Published in final edited form as:

Chem Commun (Camb). 2016 October 25; 52(87): 12806–12809. doi:10.1039/c6cc06327f.

Electrophoresis separation assisted G-quadruplex DNAzyme-based chemiluminescence signal amplification strategy on the microchip platform for highly sensitive detection of microRNA†

Jian Li^a, Jingjin Zhao^{*,a}, Shuting Li^a, Liangliang Zhang^a, Yong Huang^a, Shulin Zhao^{*,a}, and Yi-Ming Liu^b

^aState Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, Guangxi Normal University, Guilin 541004, China.

^bDepartment of Chemistry and Biochemistry, Jackson State University, 1400 Lynch St., Jackson, MS 39217, USA

Abstract

We have developed an electrophoresis separation assisted G-quadruplex DNAzyme-based chemiluminescence (CL) signal amplification strategy on the microchip platform for the detection of trace miRNA. This strategy exhibits high sensitivity and specificity for detection of target molecules.

Graphical abstract

An electrophoresis separation assisted G-quadruplex DNAzyme-based chemiluminescence signal amplification strategy on the microchip platform was developed for the detection of trace miRNA.

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

zhaoshulin001@163.com; jzhao12@163.com; Fax: +86-773-583-2294; Tel. +86-0773-21'20226.

Biomarkers are generally found in trace levels, especially in the early stages of disease progression. Reliable detection of biomarkers in organism is crucial for early diagnosis and treatment of diseases.¹ Conventional assay techniques are usually low sensitivity, labor intensive and consuming large volume of expensive reagents, thus limiting their application for early diagnosis of diseases. Microchip electrophoresis (MCE) as modern trace analysis technique can overcome some limitations of conventional assay, and exhibits numerous advantages, such as lower consumption of sample and reagent, high separation efficiency, easy operation and high throughput.^{2,3} Therefore, MCE technology has been successfully used in chemical analysis, bioassay, clinic and single cell analysis.⁴⁻⁷ However, in MCE analysis, the extremely small sample size brings a challenge to achieve high detection sensitivity.

Chemiluminescence (CL)-based detection offers advantages such as low background, high sensitivity and wide linear range.⁸ To achieve high sensitivity in MCE assay, MCE coupled with CL detection has attracted a great attention, and some MCE-CL detection methods have been successfully used for detection of biomacromolecules such as protein, enzyme, and DNA fragments.⁹ Which indicates that CL is a promising and powerful detection technique for MCE assay. However, because minimum amounts of detectable labels are used in the MCE-CL assay, the sensitivity of assay is still not satisfactory. Thus, a signal amplification system for implementing highly sensitive detection is required in MCE-CL assay.

G-quadruplex DNAzyme formed by the G-quadruplex and hemin possess specific catalytic activities. The main body structure of G-quadruplex DNAzyme is G-quadruplex, which make it easy to link functional oligonucleotides such as aptamer and hairpin DNA, and make the signal amplification techniques based on nucleic acid including rolling circle amplification, strand-displacement amplification, exonuclease III-assisted signal amplification as well as nicking enzyme assisted signal amplification become easy to implement in G-quadruplex DNAzyme-based highly sensitive sensing assay. Therefore, G-quadruplex DNAzyme has received considerable attention in recent years,¹⁰⁻¹⁴ and has served as attractive options to achieve CL signal amplification in DNA assay.^{15,16}

MicroRNAs (miRNAs) are short, endogenous, noncoding RNA of about 18-25 nucleotides (nt). The importance of miRNAs dysregulation for the development and progression of diseases, and the discovery of stable miRNAs in peripheral blood have made it an ideal class of biomarker candidates for clinical diagnosis.^{17,18} For example, the expressions of miR-30b are frequently dysregulated in the development of a variety of cancers and physiologic processes, liking colorectal cancer, oral squamous cell cancers and immune response.^{19,20} These evidences has led to a strong demand for routine quantitative detection of miRNAs in cells, tissues and blood.^{21, 22}

Herein, we report for the first time an electrophoresis separation assisted G-quadruplex DNAzyme-based CL signal amplification strategy on the microchip platform for detection of trace miRNA. The principle of proposed CL signal amplification strategy is outlined in Scheme 1.

