Introduction

Cells endure incessant DNA damage caused by intracellular or extracellular factors. DNA damage is blamed for aggravating the cell aging process through its ability to mediate cellular dysfunction. If damaged DNA remain unrepaired, it will lead to gene instabilities during the encoding of genetic information in DNA and may increase the risk of cancer. One of the repair pathways of DNA damage is referred to as nucleotide excision repair (NER). NER is initiated by a specific endonuclease that recognizes the damaged DNA and nicks the phosphodiester bond. Endonuclease V (EndoV, deoxyinosine 3'-endonuclease) is an NER enzyme that recognizes and cleaves the DNA strand containing deaminated deoxyadenosine (deoxyinosine, dI), a basic sites, base mismatches, insertion/deletion mismatches as well as other abnormal structures. EndoV cleaves the DNA strand at the second phosphodiester bond 3' to the lesions, and then opens a gate for other DNA repair enzymes to complete the subsequent repair processes. In view of this, the assay of EndoV activities represents a critical step toward the understanding of the DNA damage repairing process. Accordingly, developing a sensitive and specific strategy for the assay of EndoV activity is of fundamental importance, to provide a useful tool for the biological research, pathological study, and even early diagnosis.

Of all the existing studies on EndoV, however, most of them are concentrated on qualitative analysis, DNA mutant research and mechanism study, while few works focus on its activity assay. Moreover, most of these studies focus on radioactive labeling and gel electrophoresis, which, unfortunately, are always accompanied with tedious synthesis, indirect requirement of substrate radiolabeling, or sometimes giving false positive results. Therefore, it is a great challenge to develop a convenient and highly efficient biosensing platform for quantitative activity assay.

Fluorescent analysis methods have been widely used in bio-analytical chemistry because of their high sensitivity and widespread adaptability. However, ordinary organic dyes always suffer from short lifetime, photo bleaching, small Stokes-shift and potential bio-toxicity, which limit their further use in complex bioassays. In conventional fluorescent biosensings, there is the problem of eliminating the background noise of auto-fluorescence from biological materials and scattered light.

Keywords DNA-Tb³⁺ luminescence, Endonuclease V, Exonuclease III, deoxyinosine, guanine-rich DNA

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Since the background auto-fluorescence is transients, time-resolved luminescence (TRL) detection technique was invented to eliminate the background noise.\textsuperscript{16-19} Trivalent lanthanide ions (Ln\textsuperscript{3+}), such as terbium ion (Tb\textsuperscript{3+}), are always chosen to generate TRL in bioassays for their superior performance (sharply spiked emission bands, large Stokes-shifts, and long lifetimes).\textsuperscript{20-22} Due to their abundant 4f semi-stable state energy level, as well as the particular transition characteristics, they could possess a long-lived excited state, which will bring out delayed luminescence. Owing to the f-f transition is spin-forbidden, it is difficult to direct excitation of Ln\textsuperscript{3+} to luminesce. In order to enhance the luminescence, Ln\textsuperscript{3+} must be conjugated to the structure that both shields the Ln\textsuperscript{3+} from the quenching effects of the surrounding medium and has a sensitizing part that absorbs the excitation light and transfers it to the Ln\textsuperscript{3+}. Through the so-called antenna effect, Ln\textsuperscript{3+} luminescence is greatly enhanced by coordinating it with certain ligands such as organic molecules, nanoparticles, peptides and oligonucleotides.\textsuperscript{17,23-27} For example, guanine-rich (G-rich) ssDNA and dGMP could greatly enhance the long-lifetime, Ln\textsuperscript{3+} TRL and signal regulation technique of Exonuclease III (Exo III), a convenient and outstanding performance EndoV detection strategy was developed. Exo III specifically digests dsDNA from the 3′-OH blunt or recessed end, while it exhibits less activity on the ssDNA or 3′-protruding termini of dsDNA.\textsuperscript{29,30} Meanwhile, Exo III does not require a specific recognition site, making it an ideal candidate as a signal regulator in sensitive bioanalysis.\textsuperscript{31,32} Herein, dI base, the recognition site of EndoV, was inserted into the substrate DNA. A G-rich DNA was used to hybridize the substrate DNA to form a recessed 3′ terminus that can be recognized by Exo III. EndoV, as signal converter, nicks the phosphodiester bond near the dI and converts the heteroduplex from a 3′-recessed terminus to a 3′-protruding terminus, thus the Exo III can no longer digest G-rich DNA of the heteroduplex which then enhances the Tb\textsuperscript{3+} emission. Upon introduction of the EndoV, we can achieve a luminescent signal turn-on transformation. This strategy is simple, efficient and low cost, offering an easy operation platform to assay EndoV.

