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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^{-1} with a limit of detection of 0.01 U mL^{-1}. In order to improve the selectivity of the method, a dual-MB system has been developed to distinguish between different exonucleases. With the introduction of two differently designed MBs which respond to different exonucleases, the T5 exonuclease, Exo III and RecJf exonucleases can be easily distinguished from each other. Furthermore, fetal bovine serum and fresh mouse 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 in the ideal buffer solution. The designed MB probe could 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 the exonuclease family, the enzymatic activity of 3′-5′ exonuclease plays a key role in several important cellular and physiological processes, such as promoting genetic recombination reactions,[1] maintaining 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 greater susceptibility 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 3′-5′ exonuclease activity could be used for the diagnosis and therapy of 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 important and would be useful to develop an efficient, sensitive and easy to operate method for evaluating 3′-5′ exonuclease activity. Recently, fluorescence-based methods have been developed for the detection of different enzymes, such as polymerases, ligases, and endonucleases.[10–13] However, only a few studies have been published for the detection of 3′-5′ exonuclease activity with fluorescence-based assays.[14,15] For example, Leung *et al.* developed a label free fluorescence assay for the detection of 3′-5′ exonuclease activity based on a G-quadruplex-binding probe.[16] This assay showed great advantages over the traditional methods. However, its limit of detection was only 5 U mL^{-1}. Recently, Su *et al.* used a single-labelled DNA probe to detect the activity of Exo III with a limit of detection of 0.04 U mL^{-1}. However, a phosphorothioated base at the 3′ terminus must be modified in order to achieve selectivity to Exo III, which increased the cost and complexity of the detection system.[17] In order to overcome the limitations of these methods, we developed a fast, sensitive and cost-effective method to detect the activity of 3′-5′ exonuclease based on a traditional molecular beacon (MB).

A 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 rapidly developed 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 endonucleases,[10] polymerases,[11] ligases[12] and other enzymes which can change the nucleic acid conformation.[27] However, so far, 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 possesses the activities of 3′-5′ exonucleases to illustrate the proof-of-concept of our method. Exo III does not exist in the human body. However, other enzymes containing 3′-5′ exonuclease activity in humans are

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expected to be detected by this method because they contain the same enzymatic activity. The dual-MB system developed in this work can detect 3′-5′ exonuclease activities with a limit of detection of 0.01 U mL^{-1} in buffer and 0.04 U mL^{-1} in 10 times serum sample. This implied that the dual-MB system has the potential to be used for evaluating 3′-5′ exonuclease 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 from Fisher Scientific. The sequences for molecular beacons (MBs) were: 5′-FAMCCACCAGTTTTTTAGCTGAGGGTGG-BHQ1-3′ (FAM-MB) and 5′-Cy5-CCTCGAGTTTTTTAGCTGATCGAGGTTGTG-BHQ2-3′ (Cy5-MB). All the exonuclease enzymes (Exo III, Exo I, T7 Exo, T5 Exo, Exo T and RecJf) and 10× 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, USA). 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 1× NEBuffer 4 at 37 °C. 0.20 U Exo III was then added into the solution to incubate for 200 s. 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^{-1} to 4.00 U mL^{-1}. The fluorescence intensity of FAM at 518 nm was recorded after 200 s. 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 a concentration of 4.00 U mL^{-1}. The fluorescence intensity was also measured at 200 s for each exonuclease.

The effects of pH on the enzymatic activity of Exo III were investigated by incubating 10 nM FAM-MB in 50 μL 1× 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 the effect of divalent ions, 10 nM FAM-MB was incubated in 50 μL 1× 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 1× 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 the dual-MB system are summarized in Table 1.

