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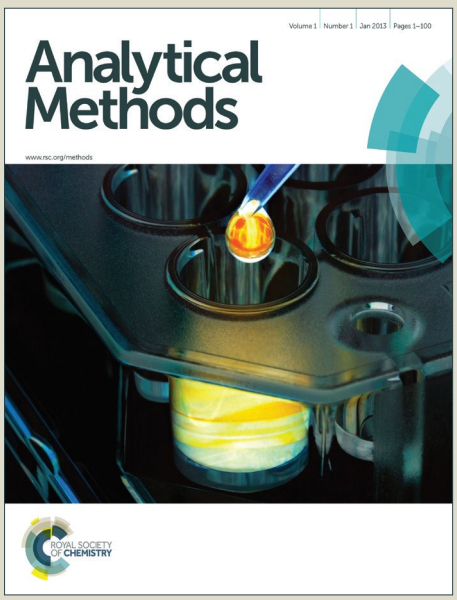

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Title: Ultrasensitive nuclease activity and inhibition assay using microchip electrophoresis with laser induced fluorescence detection

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Abstract

A novel microchip electrophoresis method with laser induced fluorescence detection (MCE-LIF) was developed for ultrasensitive detection of nuclease activity and inhibitor. S1 nuclease was chosen as the model nuclease to demonstrate the proof-of-concept of proposed approach. In this assay, the fluorescein-labeled ssDNA (FAM-ssDNA) was used as a signal reporter. In the presence of nuclease, FAM-ssDNA was digested to 5-FAM-nucleoside monophophates and short oligonucleotide fragments. However, the cleavage reaction of FAM-ssDNA with nuclease was prohibited in the presence of inhibitor. Detection of S1 nuclease activity and inhibitor can be achieved by separating FAM-ssDNA and FAM-5-nucleoside monophophates, and detecting the fluorescence intensity of FAM-5-nucleoside monophophates in the MCE-LIF platform. The calibration curve showed a linearity in the range of S1 nuclease concentrations from 0.002 to 0.2 U/mL. Based on a signal/noise ratio (S/N) of 3, the detection limit was estimated to be 0.001 U/mL, which was about 1−3 orders of magnitude more sensitive than the developed approaches. The proposed method was low-cost and simple in its operation, and the capabilities for target detection from complex fluids and screening of nuclease inhibitors were verified.

Keywords: Microchip electrophoresis; Laser induced fluorescence detection; Nuclease assay; Inhibitors screening

1 Introduction

Nucleases can hydrolyze the phosphodiester bonds in DNA or RNA. They are of significant importance in a variety of fields ranging from biotechnology to pharmacology, as well as in biological processes involving replication, recombination, DNA repair, molecular cloning, genotyping, and mapping.[1-5] Nucleases have also been recognize as promising pharmacological targets for antimicrobial and antiviral drug development.[6-9] On the basis of these biological significance, the assaying of nuclease activity and inhibition are of great importance in clinical diagnosis, modern molecular biology, and the drug-discovery process.

Traditional analytical methods for assaying nuclease activity include gel electrophoresis, high-performance liquid chromatography (HPLC), and enzyme-linked immu-nosorbent assays (ELISAs).[10-13] However, these protocols suffer from disadvantages, such as being complicated, time consuming, laborious, poor in sensitivity, or requiring radiolabeled substrates. To overcome these limitations, some intriguing techniques have been developed, including colorimetric methods based on conjugated polymers[14], gold nanoparticles[15-17] and DNAzyme[18], fluorescence assays such as fluorescence resonance energy transfer (FRET), fluorescence quenching, nanoparticle-enhanced fluorescence polarization[19-22] etc. However, most of these methods require expensive tedious synthesis procedure, polydispersity of conjugated polymers and nanoparticels, double labeled DNA substrates and usually having a high background signal. Consequently, establishing a simple and sensitive method for

nuclease analysis is greatly required.

