Biosensors and Bioelectronics 87 (2017) 552–557

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A simple G-quadruplex molecular beacon-based biosensor for highly selective detection of microRNA

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ARTICLE INFO

Article history:
Received 9 May 2016
Received in revised form
16 July 2016
Accepted 18 July 2016
Available online 19 July 2016

Keywords:
MiRNA
G-quadruplex molecular beacon
Duplex-specific nuclease
Single-base selectivity

ABSTRACT

MicroRNAs (miRNAs) family members are usually different from each other in one-base variation. The high sequence homology poses a challenge for miRNA analysis with single-base selectivity. On the basis of G-quadruplex molecular beacons (G4MB) and duplex-specific nuclease (DSN), we developed a simple and highly selective amplification biosensor for miRNA detection. G4MB with a G4 motif stem is used as recognition probe. In the present of target miRNAs, G4MB hybridizes with target miRNA perfectly and forms a G4MB-miRNA duplex. Then, DSN subsequently cleaves the G4MB of the G4MB-miRNA duplex to recycle the target miRNA, which leads to fluorescence signal amplification. In the absence of target miRNAs, DSN can not digest the stem of G4MB because of the protection of G4 motif, which eliminates the false positive signal, and produces low fluorescence background. Importantly, the powerful discriminating abilities of both G4MB and DSN make the novel sensor suitable for miRNAs detection with high single-base selectivity. Comparing with traditional linear ssDNA probe-DSN-based method, the signal response of similar miRNA sequences with one-base difference has been reduced from 24% to 6% by using this G4MB-DSN-based method. Moreover, this simple sensor also exhibits a good applicability in cancer cell samples and a multiplex capability in one sample with different miRNA targets, making it a promising strategy for clinical diagnostics.

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1. Introduction

MicroRNAs (miRNAs), single-stranded non-coding RNAs with a short length of ~22 nucleotides, play important roles as regulators in plants, animals and viruses via repression of mRNA translation (Bartel, 2004; Almeida et al., 2011). Research evidences have shown that the dysregulation of miRNAs expression is often associated with the occurrence of various diseases including cancer (Esquela-Kerscher and Slack, 2006; Leva and Croce, 2013). The actual expression levels of several miRNAs are emerging as novel biomarkers for cancer diagnosis, classification and therapy. Thus, the detection of miRNAs has attracted extensive attention. However, the unique characteristics of miRNAs, including their short length, low cellular abundance, high sequence homology and easy degradability, make the detection very challenging. Especially, the homogenous sequences of miRNA family often possess only one-base difference, demanding the miRNA analysis with single-base selectivity (Cissell et al., 2007). So far, various techniques have been developed (Várallyay et al., 2008; Chen et al., 2015; Graybill and Bailey, 2016), such as northern blotting, RT-PCR, RCA, HCR, EXPAR, *etc.* Although most of them with high sensitivity, their selectivity and simplicity are still unsatisfied in actual detection.

Duplex-specific nuclease based signal amplification (DSNSA) (Yin et al., 2012). a kind of isothermal *in vitro* technique, has become a sensitive and attractive strategy for miRNA detection in recent five years. In DSNSA, DSN digests DNA in DNA-miRNA hybrids selectively, which releases target miRNA to rehybridize with another ssDNA recognition probe, leading to significant signal amplification. Ye et al. has devised an elegant one-step DSNSA method based on linear Taqman probe, which could achieve sensitive detection of 100 fM miRNA within 30 min (Yin et al., 2012). However, this DSNSA method is difficult to discriminate similar miRNA sequences with less than 4-bases differences (Lin et al., 2013). Most DSNSA methods still exhibit low selectivity for homologous miRNAs with one-base difference, even though DSN possesses a special ability to discriminate perfectly matched short DNA duplex from non-perfectly matched one. It is mainly related

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http://dx.doi.org/10.1016/j.bios.2016.07.060
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to the design of recognition probe. In most DSNSA systems, linear ssDNA is used as the recognition probe which is difficult to recognize single-base mismatched miRNAs from complete complementary one (Yin et al., 2012; Qiu et al., 2015; Degliangeli et al., 2014; Yan et al., 2014; Xi, et al., 2014). By contrast, hairpin-structured molecular beacons (MBs) exhibit a better performance in differentiating single-base mismatched targets. By taking advantage of the powerful discriminating ability of MB and DSN, detection method can differentiate highly similar miRNA sequences with one-base difference (Lin et al., 2013). However, it is difficult to use MB in DSNSA system, because DSN can cleave the dsDNA stem of MB and generate a false positive signal (Tian et al., 2013). Lin' s research has proved that DSN can digest the stem of MB (only with 6 base pairs in its stem) and produce a 2.5-fold increase in background signal intensity (Lin et al., 2013). Some researchers overcome this difficulty by modifying the stem of MB with 2-OMe-RNA or fixing it on surface of Au electrode (Yang et al., 2014). However, some additional disadvantages occurred, such as high cost, hard synthesis and complex operation.

