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Backbone-modified molecular beacons for highly sensitive and selective detection of microRNAs based on duplex specific nuclease signal amplification†

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Based on backbone-modified molecular beacons and duplex-specific nuclease, we have developed a target recycling amplification method for highly sensitive and selective miRNA detection. The combination of a low fluorescence background of 2-OMe-RNA modified MB and nuclease-assisted signal amplification leads to ultrahigh assay sensitivity, and the powerful discriminating ability of MB enables the differentiation of highly similar miRNAs with one-base difference, both of which are of great significance to miRNA detection.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs processed from longer hairpin precursor miRNAs by the enzyme dicer.[1] MiRNAs play important regulatory roles in animals and plants by targeting mRNA for cleavage or translational repression. Nearly 30% of all human genes are regulated by miRNA to control diverse biological processes, including cell proliferation, differentiation, apoptosis, development and tumorigenesis. The dysregulation of miRNA expression is often related to the development of various diseases.[2] Thus, sensitive and selective miRNA detection is highly significant for miRNA discovery, study and clinical diagnosis.[3] However, miRNA detection is challenged by the characteristics of miRNA, including low cellular abundance and short length.[4] The miRNA family often comprises highly homogenous sequences with as few as one base difference, posing difficulty for miRNA analysis with high specificity. Currently, several methods have been developed for miRNA detection, among which northern blotting[5] is the conventional assay, but with unsatisfactory sensitivity and a tedious procedure. Microarray based techniques are attractive for high throughput and multiplex miRNA analysis,[6] but the sensitivity and the normalization of hybridization efficiency need to be improved. Homogenous detection strategies relying on signal amplification methods, such as RT-PCR,[7] RCA,[8] EXPAR,[9] *etc.*, have been proposed to improve the sensitivity. Chen *et al.*[7] developed stem-loop RT-PCR for miRNA analysis that is capable of detecting as low as 100 fM miRNA, but the method is time-consuming with high cost. A detection limit of 10 fM can be achieved with the RCA method developed by Cheng *et al.*,[8] but the analysis takes as long as 8 h. Therefore, a rapid and convenient method for miRNA detection with high sensitivity and selectivity is still in great demand.

Recently, an elegant one-step and sensitive method for miRNA detection was demonstrated by Ye *et al.*[10] based on duplex-specific nuclease signal amplification (DSNSA). Duplex-specific nuclease (DSN), a nuclease isolated from the hepatopancreas of the Kamchatka crab, shows a strong preference for hydrolyzing dsDNA and DNA in DNA–RNA hybrid duplexes rather than ssDNA or RNA.[11] A detection limit of 100 fM miRNA was obtained within 30 min by DSNSA, in which DSN was utilized to recycle the target-assisted cleavage of Taqman probes, leading to significant fluorescence signal amplification. Despite its sensitivity, the DSNSA method is limited to differentiation of similar miRNA sequences differing by at least 4-bases, making it unsatisfactory for differentiating miRNA families that possess high homology with one-base difference, *e.g.* let-7 miRNA family. Herein, we take advantage of molecular beacons (MBs) to design a target recycling signal amplification method[12] for highly selective detection of miRNA. MBs are single-stranded oligonucleotides that possess a stem-and-loop structure with a fluorophore attached to one arm and a quencher to the other, where the loop is the probe sequence complementary to the target.[13] Because of their advantages of detection-without-separation, high sensitivity, and especially excellent selectivity to differentiate single-base mismatched targets, MBs have been widely used in different areas including RNA and DNA monitoring, biosensing, and real-time gene monitoring.[13]

The working principle of the DSN-assisted target recycling signal amplification method based on molecular beacons is illustrated in Scheme 1A. In the absence of miRNA, the MB shows low fluorescence intensity since it will not be recognized and digested by DSN. Upon the addition of miRNA, the MB will bind to the target and produce a fluorescence signal. The formed MB–miRNA duplex will become the substrate for DSN cleavage. Since DSN only cleaves DNA in the duplexes, the target miRNA is subsequently released to hybridize with another MB,

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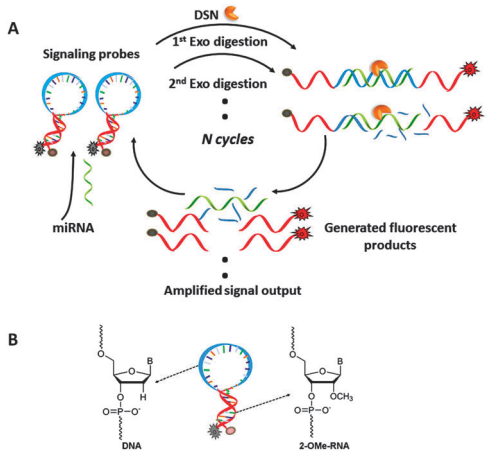

Scheme 1 (A) Working principle of the backbone-modified MB- and DSN-based target recycling signal amplification method for highly specific detection of miRNA. (B) The structure of the backbone-modified MB: the loop is made of DNA and the stem is modified with 2-OMe-RNA.

