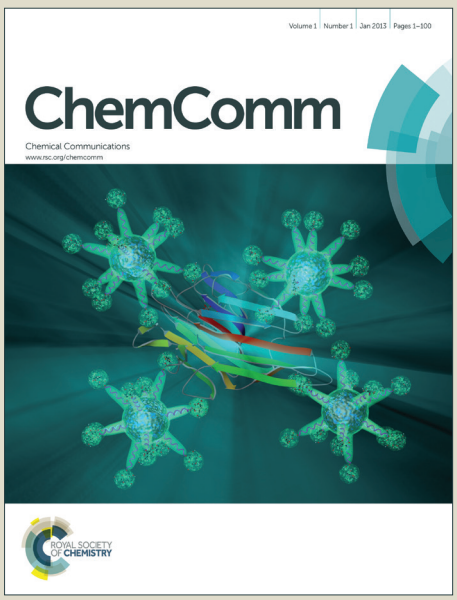
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Direct Detection of Circulating MicroRNA in Cancer Patient Serum by using Protein-Facilitated Specific Enrichment and Rolling Circle Amplification

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Cheng-Yi Hong,[a] Xian Chen,[a] Juan Li,[a] Jing-Hua Chen,*[b] Guonan Chen,[a] and Huang-Hao Yang*[a]

We have developed a simple method for direct detection of circulating microRNAs in serum by using the p19 protein-facilitated magnetic beads (PFMBs) specific enrichment and rolling circle amplification (RCA). The detection limit of microRNA is 1 fM. Therefore, the proposed method has the potential of being used in circulating microRNAs analysis and clinical diagnosis.

MicroRNAs (miRNAs) are endogenous non-coding RNAs, which consist of ~22 nt nucleotides in length. They play important roles in regulating gene expression in multitudinous cellular pathways through binding to 3' untranslated region of mRNAs to mediate mRNA cleavage or prevent protein synthesis.[1] It is reported that 3' untranslated regions are preferentially conserved and miRNAs may control up to three-fifths of all human genes.[2] Calin's group first discovered that the connection between miRNAs and human cancer, and altered expression of miRNAs has been found in many tumor types.[3] Some miRNAs act as tumor suppressors, while others act as oncogenes, either directly or indirectly.[4] Moreover, it has also been demonstrated that the circulating miRNAs are resistant to RNase digestion, extreme pH and temperature, and they can be reliably extracted and assayed in either serum or plasma[5]. These findings highlight the significance of using serum circulating miRNAs as a tool to promote the basic biomedical research progress and help cancer diagnose. Therefore, there are urgent demands of constructing sensitive and selective sensing methods for miRNA detection.

To date, many analytical approaches have been presented for miRNA detection including Northern blotting,[6] microarray-based detection,[7] capillary electrophoresis,[8] electrochemical biosensors,[9] and fluorescence-based detections (i.e., real-time quantitative polymerase chain reaction).[10] Fluorescence-based detections are the most commonly employed systems in miRNA assay for their good sensitivity and selectivity, fast analysis, and overall cost-effectiveness. Meanwhile, they offer additional flexibility by providing multiple fluorescence detection properties, including wavelength, fluorescence lifetime and intensity. Real-time quantitative polymerase chain reaction (qRT-PCR) is used as a powerful fluorescence-based detection tool for highly sensitive and accurate quantitative analysis of the miRNA. However, qRT-PCR usually needs miRNA isolated and purified from the real samples. In addition, the short length of miRNAs also increases the difficulty to design the PCR primers. In order to substitute the PCR without reducing the detection ability, various fluorescence-based signal amplification methods have been developed, such as hairpin-based amplification method,[11] duplex-specific nuclease amplification method,[12] isothermal strand-displacement polymerase reaction (ISDPR),[13] exponential amplification reaction (EXPAR)[14] and rolling circle amplification (RCA)[15]. However, most of these methods still require extraction of total RNA from real samples and purification prior to analysis, which makes the methods complicated and labor-intensive. Therefore, it is important to develop a simple method for direct detection of circulating miRNAs in real samples.