Herein, we introduce a simple and selective DSNSA assay for the quantification of miRNAs based on a novel G-quadruplex MB (G4MB). As shown in Scheme 1, the G4MB is a hairpin structure probe with a fluorophore attached to 5′ end and a quencher to 3′ end. It possesses a loop sequence that is complementary with the target miRNA and two G-rich stem sequences at both ends. In the absence of target miRNA, G-rich stems self-assemble into intramolecular G4 structure, and fluorescence is efficiently quenched upon the vicinity of the fluorophore to the quencher. Due to the resistance of G4 structure to DSN cleavage, false positive signal is eliminated, and low background signal is obtained. Upon the addition of target miRNA, G4MB perfectly hybridizes with target miRNA and forms a G4MB–miRNA duplex. In this case, DSN cleaves the G4MB DNA strand only and releases the target miRNA. The released target miRNA further hybridizes with another G4MB. As a result, fluorophores are continuously released into solution, and fluorescence signal are cyclically amplified.

2. Experimental

2.1. Materials and reagents

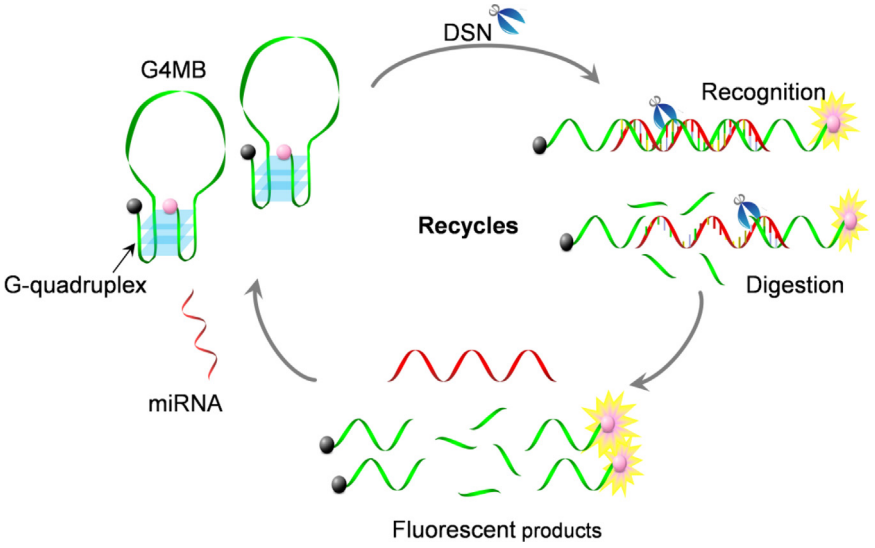
HPLC-purified RNA/DNA and RNase inhibitor were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). All oligonucleotides sequences were listed in Table S1. Duplex-specific nuclease (DSN) and were obtained from Newborn Co. Ltd. (Shenzhen, China). 4-(2-Hydroxyethyl)piperazine-1-ethanes-ulfonic acid (HEPES), 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), sodium chloride and other reagents were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA) and used without further purification. In order to create and maintain an RNase-free environment, the sterilized water (resistance $\geq$ 18.2 MΩ/cm) was treated with 0.1% DEPC and used throughout the experiments. The RNase-free tips and tubes were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

2.2. Fluorescence measurements

Each reaction sample (20 μL) containing 300 nM G4MB, 0.2 U DSN and different concentrations of target miRNAs was allowed to incubate in 1 × DSN master buffer (50 mM Tris-HCl, pH = 8.0, 5 mM $MgCl_2$ and 1 mM DTT) at 60 °C for 40 min. Another 100-μL of dilution buffer (20 mM HEPES, pH = 6.8, 20 mM KCl, 200 mM NaCl, 150 mM NH_4Cl, 0.01% TritonX-100) was injected before fluorescence measurement for each sample. The final solution was kept in room temperature for another 30 min. Unless otherwise indicated, the concentration of ODNs is that in 20 μL of reaction solution. Fluorescence measurements were performed using a LS-55 Fluorescence Spectrometer (Perkin Elmer, USA). All emission spectra were collected in the range of 500 to 650 nm with an excitation and emission slit width of 5.0 nm. The excitation and emission wavelengths were set at 490 nm and 518 nm. The emission filter is open. Photomultiplier voltage is 800 V. All experiments were carried out at least triplicates. In the multiplexed detection of the concentrations of G4MB and G4MB-21 are 300 nM in the same sample. The excitation and emission wavelengths were set at 587 nm and 610 nm.

