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The construction of a novel biosensor based on the dual-enzyme cascade amplification-induced copper nanoparticles self-assembly for ultrasensitive detection of MicroRNA153

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ABSTRACT: MicroRNAs (miRNAs) have received extensive attention because of their potential as biomarkers for cancer diagnosis and monitoring, and their effective detection is very significant. Here, a specific, one-pot, rapid, femtomolar sensitive miRNAs detection biosensor was developed based on the target-triggered three-way junction (3-WJ) and terminal deoxynucleotide transferase (TDT)/Nt.BspQI in combination to activate copper nanoparticles (CuNPs) self-assembly. To this end, a 3-WJ hairpin probe and helper probe were designed to selectively identify the target miRNA, so as to form a stable 3-WJ structure that further triggered the double-enzyme cycling to produce poly T to activate self-assembly of CuNPs. Based on the simplicity of CuNPs generation, the poly T template fluorescence CuNPs can detect the minimum detection limit of 1 fm within 1.75h. In addition, the applicability of this method in complex samples was demonstrated by analyzing the whole-blood RNA extraction from Parkinson patients, consisting with the results of commercial miRNA kits. The developed strategy performs powerful implications for miRNA detection, which may be benefit for the effective diagnostic assays and biological research of Parkinson.

Key words: CuNPs, microRNA, TDT, Nt.BspQI ,3-WJ

1. INTRODUCTION

MicroRNAs (miRNAs) are a kind of endogenous non-coding RNAs with a size of 18-24 nucleotides [1, 2]. As the tumor diagnostic and prognostic markers, candidates for therapeutic interventions and basic biomedical research, miRNAs have received extensive attentions[2-4]. However, because miRNA has such characteristics as short sequence, low expression level

and easy degradation, which puts forward higher requirements for detection methods[5]. The traditional technologies include the next-generation sequencing (NGS) [6], microarrays[7, 8], and RT-PCR[9-11]. Despite the significance of these techniques, however the complex chemical modifications and multiple experimental operations may cause the damage to the easy-degradation miRNAs, and often require professional operators and conditions, making it difficult to achieve relevant tests in poor areas.

The rapid developing spatial structure of nucleic acid[12] provided great opportunities in the fields of disease diagnosis, molecular electronics, computing, imaging and so on[13]. Particularly, DNA-assisted biological detection often displays uniform conduction without time-consuming separation and washing steps, which can well overcome the limitations of the traditional detection methods, so it has received more and more attention. Recently designed biological analysis sensors can detect proteins and nucleic acids by directional induction of DNA assembly, including DNA hydrogel[14], DNA hybridization[15], DNA-antibody Nanostructure[16], DNA Tetrahedron Nanostructure[17], etc. Among them, DNA hybridization can form special structures based on the proximity effect, such as three-way DNA junction(3-WJ)[18], which is assembled based on the simultaneous recognition of targets by two DNA-bound affinity ligands, and often referred to as affinity ligands or antibody-based or close detection of target nucleic acids[19]. Therefore, the spatial structure of nucleic acid -based sensor is an effective method with high sensitivity and easy operation.

Fluorescence probes -based detection performs high sensitivity, fast analysis speed, and visualization[20, 21], and has attracted much attention. However, most fluorescent dye and fluorescent nanoparticles -based labeling methods require complex labeling or synthetic processes, which easily caused the fluorescence quenching and influenced the activity of probes. With the development of nanoparticles[22], especially the development of metal nanoparticles[23-27], it points out a new path for fluorescence-based detection, especially for miRNA detection. Among these nanoparticles, due to the high affinity of copper ions and thymine, CuNPs can be generated by copper ions using single-chain poly (thymine) (poly T) as a template within minutes at room temperature[28-31]. Surprisingly, due to the quantum size effect, CuNPs performed high fluorescence signal, which can be further detected by several kinds of equipments[32-34]. Therefore, the in-situ synthesis of poly-T templated CuNPs is one of the most suitable fluorescent probes for biochemical detection without pre-chemical modification and fluorescence labeling.