The p19 RNA binding protein which from carnation Italian ring spot virus (19 kDa) is a RNA silencing suppressor. It binds only to double stranded RNA (dsRNA) in a size-specific and sequence-independent manner with a high affinity (nanomolar).[16] The p19 protein does not interact with ssRNA, rRNA, mRNA, ssDNA, or dsDNA. The binding affinity of the p19 protein is depends on length of dsRNA duplex region. The p19 protein binds tightly to 21-23 nt dsRNA, but progressively weaker to bind 24-26 nt dsRNA and poorly to bind 19 nt and shorter length. Recently, with the unique binding property, p19 protein-facilitated affinity capillary electrophoresis assay[8] and p19 protein binding-based electrochemical sensor[17,18] have been reported. These methods allow to detect miRNAs with high sensitivity (in the femtomole range) and selectively. However, for the real sample detection, it is difficult to optimize electrophoresis separation condition. Also, the high protein content and other blood constituents in serum may probably foul the electrode through nonspecific adsorption and generate the false signal.[9]

In this work, we developed a simple method for direct detection of circulating miRNAs in serum based on p19 protein-facilitated magnetic beads (PFMBs) specific enrichment and rolling circle amplification (RCA). RCA can amplify a short DNA primer to generate long linear single-stranded DNA with thousands of repeated units which are complementary to the circular DNA template. RCA is a powerful isothermal DNA amplification method which has been widely used as a signal amplification tool for varieties of important biomarkers detection.[19] In the present study, we chose miRNA-21 as an example. An outline of the designed procedure is shown in Fig. 1. The bifunctional Janus probe contains two regions. One is the

complementary RNA oligonucleotide sequence of miRNA-21. The other is DNA oligonucleotide sequence, which can be used as primer for RCA. In step (I), hybridization of the Janus probe with target miRNA-21 forms a dsRNA structure. This structure is then enriched through binding to the PFMBs. Subsequently, the unbound Janus probe and other RNAs were removed. Afterwards, the purified Janus probe-miRNA complex is released from the PFMBs by heating and resuspended in a small volume solution. In step (II), linear RCA was initiated by using the DNA sequence in Janus probe as the primer, generating long linear single-stranded DNA (ssDNA) with thousands of repeated units. After inactivating the phi29 DNA polymerase, the detection probes were added and hybridized to the RCA products. The dsDNA duplex can be sensitively determined in a homogeneous solution by using SYBR Green I (SG) as the fluorescence dye. Due to the miRNA-specific extraction and low nonspecific adsorption of PFMBs, additionally with RCA, the proposed method has the potential to directly detect circulating miRNA in serum.

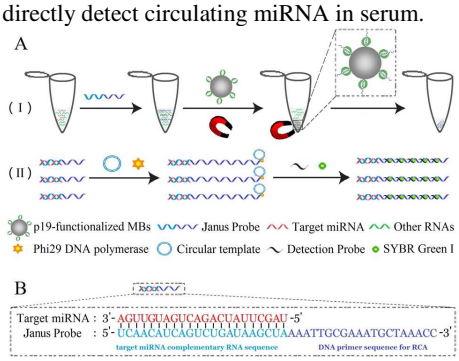


Fig. 1 (A) Schematic diagram of the process of miRNA detection. (I) PFMBs selective enrichment and separation target miRNA; (II) The hybridized Janus probe-miRNA complex is released from the magnetic beads. DNA primer sequence in Janus probe initiates RCA to assemble cascade fluorescent detection probe. (B) Sequences of the Janus probe-miRNA complex.

