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ARTICLE

Ultrasensitive Detection of 3´-5´ Exonuclease Enzymatic Activity Using Molecular Beacons

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An ultrasensitive and rapid turn-on fluorescence assay has been developed for the detection of 3´-5´ exonuclease activity of exonuclease III (Exo III) using molecular beacons (MBs). This method has a linear detection range from 0.04 to 8.00 U/mL with a limit of detection of 0.01 U/mL. In order to improve the selectivity of the method, a dual-MB system has been developed to distinguish different exonucleases. With the introduction of two different designed MBs which will response to different exonuclease, the T5 exonuclease, Exo III and RecJf exonucleases can be distinguished from each other easily. Furthermore, the fetal bovine serum and fresh mice serum were used as complex samples to investigate the feasibility of the dual-MB system for the detection of the enzymatic activity of Exo III. As a result, the dual-MB system showed a similar calibration curve for the detection of Exo III as that in the ideal buffer solution. Apparently, the designed MB probe would be a potential sensor for the detection of Exo III in biological samples.

Introduction

DNA exonucleases are enzymes that digest DNA sequences from 3´-termini or 5´-termini. In exonuclease family, the enzymatic activity of 3'- 5' exonuclease plays a key role for several important cellular and physiological processes, such as promoting genetic recombination reactions,[1] keeping fidelity of DNA replication[2] and repairing DNA double-strand breaks.[3] Both overexpression and lack of 3'- 5' exonuclease enzymatic activity will cause serious diseases and lead to more susceptible to cancers and other diseases under stress conditions.[4, 5] For example, a major 3'- 5' exonuclease named TREX1 could inhibit the innate immune response to HIV.[6, 7] Therefore, the detection of the 3'- 5' exonuclease activity could be used for the diagnoses and therapies for several diseases.

Traditional approaches for the detection of 3'-5' exonuclease activity are based on radioisotope labeled DNA,[8] which are cost-prohibitive, time-consuming, and could cause safety-concerns.[9] Therefore, it is urgent and useful to develop an efficient, sensitive and easily-operated method for evaluating the 3'- 5' exonuclease activity. Recently, fluorescence-based methods have been developed for the detection of different enzymes, such as polymerases, ligases, and endocleases.[10-13] However, only a few studies were published for the detection of 3'- 5' exonuclease activity with the fluorescence-based assay.[14, 15] For example, Leung et al. developed a label free fluorescence assay for the detection of 3'- 5' exonuclease activity based on the G-quadruplex-binding probe.[16] This assay showed great advantages over the traditional methods. However, its limit of detection was 5 U/mL only. Recently, Su et al. used the single-labelled DNA probe to detect the activity of Exo III with the limit of detection of 0.04 U/mL. However, a phosphorothioated bases in the 3' terminal should be modified in order to achieve the selectivity to the Exo III, which increased the cost and complexity of the detection system.[17] In order to overcome these limitations of these methods, we developed a fast, sensitive and cost-effective method to detect the activity of 3'-5' exonuclease based on traditional molecular beacon (MB).

MB is a single-stranded DNA sequence that can form a hairpin structure modified with a fluorophore and a quencher on the 5' - and 3'-ends, respectively. MBs have been developed rapidly for biosensor applications in the last few decades.[18, 19] Because of the low background fluorescence signal and excellent selectivity of MBs, a high signal-to-background ratio can be achieved using MBs for detection of DNA, mRNA, proteins, metal ions, and small molecules.[20-26] Moreover, MBs have been used for the detection of enzymatic activities, such as the activity of the endonuclease,[10] polymerase,[11] ligase[12] and other enzymes which can change the nucleic acid conformation.[27] However, so far, the traditional MBs have not been applied for the detection of exonuclease activities. Therefore, an ultrasensitive and ultra-rapid fluorescence assay for the detection of 3'- 5' exonuclease activity based on MBs was developed in this work. Exo III was used as a model that contains the activities of 3'- 5' exonucleases to illustrate the proof-of-concept of our method. Surely, Exo III does not exist in human body. However, other enzymes containing the 3'- 5' exonucleases activities in human are expected to be detected by this method because they contain the same enzymatic activity. This dual-MB system developed in this work can detect the 3'-5' exonucleases activities with limit of detection of 0.01 U/mL in buffer and 0.04 U/mL in 10 times serum sample. This implied that the dual-MB system has the potential to be used for evaluating the 3'- 5' exonucleases activities in order to investigate the disease generating process, diagnosis and therapy strategies.