Results and discussion

Detection of Exo III with MB

As shown in Fig. 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

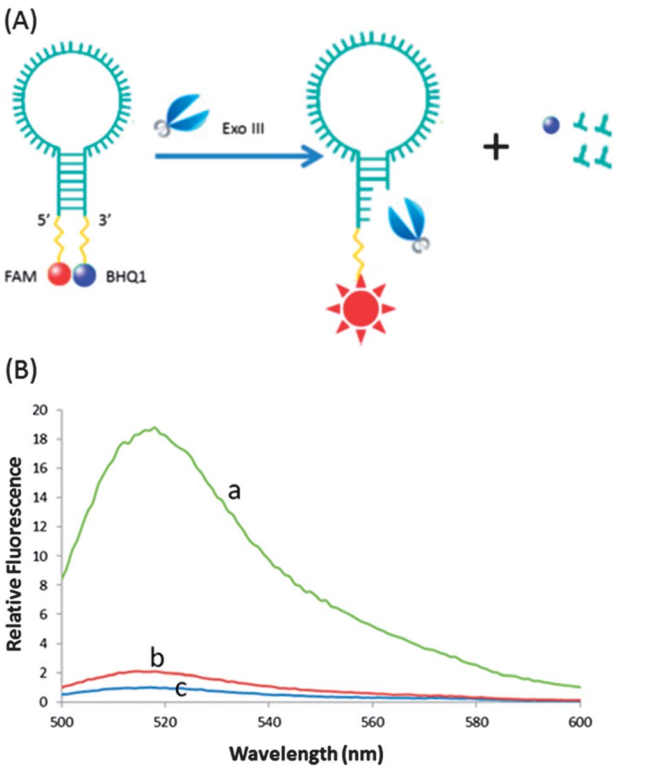

Fig. 1 (A) The working principle of the detection of Exo III enzymatic activity based on FAM-MB. (B) Fluorescence emission spectra of 10 nM FAM-MB in 50 μL 1× NEBuffer 4. (a) 4.00 U mL^{-1} Exo III; (b) 4.00 U mL^{-1} heat-inactivated Exo III; (c) no Exo III. λ_{ex} = 480 nm.

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 Fig. 1B, the fluorescence intensity of the FAM-MB solution increased by about 18 times after the reaction with 4.00 U mL^{-1} Exo III for 200 seconds (Fig. 1B, curve a) compared to the control with no Exo III (Fig. 1B, curve c), demonstrating that the designed FAM-MB could be an ultrasensitive fluorescence probe to detect the 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 for inactivation before incubation with the FAM-MB. The result showed that the fluorescence intensity of FAM-MB was significantly diminished (Fig. 1B, curve b).

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^{-1}) at 37 °C in 50 μL 1× NEBuffer 4. The fluorescence intensity of the FAM increased as the concentration of Exo III increased (Fig. 2A). The signal-to-background ratio reached 27 when the concentration of Exo III was 8.00 U mL^{-1}. 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.

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^{-1} to 8.00 U mL^{-1}. There were two linear ranges for the calibration curve. The first one was in the low concentration range (0.04 U mL^{-1}–0.40 U mL^{-1}); the calibration curve in the first linear range is shown in the top inset of Fig. 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^{-1} to 8.00 U mL^{-1}, the calibration curve of which is shown in the bottom inset of Fig. 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 units of U mL^{-1}, respectively. The limit of detection (LOD) was 0.01 U mL^{-1} based on three times 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 over other types of exonucleases. According to the properties of Exo III, any exonucleases containing duplex DNA substrates may interfere with 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 a similar exonuclease family to 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 at the same concentration as Exo III (Fig. 2C).