2.3. Peroxidase activity measurements

PS2. M sample was prepared in 10 mM PB (pH 7.4, 150 mM KCl) buffer and incubated at room temperature for 2 h to form G-quadruplex structure. G-rich probe (G4MB or Control probe or treated PS2. M) was allowed to incubate with 0.4 U DSN in 1 × DSN buffer at 60 °C for 40 min, and then 125 μM hemin was added for 2 h in buffer (20 mM HEPES, pH = 6.8, 20 mM KCl, 200 mM NaCl, 150 mM NH_4Cl and 0.01% TritonX-100). After adding of ABTS and H_2O_2, the detection was then performed by a Shimadzu UV-2700 UV–vis spectrophotometer. The final concentration of G4MB,



Scheme 1. Schematic illustration of the G4MB and DSN-based signal amplification strategy for highly specific detection of miRNA.

control probe, miRNA, PS2. M, ABTS and H_2O_2 were 1000 nM, 1000 nM, 500 nM, 150 nM, 2 mM and 2 mM, respectively.

2.4. Gel electrophoresis

Before adding loading buffer (Glycerol) for electrophoresis, the sample was prepared by mixing G4MB with DSN or miRNA in 1 × DSN master buffer and followed by incubation at 60 °C for 40 min. The concentrations of G4MB, miRNA, ssDNA and DSN were 2000 nM, 1000 nM, 1000 nM and 0.4 U, respectively. The concentrations of miRNA in lane 6 and 7 were 2000 nM. Two ladders were used to indicate the molecule weight, one was Low Range DNA Ladder (Thermo scientific, USA, cat no SM1211), and the other was microRNA marker (NEB, China, Cat no N2102S). The non-denaturing PAGE (20%) analysis of the products from the cyclic enzymatic amplification reaction was carried out at 1 W power for about 1.5 h in 1 × TBE buffer. After Stains-All staining and water eluting, the resulting gel was imaged with a Canon digital camera.

2.5. Cell culture and miRNA extraction

The human cervical cancer cell line Hela and prostate cancer cell line 22Rv1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C under a 5% CO_2 atmosphere. For lytic cell sample preparation, the Hela cells (~2×10^6 cells) were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) at 2000 rpm for 3 min. Then, 400 μL RIPA lysis buffer (Beyotime, China, cat no P0013C) was added and incubated for 40 min on ice, and then centrifuged at 12,000 rpm for 20 min at 4 °C. The cell lysate was used immediately for miRNA assay. For cellular extracts sample preparation, total microRNAs were isolated from Hela and 22Rv1 cells (~2×10^6 cells) by Total RNA Extractor (Sangon Biotech, Shanghai, China) according to the manufacturer protocols. The final supernatant was diluted with RNase-free water. The extract was used immediately for miRNA assay or stored at −80 °C. Quality and purity of total RNA were performed by using a NanoDrop 2000c spectrophotometer (Thermo, USA) according to the absorbance ratio of A_{260} nm/A_{280} nm.

3. Results and discussion

3.1. Design of G4MB

A stable G4 structure should be formed in the stem of G4MB. It is one of the prerequisite for the success of this novel G4MB DSNSA system. In order to prove it, a series of experiments had been conducted: i) we adopt the same stem sequences as that of DNAzymeMB which is reported to self-assemble into G4 and form hairpin structure (Fu et al., 2011). G4 structure can combine with hemin to form DNAzyme and catalyze a color reaction of ABTS with H_2O_2. In color reaction experiment, our G4MB also exhibits such peroxidase activity and produces a high UV–vis absorption signal(Fig. 1B). By contrast, the signal of the control probe with only one G-rich stem sequences at 5′ end, which is difficult to form G4 structure, is dramatically lower than that of G4MB; ii) the presence of the central cation is essential to the G4 structure. Moreover their stability can be enhanced by Mg^{2+} (Bourdoncle et al., 2006). In our experiment condition, the fluorescence

