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A microchip electrophoresis-based fluorescence signal amplification strategy for highly sensitive detection of biomolecules[†]

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Abstract

We have developed a microchip electrophoresis (MCE)-based fluorescence signal amplification strategy as a universal MCE method for the detection of trace biomolecules. This strategy exhibits high sensitivity and specificity for target molecules, and has been applied for the detection of interferon-gamma (IFN- γ) in human plasma with satisfactory results.

Graphical Abstract

A microchip electrophoresis-based fluorescence signal amplification strategy was developed for highly sensitive detection of biomolecules.

Microchip electrophoresis (MCE), regarded as a miniaturized version of capillary electrophoresis (CE), has become a very attractive separation assay technique.^{1–4} MCE

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exhibits numerous advantages, such as lower sample and reagent consumption, high separation efficiency, easy operation and high throughput.^{5,6} Therefore, MCE technology has been successfully used for analysis of various compounds including small molecule, metal ion, protein, and nuclease etc,^{7–10} and holds great promise for single cell analysis, biomedical research and clinical analysis.^{11–13} However, in MCE analysis, extremely small sample size (usually in the picoliter–nanoliter range) brings a challenge to achieve high detection sensitivity.

To overcome low sensitivity of MCE, various online electro-migration-based sample preconcentration techniques have been studied over the past decade. These on-chip stacking methods include large-volume sample stacking,¹⁴ multiple concentration approaches combining chitosan sweeping, field-amplified sample stacking, and reversed-field stacking.¹⁵ However, stacking method in MCE cannot meet the requirement of low detection limit for trace analytes assay in biological samples. Recently, Tang et al. developed an aptamer and nicking enzyme assisted signal amplification method based on microfluidic droplets,¹⁶ and a detection limit of 19 pM for the protein was obtained. Therefore, developing suitable non-stacking amplification method is highly expectant for enhancing the detection sensitivity of MCE assay.

Recently, some other signal amplification techniques have also been shown as a good alternative to traditional analytical methods for improving the sensitivity of biomolecules detection, such as signal-amplifiable supramolecular hydrogel,¹⁷ hyper-branched hybridization chain reaction,¹⁸ spherical nucleic acids gold nanoparticles triggered mimic-hybridization chain reaction enzyme-free dual signal amplification,¹⁹ fluorescence polarization signal amplification based on structure-switching-triggered nanoparticles enhancement,²⁰ signal amplification based on combination of magnetic separation and plasmon enhanced fluorescence,²¹ DNase I-assisted target recycling signal amplification,²² and electrophoresis separation assisted chemiluminescence (CL) signal amplification.²³

In this work, we adapt a cooperative hybridization assay²⁴ with MCE-laser induced fluorescence detection (MCE-LIF), and presented a universal MCE-based fluorescence signal amplification strategy for highly sensitive detection of biomolecules. We used interferon-gamma (IFN- γ) as model analyte in the current proof-of-concept experiments, and using proposed method quantified the IFN- γ in human plasma from normal volunteers. To the best of our knowledge, the proposed strategy is the first example for microchip electrophoresis-based fluorescence signal amplification. Compared with many reported methods for IFN- γ detection (Table S1, ESI[†]), the present signal amplification strategy possesses a simple experimental procedure and high detection sensitivity, only needs a short detection time. By simply changing the aptamer sequence of the hairpin probe, it is suitable for detecting a broader range of biomolecules in real biological samples, which exhibits improved analytical properties in biotechnological applications, and facilitates the development of point-of-care devices in clinical analysis.

[†]Electronic Supplementary Information (ESI) available: Experimental section and the optimization of the assay conditions. See DOI: 10.1039/xxxxxxx/

The principle of proposed MCE-based fluorescence signal amplification strategy is outlined in Scheme 1. The system consists of an aptamer hairpin probe, a fluorescein amidite (FAM)-labeled deoxyribonucleic acid (DNA) signal probe (FAM-DNA) and T7 exonuclease (T7 Exo). Hairpin probe contains an aptamer sequence (blue) of target and complementary sequence (green) of FAM-DNA signal probe. In the presence of target, the target combines with its aptamer to open the hairpin structure of hairpin probe because of the conformation change of the aptamer. Subsequently, the opened hairpin probe hybridizes to FAM-DNA signal probe forming DNA duplex. The formed DNA duplex triggers selective cleavage of FAM-DNA signal probe by T7 Exo, and releasing FAM-DNA segment and the target–hairpin probe complex. The released target–hairpin probe complex is able to hybridize with another FAM-DNA signal probe and the cycle starts anew. Thus, a single copy of the target generates many FAM-DNA segments. The fluorescence signal was amplified through target–hairpin probe complex circling and continuous T7 Exo enzyme cleavage. Then, the released FAM-DNA segments were separated with superfluous FAM-DNA signal probes, and detected on the MCE-LIF platform. The fluorescence intensity (peak height) of the FAM-DNA segment can be used for the quantification of target.