Capillary electrophoresis (CE) and microchip electrophoresis (MCE) have become recognized as the powerful separation technique. CE has been used in the research which relate to nuclease and nucleic acids. [23-28] Comparing with CE, MCE which is a modern trace analysis technique, has attracted increasing attention owing to its numerous advantages such as lower sample and reagent consumption, high separation efficiency, portability and the possibility of being fully automated.[29-32] However, since the channels in microfluidic devices are very narrow, a highly sensitive detection mode is required for biochemical and clinic analysis. Laser induced fluorescence (LIF) detection as a detection system is most frequently used in MCE to improve its sensitivity.[33-36] MCE-LIF platform has been proven to be a powerful tool for the separation and determination of various targets such as small molecule, metal ion, protein, and nuclease etc.[37-41] Although many MCE-LIF methods have been reported for sensing of various targets, few methods are currently available for ultrasensitive detection of nuclease activity and inhibition.

In the present work, we designed a novel strategy based on MCE-LIF platform for ultrasensitive detection of nuclease activity and inhibition. As the proof-of-concept of our approach, S1 nuclease was chosen as a model system to be investigated, and the fluorescein-labeled ssDNA (FAM-ssDNA) was used as a signal reporter. In the presence of nuclease, FAM-ssDNA was digested to 5-FAM-nucleoside monophophates and short oligonucleotide fragments. However, the cleavage reaction of FAM-ssDNA with nuclease was prohibited in the presence of inhibitor. Based on

the MCE-LIF platform, the 5-FAM-nucleoside monophophates and superfluous FAM-ssDNA were separated within 90 s, and fluorescence intensity of 5-FAM-nucleoside monophophates was used for quantification of nuclease activity and inhibition. Proposed MCE-LIF assay exhibited very high sensitivity and specificity toward S1 nuclease, and provides a new alternative for the detection of nuclease activity and screening of inhibitor.

2 Experimental

2.1 Chemicals and materials

Nuclease S1 supplied with 5×reaction buffer was provided by Thermo Fisher Scientific. The FAM-labeled 16-mer single-stranded oligonucleotides (FAM-ssDNA) with a sequence of 5'-FAM-ATGCTATAATATTAAT-3' and a completely complementary sequence with FAM-ssDNA of 5'-ATTAATATTATAGCAT-3' used in this study were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China), and purified by HPLC method. Sodium dodecyl sulfate (SDS) and sodium borate were purchased from Shanghai Chemical Reagent (Shanghai, China). Sodium pyrophosphate (PPi) was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Thrombin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Exonuclease III (Exo III), endonuclease DpnI, Nb.BbvCI and Nt.AlwI were purchased from the New England Biolabs, Inc. (Ipswich, MA, USA). All other chemicals were of analytical reagent grade and used without further purification.

Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA), and used throughout the work. All solutions were filtered through a 0.45 μm membrane filter. The electrophoresis buffer was 30 mM borate solution containing and 30 mM SDS (pH 9.4, adjusted with 1 M NaOH solution).

2.2 Apparatus and microfluidic chip

The MCE-confocal LIF detection system with 473 nm semiconductor laser was built by the Shandong Normal University. A multi-terminal high voltage power supply, variable in the range of 0−5000 V, was used for sample loading and MCE separation. The output signal was recorded and processed with a computer using a chromatography data system (Zhejiang University Star Information Technology, Hangzhou, China). A home-made glass/PDMS hybrid microfluidic chip was used for the separation of sample. The fabrication of the chip was performed according to the procedure described previously.[42] The layout and dimensions of the chip is shown in Fig. S1. All channels etched in glass substrates were 25 μm deep and 45 μm wide. The distance between sampling channel and sample waste channel is 50 μm. All reservoirs were 4 mm in diameter and 2 mm deep. The effective separation channel length was 3.2 cm. The channel between reservoirs S and SW was used for sampling, and the channel between B and BW was used for the separation.

