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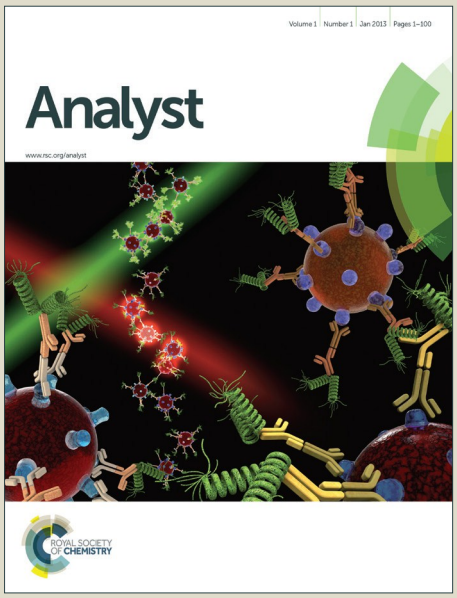

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COMMUNICATION

Label-free fluorescence detection of microRNA based on target induced adenosine$_2$-coralyne-adenosine$_2$ formation

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This study develops a simple and label-free biosensor for sensitive and selective detection of microRNA (miRNA) based on the formation of adenosine$_2$-coralyne-adenosine$_2$ complex mediated by miRNA-specific polyadenosine extension.

MicroRNAs (miRNAs) contain a large family of endogenous noncoding RNAs and play significant roles in various biological progresses such as cell proliferation, differentiation, migration, and apoptosis.[1-5] A great number of evidence have indicated that some miRNAs exhibit aberrant expression in human tissues and blood samples, which is associated with cancer initiation, tumor stage, and response of tumors to treatments.[2,6] Therefore, miRNAs can act as biomarkers for cancer diagnosis, therapeutic agents in cancer treatment, and potential targets for development of new anti-cancer drugs.[4,6-8] Accurate and quantitative analysis of miRNAs is critical for better understanding their roles in cancer and further studies on their functions in diagnosis. However, miRNA possesses several unique characteristics, such as small size, sequence similarity among the miRNA family members, and low abundance in total RNA samples, which makes their accurate detection a great challenge. Consequently, developing rapid and sensitive methods for identification and quantification of miRNAs has become imperative for the biological research, early clinical diagnosis, and screening new anticancer drugs.

There have been a few methods for analysis miRNAs.[9,10] Routine methods include Northern blotting,[11,12] microarray assay.[13] Nevertheless, these methods have a few shortcomings such as complicated operations, limited selectivity and sensitivity, or high false positive rates, which limits their application in miRNA analysis. Some new biosensor approaches have been developed to improve the detection sensitivity, robustness, adaptability and simplicity, such as fluorescent assay,[14-16] electrochemical sensor,[17-19] nanoparticle-based assay,[20,21] and the surface-enhanced Raman scattering method.[22] However, these methods also have suffered from defects. For the fluorescence-based biosensor, it requires to synthesize specific probes to each target miRNA, thus increasing the cost of the assay. The electrochemical sensor involves tedious steps of immobilization and separation. The nanoparticle-based assay needs time-consuming preparation of nanoparticle. Therefore, it still remains an unsolved problem to develop simple, selective, and sensitive methods for miRNA detection.

Coralyne (13-methyl[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo-[4,5-*i*]phenanthridium), a kind of planar alkaloid, binds specifically to polydeoxyadenosine (poly(dA)) with a stoichiometry of one coralyne per four adenine bases with an association constant of $(1.05\pm0.1)\times10^5$ M^{-1} at a neutral pH.[23] Coralyne is capable of promoting the formation of adenosine$_2$-coralyne-adenosine$_2$ (A_2-coralyne-A_2) complexes.[24] Moreover, they have been successfully used for detection of heparin in plasma and poly(A) polymerase.[25-27]

Inspired by these studies, we herein develop a label-free, sensitive and selective biosensor for miRNAs detection. This biosensor strategy relies on selective extension of target miRNA with a polyadenosine (poly(A)) tail followed by specific capture using streptavidin magnetic beads (SA-MB) and fluorescence staining mediated by the A_2-coralyne-A_2 complex. This method utilizes two specific reactions for target miRNA, a polyadenosine extension reaction and a capture reaction, which improves the selectivity of the biosensor. Moreover, the long polyadenosine tail allows selective, sensitive and label-free detection of target miRNA, making the biosensor a cost-efficient platform for selective and sensitive miRNA assay.