Herein, in this work, We designed a TDT/ Nt.BspQI co-activation CuNPs self-assembly DNA machine（ECDM）to detect Parkinson disease-related miRNA153-3p(miRNA153). A 3-WJ structure was developed to specifically identify miRNA153 by forming a 3-WJ structure with the designed hairpin probe and helper probe, resulting in the exposed cleavage

site, which can be recognized by Nt.BspQI. So with the presence of TDT and Nt.BspQI nicking enzyme, the 3-WJ structure converts the input signal (miRNA153)into poly T, and then output the fluorescent signal generated by the poly T-introducing self-assembly CuNPs. The label-free, ultra-sensitive and rapid sensor can effectively reflect the concentration of miRNA153 in the peripheral blood.

2. EXPERIMENTAL

2.1. Chemicals and Materials. Ultrapure 3-(N-morpholine) propionic acid (MOPS), magnesium chloride, copper sulfate and ascorbic acid were at least analytical grade and commercially obtained from Sangon Biotech. The reaction buffer contained 20 mM MOPS, 100 mM $MgCl_2$, pH 7.5. Deionized water was prepared through the Nano pure Infinity™ ultrapure water system. The oligonucleotides poly T40 were synthesized by Sangon Biotech (Shanghai China)， and they were purified by HPLC. DNA was dissolved in sterile deionized water. Hairpin probe and helper probe whose 3'-OH was sealed were synthesized by Takara and monomeric dTTP, TDT, and TDT buffer ($CoCl_2$ and RB10×) and Nt.BspQI were all purchased from Sangon Biotech, as well as miR153-3p, miR153-5p, miR21 and miR141. MiRNA Extraction Kit (Blood), HG TaqMan miRNA cDNA Synthesis Kit and HG has-miR-153 TaqMan qPCR Kit were purchased from Sangon Biotech (Shanghai China).

2.2. Preparation and Characterization of the CuNPs. The reaction was carried out using a solution of ssDNA (10 mM) and $CuSO_4$ (10 mM) in MOPS buffer (20 mM MOPS, 100 mM $MgCl_2$, pH 7.5), and ascorbic acid (100 mM) was used as the reducing agent. After blending completely, they were incubated at room temperature to form fluorescent CuNPs. Fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer with excitation wavelength at 340 nm and emission wavelength at 580 nm at room temperature. UV-visible absorption spectra were recorded on a C600. CuNPs with different fluorescence intensity can be detected by Thermo Scientific Microplate Reader.

2.3. The Construction of ECDM Sensor Target miRNAs were added in 20μL of 1× RB and $CoCl_2$ containing 1μM hairpin probe, 1μM helper probe, 20 units of TDT, 10 units of Nt.BspQI, and 1 mM dNTPs. Subsequently, the solutions were reacted in PCR at 37 °C for 1.5 h, the solutions than reacted at 85°C for 20 min in order to inactivate the enzyme, After the end of the reaction, 1μL $CuSO_4$ (10 mM), 50 μL MOPS buffer (100 mM $MgCl_2$, 20 mM MOPS, pH 7.5), and 1μL ascorbic acid (100 mM) were added in 10μL reaction solutions and allowed to react the dark at room temperature for 15min.

