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Investigation of the effects of metal ions in sample buffer on capillary electrophoresis coupled with laser-induced fluorescence analysis of thrombin using a dye-labeled 29-mer DNA aptamer†

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Thrombin is an important serine protease in blood and a therapeutic biomarker. The aptamer-based assays for thrombin take advantage of unique features of nucleic acid aptamers in selection, preparation, stability, and modification of functional groups. Aptamer affinity capillary electrophoresis coupled with laser induced fluorescence (CE-LIF) analysis for thrombin uses a fluorescently labeled aptamer probe and relies on the change of electrophoretic mobility of the aptamer probe caused by protein–aptamer binding. A high-affinity 29-nt DNA aptamer (Apt29) has been used as an affinity probe in CE-LIF analysis of thrombin. In this work, we made a further detailed investigation of the effects of metal ions (e.g. Na⁺, K⁺, and Mg²⁺) in sample buffer on the CE-LIF analysis of thrombin using a dye labeled 29-mer DNA aptamer probe and achieved sensitive detection of thrombin. A complex of thrombin and the Apt29 probe was well separated from the unbound probe in CE separation. We found that the addition of K⁺ in sample buffer was not preferred in CE-LIF analysis of thrombin using dye-labeled Apt29, and it caused significant decrease of complex peaks of thrombin–Apt29. The use of both Na⁺ and Mg²⁺ in sample buffer was favorable for the sensitive detection of thrombin because a larger peak area of complex peaks of thrombin and the aptamer probe was obtained. Under the optimized conditions, as low as 0.1 nM thrombin was successfully detected with good specificity. This work shows that metal ions in sample buffer have a large effect on aptamer affinity CE-LIF analysis of thrombin using an Apt29 probe.

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Introduction

Aptamers are artificial nucleic acid ligands that can bind to targets with high selectivity and binding affinity.^{1–4} They can be selected from nucleic acid libraries through a process called SELEX (systematic evolution of ligands by exponential enrichments).^{1–4} The analytical applications of aptamers are attractive due to the unique properties of aptamers, such as easy synthesis with low cost, good thermal stability, easy introduction of functional groups for labeling and immobilizations.^{3–8} Many aptamer based assays have been developed for a wide range of targets by using versatile formats.^{3–8}

Aptamer affinity capillary electrophoresis (CE) assay of proteins uses an aptamer as the affinity probe and requires CE separation of the protein–aptamer complex and the unbound aptamer.^{7,9,10} In aptamer affinity CE analysis of proteins, the

binding of the aptamer probe to protein targets usually causes a large change in electrophoretic mobility because aptamers have small size and many negative charges and proteins are relatively large and less charged.^{7,9,10} The complex of the aptamer and protein and the unbound aptamer probe are well isolated from each other during CE analysis when the aptamer has strong binding affinity to proteins.^{9,10} By using a fluorescently labeled aptamer probe, aptamer affinity CE coupled with laser induced fluorescence has been used to detect different proteins, e.g. thrombin, immunoglobulin E (IgE), platelet derived growth factor BB (PDGF-BB), *etc.*,^{9–16} showing advantages in high sensitivity and rapid separation. The analysis of multiple proteins in one CE run with aptamer probes by modulating the differences of electrophoretic mobility of protein–aptamer complexes has been achieved.^{14,17} The combination of PCR amplification and aptamer affinity CE allows the ultrasensitive detection of proteins.¹⁸ CE-coupled with chemiluminescence detection has been applied to the analysis of interactions between aptamers and proteins.¹⁹ CE is also used for the selection of aptamers against protein targets from random sequence nucleic acid libraries by separating the protein–nucleic acid complex from unbound nucleic acids.^{20–27}

