A real time S1 assay at neutral pH based on graphene oxide quenched fluorescence probe

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ABSTRACT

As the extracellular nuclease of *Aspergillus*, S1 nuclease can split single and double-stranded DNA into oligo- or mononucleotides, while preferentially digests single-stranded nucleic acids. Furthermore, the existence of S1 can be the standard to identify *Aspergillus* and used to evaluate the severity of Aspergillosis. Herein, a simple and sensitive fluorescent sensing platform for S1 assay was developed based on the S1-induced DNA strand scission and the difference in affinity of graphene oxide (GO) for single-stranded DNA containing different bases. This platform was applied to monitor S1 activity and study the kinetics in real time. Results indicated that the detection limit is 0.5 U/mL. The K_m and k_{cat} at 45 °C, are 1.4 ± 0.12 μM and 0.6 min^{-1}, respectively. Moreover, by monitoring the effect of chemical drugs on S1 activity, we found that 2 mM of erythromycin, sodium penicillin, carbenicillin disodium and ampicillin can inhibit S1 activity about 8%, 60%, 61% and 66%, respectively, while gentamycin sulfate is a stimulator. Overall, the assay platform based on graphene oxide quenched fluorescence probe is successfully constructed to study the enzymatic activity of S1 and used for screening antibiotics.

1. Introduction

The nuclease S1 from *Aspergillus oryzae* has the ability to degrade single-stranded oligonucleotides composed of either deoxynucleotides or ribonucleotides [1,2]. In addition, it has been suggested to meanwhile possess activity that can recognize the loop portion of duplex and cleaves it, leaving a mono-ribonucleotide at the 5′ terminus of DNA strands [3,4]. Thus, it can be widely used in the DNA damage detection, nuclease protection assay, in removing single-stranded tails from DNA molecules and opening hairpin loops of double-stranded cDNA [5–8]. Until now, the active site and key structural elements necessary for DNA hydrolysis have been recognized through structural analysis of S1, while biochemical evidences for the function of S1 are still much limited. In order to exploring the mechanism of enzymatic reaction catalyzed by S1, developing simple and efficient methods for monitoring reaction still remains to be done.

Several traditional methods for S1 detection include gel electrophoresis and autoradiography [9–11]. Due to the shortcomings of being time-consuming, labor-intensive and of insufficient sensitivity, great efforts have been made to develop new methods with better performance, such as fluorescence resonance energy transfer based fluorescent methods, enzymatic amplification based label-free fluorometric assay [12,13], nanomaterials [14–16] or G-quadruplex based colorimetric methods [17]. Although these methods have achieved great advances toward the S1 assay, there still are some limitations in each method. For example, dual-labeling of DNA probes with fluorescent dyes was required in the above-mentioned fluorescent methods. Multi-step modifications or washings were commonly existed in nanomaterial based assay. In G-quadruplex based assays, DNA probes need specific design. These weaknesses make the above assays expensive, complicated and/or tedious in some extent.

Recently, graphene oxide, a new generation of nanomaterial with unique physical–chemical properties, shows high potential for use in the biomedical assay. It has been confirmed that the quenching efficiency of this fluorescence quencher is higher than those of existed quenchers [18,19]. In addition, the short single-stranded DNA had weaker affinity to GO than long ssDNA [20,21]. Thus, these properties make it possible to prepare a novel bioassay platform for monitoring DNA cleavage reaction catalyzed by nuclease. However, this kind of optimal quencher can inhibit some enzymes' activity through spatial hindrance effect [19,22]. We also found that graphene oxide can inhibit S1 activity under the environment of acid pH when it was applied as the quencher of fluorescence probe (data not shown). That's maybe the reason why there was no method for monitoring S1 assay in real time. Interestingly, we found that S1 can efficiently digest graphene oxide quenched fluorescence probe in the neutral buffer containing Mg^{2+},

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which is consistent with previous report [23]. In addition, we sifted gentamycin sulfate as a strong stimulator of S1. These results provide the solid foundation for developing a simple and sensitive responsive sensor of S1 assay.