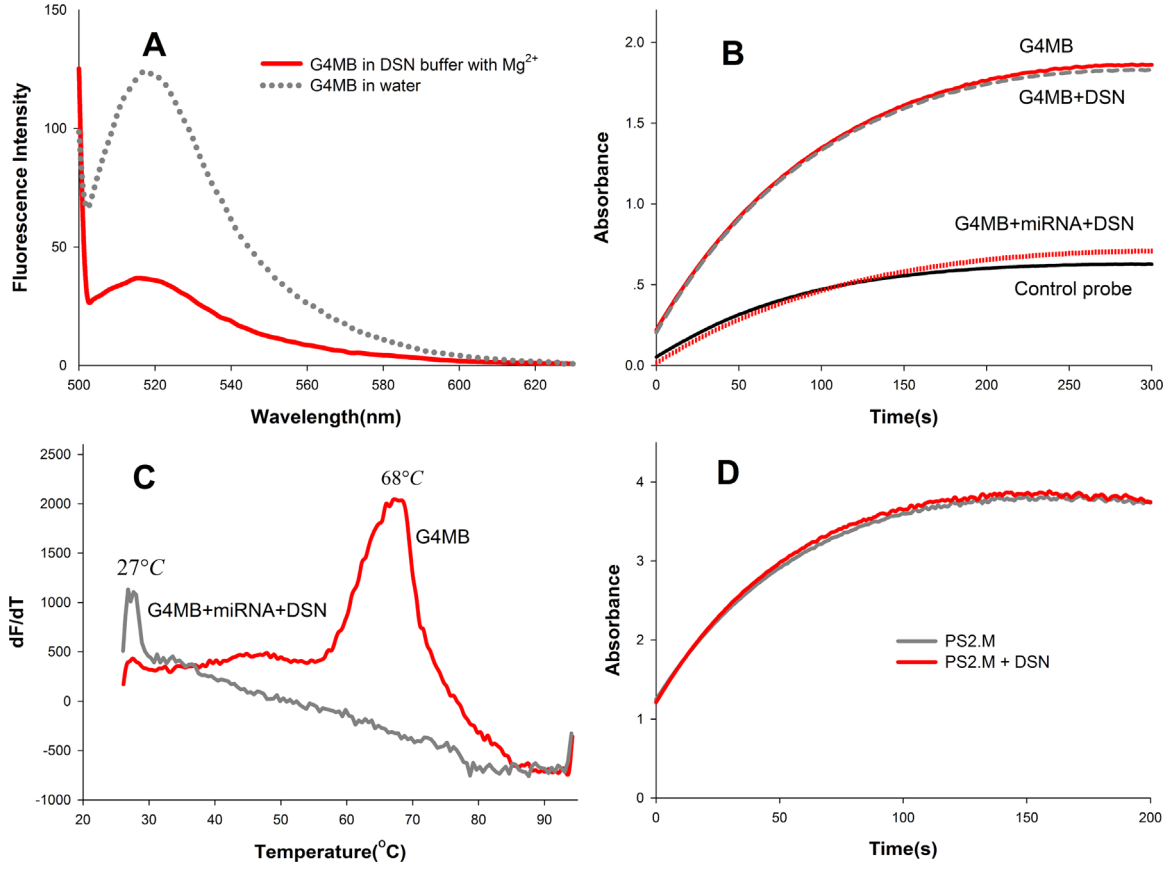


Fig. 1. (A) Fluorescence emission spectra of G4MB in water and in 1 × DSN master buffer; (B) peroxidase activity of G4MB and control probe; (C) melting curve of G4MB in 1 × DSN master buffer; (D) peroxidase activity of PS2.M before and after incubating with DSN.

intensity of G4MB is much lower than that in water, where G4 structure can not be formed (Fig. 1A); iii) the melting temperature of G4MB is about 68 °C which is higher than DSN working temperature 60 °C (Fig. 1C). All of them indicated that a stable G4 structure has been formed.