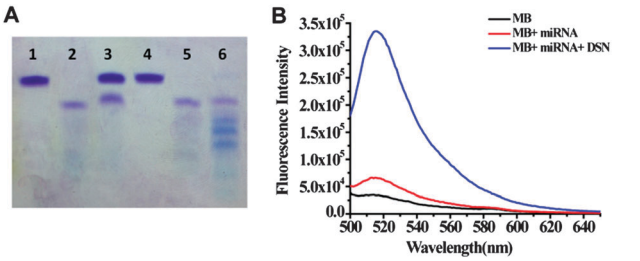

Fig. 1 (A) Denatured PAGE analysis of 2-OMe-MB and miRNA with DSN. Lane 1: MB; lane 2: miRNA; lane 3: MB and miRNA; lane 4: MB with DSN; lane 5: miRNA with DSN; lane 6: MB and miRNA with DSN. (B) Fluorescence emission spectra of MB and miRNA with DSN. The concentrations of MB and miRNA were 50 nM and 10 nM, respectively.

which leads to a cyclic reaction and fluorescent signal amplification. Theoretically, one miRNA sequence can initiate the cleavage of numerous MBs, resulting in the highly sensitive detection of miRNA. Furthermore, due to the powerful differentiating ability of MB, the method can distinguish similar miRNAs that differ by as few as one-base variation.

Since DSN can also cleave dsDNA, the stem of the MB is prone to digestion by DSN. To overcome this possible false positive signal, the MB is modified with 2-OMe-RNA on its stem. The 2-OMe-RNA differs from DNA in the 2-OMe on the pentose (Scheme 1B), which will not be recognized and cleaved by DSN. The resistance of the backbone-modified 2-OMe-MB to DSN cleavage was investigated with an unmodified DNA-MB as a negative control. As illustrated in Fig. S1A (ESI†), 2-OMe-MB remained intact in the presence of DSN without obvious cleavage (lane 2). However, DNA-MB without 2-OMe-RNA was vulnerable towards hydrolysis by DSN (lane 4) even in the absence of target miRNA. Fluorescence measurements were obtained to provide further confirmation of the DSN resistance of the modified MBs (Fig. S1B, ESI†). The unmodified DNA-MB was digested by DSN, resulting in a 2.5-fold increase in fluorescence intensity. By contrast, the fluorescence intensity change was negligible when 2-OMe-MB was incubated with DSN, confirming that 2-OMe-RNA modification of the stem of the probe can protect MB from hydrolysis by DSN.

To demonstrate the feasibility of the DSN and 2-OMe-MB (DSN-MB) based target recycling signal amplification method for miRNA detection, the let-7 miRNA family members were chosen as model targets because their sequences show a high degree of similarity and their expression levels are closely related to cell development and human cancer. The following MB sequence with the 2-OMe-RNA modified stem and the loop sequence complementary to let-7a was prepared: 5′-FAM-CGA GTC AAC TAT ACA ACC TAC TAC CTC A GA CTC G-BHQ1-3′ (The 2-OMe-RNA stem is underlined). The optimum reaction time was found to be 40 min with the optimum temperature at 65 °C (Fig. S2, ESI†), at which point the stem of 2-OMe-MB was still stable while the cleaved product was denatured (Fig. S3, ESI†). Denatured polyacrylamide gel electrophoresis (PAGE) was performed to demonstrate the protective property of 2-OMe-RNA modification towards DSN cleavage and the miRNA induced hydrolysis of 2-OMe-MB. As illustrated in Fig 1A, there was no detectable cleavage of 2-OMe-MB alone (lane 4) or miRNA alone (lane 5) after incubation with DSN. However, when miRNA was hybridized with 2-OMe-MB, the latter was subsequently cleaved by DSN, with miRNA remaining intact (lane 6). These results verified that backbone-modified 2-OMe-MB was resistant to the hydrolysis by DSN, while miRNA gave rise to the formation of a DNA–RNA hybrid duplex which was cleaved by DSN subsequently.