2. Experimental

2.1. Materials and methods

All probes and oligonucleotide strands (Table 1) were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). S1 was purchased from Takara. A $1\times$ reactive buffer [100 mM Tris–HCl, 20 mM MgCl$_2$, 1 Mm EDTA, pH 8.0, 2 mM gentamycin sulfate] was used in all reactions unless otherwise indicated. All chemicals were purchased from Sigma-Aldrich.

2.2. Fluorescence measurement

All experiments were conducted in a 500 μL quartz cell at 37 °C on a Hitachi FL-2500 spectro fluorometer (λex = 521 nm, λem = 578 nm). Excitation and emission slit widths were 5 nm and 10 nm, respectively. Background signal intensity of the solution was monitored when it kept stable. Then, S1 nuclease was added and the fluorescence signal was recorded as a function of time. The emission spectra were measured by exciting the samples at 521 nm and scanning the emission between 550 and 650 nm. Fluorescence emission intensities were measured at 578 nm.

2.3. Optimization of the ratio between TAMRA-labeled ssDNA and GO

The commercial GO was sonicated in ultra-purified water for 0.5 h to give a homogeneous black solution and stored at 4 °C for use. The working solution containing ssDNA was obtained by diluting the stock solution to a concentration of 100 nM using 100 mM Tris–HCl buffer. To optimize the ratio between TAMRA-labeled ssDNA and GO, 1 μL of the ssDNA stock solution (10 μM), and GO solution (2 mg/mL) with different volumes was mixed. The mixed solution was added with Tris–HCl buffer to a final volume of 100 μL and was incubated for 30 min at room temperature. The fluorescence intensity of the incubated solution was measured at 578 nm with excitation at 521 nm.

2.4. Real time monitoring of DNA digestion catalyzed by S1

In a standard 100 μL reaction, 100 nM different kinds of fluorescence probes were separately mixed with buffer [100 mM Tris–HCl (pH 8.0), 20 mM MgCl$_2$, 1 mM EDTA, 2 mM gentamycin sulfate] and then incubated for 10 min at 37 °C on the spectrofluorometer. Fluorescence–time curves were recorded after addition of the different amount of S1.

2.5. Performance of S1 detection in a TE buffer

The commercially provided S1 was diluted with storage buffer (50% glycerol, 20 mM Tris–HCl (pH 7.5), 30 mM NaCl, 0.5 mM EDTA, 1 mM DTT) at 4 °C. In a standard 100 μL reaction, GO-P1 was mixed with $1\times$ reactive buffer with a final concentration of 100 nM, then incubated on the spectrofluorometer for 10 min at 45 °C and fluorescence–time

curves were recorded after the addition of various concentrations of S1 and stirring for 4 s.

2.6. Kinetic study

Kinetic study of S1 was performed at 45 °C in 100 μL of the standard solution containing 25–400 nM GO-P1. Initial velocities were determined by considering only those linear portions (in the first 20 s) of 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 by the initial substrate concentration. The maximum change in fluorescence intensity for each substrate concentration was obtained by incubation with a large excess of the enzyme under testing. Values of kcat/Km were calculated from the equation $k_{cat}/K_m = V_0/[E][S]$ by measuring the initial rates at different substrate concentrations.

2.7. Effectors screening

A total volume of 100 μL standard solution that contained 100 nM GO-P1 and drug with different concentrations was initially incubated at 37 °C for 10 min. Then, fluorescence–time curves were monitored immediately at the same temperature after 5 U S1 was added. Initial velocities were calculated from the data obtained in the first 30 s of time curves. Effects of drugs on S1 were evaluated from the initial velocity variation.