Experimental section

Chemicals and Materials

DNA sequences used in this work were purchased form Fisher Scientific. The sequences for molecular beacons (MBs) were: 5'-FAMCCACCAGTTTTTTAGCTGAGGGTGG-BHQ1-3' (FAM-MB) and 5'-Cy5-CCTCGAGTTTTTTAGCTGATCGAGGTTGTG-BHQ2-3' (Cy5-MB), respectively. All the exonuclease enzymes (Exo III, Exo I, T7 Exo, T5 Exo, Exo T and RecJf) and 10X NEBuffer 4 (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM Magnesium Acetate and 1 mM Dithiothreitol pH 7.9 @ 25 °C) were purchased from New England Biolabs, Inc. The deionized water (18.3 MΩ •cm) was produced from the Millipore water purification system.

Apparatus

Fluorescence experiments were all performed using a Fluorolog-3 Spectrofluorometer with a 450 W xenon lamp (Jonin Yvon-Spex, Instruments S.A., Edison, NJ). For the FAM fluorescence detection, the excitation wavelength was 480 nm with an emission range of 500-600 nm. The fluorescence intensity at 518 nm was used to evaluate the performance of the proposed assay. For the detection of Cy5, the excitation was 645 nm with an emission range of 655-750 nm. The fluorescence intensity at 660 nm was used to evaluate the performance of the proposed assay. Both the excitation and emission slit widths were set to 5.0 nm. All the experiments were carried out at 37 °C for 200 s.

Exo III Detection Based on FAM-MB

10 nM FAM-MB was incubated in 50 µL 1X NEBuffer 4 at 37 °C. 0.20 U Exo III was then added into the solution to incubate for 200s. Finally, the fluorescence of the solution was detected. In contrast, no Exo III was added into the control solution and the fluorescence intensity was monitored for 200 s.

In order to investigate the sensitivity of the assay for Exo III, FAM-MB (10 nM) was mixed with different concentrations of Exo III varying from 0.04 U/mL to 4.00 U/mL. The fluorescence intensity of FAM at 518 nm was recorded after 200s. The calibration curve was obtained by plotting the relative fluorescence intensity *vs* the concentrations of the Exo III. The selectivity of the assay for the detection of Exo III was investigated by incubating FAM-MB (10 nM) with different exonucleases including T7 Exo, Exo I and RecJf at the concentration of 4.00 U/mL. The fluorescence intensity was also measured at 200 s for each exonulease.

The effects of pH on the enzymatic activity of Exo III were investigated by incubating 10 nM FAM-MB in 50 µL 1X NEBuffer 4 at 37 °C, with the addition of 0.20 U Exo for 200 s. The pH varied from 5.5 to 9.5. For the investigation of effect of divalent ions, 10 nM FAM-MB was incubated in 50 µL 1X NEBuffer 4 at 37 °C with addition of 0.3 mM Cu^{2+}, Ca^{2+} and Mn^{2+}, respectively. Then, 0.20 U Exo III was added into each solution to incubate for 200 s.

Distinguishing Exonucleases Based on Dual-MB System

FAM-MB (10 nM) and Cy5-MB (10 nM) were incubated in 50 µL 1X NEBuffer 4 at 37 °C. 0.20 U Exo III, T5 Exo and RecJf were then added into the solution to incubate for 200 s. Finally, the fluorescence of FAM and Cy5 were detected. Properties and expected responses of the exonucleases in dual-MB system are summarized in Table S1.

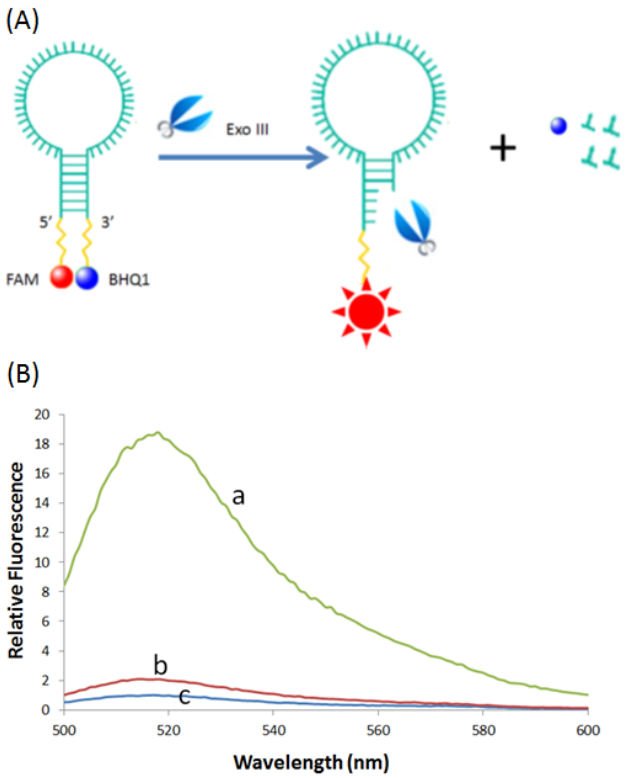

Figure 1. (A) The working principle of the detection of Exo III enzymatic activity based on FAM-MB. (B) Fluorescence emission spectra of 10 nM FAM-MB in 50 µL 1X NEBuffer 4. (a) 4.00 U/mL Exo III; (b) 4.00 U/mL heat-inactivated Exo III; (c) no Exo III. λ_{ex}= 480 nm