The Janus probe can hybrid to target miRNA-21 and form Janus probe-miRNA complex, which can selectively bind to PFMBs and achieve miRNA-21 enrichment (for detailed experimental results, see Fig. S1 in the ESI†). Therefore, we first evaluated the optimization of Janus probe concentration. The concentrations of Janus probe was varied from 0 to 200 nM on the fluorescent signal for 10 pM target miRNA-21 after RCA reaction. The results indicated that the optimum concentration of Janus probe in this study was 20 nM (Fig. S2). In this study, the signal output is based on the fluorescent readout of SG intercalated into dsDNA. Excessive circular template may significantly increase the background, which decreased the signal/background (S/N) ratio. To overcome this problem, we also optimized the circular template concentration to improve the S/N ratio of RCA. In RCA reactions step, 20 μL RCA reaction buffer containing 3 μL different concentrations of circle template (from 10 nM to 100 nM) was incubated at 30 °C for 3h. The optimum circular template concentration was 25 nM according to the best signal-to-noise level (Fig. S3).

To test the viability of our design strategy, miRNA-21 (a miRNA up-expressed in breast cancer) was selected as a model. As shown in Fig. 2, under the optimum conditions, the fluorescence intensity increased with the increasing target miRNA-21 concentrations from 0 to 100 pM. About 19-fold fluorescence enhancement is clearly observed at the concentration of 100 pM. The fluorescence ratio $(F-F_0)/F_0$ is linearly dependent on the logarithm (lg) of target miRNA-21 concentration in the ranges 10 fM-100 pM, with a correlation equation of $(F-F_0)/F_0 = 4.03 \lg(\text{miRNA}) + 58.15$ ($R^2 = 0.9935$), where F_0 and F are the fluorescence intensities at 530 nm in the absence and the presence of miRNA-21, respectively. The limit of detection based on 3σ method is 1 fM. A series of five repetitive measurements with 10 fM miRNA-21 was used for investigating the precision of the proposed method and obtained a relative standard deviation (RSD) of 3.42%, demonstrating good reproducibility of the assay. More importantly, this method is general for other target miRNA detection (Fig. S4). Currently, several novel strategies were developed to improve the sensitivity of RCA-based miRNA detection by introducing a second primer,[15a] DNAzyme,[15b] or padlock probe-based amplification.[15c] The rational introduction of these signal amplification strategies to our propose method could further improve the sensitivity of miRNA assay.

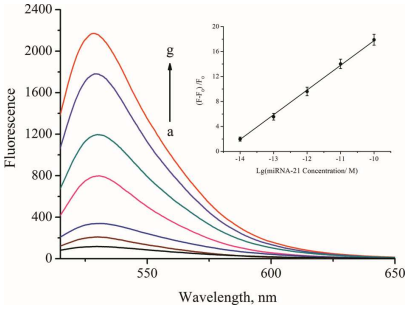


Fig. 2 Fluorescence emission spectra upon addition of different target miRNA-21 concentrations: (a) 0 M, (b) 1 fM, (c) 10 fM, (d) 100 fM, (e) 1 pM, (f) 10 pM, and (g) 100 pM. Inset is the linear relationship between fluorescence ratio $(F-F_0)/F_0$ and the logarithm of the target miRNA concentration. The illustrated error bars represent the standard deviation of five repetitive measurements.

Specificity is another essential factor to evaluate the effectiveness of an affinity assay in analyte detection. For a real-world application, the PFMBs should be able to differentiate the target miRNA from other miRNAs in a complex mixture. To evaluate the specificity, similar assay procedure was employed using target miRNA-21 and two other miRNAs (miRNA-155 and miRNA-16). As shown in Fig. 3, both miRNA-155 and miRNA-16 generated insignificant fluorescent changes when compared to the perfect match target miRNA-21. The fluorescence ratio $(F-F_0)/F_0$ from 10 fM miRNA-21 was approximately 3.25 fold higher than that of 1 pM miRNA-155 and miRNA-16. These results suggest that the proposed method with high sequence specificity has a potential application in discriminating differences of miRNA sequence.

