

Highly sensitive detection of microRNA by chemiluminescence based on enzymatic polymerization

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Abstract We have developed a new methodology for miRNA assay using chemiluminescence imaging by poly (U) polymerase catalyzed miRNA polymerization. This method is very sensitive with a 50 fM limit of detection, which is comparable to or better than current assay methods. Multiplex detection for miRNA can be easily realized by introducing different capture probes onto the biosensor array, which will make it highly versatile for various research purposes.

Keywords MicroRNA · Chemiluminescence · Array

Introduction

MicroRNA (miRNAs) is a class of small (approximately 19 to 23 nucleotides), endogenous, noncoding RNAs that are powerful transcriptional and post-transcriptional regulators of gene expression and cell development in animals, plants, and viruses [1–4]. Specific changes in miRNA expression levels are associated with a variety of diseases, including cancer [5, 6], neurodegenerative disorders [7], diabetes [8], and represents promising biomarker candidates for early cancer diagnosis [9]. For example, the highly tissue and developmental stage-specific expression of miRNA allows for the accurate molecular classification of tumors [10]. Therefore, detection of miRNAs has great significance not only for understanding their biological functions but also for clinical diagnosis of human diseases as well as the discovery

of new drugs through the use of miRNAs as targets [11, 12]. The most widely reported method for miRNA detection, Northern blotting, requires substantial amounts of starting material, is extremely laborious, and is not sensitive [13–15]. Recently, a number of new methods for microRNA detection have been reported, such as electrochemistry [16–20], fluorescence [21–24], capillary electrophoresis [25, 26], surface plasmon resonance (SPR) imaging [27, 28], and atomic force microscopy [29]. These reported methods have high sensitivity, but often at the expense of assay simplicity, multiplexing capability, or rapid analysis time.

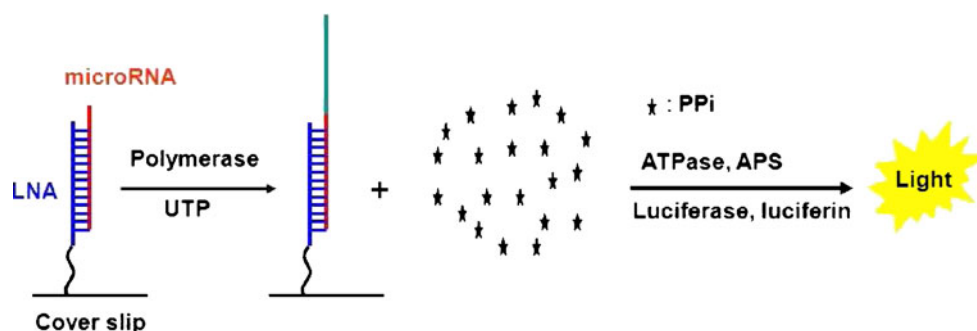
We have previously reported the use of chemiluminescence for monitoring ATP release [30], imaging of gene expression in individual bacterial cells [31, 32], and detection of single luciferase molecules [33]. In this work, we report a novel and sensitive microRNA detection method based on poly(U) polymerase polymerization by chemiluminescence microscopy imaging with an ultrasensitive intensified charge-coupled device (ICCD) camera. As shown in Scheme 1, the locked nucleic acid (LNA) probe which is complementary to the miRNA is immobilized on the cover slip. That reacts with miRNA hybrids with a complementary LNA probe by adding poly(U) polymerase, and a large amount of pyrophosphate (PPi) is released during the incorporation of UTP into the miRNA. The accumulated PPi is subsequently converted to ATP with adenosine phosphosulfate and ATP sulfurylase. The ATP levels are then determined with luciferase bioluminescence that is very sensitive.