Results and discussion

Detection of Exo III with MB

As shown in Figure. 1A, a 6-carboxyfluorescein (FAM) modified MB (FAM-MB) with blunt 3'-termini was used as the probe. Because of the hairpin structure of the MB, FAM was close to the quencher (Black Hole Quencher-1, BHQ1) modified on the 3'-termini. The fluorescence of the FAM was quenched by BHQ1 through effective Förster resonance energy transfer (FRET). However, in the presence of Exo III, whose substrate is blunt or recessed 3'-termini, the FAM-MB would be digested by Exo III from the 3'-termini. Because the quencher was modified on the last mononucleotide of the 3'-termini, with the removal of the last mononucleotide, the quencher was also removed from the MB by Exo III. As a result, the fluorescence of the FAM was restored. As shown in Figure. 1B, the fluorescence intensity of FAM-MB solution increased by about 18 times after the reaction with 4.00 U/mL Exo III for 200 seconds (Figure. 1B, curve a) compared to the control that has no Exo III (Figure. 1B, curve c), demonstrating that the designed FAM-MB could be an ultrasensitive fluorescence probe to detect 3'- 5' exonuclease activity of Exo III. In order to further confirm that the fluorescence increase is due to the addition of active Exo III, a heat-inactivated Exo III was used as the second control. The Exo III was heated at 80 °C for 30 min to be inactivated before incubation with the FAM-MB. The result showed that the fluorescence intensity of FAM-MB was significantly diminished (Figure. 1B, curve b).

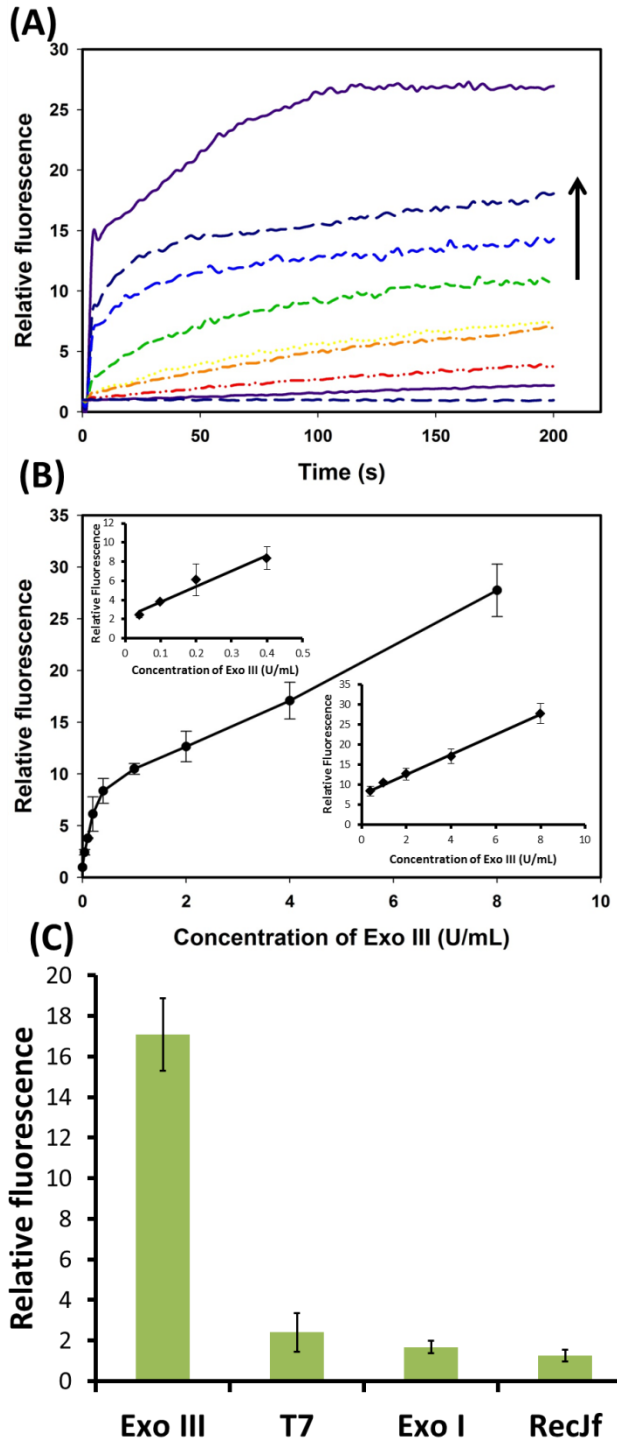

Figure 2. The detection of Exo III based on FAM-MB. (A) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.04, 0.10, 0.20, 0.40, 1.00, 2.00, 4.00, 8.00 U/mL of Exo III in 1× NEBuffer 4. (B) The plot of the fluorescence enhancement of different concentrations of Exo III. Reactions were performed in 50 µL of 1× NEBuffer 4 with 10 nM FAM-MB at 37 °C for 200 s. Inset: the two calibration curves in different concentration ranges. (C) Selectivity of the assay for Exo III over other exonucleases. Fluorescence enhancement of the FAM-MB system responded to 4.00 U/mL exonucleases.