The fluorescence measurement was conducted with a low concentration of miRNA (10 nM miRNA *vs.* 50 nM MB) to confirm the DSN-assisted target recycling process and fluorescence signal amplification. As demonstrated in Fig. 1B, 2-OMe-MB showed low fluorescence intensity. In the absence of DSN, the miRNA hybridized with 2-OMe-MB and generated a slight fluorescence increase due to the 1 : 1 binding event. In contrast, in the presence of DSN, the hybrid duplex of 2-OMe-MB and target miRNA was cleaved by DSN, followed by the release of miRNA and a cyclic cleavage reaction, which gave rise to remarkable fluorescence enhancement. The result confirmed that in the presence of a small amount of miRNA, excess MB probes can be digested, resulting in fluorescence signal amplification. The results clearly established that the DSN-MB-based method for miRNA detection is feasible.

The response of 2-OMe-MB to various concentrations of let-7a was studied. As shown in Fig. 2A, a dramatic increase in fluorescence intensity is observed as the concentration of the target increases from 0 to 25 nM. Fig. 2B shows the relationship between the fluorescence intensity and the concentration of let-7a. The fluorescence intensity exhibits a good linear positive correlation with the concentration of let-7a within the range from 0.5 pM to 500 pM. The detection limit of the method was calculated to be 0.4 pM based on 3σ of six blank sample measurements. It is noteworthy that this low detection limit was achieved within 40 min. To confirm that it is the efficient hydrolysis activity of DSN that contributes to the high sensitivity of the strategy, control experiments were carried out with different concentrations of miRNA reacting in the absence of DSN. By contrast, the limit of detection was 500 pM, three orders of magnitude higher than that of our amplification method (Fig. S4, ESI†),

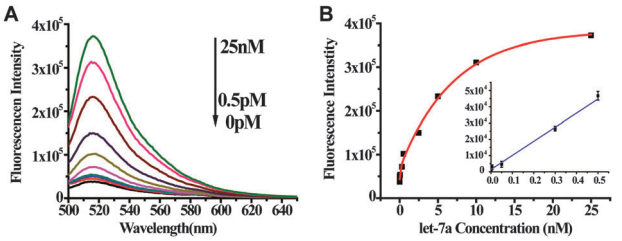

Fig. 2 (A) Fluorescence emission spectra of 2-OMe-MB with different concentrations of let-7a. (B) Scatter plot of fluorescence intensity as a function of the concentration of let-7a (0, 0.5 pM, 5 pM, 50 pM, 250 pM, 500 pM, 2.5 nM, 5 nM, 10 nM, 25 nM).

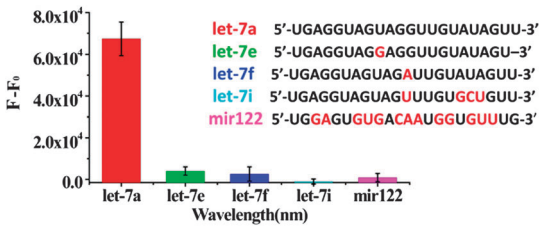

Fig. 3 Selectivity of the DSN-MB-based signal amplification method for let-7a over let-7e, let-7f, let-7i and mir122. The bases differing from those in let-7a are marked in red.

proving that target recycling assisted by DSN digestion is key to the success of this method.

The miRNA family often comprises highly homogeneous sequences with only one-base variation, posing a great challenge for distinguishing different miRNA members. The excellent ability of MBs to differentiate target sequences that differ by as few as one single nucleotide enables our method to profile the expression of different miRNA sequences with high specificity. Let-7e, let-7f, let-7i and mir122 were chosen to evaluate the selectivity of our strategy. As shown in Fig. 3A, even with 1-nt difference, the fluorescence signal of let-7e and let-7f is only 4–6% of that produced by let-7a, with a negligible signal in the presence of let-7i and mir122, which differ from let-7a by more than 4 bases. The results established that single-base selectivity was achieved to distinguish the closely related miRNA sequences by the DSN-MB-based method, while the DSN-Taqman probe-based method was unable to clearly differentiate single-base mismatch targets (Fig. S5, ESI†). Table S2 (ESI†) shows the comparison of different detection assays for miRNA. With comparable sensitivity, our method outweighs those conventional methods in terms of the high specificity capable of discriminating similar miRNAs with one-base variation, as well as the convenience of direct measurement within 40 min.