Furthermore, the G4 structure should resist the digestion of DSN. It is the other important prerequisite. We preliminarily investigated it with a G-rich sequence PS2. M (5′-GTGGGTAGGGCGGGTTGG-3′), which can self-assemble into intramolecular G4 structure and combine with hemin to act as a peroxidase (Travascio et al., 1998) (Fig. 1D). As expected, DSN can not digest such G4 structure and thus no signal change was observed. We further tested the G4MB with traditional MB as a negative control. As illustrated in Fig. S1, in the present of DSN, no detectable cleavage is observed in G4MB (lane 4 cf. lane 3), but a lower molecular weight band appears in control MB (lane 2 cf. lane 1). Fluorescence measurements provided a similar result. The control MB with 7 base pairs stem was digested by DSN, resulting in a 12.2-fold increase in fluorescence intensity. It was much higher than the 2.5-fold caused by MB with 6 base pairs (Lin et al., 2013). Comparably, G4MB displayed no more than 0.2-fold increase in the same condition. Additionally, in peroxidase activity experiment, there is also no obvious UV–vis absorption signal change of G4MB after adding of DSN (Fig. 1B). Those results confirmed that DSN can cleave the stem of MB and induce a high background. G4MB with a G4 motif stem can resist the digestion of DSN. Therefore, false positive signals were eliminated.

3.2. Feasibility of the G4MB DSNSA biosensor

The results of non-denatured PAGE electrophoresis and fluoresce experiment confirm the activation of the G4MB DSNSA system. As shown in Fig. 2, both lane 1 and lane 3 have a same G4MB band, indicating that the G4MB resist the DSN cleavage (G-rich probe should be moved more slowly than ordinary DNA probe (Mendez and Szalai, 2009; Zhou et al., 2012)). After adding half-dosage miRNA-141, a new G4MB-miRNA hybridize band was appeared in lane 2. However, both of them disappeared and were replaced by many short bands (lane 4) in the presence of DSN. We further increased the dosage of miRNA-141 and found that miRNA-141 still remain intact in the system after DSN cleavage (lane 6 cf. lane 7). This result indicated two important messages: one is that DSN can selectively cleaved G4MB in G4MB-miRNA duplex into short fragments; the other is that target miRNA-141 can be released continuously and hybridized with new G4MB, leading to a cyclic amplification. In order to confirm them, we further used a control ssDNA (with same sequences as miRNA-141) in the amplified system. By contrast, DSN can completely cleave DNA-DNA duplex. As a result, no ssDNA was released and no signal amplification was observed. Therefore, half-dosage G4MB band and short bands appeared in lane 5. Those results are consistent with the following fluoresce experiment. In the absence of DSN, the system acted as a traditional MB assay, in which target hybridized with G4MB to form 1:1 binding event. Thereby, a slight increase of fluorescence intensity was observed after adding of 10 nM miRNA-141 (line 2 cf. line 1). In the presence of DSN, the system acted as an amplification assay, in which one target miRNA-141 can trigger numerous G4MBs cleavages. Therefore, a remarkable signal enhancement was induced by 10 nM miRNA-141 (line 4 *cf.* line 3).

3.3. Sensitivity of the G4MB DSNSA biosensor

In order to achieve a better assay performance, we optimized the experimental conditions. As shown in Fig. S3, the optimum amount of DSN was found to be 0.2 U with optimum working temperature at 60 °C. The melting temperature of G4MB and G4MB-miRNA duplex are about 68 °C (shown in Fig. 1C and Fig. S2), confirming that the G4MB and G4MB-miRNA still keep hairpin structure and duplex at 60 °C, respectively. Under the selected conditions, the performance of the developed strategy for quantitative analysis of miRNA-141 was further investigated by using samples with different concentration of target. As shown in Fig. 3A, a dramatic fluorescence increase was observed as the concentration of the target increases from 0 to 25 nM. Fig. 3B exhibits a good linear relationship between the logarithm of fluorescence response (F/F_0-1) and the logarithm of miRNA-141 concentration in the range from 1 pM to 5 nM. The calibration equation is $\lg(F/F_0-1)=-0.1130+0.3244\ \lg C$, with a correlation coefficient of $R^2=0.9974$. Additionally, 1 pM miRNA-141 can also be detected (inset in Fig. 3A), which is about 500-fold lower than