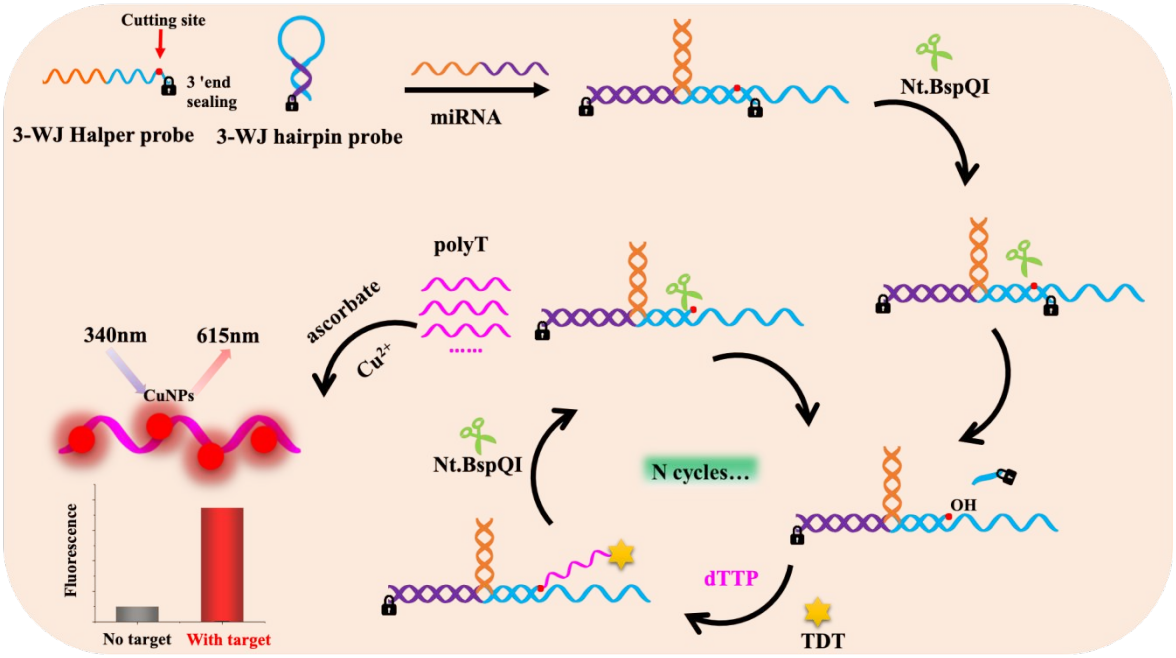
2.4. Gel Electrophoresis. Products of 3-WJ were analyzed by 3% agarose gel electrophoresis (AGE) in 1×TAE buffer (1.8g agarose, 60mL ddH_2O, pH 7.9) at a 120 V constant voltage for 40~50min at 25 ºC (room temperature). The gel was captured by C600.

2.5. Detection of MiRNAs in Human Blood Samples. MiRNAs were extracted from the whole blood of 3 normal subjects and 5 Parkinson patients by MiRNA Extraction Kit (Blood). Then cDNA was obtained by reverse transcription with HG has-miR-153 TaqMan qPCR Kit, and fluorescence quantitative PCR was performed with HG has-miR-153 TaqMan qPCR Kit. We examined miRNAs extracted from normal subjects and Parkinson patients. Finally, the fluorescence of CuNPs was compared. The whole blood samples of the patients came from the General Hospital of Tianjin Medical University, and the use of blood samples has been approved by the patients. The study protocol was in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments, and it was approved by the ethics committee of General Hospital of Tianjin Medical University.

3. RESULTS AND DISCUSSION

3.1. The Principle of ECDM and Target-triggered 3-WJ Structure

As shown in the schematic 1, the ECDM can be stimulated by the 3-WJ structure, which is formed by a hairpin probe, a helper probe and the target microRNA. The hairpin probe and helper probe each have 11 bases complementary to the target miRNA, and only have 7 bases with each other, their Tm (about 21.7 ºC) is lower than the reaction temperature (37 ºC), so they can't form a double stranded DNA structure without the target miRNA, as well as activate the ECDM. Oppositely, when target miRNA is present, the helper primer and hairpin probe can hybridize with miRNA in close proximity to form the stable 3-WJ structure, resulting in the trigger of ECDM. The cleavage site was exposed when it was on the double stranded DNA structure, and the Nt.BspQI can specially cut the helper probe to unlock the blocked 3 ends. Then TDT further extended on the unlocked 3 ends of the helper probe with dTTP to form poly T, which can be cut off again through the same cleavage site. Based on the cyclic working of TDT and Nt.BspQI, lots of free poly T are generated. With the addition of copper sulfate and ascorbic acid, the self-assembly of CuNPs can proceed. Therefore, the detection of target miRNA is turned into the detection of the fluorescent intensity of CuNPs by signal amplification and transformation of ECDM. It is worthwhile to find that the entire reaction system is a one-pot and easy-operation method without pre-fluorescence modification and labeling. The concentration can be quantified by distinguishing the fluorescence intensity of CuNPs. Compared with traditional detection methods, it has the advantages of simplicity, short time, visualization and so on.