In the investigation of sensitivity of the assay, a 10 nM FAM-MB solution was incubated with various concentrations of Exo III (0-8.00 U/mL) at 37 °C in 50 µL 1X NEBuffer 4. The FAM's fluorescence intensity increased as the concentration of Exo III increased (Figure. 2A). The signal-to-background ratio reached 27 when the concentration of Exo III was 8.00 U/mL. The total detection time is less than 200 s because the fluorescence intensity reached a plateau at 200 s after the addition of Exo III. In the following experiments, the incubation time of 200 s was chosen.

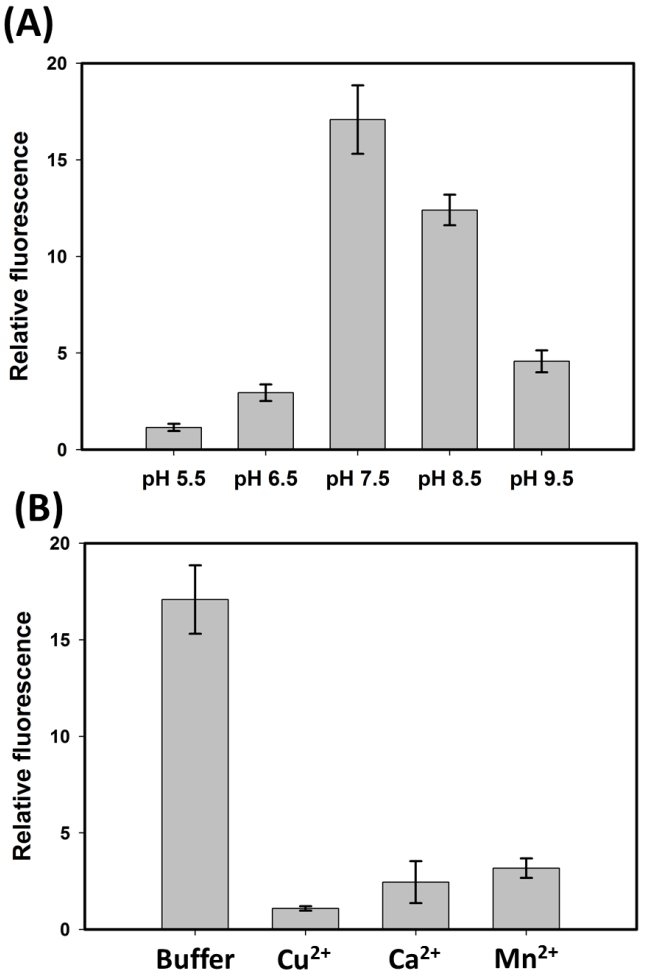

Figure 3. The stability investigation of the MB system for the detection of Exo III. (A) The effect of pH on the reaction rate of the MB system to Exo III. (B) The effect of divalent ions on the Exo III activity of the MB system to Exo III.

The fluorescence enhancement of the FAM-MB system was proportional to the concentration of Exo III. The dynamic range was from 0.04 U/mL to 8.00 U/mL. There were two linear ranges for the calibration curve. The first one was in the low concentration range (0.04 U/mL-0.40 U/mL); the calibration curve in the first linear range is showed in the top inset of Figure 2B. The regression equation was Y = 18.029X +1.6564 with a correlation coefficient of 0.9766. The second linear range was from 0.40 U/mL to 8.00 U/mL whose calibration curve was showed in the bottom inset of Figure. 2b. The regression equation was Y = 2.5011X +7.5561 with a correlation coefficient of 0.9989. Y and X represented the relative fluorescence and the concentration of Exo III in the unit of U/mL, respectively. The limit of detection (LOD) was 0.01 U/mL based on