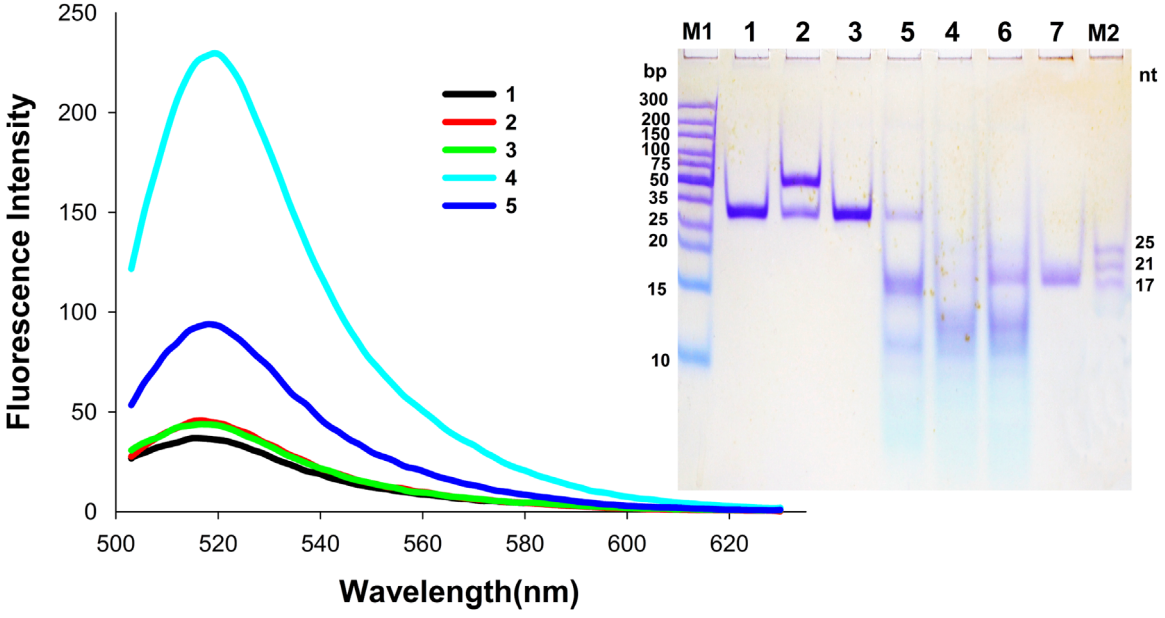


Fig. 2. Fluorescence emission spectra and non-denatured PAGE analysis of G4MB and miRNA with DSN. Line/lane 1: G4MB; line/lane 2: G4MB+miRNA; line/lane 3: G4MB+DSN; line/lane 4: G4MB+miRNA+DSN; line/lane 5: G4MB+ssDNA+DSN; lane 6: G4MB+miRNA+DSN; lane 7: miRNA; M1: DNA Marker; M2: miRNA Marker. The concentrations of G4MB, miRNA and ssDNA in fluorescence analysis were 300 nM, 10 nM and 10 nM, respectively, whereas in non-denatured PAGE analysis were 2000 nM, 1000 nM and 1000 nM, respectively. The concentrations of miRNA in lane 6 and 7 were 2000 nM.

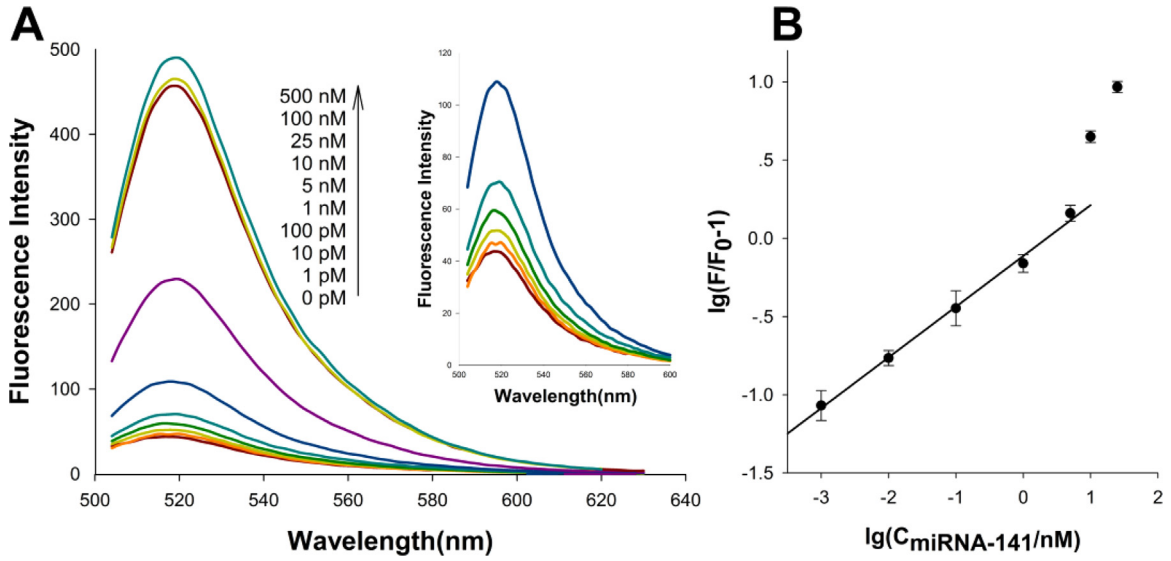


Fig. 3. (A) Fluorescence spectra of G4MB DSNSA biosensor with different concentrations of miRNA-141. (B) The relationship between the logarithm of fluorescence response and the logarithm of miRNA-141 concentration. Error bars are standard deviation of three repetitive experiments.

