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Spinach-Based Fluorescent Light-up Biosensors for Multiplexed and label-Free Detection of microRNAs

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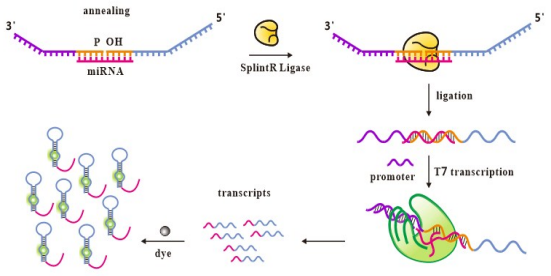
Zhan-Ming Ying, Bin Tu, Lan Liu, Hao Tang*, Li-Juan Tang, Jian-Hui Jiang*

A novel Spinach-based fluorescent light-up biosensor utilizing T7 in vitro transcription process to generate unmodified Spinach sequences for multiplexed microRNAs detection has been developed.

Recently, light-up aptamers, especially "Spinach" system have provided a simple and useful platform for construction of label-free fluorescence biosensors.[1-4] The Spinach is a RNA mimic of enhanced green fluorescent protein (eGFP), which binds and activates the fluorescence of its corresponding fluorophore, 5-difluoro-4-hydroxybenzylidene imidazolidinone (DFHBI). The Spinach-fluorophore pair represents a promising platform for developing light-up biosensors due to several factors including: high fluorescence signal-to-background ratio since the fluorophore was non-fluorescent before activation; high photo-bleaching resistance and fast fluorescence activation; applicable for various target molecules since target recognition elements can be fused with Spinach aptamer.[5] To date, Spinach-based biosensors have been adapted for analysis of various molecular targets including: small molecules such as adenosine, ATP, ADP, S-Adenosyl-L-homocysteine (SAH), S-adenosylmethionine (SAM), guanine, GTP, lead ion, cyclic di-GMP and cyclic AMP-GMP; proteins such as streptavidin, thrombin and MS2 coat protein; nucleic acids mRNA and miRNA.[6-20] Despite the successful applications of Spinach-based biosensors, several challenges are still remain. Selecting appropriate sites for recognition elements insertion remained a critical thought and the redesigned Spinach always showed reduced binding affinity toward target molecule, which might be attributed to the increased complexity of the sequence.[5] Moreover, the increased complexity of the sequence would also affect the brightness of fluorescence upon binding of fluorophore DFHBI. Current Spinach-based biosensors have rarely exploited the signal amplification strategy, which limits the sensitivity of these sensors for detection of low-abundance targets.

Herein we have explored the application of Spinach-based biosensors utilizing T7 in vitro transcription process to generate unmodified Spinach sequences for light-up fluorescence detection. We reason that utilizing unmodified Spinach aptamer for developing biosensor may be a promising approach to overcome these challenges for the following reasons: (1) Unmodified Spinach aptamer shows stronger fluorescence upon binding DFHBI than that of redesigned Spinach aptamer; (2) The target recognition module is not restricted by Spinach aptamer, which may help keep its high binding affinity; (3) Signal amplification strategy can be applied to generate a large amount of unmodified Spinach aptamer sequences, so the sensitivity of the method can be improved. In this work, we have shown advantages of this strategy by realizing multiplexed analytes detection. MiRNA-21 and miRNA-141 were chosen as model targets due to the important roles miRNA plays in biological systems.[21] Various methods have been developed for miRNAs detection.[22-26] Very recently, a redesigned Spinach aptamer termed as Panda has been developed for light-up miRNA detection by inserting a miRNA complementary sequence into Spinach aptamer, demonstrating the applicability of Spinach-based biosensors.[16] Nevertheless Panda biosensor still encountered the same challenges as discussed above. To the best of our knowledge, this work is the first time that signal amplification strategy has been applied in Spinach-based sensor for multiplexed miRNAs detection. The principle of the proposed approach was demonstrated in scheme 1.



Scheme 1 Illustration of spinach-based fluorescent light-up biosensor for multiplexed and label-free detection of microRNAs.

Institute of Chemical Biology and Nanomedicine,State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082 (P. R. China) E-mail: haotang@hnu.edu.cn, jianhuijiang@hnu.edu.cn; Tel: 86-731-88822577; Fax: 86-731-88822872.