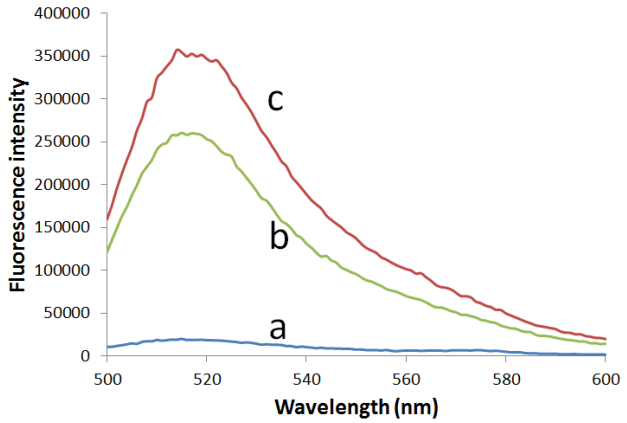

Figure 4. The fluorescence intensity responded to 10 nM FAM-MB (a), added with 4.00 U/mL T5 exoclease (b) or 4.00 U/mL Exo III (c).

three times of the signal-to-noise level. This was 500 times lower than that of the G-quadruplex-binding label-free fluorescence method.[16]

Next, we investigated whether this FAM-MB assay could selectively detect Exo III from other types of exonucleases. According to the property of Exo III, any exonucleases containing duplex DNA substrate may interference the detection of Exo III. T7 exonuclease is such an exonuclease, which acts on duplex DNA in the 5'- 3' direction. In addition, Exo I and RecJf belong to the similar exonuclease family with Exo III. Exo I can catalyze the removal of nucleotides from single-stranded DNA in the 3'- 5' direction, and RecJf can catalyze the removal of deoxynucleotide monophosphates from single-stranded DNA in the 5'- 3' direction. Therefore, these three exonucleases were chosen for investigating the selectivity of the assay. Encouragingly, the T7 exonuclease did not induce dramatic fluorescence enhancement with the same concentration of the Exo III (Figure. 2C). This may be benefited from the rapid detection of the fluorescence within 200 s of reaction time. Similarly, the fluorescence enhancement from Exo I and RedJf were limited as well (Figure. 2C).

The stability and effect of ions

The stability of the probe was also investigated. Generally, the pH not only would affect the hybridization of the MB, but also had the effect on the activity of the exonuclease. Thus, the pH value should play a crucial role in the stability of the MB probe. We choose five buffer solutions with different pH values in the range of 5.5 to 9.5 to test the fluorescence intensity of the MB probe. The results were shown in Figure. 3A. The highest signal-to-background ratio was gained when the pH was 7.5. Both the acid and alkali conditions led to the decrease of the signal-to-background ratio. Apparently, the pH value significantly affects the stability of the MB probe.

Furthermore, we investigated the effects of metal ions on the activity of exonucleases. It was reported that some divalent ions would inhibit the activity of the exonucleases to cleave DNA phosphodiester bonds.[28] Thus, the effects of three common divalent ions, including Cu^{2+}, Ca^{2+} and Mn^{2+}, on the activity of the Exo III were investigated. These cations might be present in complex

Table 1. The properties of different exonucleases. "+" indicates fluorescence enhancement; "-" indicates constant fluorescence.

Exonuclease	Substrate	Direction	FAM-MB	Cy5-MB
Exo III	dsDNA	3'- 5'	+	-
T5 Exo	dsDNA,ssDNA	5'- 3'	+	+
RecJf	ssDNA	5'- 3'	-	-

samples including serum or cells. As shown in Figure. 3B, the addition of any of these ions would dramatically affect the activity of Exo III compared to the control. Under the same concentration, the Cu^{2+} could reduce the MB probe signal over 95 %. Therefore, the designed MB probe has fairly good selectivity to the Exo III over both other types of exonucleases and metal ions.

Design of dual-MB system

However, even though the T7 exonuclease did not affect the detection of Exo III, other similar exonucleases may give a false signal if they could react fast with the MBs. We found out that the T5 exonuclease, an exonucleas has the 5'- 3' exonuclease activity, degraded DNA in the 5'- 3' direction in both single-stranded and double-stranded DNAs. When the T5 exonuclease was incubated with the FAM-MB system for 200 s, the fluorescence of the FAM