Experimental section

Materials Luciferase from firefly *Photinus pyralis*, D-luciferin, adenosine phosphosulfate (APS), adenosine

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Scheme 1 Schematic representation of detection of microRNA by chemiluminescence



triphosphate sulfurylase from *Saccharomyces* (ATPase), 3-aminopropyltriethoxysilane (APTES), and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). The 1× Hanks balanced salt solution (HBSS), and uridine triphosphate (UTP) were purchased from Life Technologies (Carlsbad, CA). Poly(U) polymerase (2,000 units/mL) was purchased from New England Biolabs (NEB, U.K.). 1× phosphate-buffered saline (PBS) buffer (pH 7.4) was purchased from Life Technologies. PBS (20×) Tween-20 buffer was purchased from Pierce Protein Research Products-Thermo Fisher Scientific (Rockford, IL). Ultrapure water from a Milli-Q system (Millipore, Billerica, MA) was used throughout the experiments. All chemical reagents were used without further purification.

DNA and RNA oligonucleotides All 3'-amino-modified LNA-DNA oligonucleotide probes (Exiqon, MA) were purified using HPLC. The amino-modified LNA probes used were: miR-20 LNA=5'-CACTATA AGCACTTT ATTTTTTTT-3', and let-7a LNA = ACAACCTAC TACCTC ATTTTTTTT-3' (all LNA bases are underlined). All synthetic miRNA sequences used were purchased from

TriLink Biotechnologies (San Diego, CA) and purified using HPLC. The miRNA sequences used in this work are: miR-20=5'-UAAAGUGCUUAUAGUG CAGGUA-3', let-7a=5'-UGAGGU AGUAGGUU GUAUAGUU-3'.

Surface derivatization and LNA probe immobilization - Cover slips with dimensions of 22×22 mm (Electron Microscopy Sciences, Hatfield, PA) were cleaned in an ultrasonic bath for 15 min in detergent and ultrapure water, 15 min in ultrapure water, and 15 min in methanol (twice). After cleaning and surface treatment, cover slips were silanated in a 1% (v/v) APTES-ethanol solution for 1 h under agitation. Next, the APTES surfaces were activated with 2% (v/v) glutaraldehyde in 1× PBS at pH 7.4 for 1 h. Amino-modified LNA probe at a concentration of 200 pM in 1× PBS was then incubated onto the activated surface for 2 h. The cover slips were then washed with 1× PBS. The remaining amine-active sites were blocked with 1% BSA solution for 1 h, and then washed with PBS Tween-20 buffer.

Detection of miRNA by chemiluminescence (a) miRNA Hybridization with LNA Probe. Reactions were performed in a volume of 20 μL in a 1× HBSS buffer solution at 37 °C for 30 min. The cover slip was then rinsed with 1× HBSS buffer

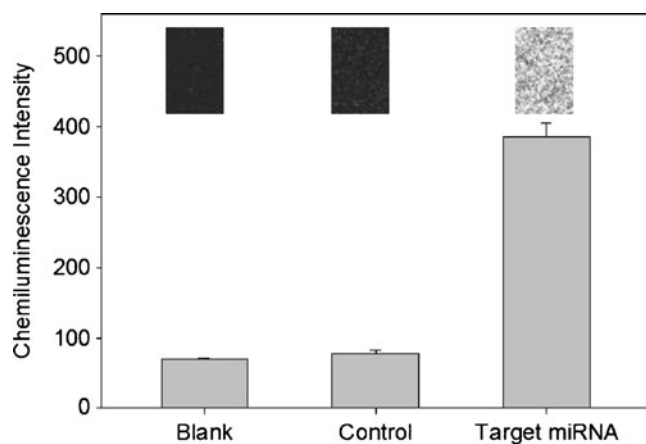


Fig. 1 Chemiluminescence of a blank (without any miRNA sample), a control (20 pM of noncomplementary let-7a miRNA), and a target miRNA (20 pM of target miR-20). The insets on top of each bar are the chemiluminescence images (10×15 μm). Exposure time: 2 s