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A pair of DNA probes A/B was designed, where probe A had a region (yellow color in scheme 1) complementary with part of miRNA target, and probe B had a region (yellow color in scheme 1) complementary with part of miRNA target. The probe A also possessed a 1st T7 promoter sequence (purple color in scheme 1) for hybridization with 2nd T7 promoter sequence. Detail information of DNA probes were listed in Table S1 in supporting information. DNA probe A was annealed to probe B in the presence of target miR-21 and SplintR Ligase. The ligation product was then acted as DNA template with 2nd T7 promoter sequence and T7 polymerase to initiate T7 in vitro transcription, so large amount of RNA transcripts was produced. The sequences of the RNA transcripts were determined by probe B and could be easily tuned to different RNA aptamers (blue color in scheme 1) for different fluorophores. The RNA transcripts activated fluorescence of their corresponding fluorophores and detection of miRNA targets was achieved. RNA aptamer termed as Broccoli and its corresponding fluorophore DFHBI-1T were employed for miR-21 detection; malachite green aptamer (MGA) and its corresponding fluorophore malachite green (MG) were employed for miR-141 detection.[4, 27] In our assay, a large amount of unmodified Broccoli and malachite green aptamer were produced, so the signals were amplified. Since these two fluorophores had different spectral properties, multiplexed detection of miRNAs could be achieved.

To validate mechanism of the approach, gel electrophoresis experiments were performed to analyze products from each reaction step. The reaction conditions were list as follows: 37 ℃, 1 h, probe A1 1 μM, B1 1 μM, miR-21 200 nM, SplintR Ligase 10 U, 2nd T7 promoter sequence 500 nM, A2 1 μM, B2 1 μM, and miR-141 200 nM. The gel was stained by GoldView and ethidium bromide, and enough DNA probes were applied to obtain suitable band images for observation. As shown in Figure 1A, in the presence of target miR-21 and SplintR Ligase, probe A1 and B1 were effectively annealed (lane 5), while without SplintR Ligase, only random hybridization products were obtained (lane 4). The SplintR Ligase was chosen due to its high ligation efficiency toward DNA-RNA substrate compared to T4 ligase.[28] The high ligation efficiency was due to the highly fidelity of SplintR Ligase to repair the "nick" and form phosphodiester bonds between 5'-phosphorylated termini and the 3'-OH termini of DNA-RNA splint substrate.[29, 30] The mixture of probe A1, B1, target miR-21, SplintR Ligase and 2nd T7 promoter sequence showed a band in lane 6 corresponding larger sequence than in lane 5, indicating 2nd T7 promoter sequence was successfully hybridized to the annealed product. Similar results of miR-141 triggered ligation and 2nd T7 promoter sequence hybridization process was observed from lane 11 to 13. We then performed T7 in vitro transcription using different hybrid DNA mixtures from lane 4, 5 and 6 as transcript template; and the 92 nt size of transcripts were only obtained using products from lane 6 (Figure S1, ESI). The results demonstrated the success of T7 in vitro transcription in the presence of probe A1, B1, target miR-21, SplintR Ligase and 2nd T7 promoter sequence. Similar results of miR-141 triggered T7 in vitro transcription was observed with products from lane 13 (Figure S1, ESI). Next investigation was subjecting the RNA transcripts obtained by T7 in vitro transcription reactions initiated by miR-21 and miR-141 to native PAGE and staining with their corresponding fluorophores. Results in Figure S2 indicated RNA transcripts of MGA and Broccoli selectively activated the fluorescence of MG and DFHBI-1T respectively.

Fluorescence responses were then investigated with different hybrid DNA mixtures according to the gel electrophoresis experiments. It was observed DFHBI-1T dye showed very weak fluorescence emission under excitation of 365 nm. The results in Figure 1B indicated in the absence of SplintR Ligase, 2nd T7 promoter sequence or target miR-21, the fluorescence of DFHBI-1T dye was not activated. While in the presence of SplintR Ligase, 2nd T7 promoter sequence and target miR-21, Spinach sequences were successfully generated by in vitro T7 transcription process, and the fluorescence of DFHBI-1T dye was activated. Strong green fluorescence was observed with a high signal-to-background ratio of ~200 (inset picture; black and red curve). The high signal-to-background ratio was attribute to the generated large amount of unmodified Broccoli aptamer sequences binding with DFHBI-1T dye. Similar strong red fluorescence(pseudocolor) and high signal-to-background ratio of ~180 were observed with miR-141 triggered in vitro T7 transcription for activation of MG dye (Figure 1C).