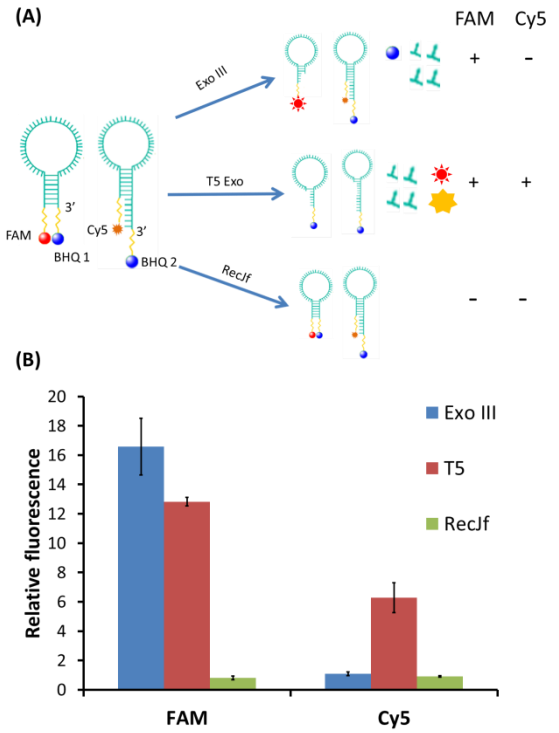

Figure 5. (A) Schematic illustration of the dual-MB system for distinguishing different exonucleases. (B) Fluorescence enhancement patterns of the dual-MB system with 4.00 U/mL Exo III, T5 exonuclease and RecJf. The dual-MBs system 20 contained 10 nM FAM-MB and 10 nM Cy5-MB in 50 GL of 1× NEBuffer 4, incubated at 37 °C for 200 s to detect the fluorescence intensity. λ_{ex} (FAM)=480 nm, λ_{em} (FAM)= 518 nm. λ_{ex} (Cy5)= 645 nm, λ_{em} (Cy5)= 660 nm.

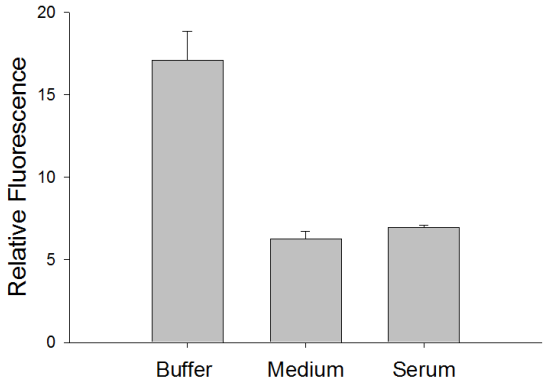

Figure 6. The fluorescence enhancements of FAM-MB in different solutions. The concentrations of Exo III were 4.00 U/mL.

increased as the addition of Exo III (Figure 4). In order to overcome this interference, a dual-MB system was developed, containing not only FAM-MB, but also a Cy5-MB with a 3´extention terminus.

As shown in Figure 5A, in the dual-MB system, the Exo III could only catalyze the digestion of the duplex DNA with blunt or recession 3'-termini to induce the fluorescence enhancement of the FAM, but not the Cy5. In contrast, the T5 exonuclease could catalyze both single-stranded DNA and double-stranded DNA from 5'-termini; as a result, it would induce the fluorescence enhancement of both FAM and Cy5. RecJf exonuclease, which can only catalyze the removal of deoxynucleotide monophosphates from a single-stranded DNA in the 5'- 3' direction, would not induce any fluorescence enhancement. The properties of the three exonucleases and anticipating fluorescence responses were summarized in the Table 1.

Using this dual-MB system, three exonucleases at the concentration of 4.00 U/mL were detected (Figure 5B). As the negative control, the RecJf exonuclease showed no fluorescence enhancement of FAM and Cy5, which meant that both FAM-MB and Cy5-MB were not digested by the RecJf exonuclease. When Exo III was added into the solution, the fluorescence of FAM increased by 16.6 times, while the fluorescence of the Cy5 did not change because this 3' extention Cy5-MB was not the substrate of Exo III. However, when T5 exonuclease was added into the solution, the fluorescence intensity of FAM increased by 12.8 times and the fluorescence intensity of Cy5 increased by 6.3 times. If the fluorescence enhancement for each dye was defined as 1, and no fluorescence enhancement defined as 0, the responses of the dual-MB system in the manner of (FAM, Cy5) for Exo III, T5 exonuclese, and RecJf were (1, 0), (1, 1) and (0, 0), respectively. From these different response patterns, Exo III, T5 exonuclease, and RecJf could be distinguished from each other.

Application of the designed probe

To test the feasibility of the designed MB probe for the detection of Exo III we applied the probe to the complex samples, including the cell culture medium and the fetal bovine serum. As shown in Figure 6, with the same concentration of Exo III, the fluorescence enhancements of FAM in the 10 times diluted RPMI 1640 medium and fetal bovine serum were lower than that of the ideal buffer