2.3 Procedures for cleavage reaction

For S1 nuclease assays, 2.5 μL of the FAM-ssDNA (4 μM), 2.5 μL of the nuclease S1

5×reaction buffer [CH_3COONa (200 mM), NaCl (1.5 M), $ZnSO_4$(10 mM), pH 4.5] and varying concentrations of S1 nuclease solution were mixed, the mixed solution was diluted with H_2O to 12.5 μL. The above prepared solution was incubated for 30 min at 37°C. After that, the mixed solution was diluted with 20 mM Tris-HCl (pH 9.0) buffer to 62.5 μL. The final concentration of FAM-ssDNA was 0.16 μM.

To study the inhibition effect of PPi, 2.5 μL of the FAM-ssDNA (4 μM), 2.5 μL of the nuclease S1 5×reaction buffer [CH_3COONa (200 mM), NaCl (1.5 M), $ZnSO_4$ (10 mM), pH 4.5], 0.2 U/mL S1 nuclease and different concentrations of PPi were mixed, and the mixed solution was diluted with H_2O to 12.5 μL. The above prepared solution was incubated for 30 min at 37°C. After that, the mixed solution was diluted with 20 mM Tris-HCl (pH 9.0) buffer to 62.5 μL.

2.4 Microchip electrophoresis

Before repetitive runs, the microfluidic channel was rinsed sequentially with 0.1 mol/L NaOH, water, and electrophoretic buffer solution for 6 min each. Prior to electrophoresis, all reservoirs were filled with the electrophoretic buffer. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoretic buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample solution, a set of electrical potentials was applied to four reservoirs: reservoir S at 650 V, reservoir B at 250 V, reservoir BW at 350 V, and reservoir SW at grounded. The sample solution was transported from reservoir S to SW in pinched mode. After 20 s, potentials were

switched to reservoir B, S and SW at 2000, 1200 and 1200 V, respectively, while reservoir BW was grounded for separation and detection.

3 Results and discussion

3.1 Design principle

Based on MCE-LIF platform, a novel homogeneous strategy for nuclease activity assay and inhibitor screening was developed. The working principle of the design is illustrated in Scheme 1. A FAM-labeled 16-mer ssDNA containing a 5'-GC-3' site was employed as the substrate for enzymatic hydrolysis. The FAM-ssDNA kept the original long state in the absence of nuclease. In the presence of nuclease, FAM-ssDNA was digested to 5-FAM-nucleoside monophophates and short oligonucleotide fragments. If the nuclease inhibitor is present, the cleavage reaction of FAM-ssDNA with nuclease was prohibited, and the fluorescence intensity of 5-FAM-nucleoside monophophates markedly decline compared with the absence of nuclease inhibitor. The 5-FAM-nucleoside monophophates and superfluous FAM-ssDNA were separated by MCE, and the fluorescences of FAM-5-nucleoside monophophates and FAM-ssDNA were detected by LIF. Thus, the fluorescence intensity of 5-FAM-nucleoside monophophates can be used for quantification of nuclease activity and inhibition.

3.2 Optimization of the cleavage reaction time

In order to achieve good performance of the proposed assay for S1 nuclease activity,

the cleavage reaction time was optimized in our studies. We investigated the effect of the cleavage reaction time in the range from 0 to 45 min on the S1 nuclease activity assay. Fig. S2 indicates the fluorescence intensity increased rapidly with the increasing cleavage reaction time up to 30 min, and then kept constant after 30 min. By weighing both the sensitivity and the total assay time, the cleavage reaction time of 30 min was selected for subsequent studies.

3.3 Optimization of separation conditions

To achieve a well separation, some important parameters such as the type of electrolyte, the concentration of electrolyte, pH of electrophoresis buffer, SDS concentration and applied voltage were optimized for the study, and the separations were characterized by resolution (Rs) values. The influence of electrolyte type on the separation was investigated with a phosphate buffer, Tris buffer or borate buffer. The results showed that the borate buffer had the best separation effect. Therefore, borate solution was selected as running buffer in this MCE separation. The pH of the buffer has an important effect on the channel-wall surface characteristics of the microchip and the effective electric charge of the analytes. The obtained results indicated that with the increase of pH value from 8.6 to 9.4, the Rs increased. Further increasing the pH resulted in the decrease of Rs value (Fig. S3). It was noted that optimum Rs was obtained at pH 9.4. The effect of the borate buffer concentration on the Rs was also investigated. Concentration of borate buffer were examined in the concentration range of 20~40 mM. The results indicated that Rs increase with the increase of borate buffer