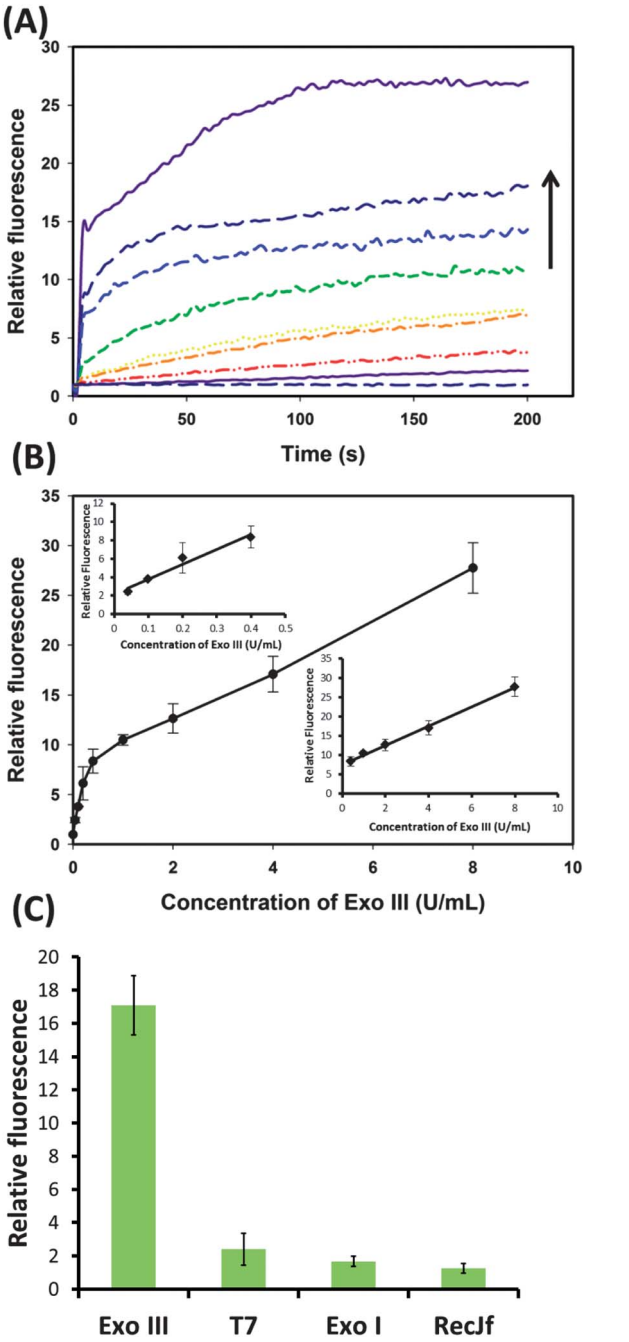

Fig. 2 The detection of Exo III based on FAM-MB. (A) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.04, 0.10, 0.20, 0.40, 1.00, 2.00, 4.00, 8.00 U mL^{-1} of Exo III in 1× NEBuffer 4. (B) The plot of the fluorescence enhancement 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 responding to 4.00 U mL^{-1} exonucleases.

This may be aided by the rapid detection of the fluorescence within 200 s of reaction time. Similarly, the fluorescence enhancement from Exo I and RedJf was limited as well (Fig. 2C).

The stability and effect of ions

The stability of the probe was also investigated. Generally, the pH would not only affect the hybridization of the MB, but also affect the activity of the exonuclease. Thus, the pH value should play a crucial role in the stability of the MB probe. We chose 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 Fig. 3A. The highest signal-to-background ratio was obtained when the pH was 7.5. Both acidic and alkaline conditions led to a decrease in the signal-to-background ratio. The pH value was found to significantly affect the stability of the MB probe.

Furthermore, we investigated the effects of metal ions on the activity of exonucleases. It has been reported that some divalent ions could 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 samples including serum or cells. As shown in Fig. 3B, the addition of any of these ions dramatically affected the activity of Exo III compared to the control. At the same concentration, Cu^{2+} could reduce the signal of the MB probe signal by over 95%. Therefore, the designed MB probe has fairly good selectivity to Exo III over both other types of exonucleases and metal ions.

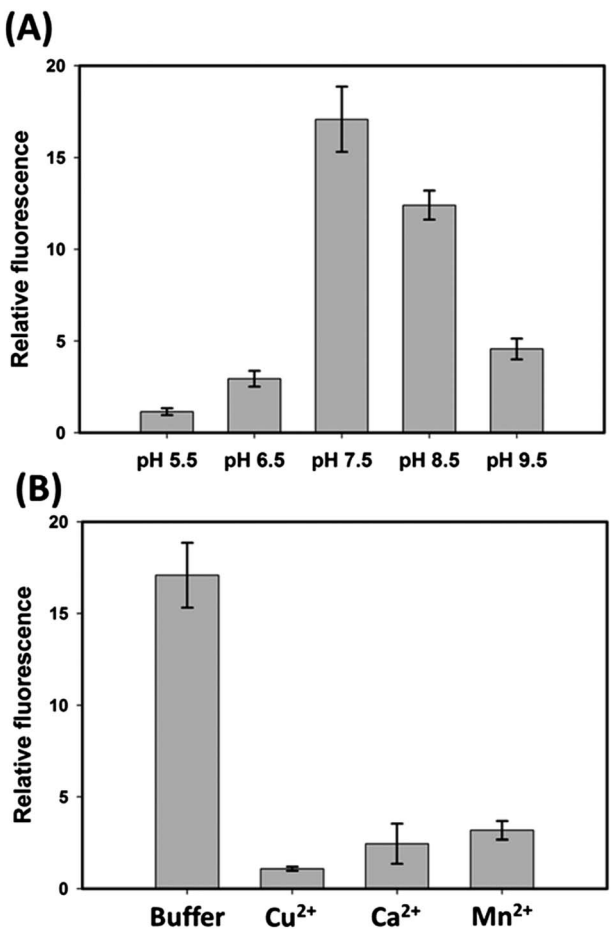

Fig. 3 The stability investigation of the MB system for the detection of Exo III. (A) The effect of pH on the reaction rate of the MB system towards Exo III. (B) The effect of divalent ions on the activity of the MB system towards Exo III.