3. Results and discussion

3.1. Design and preparation of GO-quenched probe

A new fluorescence probe, molecular beacon has been used to monitoring S1 catalyzed reaction [13]. However, the unstable background signal of the probe often influenced the reproducibility and sensitivity of the assay. In addition, the dual modification accordingly increased the cost. In this study, several kinds of GO-quenched fluorescence probes (Table 1) were used to construct S1 sensor. Fig. 1 illustrates the sensing strategy for the detection of S1. In the absence of target nuclease, TAMRA-labeled ssDNA is adsorbed onto the GO by π–π stacking making the fluorophore close proximity to GO surface; thus, GO significantly quenches the fluorescence of TAMRA. In the presence of S1, the TAMRA-labeled ssDNA is cut into small fragments and the fluorophore labeled small fragments is released from GO surface due to the weak affinity. The fluorescence intensity accordingly increases. Therefore, the fluorescence intensity of TAMRA as a function of S1 concentration can be measured simply and rapidly.

In order to demonstrate the strategy's feasibility, we first investigated the fluorescence quenching efficiency of GO to ssDNA strands with different base constituents. As shown in Fig. 2A, the quenching efficiency of GO to ssDNA increased with the concentration increase of GO. When the GO was attained at 15 mg/mL while oligonucleotides fixed at 100 nM, the quenching efficiency of GO to TAMRA-labeled probes (P1 to P4) was more than 99%. This result indicated that GO can efficiently quench the fluorescence of the fluorophore. We then investigated the fluorescence change when the GO-P1 was mixed with S1 in the commercial buffer (pH 4.5) and found that the fluorescence of GO-P1 solution only weakly increased after mixing with S1. However, the fluorescence of the same sample significantly increased when this buffer was replaced by TE buffer containing 10 mM Mg^{2+} and 2 mM gentamycin sulfate (Fig. 2B). This result provides the solid foundation for the design of GO-based fluorescence biosensor for S1 assay in real time. Subsequently, we detected the fluorescence change of GO-P1 caused by S1. Fig. 2C shows the fluorescence emission spectra of the GO-based biosensor in the presence of S1 Nuclease or not. Curve a indicated the weak emission spectra of solely GO-P1 due to the quenching

Table 1
Oligonucleotide strands with different sequences.

Name	Sequence and label fluorophore	Length (bases)
P1	5′-TAMRA-CACAAACAGGACACATGGCGAGCCGA GTTGTG-3′	31
P1′	5′-TCGGCTCGCCATGTGTCCT-3′	19
P2	5′-TAMRA-CACAA CAGGA CACAT GGCTG TCCTG TTGTG-3′	30
P3	5′-TAMRA-CACAACAGGACACATGGGGACTGTTGTG-3′	28
P4	5′-TAMRA-CACAA C AGGA CATGG C TCCT GTTGTG-3′	26
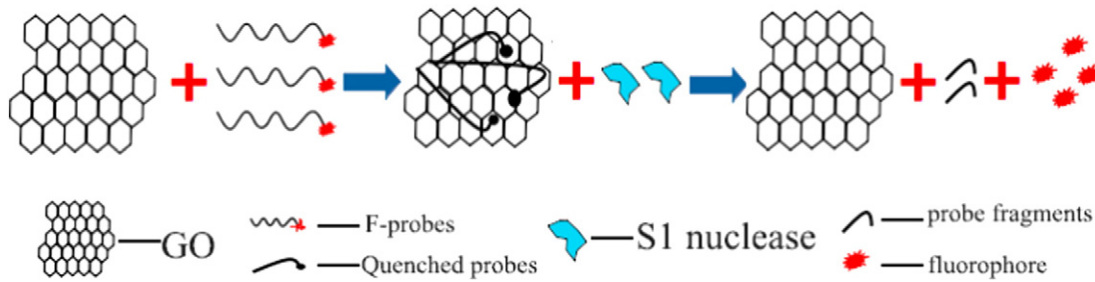
Fig. 1. The schematic of real-time monitoring of S1 using GO quenched probe as substrate. The process of nucleotide digestion catalyzed by S1 produces short fragments of fluorescence probe, which can not bind with GO, and accordingly causes a significant increase in fluorescence.

ability of GO on the TAMRA moiety, while the complete cleavage of the probe with an excess of S1 can cause fluorescence enhancement (curve b). However, the signal of the same sample kept at background level when S1 was replaced by Eco RI, a limited endonuclease (curve c). This data was further confirmed by SDS-PAGE electrophoresis (Fig. S1). These results not only indicate that the presence of S1 cannot only disrupt the interaction of GO with fluorescence probe through binging step but also suggest that the GO-probe is suitable for S1 assay sensitively.