Gel electrophoresis was used to verify the viability of proposed strategy. The results are shown in Fig. S1 (ESI[†]). The first three lanes showed the hairpin probe, FAM-DNA signal probe, and mixture of the hairpin probe with FAM-DNA signal probe, respectively. The band in lane 4 had a relatively low migration compared with lane 1, indicating the formation of hairpin probe-IFN- γ complex. A new band with lower mobility was observed in lane 5, indicating the formation of hairpin probe-IFN- γ -FAM-DNA tripartite complex. After incubating with T7 Exo, the band from the hairpin probe-IFN- γ -FAM-DNA tripartite complex disappeared in lane 6, and only one band was observed at the same migration position as that in lane 4, which demonstrated that a strand scission reaction happen. The gel electrophoresis experiments confirmed the specificity combine of hairpin probe with IFN- γ , and T7 Exo-assisted strand scission reaction.

To verify the feasibility of proposed assay strategy, the MCE-LIF performance under different conditions were investigated. Obtained electropherograms were presented in Fig. 1. As can be seen, only one peak (peak 2) was observed in trace “a” and trace “b”, and the fluorescence intensity was unchanged, indicating hairpin probe cannot hybridize with FAM-DNA signal probe in absence of IFN- γ . In trace “c”, because the hairpin probe-FAM-DNA duplex cannot be cleaved in the absence of T7 Exo, no new peak was observed. In trace “d”, a new peak (peak 1) was observed in the presence of T7 Exo, and the fluorescence intensity (peak height) of peak 2 decreased at the same time. Trace “d” indicated that FAM-DNA in hairpin probe-IFN- γ -FAM-DNA tripartite complex has been cleaved by T7 Exo, and generating the FAM-DNA segment. The fluorescence intensity (peak height) of peak 1 increased gradually with the increase of IFN- γ concentration. These results indicated that the proposed assay strategy could be used for amplified detection of IFN- γ .

In order to achieve high sensitivity, the parameters that affect the amplified reaction such as the concentration ratio of hairpin probe and signal probe, the concentration of T7 Exo and incubation time were optimized. The effect of the concentrations ratio of hairpin probe and signal probe on the amplified reaction was investigated. The results indicate that the

fluorescence intensity of the system reached maximum when the concentration of hair-pin probe and signal probe was at a concentration ratio of 1: 6.25 (Fig. S2, ESI[†]). This is due to the hairpin probe as a substrate involved in the target circling, while the signal probe was constantly being cut by T7 Exo in the cycle process, resulting in a large number of signal probe consumption. Therefore, a concentration ratio of 1: 6.25 for hairpin probe and signal probe was used in amplified reaction. The systems with different concentration of T7 Exo were also examined. As shown in Fig. S3 (ESI[†]), with increasing concentrations of T7 Exo, the fluorescence intensity increased rapidly and reached a constant at 20 U.

The incubation time has a significant influence on the specific binding of hairpin probe to IFN- γ and the cleavage reaction of T7 Exo. In order to achieve optimal sensitivity, the incubation time was examined. As shown in Fig. S4 (ESI[†]), the fluorescence intensity increased significantly with the incubation time up to 60 min. Then, there was a slight change in the fluorescence intensity with the reaction time further increased. By weighing both the sensitivity and the total assay time, 60 min was chosen as the optimal incubation time.

The parameters that affect the separation such as concentration of electrolyte, pH of electrophoresis buffer, sodium dodecyl sulfate (SDS) concentration and applied voltage were optimized, and the separations were characterized by resolution (R_s) values. The effect of the borate buffer concentration from 15 mM to 35 mM on the R_s was examined. The results showed that the R_s increased with the increase of borate buffer concentration from 15 mM to 25 mM. Further increasing the borate buffer concentration resulted in the significantly decrease of R_s because higher borate buffer concentration causes higher ion strength and excessive Joule heat, resulting in lower separation efficiency (Fig. S5, ESI[†]). The pH of the buffer solution has an important effect on the channel-wall surface characteristics of the microchip and the effective electric charge of the analytes. Therefore, the effect of pH in a range of 8.6–9.4 was investigated. The results indicate that the R_s increase as the pH value increased within 8.6–9.0, and maximum R_s was obtained when the pH value is 9.0. However, the R_s decreases as farther increase of pH value because high pH results in higher ion strength and higher electrophoresis current (Fig. S6, ESI[†]). SDS is a widely used additive in electrophoresis buffers, and can effectively restrain the adsorption of protein on the microfluidic channel. Therefore, the effect of SDS concentration on the R_s was investigated in the range of 10–50 mM. The results indicated that introducing SDS micelles into the electrophoresis buffer dramatically enhanced R_s . A maximal R_s value was obtained when the concentration of SDS was 30 mM (Fig. S7, ESI[†]). Separation voltage affected the R_s and the migration rate of analyte. It was examined in the range of 1700–2500 V. The results indicated that the migration times decrease with the increase in separation voltage. The R_s increased with the increase of the separation voltage from 1700 V to 2300 V, and reached maximum R_s when separation voltage was 2300 V (Fig. S8). Considering both the analysis time and the R_s , an optimal separation voltage was chosen to be 2300 V.

