ABSTRACT: MicroRNAs (miRNAs) modulate a variety of cellular signaling pathways and play a vital role in cell-to-cell communication. The overlapped expression of a certain miRNA is commonly reported to be related to cancers. Therefore, combined detection of multiple miRNAs is of great significance for cancer diagnosis. Herein, we developed a FeII₄L₄ tetrahedron-assisted three-way junction (3WJ) probe, which exhibited a higher stability than the normal 3WJ probe, for multiple miRNA detection. In this method, the simultaneous existence of miRNA-21 and miRNA-144 triggers the release of the Y3 sequence in the FeII₄L₄ tetrahedron-assisted 3WJ probe, which in turn triggers subsequent CRISPR-Cas12a-assisted rolling circle amplification. Based on this, simultaneous detection of miRNA-21 and miRNA-144 was achieved. Furthermore, we also applied this method to the detection of miRNAs in clinical samples and achieved good agreement with quantitative real-time polymerase chain reaction (qRT-PCR), indicating its significant potentials in early diagnosis and treatment of cancer.

INTRODUCTION

MicroRNA (miRNA) is a kind of non-coding RNA family that usually negatively regulates protein translation through the affection of messenger RNA (mRNA) stability. It modulates a variety of cellular signaling pathways and plays a vital role in cell-to-cell communication.3 In recent years, various science reports have mentioned the close relationship of miRNA with cancers due to its cancer suppressor characteristics.4,5 Therefore, miRNAs can potentially be applied as the molecular markers for cancer diagnosis and therapy.6,7 Despite the fact that the overlapped expression of one miRNA commonly occurs in many different cancers, combined detection of multiple miRNAs will contribute to a more accurate cancer diagnosis.8

Many miRNA detection methods have been developed,9 including surface plasmon resonance (SPR),10 electrochemistry,11 surface-enhanced Raman scattering (SERS),12 fluorescence,13 electrochemiluminescence, and colorimetry. Among them, the fluorescence method has the advantages of low cost, being easy to operate, quick response, and high fluorescence discrimination of different wavelengths, which not only meets the requirements of miRNA hypersensitive detection but also has a high potential application prospect in multiple miRNA detection. Despite this, few multiple miRNA detection assays have been reported.

The three-way junction (3WJ) is an uncommon DNA structure and is involved in many DNA metabolic processes, such as replication, transcription, and recombination.14,15 In addition, the 3WJ probe, consisting of three DNA strands, is considered as an ideal probe for multiple miRNA detection.16 Based on this, many 3WJ probe-based miRNA detection methods have been reported, such as a 3WJ probe-based sensitive multiple miRNA detection method by triple-input molecular AND logic gates that was reported by Miao et al.17 to achieve hypersensitivity detection of three miRNAs. Despite that, the 3WJ probe-based miRNA detection methods are also criticized for the unstable state in a complicated environment and possible false-positive detection triggered from spontaneous dissociation of the 3WJ probe.18

The loosening base pairings in the junction of the 3WJ probe may be responsible for its instability.

The FeII₄L₄ tetrahedron, a water-soluble self-assembled supramolecular that can bind to three-way DNA junctions and lead to fluorescence quenching, was used to improve the stability of the 3WJ probe (Figure S1).19,20 In this contribution, a FeII₄L₄ tetrahedron-assisted 3WJ probe was established for multiple miRNA analysis. The widely demonstrated biomarkers of non-small cell lung cancer, miR-21 and miR-141, are utilized as the examples. We have first designed a FeII₄L₄ tetrahedron-assisted 3WJ probe by hybrid-
Scheme 1. (a) Illustration of the Fe$^{II}$L$_4$ Tetrahedron-Assisted 3WJ Probe. (b) Fe$^{II}$L$_4$ Tetrahedron-Assisted 3WJ Probe for Multiple miRNA Detection and CRISPR-Cas12a-Based Signal Amplification