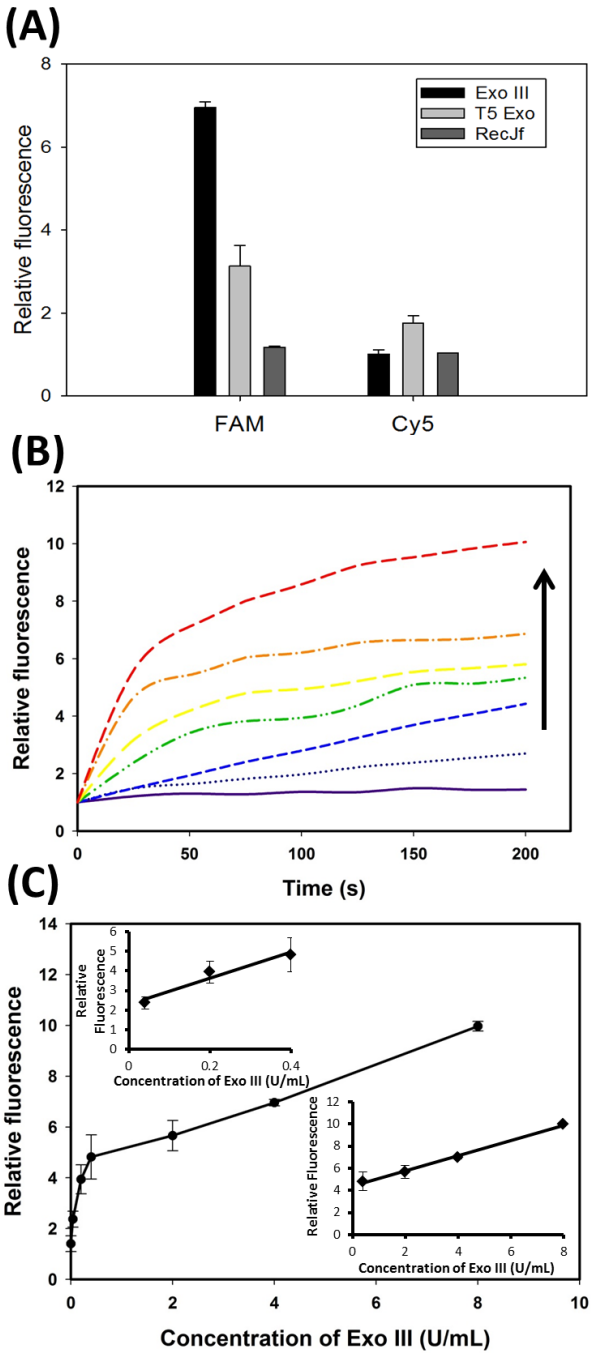

Figure 7. (A) The fluorescence enhancements of FAM-MB and Cy5-MB in 10 times diluted fetal bovine serum with the addition of 4 U/mL Exo III, T5 Exo and RecJf Exo. (B) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.04, 0.20, 0.40, 2.00, 4.00, 8.00 U/mL of Exo III in 10 times diluted fetal bovine serum. (C) The plot of the fluorescence enhancement of different concentrations of Exo III. Reactions were performed in 50 μL of 10 times diluted fetal bovine serum with 10 nM FAM-MB and 10 nm Cy5-MB at 37 °C for 200 s. Inset: the two calibration curves in different concentration ranges.

solution. The lower fluorescence enhancements of FAM in the complex samples compared with the ideal buffer might be due to the high background signal of these two matrixes when they were

excited at 488 nm irradiation. In order to show how much the other exonucleases would interfere the detection of Exo III, T5 exonuclease and RecJf were added into the dual-MB systemin the complex sample. As shown in Figure 7A, T5 exonuclease induced the fluorescence enhancement of both FAM and Cy5 in the serum sample. In contrast, RecJf exonuclease did not cause any fluorescence enhancement for these two dyes. The fluorescence intensity of FAM increased with the addition of Exo III. In contrast, the fluorescence intensity of Cy5 kept constant (Figure 7A), which was consistent with the results in the buffer solution. Therefore, this dual-MB system could eliminate the interference of other exonuclease for the detection of the 3'- 5' exonuclease enzymatic activity in complex sample by the different fluorescence enhancement combinations.

Because the fluorescence enhancements in these two complex samples were not consistent with that in the ideal buffer, the new calibration curve should be prepared for the detection of the Exo III in the complex samples. The fetal bovine serum was chosen as the example of the complex sample for the preparation of the calibration curve. As shown in Figure. 7B, the complex time fluorescence enhancements of FAM in the dual-MB system with addition of different concentrations of Exo III were monitored in 10 times diluted fetal bovine serum. Figure. 7C demonstrated the calibration curve of the dual-MB system for the detection of Exo III in the 10 times diluted fetal bovine serum. A similar curve at the concentration from 0 to 8.00 U/mL was exhibited as that in the ideal buffer except the corresponding relative fluorescence intensity was lower than those in the ideal buffer. Two linear ranges from 0 to 0.40 U/mL and 0.40 to 8.00 U/mL were observed in the complex sample, respectively. In order to further investigate the feasibility of this method for the detection of Exonuclease III activity in complex biological samples, fresh mice serum was used. As shown in Figure 8, the detection range was from 0 to 8.0 U/mL with two similar linear ranges, including 0 to 0.4 and 0.4 to 8.0 U/mL. The limit of detection in this sample was 0.03 U/mL based on three times of the signal-to-noise ratio. The detection limit is 3 times higher than that in the ideal buffer, which might be caused by the complexity of the mice serum. However, all the results indicated that this dual-MB system might be used for the detection of enzymatic activity of Exo III.