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Thrombin, a Na⁺-activated allosteric serine protease, plays multifunctional roles in physiological and pathological coagulation.^{28–30} It regulates platelet aggregation and conversion between circulating fibrinogen and fibrin monomer. It is reported that thrombin is related to Alzheimer's disease, Parkinson's disease, diabetes, and cancers.^{28–34} For the aptamer affinity CE-LIF detection of thrombin, two DNA aptamers have been used, the 15-nt DNA aptamer having weak binding affinity (Apt-15, 5'-GGT TGG TGT GGT TGG-3')³⁵ and the 29-nt DNA aptamer (Apt 29, 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3') showing a dissociation constant (K_d) value of around 0.5 nM.³⁶ A few groups reported the use of Apt29 or aptamers with a similar sequence to Apt29 as an affinity probe in the CE-LIF detection of thrombin.^{13–15} The CE-LIF assay using Apt29 enabled higher sensitivity than that using Apt15 because of the high binding affinity of Apt29.^{9,11,13–15} Usually, metal ions have effects on binding between protein and aptamers. An appropriate concentration of metal ions in buffer is required for the good binding between protein and aptamers as metal ions can influence the conformation of aptamers and binding affinity.^{11,12} In addition, metal ions also affect the electrophoretic properties of the aptamer and aptamer–protein complex. The effects of metal ions in sample buffer, *e.g.* Mg²⁺, K⁺ and Na⁺, on the CE-LIF analysis of thrombin using a dye-labeled 29-mer DNA aptamer have not been well investigated in previous study.^{13–15}

Here we made a detailed investigation of the influence of metal ions (*e.g.* Na⁺, Mg²⁺, and K⁺) in sample buffer on the CE-LIF detection of thrombin using a tetramethylrhodamine (TMR) labeled 29-nt aptamer and achieved sensitive detection of thrombin at optimized metal ion concentrations of sample buffer. We found that the presence of KCl in sample buffer significantly reduced the peak area of the complex peak of thrombin and Apt29 in CE-LIF analysis. The use of MgCl₂ or NaCl greatly improved the complex peak of thrombin and the aptamer. The simultaneous presence of MgCl₂ and NaCl in sample buffer allowed a better complex peak. Higher sensitivity of the CE-LIF detection of thrombin was achieved by using the sample buffer containing 10 mM NaCl and 1 mM MgCl₂. We achieved the detection of thrombin as low as 0.1 nM by using a 10 nM TMR-labeled aptamer under the optimized conditions. This study shows that the metal ions in sample buffer have a large effect on the performance of CE-LIF analysis of thrombin using a 29-mer DNA aptamer probe. This study will be helpful for developing affinity aptamer CE-LIF assays for proteins.

Experimental

Chemicals and reagents

Human α -thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Lysozyme, platelet derived growth factor-BB (PDGF-BB), and hemoglobin (Hb) were ordered from Sigma (St. Louis, MO, USA). Human immunoglobulin G (IgG) was obtained from Athens Research & Technology, Inc. (Athens, GA, USA). Human serum was purchased from Zhongke Chenyu Biotechnology (Beijing, China). The DNA aptamers (5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3')

with TMR labeling at the 5' end (denoted as Apt29-5'TMR) were synthesized and purified by Sangon Biotech (Shanghai, China). All solutions were prepared with 18.2 M Ω distilled deionized water (Elga Labwater system (Purelab Ultra Genetic), UK).

Instrument

A laboratory-built CE-LIF system was used to detect human thrombin.^{15,37} Briefly, we used an uncoated fused-silica capillary (365 μ m o.d.; 25 μ m i.d.) (Yongnian Company, Hebei, China) to perform CE-LIF analysis. The total length of the capillary was 40 cm, and the distance between the detection window and the inlet of the capillary was 34 cm. The capillary was activated by flushing with 100 mM NaOH for 5 hours, and then rinsed with water. A high-voltage power supply (Tianjin, China) was used to apply the voltage during CE analysis. A helium–neon laser (543.5 nm, 1 mW) (Melles Griot, Irvine, CA, USA) was employed and focused on the detection window of the capillary and to excite the fluorophore. Fluorescence emission was focused by using a microscope and passed through a pinhole. Fluorescence was filtered by using a filter (FF01-575/15-25, Semrock, USA), and then it was collected by using a photomultiplier tube (PMT, Hamamatsu Photonics, Japan). The signal from the PMT was analyzed using the chromatographic software.

CE-LIF analysis of thrombin

TMR labeled aptamer probes were mixed with varying concentrations of human thrombin in sample buffer, and a free TMR dye, used as an internal standard (IS), was also added to the sample buffer. The final concentrations of TMR labeled aptamer probes and the free TMR dye were 10 nM and 93 pM, respectively. After incubation for 1 h in ice, the sample was electrokinetically injected into the capillary by applying a positive voltage of 15 kV for 5 s. CE-LIF analysis was then performed in TG buffer (25 mM Tris, 192 mM glycine, pH 8.3), which was filtered with the 0.22 μ m filter (Millipore, Bedford, USA), by applying a positive voltage of 20 kV at room temperature (25 °C). Triplicate analyses were done. The capillary was rinsed sequentially with 20 mM NaOH for 3 min, deionized water for 2 min, and TG buffer for 2 min by applying a positive voltage of 20 kV before each injection.