Figure 1. Investigation of the Fe$^{II}$L$_4$ tetrahedron-assisted three-way junction (3WJ) probe. (a) Fluorescence spectra of the 3WJ probe, Fe$^{II}$L$_4$ tetrahedron+FAM, and Fe$^{II}$L$_4$ tetrahedron+3WJ probe. (b) Fluorescence spectrum of the 3WJ probe with the Fe$^{II}$L$_4$ tetrahedron at the binding position. (c) Fluorescence intensity of the 3WJ probe and Fe$^{II}$L$_4$ tetrahedron+3WJ probe at different temperatures. (d) Fluorescence intensity of the 3WJ probe and Fe$^{II}$L$_4$ tetrahedron+3WJ probe with different times. Data are represented as means ± SD ($n = 3$).

Principle of the Established Strategy. Details of the constructed Fe$^{II}$L$_4$ tetrahedron-assisted 3WJ probe for the multiple miRNA detection method are illustrated in Scheme 1.

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**RESULTS AND DISCUSSION**

**Principle of the Established Strategy.** The trans-cleavage activity of CRISPR-Cas12a triggered, leading to the cleavage of the surrounded fluorescent reporter probe. The proposed fluorescence detection method can provide new ideas for the detection of various miRNAs and show great potential in clinical applications.
In this method, the 3WJ probe is hybridized by three DNA sequences (Y1, Y2, and Y3). Among them, Cy3 and FAM, respectively labeled on both ends of Y3, could be quenched by BHQ at a corresponding position in the complementary chain. In general, when miR-21 and miR-141 are present simultaneously, they hybridize to Y1 and Y2 in turn, and the toehold-mediated strand displacement reaction results in the release of Y3. Meanwhile, Cy3 and FAM labeled on Y3 got away from the quenching moiety (BHQ2 and BHQ1, respectively) and the fluorescent signal appeared. The obtained fluorescent intensity of Cy3 represents the amount of miR-21 present in the system, while the fluorescent intensity of FAM is positively correlated with the amount of miR-144. In addition, the released Y3 probe can hybridize with the template in RCA and form a closed loop structure under the action of the T4 ligase and thus initiate the amplification process. The sequence obtained by RCA amplification includes a repeat sequence (C) capable of triggering the trans-cleavage characteristic of CRISPR-Cas12a. Once the single-stranded DNA products are present in the system, the reporter probes whose two terminals were labeled with a fluorescent group (Cy5) and corresponding quenching group (BHQ-2) will be cleaved to produce ssDNA products. The intensity of the signal is proportional to the total amounts of Y3. It is worth noting that the established method could not simultaneously detect the total amounts of the two miRNAs but also show the proportional relationship between the two miRNAs through detecting Cy3 and FAM, which present a very high potential application value in clinical practice.

Investigation of FeII₄L₄ Tetrahedron-Assisted Three-Way Junction (3WJ) Probe. We first investigated the quenching effect of the FeII₄L₄ tetrahedron on the fluorophore by labeling the FAM fluorophore at the 5′-terminal of the constructed 3WJ probe. The results showed that, when the FeII₄L₄ tetrahedron was mixed with the FAM-3WJ probe, the fluorescent signal was significantly reduced, and the reduction efficiency was calculated about 75%. When the FeII₄L₄ tetrahedron was mixed with the FAM dye alone, no significant change in the fluorescent intensity was observed (Figure 1a). The binding position of the FeII₄L₄ tetrahedron to the 3WJ probe was then investigated by changing the distance between the junction position of the 3WJ probe and the FAM dye. The results showed that the fluorescent intensity got stronger when the junction position was farther away from the FAM probe, and the fluorescent intensity gradually decreased when the distance got closer (Figure 1b). It was also obvious that, when the junction was 4 bp far from FAM, an optimized fluorescence quenching effect was achieved. These results indicated that the FeII₄L₄ tetrahedron binds to the junction site of the established 3WJ probe, and its fluorescence quenching effect was associated with the distance with the dyes. Next, we explored the binding ability of the FeII₄L₄ tetrahedron to some other DNA structure by mixing it with the designed double-stranded DNA (dsDNA) and four-way junction (4WJ) probe. The results showed that, when the FeII₄L₄ tetrahedron was mixed with the 3WJ probe, a much better fluorescence quenching effect was observed compared with the 4WJ probe and dsDNA probe (Figure S2). It is worth noting that the distance between the junction position that can be recognized by the FeII₄L₄ tetrahedron is the same among all the established different DNA structure probes. The results showed a favorable fluorescence quenching effect in the 3WJ probe, indicating that the constructed FeII₄L₄ tetrahedron exhibited superior binding ability to the 3WJ probe compared to dsDNA and 4WJ structure probes.