concentration from 20 to 30 mM. A further increase of the buffer concentration from 30 to 40 mM resulted in significantly decreased Rs. This decrease of Rs with increasing borate buffer concentration may be due to Joe heat dissipation. Therefore, a concentration of 30 mM borate buffer was used for further experiments. The effects of SDS concentration were tested in the range of 10~50 mM. The results indicated that introducing SDS micelles into the electrophoresis buffer dramatically enhanced Rs. An optimal SDS concentration was found to be 30 mM. Separation voltage also affected the Rs. It was examined in the range of 1100 to 2600 V. The results showed that the migration times with the increase in separation voltage. The Rs increased with the increase of the separation voltage from 1100 and 2000 V, and reached maximum Rs when separation voltage was 2000 V. Considering both the analysis time and the Rs, a separation voltage of 2000 V is considered optimal.

3.4 MCE-LIF performance

Upon optimal separation conditions, the sample solutions after cleavage reaction were analyzed, and obtained electropherograms are presented in Fig. 1. By comparing trace a and trace b, a new peak (peak 1) in trace b was observed in the presence of 0.006 U/mL S1 nuclease. Trace c indicates the peak height (fluorescence intensity) of peak 1 increased rapidly with the increasing S1 nuclease concentration, the peak height (fluorescence intensity) of peak 2 decreased at the same time. This is consistent with the fact that more active S1 nuclease is added during the nuclease cleavage reaction.

3.5 Analytical figures of merit

The proposed MCE-LIF method was evaluated for the detection of S1 nuclease activity in terms of the response linearity, limit of detection and reproducibility. The peak height (fluorescence intensity) of peak 1 was used for quantification of analytes. Under the optimized conditions, different concentrations of S1 nuclease were analyzed, the relationship between the peak height of peak 1 and the concentration of S1 nuclease was shown in Fig. 2. As can be seen from Fig.2, the peak height of peak 1 was shown to be good linear with the concentration of S1 nuclease ranging from 0.002 U/mL to 0.2 U/mL. The regression equation of calibration curve for S1 nuclease was $H = 8105.4C + 14.80$, with a correlation coefficient of 0.9965. Where H is the peak height of peak 1 (μV), and C is the concentration of S1 nuclease (U/mL). The detection limit (S/N=3) was estimated to be 0.001 U/mL. To evaluate the repeatability of the proposed assay, a standard solution of S1 nuclease (0.1 U/mL) was analyzed eight times. The results showed that the reproducibility of analyte response (RSD) of the migration time and peak height of the peak 1 were 2.6% and 3.7%, whereas for peak 2 the migration time and peak height were 2.8 % and 3.5%, respectively.

3.6 Inhibition of S1 nuclease activity

To further extend the potential application of proposed method in the inhibition assay, this MCE-LIF-based approach was used for screening the inhibitors of S1 nuclease. To evaluate this property, we investigated the prohibition effects of PPi on the S1

nuclease, which has been reported to be an effective inhibitor to prevent the cleavage of ssDNA by S1 nuclease. As shown in Fig. 3A, the peak height of peak 1 was greatly increased in the absence of PPi (trace a), indicating that the cleavage reaction of ssDNA by S1 nuclease proceed well. By comparing trace b and trace c from Fig. 3A, the electropherograms clearly show that with the increase of the PPi concentration, a gradual decrease of the peak height of peak 1 and obvious increase of the peak height of peak 2 were observed, implying that the cleavage reaction by S1 nuclease was prohibited. Fig. 3B shows that the peak height of peak 1 was continuous decreased with increasing the concentrations of PPi. The digestion of ssDNA by S1 nuclease is almost completely inhibited by PPi at 1 mM. The results indicate that the proposed method could be employed to investigate the S1 nuclease inhibition, and may further be applied to screen other S1 nuclease inhibitors.