3.2. Effect of probe length on the reaction

In order to get an optimal substrate for S1 assay, 4 kinds of fluorescence probe (P1 to P4 and P1/P1′ duplex) with different lengths were applied as substrates. By comparing the difference of the initial velocities (the fluorescence change in the first 20 s) of reaction caused by

these substrates, we found that the initial velocities of P1 to P4 didn't show significant difference to influence S1 activity (Fig. S2), while the initial velocity of the single stranded DNA is faster than that of the double-stranded DNA (P1P1′). The result suggests that single-stranded DNA is an optimal substrate for S1 assay, which is consistent with previous report [3]. And, the GO-P1 was used in the next experiments.

3.3. Effect of temperature and pH on the reaction

We then optimized the temperature on the reaction. Fig. 3A indicated that the activity profile of S1 nuclease is bell-shaped with a maximum at 45 °C. The activity at this degree was 2.6-fold higher than that of measured at 20 °C. Then, it decreased gradually as the increase of temperature. The result demonstrates that temperature can increase the initial velocity by increasing the collision frequency of molecules
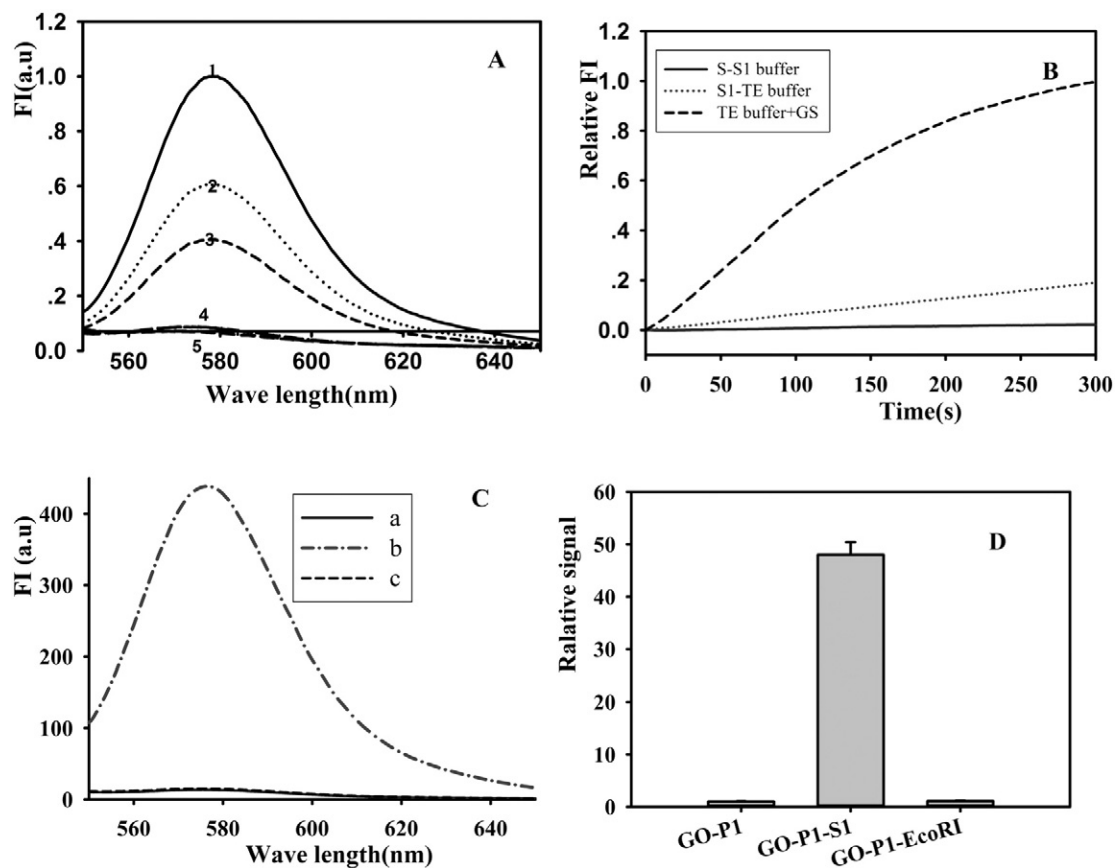