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 quickly with the MBs. We found that the T5 exonuclease, an exonuclease which has 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 increased with the addition of Exo III (Fig. 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′ extension terminus.

As shown in Fig. 5A, in the dual-MB system, Exo III could only catalyze the digestion of the duplex DNA with blunt or recessed 3′-termini to induce the fluorescence enhancement of FAM, and not Cy5. In contrast, the T5 exonuclease could catalyze both single-stranded DNA and double-stranded DNA from 5′-termini; as a result, it induced 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, did not induce any fluorescence enhancement. The properties of the three exonucleases and anticipated fluorescence responses were summarized in Table 1.

Using this dual-MB system, three exonucleases at a concentration of 4.00 U mL^{-1} were detected (Fig. 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′ extension 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

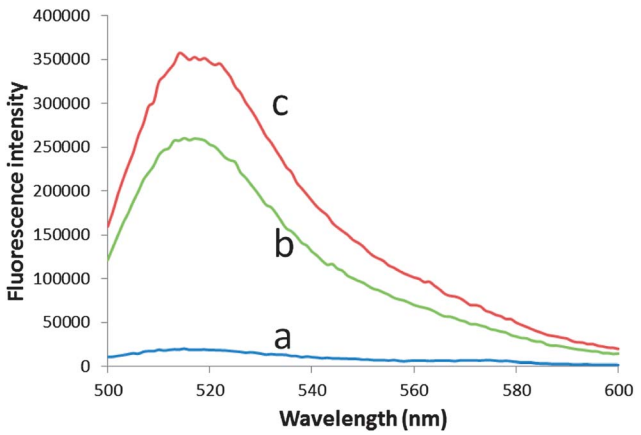

Fig. 4 The fluorescence intensity response to 10 nM FAM-MB (a), with the addition of 4.00 U mL^{-1} T5 exonuclease (b) or 4.00 U mL^{-1} Exo III (c).

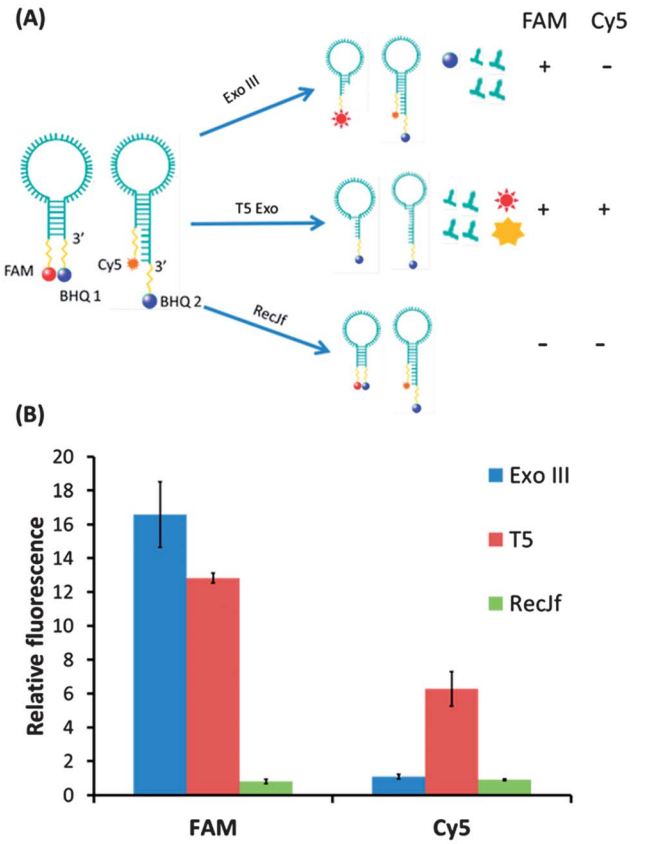

Fig. 5 (A) Schematic illustration of the dual-MB system for distinguishing different exonucleases. (B) Fluorescence enhancement patterns of the dual-MB system with 4.00 U mL^{-1} Exo III, T5 exonuclease and RecJf. The dual-MBs system contained 10 nM FAM-MB and 10 nM Cy5-MB in 50 μL of 1× NEBuffer 4, 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.