3.7 Specificity of nuclease assay

To evaluate the specificity of proposed method, we challenged the system with the double-stranded DNA (dsDNA) and some nonspecific enzymes, including endonuclease Dpn I, endonuclease Nt.AlwI, endonuclease Nt.BbvCI, exonuclease III (Exo III), and thrombin. S1 nuclease which is known to be a single-strand-specific nuclease has little effect on dsDNA. As shown in Fig. 4A, only specific ssDNA caused a dramatic fluorescence increase of peak 1, while the peak 1 was almost without observed when used perfect-matched dsDNA. In addition, the specific target led to an increase in the fluorescence intensity of peak 1, while all other nonspecific

enzymes did not lead to obvious variation of the fluorescence intensity of peak 1 (Fig. 4B). These results clearly demonstrate the high specificity of the developed MCE-LIF-based method for S1 nuclease activity assay.

4 Concluding remarks

In summary, an ultrasensitive MCE-LIF-based method was developed for the detection of S1 nuclease activity and inhibition. In contrast to previous reported methods, the developed method was low-cost and simple. In addition, this method is high sensitive with a detection limit of 0.001 U/mL, which is one of most sensitive method for S1 nuclease activity assay. Moreover, this method exhibits also a high specificity to target, and the recoveries of serum samples analysis were satisfactory. Furthermore, the proposed method not only shows the potential to screen new ssDNA-specific nucleases and monitor their cleavage reactions, but also offers a promising application in drug screening and basic research related to nucleases. By changing the sequence of the ssDNA in FAM-ssDNA, the proposed platform would allow assaying other nuclease activity and inhibition. Hence, we anticipate that the MCE-LIF platform described herein will open a new avenue for ultrasensitive detection of nuclease activity and inhibitor.

Acknowledgements

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Figure Legends

Scheme 1 Schematic illustration of MCE-LIF-based platform for nuclease activity assay and inhibitor screening.

Figure 1 Electropherograms from the nuclease leavage reaction solutions. (a) A solution containing 0.16 μM FAM-ssDNA in the absence of S1 nuclease; (b) a mixture solution containing 0.16 μM FAM-ssDNA and 0.006 U/mL S1 nuclease; (c) a solution containing 0.16 μM FAM-ssDNA and 0.1 U/mL S1 nuclease. Electrophoresis buffer was 30 mM borate (pH 9.4) containing 30 mM SDS.

Figure 2 Calibration curve between the peak height of peak 1 and the concentration of S1 nuclease. Error bars were estimated from three replicate measurements.

Figure 3 (A) Electropherograms from the solutions of inhibition effects of PPi on FAM-ssDNA cleavage by S1 nuclease. (a) a mixture solution containing 0.16 μM FAM-ssDNA and 0.2 U/mL S1 nuclease in the absence of PPi; (b) a mixture solution containing 0.16 μM FAM-ssDNA, 0.2 U/mL S1 nuclease and 0.075 mM PPi; (c) a solution containing 0.16 μM FAM-ssDNA, 0.2 U/mL S1 nuclease and 0.32 mM PPi. Electrophoresis

buffer was 30 mM borate (pH 9.4) containing 30 mM SDS. (B) Inhibition efficiency of S1 nuclease activity by PPi. The ordinate is the peak height (fluorescence intensity) of peak 1. Concentrations of FAM-ssDNA and S1 nuclease were 0.16 μM and 0.2 U/mL, respectively.

Figure 4 (A) Electropherograms for the specificity evaluation. (a) A solution containing 0.16 μM FAM-dsDNA and 0.2 U/mL S1 nuclease; (b) a solution containing 0.16 μM FAM-ssDNA and 0.2 U/mL S1 nuclease. Electrophoresis buffer was 30 mM borate (pH 9.4) solution containing 30 mM SDS. (B) The selectivity of the present strategy for S1 nuclease assay. The concentration of S1 nuclease was 0.1 U/mL and the concentrations of other enzymes were all 40 U/mL.