Considering the significance of miRNA analysis in complex biological samples, we challenged the DSN-MB-based method to detect miRNA in the lysate of the breast cancer cell MDA-MB-231, which is reported to show obviously low level expression of let-7a.[14] As demonstrated in Fig. S6 (ESI†), with the addition of increasing concentrations of miRNA, the fluorescence intensity shows a proportionate increase. The detection limit of the DSN- and MB-based signal amplification method in the cell lysate was found to be 0.5 pM, which is consistent with that obtained in the pure buffer solution. The result strongly confirmed the adaptability of our amplification approach for miRNA detection in complicated biological matrices.

In conclusion, based on backbone-modified MBs and duplex-specific nuclease, we have developed a target recycling signal amplification method for highly sensitive and specific analysis of miRNA within 40 min in homogeneous solution. Due to the resistance of 2-OMe-RNA to DSN cleavage, false positive signals were eliminated. The low background fluorescence intensity, together with the cyclic DSN-assisted signal amplification, gives rise to the high sensitivity of the assay, which is three orders of magnitude greater than that of traditional hybridization methods. More importantly, by taking advantage of the powerful discriminating ability of MB, our new method can differentiate highly similar miRNA sequences with one-base difference, a capability of great significance in miRNA detection and disease diagnosis. In addition, our method allows direct miRNA analysis in complicated biological samples without any sample pre-treatment, thereby greatly simplifying the procedure for rapid detection. Therefore, the reported amplification method based on backbone-modified MB and DSN is expected to offer a new platform for highly sensitive and specific detection of miRNA.

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Notes and references

1 (*a*) D. P. Bartel, *Cell*, 2004, **116**, 281–297; (*b*) K. K. Farh, A. Grimson, C. Jan, B. P. Lewis, W. K. Johnston, L. P. Lim, C. B. Burge and D. P. Bartel, *Science*, 2005, **310**, 1817–1821.
2 (*a*) G. A. Calin and C. M. Croce, *Nat. Rev. Cancer*, 2006, **6**, 857–866; (*b*) A. Esquela-Kerscher and F. J. Slack, *Nat. Rev. Cancer*, 2006, **6**, 259–269.
3 C. Arenz, *Angew. Chem., Int. Ed.*, 2006, **45**, 5048–5050.
4 A. W. Wark, H. J. Lee and R. M. Corn, *Angew. Chem., Int. Ed.*, 2008, **47**, 644–652.
5 (*a*) M. Lagos-Quintana, R. Rauhut, W. Lendeckel and T. Tuschl, *Science*, 2001, **294**, 853–858; (*b*) A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen and Z. Havelda, *Nucleic Acids Res.*, 2004, **32**, e175.
6 L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley and J. M. Johnson, *Nature*, 2005, **433**, 769–773.
7 C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak and K. J. Guegler, *Nucleic Acids Res.*, 2005, **33**, e179.
8 Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang and Y. Zhang, *Angew. Chem., Int. Ed.*, 2009, **48**, 3268–3272.
9 H. Jia, Z. Li, C. Liu and Y. Cheng, *Angew. Chem., Int. Ed.*, 2010, **49**, 5498–5501.
10 B. C. Yin, Y. Q. Liu and B. C. Ye, *J. Am. Chem. Soc.*, 2012, **134**, 5064–5067.
11 D. A. Shagin, D. V. Rebrikov, V. B. Kozhemyako, I. M. Altshuler, A. S. Shcheglov, P. A. Zhulidov, E. A. Bogdanova, D. B. Staroverov, V. A. Rasskazov and S. Lukyanov, *Genome Res.*, 2002, **12**, 1935–1942.
12 (*a*) X. Zuo, F. Xia, Y. Xiao and K. W. Plaxco, *J. Am. Chem. Soc.*, 2010, **132**, 1816–1818; (*b*) L. Cui, X. Lin, N. Lin, Y. Song, Z. Zhu, X. Chen and C. J. Yang, *Chem. Commun.*, 2012, **48**, 194–196; (*c*) L. Cui, G. Ke, C. Wang and C. J. Yang, *Analyst*, 2010, **135**, 2069–2073.
13 (*a*) W. Tan, K. Wang and T. J. Drake, *Curr. Opin. Chem. Biol.*, 2004, **8**, 547–553; (*b*) S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303–308.
14 F. Yu, H. Yao, P. Zhu, X. Zhang, Q. Pan, C. Gong, Y. Huang, X. Hu, F. Su, J. Lieberman and E. Song, *Cell*, 2007, **131**, 1109–1123.