Schematic1. Schematic Diagram of miRNA Detection Based on Target-Triggered 3-WJ Structure and ECDM

3.2. Design and Feasibility of the ECDM

The 3-WJ structure is formed by the hairpin probe, helper probe and target miRNA153. As TDT is a template-free DNA polymerase that can extend the 3 ends of the nucleic acid, to ensure ECDM work only in the presence of miRNA153, so the 3 ends of the hairpin probe and helper probe need be effectively blocked. In this work, we tried many kinds of sealing methods, including phosphate-terminated, 5U-C6-spacer-terminated, C3 spacer-terminated, invert dT etc. As can be seen from the Figure 1A, the best terminating method is 5U-C6spacer-terminated. Then, we optimized the structure of the hairpin probe and helper probe. Several probes with different structures are designed and the oligonucleotide sequences were list in Table S1 and Figure S1. Through comparing the signal difference (ΔFluorescence Intensity = Fluorescence Intensity of the experimental group - Fluorescence Intensity of the blank group) in Figure 1B, the combination 2 (Helper probe 1 and Hairpin probe 2 as shown in Table S1) is the most suitable pairs. So, 5U-C6 spacer-terminated hairpin probe and helper probe in combination 2 were applied for the ECDM in the following work.

The fluorescence signal of this work is generated by poly T introducing in-situ synthesis of CuNPs. The individual hairpin probe and helper probe, as well as the target miRNA153 didn't lead to the generation of CuNPs (Figure 2A).We compared different nucleotide sequences, including poly A, poly T, poly G, poly C and poly N(A, T, G, C) generated by TDT extending the unblocked 3 ends of helper probes with different nucleic acid bases. The results displayed that only poly T can promote the obvious in-situ synthesis of CuNPs

because of the strong combination of poly T and copper ions[26,31,35] (Figure 1C). In addition, the fluorescence intensity enhanced with the increasing concentration of poly T (Figure 1D). So the amount of poly T can be detected by the brightness of CuNPs, which indirectly characterizes the amount of target miRNA.