As shown in Figure 2B and Figure 7C, the Exo III calibration curves in buffer and 10 times-diluted fetal bovine serum demonstrated an initially high slope up to Exo III concentration of 0.4 U/mL followed by a decrease in slope. This might be due to the fixed substrate's concentration. Theoretically, the calibration curve should be off from the linear range when the concentration is higher than the saturated concentration. The reason for the second linear range might be from the excess residue intact the MB, which could be slowly digested by higher concentration of Exo III beyond the theoretical linear concentration limit. In this case, Exo III with a concentration of 0.40 U/mL was enough to digest most of the FAM-MB in the system. This triggered a larger slope of the calibration curve in the low concentration range. However, when the concentration of Exo III was higher than the turning point of 0.40 U/mL, even most of the FAM-MB was digested by the Exo III, the excess Exo III would no longer have enough substrate to digest. As a result, the slope of the calibration cure in the higher concentration range decreased.

Conclusions

In conclusion, an ultrasensitive, rapid and simple fluorescence method has been developed for the detection of Exo III. The dynamic range of the assay was 0.04 U/mL to 8.00 U/mL with the detection limit of 0.01 U/mL. The total incubation time for the assay was 200 s which was about 10 times faster than the label-free fluorescence method. The assay showed good selectivity over T7 exonuclease, Exo I and RecJf exonuclease. Even the T5 exonuclease would interfere the detection of Exo III, the dual-MB system was successful for eliminating the interference of other exonucleases. Finally, the designed dual-MB probe was applied to the monitoring of the enzymatic activity of Exo III in complex samples. The results suggested a great potential of the dual-MB probe for the detection of Exo III levels in complex samples. The proposed fluorescence assay is expected to be used for disease diagnosis and therapy related to the overexpression or loss of 3´-5´exonucleases and to expand the application of MBs in the enzymatic analysis.

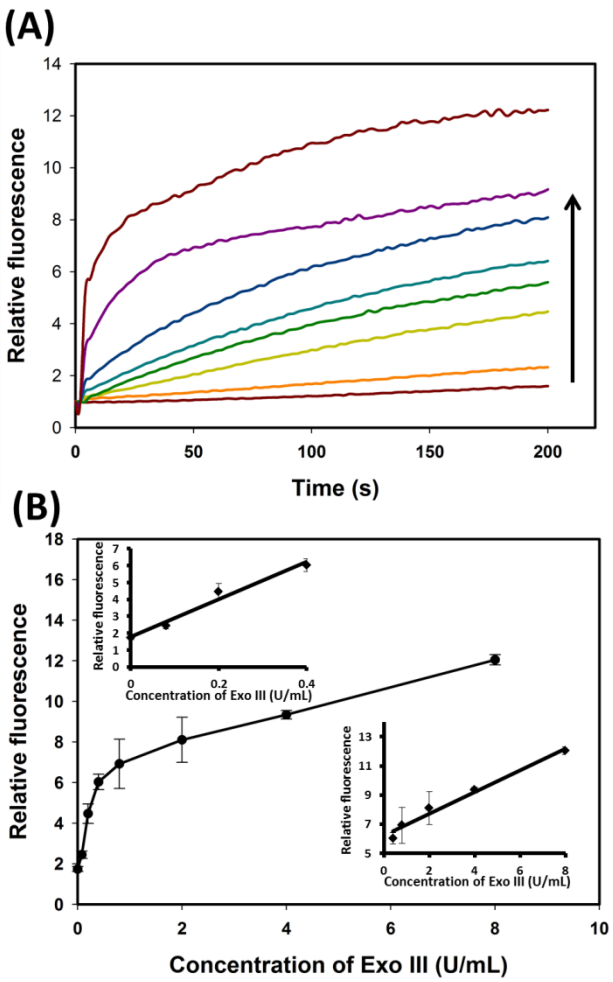

Figure 8. (A) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.08, 0.20, 0.40, 0.80, 2.00, 4.00, 8.00 U/mL of Exo III in 10 times diluted fresh mice serum. (C) The plot of the fluorescence enhancement of different concentrations of Exo III. Reactions were performed in 50 µL of 10 times diluted fresh mice serum with 10 nM FAM-MB and 10 nm Cy5-MB at 37 °C for 200 s. Inset: the two calibration curves in different concentration ranges.

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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Table of contents

An ultrasensitive and rapid fluorescence assay was developed for detection of 3´-5´ exonuclease activity using molecular beacon.