Results and discussion

Principle and complex formation during CE-LIF analysis

Fig. 1 shows the schematic principle for the detection of thrombin by affinity CE-LIF using TMR-labeled 29-nt DNA aptamers as probes. Apt29 is highly negatively charged and small in size (molecular weight about 9 kDa). The binding of TMR-labeled Apt29 to thrombin significantly changes the charge to mass ratio of the aptamer probe because thrombin has a larger size (molecular weight about 36.7 kDa) and is less charged. The thrombin–aptamer complex and free aptamer are separated due to the different charge to mass ratios of the thrombin–aptamer complex and the unbound aptamer probe. The separated species are detected by measuring fluorescence.

Fig. 1 Schematic diagram for the detection of thrombin by aptamer-based affinity CE-LIF.

First we applied 10 mM Tris-HCl (pH 7.5) as the sample buffer and the TG buffer as the separation buffer to test whether the binding of thrombin to the TMR-labeled aptamer (Apt29-5'TMR) allowed the formation of the affinity complex and the isolation of the complex from the unbound aptamer probe. We incubated 10 nM TMR-labeled Apt29 with 10 nM thrombin in the sample buffer, and then injected the sample into the capillary by applying a positive voltage of 15 kV for 5 s. CE separation was conducted in TG buffer as suggested in a previous study.¹⁰ Fig. 2 shows the electropherograms of CE-LIF analysis of Apt29-5'TMR (10 nM) in the absence of thrombin or in the presence of thrombin (10 nM) in the sample buffer. The peak of the free TMR dye (IS) appeared at around 1.7 min. In the absence of thrombin, a peak of Apt29-5'TMR was observed at around 2.5 min. When the aptamer probe was incubated with 10 nM thrombin and then tested in CE-LIF analysis, the peak area of Apt29-5'TMR decreased, and a new peak appeared at about 2.2 min, which was the complex peak of Apt29-5'TMR and thrombin. The complex of thrombin and the aptamer probe migrated faster as the complex had a relatively larger size and

Fig. 2 Electropherogram of Apt29-5'TMR (10 nM) incubated in sample buffer (10 mM Tris-HCl, pH 7.5) in the absence of or in the presence of thrombin (10 nM). Peaks 1, 2 and 3 represent the IS, aptamer-thrombin complex and free aptamer, respectively.

less negative charge to mass ratio than the fluorescently labeled Apt29. The result shows that the affinity complex of thrombin and Apt29 is well formed and isolated from the free Apt29 probe.¹³⁻¹⁵

Effect of metal ions in sample buffer on CE-LIF analysis of thrombin

We investigated the effects of different metal ions (K^+ , Na^+ , and Mg^{2+}) in sample buffer (10 mM Tris-HCl, pH 7.5) on the performance of CE-LIF analysis of thrombin. The addition of KCl in sample buffer significantly decreased the peak area of the complex peak. When 5 mM KCl existed in sample buffer, the area of the complex peak decreased almost 2-fold. When 20 mM KCl was present, the area of the complex peak was only about 30% of the peak area of the complex that was obtained in sample buffer without containing KCl. It shows that K^+ is not preferred in CE-LIF analysis of thrombin using Apt29-5'TMR as a probe (Fig. 3). This result is consistent with the previous report.¹⁵ However, Li *et al.* reported that the addition of KCl in sample buffer did not affect the CE-LIF analysis of thrombin.¹³

Fig. 3 Effects of KCl in sample buffer on CE-LIF analysis of thrombin. (A) Electropherograms corresponding to different concentrations of KCl. (B) Relationship between the complex peak area and concentration of KCl. Thrombin (10 nM) and Apt29-5'TMR (10 nM) were incubated in sample solution (10 mM Tris-HCl, pH 7.5) containing varying concentrations of KCl.