We then investigated the stability of the FeII₄L₄ tetrahedron-assisted 3WJ probe by comparing with the 3WJ probe at different temperatures and times. To make the results more intuitive, we labeled the quenching group at the 5′-end of Y3
and Cy5 at the corresponding position of the complementary strand. The results showed that the fluorescence intensity of the 3WJ probe gradually increases with the increase of temperature and comes to the peak when ambient temperature reaches 50 °C, indicating that the 3WJ probe begins to dissociate. Compared with the 3WJ probe, the fluorescence intensity of the FeII tetrahedron-assisted 3WJ probe was greatly enhanced at 60 °C, suggesting a higher stability versus temperature (Figure 1c). We then investigated the stability of the 3WJ probe toward time. As shown in Figure 1d, the intensity remained relatively low after 10 h in the FeII tetrahedron-assisted 3WJ probe. These results suggested that FeII tetrahedron-assisted 3WJ probes can be stored 5 h longer than 3WJ probes.

**Investigation of CRISPR-Cas12a-Based RCA.** The released Y3 probe is significant for signal amplification. We first explored Y3 probe-based RCA amplification by 12% PAGE electrophoresis. The results showed that, when Y3 was mixed with the template and under the action of the T4 ligase and phi29 enzyme, a highlight signal was observed in the wells mixed with the template and under the action of the T4 ligase and phi29 enzyme, a highlight signal was observed in the wells. The concentration of subsequently added miR-141 gradually increased (Figure S4). Moreover, the ratio of the obtained sample, which contained ssDNA different from that of the target sequence, suggesting a favorable trans-cleavage activity catalyzed by recognition between the ssDNA and Cas12a enzyme. Afterward, we optimized the incubation time by monitoring the fluorescence intensity with increasing time. Figure 2c demonstrates that the fluorescence intensity increased with time and peaked at an incubation time of 8 min. No more fluorescence increment was observed even with the incubation extended to 20 min, revealing that an 8 min incubation is sufficient to reach hybridization equilibrium, which is consistent with previous reports.

Interests regarding the off-target effects of CRISPR-Cas12a were raised prior to their large-scale clinical implementation. Among all the possible solutions to decrease these off-target effects, optimizing the sequence of sgRNA was shown to play an essential role in lowering these risks. In this experiment, we optimized the original sgRNA sequence by increasing its GC content to 50% (sgRNA-1) or adding chemically modified DNA nucleotides (sgRNA-2) to substitute partial RNA nucleotides at the 5′- and 3′-ends of crRNA to form DNA–RNA dimers according to the guidance of sgRNA optimization rules to reduce non-specificity caused by sgRNA off-target effects. Figure 2d shows that the fluorescence intensities of sgRNA-1 (872 ± 123.54 a.u.) and sgRNA-2 (785 ± 64.26 a.u.) were higher than those of the control group (205 ± 39.75 a.u.), indicating that both modified sgRNAs achieved an ideal fluorescence intensity toward target detection.