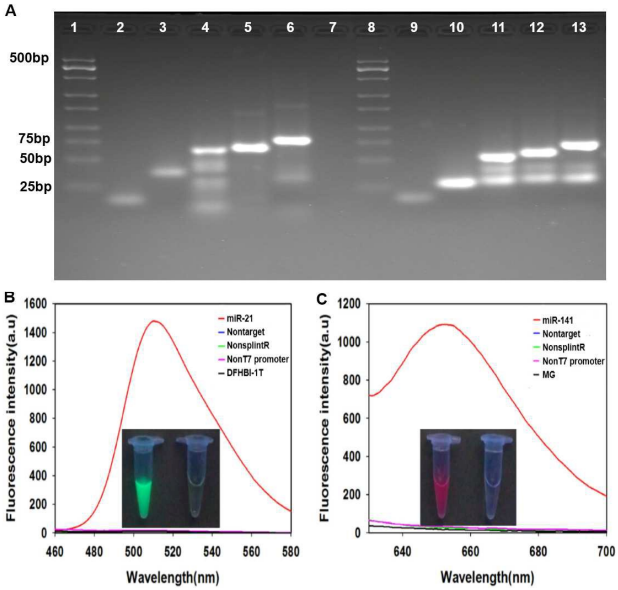

Figure 1. Validation of biosensor mechanism. (A) Agarose gel images of different samples. Lane 1 and Lane 8 : DNA ladder; Lane 2: A1; Lane 3: B1; Lane 4: A1 +B1 + miR-21; Lane 5: A1 + B1+ miR-21 + SplintR Ligase; Lane 6: A1 + B1 +miR-21 + SplintR Ligase + 2nd T7 promoter sequence; Lane 9: A2; Lane 10: B2; Lane 11: A2 + B2 + miR-141; Lane 12: A2 +B2 + miR-141 + SplintR Ligase, Lane 13: A2 + B2 + miR-141+ SplintR Ligase + 2nd T7 promoter sequence. (B) Fluorescence spectra of DFHBI-1T under different assay conditions, excitation: 440 nm. (C) Fluorescence spectra of MG under different assay conditions, excitation: 610 nm.

The reaction conditions such as temperature, pH and time were set according to classic ligation and in vitro T7 transcription protocols.[31, 32] Details of experiment conditions were shown in Supporting Information. The ability of the proposed method for quantitative detection of miRNAs was investigated by testing a series of samples with different target miRNA concentrations, and

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fluorescence responses were shown in Figure 2. For miR-21 detection, the fluorescence intensity at 512 nm increased along with the increasing concentration of the miR-21 from 0 to 10 nM, with a wide linear correlation range (10 pM to 10 nM). The detection limit was estimated to be 3 pM based on the 3σ rule. For miR-141 assay, a wide linear correlation ranges from 10 pM to 10 nM was obtained and the detection limit was estimated to be 3 pM based on 3σ rule. The proposed method demonstrated improved sensitivity for nucleic acid assay compared to previous Spinach-based fluorescent sensors.[16-20] Three to Four orders of magnitude improvement in detection limit was achieved compared to amplification-free Spinach-based fluorescent sensors (Table S2, ESI). This result may be attribute to the sensor design because unmodified Broccoli sequences were generated, and the amount of sequences were amplified by T7 transcription. Due to the advantages of the developed method, it may help expand the application of Spinach-based biosensors for investigate of low-abundance analytes. The ability of the proposed method for quantitative detection of miRNAs was investigated by testing a series of samples with different target miRNA concentrations, and fluorescence responses were shown in Figure 2.