The performance of proposed MCE-based fluorescence signal amplification strategy for the detection of IFN- γ was evaluated in terms of response linearity, limit of detection (LOD), and reproducibility. Under optimized conditions, the peak height of FAM-DNA segment (peak 1) showed a good linearity with the concentration of IFN- γ ranging from 15 pM to 2.5

nM (Fig. S9). Linear regression analysis of the data yielded the following equation: $H=928.6C+18.08$, with a correlation coefficient of 0.9985. Where H is the peak height of peak 1 (μV), and C is the concentration of IFN- γ (nM). LOD for IFN- γ was estimated to be 6.5 pM (signal/noise=3), which is much higher than that of many reported methods (Table S1, ESI[†]). This method is also one of the most sensitive methods to be applied to real sample analysis. To evaluate the repeatability of the proposed assay, a standard solution of 0.5 nM IFN- γ was analyzed seven times. The results of statistical calculation from the analysis data show that the relative standard deviations (RSDs) for the migration time and peak height of FAM-DNA segment were estimated to be 3.4% and 2.1%, respectively.

To evaluate the specificity of proposed MCE-LIF method for IFN- γ detection, we challenged the system with target (IFN- γ) and several nonspecific proteins such as platelet-derived growth factor BB (PDGF-BB), thrombin and bovine serum albumin (BSA). As shown in Fig. 2, only the target could lead to appearance of peak 1, no obvious peak 1 was observed in presence of other nonspecific proteins, and the fluorescence intensity of peak 1 was same with that of blank. These results indicate the high specificity of the developed method for IFN- γ assay.

The applicability of the proposed method in real biological samples analysis was evaluated by detecting IFN- γ in human plasma samples from four volunteers. The electropherograms obtained were shown in Fig. 3. By comparing the height of peak 1 shown in Fig. 3A and 3B, the fluorescence intensity of peak 1 increased when the plasma sample was spiked with IFN- γ standard solution, the fluorescence intensity of peak 2 decreased at the same time. The analytical results for four plasma samples were summarized in Table S2 (ESI[†]). The IFN- γ level in the plasma samples from healthy subjects was found be in the range of 163–360 pM. Recoveries of IFN- γ in the human plasma samples from healthy subjects were also studied by adding IFN- γ into the human plasma samples, and the samples were then analyzed again. Recoveries were found to be in the range of 95.7–105.8%, and the result of statistical calculation shows that RSDs were lower than 5%. These results indicated that other enzymes in the human plasma not interfere with the detection of IFN- γ because of the specificity of T7 Exo. Therefore, the proposed method was reliable for determination of IFN- γ in human plasma.

In summary, we have developed for first time a MCE-based fluorescence signal amplification strategy for highly sensitive detection of biomolecules, and have demonstrated that present method can be used for quantification of trace IFN- γ in human plasma. The method has several important features. First, this assay exhibits a high specificity to the target analyte, which make it can be applied for detection of IFN- γ in real biological samples. Second, it has very high sensitivity for IFN- γ detection. Compared with nM detection limit of traditional MCE-LIF assay, the present method could achieve a detection limit as low as pM. Third, proposed strategy for enhancing the sensitivity of MCE-LIF assay appears to be a universal approach for the detection of other target molecules by simply changing the aptamer sequence of the hairpin probe. Thus, this simple and cost-effective MCE-LIF method has a potential of becoming the major tool for biotechnological applications, and supply valuable information for biomedical research and clinical early diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Electropherograms of different samples. (a) FAM-DNA; (b) FAM-DNA+Hairpin probe; (c) FAM-DNA+hairpin probe+IFN- γ ; (d) FAM-DNA+hairpin probe+IFN- γ +T7 Exo.

Electrophoresis buffer was 25 mM borate solution (pH 9.0) containing 30 mM SDS. The concentrations of hairpin probe, FAM-DNA, T7 Exo and IFN- γ were 40 nM, 250 nM, 20 U and 1.0 nM, respectively. The sampling volume was about 110 pL.

Fig. 2.

The specificity of present strategy for IFN- γ detection. Experiment conditions were as in Fig. 1. The concentration of IFN- γ was 1.0 nM, and the concentration of other proteins was 25 nM. Each experiment was performed five times.

Fig. 3. Electropherograms obtained from the analysis of a human plasma sample diluted 6-fold (A), and a human plasma sample diluted 6-fold spiked with 0.4 nM IFN- γ (B). Experiment conditions were as in Fig. 1. The sampling volume was about 110 pL.

Scheme 1.

Schematic illustration of MC-based fluorescence signal amplification strategy for highly sensitive detection of biomolecules.