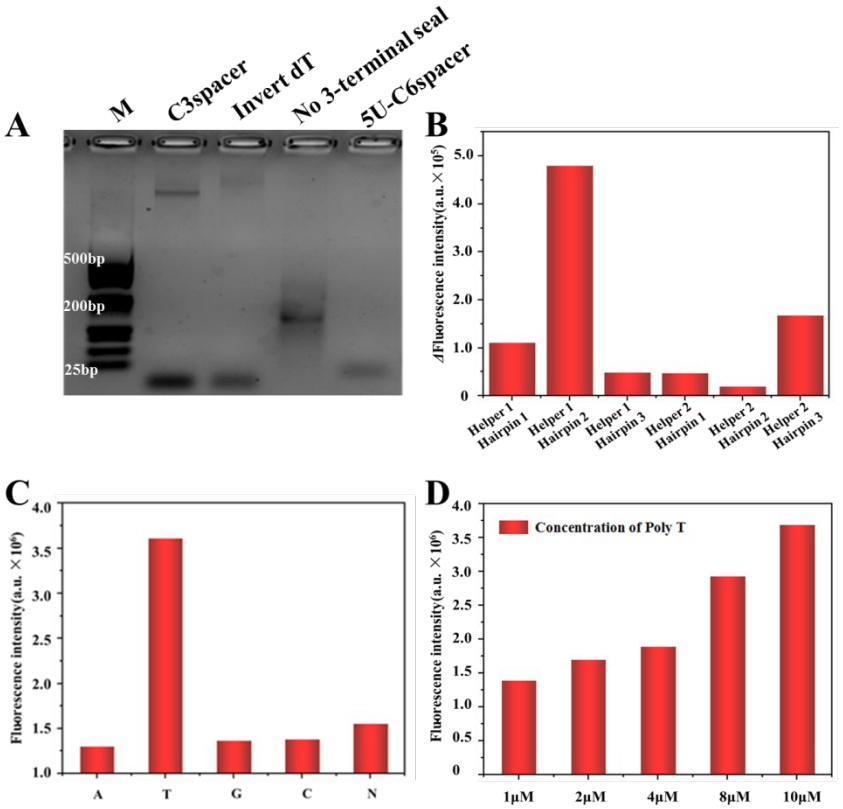


Figure 1. (A) Agarose gel (3%) electrophoresis was used to show different sealing effects. C3 spacer, invert dT and 5U-C6 spacer were used to seal the 3 ends. (B) The ΔFluorescence Intensity of Six combinations of the helper probe and hairpin probe. The concentration of every helper probe and hairpin probe was 1μM in the experimental groups and control groups. (ΔFluorescence Intensity=Fluorescence Intensity of the experimental group - Fluorescence Intensity of the blank group). Their oligonucleotide sequences were listed in Table S1. (C) The fluorescence intensity generated by different nucleotide sequences. The concentrations of A, T, G, C, N were all 1mM. (D) The fluorescence intensity generated by different concentration of poly T40. The concentration of polyT40 was 1μM, 2μM, 4μM, 8μM and 10μM respectively.

In order to realize the sensitive detection of miRNA153, the TDT and Nt.BspQI were introduced to help amplify the detection signal, which means generating numerous poly T. As shown in Figure 2B, the system included the hairpin probe, helper probe and the target miRNA153. In the absence of TDT or Nt.BspQI, there didn't exist free poly T, as well as no fluorescence signal in the corresponding tubes. Only in the presence of TDT and Nt.BspQI, the wide strip of poly T was characterized on the agarose gel, consistent with the strong fluorescence intensity of CuNPs in the fourth tube. In addition, it also can be found that, only

with the hairpin probe, helper probe and the target miRNA153, the cleavage site can be exposed on the forming double stranded 3-WJ structure and was cut off and extended cyclically by Nt.BspQI and TDT as displayed in Figure 2C. In general, ECDM can effectively work only in the presence of the target miRNA with the help of the above reagents.

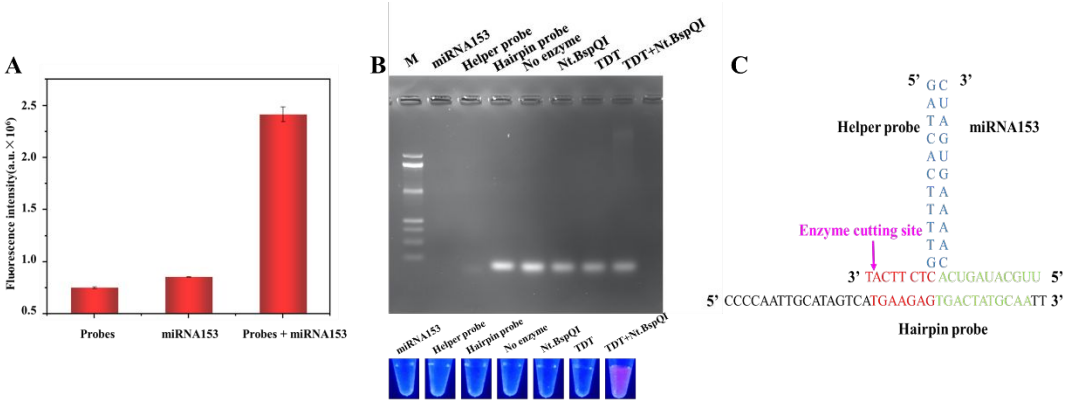


Figure 2. (A) Conditions for activating ECDM. (B) Only miRNA with TDT and Nt.BspQI was added to line 1; Only helper probe with TDT and Nt.BspQI was added to line 2; Only hairpin probe with TDT and Nt.BspQI was added to line 3; No enzyme was added to line 4; Only Nt.BspQI without TDT was added to line 5; Only TDT without Nt.BspQI was added to line 6; Both TDT and Nt.BspQI were added to Line 4. The concentration of TDT was 20U/μL, the concentration of Nt.BspQI was 10U/μL. (C) The cleavage site of Nt.BspQI.