### Experimental

**Materials and measurements**

Oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). EndoV and Exo III were purchased from New England Biolabs. TbCl\textsubscript{3}·6H\textsubscript{2}O was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemical reagents were of analytical grade and used without further purification. All solutions were prepared with ultrapure water (18.25 MΩ·cm) from a Millipore system. The time-resolved luminescence was recorded on a Biotek microplate reader (SynergyMX, USA).

Sequences of the oligonucleotides:

### Results and Discussion

It was reported that G-rich DNA could enhance the Tb\textsuperscript{3+} emission because of its triplet energy state overlapping with the resonance energy levels of Tb\textsuperscript{3+}.\textsuperscript{27} Therefore, combining with Tb\textsuperscript{3+}, dGMP and G-rich DNA were recruited to detect small molecules and nucleic acid.\textsuperscript{27,28} Interestingly, to our surprise, a remarkable distinction emerged when the Tb\textsuperscript{3+} emission is enhanced by the same concentrations of guanine in different forms. As shown in Table 1, dGMP can result in a nonlinear enhancement of Tb\textsuperscript{3+} emission with the increase of the concentrations of dGMP, but other dNTPs (N = C, A or T) cannot. Meanwhile, guanine-contained DNA also can greatly improve the Tb\textsuperscript{3+} emission. Compared with these two enhancement efficiencies, the DNA/Tb\textsuperscript{3+} emits larger enhancement of Tb\textsuperscript{3+} emission (545 nm) by different substrates (concentration of Tb\textsuperscript{3+} used here is 50 μM).

| Concentration/μM | DNA\textsuperscript{a} | dGMP | dNMP (N = CA/T) |
|------------------|------------------|------------------|------------------|
| 0.1              | 295 ± 10         | 7 ± 2            | 4 ± 1            |
| 1.0              | 2530 ± 40        | 32 ± 2           | 4 ± 1            |
| 2.5              | 5510 ± 120       | 106 ± 4          | 6 ± 1            |
| 10               | 16000 ± 500      | 490 ± 10         | 6 ± 2            |
| 25               | 24000 ± 600      | 1650 ± 30        | 9 ± 4            |
| 50               | 35200 ± 900      | 13400 ± 500      | 13 ± 2           |
| 100              | 41000 ± 900      | 26300 ± 600      | 20 ± 3           |

a. The concentration of DNA represents the concentration of guanine based on DNA.

**DNA-PROBE:** 5′-AAGCCITACCTCTCCTCACCAAGTTTAAATATAAAAAC-3′

**DNA-SIGNAL:** 5′-TATATATTTAATTTGTTGAGTGAAGGAOGTACGCGC-3′

**Assay of EndoV activity**

The reaction of EndoV with the DNA-PROBE was performed in 10 μL NEB buffer (10 mM bis-Tris-propane-\(\text{pH} 7.4\), 10 mM Mg\textsubscript{Cl2}, 1 μM DTT, 0.5 μL) was added into the EndoV system to form heteroduplex with DNA-PROBE, then Exo III and buffer was introduced to the reaction solution. The sample was cultivated at 37°C for 60 min. Then, the sample was heated at 65°C for 20 min to stop the reaction. After the EndoV cleavage reaction, DNA-SIGNAL (10 μM) was added into the EndoV system to form heteroduplex, and the mixture was left for 2 min at room temperature. The luminescence spectra from 480 to 650 nm were recorded with the excitation of 900 nm. The delay time was 100 μs, while the gate time was 1 ms.
Principle of the TRL strategy for amplified detection of EndoV activity