that of control experiment without DSN (Fig. S4). The detection limit was estimated to be 1 pM according to the 3σ rule ($n > 7$), which was comparable to some previously reported fluorescence sensors with pretty simple design for miRNAs detection (Lin et al., 2013; Wang et al., 2014; Degliangeli et al., 2014). The sensitivity is mainly attributed to the target recycling assisted by DSN digestion.

3.4. Specificity of the G4MB DSNSA biosensor

MiRNAs family members often had only one-base difference from each other. The high sequence homology posed a distinctive difficulty for miRNA analysis with high specificity. Single-base selectivity is still a challenge for DSNSA. In most DSNSA assays (Yin et al., 2012; Qiu et al., 2015; Degliangeli et al., 2014; Xi, et al., 2014), linear ssDNA, such as Taqman probe, is used as recognition probe, whereas linear ssDNA exhibits a poor single-base selectivity. In contrast, we use G4MB as recognition probe and develop a novel DSNSA system which can render a considerably improved single-base discrimination capability. As shown in Fig. 4, three members of miRNA-200 family (miRNA-141, miRNA-429, miRNA-200b), miRNA-21 and artificial synthesized miRNA (SmiRNA-141) were analyzed at the same concentration of 10 nM. All the signal ratios of control miRNAs are at least 16-fold lower than that of miRAN-141. Nearly negligible fluorescence changes were observed in the addition of control miRANs. Especially, the signal ratio of SmiRNA-141 with one mismatched base at the middle position is only about 6% of that induced by target miRNA-141. It is consistent with another 2-OMe-MB based DSNSA, and distinguish with the one linear Taqman probe based DSNSA where ~70% value is involved (Yin et al., 2012; Lin et al., 2013). We further cut off one of the G-rich stem sequences at 3′ end of G4MB, and keep the other one at 5′ end. This probe is used as a control probe, in which G4 and hairpin structure are difficult to formed. Therefore, the control probe acts as a linear ssDNA recognition probe as Taqman probe does. The test value of the single-base discrimination capability is ~24% (see Fig. S5), which is much higher than that of G4MB. These results indicate that the specificity of our method is sufficiently high to differentiate single-base mismatch targets. This single-base selectivity should be attributed to the better selectivity and higher specificity of the hairpin structure probe and DSN enzyme.

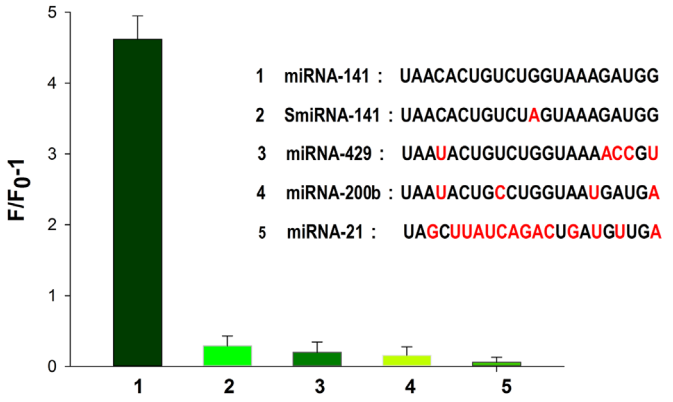


Fig. 4. Selectivity of the G4MB DSNSA biosensor for miRNA-141 over control miRNAs. The bases that differ from those in miRNA-141 are marked in red. The concentrations of G4MB, miRNA and DSN were 300 nM, 10 nM and 0.2 U, respectively. Error bars are standard deviation of three repetitive experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Applicability of the G4MB DSNSA biosensor