Fig. 2. (A) The effect of GO concentration on the quenching efficiency, the GO concentration from 1 to 5 are 2, 4, 10, 12 and 15 mg/mL, respectively; (B) The real time fluorescence curves of S1 digestion with different buffer; (C) Emission spectra of GO-quenched probe and its cleavage products, excitation: 521 nm, (a) GO-P1; (b) a + S1 nuclease; (c) a + EcoRI. (D) The S/B of GO-P1 with different treatment.
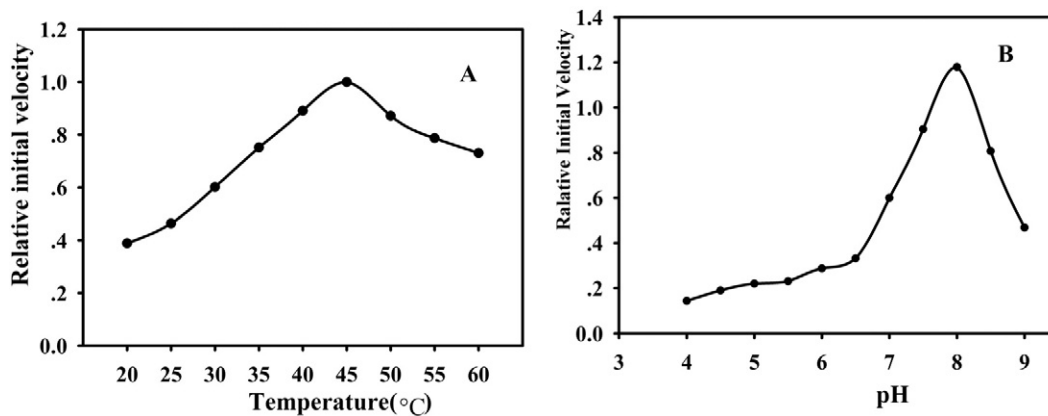
Fig. 3. The initial rates of substrate cleavage as a function of temperature (A) and pH (B). [S1] = 50 U/mL, [GO-Probe] = 100 nM.

through speeding up molecule movement in some range. We optimized another important factor of pH value, which can significantly influence S1 activity. The result demonstrates that the activity profile is bell-shaped with a maximum at pH 8.0. Activity of S1 increases as the increase of pH value from pH 5 and 9, then slowly decreases from pH 8.0. It still keeps at a high level when the pH value reaches at 10 (Fig. 3B). This profile is significantly different to most of previous reports, which is also bell-shaped with a maximal activity at pH 4.0 and 4.5 [2,3]. In fact, it has been reported that S1 efficiently digested singles-stranded DNA at pH 7.5 with Mg^{2+} as activator [3], which is

fully consistent with our data. However, the theoretical interest of this finding concerning pH medicated S1 cleavage remains open.