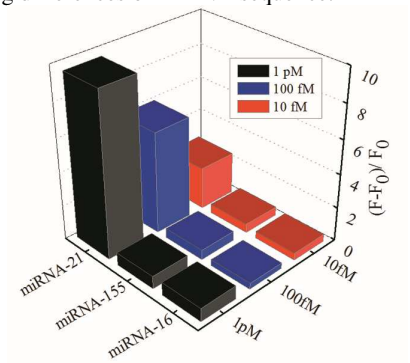


Fig. 3 Specificity of miRNA assay. Bars representing the fluorescence ratio $(F-F_0)/F_0$ from the different inputs of miRNA-21, miRNA-155 and miRNA-16 with the same concentration of 10 fM, 100 fM, and 1 pM, respectively, where F and F_0 are the fluorescence intensities at 530 nm in the presence and the absence of inputs indicated.

The expression of cancer-associated miRNA-21 in serum can be used as a biomarker of cancer progression.[20] To verify the applicability of the present method to real samples, the human serum samples from 5 newly diagnosed breast cancer patients and 5 healthy donors were tested. As shown in Fig. 4, the fluorescence intensities changes were calculated according to the following equation: $\triangle F=F-F_0$, where F and F_0 are the fluorescence intensities in the

presence and the absence of target miRNA-21, respectively. It turned out that the breast cancer patient serums (sample 1' to 5') generated higher fluorescence intensities than that obtained from the healthy donor serums (sample 1 to 5), suggesting an up-regulation of miRNA-21 expression in the breast cancer patient serums. This result is in good agreement with reported literature.[20] Furthermore, we applied a standard addition method to estimate the concentrations of miRNA-21 in the breast cancer patient serums. We choose cancer patient serum sample 1' as an example. A series of synthetic miRNA-21 at concentrations of 0, 10, 20, 40, 60, 80, and 100 fM were spiked into serum sample 1', respectively, with equal volume to establish a calibration curve. The content of miRNA-21 in the original serum sample 1' was calculated to be 60.02 fM (Fig. S5). Using the same method, the concentrations of miRNA-21 in other four breast cancer patient serums were also detected and calculated to be 73.22 fM, 84.54 fM, 89.32 fM and 115.28 fM, respectively. The qPCR analysis of miRNA-21 in the same breast cancer patient serums showed that results obtained from two methods are basically the same by considering the experimental errors, indicating that the proposed method has a promise in practical application with great accuracy and reliability for miRNA detection (Fig. S6).

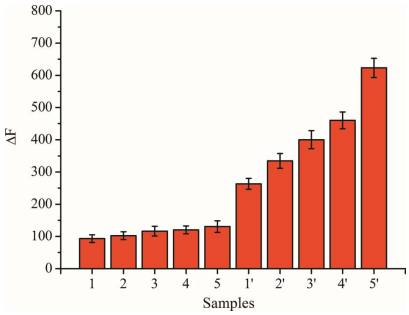


Fig. 4 The fluorescence intensities changes (△F) comparison of target miRNA-21 in the serum of healthy donors (sample 1 to 5) and breast cancer patients (sample 1' to 5'). Error bars represent standard deviations for measurements taken from at least five independent experiments.

In summary, we developed a straightforward and simple method for sensitive circulating miRNA analysis, which involves three steps: (i) The formation of the Janus probe-miRNA dsRNA structure; (ii) The target miRNA enrichment by PFMBs; and (iii) Rolling circle amplification. The detection dynamic range spans four orders of magnitude (from 10 fM to 100 pM), and the detection limit of the miRNA-21 was 1 fM. Due to the low nonspecific adsorption of PFMBs, the present method showed high selectivity and can be applied to the direct detection of target miRNA-21 in human serum samples. Therefore, the proposed method provides a simple and convenient alternative to standard approaches for quantitative detection of circulating miRNAs in real samples.

This work was supported by the National Basic Research Program of China (No. 2010CB732403), the National Natural Science Foundation of China (No. 21125524, No. 21105012), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1116), and the National Science Foundation of Fujian Province (No. 2010J06003).

