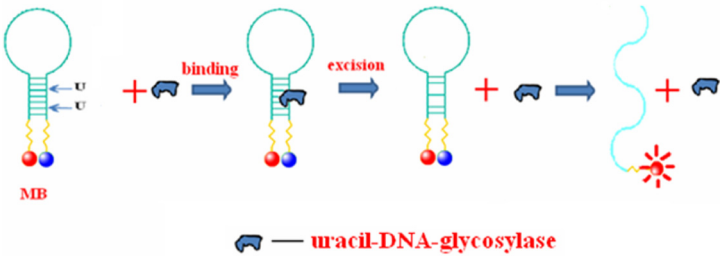
HIGHLIGHTS

- A sensitive and low-cost fluorescence detection system for UDG assay was constructed.
- A fluorescence strategy for sensing UDG activity and kinetics study was proposed.
- A low detection limit (0.005 U mL^{-1}) was obtained without any amplification.
- The strategy can be reliably applied for quantitative assay of UDG in complicated biological samples.

GRAPHICAL ABSTRACT

A real time fluorescence method with promising applications is developed for UDG assay with high accuracy and specificity using modified molecular beacons as substrates.



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ABSTRACT

As a highly conserved damage repair protein, UDG excises uracil bases through its glycosylase activity. We report here an alternative fluorescence method for UDG assay with high accuracy and sensitivity by applying uracil-modified molecular beacons as substrates. The detection limit of UDG is 0.005 U mL^{-1}. The K_M and k_{cat} are 0.89 ± 0.1 μM and 210 ± 10 min^{-1}, respectively. The method is applied to screening inhibitors and the results indicate that both of the 5-FU and cisplatin can inhibit UDG activity with the IC_{50} values of 6.1 ± 0.52 mM and 3.2 ± 0.24 mM, respectively. Furthermore, the combination of uracil-modified molecular beacons and nuclease inhibitor makes the new method possible to specifically detect UDG activity in cell-free extracts and serum. Taken together, the simple, rapid and sensitive method has potential relevance for a variety of applications, such as molecular diagnosis and screening of UDG inhibitors.

1. Introduction

The uracil residues in DNA caused by various factors in cells can be readily removed by the uracil-excision repair, which is initiated by a highly conserved damage repair protein of uracil-DNA glycosylase (UDG). As a key glycosylase, UDG can catalyze the hydrolysis of the N-glycosylic bond joining the uracil base to the deoxyribose phosphate backbone of DNA, and then coordinate with endonucleases, deoxyribophosphodiesterase, DNA polymerases and DNA ligase to facilitate the DNA repair [1–3]. Many evidences have shown that UDG is involved in the cell cycle regulation, apoptosis, tumor development, and virus proliferation [4–8]. Thus, developing new methods for UDG assay can provide a useful tool in the target drug screening and a possible aid in biomedical diagnosis. Traditionally, UDG activity is assayed by the radioactive labeling, gel electrophoresis and autoradiography technique [1,3,5,6], which is time-consuming and indirect. Furthermore, the requirement of additional separation step precludes the method for real-time monitoring enzymatic reaction. In order to take UDG assay closer to point-of-care, several heterogenous approaches,

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such as graphene oxide and gold nanoparticle based fluorescence methods have been developed [9,10]. Although promising, the requirement for tedious processes of nanomaterials preparation, the covalent labeling of DNA or the use of toxic metal ions are still the remaining obstacles of these sensitive methods. Other fluorescence alternatives, exhibiting some advantages including rapidity and simplicity, have their own important limitations of instability under the complicated environment [11–14]. Thus, development of rapid and high throughput methods for UDG detection is still necessary. As the most promising fluorescence probe, molecular beacon has been widely used for molecule detection [15–17], RNA synthesis [18], enzyme analysis [9,13,19], live cell imaging [20,21] and regulation of gene expression [22].

In the normal format, molecular beacon acts like a switch that is normally "closed", the stem structure holds the fluorophore and the quencher in close proximity. Hence, fluorescence quenching occurs as the result of resonance energy transfer. In contrast, target binding to the loop or enzymatic digestion can break the stem–loop conformation through the duplexes formation, hydrolysis of DNA strand or decreasing melt temperature by excision of bases of stem. Thus, the fluorescence is turned "on". Until now, molecular beacon-based detection system without separation is the simplest and the most specific one comparing with other fluorescence systems. These features have significant implications for the design of molecular beacons for various applications including UDG assay and image in vivo in real time [20]. However, the previously designed beacon is unsuitable for sensitive assay and rigorous kinetic study of UDG due to the existence of multiple uracil sites [19,20]. Meanwhile, modification of too many uracil sites increases the expense as well. In this paper, we designed a new kind of molecular beacon containing double uracil residues in the normal stem (6 base pairs) as the more suitable substrate for UDG assay.