3.4. S1 detection in real time in a TE buffer based on GO-sensor

In our experiment, we further used this GO-based biosensor platform to investigate the influence of S1 concentration on the initial velocity under the optimal conditions. Results in Fig. 4 showed that the initial velocity became faster as the S1 concentration gradually increased. Along with the lapse of time, the removal velocity slows down gradually
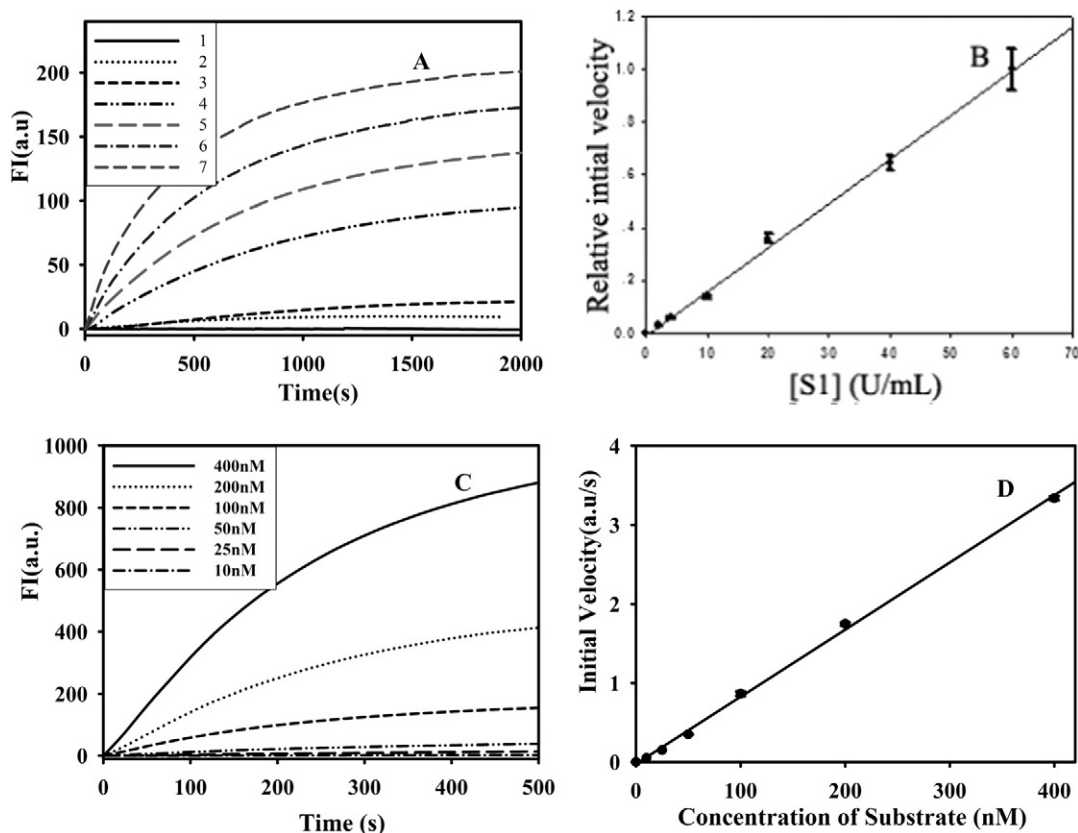

Fig. 4. Activity assay and kinetics study of S1 nuclease. Assays of A and B were performed at 45 °C in the buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM gentamycin sulfate and 100 nM GO-P1. Fluorescence intensity was measured at 578 nm upon excitation at 521 nm. Panel A shows representative reaction progress curves obtained from 1–60 U/mL S1 respectively. The lowest trace (a) was obtained from a control reaction without enzyme. Panel B shows the initial rates of substrate cleavage as a function of the concentration of S1. Panel C shows the representative reaction progress curves obtained with different concentrations of substrate. Panel D shows the Michaelis–Menten plot for S1, [GO-P1] = 10–400 nM, [S1] = 50 U/mL.

with the diminishing of reactant GO-P1. In the range from 1 to 60 U/mL, the initial velocity is directly proportional to the enzyme concentration. This relationship shows that removal kinetics obeys the Michaelis–Menten equation. The detection limit of S1 assay is 0.5 U/mL (based on three times higher fluorescence intensity than the background noise), which is lower than that of previous methods [11,13]. This is because S1 is more efficient when activated by Zn^{2+} than when activated with Mg^{2+} [23]. Considering its simplicity and rapidity and lost cost, especially for the acceptable activity at neutral condition, the new method is also hopeful for S1 application in the future.