Scheme 1

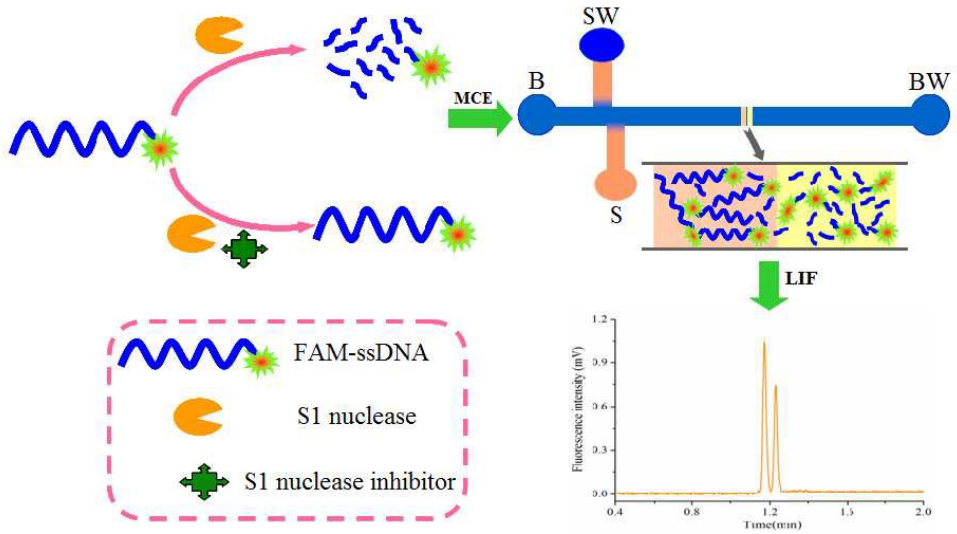


Figure 1

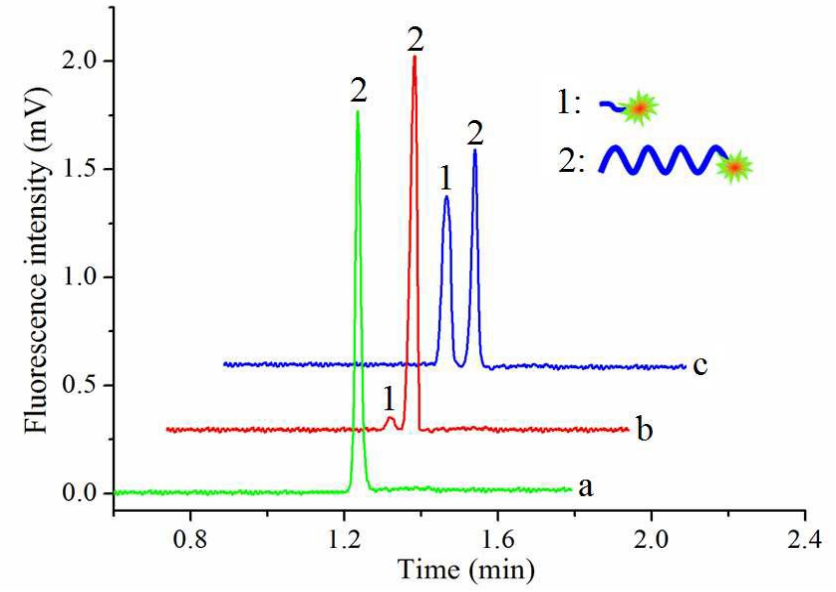


Figure 2

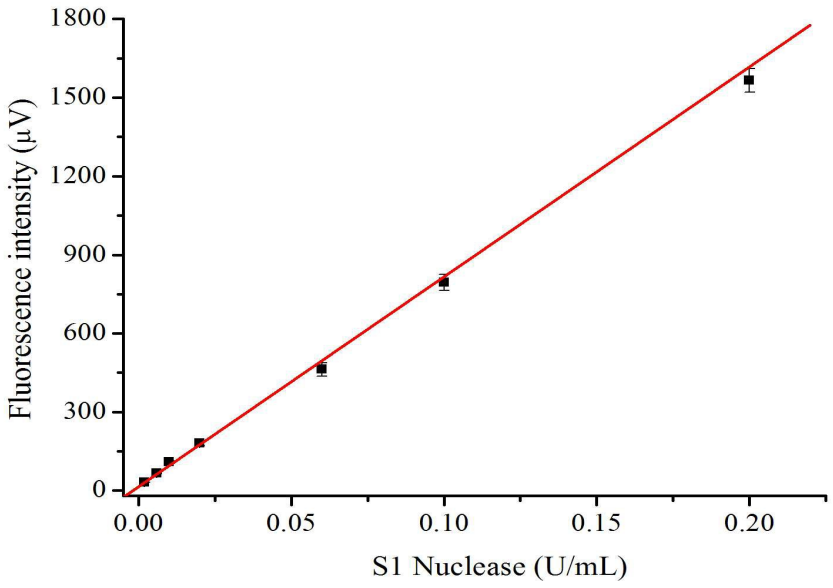


Figure 3

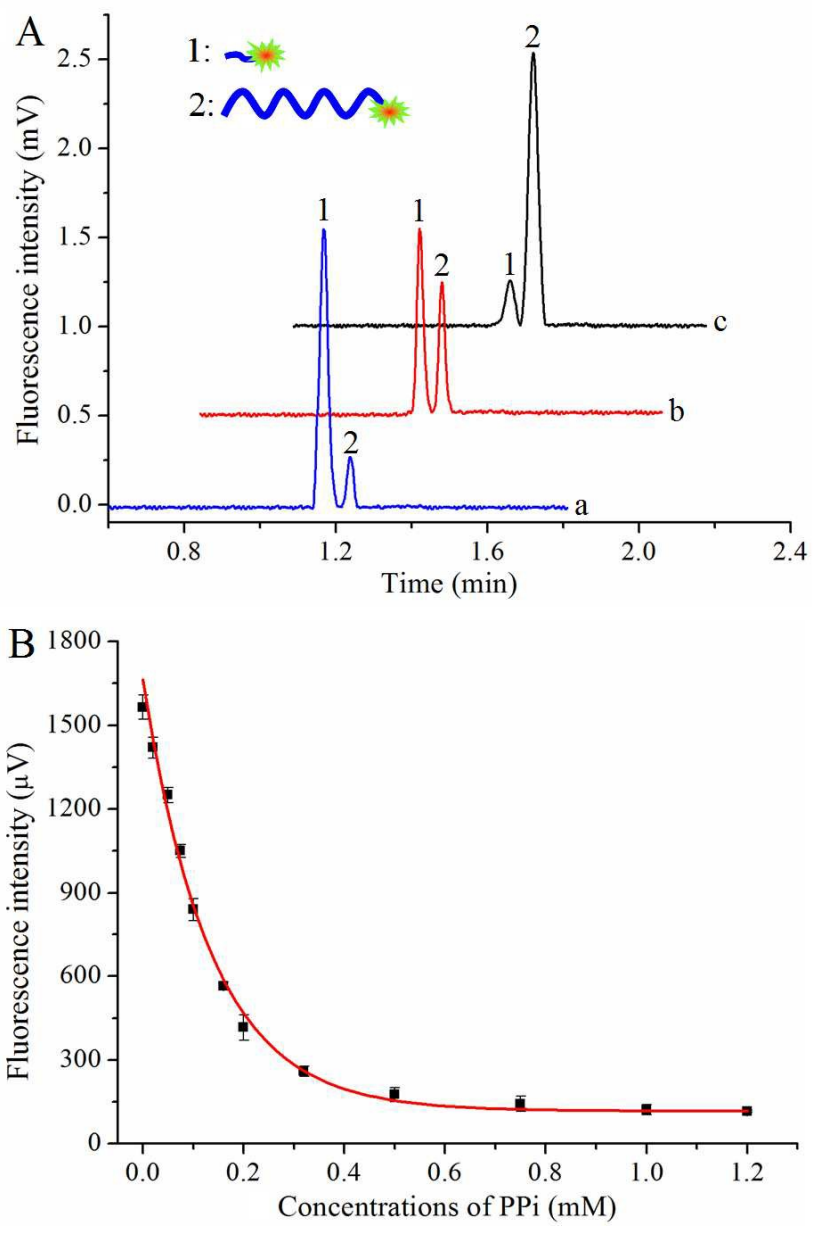
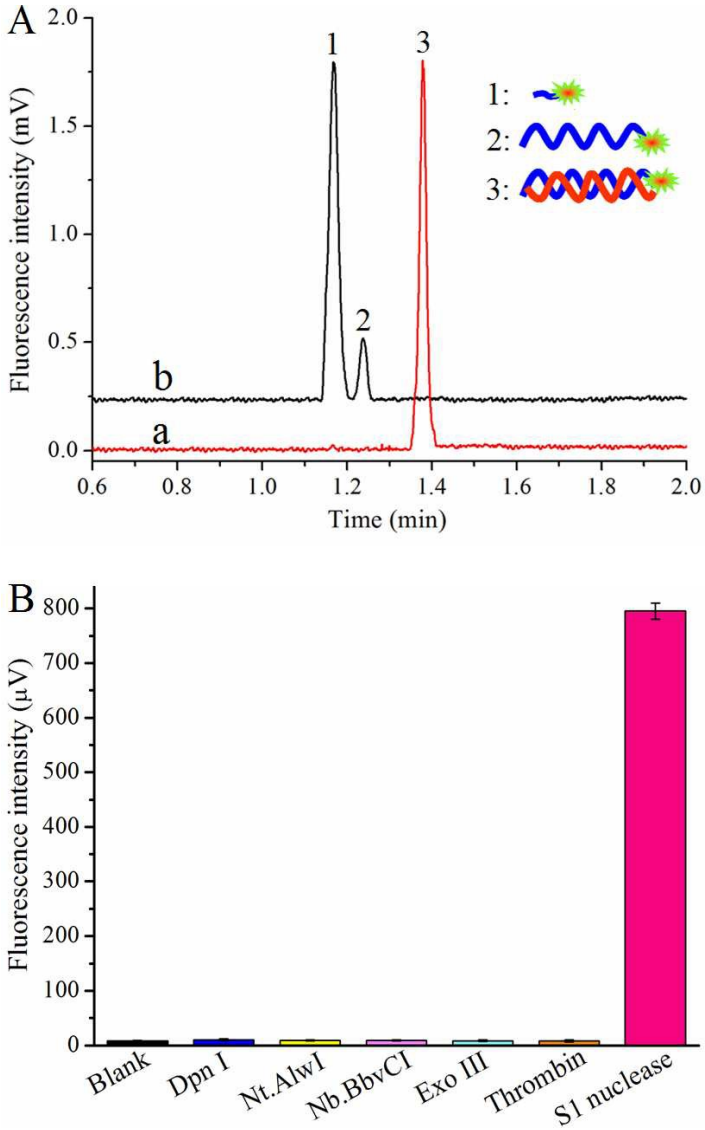


Figure 4

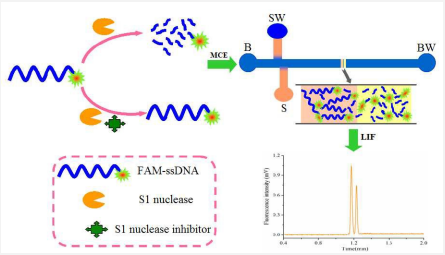


Entry for the Table of Contents

Title Text

Ultrasensitive nuclease activity and inhibition assay using microchip electrophoresis with laser induced fluorescence detection

Yingfeng Qin, Jingjin Zhao, Yong Huang, Shuting Li, and Shulin Zhao**



A novel microchip electrophoresis method with laser induced fluorescence detection was developed for ultrasensitive detection of nuclease activity and inhibitor.