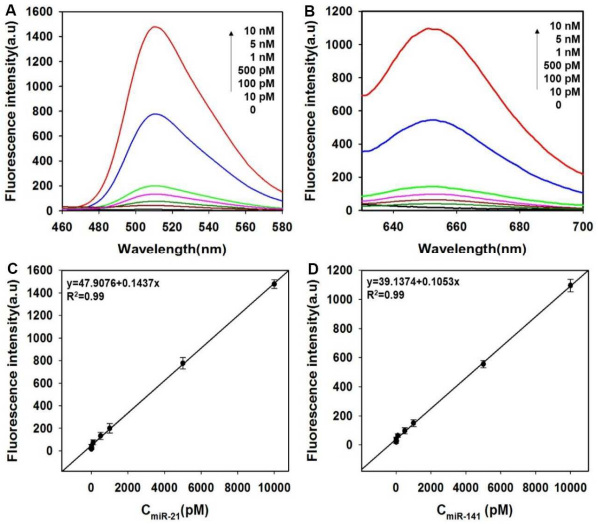

Figure 2. (A) Fluorescence spectra recorded at various concentrations of miR-21 ranging from 0 to 10 nM (B) Fluorescence spectra recorded at various concentrations of miR-21 ranging from 0 to 10 nM. (C) Fluorescence spectra recorded at various concentrations of miR-21 ranging from 0 to 10 nM. (C) Calibration curve for miR-21. (D) Calibration curve for miR-141. The error bars are standard deviations across three repetitive assays.

The selectivity of the proposed method was investigated by testing negative control cell lysate and different miRNAs including miR-222, miR-143 and let-7f. Only target miRNA showed substantial increase of fluorescence, all the other miRNAs showed very similar weak fluorescence compared to blank solution (Figure S3, ESI). These results demonstrated the biosensor provided excellent selectivity for miRNA detection. RNase inhibitor was utilized in the assay to keep the stability of the biosensor considering the degradation issue of RNA transcripts and target miRNA. The results demonstrated the biosensor had desirable stability (Figure S4, ESI). The developed biosensor was then applied for in vitro detection of cellular miRNA levels. Three different cancerous cell lines: Hela (cervical cancer cells) and MCF-7 (human breast cancer cells) and 22RV1 (human prostate carcinoma cells) were chosen to be tested. Total miRNAs were extracted from each cell line using SanPrep column microRNA mini-preps kit and verified by gel electrophoresis experiment (Figure S5, ESI). The total miRNA was then analyzed by the developed method for miR-21 and miR-141 detection. Fluorescence measurement in Figure 3 demonstrated MCF-7 had higher miR-21 level than Hela cell; 22RV1 had higher miR-141 level than MCF-7 cell. Based on the fluorescent intensity data and calibration curve in Figure 2, MCF-7 was estimated to have ~3.3 fold increase of miR-21 level than Hela cell; and 22RV1 was ~5.4 fold increase of miR-141 level than MCF-7 cell. The miRNA level differences were then measured by qPCR experiments. The qPCR analysis of the three cell lines indicated MCF-7 had ~3.1 fold increase of miR-21 level than Hela cell, and 22RV1 had ~5.8 fold increase of miR-141 level than MCF-7 cell, which were in agreement with our method. (Figure S6, S7, Table S3 and S4, ESI). These results were consistent with previous literature that miR-21 was overexpressed in MCF cell and miR-141 was overexpressed in 22RV1 cell.[33, 34]

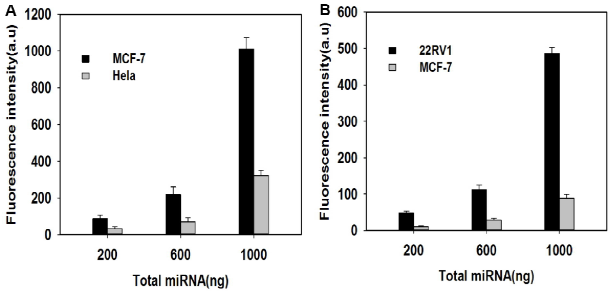

Figure 3. Fluorescence responses of miR-21 and miR-141 in different cell lines. (A) Detection of miR-21 in different amounts of total miRNA from MCF-7 and Hela cell. (B) Detection of miR-141 in different amounts of total miRNA from 22RV1 and MCF-7 cell. The error bars are standard deviations across three repetitive assays.

In conclusion, we have developed a novel Spinach-based fluorescent light-up biosensor for multiplexed and label-free detection of microRNAs. The developed approach utilized T7 in vitro transcription process to generate a large amount of unmodified RNA aptamer sequences triggered by target molecule. This method combined with amplification strategy to improve the sensitivity of Spinach-based biosensors. Highly sensitive and selective detection of miR-21 and miR-141 were achieved. By designing the sequences of DNA probe pairs, different targets can be applied to trigger the ligation process and be detected. It is envisioned the developed method may provide a new paradigm for the design of Spinach-based biosensors coupled with signal amplification strategy and hold potential for the investigation of low abundance targets by Spinach-based biosensors.

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