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′	–	–

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 was defined as 0, the responses of the dual-MB system in the manner of (FAM, Cy5) for Exo III, T5 exonuclease, 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 complex samples, including cell culture medium and fetal bovine serum. As shown in Fig. 6,

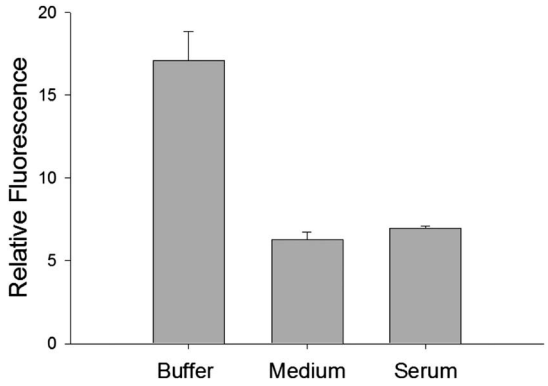

Fig. 6 The fluorescence enhancements of FAM-MB in different solutions. The concentrations of Exo III were 4.00 U mL^{-1}.

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 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 matrices when they were excited at 488 nm. In order to show how much the other exonucleases would interfere with the detection of Exo III, T5 exonuclease and RecJf were added into the dual-MB system in the complex samples. As shown in Fig. 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 remained constant (Fig. 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 samples by the different fluorescence enhancement combinations.

Because the fluorescence enhancements in these two complex samples were not consistent with those in the ideal buffer, a new calibration curve should be prepared for the detection of Exo III in complex samples. Fetal bovine serum was chosen as an example of a complex sample for the preparation of the calibration curve. As shown in Fig. 7B, the complex time–fluorescence enhancements of FAM in the dual-MB system with the addition of different concentrations of Exo III were monitored in 10 times diluted fetal bovine serum. Fig. 7C demonstrated the calibration curve of the dual-MB system for the detection of Exo III in 10 times diluted fetal bovine serum. A similar curve at concentrations from 0 to 8.00 U mL^{-1} was exhibited to that in the ideal buffer except the corresponding relative fluorescence intensity was lower than that in the ideal buffer. Two linear ranges from 0 to 0.40 U mL^{-1} and 0.40 to 8.00 U mL^{-1} were observed in the complex sample. In order to further investigate the feasibility of this method for the detection of exonuclease III activity in complex biological samples, fresh mouse serum was used as another complex sample. As shown in Fig. 8, the detection range was from 0 to 8.00 U mL^{-1}