The developed biosensor strategy is a label-free fluorescent assay based on selective interaction between coralyne and poly(A) and specific extension of poly(A) for target miRNA, as

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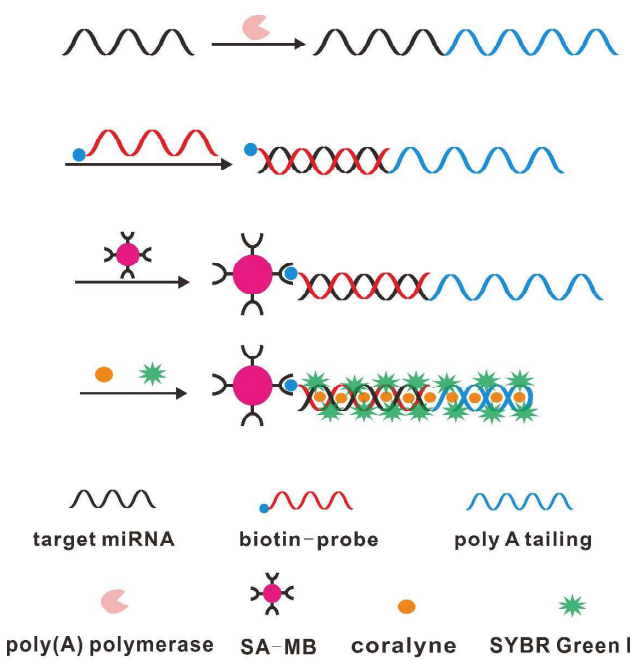

Scheme 1 Schematic illustration of fluorescence strategy based on magnetic beads and SYBR Green I for sensitivity detection of target miRNA

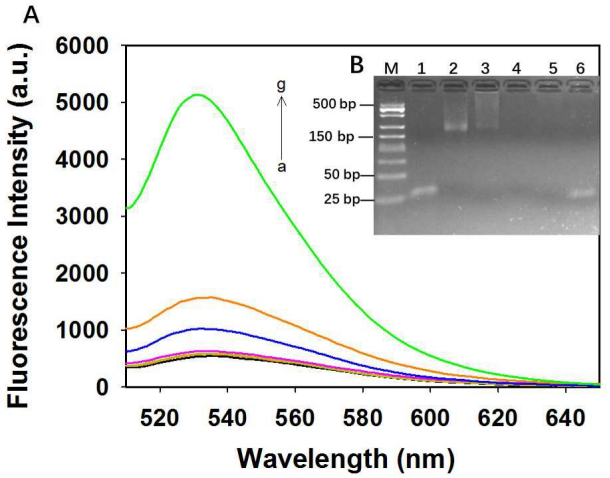

Fig. 1 (A) Fluorescence responses of the system under different conditions. (a) blank; (b) coralyne; (c) poly(A) polymerase; (d) coralyne + poly(A) polymerase; (e) miRNA; (f) miRNA+ poly(A) polymerase; (g) miRNA + coralyne + poly(A) polymerase. (B) Agarose gel electrophoresis images of miRNA. Lane M, DNA marker (25-500 bp); lane 1, 1μM miR-21; lane 2, 1μM miR-21 with 0.05 U poly(A) polymerase; lane 3, 1μM miR-21 with 0.05 U poly(A) polymerase and 4 μM coralyne; lane 4, 0.05 U poly(A) polymerase; lane 5, 0.05 U poly(A) polymerase and 4 μM coralyne; lane 6, 1μM miR-21 with 4 μM coralyne.

illustrated in Scheme 1. Target miRNA is firstly processed with poly(A) polymerase, which selectively extends the target with a long poly(A) tail at 3' end. The extended miRNA target is captured via hybridization with a biotinylated DNA probe immobilized on streptavidin-modified magnetic beads. After collected using magnetic separation, the poly(A)-tailed miRNA is treated with coralyne, which promotes the poly(A) tail to form a stable anti-parallel homoadenine duplex A_2-coralyne-A_2 with a concomitant conformational change from a random-coil structure to a hairpin structure. This hairpin structure can be stained with SYBR Green I through its intercalating into the grooves of the A_2-coralyne-A_2 complex, resulting in a greatly enhanced fluorescence signal indicating the amount of target miRNA.