An attempt to measure individually the kinetic parameters k_{cat} and K_m of S1 was performed by testing the enzyme at substrate concentrations between 50 and 400 nM. The initial cleavage velocity (V_0) was measured from the fluorescence change in the first 20 s of time curves. Plotting V_0 versus 1/[S] yields a straight concentration line (Fig. 4D). This indicates that the kinetics data fit well with the Michaelis–Menten equation. From the Lineweaver–Burk plot, the important kinetic parameters, K_m and k_{cat} are determined as $1.4 \pm 0.2\,\mu M$ and $0.6\,min^{-1}$, respectively. This implies that the affinity for the substrate of S1 is similar to fluorescence assay based on molecular beacon [13], whose K_m values for similar substrates are 6.8 μM, we are similar in the μM range.

3.5. Effects of metal ions on S1 activity

It has been reported that S1 prefers the metal ions for activity [2,23]. This means that the conformation of S1 catalytic center would change depending on the variety of the metal ion. In this experiment, we further explored the influence of heavy metal ions on S1 activity by monitoring the initial velocity change of reaction caused by the various metal ions. Fig. 5 showed the results of different metal ions on S1 activity. Among them, the presence of Na^+, K^+ and Ca^{2+} increased S1 activity in some extent, while the presence of other metal ions inhibited S1 activity in a concentration-dependent manner, which is consistent with previous reports [3]. The inhibitory ability becomes stronger according to the sequence of Pb(II), As(III), Cd(II), Cu(II) and Hg(II). Furthermore, S1 activity wholly lost when the concentration of these metal ions attained 10 μg/mL. By combing with previous results [3], we speculate that the different effect of metal ions on S1 significantly varied mainly due to the affinity difference of catalytic sites with metal ions. Although the mechanisms should be further investigated, this method still provides an alternative for studying the relation between metal ions with S1.

3.6. Effectors screening of S1

Many antibiotics are a class of regulators of nuclease enzyme [24–26]. In order to more broadly assess the applicability of this new method for the S1 effectors screening, we selected several kinds of antibiotics as

model and determined the impact of these antibiotics on S1 activity. From the results, we found that these antibiotics except cefradine didn't affect background signal of GO-P1 (data not shown) while they showed different effects on S1 activity. Among them, gentamycin sulfate, cefradine and kanamycin showed as stimulators. Especially for gentamycin sulfate, it can stimulate S1 activity in a concentration-dependent manner. The activity of S1 increases more than 10 folds when the concentration of gentamycin sulfate is at 2 mM. Then, the stimulate ability becomes weaker even when the concentration still increase (Fig. 6A). On the contrary, 2 mM of erythromycin, sodium penicillin, carbenicillin disodium and ampicillin can inhibit S1 activity about 8%, 60%, 61% and 66%, respectively (Fig. 6B). These results demonstrate that the simple, rapid and efficient method described herein provides an alternative approach for evaluating effectors targeted on S1 nuclease.