To test the efficiency of the novel method in the analysis of complex biological samples, we monitored the G4MB DSNSA system in Hela cell lysate (human cervical cancer cell with low level expression of miRNA-141 (Yin et al., 2012)). As shown in Fig. S6A, with the addition of increasing concentrations of miRNA, the fluorescence intensity shows a proportionate increase. A detectable fluorescence enhancement can be observed for samples with target miRNA concentration as low as 1.00 pM. It is consistent with that in the pure buffer solution. We further tested the applicability of the G4MB DSNSA platform in real samples. We extracted total miRNAs from 22Rv1 cells (human prostate carcinoma cancer cell with over expression of miRNA-141 (Yin et al., 2012; Porkka et al., 2007)) and Hela cells. As shown in Fig. S6B, the G4MB DSNSA platform can evidently distinguish the two kinds of cancer cell samples (Yang et al., 2014; Zhang et al., 2013). These results indicated that our method holds potential for practical applications in miRNA detection.

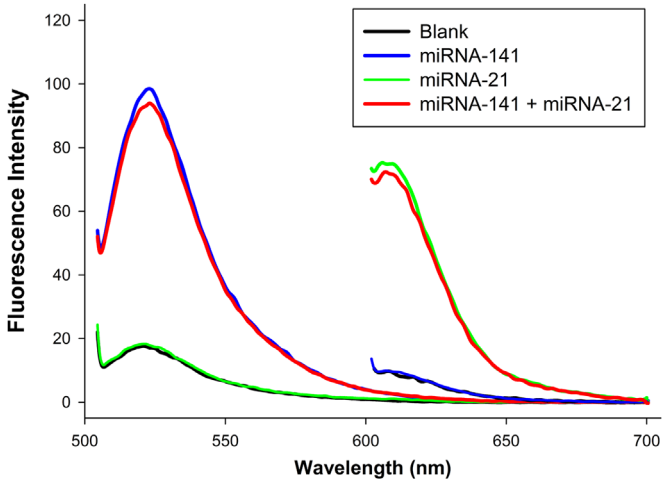


Fig. 5. Fluorescence responses of the G4MB DSNSA biosensor for different miRNAs. The concentrations of G4MB, G4MB-21, miRNA-141, miRNA-21 and DSN were 300 nM, 300 nM, 10 nM, 10 nM and 0.2 U, respectively.

3.6. *Multiplexed detection of miRNAs*

The use of miRNA method for clinical diagnostics requires simultaneously analysis of multiple miRNAs. Therefore, the multiplexing capability of this method was conducted to detect miRNA-141 and miRNA-21 in one sample. As shown in Fig. 5, G4MB with a FAM label is designed to recognize miRNA-141, and G4MB-21 with a ROX label is designed to recognize miRNA-21. Both of them are prepared in the same simple. In the absence of target miRNAs, the two peaks corresponding to miRNA-141 and miRNA-21 (FAM at 520 nm and ROX at 610 nm) are very low. Adding of target miRNA-141 leads to significant increase in the peak at 520 nm, while the peak corresponding to G4MB-21 at 610 nm remains uncharged. Similarly, the presence of miRNA-21 results in the increase of peak at 610 nm only. Importantly, when both miRNA-141 and miRNA-21 are added, both peaks increase simultaneously, and the peak intensities are close to that with miRNA-141 or miRNA-21 alone. These results indicate a good capability of our sensor for multiplexed miRNAs detection.

4. Conclusions

In summary, we demonstrated a G4MB DSNSA biosensor for miRNA detection. It displays several remarkable advantages: i) replacing traditional ssDNA, G4MB is used as recognition probe which possess more powerful single-base selectivity. By taking advantage of G4MB and DSN, our method exhibits high selectivity for discriminating miRNA family members with one-base difference; ii) for G4MB, we just need to design G-rich sequences at the stem, which is obviously simple, cost-effective and easy to synthesis. By using of G4MB, we solved the problem of false positive signals induced by digesting of DSN for dsDNA stem of MB. Therefore, low background fluorescence and high sensitivity have been obtained. Additionally, the strategy is simple and technologically easy to implement. It also exhibit a good applicability in cancer cell samples and a multiplex capability in one sample with different miRNA targets. In virtue of these advantages, the proposed strategy could be potentially used in future clinical diagnostics.

Acknowledgments

We are grateful to the National Natural Science Foundation of China Grants nos. 21405023, 81402304 and 31260206) and Jiangxi Provincial (Grant nos. 20142BAB213008 and 20161BAB203097), the State Key Laboratory of Chemical Biosensing & Chemometrics (Grant no. 2013012) and the 2014 bidding Project of Gannan Normal University for their financial support of this work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.07.060.

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