To verify the feasibility of the method, miRNA-21 (miR-21) was used as a model target in our assay. Firstly, we investigated the fluorescence emission spectra of the biosensor under different conditions, as shown in Fig. 1A. In the absence of miRNA, very weak fluorescence intensity was obtained for the collected MBs (curve a). After the addition of coralyne (curve b), poly(A) polymerase (curve c) or both of them (curve d), there were no substantial increases in the fluorescence signals. In the presence of miRNA, the collected MBs showed a slight increase of the fluorescence (curve e), which was ascribed to fluorescence staining of the DNA/RNA duplex by SYBR Green I. For collected miRNA pretreated with poly(A) polymerase, the MBs displayed a further slightly increased fluorescence intensity (curve f), which was attributed to possible non-specific staining of poly(A) tail with SYBR Green I. In contrast, for collected miRNA pretreated with poly(A) polymerase followed by co-staining with SYBR Green I and coralyne, a dramatic fluorescence enhancement was realized (curve g), indicating the formation of A_2-coralyne-A_2 complex was able to substantially amplify the fluorescence signal for the biosensor. Taken together, these results confirmed the feasibility of the proposed biosensor. Further agarose gel electrophoresis analysis revealed that only in the presence of poly(A) polymerase and target miRNA, the target miRNA extended a long poly(A) tail and folded into a duplex A_2-coralyne-A_2 with coralyne (Fig. 1B). These results implied that the extension reaction was specifically induced by target miRNA, which demonstrates that the proposed method can be used for selective detection of miRNA.

The reaction conditions such as the concentrations of poly(A) polymerase and coralyne play significant roles in the analytical process. In order to achieve the best sensing performance of the fluorescence sensor, the conditions of the reaction were optimized. The concentration of poly(A) polymerase was a vital factor, which directly affects the extension of target miRNA. Hence, the poly(A) polymerase concentration firstly investigated (Fig. S1 in ESI). It is obvious that the poly(A) polymerase concentration had an important effect on the fluorescence intensity ratio. The fluorescence intensity ratio synchronously ascended with the increase of the poly(A) polymerase concentration, from 0.0125 to 0.05 U/μL. When the concentration surpassed 0.05 U/μL, the fluorescence intensity ratio decreased. Therefore, 0.05 μL of the poly(A) polymerase was chosen as the optimum concentration for the sensor. In order to study the effect of the coralyne concentration on the detection system, various concentrations of coralyne ranging from 1 μM to 8 μM were tested (Fig. S2 in ESI). With increasing concentration, the fluorescence intensity ratio increased first and decreased after reaching the maximum. Hence, the optimal concentration of coralyne was 4 μM by considering the best fluorescence

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intensity ratio and the cost-efficiency. Moreover, other related conditions were tested.

To evaluate the sensitivity of the presented fluorescence biosensor, fluorescence spectra at various concentrations of target miRNA were investigated under the optimal assay conditions (Fig. 2A). The results obviously revealed that the fluorescence intensity increased markedly with the increasing of target miR-21 concentration. A wide dynamic range from 1 to 100 nM was achieved for this assay. The calibration curve for detection of target miRNA is shown in Fig. 2B. The fluorescence intensity F was found to have a linear correlation to the target miRNA concentration C in the range from 0 nM to 100 nM. The calibration equation was F = 600 + 44 C with a squared correlation coefficient R^2 of 0.9951. The detection limit was estimated to be 0.53 nM according to the 3σ rule. Compared to other assays, the developed method had the advantages of improved selectivity because of the introduction of poly(A) polymerase reaction, and decrease complexity and lowered cost because of its simple design of probe, label-free detection and easy operation (Table S2 in ESI).