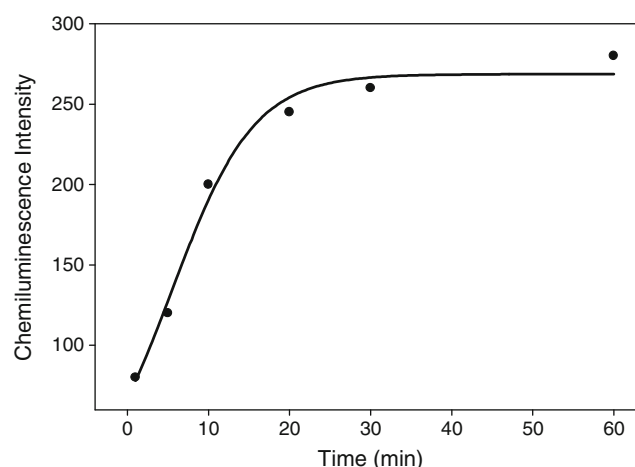


Fig. 2 Effect of poly(U) polymerase reaction time on miRNA detection. Exposure time: 2 s

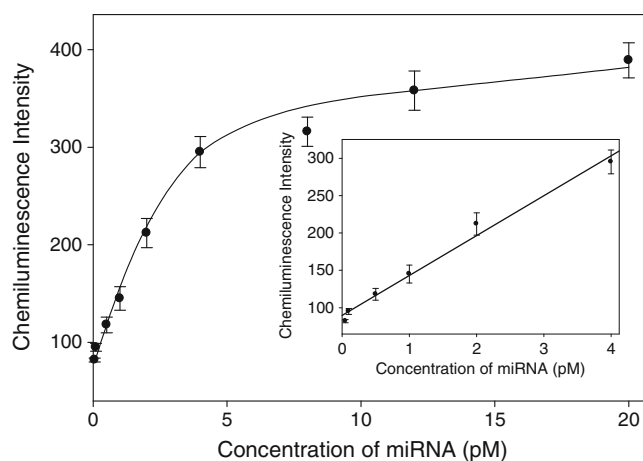


Fig. 3 Relationship between the chemiluminescence intensity and the target miRNA concentration, and (*inset*) the linear working range of miRNA. Error bars were estimate from three replicate measurements. Exposure time: 2 s

containing 10 mM MgCl_2 for 3 min. (b) Poly(U) Polymerase Reaction. After miRNA hybridization, the cover slip was reacted with a mixture of poly(U) polymerase (0.2 units/ μL) and 1 mM UTP in a volume of 15 μL in a reaction buffer of 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl_2 , and 1 mM DTT (pH: 7.9) for 30 min. (c) PPi Conversion and Bioluminometric Assay. The reaction product PPi was converted to ATP for 10 min by injection of 15 μM APS, and 0.05 unit ATP sulfurylase into the existing buffer. Finally, 1 mM D-luciferin and 1.67 μM luciferase reaction solution was injected in a final volume of 20 μL , and the bioluminescence signal was measured with chemiluminescence microscopy.

Instrumentation The imaging system consists of an inverted light microscope (Nikon Diaphot 300, Fryer, Edina, MN) and a complex electron-multiplying microchannel plate coupled ICCD (EEV 576 $\times$ 384, Princeton Instruments, Trenton, NJ) attached to the camera mount of the microscope. The ICCD camera was operated at -30°C and read out at 430 kHz with 12-bit resolution. A 100 $\times$ oil immersion objective (N/A 1.25, Nikon) was used for miRNA concentration detection, and a 10 $\times$ plano objective lens (Mitutoyo,

Japan) was used for miRNA array study. The whole system was placed in a dark box. WinView 32 (Roper Scientific, Princeton, NJ) was used for image collection, and NIH ImageJ was used to analyze and process the collected images.