The signal amplification system mainly consists of a hairpin DNA probe (H-probe), a biotin functionalized G-rich DNA probe (Bio-G4), the nicking enzyme Nb.BbvCI and streptavidin (SA). In the absence of target miRNA, H-probe maintains the shape of hairpin, so that it could not hybridize with Bio-G4. Therefore, Bio-G4 still keeps intact structure. As a result, the Bio-G4 will combine with SA to form SA-Bio-G4 complex (SA-Bio-G4). When the target miRNA was introduced into the system, the hairpin structure of H-probe was opened by the hybridization reaction between H-probe and the target to form target-H-probe complex, and the target-H-probe complex further hybridize with Bio-G4 to form a DNA duplex. The formation of DNA duplex triggers the selective cleavage of the Bio-G4 by Nb.BbvCI, resulting in the release of the G-rich DNA segments and target-H-probe complex. Released target-H-probe complex then hybridizes with another Bio-G4 to form more DNA duplexes, and initiate the release of more G-rich DNA segments by Nb.BbvCI cleavage. The G-rich DNA segments and superfluous SA-Bio-G4 can combine with hemin respectively to form two different G-quadruplex DNAzymes, and catalyze the CL reaction between hydrogen peroxide and luminol. In the MCE, two different G-quadruplex DNAzymes were quickly separated, and the CL intensity of the G-quadruplex DNAzyme from G-rich DNA segment can be used for the quantification of target.

To verify the feasibility of the proposed strategy, the MCE-CL assay of target under different conditions were investigated by using miR-30b as model analyte. As shown in Fig. 1, when hemin exists only in the sample, electropherogram showed a protuberance at 1.2 min. When the sample contained all the reagents except target miR-30b, electropherogram showed a peak at 0.8 min, which refers to the SA-Bio-G4 complex, indicating that Bio-G4 was not cleaved into two pieces. While in the presence of miR-30b, one new peak was observed in the electropherogram at 1.0 min, which refers to the G-rich DNA segment. This implies that the CL signal amplification reaction was triggered by miR-30b, and resulted in the appearance of the peak from G-rich DNA segment. When more targets were introduced into the system, the height of peak at 0.8 min decline, and that at 1.0 min increase. The results mentioned above demonstrate the feasibility of proposed the principle for CL signal amplification of miRNA assay.

The viability of proposed strategy was further investigated by gel electrophoresis. The results are shown in Fig. S1 (ESI[†]). The first five lanes showed the band from miR-30b, H-probe, Bio-G4, mixture of miR-30b and H-probe, mixture of H-probe and Bio-G4, respectively. Because the hairpin structure of H-probe was opened by target, the band from H-probe disappears while a band in slow migration appears in lane 4. Two bands appear in lane 5, and its migration position is same as that in lane 2 and lane 3, indicating there was no interaction between H-probe and Bio-G4. However, when target was introduced into the system without Nb.BbvCI, as presented in lane 6, the band from H-probe disappears and the band from Bio-G4 turns dark, while a new bright band was observed behind Bio-G4, indicating the formation of target-H-probe-Bio-G4 tripartite complex, and no Nb.BbvCI cleavage reaction happen. Compared with lane 6, a new band appears, and the band from target-H-probe-Bio-G4 tripartite complex disappears in the lane 7, which demonstrated a

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

strand scission reaction by Nb.BbvCI, and result in the generation of G-rich DNA segment and the release of target-H-probe complex.

In order to achieve good assay performance, we optimized the conditions for signal amplification reaction including the concentrations of H-probe, Bio-G4, SA and hemin, as well as the incubation time. The experimental results showed a good assay performance when 50 nM H-probe, 100 nM Bio-G4, 100 nM SA, 500 nM hemin and 2 h incubation time at room temperature were used (Fig. S2-5, ESI[†]). We also optimized the conditions for in-line CL reaction including the concentrations of luminol and H₂O₂, as well as pH value of reaction medium. It is found that the maximal CL intensity was obtained for detecting 2 nM miR-30b when 1.2 mM luminol, 110 mM H₂O₂ and the buffer solution of pH 9.5 were used (Fig. S6-8, ESI[†]).