3.3. Optimization of Important Conditions

In order to obtain better detection efficiency, the enzyme concentration, dual enzyme ratio, and dual enzyme optimal buffer should be optimized separately. As shown in Figure 3A, the higher the concentration of the Nt.BspQI and TDT, the better effect of the reaction. Unfortunately, as the enzyme concentration increases, the background will also greatly increase, so we chose 1:2 as the reaction enzyme concentration ratio, the concentration of TDT was 20U/μL, the concentration of Nt.BspQI was 10U/μL. Because the enzyme requires different reaction buffers and reaction temperatures, the most suitable reaction buffers for TDT are RB10 × and $CoCl_2$, and the most suitable reaction buffer for Nt.BspQI is NEBuffer3.1. It has been experimentally proven that RB10 × and $CoCl_2$ as reaction buffers can retain the maximum activity of both enzymes (Figure 3B), electrophoretic diagram is shown in Figure S2. According to the enzyme instructions, TDT has the greatest activity at 37 ºC, Nt.BspQI has 80% activity at 37 ºC, and both enzymes can be inactivated at 80 ºC for 20 minutes, so these two temperatures are selected in the system reaction. In addition, the optimization experiment of CuNPs mainly involves the concentration of copper ions, nucleic acid and VC. Through the optimization experiments in Figure S3, the most suitable concentration of copper ions is 0.2 mM and the concentration of ascorbic acid is 1 mM. Besides, under the most suitable conditions for CuNPs synthesis, CuNPs were examined by

transmission electron microscopy (TEM). The results are shown in Figure S4. The fluorescence and absorption spectra of CuNPs are shown in Figure S5 and S6.

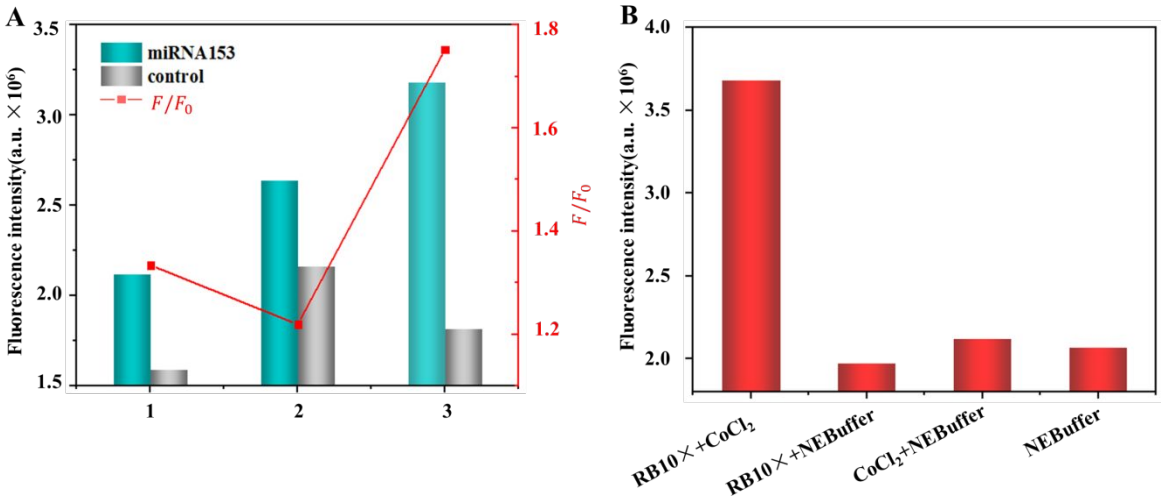


Figure 3. Optimal reaction conditions. (A) The optimum ratio of the two enzymes, TDT (10U/μL)/Nt.BspQI (20U/μL) in group 1 was 1:2, TDT (20U/μL)/Nt.BspQI (20U/μL) in group 2 was 1:1, and TDT (20 U/μL)/Nt.BspQI (10U/μL) in group 3 was 2:1. F/F_0 means the ratio of fluorescence intensity in the experimental group to that in the control group. (B) The optimum buffer for the two enzyme reactions. There were four buffers options, including RB10×+$CoCl_2$, RB10×+NEBuffer, $CoCl_2$+ NEBuffer and NEBuffer.