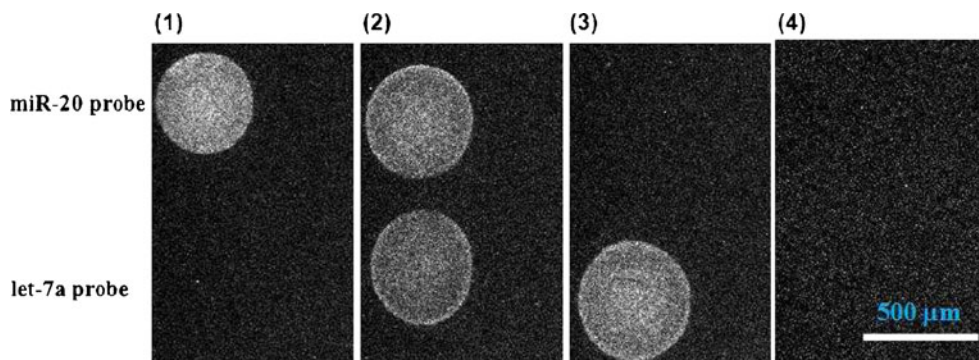
Results and discussion

Feasibility study To demonstrate this detection concept, we first evaluated the response of the biosensor with 20 pM target miRNA (miR-20) complementary to the miR-20 LNA capture probes. For comparison, a control sample was also tested by applying a noncomplementary miRNA (let-7a) for hybridization and then followed with the same procedures as that for the complementary miRNA. As illustrated in Fig. 1, a strong chemiluminescence signal was found for the target miRNA, whereas only a slight increase was observed for the control sample when compared to a blank sample (without undergoing miRNA hybridization). The result of the control sample implies that the non-hybridization-related signal from this biosensor is extremely low, which facilitates the detection of miRNA at ultralow concentrations. This may be attributed to the use of LNA modified DNA as the capture probe. LNA modifications have been used to improve detection limits and mismatch discriminations in various methods for the array detection of miRNA [13, 15, 34].

Effect of poly(U) polymerase reaction time on miRNA detection The optical amplification time was studied as shown in Fig. 2. The chemiluminescence intensities of miR-20 arising from poly(U) polymerase reaction increased within 30 min. After 30 min, the bioluminescent signals of miRNA reached its maximum and remained stable afterwards. So, 30 min was selected as being optimum for the experiments.

Detection of miRNA by chemiluminescence Under the optimized experimental conditions, the relationship between the chemiluminescence responses and the concentrations of

Fig. 4 Chemiluminescence array detection of miRNA. (1) miR-20, (2) 1:1 miR-20 and let-7a mixture, (3) let-7a, (4) blank. Exposure time: 10 s



target miRNA (miR-20) was investigated. As shown in Fig. 3, the chemiluminescence intensity increased with the increase of miRNA concentration over the range of 50 fM to 20 pM. The inset of Fig. 3 showed a linear correlation between the chemiluminescence intensity and the amount of miRNA in the concentration range of 50 fM to 4.0 pM. According to the 3 σ (standard deviation) rule, the limit of detection (LOD) was determined to be 50 fM. It is comparable to or better than the commonly used techniques, such as northern blotting (LOD=1 nM) [14], electrochemical assay (LOD=2 pM) [17], fluorometric assay (LOD=25 fM) [22], SPR imaging (LOD=10 pM) [35], and fluorescence correlation spectroscopy (LOD=500 fM) [36]. Furthermore, the LOD could be further improved in our method by increasing the exposure time to increase the signal-to-noise ratio.

Chemiluminescence array detection of miRNA To demonstrate the multiplexing capability of our platform for miRNA detection, we constructed a two component array by differentially functionalizing slides with unique LNA probes (1 and 2 are miR-20 LNA probe, 3 and 4 are let-7a LNA probe) complementary to two dissimilar miRNAs. As shown in Fig. 4, sample 1 is miR-20, sample 2 is 1:1 miR-20 and let-7a mixture, sample 3 is let-7a, and sample 4 is the blank. Sequence-specific responses are observed only when the complementary miRNA solution is exposed to the corresponding probe locations of the sensor array. The results indicate that multiplexing capability of our platform for miRNA detection was easy to implement. Furthermore, one miRNA array can be reused multiple times for different experiments since the reaction product PPI can be easily washed away while retaining 95% of the response after washing.

Conclusions

In summary, we have developed a new methodology for miRNA assay using chemiluminescence imaging by poly (U) polymerase catalyzed miRNA polymerization. This method is very sensitive with a 50 fM limit of detection, which is comparable to or better than current assay methods. Multiplex detection for miRNA can be easily realized by introducing different capture probes onto the biosensor array, which will make it highly versatile for various research purposes. Future efforts will be directed towards increasing the levels of multiplexing with microarray spotting technologies for the rapid encoding of many unique sensing targets. For example, the size of the probe spot could be reduced by perhaps 20-fold (see Fig. 4) to allow a higher density array and to accommodate smaller sample amounts.

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