The recent crystallographic analysis study has shown that the 27-mer aptamer that lacks a pair of nucleotides at terminals of Apt29 forms a duplex and pseudo-G-quadruplex motif in the complex of thrombin–aptamer.³⁸ The addition of KCl in sample buffer (10 mM Tris–HCl, pH 7.5) in CE-LIF analysis may cause the Apt29 to form a G-quadruplex structure,³⁹ which is different from the structure of aptamer binding to thrombin,³⁸ and may weaken the binding between the aptamer and thrombin.

Compared with KCl, NaCl in sample buffer showed a different influence on the CE-LIF analysis of thrombin. As shown in Fig. 4, with addition of NaCl in sample buffer, the complex peak area of thrombin and Apt29-5'TMR significantly increased. The presence of 10 or 20 mM NaCl led to about 4-fold increase of the peak area of the complex peak. In addition, with an increase of NaCl, the distance between the peak of Apt29-5'TMR and the complex peak of thrombin and Apt29-5'TMR became narrow. It suggests that NaCl also affects the separation of the complex from the unbound aptamer probe. The results show that the use of NaCl enhances the complex peak of thrombin and the aptamer.

MgCl₂ is usually involved in the selection buffer of aptamers,³⁶ and it is helpful for DNA to form a duplex secondary

structure. MgCl₂ also showed a great effect on the CE-LIF analysis of thrombin using TMR-labeled Apt29. Fig. 5 shows that when 1 mM MgCl₂ was used in sample solution (10 mM Tris–HCl, pH 7.5), the complex peak increased greatly (more than 2-fold increase) in the peak area, and then a decrease was observed with the further addition of MgCl₂. The results indicate that the addition of 1 mM Mg²⁺ in sample solution is preferred for the affinity binding of Apt29-5'TMR and thrombin. However, Song *et al.* reported that the affinity of aptamer Apt29 and thrombin became weak with increasing concentration of MgCl₂,¹⁵ which was inconsistent with our results. Perhaps, 1 mM MgCl₂ is helpful for Apt29 to form a suitable secondary structure in binding with thrombin.

We further investigated the influence of NaCl in sample buffer (10 mM Tris–HCl, pH 7.5) containing 1 mM MgCl₂ (Fig. 6). When 10 mM NaCl or 20 mM NaCl was added, a larger complex was observed. When the sample buffer contained both 1 mM MgCl₂ and 20 mM NaCl, the peak area of complex (Fig. 6) was larger than that obtained by the use of sample buffer containing only 20 mM NaCl (Fig. 4), and about 20% increase in peak area of the complex peak was obtained. The simultaneous

Fig. 4 Effects of NaCl in sample buffer on CE-LIF analysis of thrombin. (A) Electrophoregrams corresponding to different concentrations of NaCl. (B) Relationship between the complex peak area and concentration of NaCl. Thrombin (10 nM) and Apt29-5'TMR (10 nM) were incubated in sample solution (10 mM Tris–HCl, pH 7.5) containing varying concentrations of NaCl.

Fig. 5 Effects of MgCl₂ in sample buffer on CE-LIF analysis of thrombin. (A) Electrophoregrams corresponding to varying concentrations of MgCl₂ in sample buffer. (B) Relationship between the complex peak area and concentration of MgCl₂. Thrombin (10 nM) and Apt29-5'TMR (10 nM) were incubated in sample solution (10 mM Tris–HCl, pH 7.5) containing varying concentrations of MgCl₂.

Fig. 6 Effects of NaCl in sample buffer containing 1 mM MgCl_2 on CE-LIF analysis of thrombin. (A) Electropherograms corresponding to different concentrations of NaCl. (B) Relationship between the complex peak area and concentration of NaCl. Thrombin (10 nM) and Apt29-5'TMR (10 nM) were incubated in sample solution (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl_2 and varying concentrations of NaCl.

presence of NaCl and 1 mM MgCl_2 further enhanced the complex peak. Compared with the result obtained by sample buffer only containing 10 mM Tris-HCl, the peak area of the complex peak increased about 5-fold when the Tris-HCl sample buffer contained both 1 mM MgCl_2 and 10 mM NaCl. As Fig. 6 shows, the peak of the complex of Apt29-5'TMR and thrombin moved closer to the free Apt29-5'TMR peak when 20 mM NaCl was added. When NaCl concentration was higher than 20 mM, the complex peak became decreased. Therefore, the metal ions in sample buffer show great effects on the CE-LIF analysis of thrombin, and the sample buffer (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl_2 and 10 mM NaCl was preferred for CE-LIF analysis of thrombin because a large complex peak was obtained and well isolated from the unbound aptamer probe.