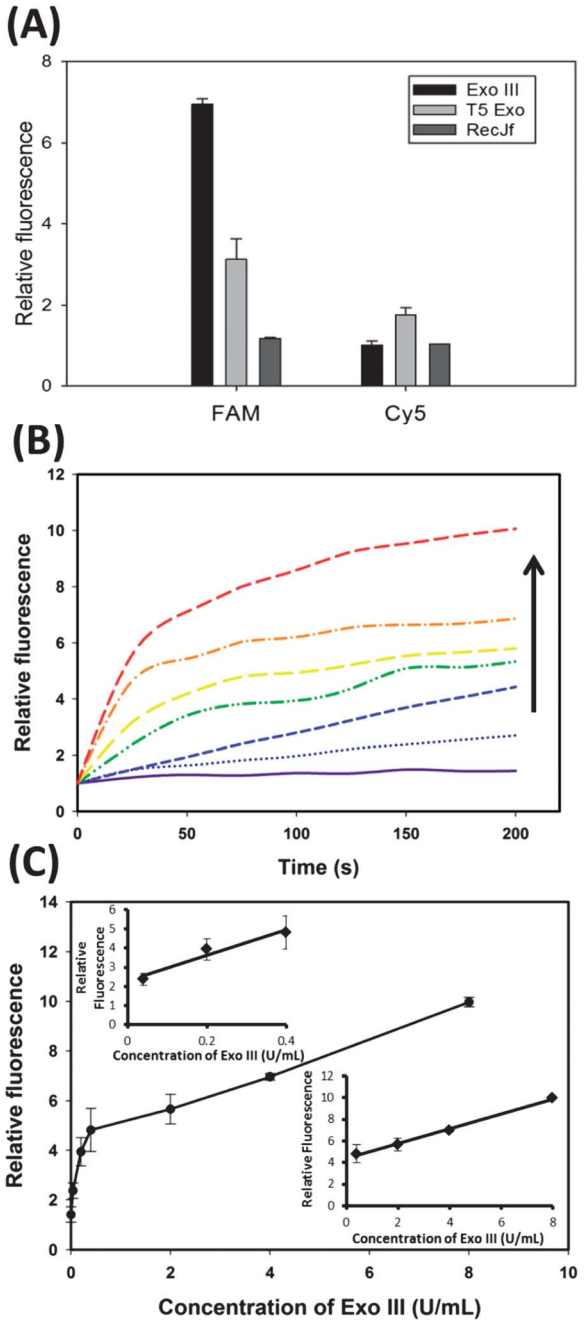

Fig. 7 (A) The fluorescence enhancements of FAM-MB and Cy5-MB in 10 times diluted fetal bovine serum with the addition of 4.00 U mL^{-1} Exo III, T5 Exo and RecJf Exo. (B) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.04, 0.20, 0.40, 2.00, 4.00, 8.00 U mL^{-1} 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.

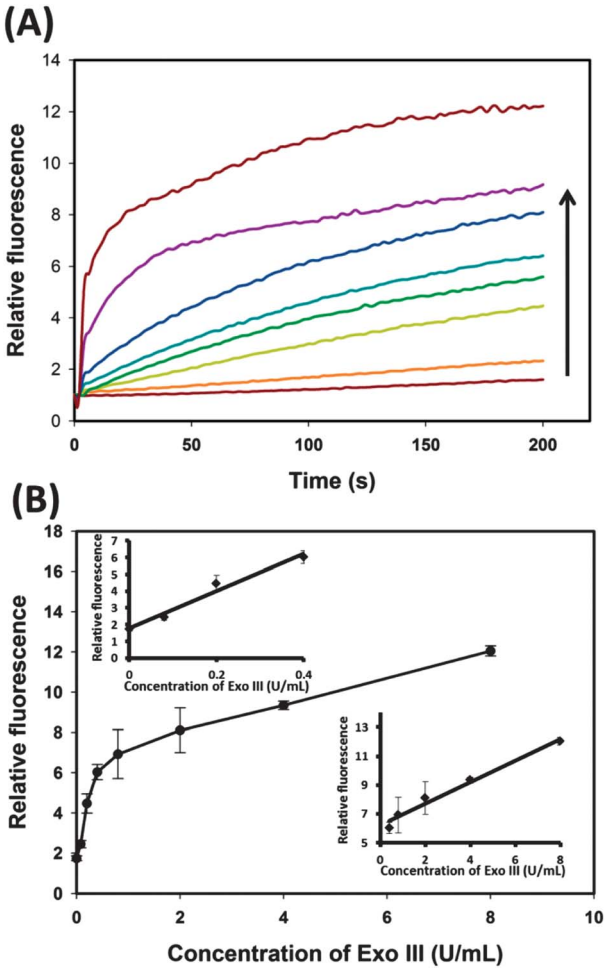

Fig. 8 (A) Time-dependent fluorescence responses in the presence of different concentrations of Exo III. From bottom to top: 0, 0.08, 0.20, 0.40, 0.80, 2.00, 4.00, 8.00 U mL^{-1} of Exo III in 10 times diluted fresh mouse serum. (C) The plot of the fluorescence enhancement of different concentrations of Exo III. Reactions were performed in 50 μL of 10 times diluted fresh mouse 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.

with two similar linear ranges, from 0 to 0.40 and 0.40 to 8.00 U mL^{-1}. The limit of detection in this sample was 0.03 U mL^{-1} based on three times the signal-to-noise level. This detection limit is 3 times higher than that in ideal buffer, which might be caused by the complexity of the mouse serum. However, all the results indicated that this dual-MB system might be used in complicated samples for the real-time detection of the enzymatic activity of Exo III.

As shown in Fig. 2B and 7C, the Exo III calibration curves in buffer and 10 times-diluted fetal bovine serum demonstrated an initially high slope up to an Exo III concentration of 0.4 U mL^{-1} followed by a decrease in slope. This might be due to the fixed substrate concentration. Theoretically, the calibration curve should deviate from the linear range when the concentration is higher than the saturated concentration. The second linear range might result from the small portion of undigested MB, which could be slowly digested by higher concentrations of Exo III beyond the theoretical linear concentration limit. In this case, Exo III at a concentration of 0.40 U mL^{-1} was sufficient 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^{-1}, most of the FAM-MB was digested by the Exo III, and the excess Exo III would no longer have enough substrate to digest. As a result, the slope of the calibration curve 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^{-1} to 8.00 U mL^{-1} with a detection limit of 0.01 U mL^{-1}. 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. Although the T5 exonuclease could interfere with 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 enzymatic analysis.

Acknowledgements

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