3.4. Sensitivity and Specificity of ECDM

After designing the appropriate 3-WJ structure and optimizing the corresponding reaction conditions, different concentrations of miRNA153 were added to the reaction system to observe the detection sensitivity of the ECDM. The relevant fluorescence intensity is shown in the Figure 4A. The fluorescence intensity is gradually increased and has a linear relationship with the logarithm of the miRNA153 concentration as shown in Figure3A. The correlation equation is y = 183609.0625x + 4343920(Figure 4A), where Y represents the fluorescence intensity and X represents the logarithm of the miRNA concentration. Derived from the equation, the limit of detection (LOD) is calculated to be 1.74× 10-17 mol/L (LOD = blank + 3SD). In addition, in order to investigate whether any other miRNA can activate the ECDM, the specific experiments were also performed. Several common miRNAs were selected as specificity comparison targets, such as miRNA141, miRNA153-5p, miRNA121 (Figure 4B). In comparison, miRNA153 performed much higher fluorescence intensity, which proves that miRNA153 can activate ECDM with perfect specificity.

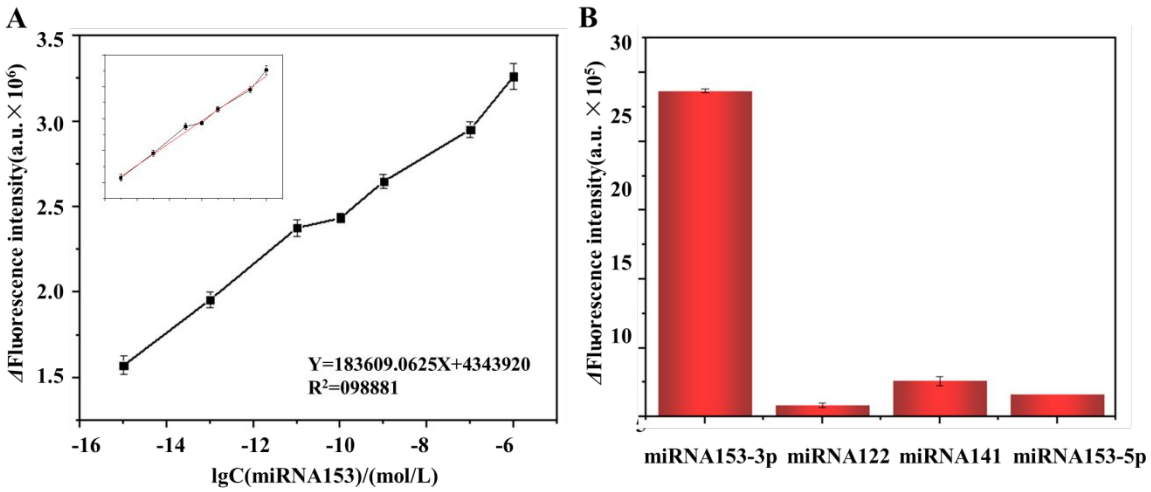
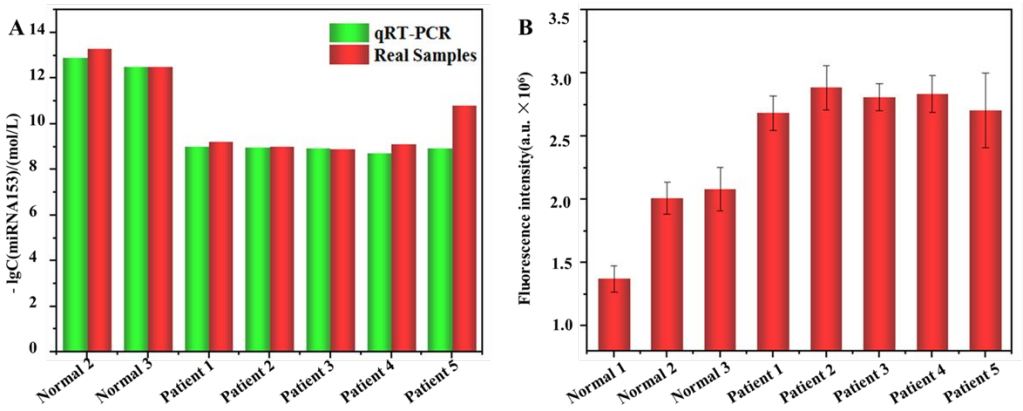