CE-LIF detection of thrombin

Under the optimized conditions, we achieved the detection of varying concentrations of thrombin with CE-LIF using Apt29-5'TMR as an affinity probe. Fig. 7 shows the electropherograms of analysis of thrombin at varying concentrations ranging from

0 nM to 200 nM in sample buffer (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl_2 and 10 mM NaCl. With an increase of thrombin concentration, the complex peak gradually increased, and the peak of the free aptamer probe decreased. The peak area of the complex peak showed linear relationship towards the concentration of thrombin in the range from 0.1 to 10 nM ($y = 129\,606x + 2292$, $R^2 = 0.989$) (Fig. 7B). As low as 0.1 nM thrombin was detected. When the concentration of thrombin was beyond 10 nM, the obtained peak area of the complex peak reached a plateau level. However, the peak of the unbound aptamer did not totally disappear at high concentrations of thrombin (higher than 20 nM), and the peak area of the unbound aptamer probe did not further decrease. This phenomenon was also observed in the previous report.¹⁵ One possible reason is that aptamer probes may contain some impurities (e.g. DNA oligonucleotides with wrong sequences). Another reason is that the synthesized aptamer probes may have different conformations though the sequences are the same. Thrombin can only bind to the aptamer that has appropriate conformation.

Fig. 7 CE-LIF detection of varying concentrations of thrombin by using Apt29-5'TMR in the sample buffer (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl_2 and 10 mM NaCl. (A) Electropherograms corresponding to varying concentrations of thrombin. The corresponding concentration of thrombin was indicated at traces. (B) Relationship between the peak area of the complex and thrombin concentration.

As a comparison, Fig. S1 in the ESI† shows the CE-LIF analysis of thrombin in sample buffer (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl₂. The complex peak area showed a linear relationship towards the concentration of thrombin in the range from 0.5 to 10 nM ($y = 51\,941x + 29\,051$, $R^2 = 0.985$), and the detection limit of thrombin was 0.5 nM. The slope of the calibration linear fitting curve is lower than that obtained in sample buffer containing 1 mM MgCl₂ and 10 mM NaCl. It shows that the use of 1 mM MgCl₂ and 10 mM NaCl improved the sensitivity of CE-LIF analysis of thrombin, and the detection limit of thrombin in our methods is lower than or comparable to that in the previous reports.^{13–15}

Specificity test

We examined the selectivity of CE-LIF analysis of human thrombin using Apt29-5'TMR by testing other proteins (10 nM) including prothrombin, Hb, human IgG and lysozyme. When Apt29-5'TMR was used, no complex peak was observed for the tested proteins (Fig. S2 in the ESI†), so these proteins did not show interference for thrombin detection.

We further tested the applicability of the CE-LIF detection of thrombin by Apt29-5'TMR in a complex sample matrix. Thrombin was spiked into a 50-fold diluted serum sample and incubated with Apt29-5'TMR. Thrombin was still be detected in the 50-fold diluted human serum sample (Fig. S3 in the ESI†). The complex peak area is linear with the concentration of thrombin ranging from 0.5 to 10 nM ($y = 64\,985x + 58\,446$, $R^2 = 0.981$) and the detection limit was 0.5 nM thrombin.

Conclusion

In this work, we tested the effects of metal ions (K⁺, Na⁺ and Mg²⁺) in sample buffer on the CE-LIF analysis of thrombin using TMR-labeled Apt29. Our results showed that the presence of K⁺ in sample buffer caused a significant decrease of the complex peak of thrombin and Apt29 in CE-LIF analysis. The presence of Na⁺ or Mg²⁺ significantly improved the peak area of the complex. A larger complex peak was observed when Na⁺ and Mg²⁺ were both present in sample buffer. Under the optimized conditions, as low as 0.1 nM thrombin was successfully detected. This assay displayed good specificity and allowed for the detection of thrombin spiked in a diluted human serum sample. This work shows that metal ions in sample buffer can cause great influence on aptamer affinity CE-LIF analysis of proteins, and it is potentially helpful for assay development.

Conflicts of interest

There are no conflicts of interest to declare.

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