Notes and references

[a] The Key Lab of Analysis and Detection for Food Safety of MOE and Fujian Provincial, College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350108, China.
E-mail: hhyang@fio.org.cn
[b] Department of Pharmaceutical Analysis, Faculty of Pharmacy, Fujian Medical University, Fuzhou 350004, China.
E-mail:cjh_huaxue@126.com

† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

1 D. P. Bartel, *Cell*, 2009, **136**, 215-233.
2 R. C. Friedman, K. K. Farh, C. B. Burge and D. P. Bartel, *Genome Res.*, 2009, **19**, 92-105.
3 G. A. Calin, C. D. Dumitru, Calin, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**, 15524-15529.
4 A. Esquela-Kerscher and F. J. Slack, *Nat. Rev. Cancer*, 2006, **6**, 259-269.
5 X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C. Y. Zhang, *Cell Research*, 2008, **18**, 997-1006.
6 A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen and Z. Havelda, *Nucleic Acids Res.*, 2004, **32**, e175.
7 J. M. Thomson, J. Parker, C. M. Perou and S. M. Hammond, *Nat. Methods.*, 2004, **1**, 47-53.
8 N. Khan, J. Cheng, J. P. Pezacki and M. V. Berezovski, *Anal. Chem.*, 2011, **83**, 6196-6201.
9 Z. Q. Gao, H. M. Deng, W. Shen and Y. Q. Ren, *Anal. Chem.*, 2013, **85**, 1624-1630.
10 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.
11 G. L. Wang and C. Y. Zhang, *Anal. Chem.*, 2012, **84**, 7037-7042.
12 (a) C. B. Yin, Y. Q. Liu and B. C. Ye, *J. Am. Chem. Soc.*, 2012, **134**, 5064-5067; (b) X. Lin, C. Zhang, Y. Huang, Z. Zhu, X. Chen and C. J. Yang, *Chem. Commum*, 2013, **49**, 7243-7245.
13 H. F. Dong, J. Zhang, H. X. Ju, H. T. Lu, S. Y. Wang, S. Jin, K. H. Hao, H. W. Du and X. J. Zhang, *Anal. Chem.*, 2012, **84**, 4587-4593.
14 H. X. Jia, Z. P. Li, C. H. Liu and Y. Q. Cheng, *Angew. Chem., Int. Ed.*, 2010, **49**, 5498-5501.
15 (a) Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang and Y. Zhang, *Angew. Chem., Int. Ed.*, 2009, **48**, 3268-3272; (b) Y. Q. Wen, Y. Xu, X. H. Mao, Y. L. Wei, H. Y. Song, Z. Chen, Q. Huang, C. H. Fan and D. Li, *Anal. Chem.*, 2012, **84**, 7664-7669; (c) H. Y. Liu, L. Li, L. L. Duan, X. Wang, Y. X. Xie, L. L. Tong, Q. Wang and B. Tang, *Anal. Chem.*, 2013, **85**, 7941-7947.
16 J. M. Vargason, G. Szittya, J. Burgyan and T. M. T. Hall, *Cell*, 2003, **115**, 799-811.
17 P. Ramnani, Y. N. Gao, M. Ozsoz and A. Mulchandani, *Anal. Chem.*, 2013, **85**, 8061-8064.
18 M. Labib, N. Khan, S. M. Ghobadloo, J. Cheng, J. P. Pezacki and M. V. Berezovski, *J. Am. Chem. Soc.*, 2013, **135**, 3027-3038.
19 H. Zhang, F. Li, B. Dever, X. F. Li and X. C. Le, *Chem. Rev.*, 2013, **113**, 2812-2841.
20 (a) S. Asaga, C. Kuo, T. Nguyen, M. Terpenning, A. E. Giuliano and D. S. B. Hoon, *Clin. Chem.*, 2011, **57**, 84-91; (b) F. J. Wang, Z. G. Zheng, J. F. Guo and X. F. Ding, *Gynecol. Oncol.*, 2010, **119**, 586-593.