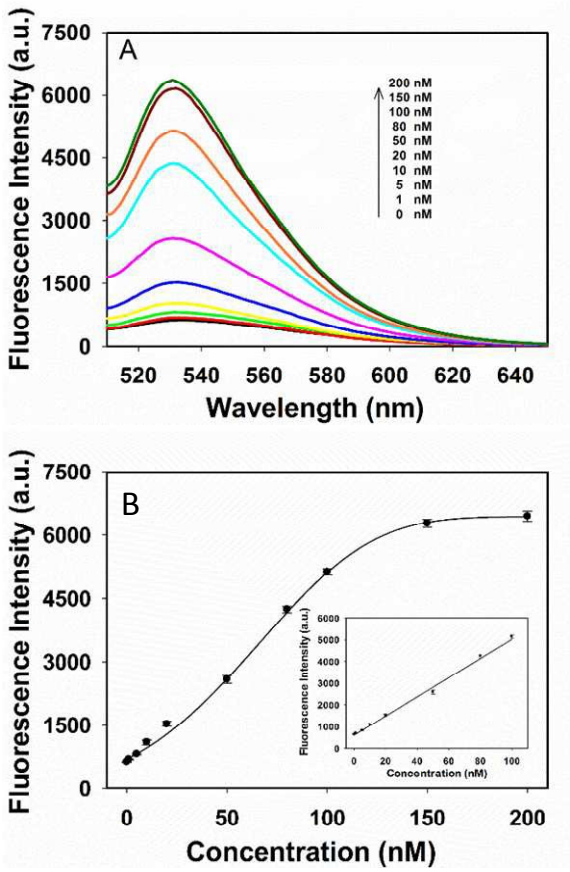

Fig. 2 (A) Fluorescence emission spectra of detection solution in the presence of various concentration of target miRNA. (B) The calibration curve of F versus the target miRNA concentration.

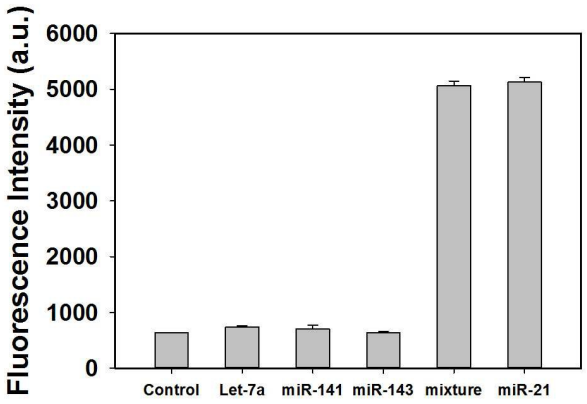

Fig. 3 Fluorescence responses of the strategy to (1) let-7a; (2) miRNA-141; (3) miRNA-143; (4) miR-21; (5) mixture of let-7a, miRNA-141, miRNA-143, and miR-21. The concentration of let-7a, miRNA-141, miRNA-143, miR-21 was 100 nM, respectively. Error bars represent the standard deviation of three measurements.

To carry out the miRNA assay with high specificity was a great challenge due to the high similarity of miRNA sequence and small size of miRNA. Because members of the same miRNA family sometimes distinguish only by one or two nucleotides. The specificity of the proposed strategy was evaluated by using other miRNA including let-7a, miRNA-141, miRNA-143 and a mixture of let-7a, miRNA-141, miRNA-143, and miR-21. As shown in Fig. 3, the fluorescence response of let-7a, miRNA-141, and miRNA-143 did not exhibit any substantial difference from the blank. Moreover, the response in the mixture showed insignificant deviation from that obtained with miR-21. These data indicated that the proposed strategy had good selectivity in miRNA detection.

To evaluate the potential of the developed method for real complex biological samples, this method was implemented for measuring the recovery of known amounts of miR-21 spiked into 10% human serum sample (Table S3 in ESI). These results indicated that the proposed method was capable of detecting miRNA in real complex samples.

In summary, we have developed a label-free, cost-effective fluorescent strategy for sensitive and selective detection of miRNAs based on the formation of an anti-parallel homoadenine duplex induced by coralyne. This approach possesses several advantages. The proposed strategy uses nucleic acid dye as fluorescent indicator and has no need of fluorescent labelling. Poly(A) based tailing introduces extra selectivity and signal amplification for miRNA detection. Magnetic enrichment of miRNA affords efficient improvement in sensitivity and selectivity. This strategy can be extended as a high throughput format for profiling miRNA expression by using a MB-based array immobilized with different capture. In consideration of these advantages, the present strategy has potential application in biomedical research and early clinical diagnostics.

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