4. Conclusion

In summary, the GO-based sensor for its ultrahigh signal to noise ratio and high stability is finding interesting applications for S1 assay. Integrated with inherited signal-transfer mechanism, the binding and cleaving elements can be used as sensitive probe for enzymatic reaction. This biosensor has merits for application in enzymatic assay and effectors screening for several reasons. First, it gives a real-time portrayal of what is happening in the reaction and avoids problems arising from stopping the reaction and taking out samples every few minutes, such as in gel electrophoresis and end-point measurement methods. Furthermore, not necessary to stop reactions in the middle with effectors can give a more precise response that can only come from the cleavage reaction of the enzyme. Second, the simple detection and analysis steps shorten the detection time for each sample and minimize environmental effects. Finally, the introduction of graphene oxide significantly decreased the cost of the assay compared with some traditional instrumental analysis based on HPLC and MS and fluorescence method based on molecular beacons. All in all, the real-time fluorescence assay based on GO sensor developed here shows great advantages in these aspects for its simple, rapid method, low cost, easy construction and high sensitivity. These properties will enable the construction of different natural substrates for S1 study in vitro and the method, coupled with other expertise, will bring more information for wholly understanding the mechanism of DNA hydrolysis catalyzed by S1 nuclease.
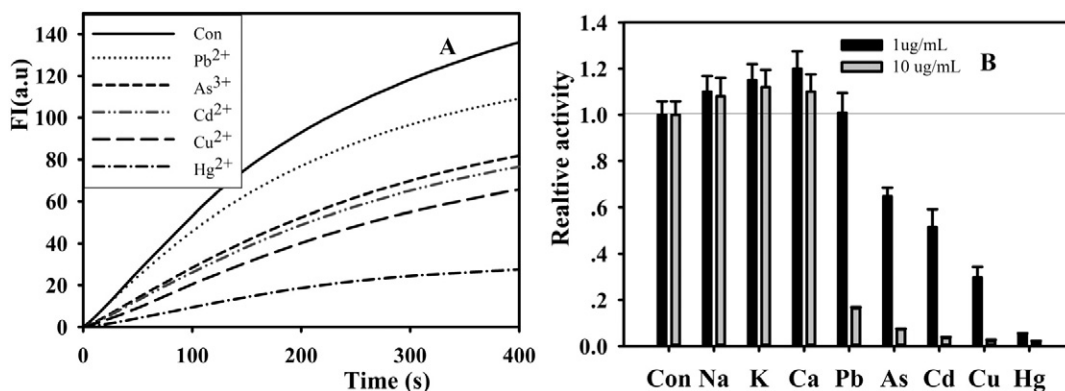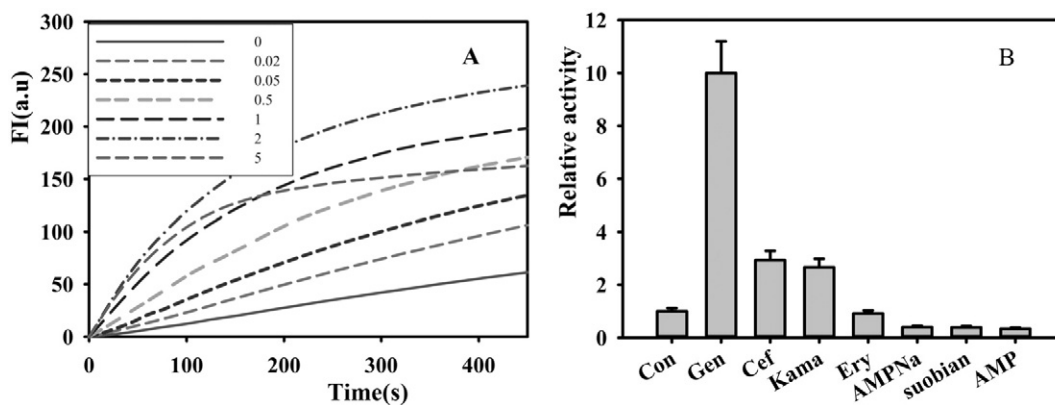
Acknowledgments

Fig. 5. The effects of metal ions on S1 activity. Panel A shows representative curves obtained with different metal ions with concentration of 1 μg/mL. Panel B shows the effects of metal ions on S1 activity. Assays were performed as described in Fig. 4. [S1] = 50 U/mL, [GO-P1] = 100 nM.

Fig. 6. Effects of antibiotics on S1 activity. Panel A shows the time–fluorescence curves of DNA hydrolysis catalyzed by S1 with different concentrations of gentamycin sulfate; Panel B shows the effects of different antibiotics with concentrations of 2 mM on S1 activity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sbsr.2015.12.002.

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