Based on the principle of proposed method, to achieve the assay performance, two different G-quadruplex DNazymes from G-rich DNA segment and SA-Bio-G4 require good separation by MCE. Therefore, the separation conditions including the concentration of borax buffer and pH value of electrophoretic buffer were investigated. The results indicated that with the increase of borax concentration from 5 mM to 20 mM, the resolution (R_s ; $R_s = 2(T_2 - T_1)/(W_2 + W_1)$, where T and W represent the migration time and the peak width at peak base, respectively) of two kinds of G-quadruplex DNazymes increased, and further increasing the borax concentration resulted in the decrease of R_s . (Fig. S9, ESI[†]). In addition, a maximal R_s was obtained when pH value of electrophoretic buffer was 9.3 (Fig. S10, ESI[†]).

To confirm the ability of proposed method for the detection of target miRNA, a series of different concentrations of miR-30b were analyzed, and the response linearity, limit of detection and the reproducibility of method were evaluated. The results indicate that the peak height (CL intensity) of G-quadruplex DNzyme from G-rich DNA segment showed a good linearity with the log miR-30b concentration from 8.0 pM to 20 nM (Fig. S11, ESI[†]). The limit of detection ($S/N=3$) was estimated to be 4.5 pM, which is same to that of amplified fluorescence polarization method for miRNA detection,²³ and better than the fluorescence assays.¹⁷ Nine repetitive measurements of 2 nM miR-30b were used for estimating the precision, and the relative standard deviation (RSD) was found to be 3.8%, suggesting good reproducibility of this miRNA assay.

To evaluate the specificity of proposed method for target miRNA detection, we challenged the assay with miR-30b and other members of miR-30 family including miR-30a, miR-30c, miR-30d, miR-30e, and non cognate miRNA sequence miR-21, Let-7d at same concentration (Fig. 3). Clearly, miR-30b could be easily differentiated with other miR-30 family members and non cognate miRNA sequence, revealing good specificity.

To demonstrate the feasibility of proposed method for the detection of miR-30b in complex biological matrix, we conducted the HepG2 cell lysate assay. Varying amounts of miR-30b were added into the cell lysate and then tested. The electropherograms obtained upon the analysis of the cell lysate samples are shown in Fig. S12 (ESI[†]). The CL intensity of G-quadruplex DNzyme from G-rich DNA segment increased upon addition of miR-30b to

cell lysate. In addition, comparable responses were found for miR-30b in both buffer solution and cell lysate (Fig. 4). These results indicated the potentiality of proposed method for miR-30b detection in real biological samples.

In summary, an electrophoresis separation assisted G-quadruplex DNAzyme-based CL signal amplification strategy on the microchip platform was developed for miRNA detection. The present method was applied for the detection of miR-30b in HepG2 cell lysate. This amplified MCE-CL assay has several excellent features. Firstly, by using the G-quadruplex DNAzyme-based CL signal amplification strategy, the sensitivity of method is at least three orders of magnitude higher than that of traditional assay. Secondly, by taking advantage of affinity and selectivity of aptamer containing in hairpin probe, the proposed MCE-CL assay exhibits very high selectivity toward target and could distinguish the target molecules from their analogues. In addition, the proposed signal amplification strategy would open new avenues on developing highly sensitive MCE-CL methods. We anticipate that this strategy can be applied readily to detect other biomarkers by simply changing relevant aptamer sequences in hairpin DNA probe, and thus finds a wide application in disease diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Natural Science Foundations of China (No. 21327007 and 21465005), and the US National Institutes of Health (GM089557), IRT1225, as well as BAGUI Scholar Program.

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Fig. 1.

Electropherograms for feasibility study. (A) Hemin; (B) blank (H-probe+Bio-G4+Nb.BbvCI +SA+hemin); (C) blank+20 pM miR-30b; (D) blank+20 nM miR-30b. Peak identification: (1) SA-Bio-G4 complex; (2) G-rich DNA segment. The concentrations of H-probe, Bio-G4, SA and hemin are 50 nM, 100 nM, 100 nM and 500 nM, respectively.

Fig. 3. Specificity of proposed electrophoresis separation assisted G-quadruplex DNAzyme-based CL signal amplification strategy. The concentration of miRNA was 2 nM each. The experimental conditions were as in Fig. 1.

Fig. 4.

Results obtained from testing of cell lysate sample and buffer solution spiked with different concentrations of miR-30b.

Scheme 1.

Schematic Illustration of the electrophoresis separation assisted G-quadruplex DNAzyme-based CL signal amplification strategy for miRNAs assay.