2. Experimental

2.1. Materials and methods

All molecular beacons and oligonucleotide strands (Table 1) were synthesized by Takara Biotechnology Co. Ltd (Dalian, China). UDG and UGI were purchased from New England Biolabs (USA). The reactive buffer (75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.3) was used in all reactions unless otherwise indicated. All other chemicals (analytical grade) were purchased from Sigma–Aldrich Corporation (USA) without any further purification.

2.2. Fluorescence measurement

All experiments were conducted in a 500 μL quartz cell at the indicated temperature on a Hitachi FL-2500 spectrofluorometer (λ_{ex} = 521 nm, λ_{em} = 578 nm). Excitation and emission slit widths were set at 5 nm and 10 nm, respectively. The background intensity of solution was monitored until it kept stable at the tested temperature. Then, UDG was added and the subsequent change in fluorescence intensity was recorded as a function of time. The emission spectra were measured by exciting samples at 521 nm and scanning the emission between 550 and 650 nm. Fluorescence emission peaks were measured at 578 nm.

Table 1
Oligonucleotide strands with different sequences.

MB (control)	5-TAMRA-CGCTGCTTCAACACCCGCCTCTCCCGGCAGCG-DABCYL-3
MMB1	5-TAMRA-CACTACAGG ACACATGGGGAGCCGAGUAGAG-DABCYL-3
MMB2	5-TAMRA-CACTACAGGACACATGGGGAGCCGAGTUGUG-DABCYL-3
Oligo1	5-CACTACAGGACACATGGGGAGCCGAGTUGUG-3
Oligo2	5-CACTACAGGACACATGGGGAGCCGAGTTGTG-3

2.3. Real time monitoring of uracil excision

Probes were dissolved in distilled water to prepare a stock solution of 100 μM. In a standard 100 μL reaction, 100 nM molecular beacons were separately suspended in the buffer (75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.3) and incubated for 10 min on the FL-2500 spectrofluorometer. When the background fluorescence was stable, UDG (5 U mL^{-1}) was added to the solution. After mixing rapidly, the time courses of fluorescence intensity were recorded and individual cases were repeated at least 3 times.

2.4. UDG activity assay

The commercially provided UDG was diluted with buffer [50% glycerol, 20 mM Tris–HCl (pH 7.5), 30 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol] at 4 °C. In a typical test, substrates (100 nM MMB2) were incubated in the buffer (75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.3). After an incubation at 37 °C for 10 min, different concentrations of UDG were added to a final volume of 100 μL. After mixing rapidly, time courses of the fluorescence intensity were recorded and individual cases were repeated at least 3 times.

2.5. Kinetic study

A kinetic study of UDG was performed at 37 °C in 100 μL of the standard solution containing 25–400 nM MMB2 and initial velocities were determined by considering linear portions (in the first 20 s) of these fluorescence curves that yielded <5% of substrate cleavage. Initial velocities in units of fluorescence intensity per second were converted to moles per liter per second by dividing by the maximum change in fluorescence intensity and multiplying the initial substrate concentration. The maximum change in fluorescence intensity for each substrate concentration was obtained by incubation with a large excess of UDG under testing. Values of k_{cat}/K_M were calculated from the equation $k_{cat}/K_M = V_0/[E][S]$ from these initial velocity data.

2.6. Inhibitor screening assay

MMB2 (100 nM) were incubated with different concentrations of candidate inhibitor (5-FU, mitomycin or cisplatin) at 37 °C, in a buffer consisting of 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 1 mM dithiothreitol. After an incubation at 37 °C for 10 min, UDG (10 U mL^{-1}) was added to a final volume of 100 μL and the time curves of fluorescence intensity were monitored with the same protocol, as previously described. Initial velocities were calculated from these time curves. To calculate the percentage inhibition, the initial velocity of reaction containing MMB2 and drugs was divided by the initial velocity of reaction with DNA alone. This value was then multiplied by 100 to give percentage inhibition.

2.7. Measurement of UDG activity in complex samples

Huh 7 and HCC20986 hepatocarcinoma cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} of penicillin and 100 mg mL^{-1} of streptomycin in a humidified incubator at 37 °C with 5% CO2. Cell-free extracts were prepared as follows: when cells were grown to 80% confluency, 1×10^6 cells were harvested by trypsin treatment after washing once with PBS, centrifuged at 1500 × g for 2 min. Cells were washed