Figure 4. Sensitivity and specificity of ECDM. (A) Fluorescence intensity with different concentrations of miRNA153 in the ECDM detection system. The concentration of miRNA153 ranged from 1fm to 1μM. (B) Three common tumor markers, miRNA122, miRNA141 and mirna153-5p, were selected as the control group, miRNA153 as the experimental group. Their concentration is 1μM.

3.5. Detection of miRNA153 Based on ECDM

We further studied the feasibility of this method by adding different amount of miRNA153 in serum. MiRNA153 was analyzed by a commercial qRT-PCR kit and our designed ECDM. Amplification results of qRT-PCR standard curve are shown in the Figure S7. As shown in Figure 5A (Normal 1 exceeded the detection limit and was not marked in Figure 5C), the results obtained by our method are close to those of the qRT-PCR kit, proving that our method is feasible and reliable.

It has been reported that miRNA153 is abnormally expressed in Parkinson's patients, which in turn can cause the expression of some functional proteins to be deregulated. Therefore, it is very helpful to observe whether miRNA153 is abnormally expressed for Parkinson diagnosis. We obtained blood from 8 individuals, including three normal individuals and five Parkinson patients. MiRNAs were extracted by miRNA Extraction Kit (Blood). After the reaction, the concentration of miRNA153 in the total miRNA was estimated from the fluorescence intensity of the CuNPs according to the formula obtained from the standard curve. Our method proves that the expression of miRNA153 is up-regulated in Parkinson (Figure 5B). To sum up, our method will provide an effective miRNA detection method for Parkinson diagnosis.



C

	Normal 2	Normal 3	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
value	-13.3	-12.5	-9.2	-9.0	-8.9	-9.1	-10.8
concentration	50.1fM	0.316pM	0.633nM	0.987nM	1.083nM	0.772nM	0.17nM

Figure 5. Real samples tested by ECDM. (A) Comparison of qRT-PCR kit and our method. (B) The fluorescence values of RNA extracted from whole blood of normal subjects and patients were measured by ECDM. (C) According to the standard curve in Figure 4 and the fluorescence value in figure 5A, the content of miRNA153 in the actual sample was calculated.

4. CONCLUSIONS

Herein, we designed a kind of label-free, ultra-sensitive and rapid ECDM sensor for the detection of Parkinson-associated miRNA153 without pre-fluorescence labeling. In the presence of the target, the designed hairpin probe and helper probe can form a stable 3-WJ structure with miRNA153, and then TDT and Nt.BspQI enzymes together initiated the self-assembly process of the CuNPs to generate fluorescence signal for the quantitative detection of miRNA153. The use of two 3-terminated probes, combined with the high accuracy of TDT and Nt.BspQI, enables the detection machine to have a low background and thus a high signal to noise ratio. The signal amplification of the double enzymes and fluorescence enhancement of CuNPs together helps realize a femtomolar sensitivity. Overall, we developed a one-pot, simple and sensitive ECDM sensor for miRNA153 detection, which may be further applied in the clinical diagnosis in the future.

SUPPORTING INFORMATION

Supplementary materials (including synthesized oligonucleotide sequences used in this

work; the electrophoretogram of the most suitable buffer; the selection of the most suitable conditions for CuNPs synthesis; the standard curve of qRT-PCR) is available.

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Notes

The authors declare no competing financial interest.

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