**Investigation of the Analytical Performances.** We then applied the established method to the detection of different concentrations of miRNAs. The results showed a gradually increasing Cy3 fluorescence intensity with the concentration of miR-21 increased (Figure S3). Based on this, as the concentration of subsequently added miR-141 gradually increased, the fluorescence intensity of FAM also gradually increased (Figure S4). Moreover, the ratio of the obtained...
fluorescence intensity of Cy3 to FAM is positively correlated with the ratio of the amount of miRNA-21 and miRNA-144 present in the system (Figure 3a). When both miRNAs existed, the fluorescent signal of Cy5 was obtained by CRISPR-Cas12a-assisted RCA. It can be seen that the fluorescence intensity of Cy5 obtained depends on the lower concentration of miRNA-21 and miRNA-144 in the system, and as its concentration increases, the fluorescence intensity gradually increases (Figure 3b). The resulting fit curve is $Y = 243.2X + 2673$ ($R^2 = 0.949$).

In practice, we can get the concentration of the lower one of the two miRNAs through the formula. Based on this, according to the results of Cy3/FAM or FAM/Cy3, the corresponding amount of the other miRNA can be obtained. To investigate the selectivity of the proposed detection systems, we have replaced miRNA inputs by three single-base mismatched miRNAs (M1, M2, and M3). As shown in Figure 3d, none of these mismatched miRNA inputs can be used for the strand displacement reaction; thus, no Cy5 fluorescence was observed. Only in the presence of the target miR-141 where a remarkable variation of the fluorescence intensity can be observed, suggesting its high specificity (Figure 3c).

Application of This Method in Clinical Sample Analysis

To further study the clinical applications of the established method in the detection of miRNAs from clinical samples, blood samples from lung cancer patients and a healthy human were collected. Latterly, miRNAs in each sample were detected separately using the proposed method and quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Figure 4, the amounts of miRNA detected by the proposed strategy and qRT-PCR. Data are represented as means ± SD ($n = 10$).

Figure 4. Analytical performances in a clinical sample. Relative level of miR-21 detected by the proposed strategy and qRT-PCR. Data are represented as means ± SD ($n = 10$).

Conclusions by this method maintained high consistency with the qRT-PCR results with $R^2 = 0.957$, demonstrating that this method has a high application potential in the detection of clinical specimens. We then compared the characteristics and principle of the proposed method with several previously developed strategies (Table S2). The table showed that the proposed method has several unique advantages over conventional approaches, such as in sensitivity and complexity.

Conclusions

In summary, we have proposed a fluorescence method capable of simultaneously detecting two miRNAs, which has great potential in the diagnosis of many diseases, especially cancer diagnosis. In this approach, we created a Fe$^{II}_{4}$L$_4$ tetrahedron to increase the temperature and time stability of the constructed 3WJ probes and applied the established Fe$^{II}_{4}$L$_4$ tetrahedron-assisted 3WJ probes for the detection of miRNA-21 and miRNA-144. Based on this, we realized the hypersensitivity detection of miRNA and calculated the amount of two miRNAs using the proportional relationship. In addition, the sequence of the 3WJ probe is flexible and has been successfully demonstrated in clinical serum samples, which indicates its significant potential applications in early diagnosis and treatment of cancer.

Associated Content

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.0c05798.

Experimental section; details of the sequences (Table S1); subcomponent self-assembly of the Fe$^{II}_{4}$L$_4$ tetrahedron in aqueous solution (Figure S1); fluorescence spectrum of the Fe$^{II}_{4}$L$_4$ tetrahedron+4WJ probe, Fe$^{II}_{4}$L$_4$ tetrahedron+3WJ probe, and Fe$^{II}_{4}$L$_4$ tetrahedron+ssDNA (Figure S2); fluorescence spectrum of the Fe$^{II}_{4}$L$_4$ tetrahedron-assisted 3WJ probe for miR-21 detection (Figure S3); and fluorescence spectrum of the Fe$^{II}_{4}$L$_4$ tetrahedron-assisted 3WJ probe for miR-141 detection (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest. Approval from Seventh People’s Hospital of Chengdu.

Acknowledgments

We really appreciate the staff of the laboratory department in the Seventh People’s Hospital of Chengdu for the sample collection, and the article is especially for the 70th anniversary of the Seventh People’s Hospital of Chengdu.

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