Inspired by the above-mentioned phenomenon, a conception of EndoV activity assay was developed by utilizing the discrepancy of Tb\(^{3+}\) fluorescence emission enhanced between G-rich DNA and dGMP. The principle of the time-resolved and turn-on assay of EndoV is illustrated in Scheme 1. Due to the quenching effect of the water molecule, Tb\(^{3+}\) ions are non-fluorescent in aqueous solutions. Once Tb\(^{3+}\) is interacted with G-rich DNA, its emission is greatly enhanced. When the G-rich DNA is digested by Exo III and converted to solo dGMP, Tb\(^{3+}\) emission is sharply decreased. Herein, a 43-nb DNA sequence containing a deoxyinosine (dI) named DNA-PROBE was designed as the substrate of EndoV. In order to form a recessed 3′ terminus heteroduplex, correspondingly a 35-nb G-rich DNA (DNA-SIGNAL) was used to hybridize with the DNA-PROBE (DNA-SIGNAL:DNA-PROBE). In the presence of Exo III, the DNA-SIGNAL on the heteroduplex (a recessed 3′ terminus) can be easily dismantled by Exo III (paths A and B, Scheme 1). Because Exo III only degrades the 3′ recessed strands, the sequence of the DNA-PROBE will then be released to bind another free DNA-SIGNAL in the solution simultaneously. In view of that, the new generated heteroduplex is identical with its predecessor, and it will undergo the same cleavage reaction. Through several such cyclic hybridization–hydrolysis processes, a DNA-PROBE strand can trigger cleavage of a large quantity of DNA-SIGNAL strands. Upon completion of the strand-digestion cycles, hardly any free DNA-SIGNAL strands would exist that could coordinate with Tb\(^{3+}\) and shield it from a state of quenching to sensitizing. Hence, nearly no fluorescence signal can be detected. Consequently, the free G-rich DNA strand (DNA-SIGNAL) in the solution can coordinate with Tb\(^{3+}\) and greatly enhance the Tb\(^{3+}\) emission. The more EndoV cut off dI on the DNA-PROBE, the stronger fluorescence signal can be acquired. Therefore, the EndoV concentration can be monitored via the change of luminescence intensity of the G-rich DNA-Tb\(^{3+}\) complex.

Feasibility study

To test the feasibility of the EndoV detection system, a polyacrylamide gel electrophoresis (PAGE) assay was used to analyze the hydrolyzation of the DNA-PROBE by EndoV and the protecting/unprotecting of the heteroduplex from being degraded by Exo III (the concentrations for all DNA are 1 μM). The cleavage of the DNA-PROBE by EndoV produces shorter oligonucleotides that migrate faster than the parental DNA substrates in a denaturing PAGE. As shown in Fig. 1, lines L1 and L3 demonstrate that EndoV can cut off the DNA-PROBE. L4 and L6 verify that Exo III can hydrolyze heteroduplex with the 3′-protruding terminus. The discrepancy between L6 and L7 verify that the EndoV can protect the heteroduplex from hydrolyzed by Exo III.

To confirm the feasibility of the sensing system, experiments performed under different conditions were investigated. Based on the different emission enhancements of Tb\(^{3+}\) by G-rich DNA and dGMP, fluorescence spectroscopy of Tb\(^{3+}\) with G-rich DNA (DNA-SIGNAL) under different conditions was carried out. The Tb\(^{3+}\) solution (50 μM) shows an extremely weak
luminescence emission under the excitation of 290 nm (line a in Fig. 1). However, when in the presence of DNA-SIGNAL (50 nM), the luminescence intensity (LI) of Tb³⁺ is increased significantly (line b), which is approximately 1000 times of that of Tb³⁺ only. Luminescence of DNA-SIGNAL-Tb³⁺ has four emission peaks, 486, 545, 582 and 620 nm, which correspond to the $5D_4 \rightarrow 7F_6$, $5D_4 \rightarrow 7F_5$, $5D_4 \rightarrow 7F_4$, $5D_4 \rightarrow 7F_2$ electron transitions, respectively.¹⁸ When the DNA-SIGNAL was hybridized with the DNA-PROBE at the ratio 10:1 (DNA-SIGNAL:DNA-PROBE), the optical signal of DNA-Tb³⁺ complex decreased to some extent (line c). When Exo III (2.67 U/mL) was added into the system solely, just as path B (Scheme 1) shows, as expected, the luminescence of the Tb³⁺ system is just 17% of that before the Exo III was added (line e). However, before the Exo III was introduced, if the DNA-PROBE was first excised by EndoV, most of the luminescence is maintained (line d) compared with that without Exo III. These results further verify the feasibility of the proposed strategy.

**Optimization of influencing factors**

With a fixed design strategy, the performance of the sensing system is still strongly influenced by the assay conditions such as the concentration of Exo III, the digestion time of Exo III and the reaction time of EndoV towards the DNA-PROBE. (i) As for the optimization of Exo III concentration, when in the absence of EndoV, the luminescence signal (normalized LI, norLI) decreases correspondingly as the Exo III concentration increases at first and then keeps steady when the concentration is higher than 2.67 U/mL (Fig. 2A). After comprehensive consideration of the consumption of Exo III and the minimum background, finally 2.67 U/mL of Exo III was selected for the follow-up experiments. (ii) A long digestion time of Exo III is expected to yield high detection sensitivity. Nevertheless, a shorter detection time should be considered at the same time. Figure 2B shows the Tb³⁺ luminescence intensity change for different Exo III reaction times. The prolonged reaction time results for Exo III show that the observed luminescence intensity gradually decreases and almost levels off at 90 min. Thus, 90 min was chosen as the optimal reaction time. (iii) The digestion time of EndoV towards the DNA-PROBE was also investigated (Fig. 2C). As expected, as time passes, the luminescence signals become more intensive, and the slope of the curve becomes smaller, suggesting that the prolongation of the digestion time can provide better protection against the hydrolysis of the DNA-SIGNAL by Exo III. A plateau in the function curve of norLI vs. time emerged after 60 min.

**Detection of EndoV activity**

Based on the pre-optimized conditions, the quantitative detection of EndoV activity was evaluated by adding a series of concentrations of EndoV into the samples. As shown in Fig. 3A, the time of 60 min was employed for the subsequent experiments.
EndoV concentrations (Fig. 3B). In a certain concentration range, the luminescence intensity (LI) increases linearly as the 0.9905, where

nonspecific protein of bovine serum albumin (BSA) and another protect the enhancement of Tb³⁺-DNA luminescence from the proposed strategy has good specificity for EndoV detection.

The results indicate that the proposed strategy can achieve high sensitivity and a low detection limit for the detection of EndoV. Therefore, we envisage that this novel strategy can be applied in complex biological media assays even in clinical applications and can be expanded for the detection of various targets. This work opens up new opportunities for the design of more novel sensing strategies and expansion of lanthanide luminescence applications in different fields.

### Selectivity of the sensing system

Furthermore, the selectivity of this EndoV activity detection platform was also evaluated with three functional proteins. A BER enzyme named Uracil-DNA glycosylase (UDG), a nonspecific protein of bovine serum albumin (BSA) and another restriction enzyme named Dpn I were selected to compare with the EndoV. From Fig. 4, it can be seen that only EndoV can protect the enhancement of Tb³⁺-DNA luminescence from destruction by Exo III. Therefore, these results demonstrate that the proposed strategy has good specificity for EndoV detection.

### Conclusion

In summary, we found that, though both G-rich ssDNA and dGMP could greatly enhance the time-resolved luminescence of Tb³⁺, their efficiencies of enhancement were considerably different. Based on such principle, for exploiting a highly sensitive and label-free EndoV sensing method, we have developed a high performance and time-resolved luminescence strategy. By introducing the Exo III, this method can obtain an extremely low detection limit for EndoV. Therefore, we envisage that this novel strategy can be applied in complex biological media assays even in clinical applications and can be expanded for the detection of various targets. This work opens up new opportunities for the design of more novel sensing strategies and expansion of lanthanide luminescence applications in different fields.

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